Lysophosphatidic Acids

III. Enhancement of Neutrophil Chemotaxis

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1-Palmitoyl-lysophosphatidic acid (LPA) was studied for its influence on the chemotaxis and ultrastructure of human neutrophils. By itself, LPA had no effect on the indices of chemotaxis or random migration of neutrophils. However, LPA on either the cellular or attractant side of Boyden chambers significantly enhanced the chemotactic responses of neutrophils to suboptimal concentrations of formyl-methionyl-phenylalanine. The enhancement of chemotaxis was achieved with concentrations of LPA (120–240 μM) that had no effect alone on neutrophil ultrastructure. The results, taken together with recent advances in knowledge of the role of the phosphatidylinositol turnover response in mediating effects of stimulating agents on cells, may provide a novel concept for understanding neutrophil chemotaxis. (Am J Pathol 1980, 100:609–618)

STUDIES OF NEUTROPHILS indicate that responses to chemoattractants and secretagogues are mediated by variations in levels of free intracellular calcium.[1–16] Evidence now suggests that responses to chemoattractants involves a localized flux of calcium at the leading edge of the neutrophil.[14] Secondary to the flux of calcium, an enrichment of microfilaments, particularly actin, occurs at the leading edge.[17–19] Interactions of these microfilaments may then be responsible for the locomotion produced.[19] Therefore, an understanding of how a localized calcium flux may be produced in response to a cell stimulus is important.

Like many other cell types, neutrophils, when stimulated, undergo a turnover of phosphatidylinositol with incorporation of ^{32}P into phosphatidic acid, and incorporation of both ^{32}P and myoinositol into phosphatidylinositol.[20–28] By analogy with other cells, it is likely that this alteration in phospholipid metabolism begins by initial breakdown of phosphatidylinositol and is linked to a flux of calcium that triggers cell activation.[29–33] Recent studies of platelets have implicated lysophosphatidic acid (LPA) as an intermediate produced during phosphatidylinositol turnover that may act as a calcium ionophore.[34–36] In the present study we have investigated

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the effect of LPA on the chemotaxis and ultrastructure of human neutrophils.

Materials and Methods

Blood was obtained by venipuncture from normal donors (with informed consent in accord with the Declaration of Helsinki), drawn into heparin, mixed 10 to 1 with 6% dextran in saline, and allowed to sediment for 45 minutes, yielding leukocyte-rich plasma. Neutrophils were separated from the leukocyte-rich plasma with Ficoll-Hypaque and washed three times in Hanks' balanced salt solution (HBSS) (Grand Island Biological Co., Grand Island, NY) by a method described previously.[37] The neutrophils were suspended to 4.4×10^6/ml in HBSS with 0.5% human albumin (Cutter Laboratory, Berkeley, Calif).

Synthetic 1-palmitoyl-lysophosphatidic acid (Serdary Research Laboratory, London, Ontario) was suspended with 2 minutes' ultrasonication to a concentration of 2.4 mM in HBSS at pH 7.4. The chemoattractant employed was N-formyl-L-methionyl-L-phenylalanine (FMP) (Sigma, St. Louis, Mo) dissolved in HBSS at 10 μg/ml. Standard Boyden-type chemotactic chambers were set up with 5-μ filters (Millipore, Worchester, Mass). The top chambers contained 2×10^6 neutrophils in 0.5 ml HBSS with albumin. The bottom chambers contained HBSS with albumin alone or with various concentrations of LPA and/or FMP (see Results). The chambers were incubated for 3 hours at 37 C; then the filters were removed, stained, and mounted for counting. The cells in 5 random high-power fields (400×) that had migrated to the far side of the filter were counted, totaled, and recorded as the migration index for each chamber. All experiments on a single day were set up in duplicate. The LPA was made up fresh each day. The FMP was made once and frozen in aliquots. The results were analyzed statistically and compared by the paired-sample *t* test.

Cells prepared for electron microscopy were incubated at 37 C for 30 minutes in HBSS with albumin alone or with LPA at 240 μM and/or FMP at 0.35 μg/ml. The initial fixation was with equal volumes of 0.2% glutaraldehyde in HBSS. After 10 minutes, the cells were separated for scanning (SEM) or transmission electron microscopic (TEM) examination. The former were fixed in suspension with 1% glutaraldehyde for 60 minutes and placed on coverslips for dehydration and critical-point drying. The dried coverslips were coated with carbon and gold and examined in a Cambridge S4-10 microscope at 20 kV. The cells for TEM examination were spun to a pellet, and fixation was completed with 1% glutaraldehyde in HBSS for 30 minutes, followed by 30 minutes in 1% osmium tetroxide/1.5% potassium ferrocyanide in distilled water. The cell pellets were dehydrated and embedded in Epon by standard methods. Sections were stained with uranyl acetate and lead citrate and examined in a Philips 301 microscope at 80 kV.

Results

Influence of Lysophosphatidic Acid on Chemotaxis

The results of these experiments, shown in Table 1, demonstrate that LPA by itself did not alter neutrophil migration. However, it did enhance the response to suboptimal levels of FMP. Previous experiments established that the optimal concentration of FMP as a chemoattractant for neutrophils was 1 μg/ml for the system employed here. This was confirmed in this series of experiments with a mean chemotactic index of 260 ± 19. When LPA was added to this level of FMP, there was no significant increase in chemotactic response. At a suboptimal level of FMP, 0.35 μg/ml, the response to the FMP alone gave a mean index of 177 ± 24. When

Table 1—Effect of Lysophosphatidic Acid (LPA) on the Chemotactic Response of Normal Human Neutrophils

Agents added to the bottom chambers:					
None	LPA	FMP*	FMP	FMP	FMP
	240 μM	0.35 μg/ml	0.35 μg/ml + LPA 120 μM	0.35 μg/ml + LPA 240 μM	1 μg/ml
9.1 ± 5.6** (8)	5.2 ± 2.3 (6)	177 ± 24 (8)	230 ± 17 (4)	239 ± 32 (10)	260 ± 19 (4)
NS		P < 0.05	236 ± 28 (14)		NS

* FMP = formyl-methionyl-phenylalanine.
† Migration indices as cells per 5 HPF; mean ± SD (N).
NS = no significant difference.

LPA at 120 or 240 μM was combined with the suboptimal concentration of FMP, neutrophil chemotaxis was enhanced to levels comparable to those with the optimal concentration of FMP. The mean for this group of experiments (236 ± 28) was significantly different ($P < 0.05$) from the response to the suboptimal level of FMP alone. When compared to matched controls, the effect of these levels of LPA was always an increase in chemotactic index, which ranged from 22% to 55% (mean 37 ± 13%). Placing the LPA in the top chamber with the cells only, or in both the top and the bottom chambers, did not alter the results. A lower level of LPA, 50 μM, did not alter the chemotactic response.

Morphologic Influence of Lysophosphatidic Acid and Formyl-Methionyl-Phenylalanine on Neutrophils

Purified neutrophils in the absence of LPA or FMP were spherical, with uniformly distributed, short surface processes. The addition of LPA alone at 240 μM did not alter this appearance as seen by either SEM or TEM examination (Figures 1 and 3). Exposure to FMP brought about distinct conformational changes in every cell. Large projections were seen extending from the body of the cell, and the small surface processes were largely lost. This appearance of the neutrophils was not further altered by the addition of LPA (Figures 2 and 4). No evidence of degranulation or other internal changes were seen following FMP or LPA or the two combined.

Discussion

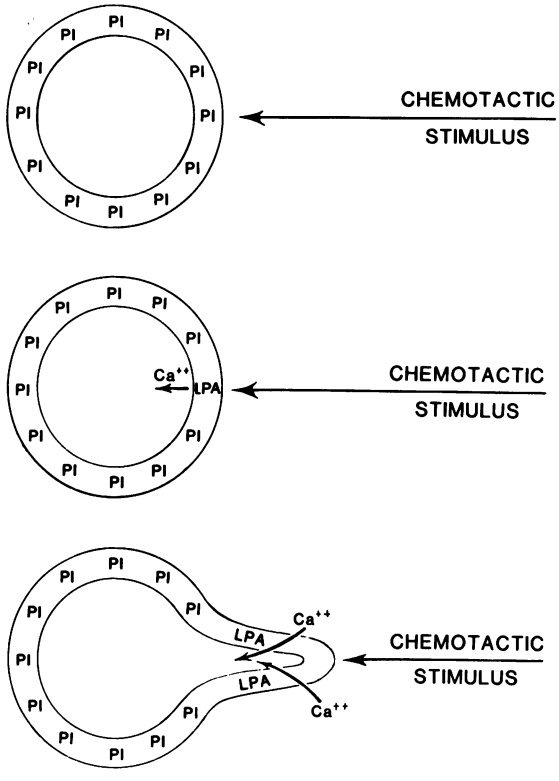
The results of the present study demonstrate that LPA can significantly enhance chemotaxis of human neutrophils. Previous studies using LPA

suggested that it might act on cells in a fashion similar to a calcium ionophore.[35,36] In neutrophils, the effect of LPA can be compared to the effect of the calcium ionophore A23187, which also enhances neutrophil chemotaxis in a similar fashion.[6] LPA may thus exert its action on neutrophils as an ionophorelike agent.

Studies in platelets have suggested that LPA is different from A23187 in that its ionophore action may be regulated by the presence of phosphatidylinositol, diphosphatidylinositol and triphosphatidylinositol, which are inhibitors of the calcium ionophore action of LPA.[36] Dog and rabbit platelets were not responsive to LPA unless they had been pretreated with adenosine diphosphate, an agent known to initiate the breakdown of phosphatidylinositol in platelets.[36] Similarly, LPA did not influence neutrophils unless it was added together with FMP. The FMP may act in part by initiating the breakdown of phosphatidylinositol, which would open a "gate" through which the LPA could be effective.

As a result of our studies with LPA in neutrophils, we would like to suggest the following model for neutrophil chemotaxis (Text-figure 1). A chemotactic agent arrives on the neutrophil surface, as shown by the arrow. The cell responds to the stimulus with a localized breakdown of phosphatidylinositol with production of phosphatidic acid and lysophosphatidic acid. The lysophosphatidic acid, and possibly also phosphatidic acid,[35,36,38,39] could act as a calcium ionophore, but only at the point on the cell where a local degradation of phosphatidylinositol has occurred, since the phosphatidylinositol elsewhere on the cell surface would inhibit the ionophore action of LPA and phosphatidic acid. The resulting localized calcium flux would, therefore, produce the localized buildup of submembranous cations at the leading edge of the neutrophil, as described by Cramer and Gallin.[14] The calcium could then mediate assembly and interaction of microfilaments leading to localized production of cell pseudopods and a directed cell movement toward the chemoattractant.[15,17-19] The local regulation achievable by the turnover of phosphatidylinositol as postulated here makes it a very attractive mechanism for specific cell responses involving only a part of the cell membrane.

It is of interest to consider the studies of Hoffstein,[16] showing that particulate stimuli cause a release of calcium from a storage site on the plasma membrane. Calcium release is local in nature and occurs only in the region of the cell membrane where the cell contacts the particles. Since phosphatidylinositol can bind calcium,[30] the calcium seen bound to the plasma membrane in the resting cell in this study could be calcium bound to phosphatidylinositol. Breakdown of phosphatidylinositol might release calcium, making it available for the LPA to transport, as well as



TEXT-FIGURE 1—A hypothesis for the mechanism of neutrophil chemotaxis. In this concept the resting neutrophil is drawn as a round cell (*top*), in which phosphatidylinositol (*PI*) exists in the membrane throughout the cell. With a chemotactic stimulus arriving from the direction shown by the *arrow*, a localized breakdown of PI occurs, producing lysophosphatidic acid (*LPA*) and releasing calcium bound to the membrane to the cell interior. With a continuing stimulus (*bottom*), continuing localized breakdown of PI occurs. The LPA now moves not only membrane calcium to a localized area inside the cell membrane but transports calcium from outside the cell as well. The localized flux of calcium could result in organization and interaction of microfilaments inside the cell membrane, leading to the extrusion of a pseudopod and directed cell movement.

releasing the inhibitory action of phosphatidylinositol on the ionophore action of the LPA. Thus LPA could transport not only calcium from the membrane, releasing it in the cytosol, but in addition, could convey calcium from outside the cell, inside. This would be consistent with results showing that membrane calcium is used for cell function, but that for optimum responses extracellular calcium is needed.[1,3,5,8,11,15]

It might be argued that the effective concentration of LPA (120–240 μM) is too high to reveal an important physiologic effect. However, the LPA was used in suspension, and the system was unstirred, so that the actual amount of LPA delivered to the neutrophil was, no doubt, quite low. Rapid metabolism of LPA reaching the cell may further lower the amount available to act as a calcium ionophore. The effect of LPA was not due to cell damage, as demonstrated by the ultrastructural studies. Thus, the present study has provided evidence that suggests a novel understanding of the mechanism of neutrophil chemotaxis.

In platelets, cell activation can occur either through a mechanism dependent on conversion of arachidonic acid to prostaglandins and thromboxanes or through a mechanism independent of such conversion (the postulated lysophosphatidic acid mechanism).[35,40] Some evidence exists that neutrophils can also be activated, either through mechanisms dependent on metabolites of arachidonic acid or independent of such compounds.[41,42] Thus, it is possible in both these cell types that a product of arachidonic acid metabolism may be a calcium ionophore in some circumstances,[40] while in other circumstances lysophosphatidic acid or phosphatidic acid fulfills this role.

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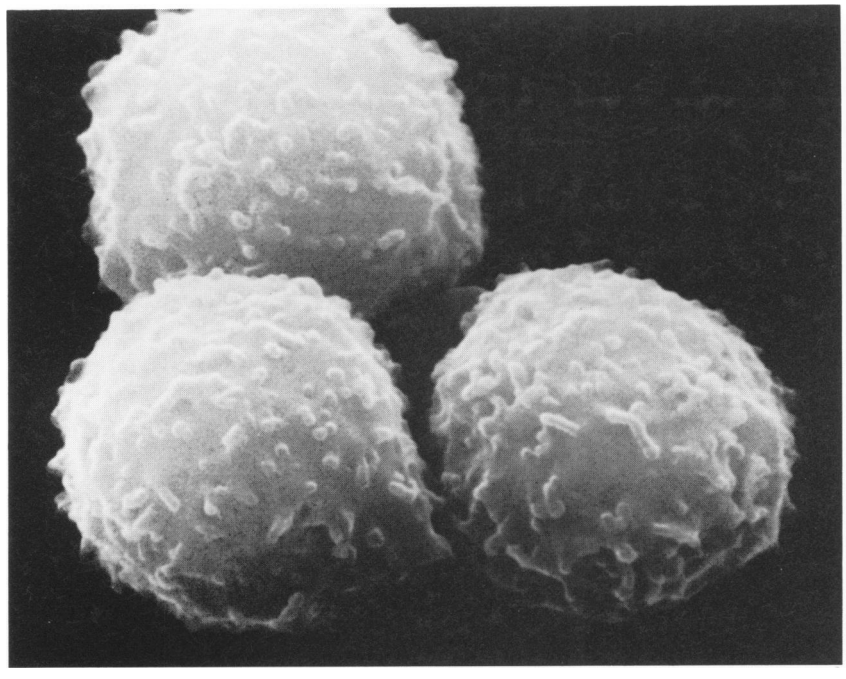

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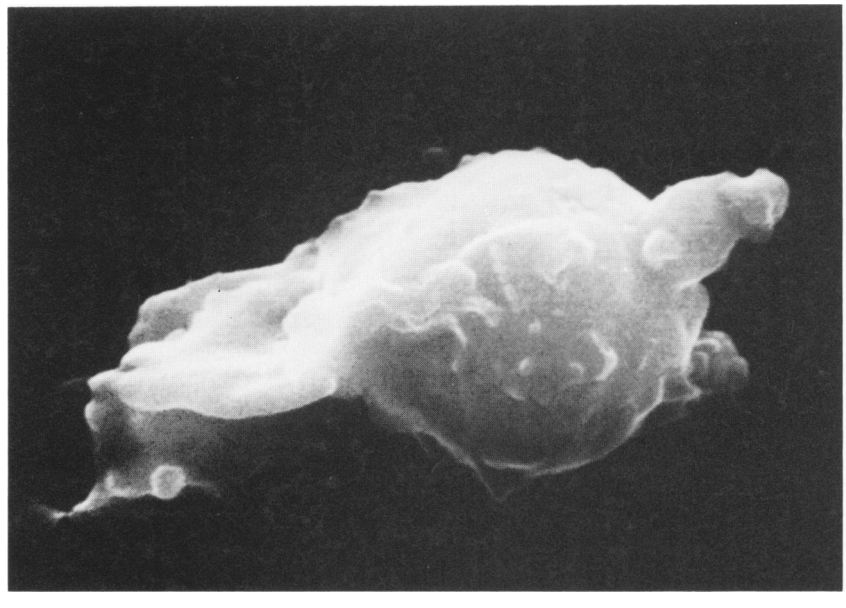

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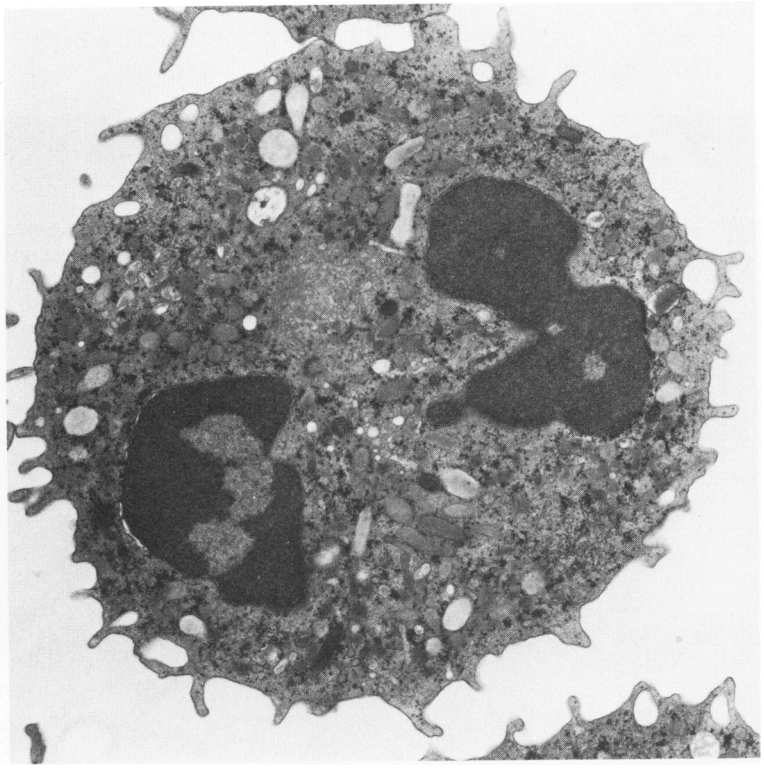
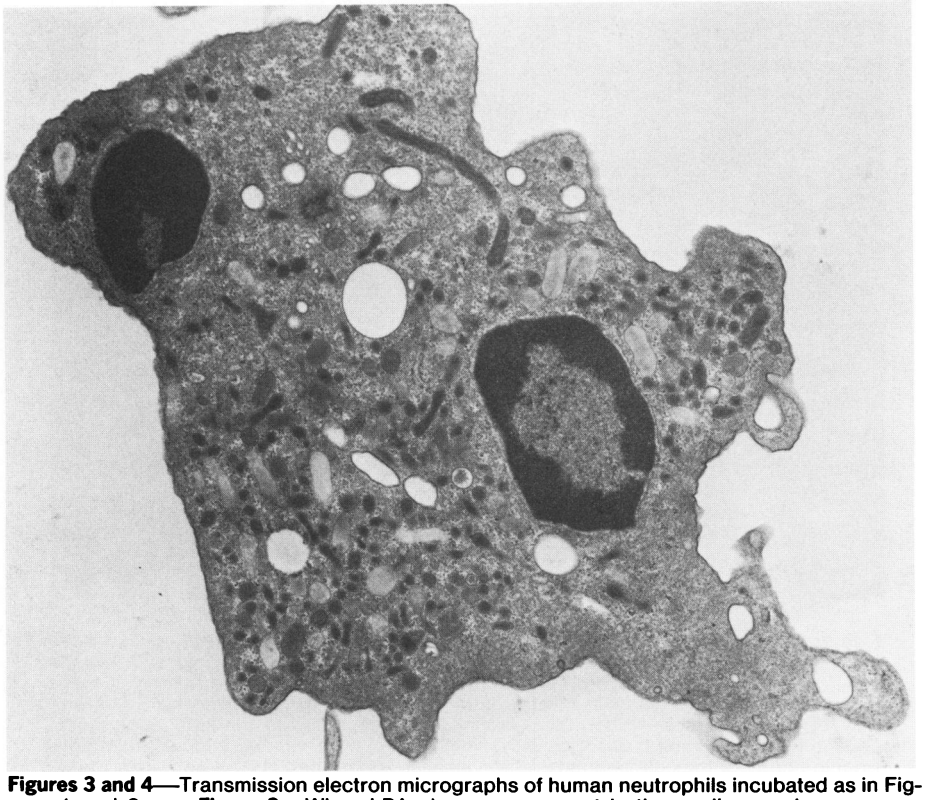
Figures 1 and 2—Scanning electron micrographs of normal human neutrophils following a 30-minute incubation at 37 C in HBSS with 0.5% human albumin and 240 μM LPA. **Figure 1**—The overall spherical shape and surface conformation of these cells are identical to the neutrophils incubated in albumin–HBSS alone (×7700) **Figure 2**—When 0.35 μg FMP/ml was added to the incubation media used in Figure 1, marked changes were seen in all cells. There were large projections from the body of the cell, and most of the smaller surface processes were lost. Identical changes were noted in cells incubated with FMP in the absence of LPA. (×7700)





Figures 3 and 4—Transmission electron micrographs of human neutrophils incubated as in Figures 1 and 2. **Figure 3**—When LPA alone was present in the media, no changes were noted, and the appearance of the neutrophil was identical to cells incubated in media alone. (×11,000) **Figure 4**—The presence of FMP caused the conformation changes described above without evidence of degranulation or other visible internal alteration. Cells exposed to FMP without LPA were morphologically identical to the one in Figure 4. (Uranyl acetate and lead citrate, ×12,000)