Arachidonyl Trifluoromethyl Ketone, a Potent Inhibitor of 85-kDa Phospholipase A2, Blocks Production of Arachidonate and 12-Hydroxyeicosatetraenoic Acid by Calcium Ionophore-challenged Platelets*

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Arachidonyl trifluoromethyl ketone (AACOCF₃) is a potent and selective slow binding inhibitor of the 85-kDa cytosolic phospholipase A₂ (cPLA₂) (Street, I. P., Lin, H.-K., Laliberté, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K., and Gelb, M. H. (1993) Biochemistry 32, 5935-5940). AACOCF₃ and a number of its structural analogues have been used to investigate the role of cPLA₂ in the cellular generation of free arachidonic acid (AA) and in eicosanoid biosynthesis. AACOCF₃ inhibited the release of AA from calcium ionophore-challenged U₉37 cells (IC₅₀ = 8 µM, 2 × 10⁶ cells ml⁻¹) and from platelets (IC₅₀ = 2 µM, 4 × 10⁶ cells ml⁻¹). Arachidonyl methyl ketone (AACH(OH)CF₃) also did not inhibit the production of AA in the ionophore-challenged cells. In addition to the release of AA, AACOCF₃ also inhibited the production of 12-hydroxyeicosatetraenoic acid (12-HETE) and thromboxane B₂, two of the major metabolites of AA produced by platelets. The inhibition of 12-HETE biosynthesis showed a dose dependence similar to that of AA release in ionophore-challenged platelets; however, when platelet 12-HETE production was stimulated with 10 µM AA to circumvent the PLA₂-dependent step, AACOCF₃ no longer inhibited the production of 12-HETE. In contrast, AACOCF₃ blocked thromboxane B₂ formation by both calcium ionophore- and AA-challenged platelets, indicating that the compound affects the cyclooxygenase pathway in addition to AA release. The crude cytosol and membrane fractions from platelets were assayed for calcium-dependent and calcium-independent PLAp activities and for the susceptibility of each to inhibition by AACOCF₃. At AACOCF₃ concentrations as high as 10 mol %, only one of the observed PLA₂ activities was inhibited by more than 25%. The AACOCF₃-susceptible PLA₂ (77% inhibition at 1.6 mol %) was found in the cytosolic platelet fraction and showed the functional characteristics of the cPLA₂. These results suggest that the cPLA₂ plays an important role in the generation of free AA for 12-HETE biosynthesis in platelets.

Eicosanoids are an important class of lipid mediators that modulate a wide variety of physiological functions. In many cell types, the rate-determining step in the generation of eicosanoids is the release of arachidonic acid (AA) from its cellular store in the phospholipid pool. A number of different pathways for the mobilization of AA have been proposed: phospholipase C in concert with glycerol lipases (1), lysophospholipase (2), and phospholipase A₂ (PLA₂). Detailed studies of the distribution and stoichiometry of metabolites have demonstrated the importance of PLA₂ as a major mediator of agonist-induced AA release in many cell types (3-5). Over the past decade a number of distinct types of PLA₂ have been isolated and characterized. The best known of these are a family of 14-kDa calcium-dependent secreted enzymes (sPLA₂), an 85-kDa cytosolic calcium-dependent enzyme (cPLA₂), and intracellular calcium-independent enzymes.

There has been a great deal of debate about the relative importance of each type of PLA₂ to the overall process of cellular AA mobilization. The 14-kDa PLA₂ require millimolar concentrations of calcium for activity, and they do not exhibit selectivity toward the fatty acid at the sn-2 position of the phospholipid. Therefore, based on these properties it appears unlikely that the role of this type of PLA₂ is to initiate AA release from inside the cell. However, evidence has been provided that sPLA₂ might play a role in the production of prostaglandins in certain cell types. Barbour and Dennis (6) reported that the antisense inhibition of the 14-kDa PLA₂ expression blocked the production of prostaglandin E₂ by P388D cells. It has also been proposed that the 14-kDa PLA₂ may be involved in AA release and prostanooid production in human umbilical vein endothelial cells (7) and mesangial cells (8).

Calcium-independent PLA₂ have been isolated from human and canine myocardium (9, 10) and P388D cells (6). The compound (E)-6-(bromomethylene)-tetrahydro-3-(1-naphthalenyl)-2H-pyrane-2-one (HEL) is a potent suicide inhibitor of the myocardial calcium-independent PLA₂ (11) and selectively inhibits this enzyme versus other known PLA₂ (12). Thus HEL has
been useful in determining the contribution made by this type of PLA₂ to AA mobilization by pancreatic islet cells (13) and aortic smooth muscle cells (14).

The cytosolic PLA₂ is thought to be involved in the production of AA for eicosanoid production since it preferentially hydrolyzes phospholipids containing AA at the sn-2 position (15, 16), responds to physiological changes in calcium concentration by translocating to membranes (15, 17), and is activated by hormonal signaling through phosphorylation of a serine residue (18, 19). However, no direct evidence for the involvement of this enzyme in the production of eicosanoids has been provided. Recently, we have described the first potent and selective inhibitor of the cPLA₂ (20). An analogue of AA in which the -COOH functionality is replaced by -COF₂ (AACOCF₂) is a potent slow tight binding inhibitor of cPLA₂. AACOCF₂ also shows 500-fold greater potency against cPLA₂ than sPLA₂ (20). Through use of AACOCF₂ and a number of its close structural analogues we report that cPLA₂ is the major mediator of AA release in calcium ionophore-stimulated platelets and U937 cells. Furthermore, we demonstrate that in platelets the AA mobilized by the action of the cPLA₂ is subsequently utilized by the 12-lipoxygenase pathway for production of 12-HETE.

MATERIALS AND METHODS

Preparation of Platelets and U937 Cells—U937 cells were grown and differentiated with Me₂SO as described by Tremblay et al. (21). Platelets were prepared from human venous blood obtained from healthy volunteers. The collected blood was immediately mixed with 0.1 volume of anticoagulant solution (65 mm citric acid, 86 mm sodium citrate, and 2% glucose) and centrifuged at 300 × g for 10 min. The supernatant was mixed with 50% (by volume) Hanks’ balanced salt solution buffered with 15 mm HEPES pH 7.4 (HBSS) and a 30% volume of the anticoagulant solution. This mixture was centrifuged at 750 × g for 10 min, and the pellet was resuspended in HBSS. Platelet concentration was determined with a Coulter counter.

Release and Measurement of AA in Platelets and U937 Cells—Human platelets were resuspended to a final density of 4 × 10⁹/ml in HBSS and ETYA added from a Me₂SO stock solution to give a final concentration of 10 μM. U937 cells were resuspended to a final density of 2 × 10⁶/ml in HBSS in which the inhibitor or of Me₂SO only was added, and the mixtures were incubated at 37 °C for 2 min. Calcium ionophore (A23187, 10 μM for U937 cells, 2 μM for platelets) was added, and incubation continued for 10 min in the case of the platelets and 3 min for the U937 cells. The cells were extracted with 1.25% Dole’s solution (10 mmol Na₂CO₃, 1 mmol sulfuric acid; 10:40:1), followed by 0.75 ml of heptane, 0.5 ml of water, and vortexing. The heptane phase was removed and dried (Na₂SO₄), and the pentafluorobenzyl esters of the fatty acids were prepared and quantified by gas chromatography-mass spectrometry with reference to an internal standard of C₁₅:0 as described by Li et al. (22).

Release and Measurement of TXB₂ and 12-HETE from Platelets—Platelets at a final concentration of 4 × 10¹⁰/ml (0.5 ml) were preincubated for 2 min at 37 °C before stimulation with 2 μM calcium ionophore (A23187) or 10 μM AA. After another 10-min incubation (1) at 37 °C, 0.25 ml of cold methanol was added to stop the reaction, and TXB₂ levels were measured by enzyme immunoassay (Miles Chemical). The procedure for 12-HETE measurements, 150 μl of the mixture was acidified with 10 μl of 3 N acetic acid, and the sample was analyzed by reverse-phase HPLC on a C₁₈ Nova-Pak column with acetonitrile/water/ acetic acid (60:40:0.1) as the solvent (23)./n

Phospholipase A₂ Assays—Samples of the purified recombinant cPLA₂ were prepared as described by Street et al. (20), and the purified recombinant 14-kDa synovial fluid-type PLA₂ was prepared as described by Tremblay et al. (23). A suspension of platelets was prepared from 60 ml of human blood as described above and centrifuged at 750 × g for 10 min. The platelets were resuspended in 20 ml of a buffer containing 10 mmol HEPES pH 7.5, 1 mmol EDTA, 100 mmol KC₃, 1 mmol phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), and the cells were broken by sonication. The suspension was centrifuged at 110,000 × g for 60 min; the cytosolic and the microsomal fractions separated, and each was assayed for PLA₂ activity. The assay procedure previously described for the recombinant 14-kDa PLA₂ was used (23) with the following modifications. The assays contained 30 μl 10-Py-FM, 50 μM Tris-HCl (pH 8.1), 2.5 mm CaCl₂ in a volume of 100 μl. Assays were incubated for 30 min at 37 °C and quenched by the addition of 20 μl EDTA and 0.9 ml of acetonitrile. The assay buffers for calcium-independent PLA₂ contained 5 mm EDTA and 50 mm Tris-HCl (pH 8.1). The activity was measured using the assay conditions described by Street et al. (20), and samples were incubated for 30 min at 37 °C. Inhibitors were added directly to the assay mixture from a Me₂SO stock solution.

Metabolism of AACOCF₂ by Cells and Hepatic Microsomes—A JEOL HX110 A mass spectrometer operating in continuous flow liquid secondary ion mass spectrometry mode (CF-LSIMS) was used to characterize the reduction of AACOCF₂ in human platelets and U937 cells. A Waters 600-MS HPLC was operated at a flow of 0.9 ml/min. The flow was split just prior to a Rhodyne injector to produce a flow of 3 ml/min through a Spherisorb 5.02 × 100-mm capillary column then flowing to the frit probe of the mass spectrometer. A linear gradient of 70–85% A in 25 min was used for the separation: A, 1.5% glycerol in CH₃CN; B, 20 mm NH₄OAc (pH 4.3), 1.5% glycerol. The mass spectrometer was operated in positive ion mode. Ions were produced by bombardment with primary Cs⁺ ions (20 keV); the accelerating voltage was 10 kV. AACOCF₂ (10 μM) was incubated with the cells at 37 °C (250 μl total volume) for the stated times, and then an equal volume of acetonitrile was added. The suspension was centrifuged and the supernatant removed. An aliquot (10 μl) of this was then injected directly onto the CF-LSIMS system. The hydrate of AACOCF₂ eluted at 16 min, while the reduction product (AACOH(OH)CF₂) eluted at 20.5 min.

Hepatic microsomes were prepared from male Sprague-Dawley rats (350 g) by standard procedure (24), and aliquots were frozen at −80 °C. Microsomal incubations were conducted with 1 mg of thawed microsomal protein; 400 μl of octofar solution containing 2.5 mm MgCl₂, 2.5 mm NADP, and 25 mm glucose 6-phosphate in 125 mm phosphate buffer (pH 7.4; 2 units of glucose-6-phosphate dehydrogenase; and 12.5 μl of water. After a 2-min incubation at 37 °C in a water bath, 12.5 μl of a Me₂SO solution of AACOCF₂ was added to give a final concentration of 200 μM AACOCF₂ in a total volume of 500 μl. Blank incubations contained no AACOCF₂, and control incubations were conducted with boiled microsomes. After 5 min, the mixtures were quenched by the addition of 500 μl of acetonitrile. The precipitated protein was removed by centrifugation, and the resulting supernatant was used for CF-LSIMS.

Synthesis of Inhibitors—AACOCF₂, AACOH(OH)CF₂, and AACOCH₂CF₂ were prepared according to the method of Street et al. (20). (E)-6-(Bromomethylen)-tetrahydro-5 H-naphthalen-2-yl-2-one was synthesized according to the method of Zupan et al. (11) except that the mesylate of 3-butyn-1-ol was used instead of 4-bromobutyne. 1H NMR (400 MHz, CDCl₃) δ 2.3–2.4 (m, 2), 2.7–2.9 (m, 2), 4.5 (t, 1, J = 7 Hz), 6.1 (s, 1), 7.4–7.43 (d, 1, J = 8 Hz), 7.4–7.6 (m, 3), 7.8 (m, 2), 7.87 (1, J = 13.8 Hz).

RESULTS

Inhibition of AA Release by Differentiated U937 Cells and Human Platelets—Stimulation of differentiated U937 cells with calcium ionophore (A23187) produced a rapid release of AA, which reached a maximum level (~150 ng/10⁶ cells) at 2–4 min (Fig. 1). At longer times, the level of free AA produced by
Inhibition of Arachidonate and 12-HETE Production

In order to measure AA release by human platelets, it is necessary to inactivate both the cyclooxygenase and the 12-lipoxygenase pathways by treatment with 10 μM ETYA (25). Challenge of the treated platelets with 2 μM A23187 resulted in a rapid burst of AA release that reached a peak level of approximately 70–120 ng of AA/10⁷ platelets 1–2 min after the addition of the calcium ionophore (Fig. 1). In contrast to the U937 cells, the level of free AA in the ETYA-treated platelets did not decline at longer times. The effect of a 2-min preincubation of the platelets with 15 μM of the arachidonoyl analogues before addition of the ionophore was determined. AACOCF₃ completely inhibited AA release at this concentration, while AAChOH and AACH(OH)CF₃ both raised the levels of AA by approximately 2-fold. The inhibition of AA release by AACOCF₃ was dose-dependent, 2 μM inhibitor producing a 50% reduction in the stimulated level of free AA (Fig. 2B). HEL was used to test if a myocardial-type calcium-independent PLA₂ also might play a role in the release of AA in ionophore-stimulated platelets. Preincubation of platelets with 10 μM HEL before stimulation resulted in less than 20% inhibition of AA release.

Inhibition of 12-HETE and TxB₂ Production by Platelets—To determine the effect of inhibition of AA release on eicosanoid biosynthesis, the production of 12-HETE and TxB₂ by activated platelets (no ETYA) in the presence of AACOCF₃ and its analogues was measured. The results are shown in Table I. 12-HETE and TxB₂ were two major metabolites of arachidonic acid produced by platelets, and on stimulation with 2 μM A23187, approximately 50 ng of 12-HETE/10⁷ platelets and 30 ng of TxB₂/10⁷ platelets were produced. Treatment of the platelets with the potent cyclooxygenase inhibitor, flurbiprofen, resulted in almost complete inhibition of TxB₂ production and a 2-fold increase in the amount of 12-HETE produced. Both AACH(OH)CF₃ and AACH(OH)CF₃ behaved in a similar manner to flurbiprofen in that addition of either compound to the platelets at a concentration of 15 μM resulted in a marked inhibition of TxB₂ synthesis (70–90%) and a 2.5- to 3-fold increase in the amount of 12-HETE produced. In contrast to the result obtained with AACH(OH)CF₃, AACH(OH)CF₃, and AACH(OH)CF₃, AACOCF₃ at a concentration of 15 μM completely inhibited both 12-HETE and TxB₂ biosynthesis. The inhibition of 12-HETE biosynthesis by AACOCF₃ was concentration-dependent (IC₅₀ = 2 μM), and as shown in Fig. 2B, the dose dependence is very similar to that of AA release in ETYA-treated platelets.

Challenge of Platelets with AA—Direct addition of AA (10 μM) to platelets without A23187 challenge allows the production of 12-HETE and TxB₂ independent of the action of phospholipase A₂. Under these conditions, flurbiprofen, AACH(OH)CF₃, AACH(OH)CF₃, and AACOCF₃ all inhibited the production of TxB₂ and all increased the levels of 12-HETE (Table I). These results establish that these compounds can inhibit TxB₂ biosynthesis by a mechanism that does not directly involve the inhibition of AA release. The results also establish that none of the arachidonoyl analogues significantly inhibit the platelet 12-lipoxygenase.

Selectivity of AACOCF₃ for Inhibition of the cPLA₂ in Platelet Subfractions—The selectivity of AACOCF₃ for inhibition of the cPLA₂ versus other platelet PLAs was demonstrated using platelet membrane and cytosolic fractions. The assays used to measure the plateau PLAs activities were optimized to differentiate between sPLA₂, cPLA₂, and calcium-independent PLAs activities. The synthetic phospholipid derivative, 10-Py-PM, was used to measure the activity of sPLA₂. The sPLA₂ requires...
free calcium for hydrolysis of 10-Py-PM, and no product was observed when EDTA was added to the assay buffer in excess of the calcium chloride (Table II). The specific activity of cPLA₂ was approximately 60,000-fold lower than that of sPLA, in the 10-Py-PM assay (Table II). The activity of the cPLA, was measured using a mixed micelle substrate of ["4C"]PAPC and Triton X-100 (20, 26). In the mixed micelle assay, the cPLA₂ required free calcium for activity (1834 pmol 30 min⁻¹ µg⁻¹), and 5 mM dithiothreitol did not inhibit the enzyme activity. The cPLA₂ was also much less active when the mixed micelles were prepared with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (29.7 pmol 30 min⁻¹ µg⁻¹). The mixed micelle assay was found to be specific for the cPLA₂ versus sPLA₂, since no detectable product was formed by 0.6 µg of sPLA₂ over a 30-min incubation.

Using the 10-Py-PM and the ["4C"]PAPC/Triton X-100 mixed micelle assays, a number of distinct PLA₄ activities were observed in the crude cytosolic and membrane fractions derived from human platelets (Table II). With the 10-Py-PM assay, most of the PLA₄ activity was found in the cytosolic fraction. The cytosolic PLA₄ activities were derived from both calcium-dependent and calcium-independent enzymes. The membrane fraction also showed significant PLA₄ activities when assayed with 10-Py-PM; in this case the observed activity was derived predominantly from calcium-dependent enzyme(s). When the ["4C"]PAPC/Triton X-100 mixed micelle assay was used, significant PLA₄ activity was observed only in the cytosolic fraction. This PLA₄ activity was calcium-dependent, was not inhibited by 5 mM dithiothreitol, and showed much lower activity on mixed micelles of Triton X-100 and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine.

The susceptibility of the various platelet PLA₄s to inhibition by AACH(OH)CF₃ was tested. The results are shown in Table II. Addition of AACH(OH)CF₃ to the ["4C"]PAPC/Triton X-100 mixed micelle assay at a concentration of 1.6 mol % inhibited the recombinant cPLA₂ by 78% and the calcium-dependent PLA₂ activity in the cytosolic subfraction of the platelets by 77%. In contrast, addition of AACH(OH)CF₃ to the 10-Py-PM assay at a higher concentration of 10 mol % produced no significant inhibition of either the calcium-dependent or the calcium-independent PLA₂ activities in the soluble extracts. At this inhibitor concentration, some inhibition of the recombinant sPLA₂ (25%) and the calcium-dependent membrane-associated PLA₂ (17%) was observed. The calcium-independent PLA₂ activity observed with the 10-Py-PM assay was not inhibited by 10 mol % of HEI, suggesting that this enzyme might be different from the myocardial calcium-independent PLA₂ described previously (12, 14, 27).

**Metabolism of AACH(OH)CF₃ in Platelets and U937 Cells**—The reduction of AACH(OH)CF₃ to AACH(OH)CF₂ in the cellular environment was followed using CF-LSIMS. The percentage of the AACH(OH)CF₃ reduction was estimated by comparing the relative peak heights due to the hydrate of AACH(OH)CF₃ (AAC(OH)CF₃ (M+H)+ = 375) and the reduction product, AACH(OH)CF₂ ((M+H)+ = 359). After a 7-min incubation of AACH(OH)CF₃ (10 µM) with U937 cells (2 × 10⁶ cells ml⁻¹), approximately 10% of the ketone was reduced. For human platelets, a 10-min incubation of AACH(OH)CF₃ (10 µM) with 4 × 10⁶ cells ml⁻¹ produced approximately 50% of the reduction product. With rat liver microsomes, a 5-min incubation of 200 µM AACH(OH)CF₃ produced about 30% of the reduction product. No reduction product of AACH(OH)CF₂ was observed when boiled microsomes were used.

**DISCUSSION**

The potential role of PLA₂ in the mobilization of AA from the cellular phospholipid pool has been the subject of a great deal of research. Human platelets produce large quantities of AA and eicosanoids upon stimulation with calcium ionophore and other agonists such as thrombin. Thus platelets provide a good system for the study of AA mobilization/utilization pathways. Platelets contain a number of different PLA₄s, the cPLA₂ (17, 28, 29), the 14-kDa secreted PLA₁ (30–32), and others (33, 34). Recent data showed that a 70% depletion of the sPLA₁ in rabbit platelets does not affect TxA₂ production, suggesting that the sPLA₁ is not required for AA liberation during platelet activation (35). This is also supported by results from Gelb and co-workers (2) who have recently tested a number of potent competitive inhibitors of sPLA₁ and found that they did not inhibit the thrombin-stimulated release of AA from platelets. The presence of the cPLA₁ in platelets and its translocation from the cytosol to the membranes in response to thrombin has recently been demonstrated (17, 36), and these results have implicated the cPLA₁ in platelet activation. To attempt to elucidate the potential role of the cPLA₁ in the AA mobilization pathways of platelets, we have employed a recently described potent slow binding inhibitor of the cPLA₁, AACH(OH)CF₃ (20). AACH(OH)CF₃ is a selective inhibitor of the cPLA₁ versus the sPLA₁ (20) and, as demonstrated here, versus other platelet PLA₄s as well. A number of distinct calcium-dependent and calcium-independent PLA₄ activities were detected in platelet fractions, and only the activity showing the functional characteristics of the cPLA₁ was significantly inhibited by AACH(OH)CF₃. In agreement with the subcellular location of the cPLA₁ in resting platelets (17), the AACH(OH)CF₃-susceptible PLA₁ activity was found only in the soluble fraction. Assays for CoA-independent transacylase activity in U937 microsomes (37) showed that AACH(OH)CF₃ is a very weak inhibitor of this activity (IC₅₀ > 50 µM).

AACH(OH)CF₃ produced a dose-dependent decrease in the calcium ionophore-stimulated production of AA from Me₃SO-dif-

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**Table II**

| PLA₁ activities observed in platelet extracts and their susceptibility to inhibition by AACH(OH)CF₃. |

| PLA₁       | Specific activity | Inhibition¹ | Specific activity | Inhibition¹ |
|------------|------------------|-------------|------------------|-------------|
| 10-Py-PM   | ±Ca²⁺            | ±Ca²⁺       | ±Ca²⁺            | ±Ca²⁺       |
| sPLA₁      | 134.683 pmol 30 min⁻¹ µg⁻¹ | 25%          | NA              | NA          |
| cPLA₁      | 2.1 pmol 30 min⁻¹ µg⁻¹  | NA          | NA              | NA          |
| Cytosol    | 1.9 pmol 30 min⁻¹ µg⁻¹  | 0%          | 0.65%           | 0%          |
| Membrane   | 0.81 pmol 30 min⁻¹ µg⁻¹  | 17%         | 0.1%            | 0%          |
| ["4C"]PAPC/Triton X-100 | Specific activity | Inhibition¹ | Specific activity | Inhibition¹ |
| sPLA₁      | 1834 pmol 30 min⁻¹ µg⁻¹  | NA          | NA              | NA          |
| cPLA₁      | 78 pmol 30 min⁻¹ µg⁻¹    | NA          | NA              | NA          |

¹ Caused by 10 mol % AACH(OH)CF₃.
² Caused by 1.6 mol % AACH(OH)CF₃.
³ NA, no activity detected.

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² M. H. Gelb, R. Apitz-Castro, and M. K. Jain, personal communication.
³ J.-P. Falgueyret and D. Riendeau, unpublished data.
differentiated U937 cells and from platelets. In contrast, AA-COCF$_3$ and AACH(OH)CF$_3$ compounds, which were more-challenged to the purified cPLA$_2$, also did not inhibit AA release by U937 cells or platelets at any of the concentrations tested (up to 40 μM). The arachidonyl analogues appear to be relatively nontoxic to cells, since none of them caused increased leakage of lactate dehydrogenase when incubated with platelets. The results presented here also point to a number of limitations for the use of AACOCF$_3$ in cell-based studies. First, AACOCF$_3$ is a slow binding inhibitor of cPLA$_2$, and although it is a potent inhibitor ($K_i < 5 \times 10^{-5}$ mol fraction) at low interfacial concentrations, the full inhibitory potency takes many minutes to develop (20). Consequently, in a cell-based assay where AA mobilization is over in less than a minute, relatively high concentrations of AACOCF$_3$ are required to inhibit cPLA$_2$ within this short period of time. Second, it was found that AACOCF$_3$ is reduced to its noninhibitory alcohol (AACH(OH)CF$_3$) on incubation with U937 cells, platelets, or rat liver microsomes. The rate of reduction was sufficiently slow that much of the AACOCF$_3$ could be recovered intact from the cells after short incubation times. However, the reduction of AACOCF$_3$ suggests that it could not be used in assays performed in cell-based systems where prolonged incubations are required. Despite these disadvantages, the results presented here show that there is a good correlation between the inhibitory potency of the arachidonyl analogues against the purified cPLA$_2$ and their ability to inhibit AA release in intact cells.

Both AACH$_2$ and AACH(OH)CF$_3$ produced an increase in the stimulated level of AA in the cell-based assays. These compounds might inhibit enzymes in the utilization and reacylation pathways of the cell, producing an increase in the steady-state level of free AA. Alternatively, the increase could be due to a direct effect of these compounds on the activity of cPLA$_2$, since AACH$_2$ and AACH(OH)CF$_3$ have been shown to increase the activity of the purified cPLA$_2$ in a mixed micelle assay (20). In this case, the increased activity of the purified cPLA$_2$ was ascribed to a more favorable partitioning of the enzyme to the lipid-water interface in the presence of these arachidonyl-like compounds (20, 35). That there is some direct stimulation of cPLA$_2$ activity in the cells is likely since both AACH$_2$ and AACH(OH)CF$_3$ also increased the stimulated level of AA in ionophore-challenged platelets. In that case, the utilization and reacylation pathways of the platelet are blocked by ETYA; consequently, the increased AA release observed in the presence of the arachidonyl analogues is probably due to the increased activity of the AA release pathway.

AACOCF$_3$ inhibited 12-HETE biosynthesis in a dose-dependent manner in calcium ionophore-stimulated platelets but not in platelets where 12-HETE production was induced by the addition of AA. This observation strongly suggests that AACOCF$_3$ inhibits 12-HETE biosynthesis at the level of AA mobilization rather than by inhibition of 12-lipoxigenase. The same dose dependencies for the inhibition of 12-HETE production and AA release also suggest that AACOCF$_3$ inhibits 12-HETE biosynthesis by decreasing the amount of free AA available for the 12-lipoxigenase. In contrast to its effect on 12-HETE production, AACOCF$_3$ inhibited biosynthesis of TxB$_2$ in both calcium ionophore- and AA-challenged platelets. Thus it is likely that in this case AACOCF$_3$ inhibits TxB$_2$ production both at the level of AA release and at a point further down the TxB$_2$ biosynthetic pathway. This is substantiated by the results obtained with structural analogues of AACOCF$_3$. Both AA-CH(OH)CF$_3$ and AACH$_2$ are noninhibitory to the purified cPLA$_2$, but both inhibited the production of TxB$_2$ in calcium ionophore- and AA-challenged platelets. This again suggests that this class of compounds can inhibit prostanooid formation at a point in the biosynthetic pathway other than AA mobilization.

Interestingly, AACH(OH)CF$_3$, and AACH$_2$ did not inhibit 12-HETE production in ionophore-stimulated platelets but increased the levels of this eicosanoid by greater than 2-fold. The stimulation of 12-HETE production by these compounds was also seen in AA-challenged platelets. It should be noted that AA was added to the platelets at a concentration that does not saturate 12-HETE production. The stimulatory effect of these compounds is likely mostly due to their inhibitory effect on the prostanooid biosynthetic pathway. It has been shown previously that the AA for both 12-HETE and TxB$_2$ biosynthesis is derived from a single phospholipid pool (39) and that AA can be shunted from one pathway to the other. In agreement with this, flurbiprofen, a potent inhibitor of cyclooxygenase, also increased the level of 12-HETE produced in calcium ionophore- and AA-challenged platelets in a manner similar to the arachidonyl analogues. The stimulation of 12-HETE by AACH$_2$ and AACH(OH)CF$_3$ was more pronounced than by flurbiprofen; this may be related to the ability of the arachidonyl analogues to directly activate the cPLA$_2$ as previously observed in a mixed micelle assay (20).

In summary, AACOCF$_3$ is a selective inhibitor of the cPLA$_2$, versus other platelet PL-A$_2$s, and this compound inhibits the release of AA in calcium ionophore-challenged U937 cells and the release of AA and 12-HETE in calcium ionophore-challenged platelets. AACOCF$_3$ also inhibits the production of TxB$_2$ in platelets, although this is likely due to a combination of its inhibitory effects on AA mobilization and at a point further along in the prostanooid biosynthetic pathway. These results are consistent with a major role for cPLA$_2$ in the liberation of AA from the phospholipid pool for eicosanoid biosynthesis.

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