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Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A_2: structure-function studies with vesicle, micelle, and membrane assays[1]

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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 A_2 (cPLA$_2$ and iPLA$_2$). 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. $Ph(CH_2)_4COCF_3$ and $Ph(CH_2)_4PO(OMe)F$ emerged as a potent inhibitor and inactivator, respectively, of iPLA$_2$, and both are poorly effective against cPLA$_2$. Of all 13 fatty alkyl trifluoromethyl ketones tested, the trifluoromethyl ketone analog of arachidonic acid is the most potent cPLA$_2$ inhibitor, and structurally similar compounds including the trifluoromethyl ketone analog of docosahexenoic acid are much poorer cPLA$_2$ inhibitors. Inactivation of cPLA$_2$ 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 A_2 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.

Keywords: Interfacial enzymology; Protein-lipid interaction; Cytosolic phospholipase A_2; Calcium-independent phospholipase A_2

1. Introduction

Phospholipases A_2 (PLA$_2$) 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 PLA$_2$ inhibitors as such components should have antiinflammatory properties [6–8]. Compared to enzymes that work in the homogeneous aqueous phase, inhibition of PLA$_2$s 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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[1] This paper is dedicated to the memory of Prof. H.M. Verheij.

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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.

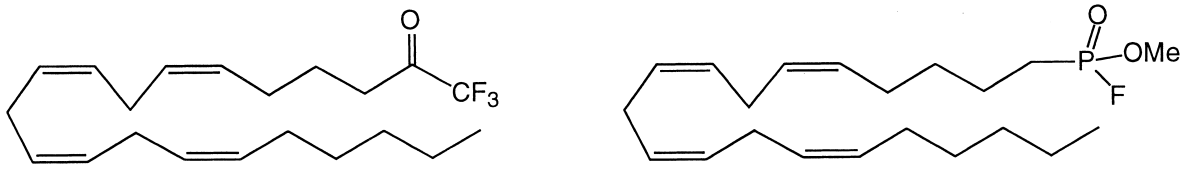
Ten groups of PLA_2s have been identified based on amino acid sequences [13,14]. The present study is focused on two intracellular PLA_2s: human group IV PLA_2, also called cytosolic PLA_2 ($cPLA_2$), and the murine group VI PLA_2, also called calcium-independent phospholipase A_2 ($iPLA_2$). Activation of $cPLA_2$ 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 $iPLA_2$ is probably as a constitutively active enzyme that removes *sn*-2 acyl chains for phospholipid remodeling [15,16].

The fatty acid analog 20:4-$COCF_3$ (also known as $AACOCF_3$, Fig. 1) has been shown to be a tight binding, reversible inhibitor of $cPLA_2$ [17] and $iPLA_2$ [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 $cPLA_2$ [19] and $iPLA_2$ [20], possibly by phosphorylation of serine. 20:4-$COCF_3$ and 20:4-PO(OMe)F do not inhibit secreted PLA_2s. In this study we have prepared a series of analogs of 20:4-$COCF_3$ and 20:4-PO(OMe)F and have measured their potencies against $cPLA_2$ and $iPLA_2$ 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 PLA_2 continue to be used by workers in the field. In addition, we sought to develop analogs of 20:4-$COCF_3$ and 20:4-PO(OMe)F that can distinguish between $cPLA_2$ and $iPLA_2$.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine ([^{14}C]PAPC) (50 Ci/mol) is from NEN, and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]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-$COCF_3$ was prepared as described [17,18]. 20:4-PO(OMe)F is from Bio-Mol. 7,7-Dimethyl-5,8-eicosadienoic acid (7,7-Me_2-5,8-20:2) is from Oxford Biomedical Research. Silica gel-60 (230–400 mesh) is from Merck. Recombinant $cPLA_2$ was purified as described [22] to near homogeneity (~80% by gel electrophoresis, specific activity of 0.033 μmol/min/mg using the previously described assay [22]). $iPLA_2$ was purified ~75 000-fold from murine P388D_1 cells through the Mono Q step as described elsewhere [23] and had a specific activity of 1.6 μmol/mg/min using the described assay [24].



20:4-$COCF_3$ 20:4-PO(OMe)F

Fig. 1. Structures of $cPLA_2$ and $iPLA_2$ inhibitor 20:4-$COCF_3$ 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-Me_2-5,8-20:2, which was prepared from the fatty acid with the standard procedure using oxalyl chloride in benzene. $Ph(CH_2)_3$-COCl and $Ph(CH_2)_4$-COCl were prepared by oxidizing the appropriate alcohols (Aldrich) with Jones' reagent followed by reaction of the carboxylic acids with 1 equivalent of PCl_5 in CH_2Cl_2 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 $\sim$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

$Ph(CH_2)_4$-PO(OMe)F was prepared as follows. To 2 g of $Ph(CH_2)_4OH$ (Aldrich) in 2 ml of CH_2Cl_2 was added dropwise 1.2 equivalents of PBr_3 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 $Ph(CH_2)_4$-$PO(OMe)_2$, 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 $Ph(CH_2)_4Br$ 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 $MgSO_4$, filtered, and concentrated to dryness in vacuo. Thin layer chromatography on a silica plate with ethyl acetate showed a single spot at $R_f = 0.3$: ^{1}H-NMR ($CDCl_3$, 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 $MgSO_4$, filtered, and concentrated to dryness in vacuo: ^{1}H-NMR ($CDCl_3$, TMS) 1.56–1.90, m, 4H; 2.62, t, 2H; 3.71, d, 3H; 7.21, m, 5H.

$Ph(CH_2)_4$-PO(OMe)OH (3.3 mmol) was fluorinated in 6 ml dry CH_2Cl_2 (distilled over CaH_2 under Ar) by dropwise addition of Et_2NSF_3 (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: ^{1}H-NMR ($CDCl_3$, 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): ^{1}H-NMR ($CDCl_3$, 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 $CH_2{=}CH(CH_2)_5PO(OMe)F$ was prepared by reacting 1-bromo-5-hexene (prepared by treating 5-hexenol with PBr_3) 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 ^{1}H-NMR.

2.4. $cPLA_2$ vesicle assay

$cPLA_2$ was assayed with DOPM vesicles containing GLU exactly as described previously using 100 μM 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 $cPLA_2$ 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, $X_I(50)$, 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. $cPLA_2$ 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 μl. 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 μl) was added to 10 μl of inhibitor in DMSO in a siliconized tube. This mixture was vortexed and then warmed to 40°C for 15 min. $cPLA_2$ (0.75 μg) in 40 μl 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.

$cPLA_2$ activity was also measured with varying times of preincubation of inhibitor with enzyme. In these experiments, 7.5 μg of $cPLA_2$ was preincubated at 21°C with 40 μl of a buffer containing all assay components except substrate to which 4 μl of inhibitor in DMSO was added. At the desired times, 4 μl of this mixture was added to 196 μl 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. $X_I(50)$ 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. $iPLA_2$ mixed-micelle assay

Assays of the $iPLA_2$ were performed with mixed-micelles composed of 100 μM [^{14}C]DPPC (200 000 cpm), 400 μM Triton X-100 in 100 mM HEPES (pH 7.5), 5 mM EDTA, and 1 mM ATP in a final volume of 500 μl. The substrate solution was prepared as described previously [23]. An aliquot of this substrate solution (445 μl) was added to 5 μl 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 μl of $iPLA_2$ 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 $cPLA_2$ mixed-micelle assay.

$iPLA_2$ activity was also measured after various times of preincubation of enzyme with inhibitor. In these experiments, 45 μl of $iPLA_2$ was preincubated at 40°C with 5 μl of DMSO containing inhibitor. After the desired times, 450 μl 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 conduced as in the $cPLA_2$ assay.

2.7. $cPLA_2$ 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 10^9 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 $50\,000 \times g$ 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 10^9 cells.

Inhibitor and U937 cell membranes (equivalent to 1×10^6 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 $CaCl_2$ for 5 min at 37°C. The reaction was initiated by the addition 1 μg $cPLA_2$ 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-[^{2}H]20:4. The mixture was extracted with chloroform, the organic layer was concentrated to dryness with a stream of N_2, 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 $Ph(CH_2)_3$-$COCF_3$ and $Ph(CH_2)_4$-$COCF_3$. The latter two compounds are analogs of the carboxyl end of 20:4-$COCF_3$ in that they contain an aromatic ring near the position occupied by the 5-double bond of 20:4. We prepared 22:6-$COCF_3$ with the thought that it would be a poor $cPLA_2$ inhibitor but retain good activity toward $iPLA_2$. This is based on the fact that phospholipids containing an *sn*-2 22:6 chain are poor $cPLA_2$ substrates [27,28]. Likewise, 11,14-20:2-$COCF_3$ and 11,14,17-20:3-$COCF_3$ were prepared because substrate specificity studies of $cPLA_2$ 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-$COCF_3$ and 9t,12t-18:2-$COCF_3$, were prepared to explore the consequence of having altered double bonds stereochemistry, and finally 7,7-Me_2-5,8-20:2-$COCF_3$ 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 $cPLA_2$ binds very tightly to these anionic vesicles [21]. Anionic phospholipids containing two saturated fatty acyl chains were avoided since $cPLA_2$ rapidly inactivates on vesicles composed of such lipids [22]. It has previously been shown that $cPLA_2$ 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 PLA_2 inhibitors [6,9]. The trifluoromethyl ketones exhibit slow binding behavior with $cPLA_2$ but not with $iPLA_2$ [17,18,24]. Thus for

Table 1
$X_I(50)$ 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)

[a]Bayburt et al. reported an $X_I(50)$ of 0.01 [21].
[b]Numbers in parentheses are the potencies of the inhibitors relative to 20:4-$COCF_3$ ($X_I(50)$ for $AACOCF_3$ divided by $X_I(50)$ for $RCOCF_3$).
[c]Conde-Frieboese et al. reported $X_I(50)$s of 0.016 and 0.015 for 20:4 and 16:0, respectively [25].
[d]Conde-Frieboese et al. reported $X_I(50)$ of 0.005 [25].
[e]Ackermann et al. reported an $X_I(50)$ of 0.028 [18].
[f]Conde-Frieboese et al. reported $X_I(50)$ of 0.006 [25].

$cPLA_2$ 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 [^{14}C]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 $cPLA_2$ to micelles preloaded with substrate and inhibitor.

Table 1 lists the mole fraction of each inhibitor required to produce 50% inhibition, designated $X_I(50)$. 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-$COCF_3$ [$X_I(50)$ for 20:4-COCF3/$X_I(50)$ for $RCOCF_3$] for each assay. For any single assay, the ratio of $X_I(50)$s for two inhibitors is equal to the ratio of the interfacial enzyme-inhibitor dissociation equilibrium constants, K_I*, and thus the relative potencies are identical to the relative $1/K_I$* values. It is difficult to compare two $X_I(50)$ 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_I* 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 $cPLA_2$ (non-neutral diluent effect [33]). However, if relative values of $X_I(50)$ for a series of inhibitors in one assay are the same as those in the other assay, then the relative values of K_I* 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 $cPLA_2$ with different rates. Thus it is best to compare the $X_I(50)$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-$COCF_3$ and 16:1-$COCF_3$ 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-$COCF_3$ 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_I* 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 $cPLA_2$ 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_I* 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 $cPLA_2$, the $X_I(50)$ 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 $X_I(50)$ 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 $cPLA_2$ 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 $iPLA_2$ 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 $cPLA_2$ versus $iPLA_2$. For example, 16:1-$COCF_3$ and $Ph(CH_2)_4$-$COCF_3$ are, respectively, 10.7-fold and 7.2-fold more potent than 20:4-$COCF_3$

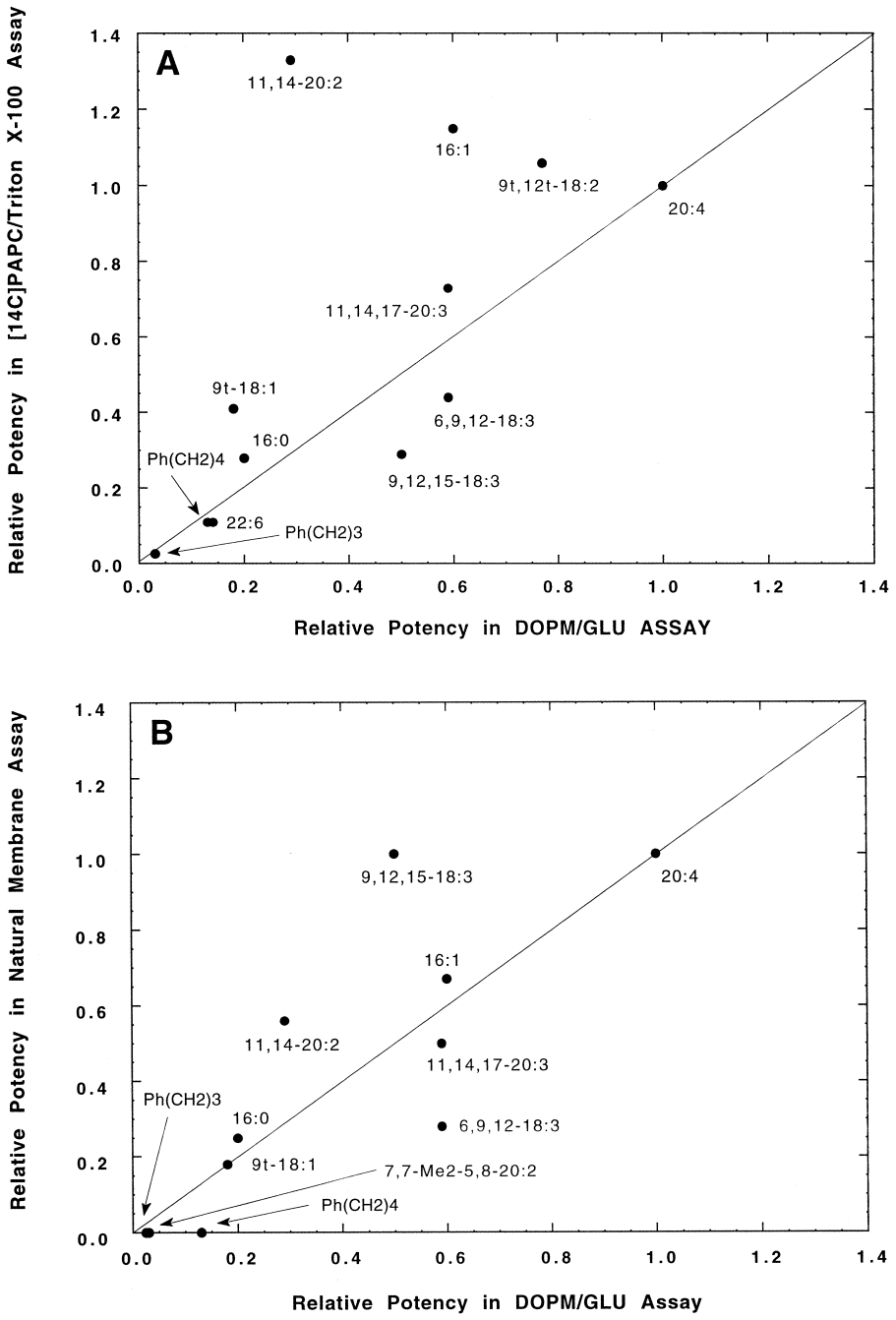


Fig. 2. In vitro inhibition of $cPLA_2$ by trifluoromethyl ketones measured with three different assays. (A) The abscissa gives the relative potency of the indicated trifluoromethyl ketones as an inhibitor of $cPLA_2$ ($X_I(50)$ for 20:4-$COCF_3$ divided by $X_I(50)$ for test compound) using the vesicle assay, and the ordinate gives the relative potency for the same set of compounds measured with the mixed-micelle assay (R group of R-$COCF_3$ 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 $iPLA_2$, whereas they are 1.7- and 7.7-fold, respectively, less potent than 20:4-$COCF_3$ against $cPLA_2$.

Among the $cPLA_2$ inhibitors, 20:4-$COCF_3$ is the best inhibitor of the series. 22:6-$COCF_3$ is 7-fold less potent than 20:4-$COCF_3$ in vesicles, which is consistent with the fact that $cPLA_2$ greatly prefers phospholipid substrates with an *sn*-2-20:4 chain over

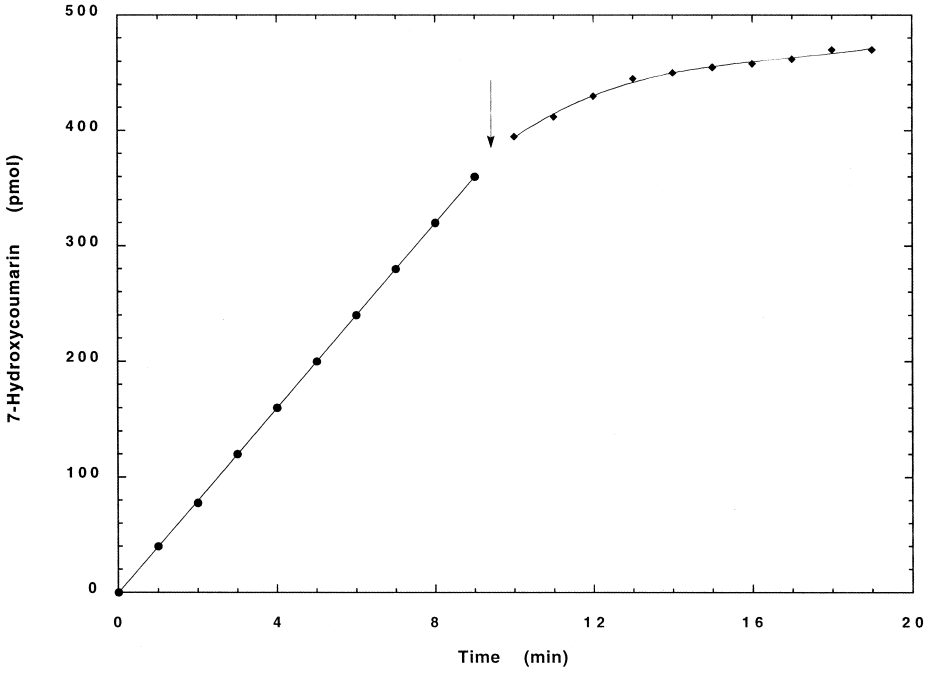


Fig. 3. Inactivation of $cPLA_2$ by 20:4-PO(OMe)F. Reaction progress curve for the hydrolysis of 0.02 mol fraction GLU in 100 mM DOPM vesicles in assay buffer (see Section 2) by 0.1 μg $cPLA_2$. 20:4-PO(OMe)F was added at a concentration of 0.005 mol fraction at the time indicated by the arrow.

those with an *sn*-2-22:6 chain (as noted above). Apparently, replacing the 5-double bond of 20:4-$COCF_3$ with an aromatic ring [$Ph(CH_2)_3$-$COCF_3$ and $Ph(CH_2)_4COCF_3$] leads to a poor fit in the active site of $cPLA_2$. Loss of the 5-double bond (11,14-20:2-$COCF_3$ and 11,14,17-$COCF_3$) results in a modest reduction in potency. Alkyl chain branching (7,7-Me_2-5,8-20:2) is not tolerated well by $cPLA_2$. Finally, shortening the chain length with removal of double bonds (16:1-$COCF_3$ and 16:0-$COCF_3$) leads to only a modest reduction in potency [18]. This is in marked contrast to previous results showing that 20:0-$COCF_3$ is much less potent as a $cPLA_2$ inhibitor compared to 20:4-$COCF_3$ [17].

$iPLA_2$ displays a very different pattern. It does not discriminate between 20:4-$COCF_3$ and 22:6-$COCF_3$, and it prefers 16:1-$COCF_3$ and 16:0-$COCF_3$ over 20:4-$COCF_3$. 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
$Ph(CH_2)_4PO(OMe)F$	0.12 ± 0.02	1.15 ± 0.3	10 800 ± 3000
$CH_2{=}CH(CH_2)_5PO(OMe)F$	0.15 ± 0.04	2.8 ± 0.7	not determined

[a]Measured from the rate of inactivation observed in real time with the DOPM/GLU assay.
[b]Measured 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 $Ph(CH_2)_4$-PO(OMe)F, that were anticipated to distinguish between $cPLA_2$ and $iPLA_2$. These compounds result in a slow loss of $cPLA_2$ 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 $cPLA_2$ 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_I^*, where K_I^* 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_I^* 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 > $Ph(CH_2)_4$-PO(OMe)-F, a trend that is consistent with the analogous series of trifluoromethyl ketones.

We also studied the rate of $cPLA_2$ 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_I, where K_I is the equilibrium constant for the reaction E•I $\rightarrow$ E+I, and k_i is the rate constant for E•I E-I (all in the aqueous phase). Values of k_i/K_I are listed in Table 2. Although k_i/K_I cannot be compared to k_i^*/K_I^* (they have different units), it is clear that $cPLA_2$ bound to the interface is much more sensitive to inactivation than enzyme in the aqueous phase. For example, for $cPLA_2$ 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 $cPLA_2$ 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_I value in Table 2). This result is in marked contrast to inactivation of secreted PLA_2s 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 $cPLA_2$ 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 $CH_2{=}CH(CH_2)_5PO(OMe)F$ and tested it as an inactivator of $cPLA_2$ using the DOPM/GLU assay (Table 2). In the aqueous phase in the absence of an interface, $cPLA_2$ is inactivated by $CH_2{=}CH(CH_2)_5PO(OMe)F$ with a second-order rate constant of 2.8 ± 0.7 M^{-1} s^{-1} (half-time for inactivation of 82 min with 50 μM inactivator). The half-time for $cPLA_2$ inactivation by 50 μM $CH_2{=}CH(CH_2)_5PO(OMe)F$ 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_I^* of 0.15 (mole fraction)$^{-1}$ s^{-1}. These results show that $CH_2{=}CH(CH_2)_5$-PO(OMe)F behaves similarly to $Ph(CH_2)_4$PO-(OMe)F as a $cPLA_2$ 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 $cPLA_2$ inactivator than are the long-chain compounds (Table 2).

Inactivation of $iPLA_2$ 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). $Ph(CH_2)_4$-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 $iPLA_2$ during

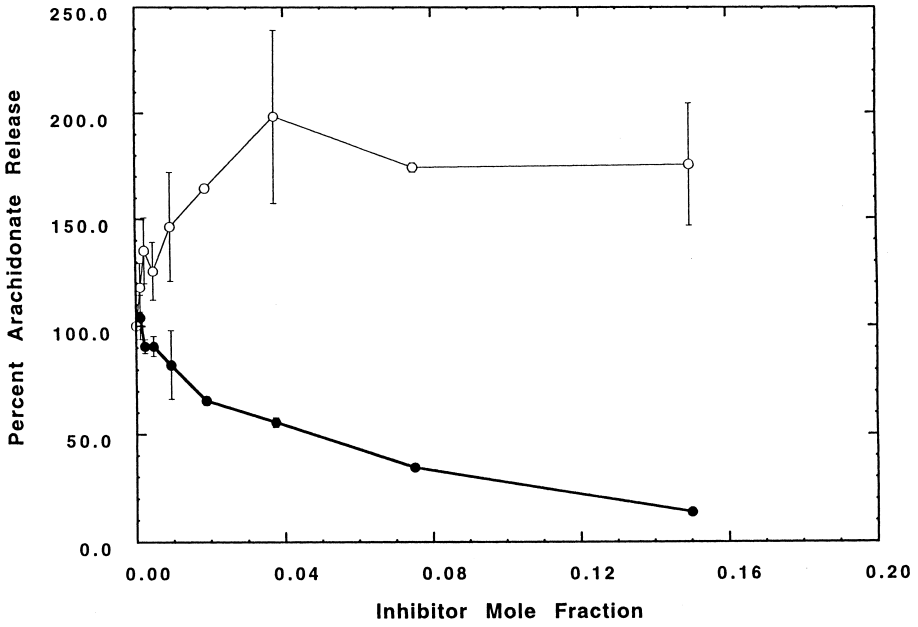


Fig. 4. Inactivation of $cPLA_2$ by fluoromethyl phosphonates in the natural membrane assay. Assays were carried out as described in Section 2 with the indicated concentrations of 20:4-PO(OMe)F (●) or $Ph(CH_2)_4$-PO(OMe)F (○). Error bars designate the standard deviation obtained from duplicate experiments. 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 $cPLA_2$ (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 $cPLA_2$ 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 $Ph(CH_2)_4$-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 $Ph(CH_2)_4$-PO(OMe)F. The reason for the approximately 2-fold increase in arachidonate production in the presence of $Ph(CH_2)_4$-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 $cPLA_2$ and $iPLA_2$ 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 $cPLA_2$ and $iPLA_2$, 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 PLA_2 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 PLA_2 can be discovered as long as the mole fraction of inhibitor in the interface is keep low (perhaps < 0.01).

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