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Fluorophore-Labeled Ether Lipids: Substrates for Enzymes of the Platelet-Activating Factor Cycle in Peritoneal Polymorphonuclear Leukocytes

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Cell-free preparations of ionophore-stimulated peritoneal rat polymorphonuclear neutrophils (PMNs) incubated with 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine (dansyllyso-PAF) converted this fluorescent lyso ether lipid into two different classes of products. In the absence of acetyl-CoA 1-(*N*-dansyl-11-amino-1-undecyl)-2-long chain acyl-*sn*-glycerol-3-phosphorylcholine (dansylalkyl-2-acyl-GPC) was the only identified new fluorescent phospholipid. In the presence of acetyl-CoA an additional new product, 1-(*N*-dansyl-11-amino-1-undecyl)-2-acetyl-*sn*-glycerol-3-phosphorylcholine (dansyl-PAF), was formed. The formation of dansyl-PAF in PMN homogenates was only transient with a maximum after about 4 min. When PMN homogenates were incubated with dansyl-PAF the formation of dansyllyso-PAF was observed prior to the formation of dansyl-2-acyl-GPC. Thus, our data indicate that enzymatically formed dansyl-PAF is completely remodeled into dansylalkyl-2-acyl-GPC by the sequential action of PAF acetylhydrolase and CoA-independent transacylase. These results demonstrate that peritoneal rat PMNs contain lyso-PAF acetyltransferase, PAF acetylhydrolase, and CoA-independent transacylase and that fluorophore-labeled ether lipids provide an easy means to assay enzymes which catalyze important enzymatic reactions involved in the biosynthesis and remodeling of platelet-activating factor.

KEY WORDS: enzymes; ether lipids; fluorescence; lipid metabolism; platelet-activating factor.

PAF,[2] now identified as 1-*O*-alkyl-2-acetyl-GPC (1–4), is a potent antihypertensive agent (3) and a mediator of anaphylaxis and inflammation (5–7). Its formation by various cell types can be triggered by different stimuli (for review, see (8)). The biosynthesis of PAF in various inflammatory cells is well established and involves liberation of lyso-PAF from 1-*O*-alkyl-2-acyl-GPC by phospholipase A_2 (9,10) and its subsequent acetylation by lyso-PAF acetyltransferase to form PAF (11–15). Recently, it was suggested that, more specifically, 1-*O*-alkyl-2-arachidonoyl-GPC might be the actual precursor of PAF biosynthesis, implying that PAF, as well as eicosanoids, may be formed from the same lipid pool (16–19).

Since the PMN has been shown to efficiently deacetylate PAF by means of PAF acetylhydrolase and preferentially reacylate the resulting lyso-PAF with arachidonic acid (16), this has prompted the formulation of a hypothetical PAF cycle (18,19). Since this cycle would essentially serve to supply both free arachidonic acid and PAF upon stimulation, there is considerable interest in investigating the metabolic regulation of enzymes involved in the remodeling of ether lipids.

Most of the assays currently used to investigate the activity of individual enzymes within this deacylation–reacylation pathway of

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[2] Abbreviations Used: PAF, platelet-activating factor; lyso-PAF, 1-*O*-alkyl-2-lyso-GPC; GPC, *sn*-glycero-3-phosphocholine; dansyllyso-PAF, 1-(*N*-dansyl-11-amino-1-undecyl)-2-lyso-GPC; dansyl-PAF, 1-(*N*-dansyl-11-amino-1-undecyl)-2-acetyl-GPC; dansyl-2-acyl-GPC, 1-(*N*-dansyl-11-amino-1-undecyl)-2-acyl-GPC; PMN, polymorphonuclear leukocytes; HBSS, Hanks' balanced salt solution.

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ether lipids rely on radiolabeled substrates and require the separation of substrates and products of the enzyme reaction prior to analysis by time-consuming and laborious lipid extraction procedures (20–24). Here we report that fluorophore-labeled ether lipids represent an interesting alternative to radiolabeled substrates to investigate these enzymes. Thus, with the appropriate 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine derivatives (dansyllyso-PAF, dansyl-PAF, and dansyl-arachidonoyl-GPC), it could be shown that ionophore-stimulated PMNs from rat possess lyso-PAF acetyltransferase, PAF acetylhydrolase, and CoA-independent transacylase, catalyzing three individual steps of the PAF cycle.

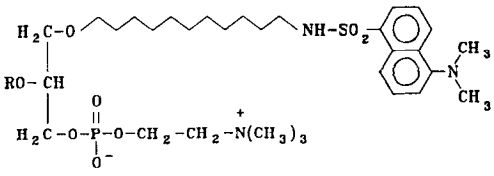
EXPERIMENTAL PROCEDURES

Synthesis of dansyl phospholipids. 1-(11-Phthalimido-1-undecyl)-*sn*-glycerol was synthesized from 2,3-*O*-isopropylidine-*sn*-glycerol (prepared from L-serine (36)) and 11-phthalimido-1-undecyl methylsulfonate by a procedure similar to that described recently (25). This product was successively tritylated at the *sn*-3 position, acylated at the *sn*-2 position with decanoyl chloride, detritylated, and converted to the choline phosphate as previously described for the synthesis of naphthylvinyl-labeled phosphatidylcholine (33). The phthalimido group was removed by hydrazinolysis and the free amine was dansylated by reaction with dansyl chloride in dry chloroform and triethylamine to give 1-(*N*-dansyl-11-amino-1-undecyl)-2-decanoyl-*sn*-glycerol-3-phosphorylcholine. This lipid was converted to dansyllyso-PAF by treatment with phospholipase A_2 (33). Phospholipase A_2 treatment was chosen since this removes any contaminating *sn*-2- and *sn*-1-glycerolphosphate isomers formed by acyl migration or incomplete enantiomeric purity, respectively, in the synthesis. These isomers are resistant to phospholipase A_2 action, and are not converted to dansyllyso-PAF. Acetylation of dansyllyso-PAF using acetic anhydride and pyridine gave dansyl-PAF. Acylation of dansyllyso-PAF with arachidonoyl anhydride in the presence of 4-pyrrolidinopyridine gave 1-(*N*-dansyl-11-amino-1-undecyl)-2-arachidonoyl-*sn*-glycerol-3-phosphorylcholine. These lipids, after purification by preparative HPLC on silica columns, gave single spots on TLC and single peaks on analytical HPLC.

Stock solutions of dansyllyso-PAF and of dansyl-PAF (1.6 mM) were prepared in water and stored at −20°C. Acetyl-CoA was from Boehringer-Mannheim (Mannheim, FRG), [1-^{14}C]Acetyl-CoA (58.1 mCi/mmol) was purchased from NEN (Boston, MA). Soy bean trypsin inhibitor was purchased from Sigma. Hanks' balanced salt solution (HBSS) was obtained from GIBCO (Karlsruhe, FRG), and sodium caseinate from Roth (Karlsruhe, FRG). All other chemicals were of reagent grade.

Isolation of peritoneal rat PMN. Peritoneal PMN from male Wistar rats were collected 14–16 h after an intraperitoneal injection of sodium caseinate (26). Briefly, cells were pelleted (200*g* for 10 min) and washed in HBSS buffer and erythrocytes were subsequently removed by osmotic shock treatment (40 s). The leukocytes were pelleted, washed once, resuspended in HBSS buffer at 10^8 cells/ml of HBSS, and then stimulated with ionophore (final concentration 3 μg/ml, 5 min, 37°C). All subsequent steps were performed at 4°C using buffer A (HBSS buffer pH 7.0 containing 0.5 mg/ml of soy bean trypsin inhibitor (27) and 25 mM NaF (28)). Cells were first washed with ice-cold buffer A and then broken by sonication at an output setting of 35 kHz (Model TG 125 sonifier, Schöller & Co., Frankfurt, FRG) for 30 s at 4°C. The resulting cell homogenate was used directly to assay individual enzyme reactions of the PAF cycle.

Enzyme assays. Except were indicated, all assays were performed using buffer A in a total volume of 80 μl. Forty microliters of crude PMN lysate, equivalent to 4×10^6 ionophore-stimulated PMNs, was used throughout the experiments as the source of enzyme.



R = H: Dansyllyso-PAF
R = acetyl: Dansyl-PAF
R = long chain acyl: Dansyl-acyl-PAF

FIG. 1. Structural formula of dansylated ether lipids used in this study.

Acetyltransferase was assayed in the presence of dansyllyso-PAF (40 μM) and acetyl-CoA (200 μM). Where indicated, [1-^{14}C]acetyl-CoA (62.5 nCi per assay) was included. *CoA-independent transacylation:* Except for the omission of acetyl-CoA from the assay, the same conditions were used as in the assay for acetyltransferase. *PAF acetylhydrolase* was assayed under the same conditions using dansyl-PAF (40 μM) as the substrate.

All incubations were carried out for up to the indicated time at 37°C and terminated by directly spotting a 5-μl aliquot of the reaction mixture onto thin-layer plates (Merck No. 5641). Separation of substrate and product(s) was performed by developing the plates using the solvent system chloroform:methanol:acetic acid:water (50:25:4.5:4.5, v/v). Quantification of individual fluorescent spots was done by scanning the plates in a fluorescence densitometer (CD 50, Desaga Heidelberg, FRG) with excitation wavelength set at 256 nm, and the emission cutoff filter set at 400 nm.

All time progression curves were analyzed by weighted fits to first-order reactions or Bateman functions with a nonlinear regression analysis program adapted from Kinfit (29).

RESULTS

The structures of dansylated ether lipids used in this investigation are shown in Fig. 1.

Nature of dansyllyso-PAF-derived reaction products. When dansyllyso-PAF was incubated with crude homogenates from ionophore-stimulated PMNs, the time-dependent formation of a single new fluorescent product (P_2) was observed (Fig. 2). P_2 was shown to represent a 1-dansylaminoalkyl-2-long chain acyl-GPC based on the following observations. First, porcine pancreatic phospholipase

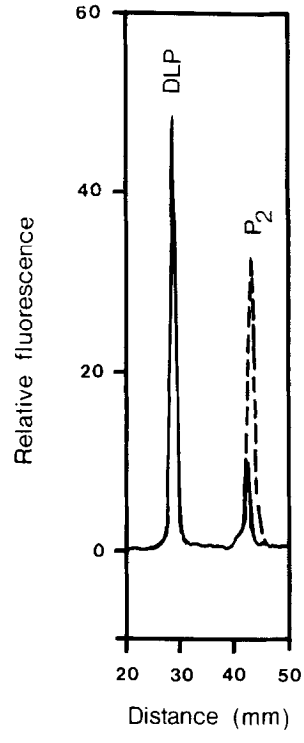


FIG. 2. Acylation of 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine by homogenates of A23187-stimulated PMN. PMN homogenate equivalent to 1×10^6 cells was incubated in the presence of dansyllyso-PAF (3.2 nmol) in 0.08 ml of buffer A for 5 min at 37°C. The incubation was terminated by spotting a 5-μl aliquot of the reaction mixture on a layer of Silica Gel 60. Fluorophore-labeled product was separated by developing the plate in chloroform:methanol:acetic acid:water (50:25:4.5:4.5, v/v) and quantitated by fluorescent densitometry with excitation set at 256 nm and emission cutoff filter set at 400 nm. Fluorescence is given in arbitrary units. The dashed line represents the densitometry scan of an identical aliquot to which synthetic 1-(*N*-dansyl-11-amino-1-undecyl)-2-arachidonoyl-*sn*-glycerol-3-phosphorylcholine was added prior to chromatography. DLP, Dansyllyso-PAF; P_2, dansyl-2-acyl-GPC.

A_2 readily hydrolyzed P_2 to dansyllyso-PAF (data not shown) consistent with the presence of an acyl chain in the *sn*-2 position of the glycerol moiety. Second, enzymatically formed P_2 co-migrated with synthetic 1-(*N*-dansyl-11-amino-1-undecyl)-2-arachidonoyl-GPC in the standard solvent system with a R_f of 0.8 indicating that the acyl portion in P_2 is a long chain fatty acid. No attempt was made to determine the exact nature of the long chain fatty acid incorporated into the P_2 fraction since this would have been beyond the scope of the present work. Since no long chain acyl donor was added to the assay mixture, the acyl components transferred to dansyllyso-PAF are derived from endogenously available phospholipids, most probably as a result of a CoA-independent transacylase reaction (24).

When PMN homogenates were incubated with dansyllyso-PAF in the presence of 200 μM acetyl-CoA, a second fluorescent product (P_1 in Fig. 3) was formed in addition to dansyl-2-acyl-GPC. The identity of P_1 with dansyl-PAF was established as follows. First, P_1 was formed only when acetyl-CoA was included in the assay. Second, enzymatically formed P_1 co-migrated with synthetic dansyl-PAF on TLC using the solvent system chloroform:methanol:acetic acid:water (50:25:4.5:4.5, v/v). Third, when the assay was run in the presence of ^{14}C-labeled acetyl-CoA, only one new radioactive spot was detectable on X-ray film which was superimposable with the fluorescent product P_1 (Fig. 3). These data are consistant with the presence in PMNs of a lyso-PAF acetyltransferase activity. The addition of soy bean trypsin inhibitor (0.5 mg/ml final concentration) to stimulated PMNs before sonication (30) was found to be crucial for good recoveries of lyso-PAF acetyltransferase in homogenates. The addition of 25 mM NaF (28) further stabilized the activity of this enzyme (unpublished observations).

Functional PAF cycle in peritoneal rat PMN. The time course of the formation of dansyl-2-acyl-GPC and dansyl-PAF from the lyso compound was investigated next. In these experiments advantage was taken from the fact that many successive aliquots could be withdrawn and analyzed from the same assay mixture allowing a quasi-continuous analysis of the enzyme reaction.

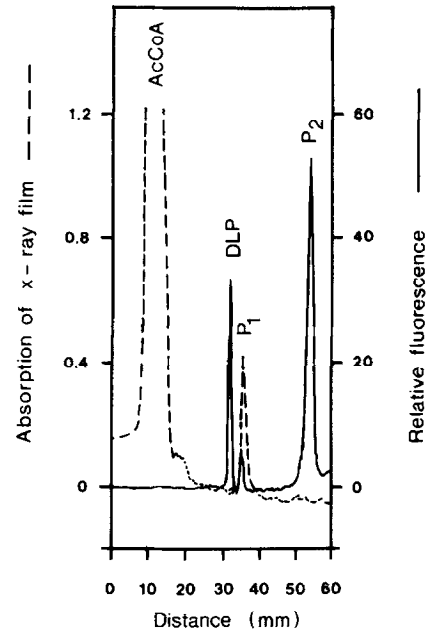


FIG. 3. Simultaneous acetylation and acylation of 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine by PMN homogenates in the presence of acetyl-CoA. PMN homogenate equivalent to 4×10^6 cells was incubated in the presence of dansyllyso-PAF (3.2 nmol) and [1-^{14}C]acetyl-CoA (62.5 nCi) in 0.08 ml of buffer A for 5 min at 37°C. Five microliters of the assay mixture was withdrawn and analyzed as described in the legend to Fig. 1. The solid line represents the fluorescence densitometric scan. The densitometric scan of X-ray film exposed to the chromatogram for 48 h is shown as a dashed line. DLP, Dansyllyso-PAF; P_1, dansyl-PAF; P_2, dansyl-2-acyl-GPC.

Figure 4 illustrates the time course of dansyl-2-acyl-GPC production from dansyllyso-PAF. It is evident from this figure that rat PMNs possess a highly active CoA-independent transacylase that effectively acylates lyso ether lipids. The initial rate of this transacylase reaction in different preparations in the standard assay was 112 pmol/min/10^6 cells (mean of four experiments).

The time course of the conversion of dan-

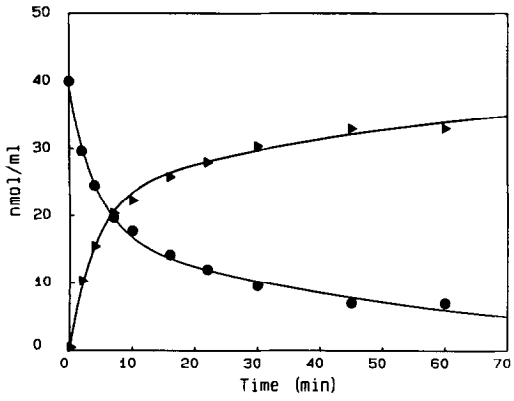


FIG. 4. Time course of the conversion of 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine to dansyl-2-acyl-GPC by PMN homogenate. PMN homogenate equivalent to 4×10^6 cells was incubated in the presence of dansyllyso-PAF (3.2 nmol) in 0.08 ml of buffer A for up to the indicated time at 37°C. Aliquots (5 μl) of the assay mixture were withdrawn and analyzed as described in the legend to Fig. 1. Dansyllyso-PAF and dansyl-2-acyl-GPC are represented by ● and ▲, respectively.

syllyso-PAF into fluorescent products by crude cell lysate in the presence of acetyl-CoA (Fig. 5) exhibited a more complex picture. The concentration of dansyllyso-PAF decreased at an initial rate of 262 pmol/min/10^6 cells (mean of four experiments). As expected, both dansyl-PAF and dansyl-2-acyl-GPC (initial rate, 182 pmol/min/10^6 cells; mean of four experiments) were formed. However, dansyl-PAF was only transiently formed reaching a sharp maximum after 4 min of incubation, but declining subsequently. This complex time course made the determination of initial velocity data for the lyso-PAF acetyltransferase reaction impossible. After 60 min of incubation essentially no dansyl-PAF could be detected in the assay mixture. Since after this time practically all of the fluorescent label resided in the dansyl-2-acyl-GPC pool, this implied that in the course of the experiment dansyl-PAF was completely remodeled into dansylated 2-long chain acyl-GPC.

In order to prove that remodeling of dansyl-PAF into dansyl-2-acyl-GPC involved removal of the acetyl group from the *sn*-2 position prior to reacylation, PMN homogenate was incubated with dansyl-PAF (40 μM) in the absence of acetyl-CoA. As shown in Fig. 6, the concentration of dansyl-PAF decreased steadily in the course of 60 min (initial rate of 58 pmol/min/10^6 cells, mean of four experiments) with a concomitant formation of both dansyllyso-PAF and dansyl-2-acyl-GPC. However, the appearance of dansyllyso-PAF clearly preceded formation of dansyl-2-acyl-GPC. Whereas dansyllyso-PAF reached a steady-state concentration of 9.7 μM after 10 min, the concentration of dansyl-2-acyl-GPC continued to increase at the same rate as dansyl-PAF decreased. This pattern is consistant with dansyllyso-PAF being an intermediate in the process of remodeling dansyl-PAF into dansyl-2-acyl-GPC.

DISCUSSION

In view of the high detection sensitivity of fluorescence the use of fluorescent labels in many cases offers an attractive alternative to

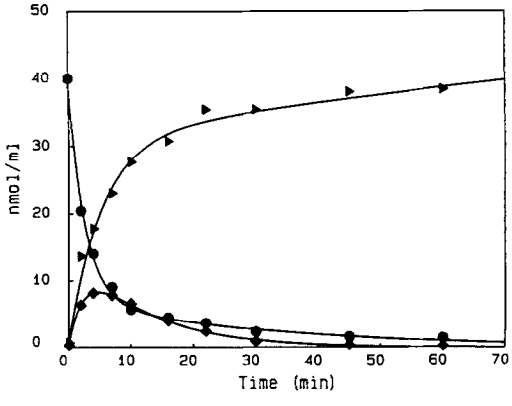


FIG. 5. Time course of the conversion of 1-(*N*-dansyl-11-amino-1-undecyl)-*sn*-glycerol-3-phosphorylcholine to fluorophore-labeled products by PMN homogenate in the presence of acetyl-CoA. PMN homogenate equivalent to 4×10^6 cells was incubated in the presence of dansyllyso-PAF (3.2 nmol) and acetyl-CoA (16 nmol) in 0.08 ml of buffer A for up to the indicated time at 37°C. Aliquots (5 μl) of the assay mixture were withdrawn and analyzed as described in the legend to Fig. 1. Dansyllyso-PAF, dansyl-PAF, and dansyl-2-acyl-GPC are represented by ●, ♦, and ▲, respectively.

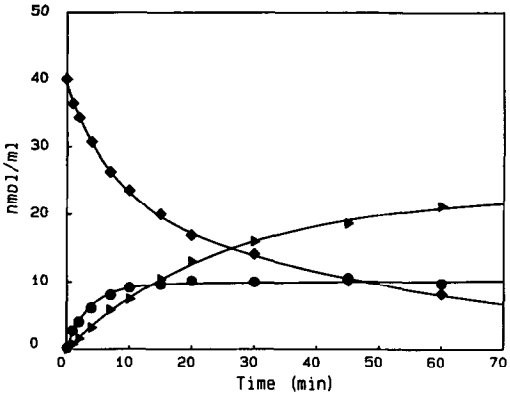


FIG. 6. Time course of the conversion of 1-(*N*-dansyl-11-amino-1-undecyl)-2-acetyl-*sn*-glycerol-3-phosphorylcholine to fluorophore-labeled products by PMN homogenate. PMN homogenate equivalent to 4×10^6 cells was incubated in the presence of dansyl-PAF (3.2 nmol) in 0.08 ml of buffer A for up to the indicated time at 37°C. Aliquots (5 μl) of the assay mixture were withdrawn and analyzed as described in the legend to Fig. 1. Dansyllyso-PAF, dansyl-PAF, and dansyl-2-acyl-GPC are represented by ●, ♦, ▲, respectively.

the use of radiolabels. However, fluorophore-labeled lipids do not seem to have found wide acceptance for the investigation of various aspects of lipid metabolism. A few reports have appeared in the literature describing the use of fluorescent phospholipids to assay phospholipase A_2 (30–33). Recently Imbs and coworkers (34) described the synthesis of a fluorescent PAF analog, 1-alkyl-2-acetyl-*sn*-glycero-3-phospho-(*N*-(9-anthrylmethyl)-*N*,*N*-dimethylethanolamine. However, no biological data on this compound were reported.

In this study we used synthetic ether lipid analogs that contained the highly fluorescent dansylamino group attached to the omega position of the *sn*-1 *O*-undecyl side chain. This position was chosen in the hope of minimizing potential perturbing effects of the bulky dansyl group on enzymes which, like acetyltransferase, transacylase, and acetylhydrolase, interact with the *sn*-2 position of the ether lipid.

The use of dansylated ether lipids as substrates was found to be more convenient than conventional radioactive assays for enzymes of the PAF cycle. First, both dansyllyso-PAF and dansyl-PAF are sufficiently soluble in water (up to 1.6 mM) to prepare stock solutions for enzyme assays without sonication or addition of bovine serum albumin. Second, substrates and reaction products present in 5-μl aliquots of the assay mixture were directly separated on TLC, thus avoiding problems of quantitative recovery of lipids after extractions into organic solvents. Third, fluorescence densitometry provided an accurate means to quantitate substrate and products. Approximately 2 pmol of individual fluorescent compounds per spot could accurately be determined. This small aliquot of the reaction mixture needed for analysis enabled us to keep the total volume of the assay mixture as low as 80 μl, still allowing removal of multiple samples from the same assay mixture for detailed time course analysis. This is in contrast to current radiolabeled assays which require individual assays for each time point of a progression curve.

The experiments reported here demonstrate that dansyllyso-PAF is a substrate for both lyso-PAF acetyltransferase and CoA-independent transacylase and that dansyl-PAF is efficiently hydrolyzed by PAF acetylhydrolase. Obviously, the presence of a dansylamino group in the alkyl moiety of these ether lipids did not significantly affect the interaction with the substrate binding site of these enzymes. This conclusion is further supported by experiments showing that kinetic parameters of lyso-PAF acetyltransferase from both human and peritoneal rat PMN microsomal preparations are rather similar when either lyso-PAF or dansyllyso-PAF is used as the substrate.[3] The only other structural analog of lyso-PAF known to be accepted to some extent by lyso-PAF acetyltransferase is 1-palmitoyl-2-lyso-GPC (11). Preliminary data on substrate specificity are available for acetylhydrolase (35). So far,

[3] P. W. Schindler and E. Ninio, manuscript in preparation.

however, no substrate analogs have been reported for CoA-independent transacylase.

Our results with homogenates from ionophore-stimulated rat peritoneal PMNs are in keeping with previous reports demonstrating that both intact PMNs (16,18,23) and PMN homogenates (24) contain all enzymes of the PAF cycle. In particular it is noteworthy that rat peritoneal PMNs exhibit high activities of both PAF acetylhydrolase and CoA-independent transacylase. As a consequence of this the determination in crude cell homogenates of initial velocity data for lyso-PAF acetyltransferase was made impossible since CoA-independent transacylase rapidly reduced the concentration of dansyllyso-PAF available for acetyltransferase. Thus, initial velocity data could not be obtained at pseudo-first-order conditions. In addition, a highly active PAF acetylhydrolase rapidly converted enzymatically formed dansyl-PAF back into dansyllyso-PAF, resulting in time progression curves clearly deviating from first-order reactions. Since these effects would not be observed when lyso-PAF acetyltransferase is assayed based on the incorporation of acetate into lyso-PAF from radiolabeled acetyl-CoA, activity data obtained with such an assay in crude PMN homogenates should be interpreted with caution.

Work is now in progress to compare radiolabeled standard assay procedures for acetyltransferase,[3] transacylase, and acetylhydrolase with our newly developed fluorophore-labeled assay and to extend our studies on phospholipase A_2 which represents another important enzyme of the PAF cycle.

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