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Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A₂: structure-function studies with vesicle, micelle, and membrane assays¹

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Abstract

A series of fatty alkyl trifluoromethyl ketones and methyl fluorophosphonates have been prepared and tested as inhibitors and inactivators of human groups IV and VI phospholipases A₂ (cPLA₂ and iPLA₂). Compounds were analyzed with phospholipid vesicle-, detergent-phospholipid mixed-micelle-, and natural membrane-based assays, and, with few exceptions, the relative inhibitor potencies measured with the three assays were similar. Ph(CH₂)₄COCF₃ and Ph(CH₂)₄PO(OMe)F emerged as a potent inhibitor and inactivator, respectively, of iPLA₂, and both are poorly effective against cPLA₂. Of all 13 fatty alkyl trifluoromethyl ketones tested, the trifluoromethyl ketone analog of arachidonic acid is the most potent cPLA₂ inhibitor, and structurally similar compounds including the trifluoromethyl ketone analog of docosahexenoic acid are much poorer cPLA₂ inhibitors. Inactivation of cPLA₂ by fatty alkyl fluoromethylphosphonates is greatly promoted by binding of enzyme to the interface. The use of both vesicles and mixed micelles to assay phospholipase A₂ inhibitors and inactivators present at low mol fraction in the interface provides reliable rank order potencies of a series of compounds that correlate with their behavior in a natural membrane assay. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Interfacial enzymology; Protein-lipid interaction; Cytosolic phospholipase A₂; Calcium-independent phospholipase A₂

1. Introduction

Phospholipases A₂ (PLA₂) are thought to liberate arachidonic acid (20:4) from the *sn*-2 position of

cellular membrane phospholipids for the biosynthesis of eicosanoids and possibly for other purposes [1–5]. There is considerable interest in PLA₂ inhibitors as such components should have antiinflammatory properties [6–8]. Compared to enzymes that work in the homogeneous aqueous phase, inhibition of PLA₂s is more difficult to study because these enzymes necessarily work at the lipid-water interface. Many previously reported reversible inhibitors of PLA₂ have been shown to operate by a non-specific

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mechanism in which an inhibitor occupies a significant fraction of the membrane surface and causes desorption of the enzyme into the aqueous phase [9]. Obviously, one desires highly specific and potent competitive inhibitors of PLA₂ which function by binding to the enzyme's active site.

Many competitive inhibitors of PLA₂s have been studied with phospholipid vesicles [10] and with phospholipid-detergent mixed micelles [11,12]. It thus becomes important to determine if both systems give reliable inhibition information. By reliable, it is meant that the relative potencies of a series of inhibitors determined with PLA₂ assays involving artificial interfaces are the same as those determined with natural membrane assays.

Ten groups of PLA₂s have been identified based on amino acid sequences [13,14]. The present study is focused on two intracellular PLA₂s: human group IV PLA₂, also called cytosolic PLA₂ (cPLA₂), and the murine group VI PLA₂, also called calcium-independent phospholipase A₂ (iPLA₂). Activation of cPLA₂ in cells by pro-inflammatory agonists leads to its calcium-dependent translocation from the cytosol to intracellular membranes and release of arachidonic acid for eicosanoid biosynthesis [2]. The function of iPLA₂ is probably as a constitutively active enzyme that removes *sn*-2 acyl chains for phospholipid remodeling [15,16].

The fatty acid analog 20:4-COCF₃ (also known as AACOCF₃, Fig. 1) has been shown to be a tight binding, reversible inhibitor of cPLA₂ [17] and iPLA₂ [18], presumably by forming a stable hemiketal with the active site serine residues of these enzymes. Another fatty acid analog 20:4-PO(OMe)F (also known as MAFP, Fig. 1) has been shown to irreversibly inactivate cPLA₂ [19] and iPLA₂ [20], possibly by phosphorylation of serine. 20:4-COCF₃ and 20:4-PO(OMe)F do not inhibit secreted PLA₂s. In this

study we have prepared a series of analogs of 20:4-COCF₃ and 20:4-PO(OMe)F and have measured their potencies against cPLA₂ and iPLA₂ using vesicle, mixed-micelle, and natural membrane assays. As noted above, comparison of these structure-activity studies using multiple assays is important given the fact that multiple types of aggregated substrates for PLA₂ continue to be used by workers in the field. In addition, we sought to develop analogs of 20:4-COCF₃ and 20:4-PO(OMe)F that can distinguish between cPLA₂ and iPLA₂.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1-¹⁴C]arachidonoyl-*sn*-glycero-3-phosphocholine ([¹⁴C]PAPC) (50 Ci/mol) is from NEN, and 1-palmitoyl-2-[1-¹⁴C]palmitoyl-*sn*-glycero-3-phosphocholine ([¹⁴C]DPPC) (50 Ci/mol) is from Amersham. The source of 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol (DOPM) and the ester formed from γ -linolenic acid and umbelliferone (GLU) is as described [21]. The other non-radioactive lipids are from Avanti Polar Lipids. 20:4-COCF₃ was prepared as described [17,18]. 20:4-PO(OMe)F is from BioMol. 7,7-Dimethyl-5,8-eicosadienoic acid (7,7-Me₂-5,8-20:2) is from Oxford Biomedical Research. Silica gel-60 (230–400 mesh) is from Merck. Recombinant cPLA₂ was purified as described [22] to near homogeneity (~80% by gel electrophoresis, specific activity of 0.033 μ mol/min/mg using the previously described assay [22]). iPLA₂ was purified ~75 000-fold from murine P388D₁ cells through the Mono Q step as described elsewhere [23] and had a specific activity of 1.6 μ mol/mg/min using the described assay [24].

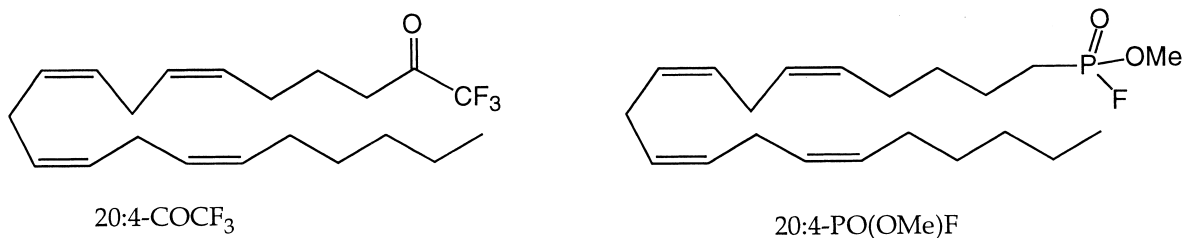


Fig. 1. Structures of cPLA₂ and iPLA₂ inhibitor 20:4-COCF₃ and inactivator 20:4-PO(OMe)F.

2.2. Synthesis of trifluoromethyl ketones

All trifluoromethyl ketones were synthesized from the appropriate acyl chlorides as described previously [17]. All fatty acyl chlorides were obtained from Nu-Chek Prep except for the acyl chloride derived from 7,7-Me₂-5,8-20:2, which was prepared from the fatty acid with the standard procedure using oxalyl chloride in benzene. Ph(CH₂)₃-COCl and Ph(CH₂)₄-COCl were prepared by oxidizing the appropriate alcohols (Aldrich) with Jones' reagent followed by reaction of the carboxylic acids with 1 equivalent of PCl₅ in CH₂Cl₂ overnight at room temperature. All compounds were purified to homogeneity by flash chromatography on silica. Compounds were judged to be pure by thin layer chromatography on silica with 10% ether in petroleum ether (*R_f* values ~0.2) and by HPLC on a C18 reverse phase column using a gradient from 0–100% acetonitrile in water (UV detection at 210 nm).

2.3. Synthesis of methyl alkyl fluorophosphonates

Ph(CH₂)₄-PO(OMe)F was prepared as follows. To 2 g of Ph(CH₂)₄OH (Aldrich) in 2 ml of CH₂Cl₂ was added dropwise 1.2 equivalents of PBr₃ with stirring, and the reaction was left at room temperature for an additional 30 min. Ether was added to the mixture and the solution was washed with ice-cold brine. The bromide was purified by flash chromatography on silica with ethyl acetate/petroleum ether.

To prepare Ph(CH₂)₄-PO(OMe)₂, 200 mg of NaH (60% dispersion in oil) was added to 0.95 ml of dry DMF. After hydrogen evolution ceased, the flask was placed under Ar, cooled in an ice-water bath, and 0.42 ml of dimethyl phosphite was added dropwise by syringe with stirring. The reaction was stirred for an additional 30 min at room temperature. The mixture was cooled on ice, and 1.1 equivalents of Ph(CH₂)₄Br were added dropwise. The reaction was stirred overnight at room temperature. Solvent was removed with a Speed-Vac (Savant Instruments), and the residue was dissolved in ether, and water was added. The ether layer was removed and combined with three additional ether extracts of the water layer. The solution was dried over anhydrous MgSO₄, filtered, and concentrated to dryness in va-

cuo. Thin layer chromatography on a silica plate with ethyl acetate showed a single spot at *R_f* = 0.3: ¹H-NMR (CDCl₃, TMS) 1.58–1.92, m, 4H; 2.62, t, 2H; 3.71, d, 6H; 7.20, m, 5H.

The dimethylphosphonate (5 mmol) was dissolved in 11.6 ml MeOH containing 1.35 g NaOH, and the mixture was refluxed at 98°C for 2 h. Water was added, and the mixture was extracted three times with ether, and the organic phases were discarded. The water layer was brought to pH 1, and the product was extracted with three portions of ether. The organic phases were combined, dried over anhydrous MgSO₄, filtered, and concentrated to dryness in vacuo: ¹H-NMR (CDCl₃, TMS) 1.56–1.90, m, 4H; 2.62, t, 2H; 3.71, d, 3H; 7.21, m, 5H.

Ph(CH₂)₄-PO(OMe)OH (3.3 mmol) was fluorinated in 6 ml dry CH₂Cl₂ (distilled over CaH₂ under Ar) by dropwise addition of Et₂NSF₃ (DAST, 5 mmol, Aldrich) with stirring at room temperature under Ar for 30 min. Thin layer chromatography on silica with ethyl acetate/petroleum ether (1/1) showed a major spot at *R_f* = 0.6. The product was purified by flash chromatography on silica with 25% ethyl acetate in petroleum ether: ¹H-NMR (CDCl₃, TMS) 1.49–2.02, m, 4H; 2.61, t, 2H; 3.82, d, 3H; 7.19, m, 5H. The product was judged to be pure by thin layer chromatography and by HPLC on a C18 reverse phase column using a gradient of 0–100% acetonitrile in water (UV detection at 210 nm).

16:0-PO(OMe)F was made as described above starting with hexadecyl bromide (Aldrich). The final product was pure as judged by thin layer chromatography on silica (*R_f* = 0.85, ethyl acetate/petroleum ether, 1/1) and by HPLC (as described above): ¹H-NMR (CDCl₃, TMS) 0.89, t, 3H; 1.28, s, 24H; 1.55–1.80, m, 2H; 1.80–2.02, m, 2H; 3.88, d, 3H.

The short-chain methyl fluorophosphonate CH₂=CH(CH₂)₅PO(OMe)F was prepared by reacting 1-bromo-5-hexene (prepared by treating 5-hexenol with PBr₃) with dimethyl phosphite followed by saponification and fluorination as described above. The product was purified by flash chromatography using hexane:ether (1:1). Final purification was carried out by preparative thin layer chromatography using chloroform, the product structure was confirmed by ¹H-NMR.

2.4. *cPLA₂* vesicle assay

cPLA₂ was assayed with DOPM vesicles containing GLU exactly as described previously using 100 μ M DOPM extruded vesicles containing 0.02 mol fraction GLU in 50 mM Tris, 150 mM NaCl, 0.3 mM CaCl_2 , 0.2 mM EGTA, 30% glycerol (v/v), pH 8.0 [21]. Inhibitors were added from stock solutions in DMSO. The concentration of organic solvent in the assay never exceeded 0.5%. The rate of GLU hydrolysis in the absence of *cPLA₂* was measured in all assays, but in all cases its value was negligible compared to the enzymatic rate, and thus no correction was needed. Control reactions were carried out without inhibitor (DMSO only). Because some of the inhibitors exhibited slow binding behavior, the reaction progress was monitored until a constant, steady-state rate was obtained (typically several min). The concentration of each inhibitor (mol fraction in the interface) required to reduce the velocity 2-fold, $X_1(50)$, was determined from dose-response curves with at least six data points.

Prior to use in these and other inhibition studies, all inhibitors were routinely checked for purity by thin layer chromatography on silica as described. Inhibitors containing unsaturated hydrocarbon chains are susceptible to air oxidation, and stock solutions were stored at -20°C under Ar.

2.5. *cPLA₂* mixed-micelle assay

Assays were performed in buffer composed of 80 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM CaCl_2 , 1 mg/ml fatty acid-free bovine serum albumin (BSA), and 1 mM dithiothreitol. The mixed-micelle assay also contained 1 mM [^{14}C]PAPC (120 000 cpm), 2 mM Triton X-100 and 30% (v/v) glycerol in a final volume of 200 μ l. The substrate solution was prepared in a solution containing all assay components except enzyme and inhibitor and at a 1.33-fold higher concentration than that desired in the final assay mixture. This solution was probe sonicated on ice for 6 min (0.5 s on and 0.5 s off cycles). An aliquot of this substrate solution (150 μ l) was added to 10 μ l of inhibitor in DMSO in a siliconized tube. This mixture was vortexed and then warmed to 40°C for 15 min. *cPLA₂* (0.75 μ g) in 40 μ l of assay buffer was then added to initiate the reaction. Assays were kept

at 40°C for 40 min. The reaction was quenched and worked-up using a modified Dole protocol as described previously [25]. The amount of radiolabeled arachidonic acid product was quantified by scintillation counting.

cPLA₂ activity was also measured with varying times of preincubation of inhibitor with enzyme. In these experiments, 7.5 μ g of *cPLA₂* was preincubated at 21°C with 40 μ l of a buffer containing all assay components except substrate to which 4 μ l of inhibitor in DMSO was added. At the desired times, 4 μ l of this mixture was added to 196 μ l of substrate in assay buffer (see above) at 40°C . The incubation and product analysis was as described above.

Control experiments were carried out using neat DMSO in place of inhibitor/DMSO. Blank reactions were carried out in the absence of enzyme, and the background hydrolysis was subtracted from the data obtained with enzyme. $X_1(50)$ values were determined by fitting the data to a dose-response curve derived from experiments with at least six different concentrations of inhibitor. Inhibitor concentrations are expressed as mol fraction inhibitor in the mixed micelle (mol of phospholipid+mol of Triton X-100).

2.6. *iPLA₂* mixed-micelle assay

Assays of the *iPLA₂* were performed with mixed-micelles composed of 100 μ M [^{14}C]DPPC (200 000 cpm), 400 μ M Triton X-100 in 100 mM HEPES (pH 7.5), 5 mM EDTA, and 1 mM ATP in a final volume of 500 μ l. The substrate solution was prepared as described previously [23]. An aliquot of this substrate solution (445 μ l) was added to 5 μ l of DMSO containing inhibitor in a siliconized tube. This mixture was agitated briefly on a vortexer and then warmed to 40°C for 15 min. Assays were initiated by the addition of 50 μ l of *iPLA₂* in mono Q buffer [23]. The mixture was incubated for 30 min at 40°C . The reaction was quenched and analyzed for product as described for the *cPLA₂* mixed-micelle assay.

iPLA₂ activity was also measured after various times of preincubation of enzyme with inhibitor. In these experiments, 45 μ l of *iPLA₂* was preincubated at 40°C with 5 μ l of DMSO containing inhibitor. After the desired times, 450 μ l of substrate solution, prewarmed to 40°C , was added to initiate the reaction. The remainder of the assay is as described

above. Blanks and controls were conducted as in the cPLA₂ assay.

2.7. cPLA₂ natural membrane assay

Human U937 cells were grown in suspension under standard conditions and harvested by centrifugation. The resulting cell pellet was resuspended in 10 ml per 10⁹ cells of ice-cold 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride. The resuspended cells were lysed by 3 cycles of nitrogen cavitation (700 psi, 4°C). The resulting lysate was centrifuged at 50 000×g for 60 min at 4°C, and the supernatant was discarded. The membrane pellet was resuspended in lysis buffer without EDTA but containing 1 mg/ml fatty acid-free BSA and then centrifuged again. The resulting membrane pellet was suspended in 10 ml of 20 mM HEPES, pH 7.4, 150 mM NaCl buffer per 10⁹ cells.

Inhibitor and U937 cell membranes (equivalent to 1×10⁶ U937 cells, ~400 μM phospholipid based on phosphate analysis) were mixed in 120 μl of 20 mM HEPES, pH 7.4, 150 mM NaCl, 3.5 mM CaCl₂ for 5 min at 37°C. The reaction was initiated by the addition 1 μg cPLA₂ in 100 μl 20 mM HEPES, pH 7.4, 150 mM NaCl, 1.8 mM EGTA (prewarmed to 37°C). After a 10 min incubation at 37°C, the reaction was quenched with 200 μl of cold methanol containing 100 μg/μl of 5,6,8,9,1,12,14,15-[²H]20:4. The mixture was extracted with chloroform, the organic layer was concentrated to dryness with a stream of N₂, and the fatty acids were derivatized in methylene chloride containing 10% (v/v) pentafluorobenzyl bromide and 10% (v/v) diisopropylamine for 20 min at room temperature. The solvent was removed in vacuo, the residue was dissolved in 200 μl decane and analyzed by gas chromatography/mass spectrometry [3]. Liberated arachidonate was quantified by monitoring the ratio of 303/311 *m/z* ratio.

3. Results and discussion

3.1. Inhibition studies with fatty alkyl trifluoromethyl ketones

Virtually any fatty acid can be converted into its

trifluoromethyl ketone analog by conversion to the acid chloride followed by reaction with trifluoroacetic anhydride in pyridine [26]. The 13 trifluoromethyl ketones prepared in this study are listed in Table 1. All compounds contain a long-chain fatty alkyl group except for Ph(CH₂)₃-COCF₃ and Ph(CH₂)₄-COCF₃. The latter two compounds are analogs of the carboxyl end of 20:4-COCF₃ in that they contain an aromatic ring near the position occupied by the 5-double bond of 20:4. We prepared 22:6-COCF₃ with the thought that it would be a poor cPLA₂ inhibitor but retain good activity toward iPLA₂. This is based on the fact that phospholipids containing an *sn*-2 22:6 chain are poor cPLA₂ substrates [27,28]. Likewise, 11,14-20:2-COCF₃ and 11,14,17-20:3-COCF₃ were prepared because substrate specificity studies of cPLA₂ showed that the 5-double bond is the most important of the four in 20:4 for recognition by this enzyme [29]. Fatty acid analogs containing *trans* double bonds, 9t-18:1-COCF₃ and 9t,12t-18:2-COCF₃, were prepared to explore the consequence of having altered double bonds stereochemistry, and finally 7,7-Me₂-5,8-20:2-COCF₃ was prepared because it has a branched fatty alkyl chain.

All inhibitors were tested using assays containing phospholipid vesicles, phospholipid-detergent mixed micelles, and crude membranes derived from U937 cells. For vesicle assays, the fluorimetric substrate, GLU, present at low mol fraction in vesicles of the anionic phospholipid DOPM was used. This assay has been well characterized, and cPLA₂ binds very tightly to these anionic vesicles [21]. Anionic phospholipids containing two saturated fatty acyl chains were avoided since cPLA₂ rapidly inactivates on vesicles composed of such lipids [22]. It has previously been shown that cPLA₂ hydrolyzes arachidonyl-containing phospholipids when dispersed in Triton X-100 micelles [30,31], and this assay was also used. Finally, a natural membrane assay was used to mimic inhibition in a natural cell.

For all assays, inhibitors were tested at low mole fraction, which helps to insure that inhibition is the result of direct binding of inhibitor to the enzyme rather than due to desorption of enzyme from the interface into the aqueous phase. The latter is a serious concern with PLA₂ inhibitors [6,9]. The trifluoromethyl ketones exhibit slow binding behavior with cPLA₂ but not with iPLA₂ [17,18,24]. Thus for

Table 1
 $X_1(50)$ values for trifluoromethyl ketones

R-COCF ₃	cPLA ₂ DOPM/GLU	cPLA ₂ mixed-micelle	cPLA ₂ mixed-micelle 4 h pre-incubation	cPLA ₂ natural membrane	iPLA ₂ mixed-micelle
20:4	0.0050 ± 0.001 ^a (1.00) ^b	0.036 ± 0.006 ^c (1.00)	0.0053 ± 0.0006 ^d (1.00)	0.05 ± 0.02 (1.00)	0.031 ± 0.006 ^e (1.00)
11,14,17-20:3	0.0085 ± 0.002 (0.59)	0.020 ± 0.002 (1.80)	0.0073 ± 0.007 (0.73)	0.10 ± 0.03 (0.50)	0.0079 ± 0.0008 (3.92)
11,14-20:2	0.017 ± 0.002 (0.29)	0.056 ± 0.009 (0.64)	0.0040 ± 0.0007 (1.33)	0.09 ± 0.02 (0.56)	0.055 ± 0.007 (0.56)
7,7-Me ₂ -5,8-20:2	0.20 ± 0.03 (0.025)	0.022 ± 0.005 (1.64)	not determined	> 0.5 (0.0)	0.091 ± 0.06 (0.34)
22:6	0.035 ± 0.005 (0.14)	0.047 ± 0.01 (0.77)	0.050 ± 0.02 (0.11)	not determined	0.029 ± 0.002 (1.07)
6,9,12-18:3	0.0085 ± 0.001 (0.59)	0.040 ± 0.01 (0.90)	0.012 ± 0.007 (0.44)	0.18 ± 0.04 (0.28)	0.055 ± 0.009 (0.56)
9,12,15-18:3	0.010 ± 0.002 (0.50)	0.029 ± 0.01 (1.80)	0.018 ± 0.01 (0.29)	0.05 ± 0.02 (1.0)	0.0091 ± 0.0009 (3.41)
9t,12t-18:2	0.0065 ± 0.001 (0.77)	0.016 ± 0.009 (2.25)	0.0050 ± 0.002 (1.06)	not determined	0.025 ± 0.005 (1.24)
9t-18:1	0.027 ± 0.002 (0.18)	0.013 ± 0.005 (2.77)	0.013 ± 0.006 (0.41)	0.27 ± 0.04 (0.18)	0.057 ± 0.002 (0.54)
16:1	0.0083 ± 0.001 (0.60)	0.010 ± 0.003 (3.60)	0.0046 ± 0.001 (1.15)	0.07 ± 0.02 (0.67)	0.0029 ± 0.0002 (10.7)
16:0	0.025 ± 0.002 (0.20)	0.022 ± 0.006 ^c (1.64)	0.019 ± 0.003 (0.28)	0.20 ± 0.03 (0.25)	0.0085 ± 0.0006 ^f (3.65)
Ph(CH ₂) ₃	0.17 ± 0.02(0.03)	0.12 ± 0.06 (0.30)	0.20 ± 0.1 (0.026)	> 0.5 (0.0)	0.28 ± 0.06 (0.11)
Ph(CH ₂) ₄	0.038 ± 0.001 (0.13)	0.040 ± 0.01 (0.90)	0.048 ± 0.002 (0.11)	> 0.5 (0.0)	0.0043 ± 0.0002 (7.21)

^aBayburt et al. reported an $X_1(50)$ of 0.01 [21].

^bNumbers in parentheses are the potencies of the inhibitors relative to 20:4-COCF₃ ($X_1(50)$ for AACOCF₃ divided by $X_1(50)$ for RCOF₃).

^cConde-Frieboese et al. reported $X_1(50)$ s of 0.016 and 0.015 for 20:4 and 16:0, respectively [25].

^dConde-Frieboese et al. reported $X_1(50)$ of 0.005 [25].

^eAckermann et al. reported an $X_1(50)$ of 0.028 [18].

^fConde-Frieboese et al. reported $X_1(50)$ of 0.006 [25].

cPLA₂ assays, it is important to record the enzymatic velocity after equilibrium between enzyme and inhibitor is reached. The DOPM/GLU assay gives a real-time progress curve, and the velocity was monitored until a constant steady-state velocity was obtained (typically several min). The assay with [¹⁴C]PAPC/Triton X-100 mixed micelles is a fixed-time point assay, and thus enzyme was preincubated with micelles containing inhibitor for 4 h prior to the addition of substrate-containing micelles to initiate the reaction. For comparison purposes, inhibition was also measured without preincubation, i.e. by addition of cPLA₂ to micelles preloaded with substrate and inhibitor.

Table 1 lists the mole fraction of each inhibitor required to produce 50% inhibition, designated $X_1(50)$. Inhibitors of lipolytic enzymes are best reported in terms of mole fraction of inhibitor in the membrane [10,32]. Table 1 also gives the potency of each trifluoromethyl ketone relative to that of 20:4-CO₂CF₃ [$X_1(50)$ for 20:4-CO₂CF₃/ $X_1(50)$ for RCO₂CF₃] for each assay. For any single assay, the ratio of $X_1(50)$ s for two inhibitors is equal to the ratio of the interfacial enzyme-inhibitor dissociation equilibrium constants, K_1^* , and thus the relative potencies are identical to the relative $1/K_1^*$ values. It is difficult to compare two $X_1(50)$ values obtained for one inhibitor measured with two different assays. This is because the degree of inhibition in the presence of any particular mole fraction of inhibitor in the interface is a function not only of K_1^* but also of the interfacial Michaelis constant for substrate, K_M^* , and the degree to which the non-substrate lipid that forms most of the interface (DOPM or Triton X-100 or natural membrane lipids) competes with the inhibitor for binding to the active site of cPLA₂ (non-neutral diluent effect [33]). However, if relative values of $X_1(50)$ for a series of inhibitors in one assay are the same as those in the other assay, then the relative values of K_1^* in the two assays will be the same. It should also be noted that the relative potencies for the inhibitors measured with the mixed-micelle assay with and without preincubation are different (Table 1), which indicates that the inhibitors bind to cPLA₂ with different rates. Thus it is best to compare the $X_1(50)$ s obtained with the vesicle assay (measured after a constant steady-state enzymatic velocity was

obtained) to those obtained with the preincubation mixed-micelle assay since equilibration between enzyme and inhibitor has occurred under these conditions.

Fig. 2A,B gives a graphical representation of the relative potencies obtained with the vesicle, preincubation mixed-micelle, and natural membrane assays. There is good agreement between the relative potencies of the trifluoromethyl ketones in all three assays, although there are a few exceptions. 11,14–20:2-CO₂CF₃ and 16:1-CO₂CF₃ are, respectively, 4.5-fold and 1.9-fold more potent in the mixed-micelle assay compared to the vesicle assay, and 9,12,15-CO₂CF₃ is 2-fold more potent in the natural membrane assay versus the vesicle assay. Overall, the data suggest that for most trifluoromethyl ketones, their relative K_1^* values are independent of the lipid matrix that forms the interface (DOPM versus Triton X-100 versus U937-derived crude membranes). Based on the likelihood that the free energy of inhibitor bound to cPLA₂ is dominated by enzyme-inhibitor interactions and the free energy of inhibitor in the interface is dominated by the hydrophobic effect, one may expect relative values of K_1^* in the different assays to be similar. The data also argue that both the vesicle and mixed-micelle assays give an accurate relative impression of how well a particular inhibitor will work in a natural membrane.

For cPLA₂, the $X_1(50)$ values for the inhibitors in both the vesicle and mixed-micelle assay (with preincubation) are within a factor of 2 of each other except as noted above. In contrast, the $X_1(50)$ values in the natural membrane assay are typically 10-fold higher. The reason for this is not known, but it is likely due to the fact that the concentration of *sn*-2-arachidonyl phospholipid substrates for cPLA₂ in natural membranes is much higher than the concentration of substrate present in DOPM vesicle and Triton X-100 micelles.

The same series of trifluoromethyl ketones was tested for inhibition of iPLA₂ using a Triton X-100 mixed-micelle assay, and the data are listed in Table 1. There is virtually no correlation between the relative potencies of the series of trifluoromethyl ketones as inhibitors of cPLA₂ versus iPLA₂. For example, 16:1-CO₂CF₃ and Ph(CH₂)₄-CO₂CF₃ are, respectively, 10.7-fold and 7.2-fold more potent than 20:4-CO₂CF₃

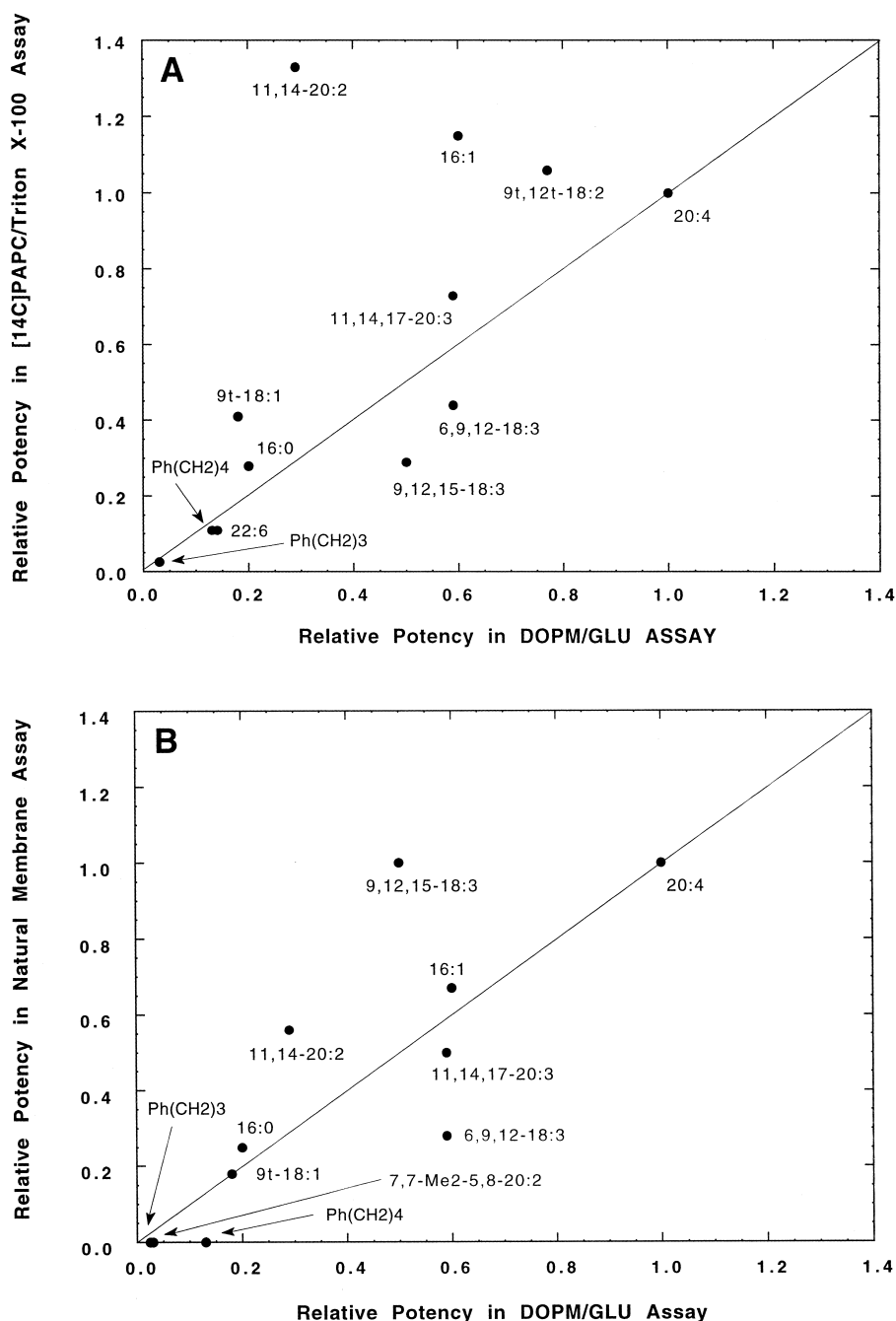


Fig. 2. In vitro inhibition of cPLA₂ by trifluoromethyl ketones measured with three different assays. (A) The abscissa gives the relative potency of the indicated trifluoromethyl ketones as an inhibitor of cPLA₂ ($X_1(50)$ for 20:4-COCF₃ divided by $X_1(50)$ for test compound) using the vesicle assay, and the ordinate gives the relative potency for the same set of compounds measured with the mixed-micelle assay (R group of R-COCF₃ given next to each data point). (B) Same as (A) but relative potency in the natural membrane assay is compared to that measured in the vesicle assay.

against iPLA₂, whereas they are 1.7- and 7.7-fold, respectively, less potent than 20:4-COCF₃ against cPLA₂.

Among the cPLA₂ inhibitors, 20:4-COCF₃ is the

best inhibitor of the series. 22:6-COCF₃ is 7-fold less potent than 20:4-COCF₃ in vesicles, which is consistent with the fact that cPLA₂ greatly prefers phospholipid substrates with an *sn*-2-20:4 chain over

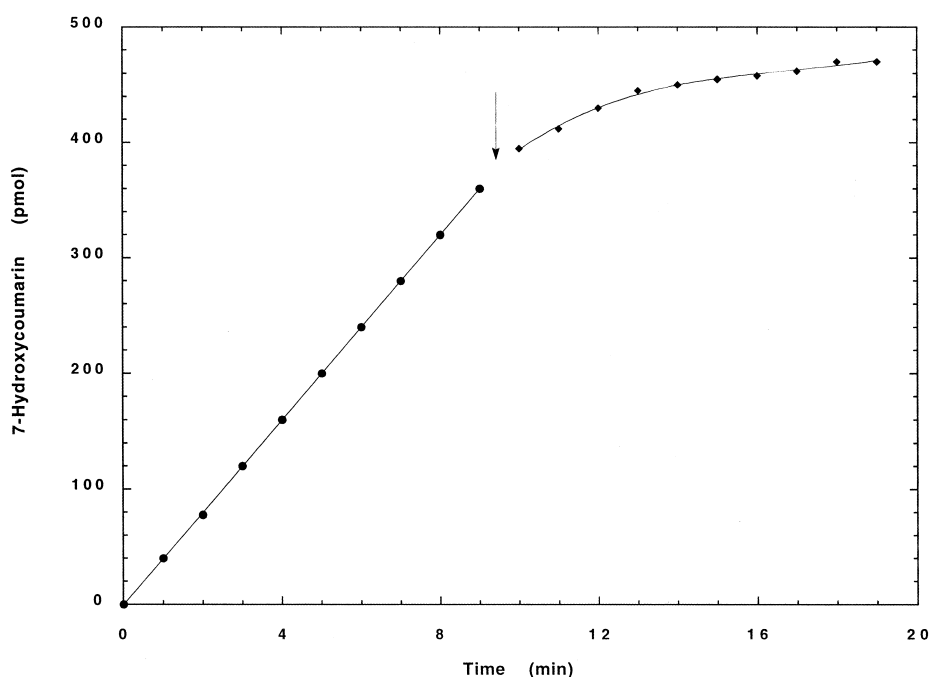


Fig. 3. Inactivation of cPLA₂ by 20:4-PO(OMe)F. Reaction progress curve for the hydrolysis of 0.02 mol fraction GLU in 100 mM DOPM vesicles in assay buffer (see Section 2) by 0.1 μg cPLA₂. 20:4-PO(OMe)F was added at a concentration of 0.005 mol fraction at the time indicated by the arrow.

those with an *sn*-2-22:6 chain (as noted above). Apparently, replacing the 5-double bond of 20:4-COCF₃ with an aromatic ring [Ph(CH₂)₃-COCF₃ and Ph(CH₂)₄COCF₃] leads to a poor fit in the active site of cPLA₂. Loss of the 5-double bond (11,14-20:2-COCF₃ and 11,14,17-COCF₃) results in a modest reduction in potency. Alkyl chain branching (7,7-Me₂-5,8-20:2) is not tolerated well by cPLA₂. Finally, shortening the chain length with removal of double bonds (16:1-COCF₃ and 16:0-COCF₃) leads to only a modest reduction in potency [18]. This is in

marked contrast to previous results showing that 20:0-COCF₃ is much less potent as a cPLA₂ inhibitor compared to 20:4-COCF₃ [17].

iPLA₂ displays a very different pattern. It does not discriminate between 20:4-COCF₃ and 22:6-COCF₃, and it prefers 16:1-COCF₃ and 16:0-COCF₃ over 20:4-COCF₃. An aromatic ring enhances inhibition when linked to the carbonyl group by a four carbon chain, but moving the ring closer by 1 methylene drastically reduces inhibition.

Table 2
Inactivation of cPLA₂ and iPLA₂ by fatty alkyl methyl fluorophosphonates

Inactivator	cPLA ₂ k_i^*/K_I^* (mol fraction) ⁻¹ s ⁻¹	cPLA ₂ k_i/K_I M ⁻¹ s ⁻¹	iPLA ₂ k_i/K_I M ⁻¹ s ⁻¹
20:4-PO(OMe)F	2.30 ± 0.03 ^a	4.8 ± 0.5 ^b	2 300 ± 800 ^b
16:0-PO(OMe)F	0.57 ± 0.02	3.7 ± 0.3	13 300 ± 3000
Ph(CH ₂) ₄ PO(OMe)F	0.12 ± 0.02	1.15 ± 0.3	10 800 ± 3000
CH ₂ =CH(CH ₂) ₃ PO(OMe)F	0.15 ± 0.04	2.8 ± 0.7	not determined

^aMeasured from the rate of inactivation observed in real time with the DOPM/GLU assay.

^bMeasured by pre-incubating enzyme with inactivator in the aqueous phase and adding mixed-micelle substrate at various times to measure the fraction of remaining active enzyme.

3.2. Inhibition studies with methyl fatty alkylfluorophosphonates

Based on the above results with trifluoromethyl ketones, we prepared two fluoromethyl phosphonates, 16:0-PO(OMe)F and Ph(CH₂)₄-PO(OMe)F, that were anticipated to distinguish between cPLA₂ and iPLA₂. These compounds result in a slow loss of cPLA₂ activity (DOPM/GLU assay), presumably due to enzyme inactivation as is the case for 20:4-PO(OMe)F [19]. Fig. 3 shows a typical cPLA₂ reaction progress curve before and after the addition of 20:4-PO(OMe)F. For these inactivators, it is best to report the observed first-order rate constant for inactivation divided by the mole fraction of inactivator in the interface. Such a quantity is proportional to k_i^*/K_1^* , where K_1^* is the mole fraction of inactivator that causes half of the interface-bound enzyme, E*, to be in the reversibly inhibited complex E*•I*, and k_i^* is the rate constant for conversion of E*•I* to the covalent E*-I complex. Values of k_i^*/K_1^* relative to that for 20:4-PO(OMe)F are listed in Table 2. Based on these measurements, the order of potency is 20:4-PO(OMe)F > 16:0-PO(OMe)F > Ph(CH₂)₄-PO(OMe)F, a trend that is consistent with the analogous series of trifluoromethyl ketones.

We also studied the rate of cPLA₂ inactivation when enzyme and inactivator were incubated for various times in the aqueous phase, and the fraction remaining active enzyme was measured by addition of substrate (in the form of mixed micelles). The observed first-order rate constant for loss of enzymatic activity divided by the bulk concentration of inactivator in the aqueous phase gives k_i/K_1 , where K_1 is the equilibrium constant for the reaction E•I → E+I, and k_i is the rate constant for E•I → E-I (all in the aqueous phase). Values of k_i/K_1 are listed in Table 2. Although k_i/K_1 cannot be compared to k_i^*/K_1^* (they have different units), it is clear that cPLA₂ bound to the interface is much more sensitive to inactivation than enzyme in the aqueous phase. For example, for cPLA₂ bound to DOPM vesicles containing 0.005 mol fraction 20:4-PO(OMe)F, inactivation occurs with a half-time of 60 s. In this experiment, the mole of inactivator divided by the total reaction volume corresponds to 0.5 μM 20:4-PO(OMe)F in the reaction. In the absence of an interface, incubation of cPLA₂ with 0.5 μM 20:4-PO(OMe)F

in the aqueous phase would lead to inactivation with a half-time of 3×10^5 s (calculated from the k_i/K_1 value in Table 2). This result is in marked contrast to inactivation of secreted PLA₂s where it has been shown that the half-times for inactivation by phenacylbromides of enzyme in the aqueous phase and bound to an interface of a neutral diluent are similar [33]. Based on theoretical arguments which have been experimentally verified, this dramatic increase in apparent inactivation potency when cPLA₂ is preincubated with methyl fluorophosphonate in aqueous solution versus presented with the inactivator bound to an interface may not be due to local concentration effects (i.e. enzyme sees a high local concentration of inactivator when both are bound to vesicles versus dispersed in the aqueous phase) [33]. Rather the results argue that the interface-bound enzyme is intrinsically more susceptible to inactivation.

We also prepared short-chain methyl fluorophosphonate CH₂=CH(CH₂)₅PO(OMe)F and tested it as an inactivator of cPLA₂ using the DOPM/GLU assay (Table 2). In the aqueous phase in the absence of an interface, cPLA₂ is inactivated by CH₂=CH(CH₂)₅PO(OMe)F with a second-order rate constant of $2.8 \pm 0.7 \text{ M}^{-1} \text{ s}^{-1}$ (half-time for inactivation of 82 min with 50 μM inactivator). The half-time for cPLA₂ inactivation by 50 μM CH₂=CH(CH₂)₅PO(OMe)F in the presence of 1000 μM DOPM vesicles (to ensure that all of the inactivator is partitioned into the interface) containing 0.02 mol fraction GLU decreased to 1.5 min corresponding to a k_i^*/K_1^* of $0.15 \text{ (mole fraction)}^{-1} \text{ s}^{-1}$. These results show that CH₂=CH(CH₂)₅-PO(OMe)F behaves similarly to Ph(CH₂)₄-PO(OMe)F as a cPLA₂ inactivator in the presence and absence of vesicles. They also show that the methyl fluorophosphonate with a short straight-chain alkyl group is a less efficient cPLA₂ inactivator than are the long-chain compounds (Table 2).

Inactivation of iPLA₂ was studied by preincubating enzyme in the aqueous phase with inactivator at 40°C followed by addition of mixed-micelle substrate to measure the fraction of remaining enzymatic activity (data in Table 2). Ph(CH₂)₄-PO(OMe)F and 16:0-PO(OMe)F show similar potencies, and 20:4-PO(OMe)F is considerably less potent. Note that nanomolar amounts of methyl fluorophosphonates are sufficient to inactivate most of the iPLA₂ during

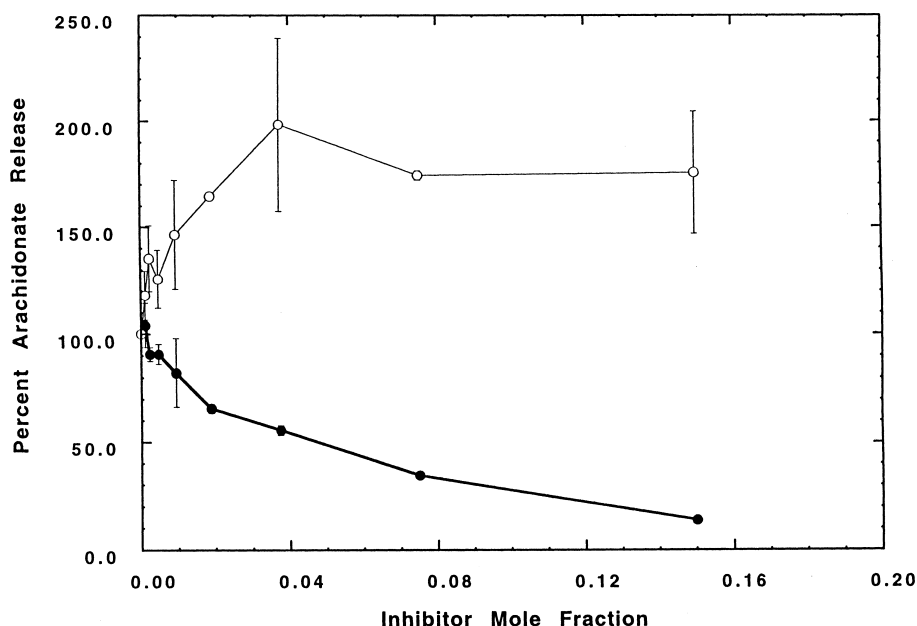


Fig. 4. Inactivation of cPLA₂ by fluoromethyl phosphonates in the natural membrane assay. Assays were carried out as described in Section 2 with the indicated concentrations of 20:4-PO(OMe)F (●) or Ph(CH₂)₄-PO(OMe)F (○). Error bars designate the standard deviation obtained from duplicate experiments. The mole fraction of inhibitor is calculated based on total membrane phospholipid (determined by phosphate analysis). Inhibition is expressed as a percentage of the arachidonate released in the absence of inhibitor (309 ± 69 pg/μl injected). All release values were corrected for the amount of arachidonate released in the absence of added cPLA₂ (54 ± 12 pg/μl injected).

preincubation in the aqueous phase for 20 min. In marked contrast, micromolar amounts of these compounds are required to inactivate most of the cPLA₂ in preincubations lasting 200 min (Table 2).

Fig. 4 shows the results of inactivation of cPLA₂ by fatty alkyl methyl fluorophosphonates present in the natural membrane assay. In this assay, various mole fractions of 20:4-PO(OMe)F and Ph(CH₂)₄-PO(OMe)F were added to natural membranes, cPLA₂ was added, and the amount of arachidonate release was quantified after a fixed incubation time. It can be seen that 20:4-PO(OMe)F is much more potent than Ph(CH₂)₄-PO(OMe)F. The reason for the approximately 2-fold increase in arachidonate production in the presence of Ph(CH₂)₄-PO(OMe)F is unknown.

Overall, the results show that the *in vitro* rank order potency of the fatty alkyl methyl fluorophosphonates as inactivators of cPLA₂ and iPLA₂ is the same as that seen with the corresponding trifluoromethyl ketones.

4. Conclusions

We have prepared an expanded set of fatty alkyl trifluoromethyl ketones and methyl fluorophosphonates that distinguish between cPLA₂ and iPLA₂, and thus should be useful to study the roles of these enzymes in cellular processes such as arachidonic acid release and lipid remodeling. Although the absolute potencies of PLA₂ inhibitors measured with different assays cannot be easily compared in a meaningful way, the relative potencies of a series of inhibitors (and inactivators) measured with vesicle, detergent-phospholipid mixed-micelle, and natural membrane assays are similar. Thus, at least for the inhibitors studied here, the free energy change for the transfer of inhibitor from the active site of the enzyme bound to the interface to the interface is approximately invariant to the type of interface used. Such a conclusion was also noted by Jain et al. in their use of a neutral diluent to measure interfacial K_M values of PLA₂s [33]. Still, the problem remains

that many investigators carry out lipolytic enzyme inhibitor studies with concentrations of inhibitors comparable to the concentration of substrate used in the assay. Under such conditions, the mole fraction of inhibitor in the interface may be quite high, and one has to be concerned that the presence of inhibitor may cause desorption of the enzyme from the interface into the aqueous phase [6]. Based on the results of this study, it may now be said that regardless of the type of assay used, vesicle, mixed-micelle, or natural membrane, true inhibitors of PLA₂ can be discovered as long as the mole fraction of inhibitor in the interface is kept low (perhaps < 0.01).

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