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Chemoattractants stimulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes

(phospholipase A_2/chemotactic peptides/lipid methylation/lysophosphatidylcholine)

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ABSTRACT When rabbit peritoneal leukocytes were treated with chemoattractants such as fMet-Leu-Phe, an apparent decrease of [^{3}H]methyl incorporation into the lipid fraction from L-[*methyl*-^{3}H]methionine was observed. This decrease was a result of increased degradation of methylated phospholipids, not of decreased synthesis. Chemotactic peptides did not affect the metabolism of the phospholipids in which [*methyl*-^{14}C]choline was incorporated. The disappearance of the [^{3}H]methyl group was associated with the release of [1-^{14}C]arachidonic acid from phospholipids prelabeled with these compounds. These findings suggested the activation by chemoattractants of phospholipase A_2, an enzyme that removes an unsaturated fatty acid from phospholipids. The order of potency of chemoattractants for the stimulated degradation of phospholipids was in good agreement with that for chemotaxis. Mepacrine (quinacrine) and hydrocortisone inhibited and a phorbol ester enhanced both chemotaxis and phospholipase A_2 activity. These results, taken together, suggest close association of the metabolism of methylated phospholipids with chemotaxis in rabbit peritoneal leukocytes.

Leukocytes respond to various chemoattractants by interaction with specific receptors on the cell surface (1). The biochemical mechanism by which stimulation of chemotactic receptors leads to directed movement of leukocytes is still poorly understood. Recently, our laboratory showed that the activation of protein carboxy-*O*-methylase in leukocytes is one of the early events in chemotaxis (2). We have also found that phospholipid methylation alters biomembrane structure and functions (3–5). Because the chemotactically responding cells show a marked polarization and have alterations in properties of membranes, we examined the effect of chemoattractants on phospholipid methylation in leukocyte membranes. Here, we report that chemotactic peptides enhance the degradation of phosphatidylcholine synthesized by the transmethylation but not by the choline pathway(s).

METHODS AND MATERIALS

Cell Preparations. Rabbit leukocytes were obtained by lavage of the peritoneal cavity of rabbits injected with 150 ml of 0.1% glycogen as described (6). When necessary, the peritoneal exudates were exposed to hypotonic saline (0.2%) in the cold for 30 sec to lyse contaminating erythrocytes and then diluted with an equal volume of 1.6% saline. After collection by centrifugation at 600 × *g* for 10 min, the cells were suspended in Gey's balanced salt solution containing 0.1% bovine serum albumin and 0.01 M Hepes buffer at pH 7.4 (modified Gey's solution), at a concentration of 8–11 × 10^6 cells per ml. A typical cell preparation contained 90% neutrophils and 10% lymphocytes and macrophages. Chemotaxis was measured in modified Boyden chambers as described (7).

Assay of Phospholipid Methylation. The phospholipid methylation was assayed by using intact leukocytes and measuring [^{3}H]methyl group from L-[*methyl*-^{3}H]methionine incorporated into the phospholipid fraction. The cells were preincubated in a total volume of 25 ml with 20 μM L-[*methyl*-^{3}H]methionine (2 μCi/nmol; 1 Ci = 3.7 × 10^{10} becquerels) or 10 μCi of [1-^{14}C]arachidonic acid (55.5 μCi/μmol) or both. After preincubation at 37°C for 30 min, the cells were washed twice and resuspended in a modified Gey's solution supplemented with 1 mM unlabeled methionine or 0.2 mg of unlabeled arachidonic acid per ml or both. Each tube contained 0.5 ml of cell suspension (5 × 10^6 cells) and the compound to be tested. The reaction was started by addition of a chemoattractant and stopped by the addition of 0.5 ml of 10% (wt/vol) trichloroacetic acid. After centrifugation at 10,000 × *g* for 10 min, the pellets were extracted with 3 ml of chloroform/methanol, 2:1 (vol/vol), as described (3, 4). The separation and identification of phospholipids were carried out by thin-layer chromatography as described (8). To measure [*methyl*-^{14}C]-choline incorporation into the lipid fraction, the cells were preincubated with 20 μM [*methyl*-^{14}C]choline (0.05 μCi/nmol) as described above and resuspended in modified Gey's solution containing 1 mM unlabeled choline. To maintain chemotactic responsiveness in the leukocytes, Gey's solution containing 0.1% bovine serum albumin was used during the procedures.

Chemicals. Gey's solution was supplied by the National Institutes of Health Media Service. L-[*methyl*-^{3}H]Methionine (71.8 mCi/μmol) was obtained from New England Nuclear. [*methyl*-^{14}C]Choline (51.5 μCi/μmol) and [1-^{14}C]arachidonic acid (55.5 μCi/μmol) were products of Amersham/Searle Corp. 21-Phosphohydrocortisone was purchased from Sigma. Mepacrine was generously supplied by Seymor Heisler (Laval University, Quebec, PQ, Canada). 12-*O*-Tetradecanoyl-phorbol-13-acetate was obtained from Consolidated Midland Corporation (Brewster, NY). fMet-Leu-Phe and fMet-Leu-Glu were obtained from Peninsula Laboratories (San Carlos, CA). Carbobenzoxy (Z) Phe-Met was prepared by R. J. Freer under National Institute of Dental Research Contract DE-52477. fPhe-Leu-Phe-Leu-Phe and Boc-Phe-Leu-Phe-Leu-Phe were kindly provided by E. Gross (National Institute of Child Health and Human Development, Bethesda, MD). All amino acids were of the L configuration. Reagents were of analytical grade.

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RESULTS

Apparent Decrease of Phospholipid Methylation by Chemoattractants. To examine the effect of chemoattractants on phospholipid metabolism, leukocytes were preincubated with L-[*methyl*-^{3}H]methionine and the reaction was initiated by the addition of fMet-Leu-Phe, a chemoattractant. This peptide caused a decrease in the amount of [^{3}H]methyl incorporated into phospholipids (Fig. 1*a*). This decrease was also observed with other chemoattractants such as fPhe-Leu-Phe-Leu-Phe and fMet-Leu-Glu but not with antagonists including Boc-Phe-Leu-Phe-Leu-Phe and Z-Phe-Met. Because both synthesis and degradation of methylated phospholipids proceed simultaneously in the cells, the L-[*methyl*-^{3}H]methionine was chased with a 50-fold excess of unlabeled methionine to measure the rate of degradation (Fig. 1*b*). In the presence of chemoattractant, the methylated phospholipids disappeared more rapidly, suggesting that the decrease of phospholipid methylation is a result of increase in degradation of the lipids.

Phosphatidylcholine, a major component of phospholipids, can be synthesized by choline pathway(s) or by transmethylation. To establish which pathway of phospholipid synthesis responded to chemoattractants, the effect of fMet-Leu-Phe on the metabolism of phospholipids labeled with [*methyl*-^{14}-C]-choline was investigated (Fig. 2). When [*methyl*-^{14}C]choline was added to the medium, approximately 50-fold more ^{14}C was incorporated, either by the CDP-choline pathway or by a base exchange (9, 10) compared to incorporation of [^{3}H]methyl from [^{3}H]methionine. In spite of the large amounts of phosphatidylcholine synthesized by these pathways, the phospholipids labeled with [^{14}C]choline did not respond to the chemoattractant in either synthesis or degradation. This observation indicates that the phospholipids synthesized by transmethylation represent the more active pool of phospholipid turnover in response to chemoattractants.

Liberation of Arachidonic Acid by Chemoattractants. To determine the pathway by which the methylated phospholipids are degraded after stimulation with chemoattractants, the leukocytes were preincubated with [1-^{14}C]arachidonic acid. This fatty acid is known to be preferentially incorporated into the 2-position of the glycerol moiety of phospholipids. When the phospholipids labeled with [^{14}C]arachidonate were separated and quantitated by thin-layer chromatography, 13, 5, 9, and 65% of the arachidonate was incorporated into phosphatidylethanolamine, monomethyl and dimethyl derivatives of phosphatidylethanolamine, and phosphatidylcholine, respectively. When the leukocytes were stimulated by chemoattractants, 5–10% of the total arachidonate in the phospholipids were released into the medium. The release of [^{14}C]arachidonate paralleled the disappearance of [^{3}H]methyl from the chloroform/methanol extractable phospholipids (Fig. 3). When the phospholipids in leukocytes stimulated with the tripeptide fMet-Leu-Phe were extracted with 1-butanol, there was a marked accumulation of lysophosphatidylcholine (data not shown). This solvent was found to be more efficient than chloroform/methanol, 2:1 (vol/vol), in extraction of the lyso form of phospholipids. These observations suggest that the degradation of the phosphatidylcholine synthesized by

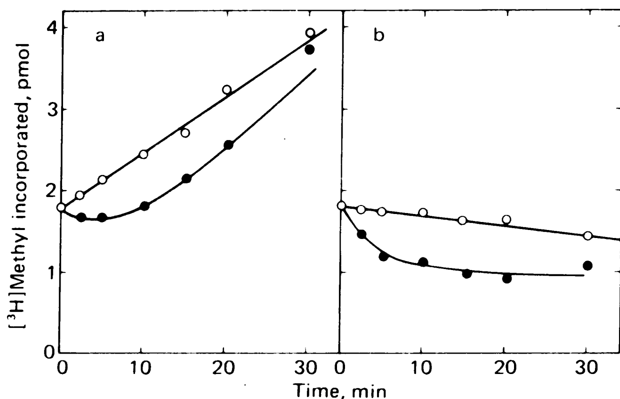

FIG. 1. Effect of chemoattractant fMet-Phe-Leu on phospholipid methylation. Rabbit peritoneal leukocytes were preloaded with 20 μM L-[*methyl*-^{3}H]methionine (2 μCi/nmol) for 30 min at 37°C. The cells were washed and resuspended in buffer containing the same components as the preincubation medium (*a*) or in modified Gey's solution containing 1 mM unlabeled methionine (*b*). The reaction was started by the addition of 5 μl of 1 μM fMet-Phe-Leu to 0.5 ml of the cell suspension (5 × 10^6 cells). Methylated phospholipids were measured. Each point represents the mean of duplicate determinations. ○, Without attractant; ●, with attractant.

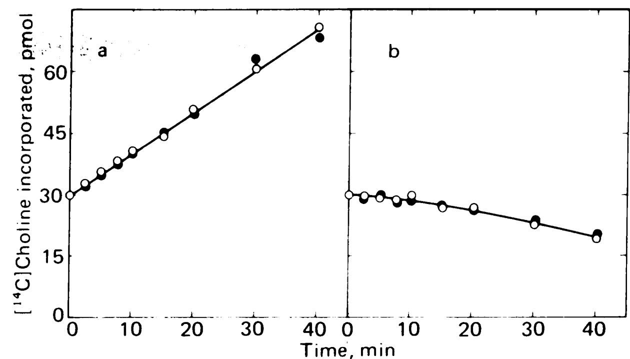

FIG. 2. Effect of fMet-Leu-Phe on incorporation (*a*) and retention (*b*) of [*methyl*-^{14}C]choline in phospholipids of leukocytes. The cells were preincubated with 20 μM [*methyl*-^{14}C]choline (110 dpm/pmol) for 30 min at 37°C. The ^{14}C radioactivity in the phospholipid fraction after stimulation of chemotaxis was measured without (*a*) and with (*b*) 1 mM unlabeled choline. ○, Without attractant; ●, with fMet-Leu-Phe.

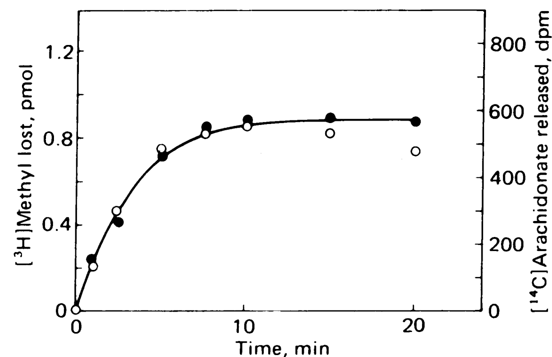

FIG. 3. Stimulatory effect of fMet-Phe-Leu on the release of [^{14}C]arachidonic acid (○) and the disappearance of [^{3}H]methyl (●) from phospholipids. Leukocytes were preincubated with 20 μM L-[*methyl*-^{3}H]methionine (2 μCi/nmol) and 0.4 μCi of [^{14}C]arachidonic acid (55.5 μCi/μmol) per ml for 30 min at 37°C. After washing, the cells were chased with 1 mM unlabeled methionine and 0.2 mg of unlabeled arachidonic acid per ml. The reaction was started by the addition of 5 μl of 1 μM fMet-Phe-Leu to 0.5 ml of cell suspension (5 × 10^6 cells) and terminated by the addition of 1 ml of cold Gey's solution. After centrifugation at 6000 × *g* for 10 min, the supernatant (1 ml) was assayed for the release of [^{14}C]arachidonic acid. The precipitate was washed with 0.5 ml of 10% trichloroacetic acid, the [^{3}H]methyl group remaining in the lipid fraction was measured, and the amount of methylated phospholipids disappearing was calculated.

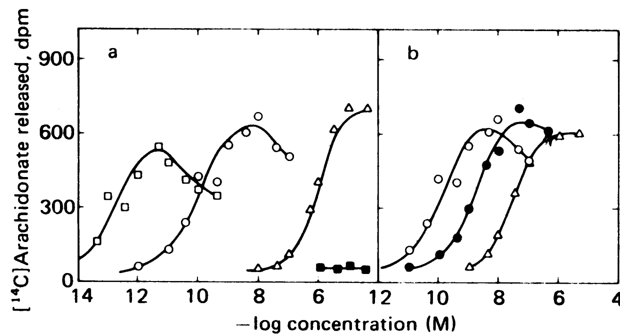

FIG. 4. Effect of chemotactic factors (*a*) and chemotactic antagonists (*b*) on the release of [^{14}C]arachidonic acid. (*a*) The release of [^{14}C]arachidonic acid from prelabeled leukocytes was measured in the presence of varying concentrations of fPhe-Leu-Phe-Leu-Phe (□), fMet-Leu-Phe (○), fMet-Leu-Glu (△), and Boc-Phe-Leu-Phe-Leu-Phe (■). (*b*) [^{14}C]Arachidonate release by varying concentrations of fMet-Leu-Phe was measured with no additions (○) or in the presence of 20 μM Boc-Phe-Leu-Phe-Leu-Phe (●) or 100 μM Z-Phe-Leu (△).

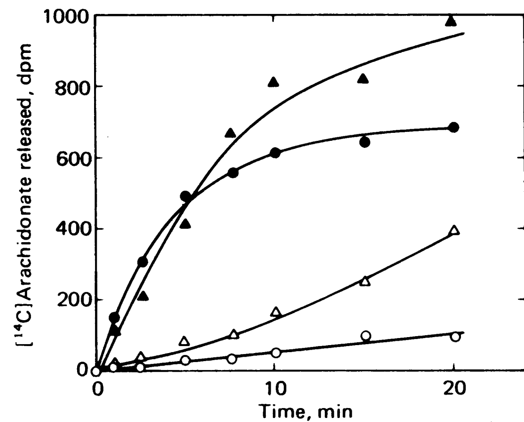

FIG. 6. Effect of a phorbol ester on the release of [^{14}C]arachidonate. [^{14}C]Arachidonate release was followed in the absence (○) and presence (△) of 12-*O*-tetradecanoylphorbol 13-acetate at 0.05 ng/ml without chemoattractants. fMet-Leu-Phe at 10 nM was added to the media with (▲) and without (●) phorbol ester.

transmethylation is initiated by the action of phospholipase A_2 because this enzyme removes a fatty acid from phosphatidylcholine to form lysophosphatidylcholine.

Specificity of Chemoattractants. To determine whether the stimulation of methylated phospholipid degradation is mediated through chemotactic receptors, the effect of chemotactic agonists and antagonists was examined. The chemotactic peptides fMet-Leu-Phe-Leu-Phe, fMet-Leu-Phe, and fMet-Leu-Glu caused the release of [^{14}C]arachidonic acid from prelabeled leukocytes (Fig. 4*a*). This release of the fatty acid was paralleled by the disappearance of [^{3}H]methyl from the phospholipids labeled with [*methyl*-^{3}H]methionine. The chemoattractants exhibited the same order of potency for release of fatty acid as for ability to promote chemotaxis (11). On the other hand, Boc-Phe-Leu-Phe-Leu-Phe and ZPhe-Leu, peptides that inhibit the binding of chemoattractants to the receptors (11), were ineffective. These antagonists added together with fMet-Leu-Phe shifted the dose–response curve of arachidonate release to the right but did not change the maximal response (Fig. 4*b*). All of these observations indicate that chemotaxis and the liberation of arachidonic acid by the activation of phospholipase A_2 are closely related.

Inhibition and Stimulation of Chemotaxis and Phospholipase A_2 by Drugs. If the chemotactic response is controlled by phospholipase A_2 activity in leukocytes, inhibition or stimulation of this enzyme by drugs should also influence chemotaxis. Mepacrine, a compound that inhibits phospholipase A in kidney cells (12), blocked both chemotaxis and release of arachidonic acid over the same concentration range (Fig. 5). Under these conditions, viability of the cells was not affected as measured by trypan blue staining. 21-Phosphohydrocortisone (3 μM) also inhibited the degradation of methylated phospholipids by approximately 55%. This steroid also prevents chemotaxis (unpublished data) and inhibits phospholipase A_2 activity in other types of cells (13).

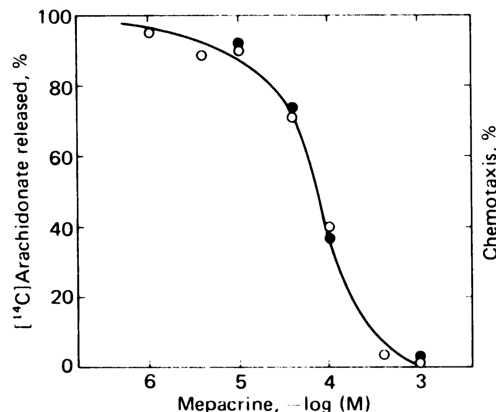

FIG. 5. Inhibition of [^{14}C]arachidonate release (○) and chemotaxis (●) by mepacrine. [^{14}C]Arachidonate release and chemotaxis in the presence of 10 nM fMet-Leu-Phe were measured with simultaneous addition of various concentrations of mepacrine.

Phorbol esters such as 12-*O*-tetradecanoylphorbol 13-acetate have been reported to amplify chemotaxis at low concentrations (14, 15). Because these compounds enhance the release of arachidonate in dog kidney (MDCK) cells (16), we examined the effect of 12-*O*-tetradecanoylphorbol 13-acetate on the release of the fatty acid from leukocytes. In the absence of chemoattractants, this compound increased the release of arachidonate (Fig. 6). The simultaneous addition of it and fMet-Leu-Phe caused a marked stimulation of arachidonate release. These findings are consistent with the previous observation that low concentrations of the phorbol ester enhances chemotaxis by fMet-Leu-Phe (14).

DISCUSSION

Our present observations clearly demonstrate a close association between chemotaxis in leukocytes and the degradation of phospholipids by phospholipase A_2. The phospholipid degraded is phosphatidylcholine and the products formed are lysophosphatidylcholine and arachidonic acid (Fig. 7). Although phosphatidylcholine is mainly synthesized by the CDP-choline pathway (9), it is the relatively minor pathway of transmethylation that is affected by chemotactic peptides. It is likely that there exist localized areas in the membrane where phospholipid methyltransferases, chemotactic receptors, and phospholipases are clustered (Fig. 7). The accumulation of lysophosphatidylcholine was observed after stimulation of chemotaxis. This phospholipid has been reported to play a crucial role in the regulation of guanylate cyclase, whose product, cyclic GMP, is considered to be closely related to chemotaxis (15, 17).

Chemotaxis of leukocytes can be inhibited by *erythro*-9-[3-(2-hydroxynonyl)]adenine plus adenosine and L-homocysteine thiolactone, which cause a marked increase of intracellular *S*-adenosyl-L-homocysteine, a competitive inhibitor of transmethylation reactions (8, 18). A similar effect can be observed with a combination of 3-deazaadenosine and homocysteine thiolactone (19). Under these conditions, both phos-

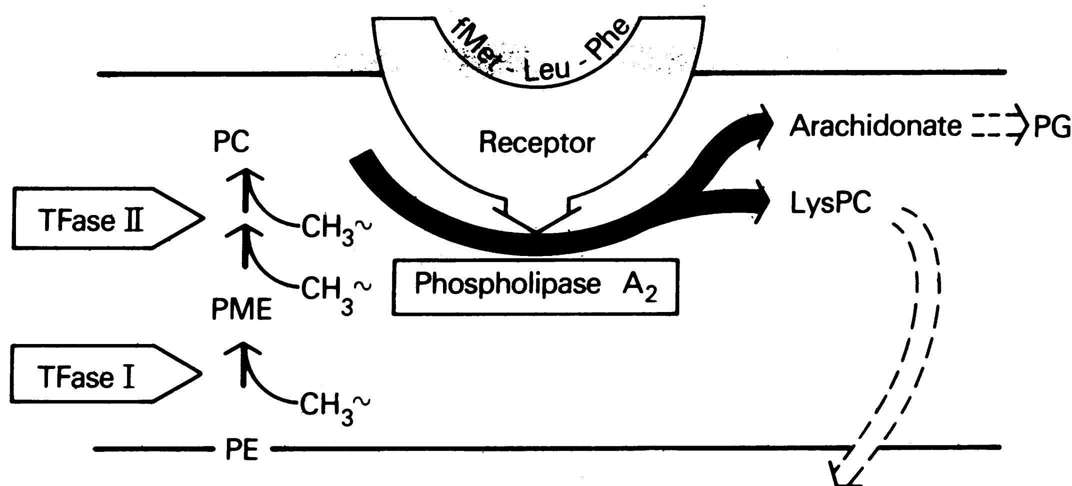

FIG. 7. Hypothetical mechanism of chemotactic receptor action. TFase I and II, phospholipid methyltransferase I and II; PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine; PC, phosphatidylcholine; LysPC, lysophosphatidylcholine; PG, prostaglandin.

pholipid methylation and protein carboxy-*O*-methylation are inhibited, indicating that both transmethylation reactions might be important in chemotaxis. Because chemoattractants cause a rapid turnover of methylated products, such as phospholipids (and proteins), inhibition of transmethylation reactions by 3-deazaadenosine would lead to the depletion of the substrate for the degradation pathway, which then could bring about the inhibition of chemotaxis. The interaction of methylation and degradation of phospholipids is suggested by the following observations. Mepacrine, a phospholipase A_2 inhibitor, also inhibits phospholipid methylation in intact leukocytes. However, this compound does not inhibit or activate phospholipid methylation in isolated membrane preparations, whose structural integrity is lost (unpublished data).

Our previous work has demonstrated that phospholipid methylation alters the structure and function of biomembranes (3-5). The methyltransferases of phosphatides are asymmetrically distributed across the membrane and permit the translocation of phospholipids (3) (Fig. 7). The accumulation of phosphatidyl-*N*-monomethylethanolamine within the membrane increases the membrane fluidity (4). Phospholipid methylation and membrane fluidity are stimulated by binding of agonists to β-adrenergic receptors, which then enhances the coupling of these receptors with adenylate cyclase in rat reticulocytes (5). It is likely that the degradation of methylated phospholipids also results in a rearrangement of these lipids across the membrane—e.g., lysophosphatidylcholine has been reported to translocate rapidly across the membrane (20). Such movements of phospholipids might regulate the dynamic functions of the membrane and contribute to the chemotaxis process.

Chemotaxis of leukocytes is one manifestation of inflammatory reactions. The release of arachidonic acid from phospholipids is potentially important because this fatty acid is a precursor of prostaglandins and prostacyclines which play a critical role in inflammation.

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1. Schiffmann, E., Corcoran, B. A. & Aswanikumar, S. (1978) in *Leucocyte Chemotaxis*, eds. Gallin, J. I. & Quie, P. G. (Raven, New York), pp. 97-107.
2. O'Dea, R. F., Viveros, O. H., Axelrod, J., Aswanikumar, S., Schiffmann, E. & Corcoran, B. A. (1978) *Nature (London)* **272,** 462-464.
3. Hirata, F. & Axelrod, J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2348-2352.
4. Hirata, F. & Axelrod, J. (1978) *Nature (London)* **275,** 219-220.
5. Hirata, F., Strittmatter, W. J. & Axelrod, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 386-390.
6. Showell, H. J. (1976) *J. Exp. Med.* **143,** 1154-1169.
7. Snyderman, R., Phillips, J. & Mergenhagen, S. E. (1970) *Infect. Immun.* **1,** 521-525.
8. Hirata, F., Viveros, O. H., Diliberto, E., Jr. & Axelrod, J. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 718-721.
9. Kennedy, E. P. & Weiss, S. B. (1956) *J. Biol. Chem.* **222,** 193-197.
10. Dils, R. R. & Hübscher, H. (1961) *Biochim. Biophys. Acta* **46,** 505-513.
11. Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B. A. & Becker, E. L. (1976) *J. Exp. Med.* **143,** 1154-1169.
12. Yorio, T. & Bentley, P. J. (1978) *Nature (London)* **271,** 79-81.
13. Hong, S. L. & Levine, L. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1730-1734.
14. Gallin, J. I., Wright, D. G. & Schiffmann, E. (1978) *J. Clin. Invest.* **62,** 1364-1374.
15. Estensen, R. D., Hill, H. R., Quie, P. G., Hogan, N. & Goldberg, N. O. (1973) *Nature (London)* **245,** 458-460.
16. Levine, L. & Ohuchi, K. (1978) *Nature (London)* **276,** 274-275.
17. Shier, W. T., Baldwin, J. H., Nilsen-Hamilton, M., Hamilton, R. T. & Thanassi, N. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1586-1590.
18. Pike, M. C., Kredich, N. M. & Snyderman, R. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3928-3932.
19. Schiffmann, E., O'Dea, R. F., Chiang, P. C., Venkatasubramanian, K., Corcoran, B. A., Hirata, F. & Axelrod, J. (1979) in *ICN-UCLA Symposium on Covalent and Noncovalent Modulation of Protein Function*, ed. Atkinson, D. (Academic, New York), in press.
20. van Zoelen, E. J. J., de Kruijff, B. & van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta* **508,** 97-108.