Alterations of New Methylated Phospholipid Synthesis in the Plasma Membranes of Macrophages Exposed to Chemoattractants

MARILYN C. PIKE and RALPH SNYDERMAN
Laboratory of Immune Effector Function of the Howard Hughes Medical Institute, Division of Rheumatic and Genetic Diseases, Departments of Medicine and Microbiology and Immunology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT Chemotactic factors have been shown to inhibit the methylation of phosphatidylethanolamine in macrophages without affecting total phospholipid synthesis. It would thus be anticipated that newly synthesized membranes of macrophages exposed to chemoattractants would have an increased ratio of phosphatidylethanolamine to its methylated derivatives. These ratios were measured directly in newly synthesized phospholipids of plasma membranes isolated from guinea pig peritoneal macrophages. The phosphatidylethanolamine:methylated phospholipid ratio in such plasma membranes was increased by 53 to 111% upon exposure of the cells to chemotactic factors. This increase was due to decreased synthesis of methylated phospholipids and not to altered formation of phosphatidylethanolamine or activation of phospholipases. Methylated phospholipid ratios were also studied in the leading front lamellipodia isolated from macrophages migrating under chemotactic and nonchemotactic conditions. The phosphatidylethanolamine:methylated phospholipid ratios were increased up to fourfold in lamellipodia of macrophages migrating towards chemotactic agents when compared to those from cells migrating randomly. Biophysical changes in the plasma membrane produced by an increase in the ratio of phosphatidylethanolamine:methylated phospholipids as a result of exposure of cells to chemoattractants may be required for sustained directed migration.

The chemotaxis of mononuclear phagocytes requires methylation reactions mediated by S-adenosyl-L-methionine (17, 24). Interestingly, chemotactic factors, when incubated with macrophages, produce a marked depression of one type of transmethylation reaction, the formation of methylated derivatives of phosphatidylethanolamine (PE) (18). Chemotactic factors do not, however, alter total phospholipid synthesis in these cells. On the basis of these observations, we have hypothesized that, when chemotactic factors bind to their cell surface receptors, phospholipid methylation is inhibited locally in the area of receptor occupancy (24). Because total phospholipid synthesis is unchanged in macrophages exposed to chemoattractants, one would expect to increase the ratio of PE to methylated phospholipids in the newly synthesized membrane lipids of such cells. Changes in the ratio of PE to methylated phospholipids could alter the biophysical characteristics in focal areas of the membranes of chemotactically responsive cells. Such changes might well be asymmetric and most pronounced at the leading front membrane where the greatest degree of chemotactic factor receptor occupancy would be expected to occur. To test this hypothesis, we have examined the effects of chemoattractants on the ratio of newly synthesized PE to total methylated phospholipids in plasma membranes isolated from guinea pig macrophages. In addition, the effects of exposure to a chemotactic gradient on these phospholipid ratios in the leading front portions (lamellipodia) of migrating macrophages were studied.

MATERIALS AND METHODS

**Chemicals**

N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) and fNle-Leu-Phe were obtained from Peninsula Laboratories (San Carlos, Calif.). PE, phosphatidyl-N'-dimethylethanolamine, lysophosphatidylcholine, and phosphatidylcholine (PC) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Phosphatidyl-N'-monomethylethanolamine was purchased from Gibco Laboratories (Grand Island Biological Co., Grand Island, N. Y.). L-[methyl-3H]methionine (10 Ci/mmole) and [1,2-14C]ethanolamine (95 mCi/mmole) were obtained from ICN (Irvine, Calif.). The chemotactic stimulants dialyzed activated guinea pig serum
incorporated into methylated phospholipids. Ratios are obtained by dividing ["C]cpm incorporated into PE by [3 H]cpm. Compound are found in extracts of macrophages treated with chemoattractants. Where indicated, results are greater than 90% of the "C label was incorporated into PE, with <10% content. Tritium and "C were counted at efficiencies of 31 and 79%, respectively. Nuclear, Boston, Mass.) or, for determination of species activity, scraped into direct into scintillation vials containing 10 ml of Aquasol (New England Nuclear). Plates were developed in chloroform: methanol and applied to a silica gel G plate (Analtech Inc., Newark, Del.) along with 251Ag of the phospholipid standards, PE, PC, phosphatidyl-N'-monomethylethanolamine, phosphatidyl-N'-N'-dimethylethanolamine and phosphatidyl-N'-monomethylethanolamine. Plates were developed in chloroform:propionic acid:n-propanol:water (2:2:3:1) (8), and phospholipid spots were visualized with iodine and marked. After vaporization of the iodine, the spots were either scraped directly into scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, Mass.) or, for determination of specific activity, scraped into test tubes and the phospholipids were eluted with 1 ml of chloroform:methanol. Portions of the eluate were assayed for radioactivity and inorganic phosphorus content. Tritium and "C were counted at efficiencies of 31 and 79%, respectively. Greater than 90% of the "C label was incorporated into PE, with <10% incorporated into the methylated derivatives of PE. Where indicated, results are expressed as the ratio of newly synthesized PE to total methylated phospholipids (17) or into PC alone (18). Portion of the "C label was incorporated into PE, with <10% incorporated into the methylated derivatives of PE. Where indicated, results are expressed as the ratio of newly synthesized PE to total methylated phospholipids (17) or into PC alone (18). The filters containing labeled lamellipodia membranes were removed and extracted in chloroform:methanol (2:1 vol/vol) for analysis of labeled phospholipids. Total methylated phospholipids (L7) or into PC alone (18). The actual cpm used to calculate the data were as follows: Buffer, 853, 1450, 609; fMet-Leu-Phe, 769, 629, 244; fNle-Leu-Phe, 867, 777, 304; and C5a, 958, 1068, 531 for PE, total methylated phospholipids and PC, respectively. Numbers in parentheses indicate the percentage of increase calculated as indicated in the legend to Fig. 2. Similar results were obtained in three other experiments.

**Preparation of Filters for Electron Microscopy**

Polycarbonate filters containing macrophage lamellipodia were fixed for 2 d at 37°C in 2% glutaraldehyde contained in 0.05 M sodium cacodylate buffer (pH 7.4). After staining for 1 h in 1% osmium tetroxide, the filters were washed in sucrose buffer and dehydrated in ethanol. The filters were critical point dried, coated with carbon followed by platinum, and examined in a Philips 301 scanning electron microscope.

**RESULTS**

**Effects of Chemotactic Factors on the Ratio of Newly Synthesized PE to Methylated Phospholipids in Macrophage Plasma Membranes**

Guinea pig macrophages were incubated for 1 h at 37°C with [methyl-3H]methionine and [14C]ethanolamine in buffer alone or in buffer containing the chemotactic factors fMet-Leu-Phe, fNle-Leu-Phe, or C5a. The doses used were maximally active for producing a chemotactic response in vitro. After incubation, the plasma membranes were isolated and the amounts of newly synthesized methylated phospholipids and PE were determined. Treatment of the macrophages with the three chemotactic agents increased the ratio of newly synth-

**Assay of Inorganic Phosphate**

Portions of chloroform:methanol extracts of macrophage membranes or of individual phospholipids isolated by thin-layer chromatography (TLC) were washed using 10% Mg(NO3)2 in absolute ethanol, and the resulting pyrophosphate was hydrolyzed in the presence of 0.5 N HCl. The resulting inorganic phosphate was assayed colorimetrically (1).
sized PE:total methylated phospholipids in the plasma membrane by 53 to 111% (Fig. 1). The PE:PC ratio was similarly increased in the plasma membranes by 59 to 132%. FMet-Leu-Phe increased the ratio of PE:methylated phospholipids in a dose-response fashion that paralleled the ability of this peptide to produce a chemotactic response (Fig. 2). The effective dose-response fashion that paralleled the ability of this peptide to produce a chemotactic response (12.5 cells per oil immersion field) induced by fMet-Leu-Phe and S in the response induced at a submaximal concentration.

The increase in the ratio of PE:methylated phospholipids produced by chemotactic factors was most pronounced in the plasma membrane-rich fraction of the cells, as compared to lysosomal membrane fractions (obtained from 36/40% sucrose interface) or pelleted material containing organelles and non-disrupted cells. For example, the increase in the PE:PC ratio produced by 10^{-8} M fMet-Met-Met-Met was 79% in the plasma membrane fraction, 50% in the pelleted cellular material, and only 28% in lysosomal membranes.

We next determined whether the increase in the newly synthesized PE:methylated phospholipid ratio produced by chemotactic factors in macrophage plasma membranes was due to increased synthesis of PE or decreased synthesis of methylated phospholipids. Macrophages were incubated in the presence or absence of 10^{-8} M fNle-Leu-Phe, the plasma membranes were isolated, and labeled phospholipids were analyzed based on total lipid phosphate content. Table I indicates that there was no significant difference in the amount of [^{14}C]-ethanolamine incorporated into PE in the presence or absence of attractant. [^{14}C]Ethanolamine incorporation was 249 ± 21 pmol/μmol lipid P; when cells were incubated with buffer alone and 219 ± 13 pmol/μmol lipid P; in the presence of fNle-Leu-Phe. In contrast, the amount of [^{3}H]methyl groups incorporated into all three methylated derivatives of PE isolated from the plasma membrane was depressed by >50% in the presence of fNle-Leu-Phe (Table I). Thus, chemotactic factors do not alter PE:methylated phospholipid ratios by affecting PE synthesis.

The increase in the ratio of PE:methylated phospholipids produced by chemotactic factors in the plasma membrane appears to be the result of decreased synthesis of methylated phospholipids. It would follow then that the ratio of the radioactivity incorporated into new methylated phospholipids to the sum total of methylated phospholipids (specific activity) is depressed in membranes of cells treated with chemotactic factors. We therefore determined the specific activity of PE and the methylated phospholipids in plasma membranes of cells that had been incubated in the presence and absence of chemotactic factors. The specific activity of methylated phospholipids in the presence of buffer alone was 283 cpm/mmol, which was decreased to 191 cpm/mmol in the presence of 10^{-8} M fMet-Leu-Phe. There was no significant difference in the specific activity of PE measured in the presence and absence of 10^{-8} M fMet-Leu-Phe.

**Effect of fMet-Leu-Phe on Preformed Derivatives of PE in Macrophage Plasma Membranes**

The observed change in PE:methylated phospholipid ratios produced by chemotactic factors could be caused by either decreased synthesis of new methylated phospholipids or enhanced degradation of preformed methylated phospholipids.

![Figure 2](image.png)

**FIGURE 2** Chemotaxis and change in the PE:methylated phospholipid ratio induced by various concentrations of fMet-Leu-Phe. Chemotaxis of guinea pig macrophages suspended in incubation buffer was performed in modified Boyden chambers using 5.0 μM polycarbonate filters and a 2-h incubation at 37°C. The percentage of maximal response is equal to [(E/C) - 1] x 100 where E is the maximal chemotactic response (12.5 cells per oil immersion field) induced by fMet-Leu-Phe and S in the response induced at a submaximal concentration. Phospholipids were analyzed in plasma membranes from guinea pig macrophages that had been incubated with various concentrations of fMet-Leu-Phe under conditions indicated in the legend to Fig. 1. The percentage of increase in the PE:methylated phospholipid ratio is equal to [(E/C) - 1] x 100 where E is the ratio obtained in the presence of fMet-Leu-Phe and C is the ratio obtained when cells were incubated with buffer alone. Similar results were noted in two additional experiments.

**Table I**

| Membranes isolated from cells incubated with: | [%^{14}C]ethanolamine incorporated into PtdEtn | [%^{3}H]methyl incorporated into methylated derivatives of PtdEtn |
| --- | --- | --- |
| fNle-Leu-Phe | pmol/μmol lipid P; | pmol/μmol lipid P; |
| Buffer | 249 ± 21 | 0.81 ± 0.18 |
| 10^{-8} M fNle-Leu-Phe | 219 ± 13 | 0.38 ± 0.02 |

Macrophages were incubated for 1 h at 37°C in the presence or absence of fNle-Leu-Phe in buffer containing 30 μCi/ml of [%^{14}C]methionine and 1.5 μCi/ml of [%^{3}H]ethanolamine. The plasma membranes were then isolated. Labeled membranes were extracted in chloroform:methanol and the labeled phospholipids were analyzed by thin-layer chromatography. Portions of the extracts were assayed for inorganic phosphorus.

* PME: phosphatidyl-N'-monomethylethanolamine
* PDE: phosphatidyl-N'-N'-dimethylethanolamine
* PtdCho: phosphatidylcholine
* LPC: lyso-phosphatidylcholine

All ± S.D.
To distinguish between these possibilities, macrophages were prelabeled with [methyl-3 H]methionine and [14 C]ethanolamine, washed extensively, and resuspended in buffer containing 1.0 mM unlabeled methionine and ethanolamine to prevent continued incorporation of radiolabel. The cells were then treated with 10^(-4) M fMet-Leu-Phe for 0, 15, or 60 min at 37°C, after which time the plasma membranes were isolated and the residual radioactivity associated with the phospholipids was analyzed. The treatment of cells with fMet-Leu-Phe did not alter the ratios of preformed PE:total methylated phospholipids or PE:PC, indicating that the chemotactic factor did not produce degradation of preformed methylated phospholipids through phospholipase activation.

Effects of Chemotactic Factors on the Ratio of Newly Synthesized PE:Methylated Phospholipids in the Lamellipodia of Macrophages

To determine whether similar changes in the ratio of newly synthesized PE:methylated phospholipids were produced in the lamellipodia of macrophages responding to gradients of chemoattractants, cells were placed in modified Boyden chambers containing 1-μm diameter pore size polycarbonate filters. This pore size is too small to allow for migration of total cells through the filter. Membrane processes of the macrophages do, however, protrude through the pores to the underside of the filters, as illustrated in the scanning electron micrograph shown in Fig. 3. These membrane processes constitute the leading front or lamellipodia of the migrating cells. These findings are similar to those reported by Wetzel et al. (27) showing that mononuclear leukocyte lamellipodia comprise the initial portion of the cells that migrate to the underside of polycarbonate filters (Nuclepore Corp.). In addition, Malech et al. (12) have used the technique of allowing lamellipodia to penetrate into undersized pores of millipore filters to study cytoskeletal changes that occur in this area of the cells when they are exposed to chemoattractants.

Macrophages were labeled in the chemotaxis chambers with [methyl-3 H]methionine and [14 C]ethanolamine. Buffer or various chemotactic factors were placed in the lower compartment of the chamber, and, after a 2-h incubation at 37°C, the chambers were emptied and the cells sheared from the top of the filters, leaving behind the lamellipodia that had penetrated to the underside of the filter. Chloroform:methanol extracts of these filters were analyzed for labeled phospholipids. The ratios of PE:total methylated phospholipids and PE:PC were increased two- to fourfold in lamellipodia derived from macrophages that had responded to dialyzed zymosan AS, C5a, or fMet-Met-Met when compared to those from cells responding to buffer alone (Fig. 4). The increase in these ratios produced by C5a were dose-dependent (Fig. 5) and occurred over the same concentration range that produced a chemotactic response in guinea pig macrophages (18).

DISCUSSION

The sequence of biochemical events initiated by chemoattractants in leukocytes that results in biological responses is poorly
doxical observations concerning methylation and chemotaxis using S-adenosyl-L-methionine as a methyl donor have biologically relevant doses of various chemotactic factors. This plasma membrane of guinea pig macrophages treated with of newly synthesized PE to methylated phospholipids in the induced by chemotactic factors. Asymmetrical changes in membrane phospholipid composition methylation reactions would depress chemotaxis by preventing methylated derivatives. If this were the case, inhibitors of would be a local accumulation of PE as compared to its portion of the membrane facing the chemotactic gradient, there is. One would expect that, under these conditions and on that under these circumstances as a consequence of inhibition of the membrane would be expected to change asymmetrically altered in a nonuniform manner. The biophysical properties of phospholipid methylation in leukocytes might be a generalized-is that local or asymmetric inhibition of phospholipid methylation in intact guinea pig macrophages (18). One explanation for these seemingly paradoxical observations concerning methylation and chemotaxis is that local or asymmetric inhibition of phospholipid methylation in macrophages is required for directed migration. Phospholipid methylation in leukocytes might be a generalized-membrane property while the cell is immobile or migrating randomly. However, when exposed to a gradient of chemotactic agents, the methylation of membrane phospholipids may be altered in a nonuniform manner. The biophysical properties of the membrane would be expected to change asymmetrically under these circumstances as a consequence of inhibition of PC formation without alterations in total phospholipid synthesis. One would expect that, under these conditions and on that portion of the membrane facing the chemotactic gradient, there would be a local accumulation of PE as compared to its methylated derivatives. If this were the case, inhibitors of methylation reactions would depress chemotaxis by preventing asymmetrical changes in membrane phospholipid composition induced by chemotactic factors.

The present study shows that there is an increase in the ratio of newly synthesized PE to methylated phospholipids in the plasma membrane of guinea pig macrophages treated with biologically relevant doses of various chemotactic factors. This increased ratio resulted from inhibition of the methylation of PE in the membranes and not from alterations in the incorporation of [14C]ethanolamine into PE. A decrease in the specific activity of methylated phospholipids but not of PE reflected the inhibition of phospholipid methylation produced by the chemotactic factors. Because the specific activity of PE is unaltered in the presence of chemotactants, it seems unlikely that chemotactants affect the decarboxylation of phosphatidylserine to form PE. In addition, it does not appear that the changes observed in methylated phospholipids in the presence of chemotactants are due to alterations in the CDP-choline pathway, because we do not detect changes in the incorporation of [3H]choline or [32P] (18) into PC in macrophages. The present study also examined the PE:methylated phospholipid ratios in lamellipodia of macrophages undergoing either random migration or chemotaxis in response to a variety of agents. These ratios are increased by up to fourfold in lamellipodia exposed to gradients of various chemotactants as compared to lamellipodia isolated from cells migrating randomly.

The increase in the newly synthesized PE:methylated phospholipid ratio appears to be due to inhibition of phospholipid methylation rather than to altered degradation of preformed methylated phospholipids by phospholipase A2. This latter mechanism has been proposed to account for the depressed levels of methylated phospholipids induced in rabbit peritoneal polymorphonuclear (PMN) leukocytes by a chemotactic peptide (9). These discrepancies with our data may reflect differences in the cell types used. It is noteworthy that the chemotaxis of PMN is less dependent upon transmethylation reactions when compared to that of macrophages (24, 28). In addition, the methylation requirements of the functional responses of leukocytes, such as phagocytosis, capping of receptors, as well as chemotaxis, also vary depending upon the species studies (4, 11, 17, 24, 25).

Activation of phospholipase C by chemotactants with subsequent degradation of phosphatidylinositol has been noted in rabbit PMNs (30) and we have observed this effect in human

**Figure 4** Effects of exposure to gradients of various chemotactic agents on the PE:methylated phospholipid ratios in leading front macrophage membranes. Macrophages were labeled in the chemotaxis chambers with [methyl-3H]methionine and [14C]ethanolamine while responding to buffer alone or the indicated chemotactant. Duplicate samples of fifteen filters each were analyzed for labeled phospholipids as indicated in Materials and Methods, and the PE:total methylated phospholipid (C) or PE:PC (C) ratios were calculated as described in the legend to Fig. 1. Similar results were obtained in at least three other experiments.

**Figure 5** Effects of exposure to gradients of various concentrations of C5a on the PE:methylated phospholipid ratios in leading front macrophage membranes. Duplicate samples of 15 filters each were analyzed for labeled phospholipids as indicated in Materials and Methods, and the PE:total methylated phospholipids (C) and PE:PC (C) ratios (± S.D.) were calculated as described in the legend to Fig. 1. Similar results were obtained in one other identical experiment.
monocytes (unpublished observations). It has not yet been determined whether activation of this pathway has any effect on phospholipid methylation.

The functional importance of the increase in newly synthesized PE:methylated phospholipid ratios produced by chemotactic factors in the plasma membranes of macrophages exposed to chemotactic gradients can only be surmised at this time. The local accumulation of PE, a molecule whose head group is both smaller than that of PC and capable of forming more hydrogen bonds, should result in local alterations in the physical state of the membrane. Although the bulk properties of the membrane may not be altered in the presence of chemotactic agents, minute, local changes in the PE:PC ratio in focal areas of newly synthesized membrane exposed to chemotactic agents could be required for altering receptor mobility, for anchoring membranous structures to cellular cytoskeletal elements, or for changing the permeability of the membrane to various ions. It can be expected that further study of phospholipid methylation in chemotactically responsive cells will lead to a greater understanding of the biochemical control of cellular motility.

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