Inhibitors of CoA-Independent Transacylase Block the Movement of Arachidonate into 1-Ether-Linked Phospholipids of Human Neutrophils†

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ABSTRACT: The enzyme CoA-independent transacylase (CoA-IT) has been proposed to mediate the movement of arachidonate between phospholipid subclasses and influence the formation of arachidonic acid metabolites and platelet-activating factor. To substantiate the critical role of CoA-IT, we have developed two structurally diverse inhibitors of CoA-IT activity, SK&F 98625 [diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydro-imidazole-1-yl)heptane phosphonate] and SK&F 45905 [2-[2-(3-4-chloro-3-(trifluoromethyl)phenyl)ureido]-4-(trifluoromethyl)phenoxy]-4,5-dichlorobenzenesulfonic acid]. These compounds were tested for their capacity to block microsomal CoA-IT activity using two assay systems, the transacylation of 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (GPC) and the transfer of [^{14}C]arachidonate from 1-acyl-2-[^{14}C]arachidonoyl-GPC to lyso-PE. Both SK&F 98625 and SK&F 45905 inhibited CoA-IT activity (IC_{50}s 6−19 μM) in these two assays. In contrast, SK&F 98625 or SK&F 45905 had little or no effect on other lipid-modifying activities, including CoA-dependent acyltransferase or acetyltransferase. Kinetic analysis revealed that both SK&F 98625 and SK&F 45905 interact directly with the enzyme and prevented the acylation of lysophospholipids in a competitive manner. In intact human neutrophils, both SK&F 98625 and SK&F 45905 completely blocked the movement of [^{3}H]arachidonate from 1-acyl-linked phospholipids into 1-alkyl-2-arachidonoyl-GPC and 1-alk-1′-enyl-2-arachidonoyl-GPE. In contrast, these compounds did not inhibit the incorporation of free arachidonic acid into cellular lipids indicating that they did not alter CoA-dependent acyl transferase activities in the intact cell. This is the first report to utilize an inhibitor to address the importance of CoA-IT in arachidonate−phospholipid remodeling. These results provide further evidence that CoA-IT mediates the movement of arachidonate into the large pools of 1-ether-linked phospholipids in human neutrophils and suggest that it may be possible to regulate AA levels in cellular phospholipids with CoA-IT inhibitors.

It is now widely recognized that arachidonic acid (AA)[1] plays an important role in inflammatory cell function (Samuelson et al., 1987). For example, AA influences signal transduction events directly and also serves as a precursor for inflammatory lipid mediators, including prostaglandins, leukotrienes, thromboxanes, and P_{450}-derived molecules. A second family of biologically active lipids, termed platelet-activating factor (PAF), consists of a variety of fatty alcohol chains at the *sn*-1 position of a 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine backbone. PAF also possesses activities that are relevant to the pathophysiology observed in inflammation (Prescott et al., 1990). Given the importance of lipid mediators in inflammation, a great deal of attention has focused on how these mediators are formed. Several years ago, a number of studies revealed an overlap in the biochemistry of eicosanoids and PAF (Wykle et al., 1986; Chilton et al., 1983, 1984; Kramer et al., 1984, 1988; Albert & Snyder, 1983; Swendsen et al., 1983; Touqui et al., 1985; Chilton, 1989). In particular, it was suggested that both lipid mediator families arise from common precursor phospholipids during cell activation. More specifically, certain 1-ether-linked, arachidonate-containing phospholipids (e.g., 1-alkyl-2-arachidonoyl-GPC) were proposed to be hydrolyzed by phospholipase A_2 (PLA_2) during cell activation providing the key intermediates, AA and lysophospholipids, for the synthesis of eicosanoids and PAF, respectively. Several findings emphasized the importance of 1-ether-linked, arachidonate-containing phospholipids. First, inflammatory cells contain high amounts of AA in 1-ether-linked phospholipids compared to noninflammatory cells (Mueller

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[1] Abbreviations: AA, arachidonic acid, 5,8,11,14-eicosatetraenoic acid; HSA, human serum albumin; CO, cyclooxygenase; GPC, *sn*-glycero-3-phosphocholine; GPI, *sn*-glycero-3-phosphoinositol; GPE, *sn*-glycero-3-phosphoethanolamine; GC/MS, gas chromatography and mass spectrometry; HBSS, Hank's balanced salt solution; 5LO, 5-lipoxygenase; lyso-PAF, 1-alkyl-2-lyso-GPC, lyso-platelet-activating factor; PLA_2, phospholipase A_2; PBS, phosphate buffered saline; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PAF, platelet-activating factor, 1-alkyl-2-acetyl-GPC; TLC, thin layer chromatography; LCAT, lecithin:cholesterol acyl transferase; ACAT, acyl-CoA:cholesterol acyl transferase.

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et al., 1983, 1984; Chilton & Connell, 1988; Sugiura et al., 1983; MacDonald & Sprecher, 1989b). Secondly, AA-containing, 1-ether-linked phospholipids supply the bulk of AA and lysophospholipids used for eicosanoid and PAF generation, respectively, in neutrophils and mast cells (Chilton & Connell, 1988; Chilton, 1989; Nakamura et al., 1991; Tessner et al., 1990; Colard et al., 1986; Albert & Snyder, 1983; Swendsen et al., 1983). Thirdly, selective depletion of AA from 1-ether-linked phospholipids leads to a marked reduction in the capacity of cells to produce eicosanoids as well as PAF (Suga et al., 1990; Ramesha & Pickett, 1986). While it is now believed that enzymes in addition to PLA_2 participate in the mobilization of AA and lysophospholipids, the fundamental concept that the turnover of 1-ether-linked, arachidonate-containing phospholipids mediates the generation of AA and its products as well as PAF has been firmly established.

Inflammatory cells appear to have mechanisms for rapidly moving arachidonate into phospholipid pools. For example, it has been recognized for some time that free AA enters into lysophospholipids such as 1-acyl-2-lyso-GPC through CoA-dependent acyl transferase reactions (Irvine, 1982; Chilton et al., 1987; Lands & Crawford, 1976; Laposata et al., 1985). More recently, evidence from several laboratories suggests that AA can be rapidly moved from 1-acyl-linked phospholipids to 1-ether linked phospholipids in a CoA-independent manner (Kramer & Deykin, 1983; Kramer et al., 1984; Chilton & Murphy, 1986; Robinson et al., 1985; Sugiura et al., 1985, 1987; Winkler et al., 1991; MacDonald & Sprecher, 1989a). This pathway appears to be highly selective for the transfer of arachidonate as evidenced by the fact that 14, 16, and 18 carbon fatty acids, which are generally more abundant than AA within inflammatory cells, are not efficiently transferred into 1-ether-linked phospholipids. Several laboratories hypothesize that the key enzyme involved in this trafficking of arachidonate between phospholipid pools is CoA-independent transacylase (CoA-IT) (Kramer & Deykin, 1983; Chilton & Murphy, 1986; Robinson et al., 1985). CoA-IT activity has been described in a variety of inflammatory cells. The commonly described characteristics of CoA-IT include fatty acyl transfer in the absence of acyl-CoAs, a marked selectivity for transferring arachidonate over shorter, more saturated fatty acids, and a preference for moving arachidonate into 1-alkyl- and 1-alk-1′-enyl-linked phospholipids (Robinson et al., 1985; Sugiura et al., 1987).

Although CoA-IT activity has been implicated in AA movement and lipid mediator formation, its direct role in these processes has been difficult to test. To investigate the role of CoA-IT in arachidonate movement, we developed two structurally diverse compounds that inhibit CoA-IT activity. These two compounds were developed utilizing two distinct lines of reasoning. Initially, it was recognized that the mechanisms of action of CoA-IT resembled that of other acyl transferase(s) and particularly those involved in cholesterol metabolism such as acyl-CoA: cholesterol acyl transferase (ACAT) and lecithin:cholesterol acyl transferase (LCAT). The first compound (SK&F 98625) is an analog of octimibate, a compound that inhibits ACAT (Rucker et al., 1988). A second series of inhibitors were designed with the idea that CoA-IT must first remove a fatty acyl moiety before it can transfer that moiety, a PLA_2-like activity (Reynolds et al., 1993). In addition, CoA-IT and PLA_2 are able to recognize similar substrate phospholipids. After examining many classes of PLA_2 inhibitors, we discovered SK&F 45905, a compound similar to one class of PLA_2 inhibitors that had less effect on PLA_2. The current study demonstrates in cell-free and intact cell assays that these compounds inhibit CoA-IT. Further, these compounds completely block the transfer of arachidonate from 1,2-diacylphospholipids into 1-alkyl- and 1-alk-1′-enyl- containing phospholipids in intact neutrophils. Together, these studies describe the first inhibitors of CoA-IT and suggest that CoA-IT inhibitors may regulate levels of AA within certain phospholipids of inflammatory cells.

MATERIALS AND METHODS

Materials. [^{3}H]Acetic acid, sodium salt (50−100 Ci/mmol), 1-[^{14}C]acyl-2-lyso-*sn*-glycero-3-phosphocholine (GPC) (40−60 mCi/mmol), 1-acyl-2-[^{14}C]arachidonoyl-GPC (40−60 mCi/mmol), [5,6,8,9,12,14,15-^{3}H]arachidonic acid (100 Ci/mmol), [^{3}H]arachidonate-labeled *Escherichia coli* (10 mCi/mmol, 4−8 nmol Pi/10 μL), and 1-[^{3}H]alkyl-2-lyso-GPC (30−60 Ci/mmol) were purchased from New England Nuclear (Boston, MA). 1-O-[1,2 ^{3}H]hexadecyl-2-lyso-GPC (60−100 Ci/mmol) was gift from Dr. Robert Wykle, Bowman Gray School of Medicine, and was used in kinetic experiments. Common laboratory chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Alkyl-2-lyso-GPC was purchased from Biomol (Plymouth Meeting, PA). Silica Gel G plates were from Analtech Inc. (Newark, DE). Essentially fatty acid-free bovine serum albumin (BSA) was obtained from Calbiochem (San Diego, CA). Silica gel columns were from Baker (Phillipsburg, NJ). All fatty acids were from Nu-Chek Prep (Elysian, MN).

SK&F 98625 is diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate (Figure 1) and was prepared by heating a solution of 3,4,5-triphenyl-1-(7-bromoheptyl)imidazole-2-one and triethyl phosphite in xylene at reflux temperature for 40 h, followed by evaporation. Found: C, 70.1; H, 7.4; N, 4.9%; $C_{32}H_{29}N_2O_4P$ requires: C, 70.3; H, 7.2; N, 5.1%. ^{1}H NMR δ ($CDCl_3$) 1.1−1.4 (12H, m, 3×CH_2, 2×CH_3), 1.45−1.65 (4H, m, CH_2CH_2P, CH_2CH_2N), 1.65−1.75 (2H, m, CH_2P), 3.45−3.50 (2H, br t, CH_2N), 4.0−4.15 (4M, m, 2×CH_2O), 6.86 (2H, d, ArH), 7.00−7.10 (3H, m, ArH), 7.17−7.23 (3H, m, ArH), 7.27−7.31 (4H, m, ArH), 7.35−7.37 (3H, m, ArH); IR η max 1696 cm^{-1}; *m/e* 546. SK&F 45905 is 2-[2-(3,4-chloro-3-(trifluoromethyl)phenyl)ureido]-4-(trifluoromethylphenoxy)-4,5-dichlorobenzene sulfonic acid (Figure 1) and was purchased from Bader Chemical Company.

Preparation of Cells. Neutrophils were prepared from venous blood collected from healthy donors and isolated as previously described (Chilton et al., 1983). The final leukocyte preparation was suspended in Hank's balanced salt solution (HBSS) and was greater than 95% viable and pure, as determined by trypan blue exclusion and histological examination.

CoA-IT Activity Assays. Microsomes were prepared from U937 monocytes, and CoA-IT activity was measured as described previously (Winkler et al., 1991). Briefly, homogenates were prepared by N_2 cavitation (750 psi, 15 min) and microsomes obtained after centrifugation (100 000*g*, 60 min). Microsomes were then diluted in phosphate buffered saline (PBS) containing 1 mM EGTA to the desired protein

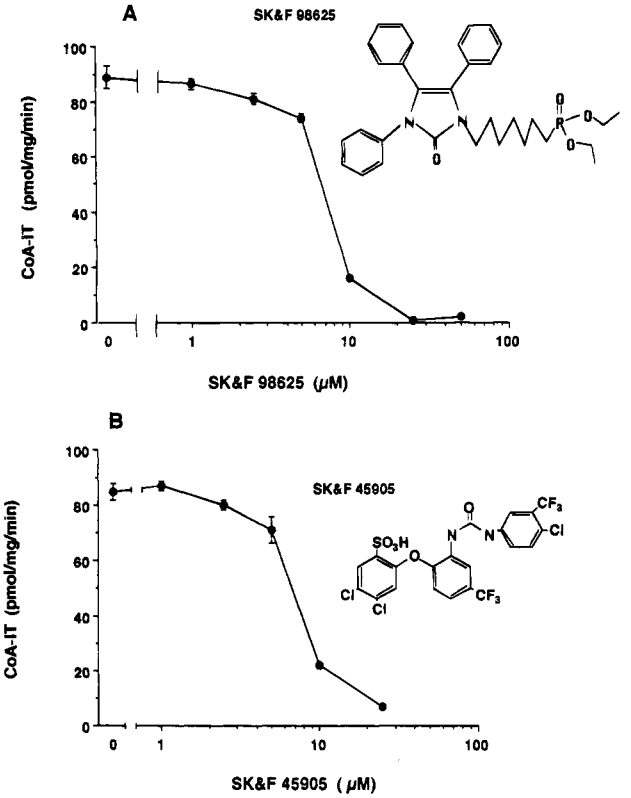

FIGURE 1: Effect of SK&F 98625 and SK&F 45905 on CoA-independent transacylase activity-acceptor assay. U937 microsomes were incubated with 1-alkyl-2-lyso-GPC as described under Materials and Methods. Reactions were terminated by extracting the lipids utilizing the method of Bligh and Dyer. The quantities of 1-alkyl-2-acyl-GPC produced in this reaction were determined and expressed as pmol/mg/min. Panel A (upper panel) and panel B (lower panel) shows the effects of SK&F 98625 and SK&F 45905, respectively, on CoA-IT activity. These data are expressed means ± SEM of 4−6 experiments.

concentration (5−20 μg of protein). The CoA-IT reaction was initiated by addition of 1-[^{3}H]alkyl-2-lyso-GPC (0.1 μCi/tube) mixed with unlabeled 1-alkyl-2-lyso-GPC (final concentration, 1 μM) in assay buffer with 0.25 mg/mL fatty acid-poor BSA. The reaction was run for 10 min at 37 °C. The reactions were stopped, the lipids were extracted, and materials from an aliquot of the chloroform extract were separated by TLC in chloroform/methanol/acetic acid/water (50:25:8:4, v/v) (system 1) and visualized by radioscanning (Bioscan, Washington, DC), and the product, 1-[^{3}H]alkyl-2-acyl-GPC, was scraped and quantified by liquid scintillation spectroscopy. In the second assay for measuring CoA-IT activity both acyl donor (1-acyl-2-[^{14}C]arachidonoyl-GPC, 0.025 μCi) and acyl acceptor (1-radyl-2-lyso-GPE, 20 μM) were mixed with U937 microsomes (50−100 μg of protein) containing CoA-IT activity (Winkler et al., 1991). After an incubation of 60 min at 37 °C, the lipids were extracted and separated by TLC in chloroform/methanol/acetic acid/water (50:25:8:2.5, v/v) (system 2). The transfer of labeled fatty acid from PC donor into PE was measured. In both assays, different concentrations of SK&F 98625 or SK&F 45905 were added to the microsomal preparation 5 min prior to the addition of substrate(s).

The effects of inhibitors on the kinetics of the CoA-IT activity reaction were also examined. In these experiments, various concentrations of SK&F 98625 (0−20 μM) or SK&F 45905 (0−20 μM) in dimethyl sulfoxide were mixed with variable concentrations of 1-alkyl-2-lyso-GPC (0.015−0.3 μM) containing 1-[^{3}H]alkyl-2-lyso-GPC. U937 microsomes containing CoA-IT activity were added and the reaction mixture incubated at 37 °C for 5 min. The reactions were stopped, and the amount of 1-[^{3}H]alkyl-2-acyl-GPC product was determined as described above. The amount of product formed was less than 5% of the added substrate.

Other Enzyme Assays. CoA-dependent acylation activity was determined using U937 microsomes that were diluted in Tris buffer (100 mM, pH 7.4) and incubated with 15 μM acyl-CoA and 0.015 μCi 1-[^{14}C]acyl-2-lyso-GPC with 1 μM unlabeled 1-acyl-2-lyso-GPC (Chilton et al., 1987). Following 10 min at 37 °C, the samples were extracted, developed on TLC plates (system I) and the amount of 1,2-diacyl-GPC product determined. Phospholipase A_2 (PLA_2) activities were determined using *Escherichia coli* membranes or using 1-acyl-2-[^{3}H]AA-GPC sonicated vesicles and measuring the liberation of free [^{3}H]AA. Free fatty acids were isolated by TLC (silica gel G) developed in hexane/ether/formic acid (90:60:6, v/v). Type II, 14 kDa PLA_2 was expressed and purified from Chinese hamster ovary cells (Stadel et al., 1992), and cytosolic 85 kDa PLA_2 was expressed and purified from baculovirus infected insect cells (Amegazie et al., 1993). Lyso-PAF:acetyltransferase activity was determined as described by Wykle and colleagues measuring the production of [^{3}H]PAF from 1-alkyl-2-lyso-GPC and [^{3}H]acetyl-CoA in neutrophil homogenates (Wykle et al., 1980).

The effect of SK&F 98625 and SK&F 45905 on CoA-IT and PLA_2 activity was also determined in neutrophil homogenates. Intact human neutrophils (50 × 10^6 cells/mL) were treated with 20 μM SK&F 98625 or SK&F 45905 or with dimethyl sulfoxide control (0.2%) for 10 min at 37 °C. The cells were then broken by sonication and the resulting homogenates used to measure enzyme activities. CoA-IT activity was measured by the transacylation of 1-[^{3}H]-alkyl-2-lyso-GPC as described above. PLA_2 activity was measured using ^{3}H-AA-labeled *E. coli*, as described above.

Incorporation of Exogenous Arachidonic Acid into Cellular Phospholipids of Intact Neutrophils. Human neutrophils in HBSS were incubated at 37 °C with [^{3}H]arachidonic acid (0.2 μCi/mL, 1 nM final concentration) complexed to HSA (0.25 mg/mL) for 60 min. Reactions were terminated by extracting lipids utilizing the method of Bligh and Dyer (1959). Phospholipid classes were separated on layers of silica gel G developed in system 2. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) were added as standards to the extraction mixture and visualized after chromatography by exposure to iodine vapor. Radioactivity on TLC plates was detected using Bioscan System 200 Imaging Scanner (Bioscan Inc., Washington DC). The amount of radioactivity migrating with PC was determined after zonal scraping followed by liquid scintillation counting.

Arachidonate Movement between Phospholipid Subclasses within Intact Neutrophils. [^{3}H]Arachidonic acid ([1 μCi] complexed to 0.25 mg/mL HSA) was added to the neutrophil suspensions (5 × 10^7) with gentle shaking at 37 °C for 5 min. Unincorporated [^{3}H]arachidonic acid was removed by three washes with HBSS containing 0.25 mg/mL HSA. The neutrophils were then exposed to various concentrations of SK&F 98625, SK&F 45905, or dimethyl sulfoxide vehicle (0.2%) and incubated without stimulation for 2 h. At that time, incubations were terminated by extraction (Bligh &

Dyer, 1959). Phospholipid classes were then separated by normal phase HPLC (Patton et al., 1982). Choline- and ethanolamine-containing phospholipids were isolated and converted into diradylglycerols by addition of 20 units (PC) or 40 units (PE) of *Bacillus cereus* phospholipase C (Sigma Type XIII) in 100 mM Tris-HCl buffer (pH 7.4) for 2.5 or 6 h, respectively. Diradylglycerols formed in the phospholipase C reaction were then converted into 1,2-diradyl-3-acetylglycerols by incubation with acetic anhydride and pyridine at 37 °C for 24 h. The resulting 1,2-diradyl-3-acetylglycerols were separated into 1-acyl, 1-alkyl, and 1-alk-1′-enyl subclasses by TLC on layers of silica gel G in benzene/hexane/ethyl ether (50:45:4, v/v) (Nakagawa et al., 1982). In some experiments, choline-containing phospholipids were separated into molecular species utilizing the method developed by Patton and colleagues. Briefly, an octadecyl reverse phase HPLC column (25 cm long, 2.1 mm internal diameter) was eluted with methanol/water/acetonitrite (90:5:7:2.5, v/v) containing 20 mmol of choline chloride at 0.40 mL/min at 40 °C (Patton et al., 1982). The effluent was monitored at 206 nm to detect end absorption of unsaturated lipids, and fractions were collected at 1.0 min intervals. The radioactivity in each fraction was then determined by liquid scintillation counting. Standards were used to assess the elution order of arachidonate-containing species of PC.

Protein Analysis. Protein concentrations were determined using the method of Bradford (1976), with reagents purchased from Bio-Rad (Hercules, CA).

Data Analysis. Each displayed experiment was performed in duplicate or triplicate and is representative of 2−4 experiments performed on different donors. The results are the means ± SE, with statistical analysis performed on original data by student's test or ANOVA with Scheffe post-hoc tests.

RESULTS

To study the role of CoA-IT in arachidonate−phospholipid remodeling, we searched for tool compounds that would inhibit CoA-IT activity without affecting the activities of other enzymes involved in arachidonate metabolism. Initial studies monitored CoA-IT activity by measuring the acylation of 1-[^{3}H]alkyl-2-lyso-GPC (acceptor assay) to form 1-[^{3}H]-alkyl-2-AA-GPC in U937 microsomes (Winkler et al., 1991). SK&F 98625 and SK&F 45905 were discovered to inhibit CoA-IT activity with IC_{50}s of 9 and 6 μM, respectively, with complete inhibition by both compounds observed at >25 μM (Figure 1). As a second measure of CoA-IT activity, the transfer of [^{14}C] arachidonate from 1-acyl-2-[^{14}C]arachidonoyl-GPC to 1-radyl-2-lyso-GPE was monitored in the presence and absence of inhibitors (donor assay). This second assay system was important because both substrates of the CoA-IT reaction were provided; thus, inhibitors that have effects on transacylase activity in this assay are likely acting independently of any effects on endogenous microsomal lipids. As was demonstrated in the acceptor assay (above), both SK&F 98625 and SK&F 45905 inhibited CoA-IT activity in the donor assay in a dose-dependent manner with IC_{50}s of 11 and 19 μM, respectively (Figure 2 and Table 1).

Once inhibitors of CoA-IT activity had been discovered, the effects of these compounds on enzyme activities other

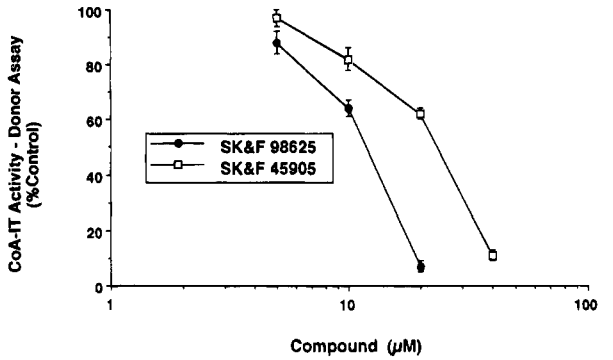

FIGURE 2: Effect of SK&F 98625 and SK&F 45905 on CoA-independent transacylase activity-donor assay. Both acyl donor (1-acyl-2-[^{14}C]arachidonoyl-GPC) and acyl acceptor (1-radyl-2-lyso-GPE) were added to U937 microsomes as described under Materials and Methods. Reactions were terminated by extracting the lipids by the method of Bligh and Dyer and the transfer of labeled fatty acid from the PC donor to the PE acceptor was measured. These data are expressed as means ± SEM of three experiments.

Table 1

	98625	45905
CoA-IT (acceptor assay)	9 μM	6 μM
CoA-IT (donor assay)	11 μM	19 μM
bovine pancreatic PLA_2	NE @ 50 μM	NE @ 50 μM
synovial (group II) PLA_2	NE @ 50 μM	NE @ 50 μM
CoA-dependent acyltransferase	35 μM	50 μM
acetyltransferase	NE @ 50 μM	NE @ 50 μM

than CoA-IT were determined. PLA_2 activities were initially measured utilizing purified recombinant group II PLA_2, $cPLA_2$, and neutrophil homogenates as enzyme sources and [^{3}H]AA-labeled *E. coli* as the enzyme substrate. Group II PLA_2 activity was not inhibited with SK&F 98625 or SK&F 45905 in this assay at concentrations as high as 50 μM. In contrast, $cPLA_2$ was inhibited 68 ± 8.1 and 33 ± 10.6 with SK&F 45905 and SK&F 98625, respectively, at 20 μM. The effect of these compounds on $cPLA_2$ was examined further using sonicated vesicles containing 1-acyl-2[^{3}H]arachidonoyl-GPC as a substrate. In these experiments, constant amounts of compounds were added to reaction mixtures containing varying quantities of substrate. SK&F 94505 was found to inhibit $cPLA_2$ by greater than 80% and was independent of the ratio of drug to vesicle substrate over a 0.4−40 range (data not shown). SK&F 98625 had only a modest (30%) effect on $cPLA_2$ at the highest drug to substrate ratio (e.g., 40). As a further check of specificity of the putative CoA-IT inhibitors against PLA_2, intact neutrophils were exposed to 20 μM of either SK&F 98625 or SK&F 45905 and then cellular CoA-IT or PLA_2 activities were measured in broken cell homogenates. These results demonstrated that both SK&F 98625 and SK&F 45905 significantly block CoA-IT activity in these homogenates when compared to untreated homogenates. However, neither SK&F 98625 or SK&F 45905 induced a diminution of acyl hydrolytic activity in neutrophil homogenates. The significance of these results was to demonstrate the lack of effect of these inhibitors on PLA_2 activity in whole cells under conditions where CoA-IT is inhibited. The aforementioned experiments point out a number of the complexities of using different phospholipid substrates and lipophilic inhibitors to determine inhibitor specificity profiles. Taken together, these data suggest that SK&F 98625 and SK&F 45905 do not block group II PLA_2 or acyl hydrolytic activities in neutrophil homogenates.

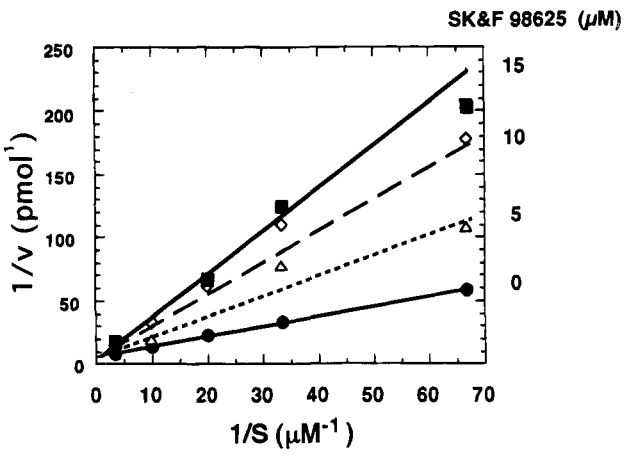

FIGURE 3: Kinetics of CoA-independent transacylase inhibition by SK&F 98625. U937 microsomes were incubated with various concentrations of SK&F 98625 (0−20 μM) and 1-[^{3}H]alkyl-2-lyso-GPC as described under Materials and Methods. The quantities of 1-[^{3}H]alkyl-2-acyl-GPC produced in the reactions were determined. A double-reciprocal plot of 1/velocity verses 1/[1-alkyl-2-lyso-GPC] is shown.

However, SK&F 45905 appears to inhibit cPLA$_2$ in all systems tested.

In addition, SK&F 98625 and SK&F 45905 failed to inhibit the activity of a variety of other enzymes including pancreatic PLA$_2$, cyclooxygenase (data not shown), and acetyltransferase (Table 1). Both SK&F 98625 and SK&F 45905 were found to show some inhibition of CoA-dependent acyl transferase activity, with IC$_{50}$s of 35 and 50 μM, respectively. The ability of SK&F 98625 and SK&F 45905 to affect additional enzymes remain to be gauged.

To further explore the interaction of these putative inhibitors with CoA-IT, kinetic analysis experiments were performed. Initially the effects of various concentrations of SK&F 98625 on the velocity of the CoA-IT reaction at variable concentrations of 1-alkyl-2-lyso-GPC substrate, (0.015−0.3 μM) were examined (Figure 3). Analysis of these results indicated that the inhibition of CoA-IT activity by SK&F 98625 can be best fit to a model of competitive inhibition versus the 1-acyl-2-lyso-GPC substrate, using eq 1 from Cleland (1979):

$$v + V_m s/[K_m(1 + I/K_i) + s] \quad (1)$$

The apparent K_m of the substrate was 0.13 ± 0.02 μM, and the K_{is} of SK&F 98625 was 4.6 ± 0.5 μM. Similarly, various concentrations of SK&F 45905 were mixed with variable concentrations of 1-alkyl-2-lyso-GPC, and reaction velocity was measured. As with SK&F 98625, SK&F 45905 appeared to be inhibiting the CoA-IT reaction in a competitive manner with a K_{is} of 1.9 + 0.9 μM (data not shown). Together these data strongly suggested that these inhibitors were interacting with the CoA-IT enzyme in a specific and predictable manner and that the inhibition of CoA-IT was not caused by a nonspecific effect, such as detergent effect on the microsomal membranes. On the basis of the profile of these compounds in cell free assays, they were determined to be appropriate tools to examine the effects of inhibition of CoA-IT within intact cells.

Influence of CoA-IT Inhibitors on Arachidonate Remodeling in Human Neutrophils. Human neutrophils have a number of characteristics which make them well-suited for this study of arachidonate−phospholipid remodeling. They contain high amounts of CoA-IT activity, and the bulk of their arachidonate is located in 1-ether-linked phospholipids. In addition, AA is initially incorporated into 1-acyl-linked phospholipids and then is slowly transferred into 1-ether-linked phospholipids. It was our hypothesis that this slow transfer into 1-ether-linked phospholipids was accomplished by the enzyme CoA-IT. To test this hypothesis, the effects of SK&F 98625 on the transfer of arachidonate from 1-acyl into 1-alkyl and 1-alk-1-enyl-linked phospholipids was examined. Neutrophils were pulse-labeled with [^{3}H]arachidonic acid to allow incorporation of this label into 1-acyl-linked phospholipid. Neutrophils were then treated with various concentrations of SK&F 98625, and the movement of arachidonate between phospholipid subclasses was assessed over a 2 h period in the absence of cell stimulation. Figure 4 shows that SK&F 98625 produced a concentration-dependent blockage of movement of [^{3}H]arachidonate into the two major 1-ether-linked phospholipids of human neutrophils, 1-alkyl-2-arachidonoyl-GPC and 1-alk-1-enyl-2-arachidonoyl-GPE. The concentrations of SK&F 98625 needed to block the transfer of arachidonate were similar to that observed for the inhibition of CoA-IT activity in broken cell preparations. It was important to note that SK&F 98625 could completely block the arachidonate movement, suggesting that not only did CoA-IT mediate this movement but no other enzyme was utilized within the intact cell to compensate for the blockage of CoA-IT.

Similar experiments were carried out with the second CoA-IT inhibitor, SK&F 45905. SK&F 45905 had comparable effects with SK&F 98625 in its capacity to block the movement of AA into 1-alkyl-2-arachidonoyl-GPC and 1-alk-1-enyl-2-arachidonoyl-GPE (data not shown). Figure 5 illustrates the movement of labeled AA from 1-acyl-2-AA-GPC molecular species to 1-alkyl-2-AA-GPC molecular species during the 2 h chase period in the presence or absence of both CoA-IT inhibitors. After the pulse, [^{3}H]arachidonate was located predominantly in 1-acyl-linked PC molecular species containing 16:0, 18:1, and 18:0 fatty acyl chains. During the 2 h chase [^{3}H]arachidonate moved into 1-alkyl-linked PC molecular species (16e/20:4, 18:1e/20:4, and 18:0e/20:4). Both SK&F 45905 and SK&F 98625 inhibited the remodeling of arachidonate from 1-acyl- to all 1-alkyl-linked molecular species of PC.

Influence of CoA-IT Inhibitors on the Incorporation of Arachidonic Acid into Cellular Phospholipids. Because these inhibitors had the capacity to completely block the incorporation of arachidonic acid into 1-ether-linked phospholipid and had a slight effect on CoA-dependent acyl transferase activity in broken cell systems, it was important to assure that these compounds were not inhibiting CoA-dependent acyltransferase reactions within the intact cell, especially as they relate to arachidonic acid. To test this, neutrophils were incubated with labeled arachidonic acid, and the incorporation of this arachidonic acid into PC was measured in the presence and absence of inhibitors. As illustrated in Figure 6, neither SK&F 98625 nor SK&F 45905 blocked the initial incorporation of labeled AA into membrane phospholipids of the human neutrophil. These data together with the assays in the cell-free systems provided confidence that the putative inhibitors of CoA-IT were inhibiting only CoA-independent acylation reactions and these reactions were critical to placing arachidonate in 1-ether-linked phospholipids.

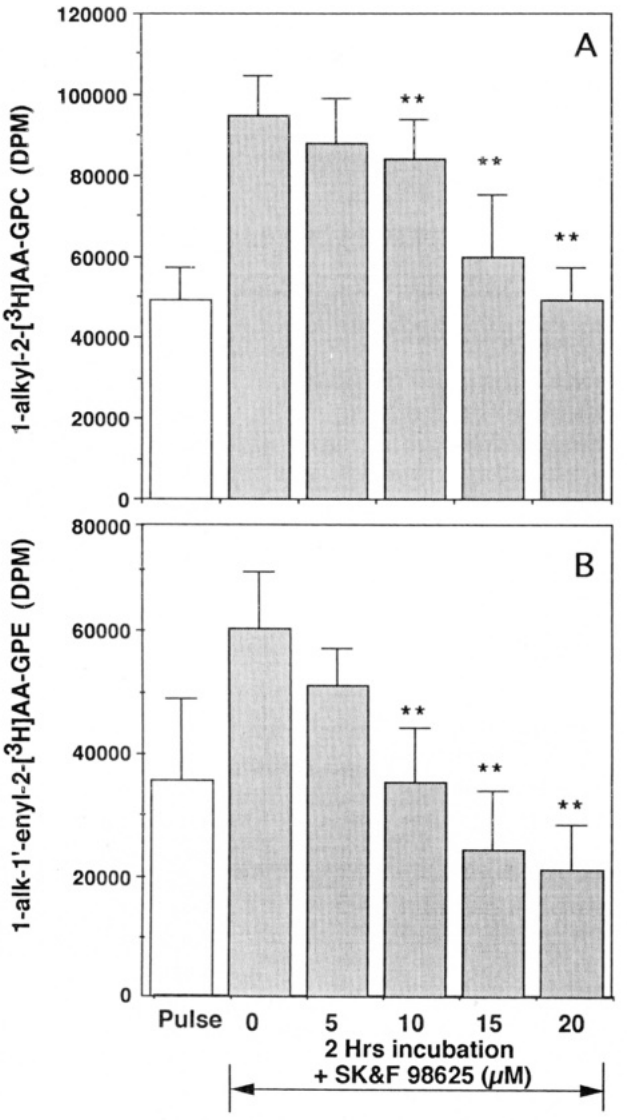

FIGURE 4: Effect of SK&F 98625 on the transfer of arachidonate into 1-alk-1-enyl-2-AA-GPE and 1-alkyl-2-AA-GPC in human neutrophils. Neutrophils were pulse-labeled with [^{3}H]AA for 5 min, washed, and then incubated in the absence or presence of SK&F 98625 for 2 h. Reactions were terminated by extracting the lipids utilizing the method of Bligh and Dyer and phospholipid subclasses isolated as described under Materials and Methods. Panels A and B show the quantities of [^{3}H]AA in 1-alkyl-2-AA-GPC and 1-alk-1-enyl-2-AA-GPE, respectively, under different experimental conditions. These data are means ± SEM for three separate experiments. "**" indicates $p < 0.05$.

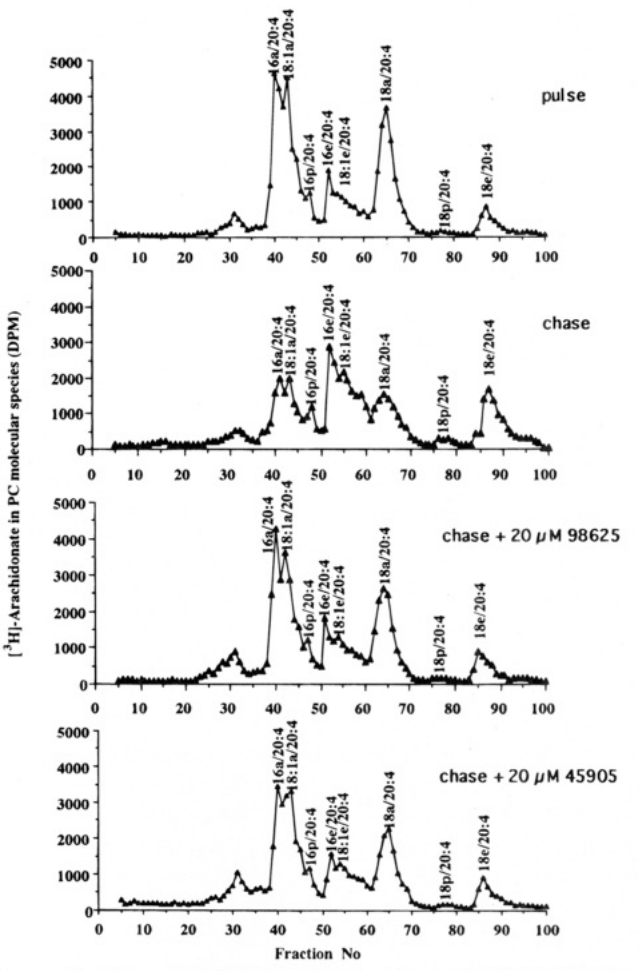

FIGURE 5: Effect of SK&F 98625 (20 μM) and SK&F 45905 (20 μM) on the transfer of arachidonate into PC molecular species. Neutrophils were pulse labeled with [^{3}H]AA for 5 min (pulse) and then incubated in the presence of absence of SK&F 98625 (20 μM) or SK&F 45905 (20 μM) for 2 h. Reactions were terminated by extracting lipids by the method of Bligh and Dyer. PC was isolated by normal phase HPLC and PC molecular species isolated by reverse phase HPLC as described under Materials and Methods. "a" represents an acyl linkage at the *sn*-1 position, "e" represents an alkyl linkage at the *sn*-1 position, and "p" represents an alk-1-enyl linkage at the *sn*-1 position (e.g., 16e/20:4 is 1-*O*-hexadecyl-2-AA-GPC). These data are representative of two separate experiments.

DISCUSSION

A route detailing the movement of arachidonate through different phospholipids molecular species has been proposed for several years. Initially, arachidonate as arachidonoyl-CoA is thought to be incorporated into 1-acyl-linked phospholipids by CoA-dependent acyltransferase(s). This arachidonate in 1-acyl-linked phospholipids is then proposed to be transferred to 1-ether-linked phospholipids such as 1-alkyl-2-arachidonoyl-GPC and 1-alk-1-enyl-2-arachidonoyl-GPE by the enzyme CoA-IT. While most of the data in the current literature are consistent with such a pathway, it has not been possible to directly test this hypothesis within intact cells. In the current paper, we have discovered and then utilized two inhibitors of CoA-IT activity to probe the role of this enzyme in arachidonate−phospholipid metabolism within the human neutrophil.

The two inhibitors of CoA-IT, SK&F 98625 and SK&F 45905, were developed from two structurally distinct classes of compounds. One class of compounds typified by SK&F 98625 is an analog of compounds that inhibit ACAT. The second class of compounds typified by SK&F 45905 is an analog of compounds that block PLA_2 activity. SK&F 45905 also has some effect (in the concentration range used in these experiments) on $cPLA_2$ activity. Both compounds also appear to have small effects on other enzyme activities which work at the *sn*-2 position of membrane phospholipids. Thus these compounds are not totally selective for CoA-IT. However, while these different classes of compounds have distinct profiles for inhibition of other enzymes, they share inhibition of CoA-IT as their unifying feature. For this reason, it was important to utilize two structurally diverse CoA-IT inhibitors to probe enzymatic reactions in the complex milieu of the intact cell. In these studies, no property was ascribed to CoA-IT unless that property could be blocked in the intact cell by both classes of CoA-IT inhibitors. This approach was warranted by the complete

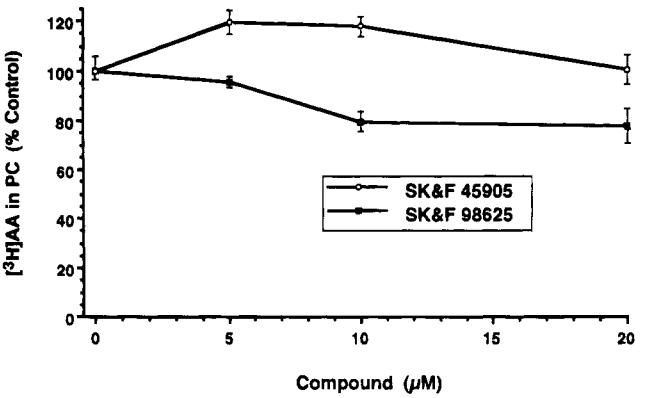

FIGURE 6: Effect of SK&F 98625 and SK&F 45905 on the incorporation of arachidonic acid into human neutrophils. Human neutrophils were provided AA (complexed to albumin) for 60 min in the absence and presence of SK&F 98625 and SK&F 45905 as described under Materials and Methods. Reactions were terminated by extracting the lipids utilizing the method of Bligh and Dyer and the incorporation of [^{3}H]AA into PC determined by TLC. These data are means ± SEM of three separate experiments.

lack of knowledge about the effects of inhibition of CoA-IT activity.

It was also important to demonstrate that the inhibitors were interacting directly with the enzyme and not influencing properties or quantities of the phospholipid substrate acted upon by the enzyme. Two sets of studies suggested that the inhibitors were interacting with enzyme. (1) Both inhibitor classes blocked two different assays of CoA-IT where different donor and acceptor substrates were provided to the enzyme. (2) Kinetic analysis revealed that both classes of inhibitors were preventing the CoA-IT reaction by competing with substrate in the active site of the enzyme. The active site of CoA-IT is unknown at this time. It is our hypothesis, however, based on work using a number of nonspecific inhibitors, that arachidonate from a donor phospholipid binds a serine residue in the active site of CoA-IT. This is a reaction typified by other acyl transferase activities such as LCAT. Further experiments will be necessary to determine the molecular mechanism of action of CoA-IT and how these two classes of inhibitors compete with the proposed substrates for CoA-IT.

This is the first report to utilize inhibitors within intact cells to test the role of CoA-IT in arachidonate–phospholipid remodeling. Both CoA-IT inhibitors, SK&F 98625 and SK&F 45905, blocked in a dose-dependent manner the movement of arachidonate into 1-ether-linked phospholipids. It was also important to note that the incorporation of free arachidonic acid into membrane phospholipids was not influenced by either inhibitor class. Taken together these findings strongly support the hypothesis that arachidonate is transferred into the largest pools (i.e., 1-alkyl-2-arachidonoyl-GPC and 1-alk-1-enyl-2-arachidonoyl-GPE) within the cell by the enzyme CoA-IT and other enzymes such as CoA-dependent acyl transferases probably have only a minimal role in this transfer event.

These studies raise the possibility that blocking the enzyme CoA-IT may modulate cellular levels of arachidonate. Several dietary reduction and supplementation strategies have been utilized in animals and humans in an attempt to reduce cellular and tissue levels of arachidonate with concomitant alterations in eicosanoid generation and clinical outcomes (Friedman et al., 1978; Prickett et al., 1981; Kelley et al., 1985; Lefkowith et al., 1990; Rovin et al., 1990; Hurd et al., 1981; Surette et al., 1992a,b). In particular, there are several studies in which animals that were placed on essential fatty acid-depleted diets had dramatically reduced levels of arachidonate within inflammatory cells. This led to decreased levels of eicosanoids and PAF and amelioration of inflammation in models which mimicked arthritis, asthma, or glomerulonephritis. The current studies demonstrate that CoA-IT blocks the incorporation of arachidonate into the largest arachidonate pools within inflammatory cells. It is our hypothesis that a CoA-IT inhibitor given long-term will preferentially deplete arachidonate in 1-ether-linked phospholipids of inflammatory cells. Preliminary experiments utilizing a long-term treatment of mast cells with SK&F 45905 support this hypothesis (data not shown). Recent studies also suggest that the rapid movement of AA between phospholipid subclasses is a step required for lipid mediator generation (Fonteh & Chilton, 1992; Uemura et al., 1991; Venable et al., 1991; Winkler et al., 1994a). Therefore, short-term blockage of arachidonate–phospholipid remodeling may also inhibit lipid mediator generation. Both platelet-activating factor and arachidonic acid mobilization are blocked in experiments, utilizing a short-term treatment of mast cells and neutrophils with either SK&F 45905 or SK&F 98625 (Winkler et al., 1994b). While the use of pharmacologic agents to modulate arachidonate–phospholipid remodeling has been an unexplored area, these data support the concept that CoA-IT may be a new therapeutic target to regulate arachidonate levels and lipid mediator generation.

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