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. These, 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. Suramin 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.

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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 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 fMLP1 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-acyl-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,1 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

1 The abbreviations used are: fMLP, formylmethionylleucylphenylalanine; PLD, phospholipase D; PA, phosphatidic acid; DG, 1-acyl-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-dioctanoyl-sn-phosphatidic acid; 14:0-PA, 1,2-dimyrhistoyl-sn-phosphatidic acid; 18:0,20:4-PA, 1-stearoyl-2-arachidonoyl-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.
monolayers of mesothelial cells (37), actin stress fibers assembly (38, 39), and to activate effector enzymes, such as PLD (40, 41), cytoplasmic nuclear accumulation of focal adhesion kinase (42), 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 suramin. Since LysoPA was 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-oleoyl-2-acetylated-3-sn-phosphatidic acid (18:0,20:4-PA), 1-deoxy-2-lyso-phosphatidic acid (18:1-LPA), phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylglycerol, phosphatidylinositol, lysophosphatidylserine, 1,2-diactanoyl-3-glycerol (8:0-DG), 1,2-diacyl-glycerol (18:1-DG). The following were from Serdary Research Laboratories, Inc. (Port Huron, MI): 1,2-dioleoyl-3-sn-phosphatidylcholine, 1,2-distearoyl-3-phosphatidylcholine (16:0-PA), 1-palmitoyl-2-lysophosphatidic acid (16:0-LPA), phosphatidylinositol, lysophosphatidylcholine, 1,2-dimyristoyl-3-phosphatidylcholine (14:0-PA), 1-oleoyl-2-lysophosphatidic acid (18:1-LPA), phosphatidylinositol, lysophosphatidylserine, 1,2-diactanoyl-3-glycerol (8:0-DG), 1,2-diacyl-glycerol (18:1-DG). The following were from Du Pont de Nemours (Dreiech, Germany): human recombinant MCP-1 (MCP-1), fMLP. The following were from Calbiochem: 1,6-diphenyl-1,3,5-hexatriene (Janssen, Beerse, Belgium) as probe. Percol-purified monocytes (10⁶/ml) were resuspended in RPMI 1640 and incubated with 1 μM fMLP acetoxymethyl ester (Calbiochem) at 37°C for 15 min. After incubation, monocytes were washed and resuspended in Hanks’ buffered salt solution (Biochrome containing 1.2 mM CaCl₂) 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 × 10⁶/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 Shinitchi and Barenholz (56) using 1,6-diphenyl-1,3,5-hexatriene (Janssen, Beerse, Belgium) as probe. Percoll-purified monocytes (10⁶/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. Lymphocytes were added in chloroform (0.1 μl/ml); this concentration of the solvent was without effect on membrane fluidity values.

Measurement of F-actin—F-actin levels were determined as described previously (57). Briefly, Percoll-purified monocytes (5 × 10⁶/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% formaldehyde for 10 min. Following permeabilization with 50 ng/ml lysophosphatidic acid (Sigma), cells were stained with 0.165 μM N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-NBD-phallacidin (Molecular Probes Inc., Eugene, OR). Cells were then washed, and fluorescence was evaluated by a FACStarPlus (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 means ± S.D. of a representative experiment or as means ± 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 μM. 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 vitro chemotactic assays. As reported in Table 1, at the optimal concentration of 1 μM, 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” chemotactant fMLP (10⁻⁸ M) and ~80% of the migration observed with a similar concentration of the most active molecule.
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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 dipalmitoyl 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

| Chemoattractant | Concentration | % Input | Chemotactic Index | Relative Efficacy |
|-----------------|---------------|---------|-------------------|------------------|
| IMLP (13)       | 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 (2)          | 10^(-6)       | 12.5     | 3.1               | 82               |
| 18:0,20:4-PA (12)| 10^(-3) | 9.7 ± 4  | 2.6 ± 1           | 67               |
| LTB4 (3)        | 3 × 10^(-7)   | 9.1 ± 5  | 2.2 ± 0           | 56               |
| PAF (5)         | 10^(-7)       | 8.1 ± 2  | 1.5 ± 0           | 38               |

* % migrated monocyte with respect to the input (7.5 × 10^6 monocytes/well).
* No. of experiments.
* AA, arachidonic acid.

Table II

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

a p < 0.01 versus migration to control medium (above and below the filter) by Student’s t test.

Table II shows that monocyte migration in response to 18:0,20:4-PA is chemotaxis (directional migration) rather than chemokinesis (activated random migration).

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. 2A). On the contrary, all the other phospholipids and lyso phospholipids tested were not active (Fig. 2B).

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 than 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, ~2% 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 a 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. 3A 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. 3A) 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 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. 3A, in these conditions no monocyte migration was observed either in the absence or in the presence of the respective PA.
motactic concentration of PA or lysoPA, washed, and then experiments, monocytes were first exposed to an optimal concentration 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 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 compartment 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-dioleoylglycerol (18:1-DG), dioleoyl PA (18:0), dielyl lysoPA (18:1-LPA), dipalmitoyl PA (16:0-PA), and palmitoyl lysoPA (16:0-LPA); panel B, 1,2-stearoyl-2-arachidonyl PA (18:0,20:4-PA), phosphatidylinositol (PI), lysophosphatidylglycerol (LPG), lyso phosphatidylglycerol (LPG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylethanolamine (LPE), phosphatidylserine (PS), lysophosphatidylserine (LPS), phosphatidylcholine (PC), phosphatidylcholine (LPC).

concentration in the lower well. Similar results were obtained when lysoPA was used (Fig. 3B). Table III also shows that in the same experimental conditions, fMLP was only minimally able to promote monocyte haptotaxis, in agreement with a previous report (52). On the contrary, MCP-1, which shares 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/mm² (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 chemotactant. 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. 5A 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. 5B). 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...
TABLE III
Haptotactic activity of PA and lysoPA: comparison with other chemotactic agonists

| Agonist    | Chemotactic in the lower well | Fibronectin coating | No. cell migrated | Ratio¹ |
|------------|-------------------------------|---------------------|------------------|-------|
| 18:0:20:4-PA | –                             | –                   | 69 ± 6           |       |
| 16:0-LPA    | –                             | –                   | 70 ± 3           | 1.0   |
| 8:0-DG      | –                             | –                   | 58 ± 3           | 1.0   |
| fMLP        | –                             | –                   | 54 ± 4           | 1.0   |
| rMCP-1      | –                             | –                   | 54 ± 4           | 1.4   |
| 18:0:20:4-PA | –                             | +                   | 28 ± 4           | 0.2   |
| 16:0-LPA    | +                             | +                   | 68 ± 1           | 0.4   |

¹ 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 chemotactants, in the lower well, at their optimal concentration (1 mM for PA, lysoPA, and DG, 10⁻⁹ M fMLP, and 6 × 10⁻⁹ M MCP-1).

² Haptotactic response chemotactic (+ haptotactic) response ratio.

³ 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 × 10⁶/ml in PBMC) were preincubated with 1 mM 1-stearoyl-2-arachidonyl-PA (PA), 1 mM oleyl 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.

Fig. 5. Effect of PTox and CTox pretreatment on monocyte migration. Monocytes (1.5 × 10⁶/ml in PBMC) were incubated at 37°C with different concentrations of the toxins for 90 min. At the end of the incubation, monocytes 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-diocatanylglycerol (DG), and oleyl 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 (10⁶/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 [Ca²⁺] (Fig. 7, A and B). At both concentrations the effect was biphasic with a very rapid increase of [Ca²⁺], followed by a slower phase which peaked about 10 s after stimulation and declined very slowly. The concentration of 0.1 μM, active in inducing monocyte mi-
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**FIG. 7.** Effect of PA, LysoPA, and DG on \( [Ca^{2+}] \). Percoll-purified monocytes \((10^6/ml)\) were incubated with Fura-2-acetoxymethyl ester \((1 \mu M)\) at 37 °C for 15 min, washed, and then exposed in cuvette \((5 \times 10^6/ml)\) to different concentrations of the agonists (1-stearoyl-2-arachidonyl PA (PA), 1,2-dioctanoylglycerol (DG), lyso deoxy-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 \((380 \text{ and } 380 \text{ nm})\) and emission at 487 nm.

**FIG. 8.** Effect of Suramine on PA-, LysoPA-, DG-, and MCP-1-induced monocyte migration. Human monocytes \((1.5 \times 10^6/ml \text{ in PBMC})\) were tested for their ability to migrate across a polycarbonate filter in response to a fixed concentration \((1 \mu M)\) of 1-stearoyl-2-arachidonyl PA (PA), 1,2-dioctanoylglycerol (DG), lyso deoxy-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 \((90 \text{ min})\), 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.

**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 \((67)\), CSa \((68)\), and interleukin-8 \((51, 52)\). In addition, membrane-bound molecules, such as platelet-activating factor \((69)\), MIP-1α, 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 \((47, 48, 73)\),
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 (80%) homologous desensitization and only a partial (38%) cross-desensitization with DG (Fig. 4); (ii) PA-induced monocyte chemotaxis was much more sensitive to the effect of suramine than DG (IC50 = 1.4 μM); (iii) PA and DG induced a 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 (EC50 = 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 [Ca2+]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. 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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REFERENCES

1. Mantovani, A., and Dejana, E. (1989) Immunol. Today 10, 370–375
2. Springer, T. A. (1990) Cell 68, 341–350
3. Mantovani, A., Bussolino, F., and Dejana, E. (1992) FASEB J. 6, 2591–2599
4. Butcher, E. C. (1991) Cell 67, 1033–1036
5. Rot, A. (1992) Immunol. Today 13, 291–294
6. McPhail, L. C., and Harvath, L. (1993) In The Natural Immune System: The Neutrophil (Wheeler, J. G., and Abramson, J., eds) pp. 63–107, Oxford University Press, Oxford.
7. Murthy, P. M. (1994) Annu. Rev. Immunol. 12, 593–633
8. Baggiolini, M., Dewald, B., and Moser, B. (1994) Adv. Immunol. 55, 99–179
9. Sozzani, S., Locati, M., Zhou, D., Rieppi, M., Luini, W., Lanotte, G., Biandri, G., Polentarutti, N., Allavena, P., and Mantovani, A. (1995) J. Leukoc. Biol. 57, 788–794
10. Prescott, S. M., Zimmerman, G. A., and McIntyre, T. M. (1990) J. Biol. Chem. 265, 13781–13784
11. Hoffman, R. D., Kligerman, M., Sundt, T. M., Anderson, N. D., and Shin, H. S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3285–3289
12. Quimby, M. T., Pastharis, S., and Steinberg, D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 2805–2809
13. Wright, T. M., Hoffman, R. D., Nishiijima, J., Jakol, L., Snyderman, R., and Shin, H. S. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 1869–1873
14. Bishop, W. R., and Bell, R. M. (1988) Annu. Rev. Cell Biol. 4, 579–610
15. Exton, J. H. (1990) J. Biol. Chem. 265, 1–4
16. Billah, M. M. (1993) Curr. Opin. Immunol. 5, 114–123
17. Kaul, O., and Hauser, G. (1987) Arch. Biochem. Biophys. 253, 453–461
18. Martin, T. W. (1988) Biochim. Biophys. Acta 962, 282–296
19. Truett, A. P., Bockino, S. B., and Murray, J. J. (1992) FASEB J. 6, 2720–2725
20. Agwu, D. E., McPhail, L. C., Sozzani, S., Bass, D. A., and McCall, C. E. (1991) J. Clin. Invest. 88, 531–539
21. Billah, M. M., Ekedal, S., Mullmann, T. J., Egan, R. W., and Siegel, M. I. (1989) J. Biol. Chem. 264, 17069–17077
22. Agwu, D. E., McPhail, L. C., Chabot, M. C., Daniel, L. W., Wykle, R. L., and McCall, C. E. (1989) J. Biol. Chem. 264, 1405–1413
23. Mullmann, T. J., Siegel, M. I., Egan, R. W., and Billah, M. M. (1990) Nature...
