Slow- and Tight-Binding Inhibitors of the 85-kDa Human Phospholipase A_2[†]

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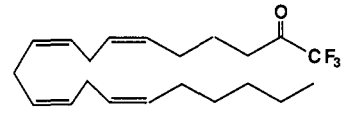
ABSTRACT: A trifluoromethyl ketone analogue of arachidonic acid in which the COOH group is replaced with $COCF_3$ ($AACOCF_3$) was prepared and found to be a tight- and slow-binding inhibitor of the 85-kDa cytosolic human phospholipase A_2 ($cPLA_2$). Enzyme inhibition was observed when $AACOCF_3$ was tested in assays using either phospholipid vesicles or phospholipid/Triton X-100 mixed micelles. The fact that the inhibition developed over several minutes in both assays establishes that $AACOCF_3$ inhibits by direct binding to the enzyme rather than by decreasing the fraction of enzyme bound to the substrate interface. From the measured values of the inhibitor association and dissociation rate constants, an upper limit of the equilibrium dissociation constant for the Ca^{2+}·$AACOCF_3$·$cPLA_2$ complex of 5×10^{-5} mole fraction was obtained. Thus, detectable inhibition of $cPLA_2$ by $AACOCF_3$ occurs when this compound is present in the assay at a level of one inhibitor per several thousand substrates. Arachidonic acid analogues in which the COOH group is replaced by $COCH_3$, $CH(OH)CF_3$, CHO, or $CONH_2$ did not detectably inhibit the $cPLA_2$. The arachidonyl ketones $AACOCF_2CF_3$ and $AACOCF_2Cl$ were found by ^{19}F NMR to be less hydrated than $AACOCF_3$ in phospholipid/Triton X-100 mixed micelles, and compared to $AACOCF_3$ these compounds are also weaker inhibitors of $cPLA_2$. In keeping with the fact that $cPLA_2$ displays substrate specificity for arachidonyl-containing phospholipids, the arachidic acid analogue $C_{19}H_{39}COCF_3$ is a considerably less potent inhibitor compared to $AACOCF_3$. $AACOCF_3$ is about 4 orders of magnitude less potent as an inhibitor of the human nonpancreatic secreted 14-kDa phospholipase A_2. This fact together with the likelihood that $AACOCF_3$ is cell-permeable suggests that this compound may be useful in studying the role of the $cPLA_2$ in cellular processes that involve arachidonic acid liberation.

Phospholipases A_2 (PLA_2)[1] (EC 3.1.1.4) catalyze the hydrolysis of the ester linkage at the *sn*-2 position of phospholipids to produce a free fatty acid and a lysophospholipid (van Deenan & de Haas, 1963). Most of the PLA_2s purified and characterized so far are from the family of low molecular weight secreted enzymes (Verheij et al., 1981; Dennis, 1983; Seilhamer et al., 1989; Kramer et al., 1989). Recently, a new type of PLA_2 found in the cytosol of cells ($cPLA_2$) that shows no sequence homology with any of the secreted enzymes has been purified from various sources such as platelets (Kramer et al., 1987; Kim et al., 1991), macrophages (Leslie et al., 1987; Wijkander & Sundler, 1989), spleen (Wijkander & Sundler, 1991), kidney (Gronich et al., 1990), and the human monocytic cell line U937 (Clark et al., 1990, 1991; Diez & Mong, 1990; Kramer et al., 1991). The $cPLA_2$ has a high molecular mass (85 kDa), is activated by submicromolar concentrations of calcium (Diez & Mong, 1990; Clark et al., 1991; Kramer et al., 1991; Tremblay et al., 1992), and shows some selectivity toward the hydrolysis of arachidonyl-containing phospholipids (Diez & Mong, 1990; Clark et al., 1991; Kramer et al., 1991). The $cPLA_2$ has been implicated in the process of arachidonic acid release from its cellular store in the phospholipid pool in response to a number of different cellular stimuli (Lin et al., 1992). Since arachidonic acid release is thought to be the rate limiting step in the biosynthesis of leukotrienes and prostaglandins, it is likely that the $cPLA_2$ plays an important regulatory role in the production of these potent proinflammatory mediators.

Little is known about the catalytic mechanism of the $cPLA_2$. The available evidence suggests that there are significant mechanistic differences between the $cPLA_2$ and the secreted enzymes. Although both types of PLA_2 require calcium for activity, it is likely that the function of this cofactor is different for each type of enzyme (Scott et al., 1991; Wijkander & Sundler, 1992; Ghomashchi et al., 1992). Furthermore, although several classes of potent substrate/transition-state analogue inhibitors are available for the secreted enzymes (Yuan et al., 1987; Jain et al., 1989, 1991a; Ransac et al., 1990; Yu et al., 1990), most of these compounds have little, if any, inhibitory activity against the $cPLA_2$ (Street and Gelb, unpublished results). In this paper we report that the trifluoromethyl ketone analogue of arachidonic acid ($AACOCF_3$) is a potent and selective slow-binding inhibitor of the $cPLA_2$.



AACOCF3

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[1] Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, 85-kDa cytosolic phospholipase A_2; $AACOCF_3$, $AACOCH_3$, $AACOCF_2Cl$, and $AACOCF_2CF_3$, analogues of arachidonic acid in which the OH is replaced with, CF_3, CH_3, CF_2Cl, and CF_2CF_3, respectively; PAPC, 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; SAPC, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphocholine; DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol.

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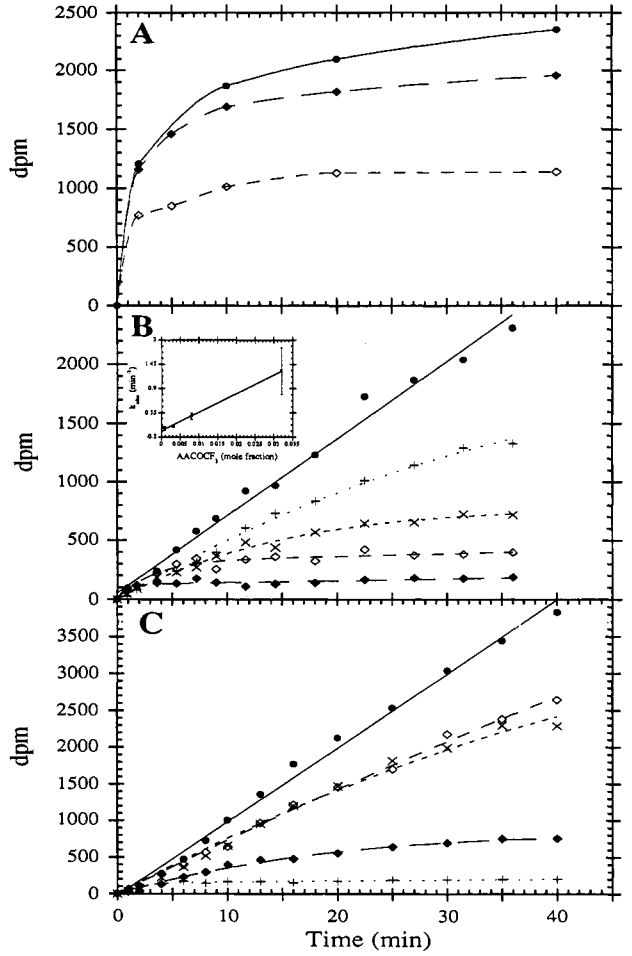


FIGURE 1: Inhibition of $cPLA_2$ by hydrated ketones. (A) Reaction progress curves for the action of $cPLA_2$ on vesicles of [^{14}C]SAPC (54 mCi/mmol) containing 33 mol % of dioleoyl-*sn*-glycerol. Each assay contained 9.9 μM total lipid in 50 μL of 10 mM HEPES, pH 8.0, 0.1 mM EDTA, and 0.6 mM $CaCl_2$ at 37 °C. Symbols: (●) no inhibitor; (◆) 7 mol % $C_{19}H_{39}COCF_3$; (◇) 7 mol % $AACOCF_3$. (B) Reaction progress curves for the action of $cPLA_2$ on mixed micelles of [^{14}C]PAPC (50 mCi/mmol)/Triton X-100. Each assay contained 100 μM [^{14}C]PAPC, 200 μM Triton X-100, and the indicated concentrations of arachidonyl ketone, all in 80 mM glycine, pH 9.0, 5 mM $CaCl_2$, and 50% glycerol. Symbols: (●) 3.2 mol % $AACOCH_3$; (+) 3.12 mol % $AACOCH_3$ and 0.08 mol % $AACOCF_3$; (×) 2.88 mol % $AACOCH_3$ and 0.32 mol % $AACOCF_3$; (◇) 2.39 mol % $AACOCH_3$ and 0.81 mol % $AACOCF_3$; (◆) 3.2 mol % $AACOCF_3$. (C) Reaction progress curves for the action of $cPLA_2$ on mixed micelles of [^{14}C]PAPC/Triton X-100 containing the following: (●) 3.2 mol % $AACOCH_3$; (×) 1.6 mol % $AACOCH_3$ and 1.6 mol % $C_{19}H_{39}COCF_3$; (◇) 1.6 mol % $AACOCH_3$ and 1.6 mol % $AACOCF_2CF_3$; (◆) 1.6 mol % $AACOCH_3$ and 1.6 mol % $AACOCF_2Cl$; (+) 1.6 mol % $AACOCH_3$ and 1.6 mol % $AACOCF_3$.

MATERIALS AND METHODS

A detailed description of the procedure used for purification of the recombinant human $cPLA_2$, assay procedures, synthetic methods, and spectroscopic data is available as supplementary material (see paragraph at end of paper). The recombinant human $cPLA_2$ used in the following experiments was judged to be greater than 80% pure by gel electrophoresis. Inhibitor concentrations are expressed as a mole percent of the total concentration of lipid present in the assay (mixed-micelle assay, 10 μM arachidonyl ketone, 100 μM substrate, 200 μM Triton X-100).

RESULTS

Inhibition of $cPLA_2$ in Vesicles. Figure 1A shows the reaction progress curves for the action of the $cPLA_2$ on sonicated substrate vesicles of [^{14}C]SAPC that contain 33 mol % 1,2-dioleoyl-*sn*-glycerol either with or without 7 mol % trifluoromethyl ketone. The reaction progress curves are complex in that a fast phase is seen initially, followed by a slower steady-state phase (Leslie et al., 1987; Wijkander & Sundler, 1991; Ghomashchi et al., 1992). Previous studies have shown that, during the initial phase, the $cPLA_2$ is operating in a mode involving intervesicle exchange (Ghomashchi et al., 1992). As the reaction products are generated in the vesicles, the enzyme becomes trapped on product-containing vesicles and the reaction slows considerably (Ghomashchi et al., 1992). In the absence of the diglyceride, the reaction progress during the steady-state phase slows to near zero velocity, and thus the diglyceride is an apparent activator of the reaction presumably by weakening the trapping of the enzyme on product containing vesicles (Ghomashchi et al., 1992).

It can be seen from Figure 1A that the addition of $AACOCF_3$ to the vesicles leads to inhibition of the progress of the reaction. The inhibition pattern suggests that $AACOCF_3$ is a slow-binding inhibitor in that little inhibition is seen at the first time point (2.5 min), but then the reaction progress completely ceases. The importance of the trifluoromethyl ketone group for inhibition is apparent from the fact that replacement of this group with $CONH_2$, CHO, $COCH_3$, or $CH(OH)CF_3$ leads to a loss in inhibitor potency (less than 10% inhibition at all time points; not shown). In addition, the double bonds in the acyl chain of $AACOCF_3$ are also important for inhibition in that the saturated analogue ($C_{19}H_{39}COCF_3$) is considerably less potent (Figure 1A). No inhibition or inactivation was detected with the halomethyl ketone $C_{19}H_{39}COCH_2Cl$.

Kinetics of Inhibition in Mixed Micelles. Because of the nonlinear reaction progress curve observed with the $cPLA_2$ in the vesicle assay, it was difficult to make any quantitative assessment of the binding parameters for $AACOCF_3$. To better characterize the slowbinding inhibitors of the $cPLA_2$, experiments were carried out in mixed micelles in which the reaction progress curve has a prolonged pseudo-zero-order phase. An adaptation of the Triton X-100/phospholipid/glycerol mixed-micelle assay described by Ulevitch et al. (1988) was used [also see Clark et al. (1990)].

In preliminary experiments it was found that addition of low concentrations of $AACOCH_3$ or $AACH(OH)CF_3$ to [^{14}C]PAPC/Triton X-100 mixed micelles caused a 2–10-fold increase in the rate of the $cPLA_2$-catalyzed hydrolysis of the substrate. This activating effect was also observed with other compounds that contained an arachidonyl-like group, including $AACOCF_3$. However, in the case of $AACOCF_3$ the activating effect was only observed at early time points in the reaction progress curve, since at later times the reaction slows considerably due to inhibition of the $cPLA_2$. It has been shown previously that arachidonic acid increases the affinity of the $cPLA_2$ for the lipid–water interface (Ghomashchi et al., 1992). Therefore, it is possible that the activating effect of $AACOCH_3$ and $AACH(OH)CF_3$ in the mixed-micelle assay is due to the arachidonyl-like moiety of these compounds increasing the fraction of the total enzyme bound to the surface of the micelles. To overcome these problems, a system was developed in which the mole ratio of $AACOCF_3$ to $AACOCH_3$ was altered but the total mole amount of arachidonyl ketone was maintained at a constant value. This system allowed observation of the $cPLA_2$ activity at various interfacial concentrations of the inhibitor while the physical properties of the micelles were maintained as constant as possible (Ransac et al., 1990).

The results obtained with mixed micelles containing $AACOCF_3/AACOCH_3$ are shown in Figure 1B. In the

control reaction (3.2 mol % $AACOCH_3$), the progress curve was linear until greater than 10% of the total substrate had been hydrolyzed. Addition of $AACOCF_3$ to the mixed micelles had little effect on the initial rate, but as time progressed the rate of hydrolysis decreased in a first-order manner until a steady state was obtained. Using nonlinear least squares regression analysis, eq 1 was fit to the data. The lines shown in Figure 1B, C were calculated from eq 1 using the values of k_{obs}, v_o, and v_s obtained from least squares regression analysis of the corresponding data set.

$$P = v_s t + (v_0 - v_s)[1 - \exp(-k_{obs}t)]/k_{obs} \quad (1)$$

P is the product concentration, v_o is the initial reaction velocity, v_s is the final steady-state velocity, and k_{obs} is the first-order rate constant for the approach to steady state (Morrison, 1982). The data in the inset to Figure 1B show that the value of k_{obs} increases linearly with the mole fraction of $AACOCF_3$ present in the micelle over the range 0.08–3.2 mol %. For slow-binding inhibitors, the formation of the stable enzyme–inhibitor complex is usually preceded by the formation of a less stable complex that is in rapid equilibrium with the free enzyme and inhibitor (Morrison, 1982). Thus, the data suggest that the dissociation constant for the rapidly reversible $cPLA_2$–$AACOCF_3$ complex is higher than the highest inhibitor concentration tested since the value of k_{obs} versus the inhibitor concentration did not show saturation behavior. The observation that the inhibition increases as time progresses would strongly suggest that inhibition of $cPLA_2$ by $AACOCF_3$ is due to the direct binding of the inhibitor to the enzyme rather than due to a decrease in the fraction of the $cPLA_2$ bound to the micelles or to a decrease in the rate of intermicelle exchange of the $cPLA_2$ or substrate (Jain et al., 1992).

A number of other ketones were also evaluated as inhibitors of the $cPLA_2$ in the mixed-micelle assay. The compounds were tested at a single concentration (1.6 mol %), and the results (k_{obs} and percent inhibition at the steady state) were compared to those obtained with $AACOCF_3$ (Figure 1C). Under these conditions $AACOCF_3$ (k_{obs} = 0.25 min^{-1}) produced greater than 95% inhibition of the $cPLA_2$ at the steady state. $AACOCF_2Cl$ (k_{obs} = 0.04 min^{-1}) also produced greater than 95% inhibition; however, the time taken to attain the final steady-state velocity was considerably greater than for $AACOCF_3$. Both $AACOCF_2CF_3$ and $C_{19}H_{39}COCF_3$ are relatively poor inhibitors in that a steady-state rate was not established during the time of the experiment.

Stability of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ Complex. To determine the stability of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex, the $cPLA_2$ was inhibited in mixed micelles containing 3.0 mol % $AACOCF_3$, 5.0 mol % 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol (DTPM), and Triton X-100. In preliminary experiments it was found that addition of DTPM to the Triton X-100/$AACOCF_3$ mixed micelles increased the rate at which the $cPLA_2$ was inhibited. The effect of DTPM is probably due to an increase in the fraction of the $cPLA_2$ bound to the micelles. Previously, Leslie and Channon (1990) demonstrated that anionic phospholipids activate the $cPLA_2$-catalyzed hydrolysis of neutral phospholipids. A control reaction was also performed in which $AACOCH_3$ replaced $AACOCF_3$ in the preincubation mixture. An aliquot of the inhibited enzyme was then diluted (1500-fold) into the [^{14}C]PAPC/Triton X-100 mixed-micelle assay, and at various times after dilution the amount of product formed was measured. As shown in Figure 2, the amount of product released by the $AACOCH_3$-treated enzyme initially increased linearly with time, but then the rate slowly decreased as the incubation time progressed. The slow decrease in the activity

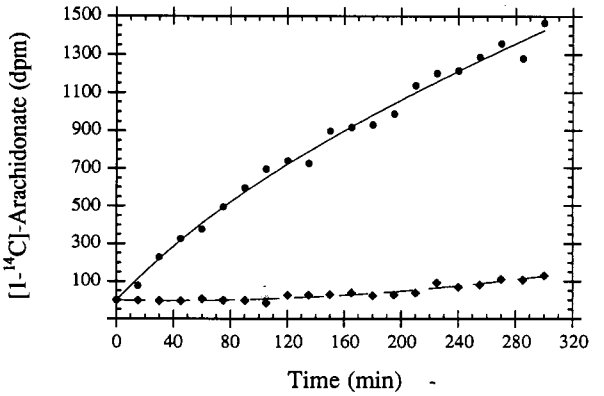


FIGURE 2: Dissociation of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex in the presence of calcium. The $cPLA_2$ was incubated in a buffer containing 370 μM Triton X-100, 20 μM DTPM, and 10 μM $AACOCF_3$ or 10 μM $AACOCH_3$, plus 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.3 mM $CaCl_2$. After 10-min incubation a sample of the treated $cPLA_2$ was diluted 1500-fold into the Triton X-100/[^{14}C]PAPC mixed-micelle assay, and at various times along the reaction progress curve the amount of product released was measured. [1-^{14}C]Arachidonic acid was released by (●) $AACOCH_3$-treated $cPLA_2$ or (◆) $AACOCF_3$-treated $cPLA_2$.

of the $cPLA_2$ is probably due to denaturation of the enzyme. In contrast, the rate of product release from the sample of $cPLA_2$ inhibited with $AACOCF_3$ was initially near to zero but increased slightly after several hours incubation. In a separate experiment it was determined that the amount of $AACOCF_3$ carried over from the preincubation (3.2×10^{-5} mole fraction) decreased the amount of product formed in the control reaction by less than 10% over 5 h (data not shown). At the end of the 5 h incubation period, a comparison of the rate of product release by the $AACOCF_3$-treated enzyme with that of the control reaction suggests that approximately 14% of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex dissociated to produce free and active enzyme.

To determine if calcium plays a role in stabilizing the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex, a reactivation experiment was carried out in the presence of EDTA. The inhibited enzyme, formed as described above in the presence of calcium, was incubated in a buffer containing EDTA in excess of the calcium and containing Triton X-100/[^{14}C]PAPC mixed micelles. At various times of incubation calcium was added in excess of the EDTA to initiate the hydrolysis of the substrate. Again a control reaction in which $AACOCH_3$ replaced $AACOCF_3$ in the preincubation mixture was performed. The results shown in Figure 3 establish that in the presence of excess EDTA the dissociation rate of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex was relatively rapid compared to that in the presence of calcium. The activity of the $AACOCF_3$ inhibited enzyme was initially much lower than that of the $AACOCH_3$-treated enzyme, but as time progressed the $cPLA_2$ activity in the $AACOCF_3$-treated sample increased until almost the same level of activity as in the control reaction was obtained. These results establish that inhibition of the $cPLA_2$ by $AACOCF_3$ is not due to irreversible modification of the enzyme by the inhibitor.

Selectivity of $AACOCF_3$. A 14-kDa phospholipase A_2 has been isolated from platelets, rheumatoid synovial fluid, and other sources [see, for example, van den Bosch et al. (1992)]. This human nonpancreatic secreted enzyme has been recently assayed on vesicles composed of the anionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol (Bayburt et al., 1993). In this assay, the enzyme remains irreversibly bound to the substrate interface. Thus, true competitive inhibitors of the interfacial catalysis that operate by binding directly to the enzyme rather than by decreasing the fraction of enzyme

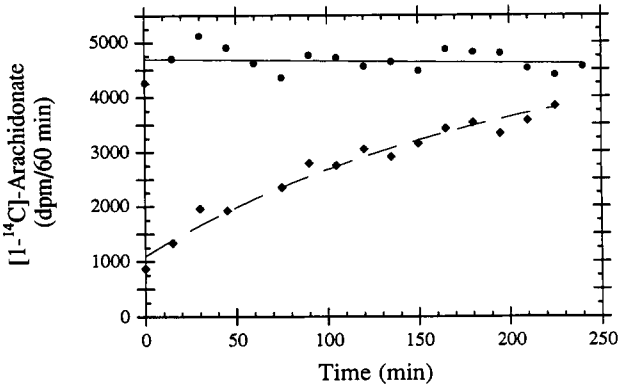


FIGURE 3: Dissociation of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex in the presence of excess EDTA. The $cPLA_2$ was incubated with 5 μM $AACOCF_3$, or 5 μM $AACOCH_3$, for 20 min in Triton X-100/DTPM mixed micelles. The treated enzyme was then diluted into the Triton X-100/[^{14}C]PAPC mixed-micelle assay except that $CaCl_2$ was omitted from the buffer. At various times 50-μL samples were removed, and 5 mM $CaCl_2$ was added to initiate hydrolysis of the substrate. After 1 h at 30 °C the reaction was quenched and the product measured. Product was released by (●) $AACOCH_3$-treated $cPLA_2$ or (◆) $AACOCF_3$-treated $cPLA_2$.

bound to the interface can be identified and characterized (Jain et al., 1991b). Under these conditions, the reaction progress curve for the action of the 14-kDa enzyme on the anionic vesicles is linear for about 20 min, and then there is a slow decrease in the rate due to substrate depletion and product inhibition (Bayburt et al., 1993). No inhibition (<10%) could be detected over a 40-min period when $AACOCF_3$ was present in the substrate vesicles up to 5 mol %. At higher concentrations of $AACOCF_3$ (10–15 mol %), a slow onset of inhibition could be detected. After about 40 min, a steady-state velocity was reached that was about 50% of the initial enzyme velocity measured in the absence of $AACOCF_3$. The presence of up to 15 mol % $AACOCH_3$ did not detectably alter the reaction progress.

Hydration of the Fluoro Ketones. ^{19}F NMR spectra were obtained for the various fluoro ketones present in phospholipid/Triton X-100 mixed micelles in order to estimate the fraction of the inhibitor which is hydrated. The results are summarized in Figure 4. Both $AACOCF_3$ and $C_{19}H_{39}COCF_3$ are at least 90% hydrated. In contrast, both $AACOCF_2Cl$ and $AACOCF_2CF_3$ are approximately 60% hydrated.

DISCUSSION

Hydrated ketones are potent inhibitors of serine proteases (Imperiali & Abeles, 1986; Stein et al., 1987; Peet et al., 1990; Parisi & Abeles, 1992), where it has been demonstrated that a stable hemiketal is formed between the trifluoromethyl ketone inhibitor and the active site serine residue (Imperiali & Abeles, 1986; Stein et al., 1987; Parisi & Abeles, 1992). However, hydrated ketones are also potent inhibitors of zinc metalloproteases and aspartylproteases (Gelb et al., 1985). For both of these latter enzymes, it is the tetrahedral hydrate which is the inhibitory species since it is a structural mimic of the intermediate that is formed during the hydrolysis of substrates. From the data available at present, it is not possible to know if the hydrated form of $AACOCF_3$ is bound to the $cPLA_2$ or whether a hemiketal is formed with an enzymatic nucleophile. However, on the basis of simple mass action arguments it can be shown that, in either case, the potency of the inhibitor will increase as the fraction of the hydrated ketone in the interface increases.

The importance of the $COCF_3$ group for inhibition of the $cPLA_2$ is apparent in that reduction of this group to a secondary alcohol [$AACH(OH)CF_3$], or its replacement by CHO, $CONH_2$, or $COCH_3$, dramatically reduces the inhibitory potency. Furthermore, within the series of arachidonyl ketones there is a correlation between the fraction of the inhibitor which is present as the hydrate and the inhibitory potency against the $cPLA_2$. As observed by ^{19}F NMR in a mixed-micelle system, $AACOCF_3$ was >90% hydrated while the

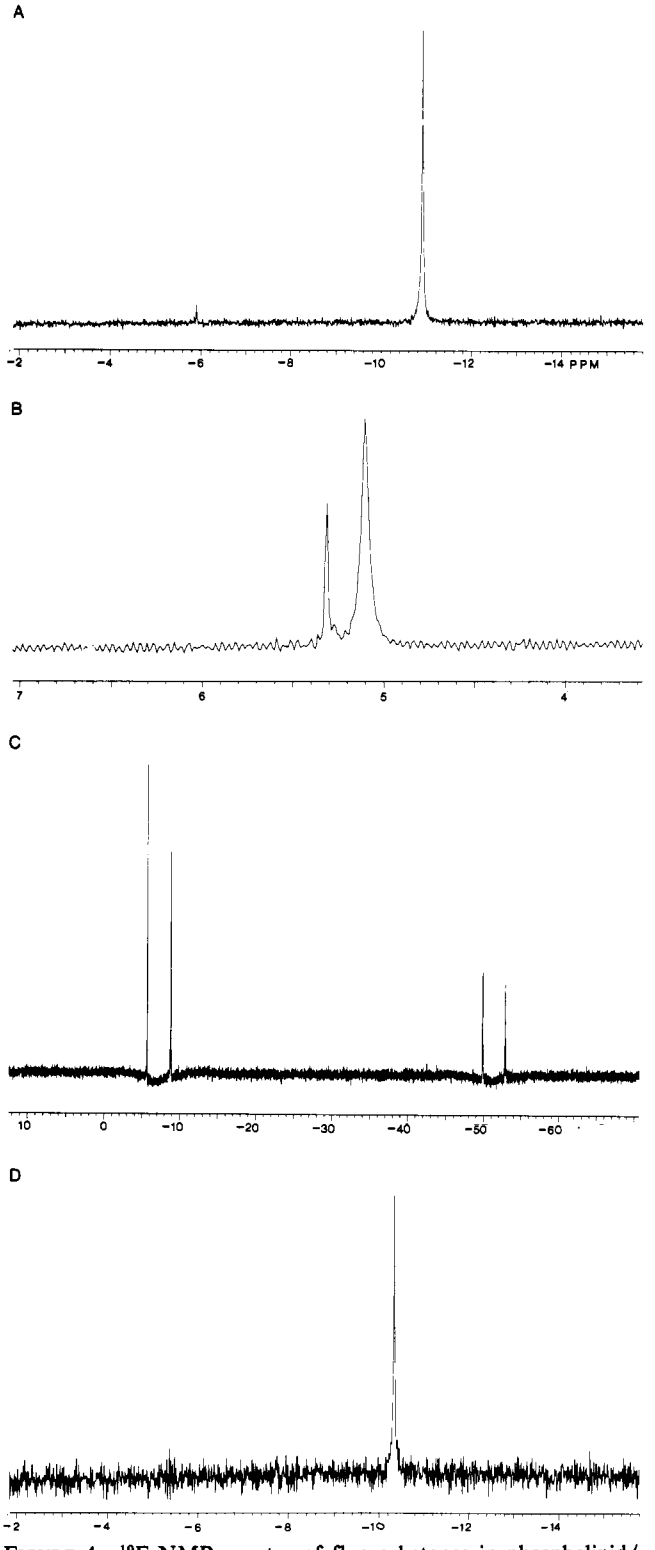
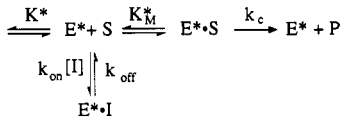


FIGURE 4: ^{19}F NMR spectra of fluoro ketones in phospholipid/Triton X-100 mixed micelles. Each sample contained 40 mM Triton X-100, 5 mM 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, and 2 mM each of the following fluoro ketones: (A) $AACOCF_3$ (ketone, −5.91 ppm; hydrate, −10.90 ppm); (B) $AACOCF_2Cl$ (ketone, 5.31 ppm; hydrate, 5.10 ppm); (C) $AACOCF_2CF_3$ (ketone, −8.80 and −49.92 ppm; hydrate, −5.69 and −52.87 ppm); (D) $C_{19}H_{39}COCF_3$ (ketone, −5.37 ppm; hydrate, −10.35 ppm). All samples were prepared in a buffer containing 10 mM $CaCl_2$ at pH 8.0.

Scheme I



weaker inhibitors, $AACOCF_2Cl$ and $AACOCF_2CF_3$, were only approximately 60% hydrated. However, the extent of hydration alone cannot account for the low potency of $AACOCF_2CF_3$ compared to $AACOCF_2Cl$, and in this case it is possible that unfavorable steric interactions between the larger CF_2CF_3 substituent and the binding site of the $cPLA_2$ might contribute to the reduction in inhibitor potency.

Other structural features of the trifluoromethyl ketones are also important for inhibition of $cPLA_2$; in particular, a marked selectivity for inhibition by polyunsaturated trifluoromethyl ketones was observed. A comparison of the ^{19}F NMR spectra of $AACOCF_3$ and $C_{19}H_{39}COCF_3$ recorded in phospholipid/Triton X-100 mixed micelles shows that both compounds have a similar degree of hydration (>90%); however, $AACOCF_3$ is a much more potent inhibitor of $cPLA_2$ than $C_{19}H_{39}COCF_3$. This result is in good agreement with the reported specificity of the $cPLA_2$ for arachidonyl-containing phospholipids (Diez & Mong, 1990; Clark et al., 1991; Kramer et al., 1991).

Kinetic studies with phospholipases are complicated by the fact that binding of the enzyme to the lipid–water interface must precede catalysis. Thus the affinity of the enzyme for the lipid–water interface can affect the apparent rates of substrate turnover. Furthermore, in a mixed-micelle system the apparent rate of substrate turnover can also be affected by the intermicelle exchange of components since replenishment of substrate in enzyme-containing micelles is required for steady-state hydrolysis (Jain et al., 1992). It is very likely that $AACOCF_3$ inhibits the lipolysis reaction by direct binding to the enzyme rather than by altering the fraction of the enzyme at the interface or the intermicelle exchange rates. If the latter were true, the inhibition would be observed at the earliest time points, and this is in contrast to the slow onset of inhibition. Scheme I shows a minimal kinetic mechanism describing the inhibition of $cPLA_2$ by $AACOCF_3$. In this scheme, K^* is the equilibrium constant for the dissociation of the enzyme from the lipid–water interface into the aqueous phase, E* is the free enzyme bound to the lipid–water interface, K_M^* is the interfacial Michaelis constant for the substrate, k_{on} is the second-order rate constant for the association of enzyme and inhibitor, k_c is the rate-constant for the rate determining step in catalysis, and k_{off} is the first-order rate constant for dissociation of the enzyme–inhibitor complex. Under conditions where all of the enzyme is bound to the interface and interaction of the enzyme with the inhibitor and the substrate occurs only on the interface, then the rate expressions associated with this mechanism are given in eq 2–4 [for a derivation of these expressions, see Morrison (1982), Morrison and Walsh (1988), and Stein et al. (1987)].

$$v_s = \frac{k_c[E^*][S]}{K_M^*(1 + [I]/K_I^*) + [S]} \quad (2)$$

$$K_I^* = k_{off}/k_{on} \quad (3)$$

$$k_{obs} = \frac{k_{on}[I]}{(1 + [S]/K_M^*)} + k_{off} \quad (4)$$

K_I^* is the interfacial equilibrium dissociation constant for the enzyme–inhibitor complex. The mechanism described by Scheme I requires that the inhibitor compete with the substrate for E* (competitive inhibition). However, it is also possible that the inhibitor could bind to both E*·S and E* (noncompetitive inhibition). To distinguish between these two possibilities, the interfacial concentration of the substrate must be varied; unfortunately, for many interfacial systems it is not trivial to achieve this without affecting the physical structure of the system. For example, in the mixed-micelle assay used in the present study the substrate constitutes almost 50 mol % of the lipid–water interface. Therefore, it is probably unreasonable to assume that the substrate concentration could be varied over a wide range without dramatically affecting the total interfacial area and consequently the fraction of bound enzyme. Given these limitations, it is not possible at present to distinguish between the competitive and noncompetitive models, and true values for K_M^* and K_I^* cannot be determined.

However, from the available data an upper limit for the value of K_I^* can be estimated. An upper limit for the value of k_{off} can be determined from the reactivation experiment carried out in the presence of calcium. Approximately 14% of the Ca^{2+}·$cPLA_2$·$AACOCF_3$ complex dissociated to produce free and active enzyme over a period of 5 h; thus the value of k_{off} must be less than 2×10^{-3} min^{-1} (half-life of greater than 5 h). Since $k_{off} \ll k_{obs}$, then from eq 4 and the linear variation of k_{obs} with $AACOCF_3$ concentration, a lower limit for the value of k_{on} can be determined as 42 mole fraction^{-1} min^{-1}. If the inhibitor is competitive with substrate, then this value will be smaller than the true value of k_{on} by a factor of $1/(1 + [S]/K_M^*)$. Thus by applying the limits of k_{off} and k_{on} to eq 3, an upper limit for the value of K_I^* is 5×10^{-5} mole fraction. Since the estimates of both k_{on} and k_{off} were determined under conditions where most of the enzyme was bound to the lipid–water interface (in the presence of calcium), then the value of 5×10^{-5} mole fraction represents an estimate of the value for the equilibrium dissociation constant of the enzyme–$AACOCF_3$ complex in the interface to give the free enzyme and the free inhibitor in the interface. Thus detectable inhibition of $cPLA_2$ by $AACOCF_3$ occurs when this compound is present in the assay at a level of one inhibitor per several thousand lipid molecules. The value of 5×10^{-5} mole fraction is a very conservative estimate of the true interfacial equilibrium dissociation constant, since not only is it likely that the value of k_{off} is much smaller than 2×10^{-3} min^{-1} but also in most cases competition from the substrate for the inhibitor binding site will tend to decrease the estimated value of k_{on}.

Relatively high concentrations of $AACOCF_3$ were needed to observe the inhibition of the $cPLA_2$ in the vesicle assay. The reasons for this are probably twofold. First, in this assay the enzymatic reaction slows abruptly after just a few minutes. Since $AACOCF_3$ binds slowly to the enzyme, relatively large amounts of the compound are required in vesicles to observe inhibition before the reaction slows. Second, the data in Figure 1A clearly show that inclusion of $AACOCF_3$ in the substrate vesicles leads to a complete cessation of the reaction progress at later times. Without knowing the value of the K_M^* for [^{14}C]SAPC in the vesicle assay, it is not possible to obtain K_I^* for $AACOCF_3$; however, an approximate K_I^* value can be obtained in the following way. In a previous study (Diez et al., 1992), it was found that variation of the mole fraction of the $cPLA_2$ substrate 1-*O*-hexadecyl-2-arachidonyl-*sn*-glycero-3-phosphocholine in vesicles of the nonhydrolyzed phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol led to a hyperbolic response for the initial velocity of the reaction with an apparent K_M^* of 0.34 mol %. If it is assumed that $AACOCF_3$ is a competitive inhibitor, that the degree of inhibition in the

vesicle assay reaches >90% (Figure 1A), and that K_M^* for [^{14}C]SAPC in the vesicle assay is 0.34 mol %, then an upper limit of K_I^* of 4×10^{-4} mole fraction is calculated using the standard steady-state equation for a competitive inhibitor of interfacial catalysis [equation A19 of Jain et al. (1991c)] in the presence of substrate at a mole fraction near unity. This value of K_I^* is in good agreement with the value obtained with the mixed-micelle assay.

Experiments were carried out to determine the selectivity of $AACOCF_3$ as an inhibitor of $cPLA_2$ versus the human 14-kDa nonpancreatic secreted PLA_2. Unfortunately, an assay system in which both enzymes showed reasonable levels of activity was not available, and therefore a direct comparison of the two enzymes could not be made. However, as stated above, K_I^* is the interfacial equilibrium dissociation constant for the release of the inhibitor from the enzyme in the interface, to give the free enzyme and the free inhibitor in the interface. Thus the value of K_I^* is independent of substrate concentration, and a comparison of the K_I^* values obtained with $AACOCF_3$ and the two different enzymes provides a reasonable measure of the compound's selectivity. The K_I^* for the action of $AACOCF_3$ on the 14-kDa human nonpancreatic secreted phospholipase A_2 is approximately equal to the mole fraction of inhibitor in the interface that causes 50% inhibition since the K_M^* for the substrate, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol, is larger than 1 mole fraction (Bayburt et al., 1993). Thus, a K_I^* of 0.1 mole fraction is obtained, and this is about 4 orders of magnitude larger than the K_I^* of $AACOCF_3$ acting on the $cPLA_2$.

$AACOCF_3$ is the first reported potent and specific inhibitor of the $cPLA_2$. On the basis of its physical properties, $AACOCF_3$ is expected to readily incorporate into cell membranes and to flip to the inner leaflet where it can interact with the intracellular $cPLA_2$. Thus, it may be possible to use $AACOCF_3$ and some of its analogues to determine the role of the $cPLA_2$ in processes such as the generation of free arachidonic acid for eicosanoid biosynthesis. Further work will also be needed to better understand the mode of inhibition of the $cPLA_2$ by $AACOCF_3$ and to prepare analogues of even greater potency.

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SUPPLEMENTARY MATERIAL AVAILABLE

A detailed description of the $cPLA_2$ purification, assay procedures, synthetic methods, and spectroscopic data for characterization of the inhibitors (10 pages). Ordering information is given on any current masthead page.

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