Activated Ketones as Inhibitors of Intracellular Ca^{2+}-Dependent and Ca^{2+}-Independent Phospholipase A_2

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Abstract: Studies are reported on two different types of activated ketones as inhibitors of two important intracellular phospholipase A_2s (PLA_2): the group IV 85 kDa Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$) and the $P388D_1$ Ca^{2+}-independent phospholipase A_2 ($iPLA_2$). In a mixed micelle assay, we observed that the reaction progress curve of $cPLA_2$ in the presence of a trifluoromethyl ketone (TFMK) is linear at pH 7.4, while at pH 9.0 it is nonlinear and slows with time. An investigation of this discrepancy demonstrated that the TFMKs are slow, tight-binding inhibitors of the $cPLA_2$ at both pH's, that the rate of dissociation of the enzyme−inhibitor complex is the same at both pH's, but that the rate of association of enzyme and inhibitor is slower at pH 7.4 than at pH 9.0. A novel group of activated ketone inhibitors has been synthesized that contain a fatty acyl tricarbonyl. These compounds also inhibit the $cPLA_2$ in the mixed micelle assay. The inhibition of $cPLA_2$ by the tricarbonyls is readily reversible upon dilution and does not involve slow binding. For both types of inhibitor, no preference for fatty acid chain was observed as the palmityl analogs inhibited as well as the arachidonoyl analogs, despite the fact that the $cPLA_2$ shows a strong preference for arachidonoyl-containing phospholipid substrates over palmitoyl-containing substrates. With the $iPLA_2$, the inhibition by TFMKs is reversible and does not involve slow or tight binding. The tricarbonyls also inhibited the $iPLA_2$, but were less potent than the TFMKs. Unlike the $cPLA_2$, the $iPLA_2$ does exhibit a fatty acid preference as the palmityl analogs of both compounds inhibit better than the arachidonoyl analogs. The palmityl TFMK displayed a 10-fold lower IC_{50} at pH 9.0 than at pH 7.5, whereas the potency of the tricarbonyl was unchanged in this range. Thus, the TFMKs inhibit both the $cPLA_2$ and the $iPLA_2$, but the mechanism of inhibition of the two enzymes appears to be quite different. The tricarbonyls also inhibited both enzymes, but in both cases in a reversible manner and as such appear to be poorer inhibitors than the TFMKs.

Ketones with enhanced electrophilic reactivity (hydrate-forming ketones), such as trifluoromethyl ketones (TFMKs), α-diketones, α-keto esters, and α,*β*-dioxo esters or vicinal tricarbonyls,[1,2] are a well-established class of inhibitors for proteases, lipases, and other serine esterases. This type of inhibitor has now been used to study phospholipase A_2s (PLA_2), a class of esterases which carry out a reaction similar to that of lipases. Of particular interest are two intracellular PLA_2s, the Group IV, 85 kDa, Ca^{2+}-dependent PLA_2 ($cPLA_2$)[3−5] and the murine $P388D_1$ 80 kDa, Ca^{2+}-independent PLA_2 ($iPLA_2$).[6] The mechanisms of action of these two PLA_2s are not well established, although the $cPLA_2$ reaction is believed to proceed through an acyl-enzyme intermediate.[7,8] Sharp et al.[9] have shown that mutagenesis of a single serine residue at position 228 of $cPLA_2$ causes a loss of both the enzyme's PLA_2 and lysophospholipase (lysoPLA) activities. Thus, this enzyme is presumably a serine esterase. $cPLA_2$ is inhibited by a TFMK analog of arachidonic acid.[10] This arachidonoyl trifluoromethyl ketone (AATFMK) appears to be a slow, tight-binding inhibitor of $cPLA_2$, consistent with a serine esterase mechanism.

Much less is known about the mechanism of $iPLA_2$, except that this enzyme is inactivated by the haloenol lactone (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one,[11] a compound which was first introduced as an inhibitor of chymotrypsin.[12] While this enzyme is also inhibited by fatty acyl TFMKs, this inhibition appears to be readily reversible and not slow or tight-binding.[11] We have now compared the inhibition of the $cPLA_2$ and the $iPLA_2$ by TFMKs. We have also studied the inhibition of these enzymes by a novel group of vicinal tricarbonyl compounds (Chart 1), whose synthesis is reported herein. Since the two PLA_2s differ in their substrate

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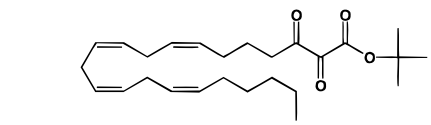
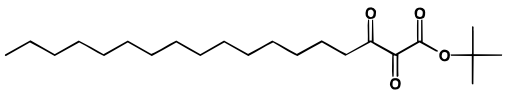
Chart 1



ARACHIDONYL TRICARBONYL (AA-TC)



PALMITOYL TRICARBONYL (PA-TC)

specificity with respect to the fatty acid hydrolyzed, the specificity of the enzymes for the fatty acid in the inhibitor was compared.

Experimental Section

Materials. L-α-1-Palmitoyl-2-[^{14}C]arachidonoyl phosphatidylcholine (57 mCi/mmol) was purchased from DuPont New England Nuclear. L-α-1-Palmitoyl-2-[^{14}C]palmitoyl phosphatidylcholine (58 mCi/mmol) was purchased from Amersham. Nonradioactive lipids were purchased from Avanti Polar Lipids. Ultrol grade 4-(2-hydroxyethyl)-1-piperazinylethanesulfonic acid (Hepes, free acid) and Triton X-100 were from Calbiochem. The silica gel used in Dole assays was Davisil grade 633 (200−425 mesh) from Fisher Scientific. Fatty acid free bovine serum albumin (BSA) was from Sigma. Purified, recombinant human cPLA$_2$ was provided by Dr. Ruth Kramer, Lilly Research Laboratories. iPLA$_2$ was purified from P388D$_1$ cells as described previously[6] and mono-Q eluents (purified 100000-fold) with a specific activity of about 1.3 μmol/(min mg) were utilized. Pentadecyl trifluoromethyl ketone (palmityl trifluoromethyl ketone; PATFMK) and *all-cis-5*,8,11,14-nonadecatetraenyl trifluoromethyl ketone (arachidonoyl trifluoromethyl ketone; AATFMK) were synthesized as described elsewhere.[11]

General Methods. Thin-layer chromatography (TLC) was carried out on Analtech silica gel GHLF 250-μm plates. NMR spectra were obtained on a GE QE 300-MHz NMR spectrometer or a Y 490-MHz NMR spectrometer with chemical shifts recorded in parts per million (ppm) from tetramethylsilane and referenced to residual solvent ($CHCl_3$: 7.24 ^{1}H-NMR; 77.0 ^{13}C-NMR). Infrared (IR) spectra were recorded on a Nicolet FTIR spectrometer. High-resolution mass spectra (HRMS) were performed by the University of Illinois, Champaign−Urbana, Mass Spectroscopy Laboratory (Urbana, IL). Radioactivity was measured with a Packard Tri-Carb model 1600 TR liquid scintillation analyzer using a Biosafe II scintillation cocktail (Research Products International, Corp.).

2,3-Dioxo-*all-cis*-7,10,13,16-docosatetraenoic Acid *tert*-Butyl Ester Monohydrate (AA-TC). Arachidonic acid (421 mg, 1.38 mmol, 70% from Sigma) was stirred at 25 °C in 7.0 mL of anhydrous dichloromethane under nitrogen. To this solution were added 780 mg (2.1 mmol, 1.5 equiv) of *tert*-butyl 2-(triphenylphosphoranylidenyl)acetate and 795 mg (2.5 mmol, 1.8 equiv) of EDCl (1-(3-(dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride). After approximately 5 min, a catalytic amount of DMAP (*N*,*N*-dimethylaminopyridine) was added. The reaction was then stirred at 25 °C for 8 h. The coupling reaction was monitored by TLC (silica; 1:1, ethyl acetate−hexane; R_f(arachidonic acid) = 0.6, R_f(product) = 0.9). Extended reaction times (>12 h) result in the formation of a lower R_f material, most likely the result of decomposition of the desired product, as this material does not react under the above coupling reaction conditions. The reaction was quenched by the addition of 3 mL of brine and washed with 3 × 10 mL of dichloromethane. The combined organic layers were then dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The crude oil was purified by silica gel chromatography (20:1, ethyl acetate−hexane) and provided 555 mg (0.77 mmol, 86%) of the desired ylide. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.4−7.7 (m, 15H), 5.32 (m, 8H), 2.80 (m, 6H), 2.05 (m, 2H), 1.55 (m, 4H), 1.29 (m, 6H), 1.05 (s, 9H), 0.87 (t, 3H, J = 7.3 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 197.0, 174.1, 133.1, 132.9, 132.2, 131.2, 131.5, 130.6, 130.5, 128.8, 128.7, 128.6, 128.5, 128.4, 128.1, 128.0, 127.9, 127.2, 127.6, 126.6, 40.0, 39.8, 31.7, 29.5, 28.3, 28.2, 25.9, 22.8, 14.0. HRMS: $C_{44}H_{56}O_3P$ requires 663.3967 amu, observed 663.3936 amu. IR (cm^{-1}): 3442.9, 2920.5, 2856.7, 1732.4, 1670.0, 1469.9, 1440.5, 1368.9, 1305.7, 1255.1, 1128.6, 1088.0.

Two hundred and fifty milligrams (0.38 mmol) of the ylide derived from arachidonic acid (above) was dissolved in 2.0 mL of anhydrous tetrahydrofuran (THF). To this solution were added 0.4 mL of deionized water and 465 mg (0.76 mmol, 2.0 equiv) of OXONE. After the mixture was stirred at 25 °C for approximately 2 h, the reaction was quenched by the addition of 3 mL of brine. The crude tricarbonyl compound was obtained after washing the reaction mixture with 3 × 8 mL of dichloromethane, drying the combined organic layers over $MgSO_4$, filtering, and concentrating *in vacuo*. Purification by silica gel chromatography (20:1 to 10:1, ethyl acetate−hexane) provided 160 mg (0.36 mmol, 95%) of the desired product as a pale yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.38 (m, 8H), 4.93 (s, OH), 2.80 (m, 6H), 2.56 (t, 2H, J = 7.5 Hz), 2.06 (m, 4H), 1.71 (m, 6H), 1.48 (s, 9H), 1.29 (m, 6H), 0.87 (t, 3H, J = 7.3 Hz). ^{13}C-NMR (75 MHz, CD_3OH): δ 205.9, 168.3, 131.1, 130.1, 129.7, 129.4, 129.1, 129.0, 128.8, 128.7, 84.5, 51.0, 37.2, 32.7, 30.5, 28.2, 28.1, 27.6, 26.6, 24.4, 23.7, 14.5. IR (cm^{-1}): 3452.3, 3008.3, 2954.3, 2929.9, 2862.2, 1733.6, 1729.5, 1457.6, 1395.6, 1366.3, 1280.0, 1255.5, 1152.2, 1103.5, 1097.7. HRMS or C, H analysis was not performed due to the instability of the compound; AA-TC decomposes at −20 °C in several days if stored neat or in neutralized $CDCl_3$. However, when stored in methanol only slight decomposition occurred over several weeks at −20 °C.

2,3-Dioxooctadecanoic Acid *tert*-Butyl Ester Monohydrate (PA-TC). PA-TC was prepared from palmitic acid using the procedure described above. The analytical data for the ylide (51% yield) are as follows: ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.42−7.68 (m, 15H), 2.88 (t, 2H, J = 7.3 Hz), 1.61 (m, 2H), 1.26 (m, 24H), 0.89 (t, 3H, J = 6.7 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 198.3, 168.2, 133.2, 133.1, 131.5, 128.6, 128.4, 127.5, 126.3, 70.3, 49.5, 40.4, 31.9, 29.8, 29.5, 26.0, 22.8, 14.2. HRMS: $C_{40}H_{56}O_3P$ requires 615.3967 amu, observed 615.3973 amu. The analytical data for PA-TC (90% yield) are as follows: ^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.01 (s), 2.56 (t, 2H, J = 8.6 Hz), 1.49 (s, 9H), 1.25 (s, 26H), 0.88 (t, 3H, J = 6.9 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 203.7, 168.3, 92.5, 84.9, 35.7, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 27.7, 23.4, 22.7, 14.2. HRMS: $C_{20}H_{41}O_4$ requires 369.3004 amu, observed 369.2998 amu.

Standard cPLA$_2$ Assay. cPLA$_2$ activity was measured in a PC mixed micelle assay in a standard buffer composed of 80 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM $CaCl_2$, 1 mg/mL BSA, and 1 mM dithiothreitol (DTT). The assay also contained 1 mM 1-palmitoyl-2-arachidonoylphosphatidylcholine (PAPC) (with 100000 cpm [^{14}C]-PAPC), 2 mM Triton X-100, 30% glycerol, and 3.75 μg/mL cPLA$_2$ in a volume of 200 μL. To prepare the substrate, an appropriate volume of cold PAPC in chloroform and [^{14}C]PAPC in toluene/ethanol 1:1 solution was evaporated to dryness under a stream of nitrogen. Triton X-100 (8 mM) in 3X assay buffer (240 mM Hepes (pH 7.4), 450 mM NaCl, 30 mM $CaCl_2$, 3 mg/mL BSA) was added to the dried lipid to give 4-fold concentrated substrate solution (4 mM PAPC). This solution was probe-sonicated on ice (0.5 s on, 0.5 s off for 6 min). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) at a concentration twenty times the desired assay concentration.

The substrate solution (50 μL) was placed in a siliconized glass test tube[7] containing 100 μL of 60% glycerol and 10 μL of the inhibitor/DMSO solution. The control contained DMSO alone, and no inhibitor. The mixture was vortexed and then bath sonicated for about 10 s. The reaction was initiated by the addition of 40 μL of enzyme solution (18.75 ng/μL in a 1.25X assay buffer) and incubated at 40 °C for 40 min. The reaction was quenched by the addition of 2.5 mL of Dole reagent (2-propanol, heptane, 0.5 M H_2SO_4; 400/100/20, v/v/v).[13] The amount of hydrolysis was determined using a modified Dole procedure[14] as described earlier.[15] Blank reactions lacking enzyme were routinely run and the resulting background hydrolysis was subtracted from the

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reported activities. Inhibitor concentrations expressed as mole fractions (χ_s)[16] were calculated utilizing the total concentration of lipid present in the assay (*i.e.* PC plus Triton X-100 plus inhibitor).

cPLA$_2$ Assay after Preincubation. The activity of cPLA$_2$ in the presence of AATFMK was also determined in the cPLA$_2$ assay after a 4-h preincubation. Solutions were prepared as described for the standard cPLA$_2$ assay above, except that the buffer also contained 80 mM glycine. The enzyme solution, 40 μL was incubated in the presence of inhibitor in 5 μL of DMSO and 100 μL of 60% glycerol for 4 h prior to the initiation of the reaction by the addition of 50 μL of substrate solution. The inhibitor concentrations reported represent that in the final assay.

Progress Curves. Time courses for the cPLA$_2$ were performed similar to the standard assay but utilized a mixed buffer, prepared by adding 80 mM glycine to the standard assay buffer, so that time courses at both pH 7.4 and 9.0 were performed in similar buffers. Higher amounts of radioactivity were also utilized (300000 cpm/200 μL) so that the rates at the earlier time points could be more accurately measured. Other assay components were the same as described above, except that reactions were initiated in a volume of 2.0 mL. At each time point a 200-μL aliquot was removed, quenched by being added to a test tube containing 2.5 mL of Dole reagent, and then extracted as described above.

iPLA$_2$ Assay. Assays for the iPLA$_2$ contained 100 μM 1,2-dipalmitoylphosphatidylcholine (DPPC) (with 200000 cpm [^{14}C]-DPPC), 400 μM Triton X-100, 100 mM Hepes (pH 7.5), 5 mM EDTA, and 0.1 mM ATP in a final volume of 500 μL. The substrate was prepared as described previously.[6] The inhibitors, in 5 μL of DMSO, were added to 445 μL of the mixed micellar substrate, vortexed for 10 s, bath sonicated for 10 s, and vortexed again for 10 s. Assays were initiated by the addition of 50 μL of iPLA$_2$ (an amount sufficient to generate 2000 cpm in 30 min). Assay solutions were incubated at 40 °C for 30 min, then quenched and extracted using the Dole procedure described above for the cPLA$_2$ assay. Assays at pH 9.0 were run in a mixed buffer composed of the assay buffer with 100 mM glycine added. For time courses, the reactions were initiated in a volume of 5 mL and at each time point 500 μL of the assay mix (containing 200000 cpm [^{14}C]-DPPC) were removed, quenched with Dole reagent, and extracted as before.

Inhibition Recovery Curves. For experiments monitoring the recovery of cPLA$_2$ from inhibition, 1 mg/mL of enzyme was incubated for 1 h at 40 °C with 1 mM AATFMK or PA-TC in a volume of 50 μL. The buffer was a combination of enzyme storage buffer and assay buffer and was composed of 160 mM glycine, 160 mM Hepes, 6.25 mM Tris-HCl, 338 mM NaCl, 20 mM $CaCl_2$, 0.5 mM EDTA, 2.25 mM DTT, 2 mg/mL BSA, 8.75% glycerol, and 6.5% DMSO at pH 7.4 or 9.0. After preincubation, enzymatic activity was followed as described above for the progress curve experiments. To initiate the reaction, 3 μL of the preincubation solution was added to 3 mL of the standard cPLA$_2$ assay mixture (at the same pH as the preincubation) resulting in a 1000-fold dilution of enzyme and inhibitor into the assay. From this solution, 200 μL were removed at appropriate times and the amount of product quantified as described above.

To determine the reversibility of iPLA$_2$ inhibition by PATFMK, the iPLA$_2$ was first concentrated 10-fold using a Centricon-10 concentrator from Amicon. The concentrated iPLA$_2$ (20 μL) in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM Triton X-100, 1 mM DTT, 1 mM ATP, and 10% glycerol was preincubated with either 30 μM PATFMK in DMSO or DMSO alone (2 μL) for 1 h at 40 °C. A 2-μL aliquot was then removed and diluted 1500-fold into 3 mL of assay mixture containing 100 μM DPPC (with 400000 cpm per 100 μL), 400 μM Triton X-100, 5 mM EDTA, 1 mM DTT, 0.8 mM ATP, 100 mM Hepes, and 100 mM glycine at pH 9.0. At selected time points, a 100-μL aliquot was removed and the amount of product was quantified as described above.

Data Analysis. Linear least-squares lines were drawn through all linear data, and the correlation coefficients were all greater than 0.99. For nonlinear plots the nonlinear regression algorithms of the Sigmaplot Graphing program (Jandel Scientific) were used to fit the data to the following equations. For Figures 2 and 5 the IC$_{50}$s were determined with:

$$\frac{v_i}{v_o} = \frac{IC_{50}}{IC_{50} + [I]} \quad (1)$$

Where v_i is the inhibited velocity, v_o is the non-inhibited velocity, [I] is the inhibitor concentration, and IC$_{50}$ is the concentration of the inhibitor that produces 50% inhibition.

For the progress and recovery experiments (Figures 3 and 4), the data were fit to eq 2.[17]

$$P = v_s t + \frac{v_o - v_s}{k_{obs}}(1 - e^{-k_{obs}t}) \quad (2)$$

Where P is the product, v_s is the equilibrium velocity, v_o is the initial velocity, k_{obs} is the apparent rate constant for the approach to equilibrium, and t is time.

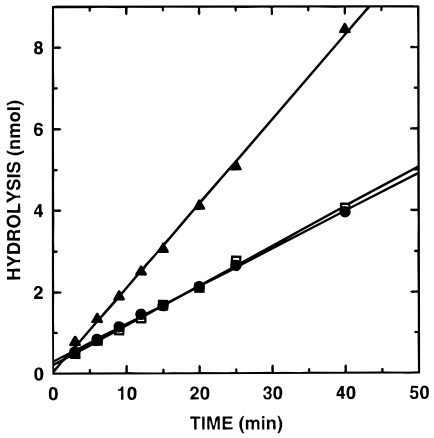

Figure 1. Reaction progress curves of cPLA$_2$ at pH 7.4 in the presence of PA-TC and AATFMK. The cPLA$_2$ was assayed in the cPLA$_2$ assay at pH 7.4 in the absence of inhibitor (▲) or in the presence of either 50 μM AATFMK (●) or 50 μM PA-TC (□). Each point represents the average of duplicates.

Results

cPLA$_2$. Using the cPLA$_2$ assay, we investigated the action of several fatty acid analogs as inhibitors of cPLA$_2$. Figure 1 shows the reaction progress curve for cPLA$_2$ at pH 7.4 along with the progress curves observed in the presence of 50 μM PA-TC or 50 μM AATFMK. All three reactions are linear with time for over 40 min. This result is in striking contrast to the nonlinear reaction progress curve observed previously with AATFMK[10] which was indicative of a slow, tight-binding inhibition.

The concentration-dependent inhibition of cPLA$_2$ by the fatty acid tricarbonyls and the fatty acid TFMKs is shown in Figure 2. IC$_{50}$s were determined by fitting the data to eq 1 using nonlinear regression and are as follows: AATC = 67 ± 3 μM (0.022 mole fraction), PATC = 57 ± 8 μM (0.019 mole fraction), AATFMK = 50 ± 6 μM (0.016 mole fraction), and PATFMK = 45 ± 7 μM (0.015 mole fraction). The potency of the tricarbonyl inhibitors was approximately the same as that of the trifluoromethyl ketone inhibitors. Note that for both types of compounds, the palmitic acid analog inhibited as well as the arachidonic acid analog. Thus, this enzyme did not show a preference for the fatty alkyl chain attached to the functional group. Since the reaction progress curves in Figure 1 were linear, the data shown in Figure 2 were obtained without first preincubating the enzyme with the inhibitor; preincubation of the enzyme with the inhibitors for 15 min prior to assaying did not affect the amount of inhibition observed. The data shown

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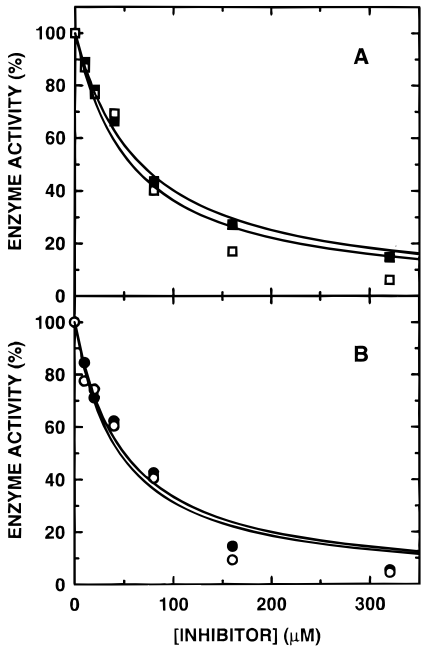

Figure 2. Concentration-dependent inhibition of $cPLA_2$ by tricarbonyls and trifluoromethyl ketones. $cPLA_2$ was assayed in the $cPLA_2$ assay at pH 7.4 in the presence of increasing concentrations of inhibitor: (A) PA-TC (□) or AA-TC (■); (B) PATFMK (○) or AATFMK (●). The enzyme activity is plotted as the percentage of the control activity, which was assayed in the absence of inhibitor; 100% activity represents 0.15 μmol/(min mg). Each point represents the average of triplicates.

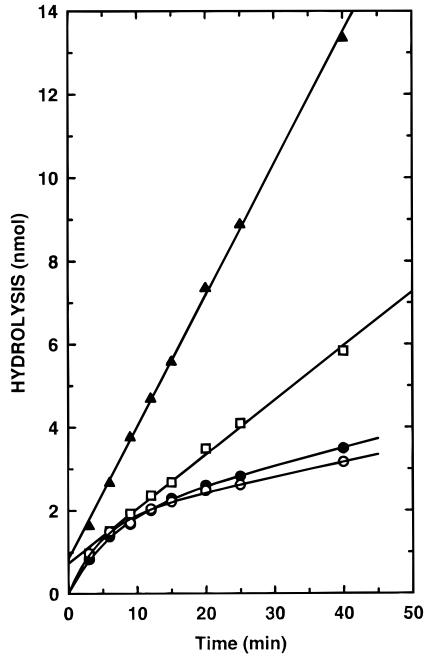

Figure 3. Reaction progress curves of $cPLA_2$ at pH 9.0 in the presence of PA-TC, PATFMK, and AATFMK. $cPLA_2$ was assayed in the $cPLA_2$ assay at pH 9.0 in the absence of inhibitor (▲) or in the presence of either 50 μM AATFMK (●), PATFMK (○) or PA-TC (□). Each point represents the average of duplicates.

in this figure were obtained in the standard buffer but in the absence of DTT; when the experiments were repeated in the presence of DTT the results were the same. Also, since fatty acids bind to BSA we were concerned that the inhibitors under study might also bind to BSA. However, experiments run in the absence of BSA showed the same IC_{50}s.

To determine if the pH of the assay affects the nature of the inhibition, we analyzed the reaction progress curves at pH 9.0. As shown in Figure 3, the control and PA-TC displayed linear progress curves at pH 9.0. The presence of 50 μM PA-TC caused a 58% inhibition of the control activity which was similar to the 53% inhibition observed at pH 7.4 (Figure 1). Thus, the potency of this compound is not affected by the pH of the assay.

The reaction progress curves with both AATFMK and PATFMK are not linear at pH 9.0 since the reaction rates decrease with time; this is similar to the results reported by Street et al.[10] The parameters were obtained from the nonlinear regression of the AATFMK data to eq 2 and are $v_o = 0.32 \pm 0.01$ nmol/min, $v_s = 0.41 \pm 0.03$ nmol/min, and $k_{obs} = 0.16 \pm 0.01$ min^{-1} while those for PATFMK are $v_o = 0.41 \pm 0.03$ nmol/min, $v_s = 0.036 \pm 0.003$ nmol/min, and $k_{obs} = 0.22 \pm 0.02$ min^{-1}. Thus, pH does affect the nature of the inhibition by these compounds.

Since the reaction progress curve of $cPLA_2$ in the presence of AATFMK was nonlinear at pH 9.0 but linear at pH 7.4, the mechanism of AATFMK inhibition is ambiguous. Thus, we investigated further the behavior of these inhibitors at pH 7.4 and 9.0. The reversibility of $cPLA_2$ inhibition by AATFMK and PA-TC at pH 7.4 and 9.0 is shown in Figure 4. The enzyme was preincubated with a high concentration of inhibitor (1 mM) for 1 h and then diluted 1000-fold into an assay mixture. The hydrolysis of substrate with time following dilution is shown. The inhibition of $cPLA_2$ by PA-TC was readily reversible at both pH's and full enzymatic activity was recovered immediately after dilution. In contrast, the TFMK exhibited slow-binding at both pH's and little activity was observed immediately after

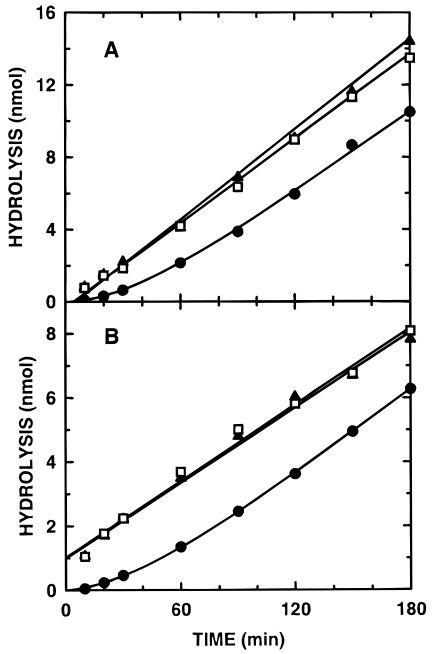

Figure 4. Reversibility of $cPLA_2$ inhibition by PA-TC and AATFMK: panel A, pH 7.4; panel B, pH 9.0. $cPLA_2$ was incubated for 1 h in the absence of inhibitor (▲) or in the presence of PA-TC (□) or AATFMK (●). After preincubation, the enzyme was diluted 1000-fold into a substrate solution and the amount of product formed was monitored with time. Each point represents the average of duplicates.

dilution. The initial velocity, v_o, in the recovery experiments was $<10^{-10}$ nmol/min (because of the low value the error in determining this value was very high) at pH 7.4 and 0.004 ± 0.002 nmol/min at pH 9. Enzyme activity recovered with time following dilution and full enzymatic activity was observed within 1 h. The final equilibrium velocities were close to the controls, 0.075 ± 0.003 nmol/min at pH 7.4 and 0.045 ± 0.0008 nmol/min at pH 9.0. Note that this rate of recovery is considerably faster than that observed by Street et al.,[10] who saw

only 14% recovery of activity in 4 h after dilution. This faster recovery rate could be due to the higher substrate and Triton concentrations used in this current study as well as to the lack of 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol (DTPM) which was present in the previous study and which may cause an increase in the fraction of $cPLA_2$ bound to the micelles.[10]

In Figure 4, enzymatic activity is recovered very rapidly after the 1000-fold dilution and appears to reach equilibrium. The rate constant of this recovery (k_{obs}) is given by eq 3:[17]

$$k_{obs} = k_{off} + \left(\frac{k_{on}}{1 + [A]/K_A}\right)[I] = k_{off} + k'_{on} \qquad (3)$$

where K_A is the Michaelis constant for the substrate whose concentration is A, k_{on} is the association rate constant, k'_{on} is the apparent association rate constant, and k_{off} is the dissociation rate constant. Since the enzyme−inhibitor complex is diluted 1000-fold into the assay mix, the concentration of inhibitor I present during recovery is 1 μM which is well below the estimated IC_{50} of 50 μM. Therefore the term $\{k_{on}/(1 + [A]/K_A)\}[I]$ should be small compared to k_{off}. Thus, k_{obs} is approximately equal to k_{off}. The k_{obs} obtained from the recovery of $cPLA_2$ from AATFMK inhibition is 0.025 ± 0.007 min^{-1} at pH 7.4 and 0.021 ± 0.003 min^{-1} at pH 9.0. Thus, the off rate for AATFMK is the same at both pH 7.4 and 9.0.

At pH 7.4, the progress and the recovery experiments for AATFMK do not seem to agree. The progress experiment indicates that AATFMK is not a slow-binding inhibitor while the recovery experiment indicates that it is. One way in which these differences can be reconciled is if AATFMK reaches equilibrium much faster or much slower than the rate of substrate hydrolysis, i.e. equilibrium is achieved either before the first assay data point or long after the last data point is taken so that during the assay no appreciable change in rate is observed. To distinguish between these two possibilities, the concentration-dependent inhibition of $cPLA_2$ by AATFMK was repeated as described above with a 4-h preincubation of enzyme and inhibitor prior to assay. At pH 9.0, after preincubation the apparent IC_{50} was 5.6 ± 0.7 μM; at pH 7.4, preincubation lowered the apparent IC_{50} from 50 ± 6 to 15 ± 1 μM (data not shown). The decrease in IC_{50} after the long preincubation demonstrates that the amount of inhibition is slowly increasing and indicates that the rate of reaction of AATFMK with $cPLA_2$ is very slow at physiological pH. Since the k_{off} has been shown to be the same, this change in k_{obs} is due to the association rate being slower at pH 7.4, slow enough so that no decrease of the enzymatic rate is perceived during the reaction progress curve in Figure 1, thus implying that k'_{on} at pH 7.4 is less than at pH 9.0.

$iPLA_2$. In addition to inhibiting the $cPLA_2$, we have previously demonstrated that the fatty acid TFMKs also inhibit the mouse macrophage $iPLA_2$.[11] Unlike the $cPLA_2$, the $iPLA_2$ showed a preference for the fatty acid moiety and was about 4-fold more potent on PATFMK than on AATFMK.[11] These previous studies with the $iPLA_2$ were performed at pH 7.5 where the enzyme displays a linear progress curve in the presence of the TFMKs. In light of the results described above for $cPLA_2$, the behavior of $iPLA_2$ was investigated with the TFMKs at pH 9.0. Also, the ability of the tricarbonyls to inhibit the $iPLA_2$ was tested. The concentration-dependent inhibition of $iPLA_2$ by PATFMK and PA-TC is shown in Figure 5 at pH 7.5 (A) and 9.0 (B). As before,[11] PATFMK inhibited the $iPLA_2$ at pH 7.5 with an IC_{50} of 3.3 ± 0.3 μM (0.006 mole fraction). In surface concentration terms, this IC_{50} is 2−3-fold lower than that observed by the TFMKs with the $cPLA_2$. Thus, at pH 7.5 without preincubation the inhibitor appears to work equally well on both enzymes. The tricarbonyl was a poorer inhibitor for the $iPLA_2$ than the TFMK, with an IC_{50} of 16 ± 3 μM (0.031 mole fraction). The AA-TC (not shown) was an even weaker inhibitor with an IC_{50} of 52 μM (0.094 mole fraction), consistent with the fatty acid preference observed with the TFMKs. At pH 9.0 the IC_{50} of the tricarbonyl is unchanged, 20 ± 2 μM. The TFMK, however, was significantly more potent at this pH with an IC_{50} of 0.36 ± 0.02 μM (0.0006 mole fraction), over 10-fold lower than was observed at pH 7.5.

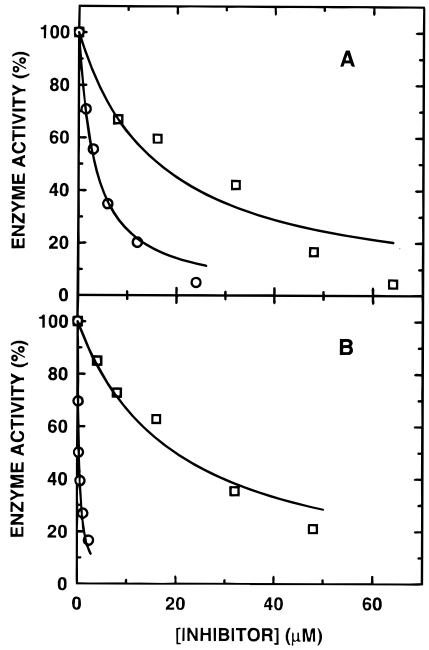

Figure 5. Concentration-dependent inhibition of $iPLA_2$ by PA-TC and PATFMK. $P388D_1$ $iPLA_2$ was assayed at pH 7.5 (A) or 9.0 (B) in the $iPLA_2$ assay in the presence of increasing concentrations of PATFMK (○) or PA-TC (□). The enzyme activity is plotted as the percentage of the control activity, which was assayed in the absence of inhibitor; 100% activity represents 20 pmol/min in both A and B. Each point represents the average of duplicates.

The reaction progress curve for $iPLA_2$ in the presence of the fatty acyl tricarbonyls was linear with time at both pH 7.5 and 9.0, as shown in Figure 6. The reaction progress was also monitored at pH 9.0 in the presence of PATFMK. This reaction was also linear with time. This is in contrast to the time-dependent inhibition of $cPLA_2$ at this pH. Also, after 1 h of preincubation the inhibition of $iPLA_2$ by PATFMK was fully reversible at pH 9.0 immediately following dilution (Figure 7). Thus, the mechanism of inhibition of $iPLA_2$ by the trifluoromethyl ketones appears to be different from that of the $cPLA_2$.

Discussion

The use of trifluoromethyl ketones as inhibitors of proteases and other hydrolases was first described by Abeles and co-workers.[18,19] These activated ketones form hydrates in aqueous solution. They can also form an enzyme-bound hemiketal upon reaction with the hydroxyl group of a serine residue. Both the hemiketal and the hydrate forms of the TFMKs have been observed with the proteases; the hemiketal form inhibits the serine proteases[19,20] and the tetrahedral hydrate

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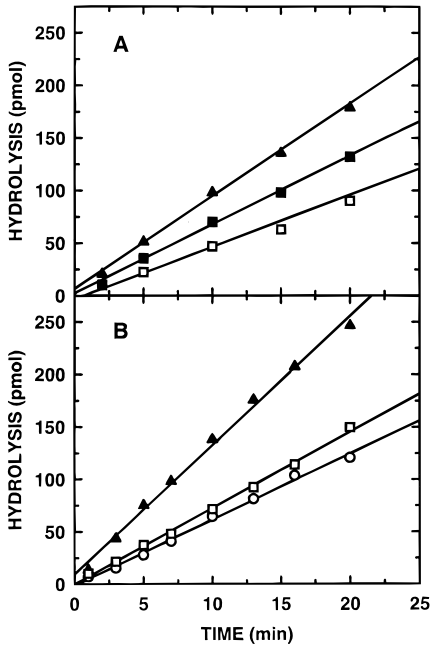


Figure 6. Reaction progress curves of $iPLA_2$ in the presence of tricarbonyls and trifluoromethyl ketones. $iPLA_2$ activity was measured at pH 7.5 (A) in the absence of inhibitor (▲) or in the presence of 32 μM AA-TC (■) or 32 μM PA-TC (□) or at pH 9.0 (B) in the absence of inhibitor (▲) or in the presence of 24 μM PA-TC (□) or 0.4 μM PATFMK (○). Each point represents the average of duplicates.

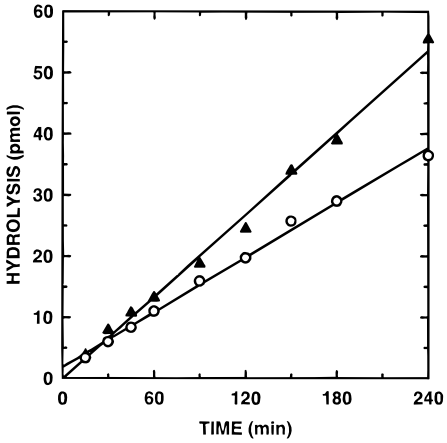
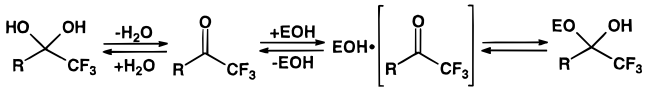


Figure 7. Reversible inhibition of $iPLA_2$ by PATFMK. $iPLA_2$ was preincubated for 1 h in the absence (▲) or presence (○) of 30 μM PATFMK and then diluted 1500-fold into an assay mixture at pH 9.0. At the indicated time points, an aliquot was removed and the amount of product formed was measured. Each point represents the average of duplicates.

form inhibits the aspartyl proteases and the zinc metalloproteases.[18] At basic pH, an anion of the hydrate or hemiketal can be formed. The TFMKs have recently been applied to the study of phospholipases by Street et al.,[10] who demonstrated that AATFMK is a reversible, slow, tight-binding inhibitor of the Group IV cytosolic, 85 kDa, Ca^{2+}-dependent PLA_2. NMR studies[8] suggested that the inhibitor was bound as the anion of the hemiketal; however, the possibility that the bound species was the monoanion of the hydrate could not be excluded.

Our studies with activated ketone inhibitors of $cPLA_2$ began with the evaluation of a novel set of fatty acyl tricarbonyl compounds. These compounds were designed based on the peptidyl tricarbonyls which have previously been described as inhibitors of the proteases.[1] The fatty acyl TFMKs were initially utilized as controls for comparison with the fatty acyl tricarbonyls; however, the unexpected linear reaction progress curve with the TFMKs at pH 7.4 led us to investigate this inhibition further. We have found that AATFMK is a slow, tight-binding inhibitor of $cPLA_2$ at both pH 7.4 and 9.0, but at pH 7.4 the inhibition is considerably slower than is observed at pH 9.0.

Scheme 1



Our results indicate that the rate of dissociation of the enzyme−inhibitor complex is the same at pH 7.4 as at pH 9.0. This result is similar to that observed for the dissociation of the chymotrypsin/TFMK complex[21] where k_{off} showed no pH dependence between pH 4.0 and 9.5 indicating the lack of an ionizable group in the enzyme−inhibitor complex in this pH range. Unlike k_{off}, the apparent association rate constant (k'_{on}) of AATFMK with $cPLA_2$ is smaller at pH 7.4 than at pH 9.0. As shown in Scheme 1, the binding of the inhibitor to the enzyme involves several steps:[22] the dehydration of the hydrate form of the inhibitor (which is predominant in aqueous solution) to the free ketone, the initial binding of the inhibitor to the enzyme, and a second enzymatic step, presumably the formation of a covalent enzyme−inhibitor hemiketal. k'_{on} is a function of all these steps. The different association rates observed at different pH's could reflect an effect of pH on any of these steps, particularly on the rate of dehydration of the hydrate to the ketone form of the inhibitor or on the ionization of certain groups in the enzyme active site. The effect of pH may also be on substrate binding to the active site since k'_{on} contains the term $1 + [A]/K_A$.

The fatty acyl tricarbonyls appeared to behave similarly to the TFMKs. In particular, the reaction progress curves and the concentration-dependent inhibition by the tricarbonyls at pH 7.4 (in the absence of preincubation) were practically superimposable with those of the TFMKs. However, the linear reaction progress curve at pH 9.0 and the immediate reversibility of PA-TC inhibition following preincubation clearly differentiate these inhibitors from the TFMKs and indicate that the tricarbonyls are not slow, tight-binding inhibitors.

According to Morrison and Walsh,[23] the majority of slow, tight-binding inhibitors involve the rapid formation of an inhibitor−enzyme complex followed by a slower isomerization or conformational change. In the case of the TFMKs, the inhibitor could bind rapidly followed by the slower formation of the enzyme−inhibitor hemiketal as shown by Street et al.[10] With the tricarbonyls, the inhibitor may bind quickly but be unable to form the hemiketal. As evidenced by ^{13}C-NMR data, the tricarbonyls are isolated as stable hydrates of the central ketone, similar to that observed previously with the peptidyl tricarbonyls.[1,2,24] The most reactive carbonyl for this inhibitor may not be positioned properly in the active site for attack by the serine residue. Such a mechanism would explain why the TFMKs and tricarbonyls appear to behave so similarly at pH 7.4 in the absence of preincubation. Alternatively, it is possible that the tricarbonyls bind differently in the active site or that they do not act at the catalytic site but rather bind to another site on the enzyme or interfere with the binding of the $cPLA_2$ to the surface.

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One striking fact observed with the TFMKs and tricarbonyls is that for both types of inhibitors the palmityl analog inhibited as well as the arachidonoyl analog. $cPLA_2$ has been shown by several groups to prefer arachidonoyl-containing phospholipids as substrates.[25−28] However, the $cPLA_2$ also exhibits a lysophospholipase activity[7,29] and readily hydrolyzes palmitoyl lysophosphatidylcholine in a Triton/phosphatidylcholine/lysophosphatidylcholine mixed micelle assay at a rate comparable to the PLA_2 activity (K. Conde-Frieboes and E. A. Dennis, unpublished data). Since the enzyme will hydrolyze both fatty acid esters, it is reasonable that a fatty acid chain preference for these inhibitors was not observed.

In preliminary experiments with the $iPLA_2$ from the murine macrophage-like cell line, $P388D_1$, we found that the inhibition of the enzyme by TFMKs did not appear to involve slow binding. In light of the results with the $cPLA_2$, we have now reexamined this inhibition under more stringent conditions. We show here that even at pH 9.0 the reaction progress curve is linear in the presence of PATFMK and this inhibition is readily reversible after a 1-h preincubation. Also, we found the TFMK to be 10-fold more potent at pH 9.0 than at pH 7.5. Thus, the mechanism of inhibition of the fluoromethyl ketones with the $iPLA_2$ differs from that observed with the $cPLA_2$. While a serine hemiketal of AATFMK appears to be the form of inhibition with the $cPLA_2$,[8] it is possible that the $iPLA_2$ is inhibited by the tetrahedral hydrate, or perhaps it forms a hemiketal with a different amino acid residue.

In comparison with the TFMKs, the fatty acyl tricarbonyls were weaker inhibitors for the $iPLA_2$. The palmityl tricarbonyl inhibited better than the arachidonoyl tricarbonyl, consistent with the TFMKs and with the previously noted substrate preferences for this enzyme.[6] As with the TFMKs, the reaction progress curves with the tricarbonyls were linear; however, the potency of the tricarbonyls was unaffected by pH. Thus, the tricarbonyls were poorer inhibitors than the TFMKs for both the $cPLA_2$ and the $iPLA_2$.

An important conclusion of this study is that compounds that were thought to be specific inhibitors of the $cPLA_2$ are also capable of inhibiting the $iPLA_2$. Thus, particular caution should be taken when using $cPLA_2$ or $iPLA_2$ inhibitors in whole cell studies, as these inhibitors may affect both enzymes. Also, remarkably, while the $cPLA_2$ is specific for arachidonoyl-containing phospholipid substrates, arachidonoyl-containing inhibitors in these studies were no better than palmityl-containing inhibitors.

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