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INHIBITION OF PLATELET AND NEUTROPHIL PHOSPHOLIPASE A_2 BY HYDROXYEICOSATETRAENOIC ACIDS (HETES)

A NOVEL PHARMACOLOGICAL MECHANISM FOR REGULATING FREE FATTY ACID RELEASE

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Abstract—The present study demonstrated that acid-extracted platelet phospholipase A_2 (PLA_2) exhibited marked hydrolytic activity against both [1-^{14}C]oleic acid- and [1-^{14}C]arachidonic acid-labeled *Escherichia coli*. The rate of hydrolysis was linear up to 30 min and was directly proportional to the amount of enzyme added to the reaction mixture. The data further indicated that 5-hydroxy-6,8,11,15-eicosatetraenoic acid (5-HETE) inhibited platelet PLA_2 in a dose-dependent manner ($IC_{50} = 42\ \mu M$), whereas 5-lactone HETE had no inhibitory effect up to 100 μM. The degree of inhibition of PLA_2 activity was unaffected by Ca^{2+} concentrations but was reduced in the presence of increasing amounts of *E. coli* substrate. Both 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) also inhibited platelet PLA_2 activity ($IC_{50} = 26$ and 72 μM respectively). Furthermore, the inhibitory effects of these monoHETEs were confirmed with a PLA_2 preparation derived from rat neutrophils. Thus, these data suggest a novel pharmacological action of HETEs on PLA_2 which may have potential ramifications in the regulation of arachidonic acid metabolism.

Recent studies suggest that lipoxygenase products such as hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs) play a feedback role in the regulation of arachidonic acid metabolism (see review, Ref. 1). In 1976, Moncada *et al.* [2] reported that 15-HPETE inhibited prostaglandin I_2 (PGI_2) synthesis by pig aortic microsomes and hypothesized that endogenous lipid peroxidation may inhibit the formation of the antiaggregatory substance, PGI_2, from endothelial cells. Recent evidence further suggests that HETEs, the more stable products of HPETEs, also inhibit the synthesis of lipoxygenase products such as 5-HETE, leukotriene B_4 (LTB_4) and LTC_4. For example, 5-HETE, 12-HETE and 15-HETE reduce leukotriene synthesis by rabbit neutrophils [3, 4], and 5-HETE and 15-HETE, but not 12-HETE, inhibit the endogenous formation of 12-HETE by rabbit platelets [5]. The inhibitory effect of 15-HETE has since been confirmed using human neutrophils and platelets [6]. Interestingly, 12-HPETE but not 12-HETE activates 5-lipoxygenase reactions [7]. Thus, the synthesis of arachidonic acid metabolites may be regulated by feedback mechanisms operating within the cascade.

It is generally accepted that release of free arachidonic acid from membrane phospholipids is the critical first step in the initiation of eicosanoid synthesis. Indeed, the antiinflammatory steroids are thought to inhibit eicosanoid synthesis by inducing the synthesis of a phospholipase A_2 (PLA_2) inhibitory protein termed macrocortin or lipomodulin [8, 9]. Two major pathways of arachidonic acid release have been proposed to account for phospholipid hydrolysis. First, the PLA_2-mediated cleavage of arachidonic acid from the 2-position of phosphatidylcholine and phosphatidylethanolamine occurs during platelet activation [10]. In support of this, it has been demonstrated that, during platelet activation by thrombin, there is a loss of radioactivity associated with phosphatidylcholine and phosphatidylethanolamine previously prelabeled with [1-^{14}C]arachidonic acid [11–14]. Recent findings further indicate that lysophosphatidylcholine and lysophosphatidylethanolamine are formed within seconds after thrombin-induced platelet aggregation [15, 16].

Second, phosphatidylinositol, which turns over very rapidly, may also serve as the initial source for arachidonic acid; however, the mechanism of release of free arachidonic acid released from this phospholipid is controversial. Lapetina [17] proposed that the intermediate, phosphatidate, formed through the action of a phosphatidylinositol specific phospholipase C and diglyceride kinase, serves as the substrate for phospholipase A_2. In contrast, others have argued that diglyceride is cleaved directly by a diglyceride lipase to release arachidonic acid [18]. It is, of course, possible that arachidonic acid release occurs by a combination of these mechanisms as has been suggested recently [16].

In spite of the importance of PLA_2 in arachidonic acid release, several laboratories failed to demonstrate sufficient PLA_2 activity in isolated platelet extracts to account for the amount of free arachidonic acid required for conversion to cyclooxygenase and lipoxygenase products [19, 20]. A recent study by Ballou and Cheung [21], however, has suggested

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that, under normal isolation procedures, PLA_2 may be suppressed by an endogenous inhibitor. The exact nature of the inhibitor is unknown but appears to be related to unsaturated fatty acids [22]. We now show that HETEs inhibit PLA_2 activity. This inhibitory activity has not been reported previously and suggests that PLA_2 activity may be inhibited by lipids derived from the lipoxygenase pathway.

MATERIALS AND METHODS

Materials. All solvents were of reagent grade and purchased from Fisher Scientific, King of Prussia, PA. Aluminium-backed thin-layer chromatographic plates were purchased from VWR, San Francisco, CA. Autoclaved [1-^{14}C]oleic acid- and [1-^{14}C]arachidonic acid-labeled *Escherichia coli* were prepared for us by Dr. Richard C. Franson, Department of Biochemistry, Virginia Commonwealth University, Richmond, VA. 5-HETE and 5-lactone HETE were synthesized at Wyeth Laboratories by Dr. A. Kreft. 15-HETE was a gift from Dr. Jack Y. Vanderhoek of George Washington University, Washington, DC. 12-HETE was purchased from Seragen, Boston, MA. Hydrofluor was purchased from National Diagnostics, Somerville, NJ.

Isolation of PLA_2. Membrane-associated PLA_2 was isolated and solubilized as previously described [23]. Briefly, expired human platelets obtained from the blood bank were centrifuged for 15 min at 200 *g* to remove red blood cells. The platelet plasma was removed and centrifuged at 2500 *g* for 15 min to obtain a platelet pellet. After removal of the plasma, a suitable volume of cold 0.18 N sulfuric acid (4 ml/unit) was added, and the pellet was homogenized with a Potter–Elvehjem homogenizer. The homogenate was left for 1 hr at 4°, homogenized again, and subsequently centrifuged for 15 min at 10,000 *g*. The resulting supernatant fluid enriched in PLA_2 activity was removed, and the amount of protein was determined by standard procedures and either kept at −20° or dialyzed extensively against acetate buffer (pH 4.5). Since no difference in terms of enzyme activity was observed between the acid extract and the dialyzed material, most experiments were performed using the acid extract. Confirmation of the specificity of the phospholipase present in the acid extract has been demonstrated previously [23]. PLA_2 extracts were similarly prepared from rat glycogen-elicited neutrophils. Rat peritoneal neutrophils were obtained from Wistar rats (150–250 g) that received an intraperitoneal injection of 6% glycogen (10 ml). After 24 hr, rats were killed by CO_2 asphyxiation, and peritoneal cells were harvested by peritoneal lavage using Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. The peritoneal exudate was then centrifuged at 400 *g* for 10 min, the lavaged fluid was removed and the cell pellet (36×10^7 cells) was homogenized in 2 ml of cold 0.18 N sulfuric acid. Confirmation of the specificity of the phospholipase present in neutrophil acid extract has been demonstrated previously [24].

Assay for PLA_2 activity. PLA_2 activity was measured by the hydrolysis of [1-^{14}C]oleic acid-labeled autoclaved *E. coli* that was prepared by the method of Franson [25]. Unless otherwise stated, incubation mixtures routinely contained the following: 100 mM Tris buffer, 5 mM $CaCl_2$, 2.5×10^6 *E. coli* substrate (equivalent to 5 nmoles of phospholipid), platelet extracts (100–115 μg protein), and the appropriate concentrations of drug or drug solvent. Incubations were carried out at 37° in a shaking water bath for 30 min. Preliminary experiments showed that the rate of hydrolysis was linear up to 30 min. The reaction was terminated by the addition of 3 vol. of $CHCl_3/CH_3OH$ (1:2, v/v) and vortexed. Samples were then placed in a shaking water bath at 37° for an additional 15 min. Subsequently, 1.5 ml of water and 1 ml of $CHCl_3$ were added to each sample. The lower $CHCl_3$ phase was removed and evaporated under N_2. The dry residue was redissolved in 50 μl of $CHCl_3/CH_3OH$ (9:1, v/v), spotted on aluminium-backed thin-layer chromatographic plates, and developed in a solvent system consisting of petroleum ether–diethyl ether–acetic acid (80:20:1). Lipids were visualized by exposure to I_2 vapor, and the radioactive spots corresponding to authentic phospholipids or free fatty acid were cut from the plate. Radioactivity on the strip was determined by the addition of 1 ml of CH_3OH followed by 10 ml of hydrofluor and counting in a liquid scintillation counter.

The percentage hydrolysis was calculated by the following equation:

$$\% \text{ Hydrolysis} = \frac{\text{free fatty acid (dpm)}}{\text{total phospholipid and free fatty acid (dpm)}}$$

$$\text{Rate of hydrolysis (nmoles/min)} = \frac{\% \text{ hydrolysis} \times \text{phospholipid (5 nmoles)}}{\text{incubation time (min)}}$$

RESULTS

Effects of time and protein concentration on platelet PLA_2 activity. Figure 1A shows that platelet PLA_2 extracts hydrolyzed [1-^{14}C]oleic acid-labeled *E. coli* in a time-related fashion. The hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli* by platelet extracts (115 μg protein) was rapid and linear up to 30 min and the mean rate of hydrolysis was calculated to be 20 nmoles phospholipids/hr/mg protein. Figure 1B shows that the release of free oleic acid from *E. coli* phospholipids was also linear with protein concentration up to 230 μg of protein.

Effect of HETEs on platelet PLA_2 activity. Figure 2 demonstrates the effects of HETEs on the hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli* by platelet PLA_2. The addition of 5-HETE to the reaction mixture inhibited PLA_2 activity dose dependently; at 100 μM, 5-HETE reduced PLA_2 activity by 90% and the calculated IC_{50} was 42 μM. In contrast, 5-lactone HETE up to 100 μM did not inhibit PLA_2 activity. In light of the fact that 5-HETE is not the only HETE that can be synthesized by mammalian cells, we also examined two other major isomers of HETE that can be derived from lipoxygenase reactions, namely 12-HETE and 15-HETE. Figure 2 shows that both 12-HETE ($IC_{50} = 26$ μM) and 15-HETE ($IC_{50} = 72$ μM) were effective inhibitors of platelet PLA_2 activity. It should also be noted that both PGE_2 and

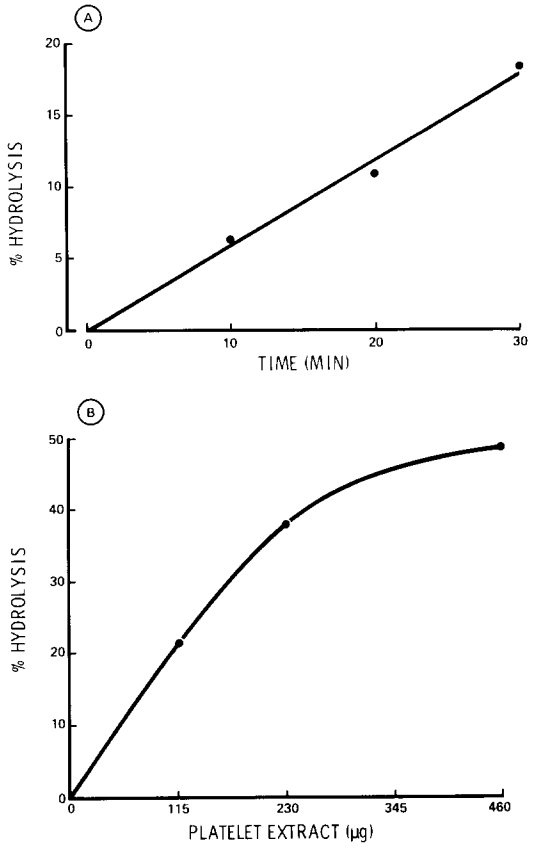

Fig. 1. Effects of time and platelet protein concentration on the hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli*. Platelet PLA_2 was solubilized and isolated by acid treatment as described in Materials and Methods. Panel A depicts the rate of hydrolysis with respect to time by a fixed protein concentration of platelet lysates (115 μg). Panel B shows the degree of hydrolysis of *E. coli* substrate when incubated with various concentrations of platelet lysates for 30 min at 37°. All values are expressed as the mean percent hydrolysis of substrate of two separate determinations.

LTB_4 had no effect on the hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli* by platelet PLA_2 (data not shown).

Since arachidonic acid and not oleic acid is the natural substrate for subsequent conversion to prostaglandins and leukotrienes, the ability of 5-HETE to inhibit the hydrolysis of [1-^{14}C]arachidonic acid-labeled *E. coli* was examined. 5-HETE (12.5 to 100 μM) also inhibited the hydrolysis of [1-^{14}C]-arachidonic acid-labeled *E. coli* by platelet PLA_2 dose dependently (IC_{50} = 38 μM).

Effect of Ca^{2+} *and substrate concentration on 5-HETE-induced platelet* PLA_2 *inhibition.* Because PLA_2 activity was affected by decreasing concentrations of Ca^{2+}, the effect of Ca^{2+} on the inhibitory activity of 5-HETE was investigated in comparison to the control PLA_2 activity at each Ca^{2+} concentration. At 0.1 mM Ca^{2+}, 5-HETE (50 μM) reduced PLA_2 activity by 68% (4.4 ± 0.2 vs 13.6 ± 0.2 nmoles phospholipids/hr/mg protein). Similarly, 5-HETE was effective in reducing PLA_2

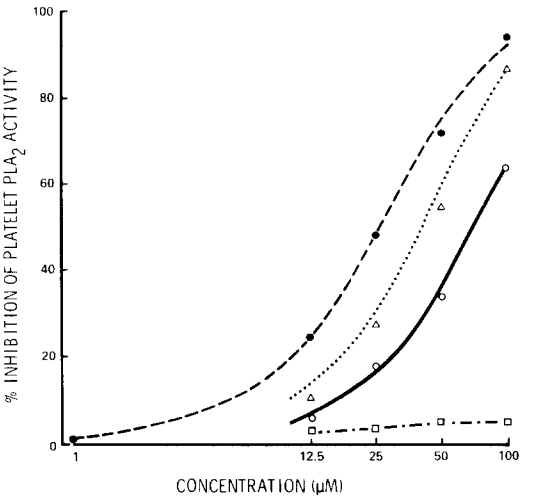

Fig. 2. Dose–response curves of 5-HETE, 12-HETE and 15-HETE for inhibiting human platelet PLA_2 activity. Platelet PLA_2 was isolated by acid treatment as described in Materials and Methods. An aliquot of platelet extract (115 μg protein) was added to [1-^{14}C]oleic acid-labeled autoclaved *E. coli* and incubated with various concentrations of 5-HETE (△), 5-lactone HETE (□), 12-HETE (●), and 15-HETE (○). Values are expressed as the mean percent inhibition of *E. coli* substrate hydrolysis of at least four determinations.

activity by 63% (6.8 ± 0.2 vs 18.1 ± 0.9 nmoles phospholipids/hr/mg protein) in incubations containing 5 mM Ca^{2+}.

To define further the mechanism of inhibition of PLA_2 activity, experiments using various amounts of [1-^{14}C]oleic acid-labeled *E. coli* substrate were performed. It can be seen in Fig. 3 that the addition

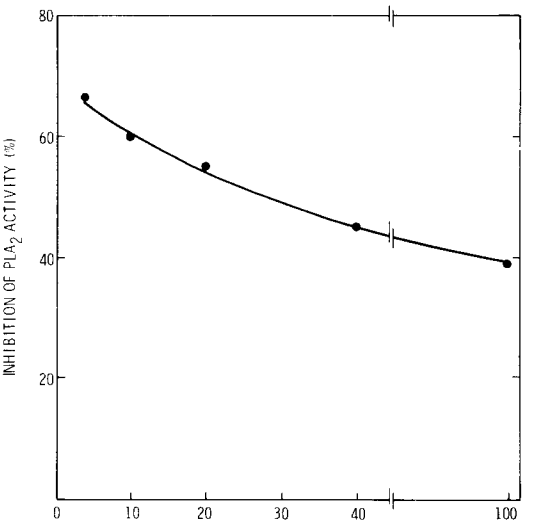

Fig. 3. Effect of substrate concentration on the inhibitory activity of 5-HETE on human platelet PLA_2 activity. Incubations were carried out in the absence or presence of 5-HETE using the indicated concentrations of [1-^{14}C]oleic acid-labeled autoclaved *E. coli*. A fixed concentration of 5-HETE (50 μM) was added to the reaction mixture, and percent hydrolysis was determined. Results are expressed as the mean percent inhibition of hydrolysis and do not vary by more than 10%.

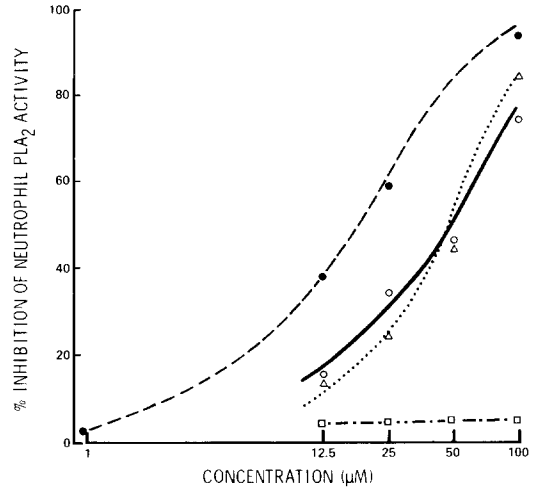

Fig. 4. Dose–response curves of 5-HETE, 12-HETE and 15-HETE for inhibiting rat neutrophil PLA_2 activity. Neutrophil PLA_2 was isolated by acid treatment as described in Materials and Methods. An aliquot of neutrophil extract (115 μg protein) was added to [1-^{14}C]oleic acid-labeled autoclaved *E. coli* and incubated with various concentrations of 5-HETE (△), 5-lactone HETE (□), 12-HETE (●), and 15-HETE (○). Values are expressed as the mean percent inhibition of *E. coli* substrate hydrolysis of at least four determinations.

of increasing amounts of substrate decreased the inhibitory effect of 5-HETE. For example, whereas 5-HETE (50 μM) reduced PLA_2 activity by 66% in the presence of a 10 μM concentration of substrate, 5-HETE at the same concentration reduced PLA_2 activity by only 38% when the reaction mixture contained 100 μM substrate.

Effect of 5-HETE, 12-HETE and 15-HETE on neutrophil PLA_2 activity. Figure 4 shows that PLA_2 isolated under identical conditions from rat neutrophils was also inhibited by 5-HETE, 12-HETE and 15-HETE. As observed in the experiments using platelet PLA_2, 5-HETE (IC_{50}) = 48 μM), 12-HETE (IC_{50} = 18 μM) and 15-HETE (IC_{50} = 50 μM) inhibited the hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli* by neutrophil PLA_2. The rank order of potency was 12-HETE > 5-HETE ≥ 15-HETE.

DISCUSSION

In the present study, our data show that acid extraction of platelet homogenates yielded a supernatant fraction that was enriched in PLA_2 activity, as previously demonstrated [23]. We further demonstrated that the rate of hydrolysis by this PLA_2 preparation was rapid, linear with time up to 30 min, and directly proportional to the amount of protein added to the reaction mixture. Although not investigated in the present study, previous reports have demonstrated that the phospholipase found in platelet acid extracts is of the A_2 type since incubation with [1-^{14}C]stearyl-2-acyl-3-sn-phosphatidylethanolamine yielded only radioactive lysophosphatidylethanolamine [23]. It is interesting to note that the acid-extracted enzyme is extremely active when compared to previously published PLA_2 activity found in platelet homogenates prepared under neutral pH. For example, in two reports where PLA_2 was initially prepared in a neutral pH buffer, the rate of hydrolysis ranged from 0.04 to 0.6 nmoles/hr/mg protein [19, 20] and is at least 33-fold less active than that observed with our preparation. It is, therefore, possible to markedly improve the recovery of platelet PLA_2 activity when the cells are extracted by acid.

This report also presents the first direct evidence that 5-HETE, an important product derived from 5-lipoxygenase reactions, inhibits PLA_2-induced hydrolysis of [1-^{14}C]oleic acid-labeled *E. coli*. The data showed that 5-HETE inhibited both neutrophil and platelet PLA_2 in a dose-dependent manner, whereas 5-lactone HETE, the cyclized derivative of 5-HETE, failed to inhibit PLA_2 up to 100 μM. In addition, 5-HETE is also able to prevent the hydrolysis of [1-^{14}C]arachidonic acid-labeled *E. coli* by platelet PLA_2 (unpublished observations). The ability to inhibit PLA_2 activity appears to be a general property of monoHETEs since both 12-HETE and 15-HETE also inhibited PLA_2. Whereas 12-HETE was twice as potent as 5-HETE, 15-HETE was 1.7 times less potent than 5-HETE in terms of inhibitory activity. These results suggest that the inhibitory potency is dependent on the position of the hydroxyl group.

To define further the mechanism of action of monoHETEs, the effects of Ca^{2+} and substrate concentration were investigated. No significant changes in the inhibitory potency of 5-HETE were observed when the Ca^{2+} concentration in the reaction mixture was varied from 0.1 to 5 mM. Therefore, it is unlikely that 5-HETE is acting as a calcium chelator or a calmodulin antagonist. In contrast, increasing concentrations of *E. coli* substrate reduced the inhibitory action of 5-HETE. For example, the addition of the highest substrate concentration tested (100 μM) reduced the degree of inhibition seen with 50 μM 5-HETE by 23%, suggesting that 5-HETE is a reversible substrate competitor rather than a direct enzyme inhibitor.

The inhibition of PLA_2 activity by monoHETEs may provide an explanation for the low PLA_2 activity found in platelet homogenates by previous studies [19, 20]. Since monoHETEs are labile in acid and acid treatment improves the recovery of PLA_2 activity, it is tempting to speculate that the low PLA_2 activity detected under normal conditions is due to the presence of monoHETEs (especially 12-HETE) which are likely to be produced during homogenization of platelets [26]. A corollary to this is that the lack of PLA_2 activity in platelet homogenates cannot be used as an argument against the contribution of the PLA_2 pathway to arachidonic acid release [18]. The existence of endogenous lipids that inhibit PLA_2 activity has been suggested recently by the work of Ballou and Cheung [21] and, in abstract form [22], the same workers suggested that the identities of these lipids are unsaturated fatty acids.

Inhibition of PLA_2 by 12-HETE could also provide a mechanism by which the availability of the substrate, arachidonic acid, is reduced for subsequent conversion to leukotrienes. In light of the observation that 12-HPETE, the precursor of 12-HETE, has an opposing effect, i.e. increases leukotriene synthesis [7], it is tempting to speculate that

the endogenous regulation of leukotriene synthesis may in part be determined by the relative contributions of 12-HETE and 12-HPETE.

In conclusion, the present study describes a novel pharmacological action of monoHETEs on PLA_2 activity; however, it is not known whether monoHETEs exert such actions endogenously. Until intracellular concentrations of HETEs are determined, the physiological significance of the ability of monoHETEs to inhibit PLA_2 remains to be determined. Nevertheless, we speculate that in addition to the recently described PLA_2 inhibitory proteins, termed lipomodulin or macrocortin [8, 9], monoHETEs can provide an alternative mechanism for regulating PLA_2 activity.

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REFERENCES

1. P. Borgeat, B. F. DeLaclos and J. Maclouf, *Biochem. Pharmac.* **32**, 381 (1983).
2. S. Moncada, R. J. Gryglewski, S. Bunting and J. R. Vane, *Prostaglandins* **12**, 715 (1976).
3. J. Y. Vanderhoek, R. W. Bryant and J. M. Bailey, *J. biol. Chem.* **255**, 5996 (1980).
4. J. Y. Vanderhoek, R. W. Bryant and J. M. Bailey, *J. biol. Chem.* **255**, 10064 (1980).
5. J. Y. Vanderhoek, R. W. Bryant and J. M. Bailey, *Biochem. Pharmac.* **31**, 3463 (1982).
6. B. F. DeLaclos, P. Braquet and P. Borgeat, *Prost. Leuk. Med.* **13**, 47 (1984).
7. J. Maclouf, B. F. DeLaclos and P. Borgeat, *Proc. natn. Acad. Sci. U.S.A.* **79**, 6042 (1983).
8. R. J. Flower and G. J. Blackwell, *Nature, Lond.* **278**, 456 (1979).
9. F. Hirata, E. Schiffman, K. Verkatasubramanian, D. Salomon and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2533 (1980).
10. T. K. Bills, J. B. Smith and M. J. Silver, *Biochem. biophys. Acta* **424**, 303 (1976).
11. T. K. Bills, J. B. Smith and M. J. Silver, *J. clin. Invest.* **60**, 1 (1977).
12. E. G. Lapetina, K. Chandrabose and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **75**, 818 (1978).
13. S. Rittenhouse-Simmons, F. A. Russell and D. C. Deykin, *Biochim. biophys. Acta* **488**, 379 (1977).
14. M. Guichardant and M. Lagarde, *Thromb. Res.* **18**, 285 (1980).
15. M. L. McKean, J. B. Smith and M. J. Silver, *J. biol. Chem.* **256**, 1522 (1981).
16. M. J. Brockman, J. W. Ward and A. J. Marcus, *J. clin. Invest.* **66**, 275 (1980).
17. E. G. Lapetina, *Trends pharmac. Sci.* **3**, 115 (1982).
18. R. L. Bell, D. A. Kennerly, N. Stanford and P. W. Majerus, *Proc. natn. Acad. Sci. U.S.A.* **76**, 3238 (1979).
19. G. J. Blackwell, W. C. Duncombe, R. J. Flower, M. F. Parsons and J. R. Vane, *Br. J. Pharmac.* **59**, 353 (1977).
20. S. Darkson and P. Cohen, *J. biol. Chem.* **250**, 9342 (1975).
21. L. R. Ballou and W. Y. Cheung, *Proc. natn. Acad. Sci. U.S.A.* **80**, 5203 (1983).
22. L. R. Ballou and W. Y. Cheung, *Fedn Proc.* **43**, 1463 (1984).
23. R. L. Jesse and R. C. Franson, *Biochim. biophys. Acta* **575**, 467 (1979).
24. R. Franson, P. Patriaca and P. Elsbach, *J. Lipid Res.* **15**, 380 (1974).
25. R. C. Franson, in *Liposomes: from Physical Structure to Therapeutic Applications* (Ed. G. Knight), p. 349. Elsevier, New York (1979).
26. D. H. Nugteren, *Biochim. biophys. Acta* **380**, 299 (1975).