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Inhibition of Macrophage Ca^{2+}-independent Phospholipase A_2 by Bromoenol Lactone and Trifluoromethyl Ketones*

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A novel Ca^{2+}-independent phospholipase A_2 (PLA_2) has recently been purified from the murine macrophage-like cell line $P388D_1$ (Ackermann, E. J., Kempner, E. S., and Dennis, E. A. (1994) *J. Biol. Chem.* 269, 9227–9233). This enzyme is now shown to be inhibited by palmitoyl trifluoromethyl ketone ($PACOCF_3$), arachidonyl trifluoromethyl ketone ($AACOCF_3$), and a bromoenol lactone (BEL). Both $PACOCF_3$ and $AACOCF_3$ were found to inhibit the macrophage PLA_2 in a concentration-dependent manner. $PACOCF_3$ was found to be ~4-fold more potent than $AACOCF_3$, with IC_{50} values of 3.8 μM (0.0075 mol fraction) and 15 μM (0.028 mol fraction), respectively. Reaction progress curves in the presence of either inhibitor were found to be linear, and the $PACOCF_3$·PLA_2 complex rapidly dissociated upon dilution.

BEL was also found to inhibit the macrophage PLA_2 in a concentration-dependent manner, with half-maximal inhibition observed at 60 nM after a 5-min preincubation at 40 °C. Inhibition was not reversed after extensive dilution of the enzyme into assay buffer. Treatment of the PLA_2 with BEL resulted in a linear, time-dependent inactivation of activity, and the rate of this inactivation was diminished in the presence of $PACOCF_3$. In addition, PLA_2 treated with [^{3}H]BEL resulted in the covalent labeling of a major band at M_r 80,000. Inactivation of the PLA_2 by 5,5′-dithiobis(2-nitrobenzoic acid) prior to treatment with [^{3}H]BEL resulted in the near complete lack of labeling consistent with covalent irreversible suicide inhibition of the enzyme. The labeling of a M_r 80,000 band rather than a M_r 40,000 band upon treatment with [^{3}H]BEL distinguishes the macrophage Ca^{2+}-independent PLA_2 from a previously identified myocardial Ca^{2+}-independent PLA_2 and provides strong evidence that the M_r 80,000 protein is the catalytic subunit.

Phospholipase A_2 (PLA_2)[1] has been the focus of considerable research over the years due to its potential involvement in the release of arachidonic acid from membrane phospholipids and the subsequent production of prostaglandins and leukotrienes (for review, see Ref. 1). PLA_2 are also thought to play key roles in phospholipid metabolism, digestion, and various disease states. Even though it is now evident that they represent a very large and diverse family of enzymes (for review, see Ref. 2), the vast majority of the structural and mechanistic information available is from the Ca^{2+}-dependent Group I, II, and III secreted PLA_2 ($sPLA_2$). These enzymes are characterized by their low molecular weight, high disulfide bond content, conserved three-dimensional structures, and a requirement for calcium during hydrolysis (for reviews, see Refs. 3 and 4).

Recently, a number of unique intracellular cytosolic PLA_2 have been identified and purified that are distinct from the $sPLA_2$. Two well studied examples are the 85-kDa Group IV cytosolic PLA_2 ($cPLA_2$) (5, 6) and the M_r 40,000 myocardial Ca^{2+}-independent PLA_2 ($iPLA_2$) (7). In addition, we have also reported the purification of an apparent M_r 80,000 cytosolic Ca^{2+}-independent PLA_2 from the macrophage-like cell line $P388D_1$ (8). Unlike the $sPLA_2$, very little is known about the catalytic mechanisms of these enzymes, their intracellular roles, or their relationships to one another. This is especially true in the case of the myocardial $iPLA_2$ and the macrophage $iPLA_2$. These two enzymes are unique among the known PLA_2 in that they are both modulated by ATP and they both form high molecular weight complexes of ~400,000 (8, 9). Because of these similarities, there has been some uncertainty as to whether they represent similar enzymes modulated by the same regulatory protein (10) or whether they are truly distinct enzymes. Unfortunately, sequences have not been available for either of these two $iPLA_2$.

One advantageous method for studying and comparing kinetic and chemical mechanisms between enzymes is through the use of inhibitors. Recently, two active site-directed inhibitors have been reported in the literature, arachidonyl trifluoromethyl ketone ($AACOCF_3$), which reportedly displays specificity for the Group IV $cPLA_2$ *versus* the Group II $sPLA_2$ (11), and a bromoenol lactone (BEL), (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one, which reportedly displays specificity for the myocardial $iPLA_2$ *versus* both Group I and III $sPLA_2$ (12). In this study, we have investigated the action of each of these inhibitors as well as several additional compounds including a new potent inhibitor, palmitoyl trifluoromethyl ketone ($PACOCF_3$), toward the purified macrophage $iPLA_2$, and we have utilized [^{3}H]BEL to help identify the catalytic subunit of the macrophage $iPLA_2$.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine was purchased from Amersham Corp., 1-[1-^{14}C]palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine was purchased from DuPont NEN, and unlabeled phospholipids were purchased from Avanti Polar Lipids, Inc. All other reagents were analytical grade or better. (*E*)-6-(Bromomethylene)tetrahydro-3-(1-[4-^{3}H]naphthalenyl)-2*H*-pyran-2-one ([^{3}H]BEL) was the generous gift of Randy H. Weiss,

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[1] The abbreviations used are: PLA_2, phospholipase(s) A_2; $sPLA_2$, secreted Ca^{2+}-dependent PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; $AACOCF_3$, arachidonyl trifluoromethyl ketone; $PACOCF_3$, palmitoyl trifluoromethyl ketone; BEL, bromoenol lactone; DPPC, dipalmitoylphosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

and Philip Needleman (Monsanto Co., St. Louis, MO) (13). Anandamide (arachidonylethanolamide) (14) was kindly provided by Ester Fride and Raphael Mechoulam (Hebrew University, Jerusalem, Israel). The two palmitoyl anandamide analogs (*N*-(2-hydroxyethyl)hexadecanesulfonamide and 3-(hexadecanesulfonyl)-2-methoxy-1,3-oxazolidine) were synthesized and will be described elsewhere.[2] Synthesis of the phospholipid substrate analogs 1-(hexylthio)-2-(hexanoylamine)-1,2-dideoxy-*sn*-3-phosphocholine and 1-(hexylthio)-2-(hexanoylamine)-1,2-dideoxy-*sn*-3-phosphoethanolamine is described elsewhere (15), and they were provided by Scott Boegeman.

AACOCF$_3$—AACOCF$_3$ was prepared according to the general procedure of Boivin *et al.* (16) with the following modifications. Instead of using an acylchloride, a mixed anhydride was formed in the reaction mixture by the following procedure. 0.50 g of arachidonic acid (1.6 mmol; Aldrich) was dissolved in 25 ml of dichloromethane under a nitrogen atmosphere. 1.3 ml of pyridine (16.4 mmol) and 1.9 ml of trifluoroacetic anhydride (13.1 mmol) were added at room temperature, and the mixture was stirred for 1 h. The solution was cooled in a ice bath followed by the addition of 10 ml of water. Workup with 100 ml of water, extraction of the aqueous phase with 3 × 30 ml of dichloromethane, and evaporation of the organic phase after drying with sodium sulfate gave 0.35 g (60%) of AACOCF$_3$ after chromatography with ether/hexane (1:2) on silica gel (R_F = 0.67). Mass spectroscopy (fast atom bombardment) M − 1: 355.2248 (theoretical), 355.2260 (found). ^{1}H and ^{13}C NMR data were consistent with previously published results (11).

PACOCF$_3$—PACOCF$_3$ was synthesized in the same manner as described above for the arachidonoyl analog. Using 0.95 g (3.7 mmol) of palmitic acid as starting material yielded 0.68 g (60%) of PACOCF$_3$ (R_F = 51; ether/hexane (1:2)). ^{1}H NMR ($CDCl_3$, 300.0 MHz) δ 0.88 (t, 3H), 1.2–1.4 (m, 24H), 1.6–1.7 (m, 2H), and 2.69 (t, 2H); ^{13}C NMR ($CDCl_3$, 75.48 MHz) δ 14.1, 22.4, 22.8, 28.8, 29.3, 29.5, 29.6, 29.7, 29.8, 32.0, 36.4, 115.7 (q, J = 292 Hz), and 191.5 (q, J = 35 Hz).

BEL and 6-Bromo-2-(1-naphthyl)-5-oxohexanoic Acid (Bromomethyl Ketone)—BEL was synthesized as described previously by Daniels *et al.* (17). For the bromomethyl ketone, 0.10 g (0.3 mmol) of BEL was dissolved in 5 ml of tetrahydrofuran, and 1 ml of 5 M HCl was added. After 1 h of stirring at room temperature, 50 ml of water was added to the reaction mixture, and the aqueous phase was extracted three times with 50 ml of dichloromethane. Drying the organic phase over sodium sulfate, removing the solvent, and chromatography on silica gel gave 14 mg of a clear oil. ^{1}H NMR ($CDCl_3$, 300.0 MHz) δ 2.2–2.7 (m, 4H), 3.9 (s, 2H), 4.4 (t, 1H), 7.3–7.6 (m, 4H), 7.7–7.9 (m, 2H), and 8.1 (m, 1H).

Preparation of P388D$_1$ Ca^{2+}-independent Phospholipase A_2—The P388D$_1$ Ca^{2+}-independent PLA$_2$ was purified utilizing an ammonium sulfate precipitation of the whole cell homogenate followed by sequential column chromatography as described previously (8). Mono Q eluents (purified 100,000-fold with a specific activity of ~1.3 μM/min/mg (8)) were utilized for most experiments. ATP-agarose eluents (purified 26,000-fold with a specific activity of ~0.32 μM/min/mg (8)) were utilized for the time-dependent inactivation experiments because the Tris buffer present in the Mono Q eluents catalyzed the hydrolysis of BEL (17).

Phospholipase A_2 Assay—Each PLA$_2$ assay contained 400 μM Triton X-100, 100 μM dipalmitoylphosphatidylcholine (DPPC) (containing 200,000 cpm 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine), 100 mM Hepes, pH 7.5, 5 mM EDTA, and 0.1 mM ATP in a final volume of 500 μl. The mixed micellar substrate was prepared as described previously (8). Assays were incubated at 40 °C for 30 min with agitation (unless otherwise indicated), and the reaction was stopped 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)) (18). The product mixture was subsequently processed according to the modified (19) Dole extraction system (18) as described previously (20). Control reactions lacking enzyme were routinely carried out and subtracted from the reported activities.

Inhibition with Trifluoromethyl Ketones—Stock solutions and serial dilutions of inhibitors and fatty acids were prepared in Me_2SO. Each assay tube was prepared by the addition of 5 μl of the appropriate inhibitor to 445 μl of the mixed micellar substrate followed by vortexing, bath sonication, and vortexing (30 s each). Assays were initiated by the addition of 50 μl of the P388D$_1$ Ca^{2+}-independent PLA$_2$ (sufficient to produce ~3000 counts in 30 min) to the substrate mixture and were incubated at 40 °C for 30 min. Time course experiments were carried out in a similar fashion with variable incubation times. Inhibitor concentrations expressed as mole fractions were calculated utilizing the total concentration of lipid present in the assay (*i.e.* 100 μM DPPC and 400 μM Triton X-100 plus the inhibitor concentration). Because under our assay conditions the monomeric concentration of Triton X-100 is not known, we did not take it into account in calculating the mole fractions reported herein (21).

Inhibition with BEL—Stock solutions and serial dilutions of BEL were prepared in Me_2SO. The P388D$_1$ Ca^{2+}-independent PLA$_2$ (sufficient to produce 3000 cpm in 30 min) was preincubated with the indicated amounts of BEL in 10 mM Tris (or 10 mM Hepes), pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM Triton X-100, and 10% glycerol. Preincubations were carried out at 40 °C for 5 min with agitation in a volume of 55 μl. The remaining enzyme activity was assayed by the addition of 445 μl of substrate mixture followed by incubation at 40 °C for 30 min. The reactions were stopped and processed as described above. The time course of inactivation was carried out in the same manner, except that preincubation times were varied as indicated.

For the competition experiments, PACOCF$_3$ and BEL were added simultaneously to the enzyme and incubated as described above. Upon dilution into the assay mixture, the concentration of PACOCF$_3$ was ~1.1 μM, a concentration that results in ~16% inhibition of the PLA$_2$ activity (see Fig. 1). For a control, the PLA$_2$ was routinely preincubated in the presence of PACOCF$_3$ alone, and the resulting activity (measured after dilution into the assay mixture) was defined as 100% of control for all assays in which PACOCF$_3$ was present.

For dilution experiments, the PLA$_2$ was concentrated ~6-fold using a Centricon 10 apparatus (Amicon, Inc.). The resulting enzyme preparation was preincubated at 40 °C for 5 min with either Me_2SO alone or 10 μM BEL and Me_2SO. After preincubation, a 2-μl aliquot was removed and diluted 1500-fold into 3 ml of assay buffer containing 400 μM Triton X-100, 100 μM DPPC (with 200,000 cpm 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine/50 μl of assay buffer), 100 mM Hepes, pH 7.5, 0.8 mM ATP, 1.0 mM dithiothreitol, and 5 mM EDTA. At the indicated time intervals, a 50-μl aliquot was removed, and the released radiolabeled fatty acid was measured as described above.

Covalent Modification of P388D$_1$ Ca^{2+}-independent PLA$_2$ by [^{3}H]BEL—~2–3 μg of the P388D$_1$ Ca^{2+}-independent PLA$_2$ was incubated with 2 μM [^{3}H]BEL for 30 min at 40 °C. Excess unreacted [^{3}H]BEL along with the Triton X-100 was removed through four cycles of concentration and dilution utilizing a Centricon 10 apparatus. Samples were subsequently lyophilized, resuspended in sample buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a prepoured 10% gel (Novex). Gels were fixed with 50% methanol and visualized by autoradiography. Experiments utilizing the DTNB-inactivated enzyme (P388D$_1$ PLA$_2$ preincubated with 1.0 mM DTNB at 40 °C for 15 min) were carried out in a similar fashion, expect that 1–2 μg of enzyme was utilized and the fixed gels were soaked for 30 min in Amplify (Amersham Corp.) prior to visualization.

Control experiments were also carried out that demonstrated that under these conditions, [^{3}H]BEL inhibited ~83% of the PLA$_2$ activity. For these experiments, the PLA$_2$ activity was assayed utilizing a double-labeled phospholipid (1-[1-^{14}C]palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine), and the activity was measured by following the release of 1-[1-^{14}C]palmitoyl-2-lyso-*sn*-glycero-3-phosphorylcholine separated by TLC as described previously (8). This method was used because the presence of [^{3}H]BEL would have interfered with the detection of released radiolabeled fatty acid in the Dole extraction method.

RESULTS

Inhibition by Trifluoromethyl Ketones—Fig. 1 shows the concentration-dependent inhibition of the P388D$_1$ Ca^{2+}-independent PLA$_2$ by trifluoromethyl ketone analogs of palmitic acid (PACOCF$_3$) and arachidonic acid (AACOCF$_3$) using a mixed micelle assay system with 400 μM Triton X-100 and 100 μM DPPC (4:1 molar ratio of Triton to phospholipid). The saturated analog of AACOCF$_3$ was also synthesized, but it could not be utilized in these experiments due to its low solubility. Also shown in Fig. 1 are the results obtained with the free fatty acids palmitate and arachidonate, which were not inhibitory. Interestingly, PACOCF$_3$ was found to be ~4-fold more potent than AACOCF$_3$, with an IC_{50} of 3.8 μM (0.0075 mol fraction) compared with 15 μM (0.028 mol fraction) for AACOCF$_3$. This selectivity is consistent with the substrate preference found earlier utilizing mixed micellar substrates in which the rate of hydrolysis for dipalmitoylphosphatidylcholine was ~4-fold faster than that for 1-palmitoyl-2-arachidonoyl-*sn*-

[2] K. Conde-Frieboes and E. A. Dennis, manuscript in preparation.

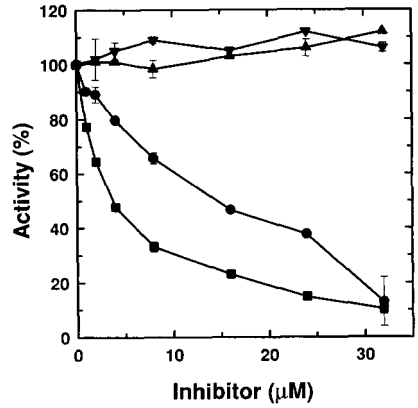

FIG. 1. **Concentration-dependent inhibition of the P388D_1 Ca^{2+}-independent PLA_2 by $PACOCF_3$ and $AACOCF_3$.** The P388D_1 PLA_2 was assayed in the presence of increasing concentrations of $PACOCF_3$ (■), $AACOCF_3$ (●), palmitic acid ([▼), or arachidonic acid ([▲) utilizing a mixed micelle assay containing Triton X-100 and DPPC. The enzyme activity is plotted as the percentage of the control enzyme assayed in the absence of inhibitor. Each point represents the average of duplicates.

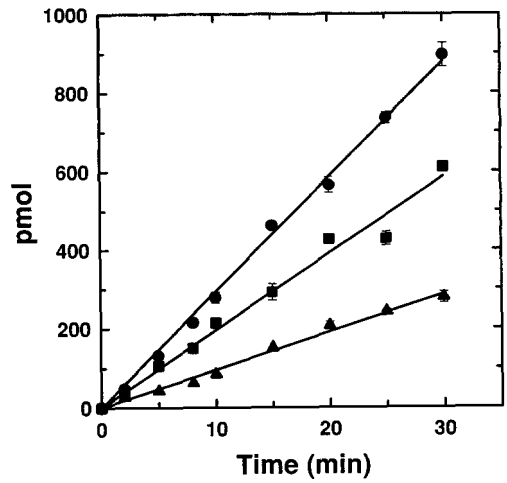

FIG. 2. **Reaction progress curves of the P388D_1 Ca^{2+}-independent PLA_2 in the presence of trifluoromethyl ketone inhibitors.** The P388D_1 PLA_2 was assayed under standard assay conditions in the absence of inhibitor (●) or in the presence of either 12 μM $AACOCF_3$ (■) or 8 μM $PACOCF_3$ ([▲). Each point represents the average of duplicates.

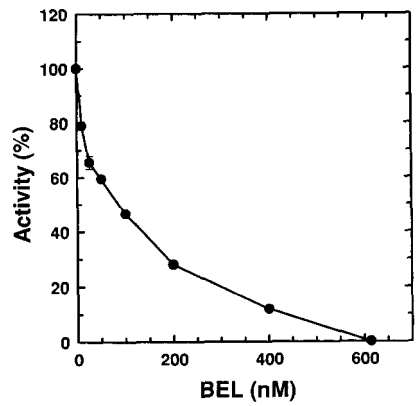

FIG. 3. **Concentration-dependent inhibition of the P388D_1 Ca^{2+}-independent PLA_2 by BEL.** The P388D_1 PLA_2 was incubated with the indicated concentrations of BEL for 5 min at 40 °C in buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM Triton X-100, 10% glycerol, and 1 mM ATP. After this preincubation, the enzyme was diluted ~10-fold into the assay mixture, and the remaining activity was measured. Each point represents the average of duplicates and is plotted as the percent of control (enzyme preincubated in the absence of inhibitor).

glycerol-3-phosphorylcholine (8).

Because $AACOCF_3$ has recently been shown to be a slow, tight-binding inhibitor of the Group IV $cPLA_2$ (11), we examined the time course and reversibility of inhibition with the P388D_1 Ca^{2+}-independent PLA_2. The P388D_1 PLA_2 was assayed in the presence of either 8 μM $PACOCF_3$ or 12 μM $AACOCF_3$ or in the absence of inhibitor. As shown in Fig. 2, linear progress curves were observed under all three conditions, with each curve passing through the origin, indicating that these are not slow binding inhibitors. In addition, preincubation of the P388D_1 Ca^{2+}-independent PLA_2 with 300 μM $PACOCF_3$ for 5 min at 40 °C followed by a 1500-fold dilution into assay buffer resulted in near complete recovery of the PLA_2 activity at all time points measured (15–240 min). This is in contrast to the slow dissociation rate observed for the Ca^{2+}·$AACOCF_3$·$cPLA_2$ complex, where under similar conditions, only 14% of the complex dissociated over a 5-h period (11). Thus, the association/dissociation rates observed between these inhibitors and the P388D_1 PLA_2 appear to be much faster than the rates observed between the Group IV $cPLA_2$ and $AACOCF_3$.

Concentration-dependent Inhibition of P388D_1 Ca^{2+}-independent PLA_2 by BEL—BEL was found to be a potent inhibitor of the purified P388D_1 Ca^{2+}-independent PLA_2. As shown in Fig. 3, preincubation of the P388D_1 PLA_2 for 5 min at 40 °C with increasing amounts of BEL resulted in a concentration-dependent inhibition of activity. Half-maximal activity was found at ~60 nM BEL. In contrast, similar experiments carried out utilizing the hydrolyzed form of the inhibitor (bromomethyl ketone) did not result in any appreciable inhibition of the PLA_2 activity at concentrations up to 700 nM (see "Discussion"). Because BEL has been shown to be a suicide inhibitor of the myocardial $iPLA_2$, we investigated the mechanism of inhibition with the P388D_1 $iPLA_2$.

Irreversible Inhibition—The reversibility of inhibition could not be assessed using either gel filtration or dialysis techniques due to the presence of Triton X-100 with the enzyme, which was necessary for stabilization of the PLA_2 activity (8). Instead, the P388D_1 PLA_2 was preincubated in the presence or absence of BEL and subsequently diluted 1500-fold into assay buffer. Aliquots were removed at various time intervals, and the enzyme activity was determined. As shown in Fig. 4, enzyme not treated with inhibitor resulted a linear time course over the entire assay period, while enzyme pretreated with BEL did not regain any measurable activity in the 4 h following dilution, indicating tight or covalent inhibition of the enzyme by BEL. It should be noted that the enzyme used for the dilution studies was initially concentrated ~6-fold, and therefore, the Triton X-100 present with the enzyme was also concentrated. We have found that in the presence of high concentrations of Triton X-100, higher concentrations of BEL were required in order to achieve full inhibition of the P388D_1 PLA_2 activity. Consequently, 10 μM BEL was utilized in these experiments to ensure complete inactivation of the enzyme. Whether this phenomenon is due to a protection of the enzyme by Triton X-100 or a partitioning effect is unknown at this time.

Time-dependent Inactivation and Protection by $PACOCF_3$—To explore the characteristics of this inhibition, the time course of inactivation was examined. The P388D_1 PLA_2 was preincubated with BEL for periods of 1–30 min, followed by dilution into the assay mixture and quantification of remaining activity. A semilogarithmic plot of the remaining activity *versus* preincubation time resulted in a linear inactivation time course (Fig. 5) for each concentration tested up to at least 7 min, indicating pseudo first-order kinetics. This time-dependent inactivation is

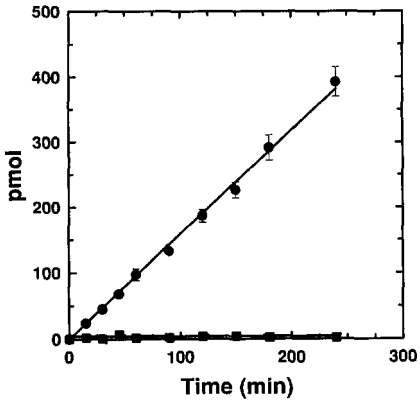

FIG. 4. **Irreversible inhibition of the P388D_1 Ca^{2+}-independent PLA_2 by BEL.** The P388D_1 PLA_2 was preincubated at 40 °C for 5 min with either 10 μM BEL in Me_2SO (■) or Me_2SO alone (●). After preincubation, a 2-μl aliquot was removed and diluted 1500-fold into 3 ml of assay buffer. At the indicated time intervals, a 50-μl aliquot was removed, and the amount of released radiolabeled fatty acid was determined. Each point represents the average of duplicates.

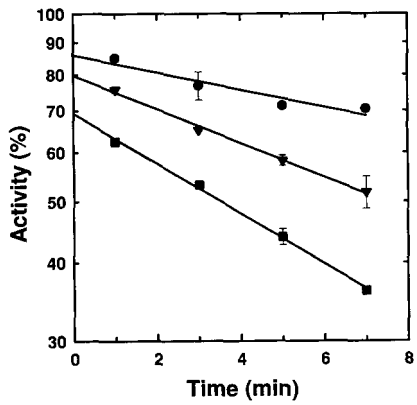

FIG. 5. **Time-dependent inactivation of the P388D_1 Ca^{2+}-independent PLA_2 by BEL.** The P388D_1 PLA_2 was preincubated with 30 nM BEL (●), 60 nM BEL (▼), or 100 nM BEL (■) for the indicated time periods at 40 °C, followed by dilution into assay buffer and quantification of remaining activity. Each point represents the average of duplicates and is plotted on a semilogarithmic plot as the percent of control enzyme incubated in the absence of inhibitor.

indicative of a direct binding of the inhibitor to the enzyme. At longer preincubation times (7–30 min), first-order kinetics were no longer observed. This is most likely due to a depletion of the inhibitor concentration to a point below or equal to the enzyme concentration (22). In addition, the initial steady-state rate was apparently preceded by a burst of inhibition as evidenced by the lack of intersection at 100% activity for any of the concentrations tested. Suicide inhibition of both the myocardial PLA_2 and chymotrypsin by BEL also resulted in a similar burst of inhibition (12, 23).

Time-dependent experiments were also carried out in the presence of the reversible inhibitor $PACOCF_3$. The P388D_1 PLA_2 was preincubated with BEL alone or with BEL and 10 μM $PACOCF_3$. As shown in Fig. 6, the rate of inactivation in the presence of $PACOCF_3$ was significantly less than that observed in its absence. This protection afforded by $PACOCF_3$ suggests that the binding sites on the enzyme for these two inhibitors are at least partially overlapping.

Covalent Labeling of P388D_1 Ca^{2+}-independent PLA_2 with [^{3}H]BEL—Finally, we examined the ability of [^{3}H]BEL to covalently label the P388D_1 PLA_2. The P388D_1 PLA_2 was preincubated with [^{3}H]BEL, the excess inhibitor was removed, and the preparation was separated by SDS-PAGE. As shown in Fig. 7*A*, a single major band was visualized upon autoradiography

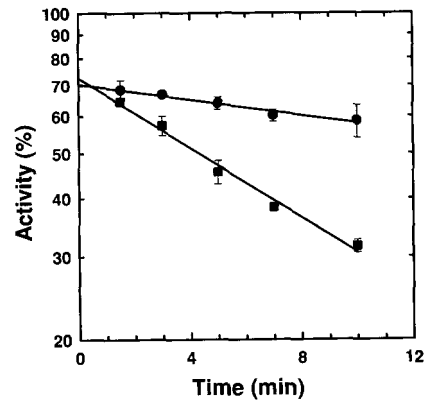

FIG. 6. **Protection of the P388D_1 Ca^{2+}-independent PLA_2 from inhibition by BEL.** The P388D_1 PLA_2 was preincubated with 100 nM BEL in the presence (●) or absence (■) of 10 μM $PACOCF_3$ for the indicated time periods at 40 °C, followed by dilution into the assay mixture and quantification of remaining activity. These results are expressed relative to the control rate measured in the presence of Me_2SO (for ■) or Me_2SO plus $PACOCF_3$ (for ●) and are plotted on a semilogarithmic plot. Each point represents the average of duplicates.

at M_r 80,000. This molecular weight correlated with that observed previously in purified preparations of the P388D_1 PLA_2 after SDS-PAGE and silver staining (8). This result is in contrast to that obtained with the myocardial PLA_2 in which [^{3}H]BEL labeled the myocardial M_r 40,000 catalytic subunit (12). In addition to the M_r 80,000 band, we also observed a diffuse band near the top of the autoradiogram. This band was located near the junction between the stacking and running gels and therefore is most likely due to aggregated protein that did not enter the gel.

A similar experiment was also carried out utilizing enzyme that had been treated with DTNB prior to inhibition with [^{3}H]BEL. Treatment of the P388D_1 Ca^{2+}-independent PLA_2 with 1 mM DTNB resulted in the complete loss of PLA_2 activity. As shown in Fig. 7*B*, utilization of this DTNB-inactivated enzyme resulted in the near complete lack of covalent binding of [^{3}H]BEL to the M_r 80,000 protein, as well as the complete lack of the label at the top of the gel, suggesting the necessity for a catalytically competent enzyme for incorporation of label. It should be noted that visualization for the experiment shown in Fig. 7*B* was carried out utilizing fluorography and therefore resulted in a much higher degree of sensitivity than that observed in Fig. 7*A*. As can be seen, under these more sensitive conditions, the M_r 80,000 band appears much darker and even overloaded, and there is also a previously unobserved very faint band near the bottom of the gel at M_r ~36,000. Because this band is only a very small fraction of the total label and was only detected under these more sensitive conditions, it is most likely the result of a degradation product or contamination with another protein that reacts with BEL.

Other Inhibitors—During the course of our studies, we have also investigated the action of several other potential inhibitors on the P388D_1 Ca^{2+}-independent PLA_2. Each of these compounds gave an estimated IC_{50} value of >50 μM (~10 mol %) and therefore were not pursued further. They include nordihydroguaiaretic acid, anandamide (arachidonylethanolamide), two analogs of anandamide (*N*-(2-hydroxyethyl)hexadecanesulfonamide and 3-(hexadecanesulfonyl)-2-methoxy-1,3-oxazolidine), and two phospholipid substrate analogs (1-(hexylthio)-2-(hexanoylamine)-1,2-dideoxy-*sn*-3-phosphocholine and the corresponding phosphoethanolamine), which are potent inhibitors of the Group I PLA_2 (15).

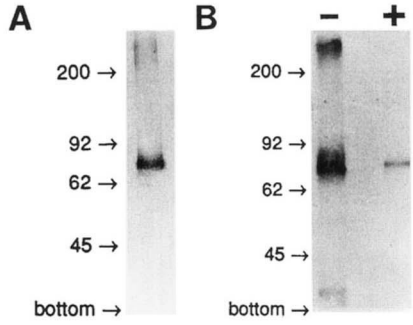

FIG. 7. **Covalent modification of the P388D_1 Ca^{2+}-independent PLA_2 by [^{3}H]BEL and its attenuation with DTNB-inactivated enzyme.** *A*, ~2–3 μg of the P388D_1 PLA_2 was incubated with 2 μM [^{3}H]BEL for 30 min at 40 °C, followed by separation by SDS-PAGE on a 10% gel and visualization by autoradiography. *B*, control (−) or DTNB-inactivated (+) P388D_1 PLA_2 (1–2 μg) was incubated with 2 μM [^{3}H]BEL for 30 min at 40 °C. Samples were subsequently separated by SDS-PAGE on a 10% gel and visualized by fluorography.

DISCUSSION

Trifluoromethyl Ketones: P388D_1 iPLA$_2$ Versus Group IV cPLA$_2$ Specificity—Recently, Street *et al.* (11) have reported the inhibition of the 85-kDa cytosolic Group IV $cPLA_2$ by a trifluoromethyl ketone analog of arachidonic acid ($AACOCF_3$). $AACOCF_3$, which presumably binds directly to the active site of the $cPLA_2$, was found to be a slow and tight-binding inhibitor as demonstrated by nonlinear progress curves and a very slow dissociation rate. From these experiments, an upper estimate for the K_i value was estimated at 5×10^{-5} mol fraction. When compared with the 0.1 mol fraction estimated for the $sPLA_2$, it was apparent that $AACOCF_3$ is a selective inhibitor for the $cPLA_2$ *versus* the $sPLA_2$.

Results reported herein demonstrate that the P388D_1 PLA_2 is also inhibited by $AACOCF_3$ as well as a new analog, $PACOCF_3$. Inhibition was found to be concentration-dependent, with IC_{50} values of ~0.028 and 0.0075 mol fractions, respectively. Competition experiments carried out in the presence of BEL demonstrate that this inhibition is mediated through a direct binding of the inhibitor to the enzyme, most likely at the active site, consistent with results obtained with the $cPLA_2$. However, in contrast to the Group IV $cPLA_2$, kinetic experiments reveal linear progress curves in the presence of both inhibitors, and the $PACOCF_3 \cdot PLA_2$ complex was found to rapidly dissociate upon dilution. Taken together, these data are consistent with a classical mechanism of reversible inhibition.

These data represent the first report of inhibition of a Ca^{2+}-independent PLA_2 by trifluoromethyl ketone inhibitors. It is difficult to directly compare the exact potency of inhibition between the macrophage $iPLA_2$ and the $cPLA_2$ due to the different types of inhibition observed. In addition, it should be kept in mind that the IC_{50} values reported for the macrophage $iPLA_2$ are a function of the assay conditions utilized and should only be taken as an upper limit estimate of the true K_i value, which may be much lower. In any case, the ability of these compounds to inhibit multiple intracellular PLA_2 in the low micromolar range indicates that their use as specific inhibitors for *in vivo* studies should be carried out with some caution. For example, the P388D_1 macrophages contain a Group IV $cPLA_2$, a Group II $sPLA_2$, and the Ca^{2+}-independent $iPLA_2$ (24), and therefore, these inhibitors cannot be used indiscriminately in this cell type to inhibit specifically either the Group IV $cPLA_2$ or the Ca^{2+}-independent $iPLA_2$. On the other hand, $AACOCF_3$ has been used recently in calcium ionophore- and thrombin-stimulated platelets to implicate the involvement of the $cPLA_2$ rather than the $sPLA_2$ in arachidonic acid release (25, 26). Structure/function studies were also carried out with several different compounds besides $AACOCF_3$ to help eliminate nonspecific inhibition effects (25, 26).

BEL: P388D_1 PLA$_2$ Versus Myocardial PLA$_2$—The macrophage $iPLA_2$ and the myocardial $iPLA_2$ are unique among the known PLA_2 in that they are both modulated by ATP, and they both form oligomeric complexes of M_r ~400,000 (8, 9). Despite these similarities, there has been some uncertainty as to whether they are similar enzymes modulated by the same regulatory protein or whether they are truly distinct enzymes. For example, in the case of the myocardial PLA_2, these characteristics are thought to be due to the regulation of the M_r 40,000 catalytic PLA_2 by phosphofructokinase (10), a tetrameric enzyme composed of M_r 85,000 subunits. Together, the myocardial PLA_2 and the phosphofructokinase are thought to form an ATP-sensitive regulatory complex, composed of one M_r 40,000 PLA_2 subunit and four M_r 85,000 phosphofructokinase regulatory subunits. The P388D_1 PLA_2, on the other hand, is thought to be a M_r 80,000 protein that is active as a tetramer (radiation inactivation experiments indicated a catalytically active complex of 337 ± 25 kDa), and it is thought to bind ATP directly (8). However, in our previous studies (8), we were unable to completely rule out a model in which the M_r 80,000 P388D_1 protein is actually phosphofructokinase that was purified along with an undetected M_r 40,000 catalytic subunit, in analogy with the myocardial PLA_2.

Recently, BEL has been shown to be a potent suicide inhibitor of the myocardial PLA_2 (12, 27). This inhibition was found to be at least 1000-fold more potent toward the myocardial PLA_2 than toward the Group I or III $sPLA_2$ (12). Because of the similarities between the myocardial PLA_2 and the macrophage PLA_2, we examined the ability of BEL to inhibit the P388D_1 PLA_2 in the hope of gaining insight into the relationship between these two enzymes. We have found that (*a*) BEL is a potent inhibitor of the P388D_1 $iPLA_2$, with half-maximal activity found at 60 nM after a 5-min preincubation at 40 °C; (*b*) inhibition is irreversible when subjected to a 1500-fold dilution and covalent as demonstrated by the incorporation of [^{3}H]BEL, which persisted through SDS-PAGE treatment; (*c*) inhibition is time-dependent and shows pseudo first-order kinetics; (*d*) this time-dependent inactivation is slowed in the presence of the reversible inhibitor $PACOCF_3$; and (*e*) a catalytically active enzyme is necessary for covalent modification as DTNB-inactivated PLA_2 had a greatly diminished capacity for incorporation of label.

Taken together, these data indicate that BEL is a covalent irreversible inhibitor of the P388D_1 PLA_2, with inactivation proceeding through an enzyme-mediated process. These results are consistent with the documented cases of suicide inhibition utilizing BEL with both the myocardial PLA_2 (12) and chymotrypsin (17). In addition, we have found that the hydrolyzed form of BEL (bromomethyl ketone) is not inhibitory. This indicates that the bromomethyl ketone (which is proposed to be the reactive species responsible for irreversible modification of both the myocardial PLA_2 and chymotrypsin) is not released from the enzyme prior to irreversible inactivation, *i.e.* ruling out a metabolically activated mechanism (22). Furthermore, the sensitivities and characterizations of BEL inhibition observed with the P388D_1 PLA_2 were strikingly similar to those observed with the myocardial PLA_2. Both enzymes were inhibited by BEL in the nanomolar range, both showed an initial burst of inhibition followed by a first-order time-dependent inactivation, and both enzymes lost their ability to incorporate [^{3}H]BEL label after inactivation with DTNB. These data are intriguing in that they suggest that these two enzymes may share very similar active-site environments.

However, despite these similar sensitivities to BEL, treat-

ment of the macrophage PLA_2 with [^{3}H]BEL resulted in the labeling of a single major M_r 80,000 protein, as opposed to the M_r 40,000 band documented with the myocardial PLA_2. These data distinguish the macrophage PLA_2 from the myocardial PLA_2 and provide strong evidence that the M_r 80,000 protein is the catalytic subunit, and not phosphofructokinase. Thus, the ATP activation and oligomerization observed with both of these enzymes appear to be a function of distinct regulatory mechanisms.

In conclusion, we have demonstrated that $PACOCF_3$, $AACOCF_3$, and BEL inhibit the macrophage Ca^{2+}-independent PLA_2 activity. Based on the data presented herein and in analogy with results obtained with the $cPLA_2$ and the myocardial $iPLA_2$, we propose that the trifluoromethyl ketones are classical reversible inhibitors of the macrophage $iPLA_2$ and that BEL is a suicide inhibitor. More important, these data demonstrate that the myocardial $iPLA_2$ and the macrophage $iPLA_2$ are distinct enzymes and provide new evidence that the M_r 80,000 protein is the macrophage $iPLA_2$ catalytic subunit.

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