Phospholipid Metabolism, Calcium Flux, and the Receptor-mediated Induction of Chemotaxis in Rabbit Neutrophils

DONNA LYNN BAREIS, FUSAO HIRATA, ELLIOTT SCHIFFMANN, and JULIUS AXELROD
Laboratory of Clinical Science, National Institute of Mental Health; and the Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, Bethesda, Maryland 20205. Dr. Bareis's present address is the Basic Pharmacology Branch, U. S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 21010.

ABSTRACT Rabbit neutrophils were stimulated with the chemotactic peptide fMet-Leu-Phe in the presence of the methyltransferase inhibitors homocysteine (HCYS) and 3-deazaadenosine (3-DZA). HCYS and 3-DZA inhibited chemotaxis, phospholipid methylation, and protein carboxymethylation in a dose-dependent manner. The chemotactic peptide-stimulated release of [^{14}C]arachidonic acid previously incorporated into phospholipid was also partially blocked by the methyltransferase inhibitors. Stimulation by fMet-Leu-Phe or the calcium ionophore A23187 caused release of arachidonic acid but not of previously incorporated [^{14}C]-labeled linoleic, oleic, or stearic acids. Unlike the arachidonic acid release caused by fMet-Leu-Phe, release stimulated by the ionophore could not be inhibited by HCYS and 3-DZA, suggesting that the release was caused by a different mechanism or by stimulating a step after methylation in the pathway from receptor activation to arachidonic acid release. Extracellular calcium was required for arachidonic acid release, and methyltransferase inhibitors were found to partially inhibit chemotactic peptide-stimulated calcium influx. These results suggest that methylation pathways may be associated with the chemotactic peptide receptor stimulation of calcium influx and activation of a phospholipase A_2 specific for cleaving arachidonic acid from phospholipids.

Leukocytes respond with a directed migratory movement, chemotaxis, to a number of peptides derived from complement (1), crude bacterial factors (2), and well-defined synthetic formylated peptides (3, 4). The chemotactic response to the formylated peptides involves interaction with specific receptors on the cell surface (5). However, the biochemical events translating receptor activation into directed cell movement remain poorly understood. It has been shown in this and other laboratories that some early events in chemotactic peptide-stimulated cell movement are: activation of protein carboxy-o-methylase (6), degradation of phosphatidylcholine synthesized by transmethylation of phosphatidylethanolamine (7), release of arachidonic acid (7, 8) which can be metabolized to hydroxyeicosatetraenoic acids that are also chemoattractants (9, 10) and influx and redistribution of calcium ions (11, 12, 13). Inhibition of any one of these processes inhibits receptor-activated neutrophil chemotaxis (7, 14, 15, 16, 17, 18). However, little is known about the interrelationships between peptide receptor, methyltransferase reactions, calcium ion fluxes, and phospholipase A_2 activation leading to induced neutrophil migration. Studies on other receptor-mediated stimulations of T lymphocytes, mast cells, and rat basophilic leukemia cells have suggested that phospholipid methylation is involved in calcium ion movement, activation of phospholipase A_2, and the specific cellular response (19, 20, 21). We report here that methyltransferase inhibitors partially block receptor-mediated calcium influx, phospholipase A_2 activation, and chemotaxis in rabbit neutrophils. Furthermore the chemotactic peptide fMet-Leu-Phe and the calcium ionophore A23187 specifically stimulate the release of arachidonic acid and no other fatty acid examined.

MATERIALS AND METHODS

Cell Preparations

Rabbit leukocytes were obtained by lavage of the peritoneal cavity of rabbits injected 8 to 12 h earlier with 150 ml of 0.1% glycogen (4). After collection by centrifugation at 600 *g* for 10 min, the cells were suspended in Gey's balanced salt solution containing 1% essentially fatty acid-free bovine serum albumin (BSA) (to maintain chemotactic responsiveness) and 0.015 M HEPES buffer at pH 7.4 (Gey's buffer). BSA in the media was essential to maintain chemotactic

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responsiveness and for reproducible results. A typical cell preparation contained 90% neutrophils and 10% lymphocytes and macrophages. Chemotaxis was measured in modified Boyden chambers as described (1) using 1 nM fMet-Leu-Phe as the chemoattractant and incubating the chambers for 2 h at 37°C.

Assays of Phospholipid Methylation and Protein Carboxymethylation

Intact leukocytes at a concentration of 15×10^6 cells/ml in Gey's buffer were incubated with 10 μM L-[methyl-^{3}H]methionine in small plastic tubes for 1 h at 37°C. Each tube contained 0.2 ml of cell suspension with or without additions. The reaction was stopped by the addition of 0.5 ml of 10% wt/vol trichloroacetic acid (TCA) containing 1 mM unlabeled methionine. After centrifugation at 27,000 *g* for 10 min, the pellets were extracted with 3 ml of chloroform/methanol, 2/1 (vol/vol) for determination of phospholipid methylation (19, 22). For protein carboxymethylation, the TCA-precipitated protein carboxymethylesters were hydrolyzed for 30 min with 1 ml of 1.0 M borate buffer at pH 11 containing unlabeled methanol 1% vol/vol as carrier, and the hydrolytic product, [^{3}H]-methanol, was extracted with 3 ml of a 3/2 mixture (vol/vol) of toluene and isoamyl alcohol as previously described (6, 23).

Fatty Acid Incorporation and Release

Leukocytes (10×10^6 cells/ml) were preincubated for 30 min at 37°C in Gey's buffer with 4.4 μM [1-^{14}C]arachidonic acid, [1-^{14}C]-stearic acid, [1-^{14}C]oleic acid or [1-^{14}C]linoleic acid in a final concentration of 0.5% vol/vol ethanol which was used to suspend the fatty acids. The cells were then washed twice and resuspended in Gey's buffer. Under these conditions of preincubation, arachidonic acid may be metabolized to products which increase neutrophil calcium permeability (24); however, no degranulation occurred and, after washing, the cells retained their responses to fMet-Leu-Phe. For experiments using homocysteine thiolactone and/or 3-deazaadenosine, these labeled cells were then incubated for 30 min at 37°C with or without the inhibitors, centrifuged, and the pellets were resuspended in Gey's buffer with or without the drugs. Each tube contained 0.5 ml of cell suspension (5×10^6 cells). The reaction was started by the addition of 10 nM fMet-Leu-Phe, a concentration which produces maximal stimulation of both chemotaxis and arachidonic acid release (4, 7), and stopped by the addition of 1 ml of cold Gey's buffer. After centrifugation at 6,000 *g* for 10 min, 0.5 ml of supernatant was counted with a liquid scintillation spectrometer for the release of [^{14}C] fatty acid. To identify the released radioactive products after [^{14}C]-arachidonic acid incorporation, an additional 0.5 ml of supernatant was acidified to pH 3 with 1N formic acid, extracted with 6 vol of ethyl acetate, and chromatographed on a silica gel G plate developed in the upper phase of ethyl acetate/2,2,4-trimethylpentane/glacial acetic acid/water (90/50/20/100). This solvent system separates hydroxyeicosatetraenoic acids (HETES) from the prostaglandin products of arachidonic acid (10, 25). To measure the incorporation of [^{14}C]arachidonic and other fatty acids into phospholipids, the pellet obtained after centrifugation was suspended in 0.5 ml of 10% (wt/vol) TCA, centrifuged at 27,000 *g* for 10 min, and extracted with chloroform/methanol as described above. The separation and identification of phospholipids was carried out by thin-layer chromatography (TLC) of the washed chloroform phase (dehydrated overnight with anhydrous sodium sulfate and evaporated under a stream of N_2 gas) on a silica gel G plate developed in chloroform/methanol/water (65/25/4; vol/vol) (26). Thin-layer chromatograms were scanned for radioactivity using a BID System 100 radiochromatogram imaging system (Bioscan, Inc., Washington, D.C.).

Calcium Flux

Leukocytes at a concentration of 5×10^6 cells/ml in Gey's buffer (including 1 mM $CaCl_2$) were preincubated for 30 min at 37°C with 10 μCi/ml $^{45}CaCl_2$ with or without homocysteine thiolactone and 3-deazaadenosine (14). The incubation was then continued in the presence or absence of 10 nM fMet-Leu-Phe. Each tube contained 0.5 ml (2.5×10^6 cells) and the flux was stopped with the addition of 0.75 ml of ice-cold 0.9% NaCl containing 5 mM $CaCl_2$ and 10 mM HEPES (pH 7.4). The cells were immediately centrifuged for 30 s in a Beckman Microfuge B (Beckman Instruments, Inc., Fullerton, CA) and resuspended and washed twice with ice-cold saline-$CaCl_2$-HEPES. The cells were then lysed by resuspension in 5 mM $CaCl_2$, the membranes were centrifuged at 20,000 *g* for 10 min, and the supernatant was counted by liquid scintillation spectrometry.

Data Presentation

Because of large variations in the day-to-day response to fMet-Leu-Phe of different preparations of rabbit neutrophils, the figures and tables shown here generally represent means of two to three determinations from a single typical experiment. Standard error of the mean (SEM) of replicate values did not exceed 10%. The number of experiments performed and range of amount of stimulation and/or inhibition found are given in legends.

Materials

Chemicals were obtained from the following sources: L-[methyl-^{3}H]methionine (15.0 Ci/mmol) and $^{45}CaCl_2$ (0.5 to 3 Ci/mmol) from New England Nuclear, Boston, MA; [1-^{14}C]arachidonic acid (56.4 mCi/mmol), [1-^{14}C]-stearic acid (56.5 mCi/mmol), [1-^{14}C]oleic acid (53 mCi/mmol) and [1-^{14}C]-linoleic acid (52 mCi/mmol) from Amersham Corp., Arlington Heights, IL; fMet-Leu-Phe from Peninsula Laboratories, San Carlos, CA; L-homocysteine thiolactone from Sigma Chemical Co., St. Louis, MO; 3-deazaadenosine from Southern Research Institute, Birmingham, AL; A23187 from Eli Lilly and Company, Indianapolis, IN; phospholipid standards from Grand Island Biological Company, Grand Island, NY; silica gel G chromatography plates from Analtech, Inc., Newark, DE; and Gey's balanced salt solution from the National Institutes of Health Media Service.

RESULTS

Inhibition of Methyltransferases and Chemotaxis

Previous work has shown that inhibitors of methyltransferases block chemotactic processes in a number of eucaryotic cells, including neutrophils, monocytes and macrophages (17, 27, 28, 29). Both protein carboxy-O-methylation and phospholipid methylation have been implicated in chemotactic peptide-stimulated movement in neutrophils (6, 7, 17, 30). To determine whether protein carboxymethylation and/or phospholipid methylation are involved in the directed movement of rabbit neutrophils induced by the peptide fMet-Leu-Phe, various concentrations of 3-deazaadenosine (3-DZA) and homocysteine (HCYS) were used to selectively inhibit the different methyltransferases. Incubation of cells with 3-DZA and HCYS leads to the accumulation of S-adenosylhomocysteine and 3-deazaadenosylhomocysteine which then results in the inhibition of S-adenosylmethionine-requiring methyltransferases (31, 32). HYCS and 3-DZA inhibited both phospholipid methylation and protein carboxymethylation to about the same extent in a dose-dependent manner (Fig. 1). fMet-Leu-Phe-induced chemotaxis was also reduced by the methyltransferase inhibitors in the same dose range, possibly implicating one or both of these methylation reactions in chemotaxis. HCYS potentiates the inhibition by 3-DZA, particularly at low 3-DZA concentrations, and maximal inhibition of phospholipid methylation (85–90% inhibited) was obtained using 1 mM HCYS and 0.1 mM 3-DZA; these were the concentrations used in subsequent experiments on fMet-Leu-Phe-stimulated arachidonic acid release and calcium flux. Very little inhibition of nucleic acid methylation could be demonstrated at the concentrations found to inhibit phospholipid methylation, protein carboxymethylation, and chemotaxis (data not shown).

Phospholipid Methylation and Phospholipase A_2 Activation

Recent work in our laboratory has shown that stimulation of rabbit neutrophils by chemotactic peptides produces a receptor-mediated activation of phospholipase A_2 as shown by the release of arachidonic acid previously incorporated into phospholipids (7). In rat basophilic leukemia cells, it has been found that the receptor-mediated increase in phospholipid methylation is associated with phospholipase A_2 activation and histamine release (21). To determine whether phospholipid methylation is associated with phospholipase A_2 activation and subsequent release of arachidonic acid, neutrophils were prelabeled by incubation with [^{14}C]arachidonic acid. This fatty acid is known to be preferentially incorporated into the

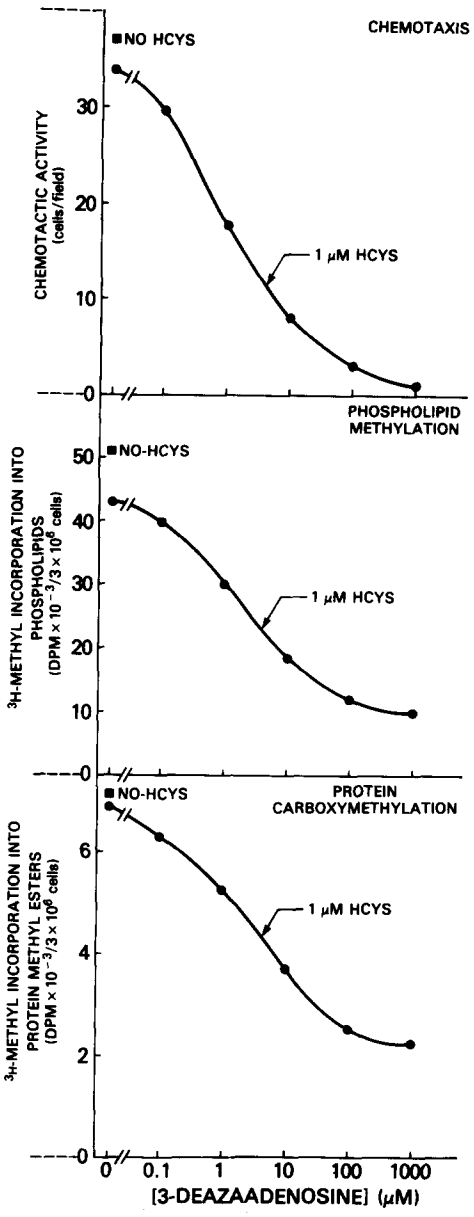

FIGURE 1 Dose response curves for inhibition of fMet-Leu-Phe-stimulated chemotaxis, phospholipid methylation, and protein carboxymethylation by 3-deazaadenosine in rabbit neutrophils. Chemotaxis incubations were done for 2 h at 37°C in modified Boyden chambers containing 2 × 10^6 cells and 1 nM fMet-Leu-Phe and were performed after 30-min preincubation with various concentrations of 3-deazaadenosine and 1 μM homocysteine thiolactone (HCYS). Chemotaxis results are expressed as means of duplicate determinations from two experiments. Methyltransferase assays were incubated for 1 h at 37°C without chemoattractant. Methylation data are expressed as means of duplicate determinations which did not vary by >5%; the experiment was performed three times. Blanks for methyltransferase reactions were kept at 0°C and were <15% of uninhibited samples incubated at 37°C. Abscissa is logarithmic.

2-position of the glycerol moiety of phospholipids. After a 30-min incubation with labeled fatty acid, the lipids were extracted as described in Materials and Methods and separated by TLC (Fig. 2). Most of the [^{14}C]arachidonic acid was incorporated into phosphatidylcholine; a smaller amount was present in phosphatidylethanolamine and a negligible amount of [^{14}C]-arachidonic acid was found in phosphatidylinositol or phosphatidylserine. The remainder of the radioactivity migrated with nonpolar lipids. Stimulation with fMet-Leu-Phe caused a release of radioactivity from rabbit neutrophils. >90% of the radioactivity comigrated with arachidonic acid on thin-layer chromatography to separate arachidonic acid metabolites (data not shown), suggesting that these compounds (mostly HETEs) are reincorporated into the cell as previously reported (10). To examine the effect of inhibition of methyltransferase reactions on peptide-induced release of arachidonic acid, cells were incubated for 30 min at 37°C with [^{14}C]arachidonic acid, then washed and incubated for an additional 30 min with HCYS and 3-DZA. The subsequent stimulation of the cells with fMet-Leu-Phe resulted in ~60% inhibition of arachidonic acid release as compared to cells incubated without methyltransferase inhibitors (Fig. 3). This inhibition of release of arachidonic acid by methyltransferase inhibitors was found to be concentration-dependent (Fig. 4). The methyltransferase inhibitors had no effect on unstimulated release of [^{14}C]arachidonic acid (data in Table V, line 1) or on viability of the cells as measured by trypan blue staining. These results suggest that the arachidonic acid released by the chemotactic peptide may partially arise from phospholipids synthesized by the methylation pathway.

Specificity of fMet-Leu-Phe-Stimulated Fatty Acid Release

Several recent studies have suggested that different compartments of phospholipases A_2 may exist which are either closely coupled to receptor activation or respond to more general stimulation (33–36). In mouse fibroblasts and rat leukemic basophils, receptor-mediated activation of phospholipase A_2 specifically releases arachidonic acid from phospholipids, while stimulation with the calcium ionophore A23187 results in the nonspecific release of several different fatty acids (35, 37). To determine whether the phospholipase activated by the chemotactic peptide, fMet-Leu-Phe, selectively releases arachidonic acid, neutrophils were prelabeled by incubation for 30 min at 37°C with either [^{14}C]arachidonic, [^{14}C]linoleic,

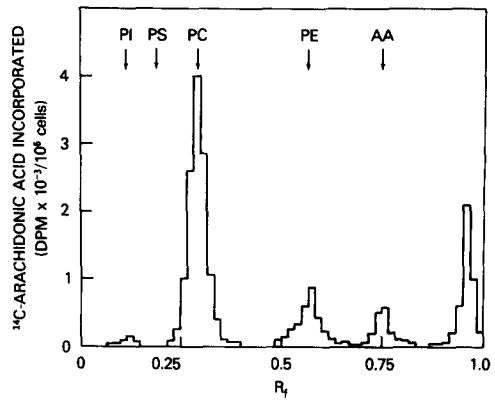

FIGURE 2 Histogram of [^{14}C]arachidonic acid incorporated into phospholipids. Neutrophils were incubated for 30 min at 37°C with 4.4 μM arachidonic acid. Lipids were extracted as described in Materials and Methods and separated by thin-layer chromatography in chloroform/methanol/water (65/25/4, vol/vol). Approximately 35–50% of added arachidonic acid was incorporated in four experiments. In this experiment, 5.5 × 10^4 DPM of arachidonic acid was added per 10^6 cells, and 2.15 × 10^4 DPM was incorporated into lipid (39%). Blanks were kept at 0°C and averaged ~25% of the 37°C values. *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *AA*, arachidonic acid.

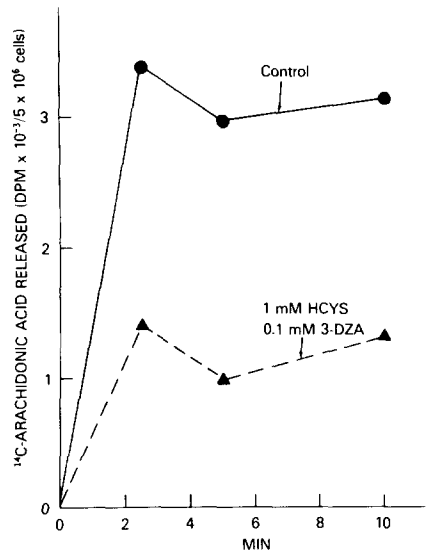

FIGURE 3 Stimulation of [^{14}C]arachidonic acid release by fMet-Leu-Phe in the presence and absence of homocysteine (HCYS) and 3-deazaadenosine (3-DZA). Cells were preincubated with arachidonic acid for 30 min at 37°C, washed, and preincubated with or without 1 mM HCYS and 0.1 mM 3-DZA for 30 min at 37°C before stimulating with 10 nM fMet-Leu-Phe. HCYS and 3-DZA did not affect unstimulated release of [^{14}C]arachidonic acid (see Table IV, line 1 for data). Results are expressed as means of duplicate determinations from a single typical experiment. The experiment was repeated five times. The range of stimulation of arachidonic acid release was 100–620% over untreated blanks kept at 37°C, and the amount of inhibition by HCYS and 3-DZA was 55–75%. (●——●) control (stimulated by fMet-Leu-Phe, no inhibitors); (▲------▲) 1 mM HCYS, 0.1 mM 3-DZA (stimulated by fMet-Leu-Phe).

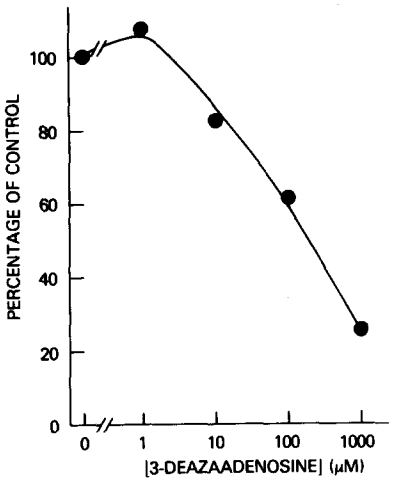

FIGURE 4 Dose - response curve for inhibition of fMet-Leu-Phe-stimulated [^{14}C]arachidonic acid release by 3-deazaadenosine. Neutrophils were preincubated with arachidonic acid for 30 min at 37°C, washed, and preincubated with various concentrations of 3-deazaadenosine and 100 μM homocysteine for 30 min at 37°C before stimulating for 5 min with 10 nM fMet-Leu-Phe. Results are expressed as means of triplicate determinations. Uninhibited release was 480% over unstimulated samples (stimulated release = 3,080 DPM/5 × 10^6 cells; unstimulated release = 645 DPM/5 × 10^6 cells). Abscissa is logarithmic.

[^{14}C]oleic or [^{14}C]stearic acid. Under these conditions a similar amount of each fatty acid was incorporated into lipid, and after extraction and separation by TLC a considerable portion of the fatty acid was found to be incorporated into phosphatidylcholine (Table I). However, upon stimulation with either fMet-Leu-Phe or A23187 or both, only arachidonic acid was released to any appreciable extent (Table II). A23187 also released ~10-fold less linoleic acid. Either stimulant appeared able to stabilize oleic acid-containing lipids, inhibiting its release. It would appear that in rabbit neutrophils both the receptor-activated phospholipase A_2 and the enzyme stimulated by increased calcium flux caused by the ionophore are quite specific for the release of arachidonic acid.

Phospholipid Methylation, Calcium Influx, and Arachidonic Acid Release

Several studies have demonstrated a requirement for calcium fluxes in neutrophil chemotaxis measured in Boyden chambers (14, 15, 38, 39). Treatment of rabbit neutrophils with fMet-Leu-Phe stimulates calcium influx (11–13), and this ion has also been found to activate phospholipase A_2 (40–43). To determine whether extracellular calcium is required for stimulation of arachidonic acid release by the chemotactic peptide, rabbit neutrophils were incubated with or without calcium or without calcium and including EDTA (Table III). In the absence of calcium there was negligible release of arachidonic acid after treatment with fMet-Leu-Phe.

We have shown that the methylation of phospholipids en-

TABLE I

Incorporation of [^{14}C]-Labeled Fatty Acids into Phospholipids of Rabbit Neutrophils

Fatty acid	Total incorporated into lipid	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylinositol	Neutral Lipids
	DPM	%	%	%	%
Arachidonic	16.4 × 10^4	53.6	14.3	1.3	23.2
Linoleic	16.9 × 10^4	42.6	8.1	0.8	45.0
Oleic	12.8 × 10^4	20.5	6.6	0.8	68.3
Stearic	10.2 × 10^4	27.1	12.6	1.0	52.2

Results are expressed as amount of radioactive fatty acid incorporated into lipid in 30 min at 37°C per 5 × 10^6 cells and represent means of two experiments. Blanks were kept at 0°C and ranged from 25 to 35% of values at 37°C.

TABLE II

Specificity of Stimulated Fatty Acid Release from Rabbit Neutrophils

Treatment	Arachidonic acid	Linoleic acid	Oleic acid	Stearic acid
	DPM	*DPM*	*DPM*	*DPM*
None	1910	2140	2770	1710
10 nM fMet-Leu-Phe	4400	1770	1450	1460
1 μM A23187	6320	2580	2030	1810
10 nM fMet-Leu-Phe +1 μM A23187	8850	2390	1860	1620

Results are expressed as DPM [^{14}C]fatty acid released by treatment in 5 min per 5 × 10^6 cells and represent means of two experiments. The range of fMet-Leu-Phe-stimulated arachidonic acid release was 90–185%.

TABLE III

Calcium Requirement for Stimulation of Arachidonic Acid Release from Rabbit Neutrophils by fMet-Leu-Phe

Condition	Unstimulated	fMet-Leu-Phe-stimulated
	DPM	*DPM*
1 mM $CaCl_2$	260	2100
No Ca	200	210
No Ca + 2 mM EDTA	140	130

Results are expressed as DPM [^{14}C]arachidonic acid released in 5 min per 5 × 10^6 cells with or without stimulation by 10 nM fMet-Leu-Phe and represent means of three determinations. Cells were preincubated with or without Ca or with EDTA for 15 min at 4°C after labeling with arachidonic acid and before stimulating with chemoattractant.

hances calcium flux in IgE receptor-mediated release of histamine from mast cells and rat basophilic leukemia cells (20, 37). This prompted an examination of the effect of inhibition of methylation on the 75% increase in intracellular calcium measured after treatment with fMet-Leu-Phe. Rabbit neutrophils were pretreated with methyltransferase inhibitors and the effect of these compounds on stimulated calcium movement was measured. HCYS and 3-DZA had no effect on unstimulated equilibration of ^{45}Ca in the cells. However, the methyltransferase inhibitors blocked the influx of ^{45}Ca by ~40% (Fig. 5). Inhibition of fMet-Leu-Phe-stimulated ^{45}Ca movement was seen at 3-DZA concentrations as low as 10 μM (Table IV). These results implicate phospholipid and/or protein carboxymethylation in the partial regulation of calcium movements stimulated by fMet-Leu-Phe. It should be noted that the net calcium movement measured included both intracellular displacement from membrane binding sites and the opening of calcium channels to increase membrane permeability to the ion. Methyltransferase inhibitors would not be expected to interfere with all the processes measured using our procedure and therefore the 40% inhibition obtained can be regarded as quite substantial.

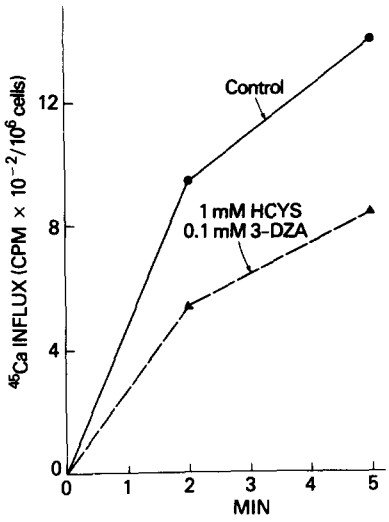

FIGURE 5 Stimulation of ^{45}Ca influx by fMet-Leu-Phe in the presence and absence of homocysteine (HCYS) and 3-deazaadenosine (3-DZA). Cells were preincubated for 30 min at 37°C with 10 μCi/ml $^{45}CaCl_2$ in 1 mM $CaCl_2$ and with or without 1 mM HCYS and 0.1 mM 3-DZA before stimulating with 10 nM fMet-Leu-Phe. HCYS and 3-DZA had no effect on unstimulated equilibration of ^{45}Ca. Results are expressed as means of duplicate determinations from a single typical experiment. The experiment was repeated three times. The range of stimulation of ^{45}Ca influx was 35–95% over untreated blanks kept at 37°C (typical blank value was 1,800–2,000 CPM/10^6 cells), and the amount of inhibition by HCYS and 3-DZA was 20–45%. (●——●) control (stimulated by fMet-Leu-Phe, no inhibitors); (▲------▲) 1 mM HCYS, 0.1 mM 3-DZA (stimulated by fMet-Leu-Phe).

TABLE IV

Effect of Various Concentrations of 3-Deazaadenosine (3-DZA) on fMet-Leu-Phe-Stimulated ^{45}Ca Movement into Rabbit Neutrophils

[3-DZA]	Unstimulated	10 nM fMet-Leu-Phe
μM	CPM	CPM
0, no HCYS	1630	2830
1	1530	3010
10	1550	2100
100	1730	1980
1000	1890	2070

Cells were preincubated for 30 min at 37°C with ^{45}Ca, 3-DZA, and 1 μM homocysteine thiolactone (HCYS) and then stimulated for 5 min with chemoattractant as described in Materials and Methods. Results are expressed as means of three determinations of intracellular ^{45}Ca.

TABLE V

Effect of Methylation Inhibitors on Stimulation of Arachidonic Acid Release from Rabbit Neutrophils by fMet-Leu-Phe and/or A23187

Treatment	Control	1 mM Homocysteine 0.1 mM 3-Deazaadenosine
	DPM	DPM
None	290	310
10 nM fMet-Leu-Phe	2500	840
1 μM A23187	2490	2470
10 nM fMet-Leu-Phe +1 μM A23187	6900	5320

Results are expressed as DPM [^{14}C]arachidonic acid released by treatment in 5 min per 5 × 10^6 cells and represent means of two determinations from a single experiment. The experiment was performed three times. The range of stimulation of arachidonic acid release was 110–750% over untreated samples. The range of inhibition of peptide-stimulated arachidonic acid release by homocysteine and 3-deazaadenosine was 60–75%.

As shown above, methyltransferase inhibitors partially prevented the chemotactic peptide-stimulated calcium influx and arachidonic acid release, thus implicating methylation as one factor involved in receptor stimulation of both processes. We then tested the effect of HCYS and 3-DZA on arachidonic acid release caused by direct stimulation of calcium fluxes using the ionophore A23187 (Table V). While the addition of methyltransferase inhibitors considerably reduced the effect of the chemotactic peptide, there was no effect on the release of arachidonic acid by the ionophore. This suggests that direct stimulation of calcium fluxes by the ionophore bypasses any requirement for the methyltransferase enzymes.

DISCUSSION

The results presented here suggest that peptide receptor-mediated chemotaxis may involve phospholipid methylation, calcium influx, and arachidonic acid release, and these processes appear to be associated in a cascade of events required for activation of directed movement in rabbit neutrophils. Our findings are consistent with a sequence in which receptor activation is followed by phospholipid methylation (or protein carboxymethylation as discussed below) which stimulates the influx or membrane redistribution of calcium ions, and increased calcium availability leads to the activation of phospholipase A_2, an enzyme generating arachidonic acid from phospholipids. Other studies have shown that released arachidonic acid is metabolized by a lipoxygenase to products which are reincorporated into the cell (10, 44) and this metabolism may be essential for chemotaxis (16, 18). A similar cascade has also been found for other receptor-mediated processes, notably IgE stimulation of histamine release from rat mast cells and basophilic leukemia cells (20, 21, 37) and concanavalin A stimulation of T-lymphocyte mitogenesis (45).

Specifically, we have shown that inhibition of methyltransferase reactions by homocysteine and 3-deazaadenosine also inhibits fMet-Leu-Phe stimulation of calcium movement, arachidonic acid release, and chemotaxis. Furthermore, increasing the calcium availability directly by using the calcium ionophore A23187 activates a phospholipase with the same specificity for arachidonic acid as the enzyme stimulated by fMet-Leu-Phe. These data suggest that methyltransferase reactions precede and are required for normal levels of chemotactic factor-stimulated calcium flux and arachidonic acid

release, and it may be the increased calcium availability which then activates arachidonic acid release. Since methyltransferase inhibitors do not block calcium ionophore-stimulated arachidonic acid release, it may be that methylation is not directly required for phospholipase activation but is involved in the calcium redistribution required to release arachidonic acid. However, it has been shown that arachidonic acid released by fMet-Leu-Phe stimulation at least partially arises from increased degradation of phospholipids synthesized through the methylation pathway (7). Therefore phospholipid methylation may also provide a substrate for activated phospholipase A_2 in addition to stimulating calcium availability.

Previous work in this and other laboratories has indicated that each of the biochemical events studied here could be required for neutrophil chemotaxis, but the sequence of these processes is probably much more complicated than our data alone would seem to suggest. Inhibition of methyltransferases inhibits chemotaxis in several eucaryotes (17, 27–29). Calcium fluxes have been demonstrated to be necessary for efficient neutrophil chemotaxis in Boyden chambers (14, 15, 38, 39), while mepacrine, an inhibitor of phospholipase A_2 (46–50), decreases both arachidonic acid release and chemotaxis over the same dose range (7). Calcium is required for stimulation of phospholipase A_2 in this and other systems (40–43). However, there is also evidence that arachidonic acid metabolites increase membrane permeability to calcium, causing increased calcium influx (24). Since calcium movement stimulated by fMet-Leu-Phe occurs faster than the flux stimulated by arachidonic acid (11, 24), more than one mechanism may be involved. The very rapid influx in the first 2–5 min seen with fMet-Leu-Phe may in fact be due to release of calcium from a membrane-bound pool to the cytoplasmic side of the membrane (12). This calcium could activate phospholipase A_2, releasing arachidonic acid, whose metabolites then cause the plasma membrane to become more permeable to calcium ions. This process could account for fMet-Leu-Phe-stimulated increases in intracellular $^{45}Ca^{2+}$ content after 5 min. While the first phase (redistribution of calcium from the membrane) may activate phospholipases, the second phase (permeability increase) may be essential for other calcium requiring events in neutrophil function, such as microtubule assembly and lysosomal enzyme release. Our assay for calcium movement counted only the nonmembrane bound intracellular $^{45}Ca^{2+}$ content and can not differentiate between increased influx and membrane redistribution. Clearly, much more work needs to be done on the biochemistry of fMet-Leu-Phe-stimulated calcium mobilization and arachidonic acid release and metabolism to unambiguously determine the sequence of these events during activation of chemotaxis.

It is not clear how methylation of phospholipids or proteins can stimulate calcium movement. One possibility is that phospholipid methylation changes the local lipid environment around the calcium channel or binding site, increasing fluidity and allowing calcium to be released or the channel to open. A decrease in membrane viscosity measured with fluorescent and spin-labeled probes has been associated with phospholipid methylation in erythrocytes (22, 51). This fall in viscosity may allow mobility of membrane proteins. Alternatively, calcium binding sites on membrane proteins may be carboxymethylated. Although methyltransferase inhibitors only partially block chemotactic factor-stimulated calcium mobilization, this may be sufficient to reduce calcium availability below the critical level needed for phospholipase A_2 activation. Also, degradation of methylated phospholipids to release arachidonic acid may be further hampered by lower availability of substrate in the presence of methyltransferase inhibitors.

The demonstration that chemotactic factors enhance arachidonic acid release by specifically stimulating the degradation of phosphatidylcholine synthesized from the methylation pathway and not the CDP-choline pathway (7) suggests that phospholipid methylation may be the methyltransferase reaction of most interest in understanding receptor activation of neutrophil chemotaxis. However, in these studies the methyltransferase inhibitors were used in concentrations that inhibited protein carboxymethylation as well as phospholipid methylation. Protein carboxymethylation has been shown to be essential for bacterial chemotaxis (52, 53) and in neutrophils can be transiently stimulated by the chemotactic peptide fMet-Leu-Phe (6). The experiments presented here do not rule out the possibility that protein carboxymethylation plays a role in chemotactic factor receptor activation of calcium mobilization and phospholipase A_2. It is possible that both methyltransferases are involved in the complex series of events leading to directed movement of neutrophils.

Chemotactic factor-induced arachidonic acid release was less sensitive than phospholipid methylation, protein carboxymethylation, calcium mobilization, or chemotaxis to inhibition by low concentrations of homocysteine and 3-deazaadenosine. This may have resulted from any one or a combination of several factors. First, as discussed above, methylation of phospholipids or proteins may be only indirectly linked to phospholipase A_2 activation by either increasing calcium availability or providing a substrate for degradation by the phospholipase. Second, arachidonic acid undoubtedly arises from sources other than methylated phospholipids, such as a small pool of phosphatidylinositol. Rapid turnover of this lipid is also stimulated by fMet-Leu-Phe; however, in neutrophils this has not been associated with either chemotaxis or lysosomal enzyme release (54, 55). Another problem arises when considering the limitations of our measurement of arachidonic acid release. Only extracellular arachidonic acid was counted in our assay, and >90% of that was found not to be metabolized. The arachidonic acid released by fMet-Leu-Phe stimulation is known to be further metabolized by lipoxygenase to leukotrienes and by cyclooxygenase to prostaglandins (44). These metabolites are then taken up by the neutrophil and reincorporated into cellular lipids (10). Therefore, the measured extracellular arachidonic acid does not include the reincorporated metabolites produced, and probably underestimates the total amount of arachidonic acid released. Since the metabolized pool is probably most closely associated with receptor-stimulated release, production of arachidonic acid metabolites upon fMet-Leu-Phe stimulation may prove to be more sensitive to blockade by methyltransferase inhibitors than the release of untransformed arachidonic acid. Metabolites of the lipoxygenase pathway such as hydroxyeicosatetraenoic acids (HETEs) and, more recently, the leukotrienes have been shown to possess potent chemotactic activity (8, 56, 57). Inhibition of lipoxygenase and cyclooxygenase with eicosatetraynoic acid (ETYA) or nordihydroguaiaretic acid inhibits chemotaxis (16, 18). Prostaglandin E, though not itself a strong chemoattractant, has been implicated as a possible modulator of the chemotactic potency of the lipoxygenase products (58).

Arachidonic acid was the only fatty acid released by stimulation with either fMet-Leu-Phe or A23187. While other receptor-mediated activations of phospholipases have demonstrated similar fatty acid specificity (34–36), A23187 stimulation has

generally resulted in nonspecific release of several fatty acids in these systems, suggesting the presence and activation of several phospholipase A_2 compartments (34, 37). In the rabbit neutrophil, however, the specificity for arachidonic acid release suggests that fMet-Leu-Phe and A23187 may be activating the same pool of phospholipases. In neutrophils, it appears that the calcium-activated membrane phospholipases are specific for arachidonic acid-containing phospholipid. However, specificity for one fatty acid does not necessarily have to arise from chemical specificity of the enzyme but may be due to topological localization within the membrane. In our proposed model for receptor-activated arachidonic acid release, the receptor, phospholipid methyltransferases, calcium channel or binding site, phospholipase A_2, and perhaps also a reacylase could be closely associated in a cluster within the membrane. In rat basophilic leukemia cells, phosphatidylcholine rich in arachidonic acid arises from the methylation pathway which supplies the phospholipids for metabolism by a phospholipase A_2 stimulated by IgE and presumably located nearby (37).

In conclusion, the data presented here suggest that several processes which have previously been implicated in neutrophil chemotaxis may be associated in a sequence starting with activation of the chemotactic receptor and eventually resulting in directed movement. Methylation of phospholipids or proteins or both appears to be an early event in the sequence because methyltransferase inhibitors also partially block fMet-Leu-Phe-stimulated calcium movement, arachidonic acid release, and chemotaxis. Increased calcium availability may be the event which activates phospholipase A_2 to release arachidonic acid from methylated phospholipids, since stimulation by calcium ionophore A23187 alone also causes specific release of arachidonic acid. However, this association between the chemotactic factor receptor, methyltransferase enzymes, calcium mobilization, phospholipase A_2, and chemotaxis does not appear to be absolute, and future investigations are needed to further define the relationship between these events.

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