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The Formation of Diarachidonyl Diglyceride by Rat Neutrophils

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SUMMARY

Neutrophils isolated from the pleural cavity of rats 3 hr after the intrapleural injection of carrageenan metabolize exogenously added arachidonic acid via cyclooxygenase and lipoxygenase. In addition, these cells esterify arachidonic acid to produce diarachidonyl diglyceride. The structure of the diglyceride was determined with the use of various chemical and enzymatic digestions, gas chromatography-mass spectrometry, and ^{252}Cf plasma-desorption mass spectrometry. The formation of this unique diglyceride is stimulated by the presence of nonsteroidal anti-inflammatory drugs. Some of the possible consequences of diarachidonyl diglyceride production are discussed.

INTRODUCTION

There have been numerous attempts to correlate the effects of nonsteroidal anti-inflammatory drugs with their abilities to affect the production of endogenous substances or mediators. This is especially true with respect to arachidonic acid metabolism since the classic studies of Vane (1) and Ferreira *et al.* (2) demonstrated that aspirin and indomethacin inhibited cyclooxygenase activity. These results offered the most logical explanation for the mechanism of action of nonsteroidal anti-inflammatory drugs. However, it has not been entirely clear whether the whole spectrum of pharmacological activities of these anti-inflammatory drugs can be explained by inhibition of prostaglandin biosynthesis.

In this paper, neutrophils isolated from the pleural cavity of rats 3 hr after the intrapleural injection of carrageenan (3, 4) have been utilized to investigate the effects of nonsteroidal anti-inflammatory drugs on the metabolism of exogenously supplied arachidonic acid via pathways other than cyclo-oxygenase and lipoxygenase. It was of interest to investigate the utilization of arachidonic acid in these cells since they are presumably actively involved in acute inflammatory responses.

MATERIALS AND METHODS

Isolation of pleural cavity neutrophils. Rat neutrophils were harvested essentially as previously described (4). Neutrophils (85–95% pure) were isolated from the pleural cavity of male Sprague-Dawley rats 3 hr after the intrapleural injection of 500 μg of carrageenan (Marine Colloids Inc., Lot RENJ 8254) in 0.25 ml of pyrogen-free water (3). The neutrophils were isolated from the pleural exudates by centrifugation at 4° for 10 min at 200 × *g*. The cell pellet was resuspended in 17 mM Tris·HCl (pH 7.2) containing 0.75% NH_4Cl to lyse contaminating erythrocytes (5), followed by centrifugation at 4° for 5 min at 200 × *g*. The pelleted neutrophils were resuspended in 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA.

Isolation of peripheral blood neutrophils. Heparinized venous blood was obtained from male Sprague-Dawley rats weighing 160–180 g. A 1.6% (w/v) solution of Dextran T-500 (Pharmacia Fine Chemicals, Piscataway, N. J.) was prepared using a modified GBSS[3] in which the divalent cations were not added. Equal volumes of pooled blood and Dextran T-500 were mixed by inversion. Red blood cell rouleaux were allowed to sediment at unit gravity for 1 hr. The upper plasma layer containing the leukocytes was removed and centrifuged for 12 min at 1300 rpm in a Sorvall GLC-1 table-top centrifuge. The cell pellet was washed with 30 ml of modified GBSS and resuspended in 25 ml of the same buffer. The resuspended cells were layered over a solution containing 25 ml of Ficoll-Paque (Pharmacia Fine Chemicals) and 5.40 ml of metrizoate sodium (Accurate Chemical and Scientific Corporation, Hicksville, N. Y.) having a density of 1.145 g/ml. The gradient was centrifuged for 60 min at 2000 rpm. The lymphocyte fraction at the GBSS interface and the Ficoll layer were removed by suction down to the cell

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[3] The abbreviations used are: GBSS, Gey's balanced salt solution; BW 755C, 3-amino-1-*m*-trifluoromethyl-2-pyrazoline; HPLC, high-pressure liquid chromatography; GC, gas chromatography; MS, mass spectrometry; $^{252}CfPDMS$, ^{252}Cf plasma desorption mass spectrometry; BSTFA, *N,O*-bis(trimethylsilyl)fluoroacetamide; TLC, thin-layer chromatography; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, hydroxyicosatetraenoic acid.

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pellet at the bottom of the tube. The sides of the tube were wiped clean of adherent lymphocytes before addition of modified GBSS to resuspend the granulocyte pellet. These cells were washed and viability was determined to be greater than 90% by trypan blue exclusion. Total and differential white blood cell counts were performed on the purified neutrophils.

Assay for arachidonic acid metabolism. Arachidonic acid metabolism in these rat neutrophils was determined by incubating 7×10^6 intact cells at 37° with [1-^{14}C] arachidonic acid (Amersham Corporation, Arlington Heights, Ill., 55 μCi/μmole) in 0.1 ml of 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl and 1 mM EDTA. Assays (in duplicate) were terminated by the addition of 0.9 ml of 0.1% formic acid and 2.4 ml of a chloroform-methanol (1:1, v/v) mixture. The suspension was vortexed, immediately cooled in ice, and centrifuged, and the organic layer was withdrawn. The evaporated extract was resuspended in a minimum volume of chloroform-methanol (1:2, v/v) and spotted on silica thin-layer plates (Sil G-25, without gypsum; Brinkmann Instruments, Westbury, N. Y.). Chromatograms were developed with an ascending solvent of petroleum ether-diethyl ether-glacial acetic acid (40:60:1, v/v/v). Products were located by autoradiography and the appropriate regions of the thin-layer plates were scraped and counted in a liquid scintillation counter. Metabolites were identified by co-chromatography with authentic standards on thin-layer plates and by HPLC (6–9).

Aspirin and BW 755C were obtained from Burroughs Wellcome (Research Triangle Park, N. C.). Indomethacin was obtained from Merck, Sharp and Dohme (Rahway, N. J.). All other reagents were of the highest grade available.

GC/MS. A Varian Aerograph 1700 gas chromatograph coupled to a Varian MAT CH5-DF double-focussing mass spectrometer was used. The interface between the GC and MS was a single-stage glass jet separator from SGE Corporation. The data generated were processed by a VG Multispec data system. The mass spectrometric conditions were ionization energy 70 eV, trap current 300 μA, accelerating voltage 3 kV, multiplier voltage 1.7 kV, and ion source temperature 250°. GC was performed on a 6-foot, 2-mm internal diameter glass column containing 3% OV-1 on Supelcoport 100–200 mesh. The helium carrier gas flow was 30 ml/min. The temperature program consisted of holding for 2 min at 100° followed by 10°/min temperature rise to 250°. All temperatures after the column through the ion source were maintained at 250°.

GC of ^{14}C-labeled metabolites. A Perkin-Elmer Model 900 gas chromatograph equipped with a flame ionization detector and a laboratory-constructed [^{14}C]CO_2 detector was employed for GC. The column and conditions described above for the GC-MS work were also utilized in the GC experiments. The [^{14}C]CO_2 detector was connected in series with a flame ionization detector. After being combusted in the flame the effluent from the GC was introduced into the top of a modified spinning-band column. Oxifluor-CO_2 from New England Nuclear Corporation (Boston, Mass.), which contains a strong base to absorb CO_2, was pumped into the top of the column at 60 ml/min. The effluent from the bottom of the spinning-band column was collected in 6-ml fractions. The vials were subsequently counted in a Packard 3320 liquid scintillation spectrometer to determine [^{14}C]CO_2. A similar apparatus has been described previously (10).

^{252}CfPDMS. A detailed description of ^{252}CfPDMS has been reported (11). The putative diglyceride was delivered by air freight to the ^{252}CfPDMS laboratory in hexane cooled in Dry Ice. Upon arrival 12 hr later, the sample was immediately prepared for analysis by evaporation of the solution under dry argon onto an aluminized Mylar target. The sample was loaded onto the target stick and transferred under dry nitrogen to the mass spectrometer. A spectrum was obtained 1 hr after the sample was placed in the mass spectrometer.

Preparation of samples for GC and GC/MS. Samples were methylated by the addition of a diethyl ether solution of diazomethane until a faint yellow color persisted and evolution of nitrogen gas ceased. Samples were also derivatized by the addition of 200 μl of BSTFA containing 1% trimethylchlorosilane followed by heating to 110° for 15 min.

RESULTS

While investigating the metabolism of arachidonic acid via lipoxygenase and cyclooxygenase in neutrophils obtained 3 hr after the injection of carrageenan into the pleural cavity of rats (4), the formation of an unknown metabolite of arachidonate was observed. Incubation of rat pleural cavity neutrophils with [1-^{14}C]arachidonic acid yields a compound, 1, with an R_F of 0.47 on Sil G-25 thin-layer plates developed with an ascending solvent of 40:60:1, v/v/v, petroleum ether-diethyl ether-acetic acid (solvent system I) and an R_F of 0.83 on Sil G-25 developed with an ascending solvent composed of the upper phase of 90:50:20:100, v/v/v/v, ethyl acetate-isooctane-acetic acid-water (solvent system II).

Formation and isolation of the arachidonic acid metabolite. As illustrated in Fig. 1, the incorporation of [1-^{14}C]arachidonic acid into this arachidonic acid metabolite is linear for approximately 2 min at 37° but the amount

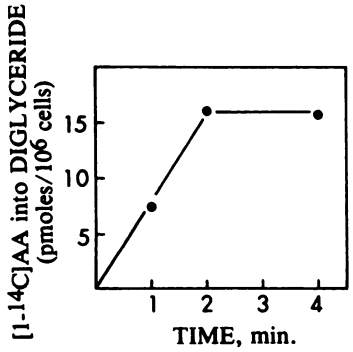

FIG. 1. *Time course of arachidonic acid incorporation into diglyceride*

Neutrophils isolated from the pleural cavity of rats 3 hr after the intrapleural injection of 500 μg of carrageenan were washed and assayed with 50 μM [1-^{14}C]arachidonic acid (*[1-^{14}C]AA*) for various times as described under Materials and Methods. These results are from one of three replicate experiments, and values are ±10%.

recovered remains constant at longer times. The amount of this metabolite was proportional to the number of cells in the incubation up to approximately 10^9 neutrophils/ml. Heat-denatured cells did not produce this or any other arachidonic acid metabolite (data not shown). The amount of exogenously added arachidonic acid necessary to yield a half-maximal rate of formation of this metabolite is approximately 27 μM (Fig. 2).

Although rat neutrophils isolated from the pleural cavity 3 hr after carrageenan injection appear to form this metabolite, those isolated from the peripheral blood from normal or carrageenan-injected rats do not (data not shown). Following preparation by the same procedure utilized to prepare peripheral cells (see Materials and Methods), rat neutrophils isolated from the pleural cavity after carrageenan injection still produce this metabolite from exogenous arachidonic acid. Therefore, this metabolite appears to be related to the fact that neutrophils have arrived in the pleural cavity as a result of the presence of an activator or chemotactic signal formed following carrageenan injection.

In order to obtain enough material to elucidate the structure of this arachidonic acid metabolite, 10^9 neutrophils, isolated from the pleural cavity of rats 3 hr after the injection of carrageenan, were incubated for 2 min at 37° with 37 μM [1-^{14}C]arachidonic acid and the assay mixture extracted with chloroform-methanol as described under Materials and Methods. After the organic solvent extract was dried under N_2, the residue was redissolved in 1 ml of hexane-diethyl ether, 9:1 (v/v). The solution was applied to a dry sialic acid column (0.6 × 4.0 cm) (Unisil, Clarkson Chemical Company, Williamsport, Pa.). Following washing of the column with 10 ml of hexane-diethyl ether, 9:1 (v/v), the metabolite was eluted with 20 ml of hexane-diethyl ether, 4:1 (v/v). After drying under N_2, the ^{14}C-labeled compound was suspended in a minimal volume of hexane-isopropanol-acetic acid, 991:8:1 (v/v/v), and chromatographed by HPLC on silica (Fig. 3). Since the radioactive material in Fractions 39–45 co-chromatographed with the unknown metabolite on thin-layer plates, these fractions were pooled, evaporated to dryness under N_2, and redissolved in a minimal volume of acetonitrile-water, 19:1 (v/v), containing 0.01% acetic acid. This solution was then chromatographed by HPLC on a reverse-phase column (Fig. 4). The radioactive material in Fractions 20–22 co-chromatographed with the unknown metabolite, **1**, on thin-layer systems. This preparation of radiochemically pure material was utilized to elucidate the structure of the rat neutrophil arachidonic acid metabolite.

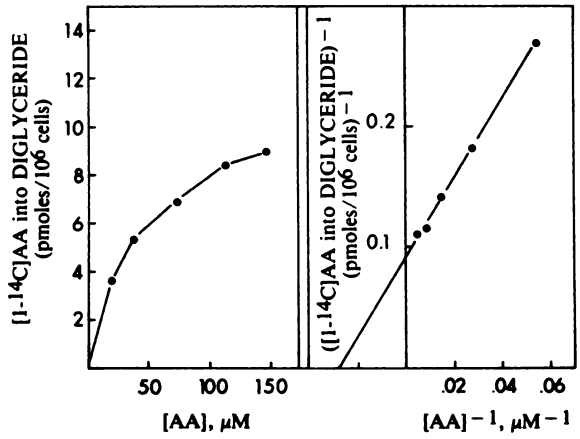

FIG. 2. *Concentration dependence of arachidonic acid incorporation into diglyceride*

Neutrophils were assayed with various concentrations of [1-^{14}C] arachidonic acid (*[1-^{14}C]AA*) for 1 min at 37° and the products were analyzed as described under Materials and Methods. These results are from one of three replicate experiments, and values are ±10%.

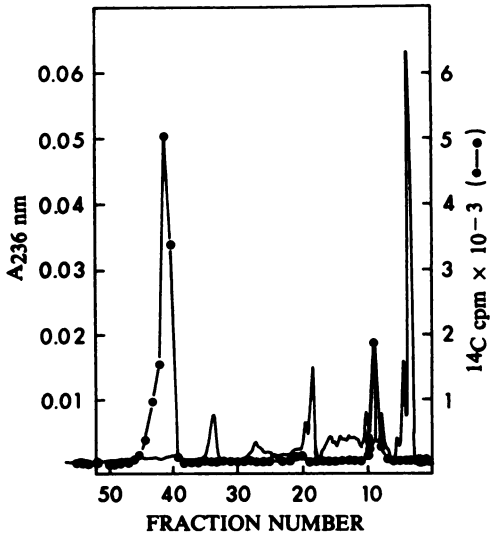

FIG. 3. *Chromatography of diglyceride on μPorasil*

Partially purified ^{14}C-labeled diglyceride was injected into a μPorasil column (0.4 × 30 cm) (Waters Associates) attached to a Waters U6K injector, Model 6000A pumps, and Model 450 UV monitor. The column was equilibrated with 991:8:1, v/v/v, hexane-isopropanol-acetic acid, and eluted at 1 ml/min with the same solvent. Fractions (1-ml) were collected and 10-μl aliquots were counted in a liquid scintillation spectrometer to determine ^{14}C content.

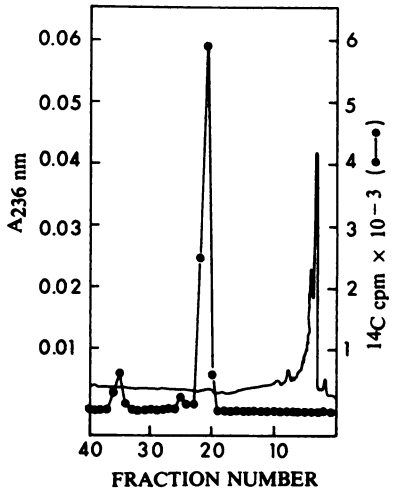

FIG. 4. *Reverse-phase chromatography of diglyceride*

The partially purified diglyceride from μPorasil chromatography (Fig. 3) was injected onto a μBondapak C-18 column (0.4 × 30 cm) (Waters Associates) on the HPLC described in Fig. 3. The sample was eluted at 1 ml/min with 19:1 (v/v) acetonitrile-water containing 0.01% acetic acid. Fractions (1-ml) were collected and 10-μl aliquots were counted in a liquid scintillation spectrometer to determine ^{14}C content.

Identification of the arachidonic acid metabolite, 1: The neutrophil-generated metabolite was not amenable to direct-probe analysis using electron-impact MS. Treatment of 1 with diazomethane and BSTFA did not improve the mass spectrometric results. In addition, the derivatized sample was also examined by GC with flame ionization and radiochemical detectors as described under Materials and Methods. No radioactive product eluted using a temperature program of 100°–300° at 20°/min.

In order to characterize the arachidonic acid metabolite, the HPLC-purified material was subjected to various chemical and enzymatic treatments, the results of which were analyzed by GC using a flame ionization detector and ^{14}C labeling. When a volatile radioactive product was obtained, GC-MS was used to characterize the material. The product of incubation of the HPLC-purified 1 with pancreatic lipase [EC 3.1.1.3, Type VI, Sigma Chemical Company), 100 μg/ml, in 50 mM Tris·HCl (pH 7.7) or with 0.1 N KOH in 80% methanol was derivatized with diazomethane and BSTFA. The GC of this material produced a single radioactive peak. GC-MS studies identified the material as [1-^{14}C]arachidonic acid methyl ester (Fig. 5). Moreover, the only fatty acid of chain length C_{12}–C_{22} was labeled arachidonic acid. Materials giving peaks *b* and *e* were added as chemical markers. Peak *f* was identified as a phthalate impurity. Peaks *a* and *c* were not methyl esters of fatty acids; i.e., they did not possess significant m/z 74 ions and did not match fatty acid methyl esters in a library search of the standard National Bureau of Standards mass spectral library.

Since the [1-^{14}C]arachidonic acid provided to the cells contained more than 91% ^{14}C at position 1, it is interesting to note that the molecular ion observed was m/z 320 with a relative intensity of 1% whereas the m/z 318 ion could not be distinguished from background. Therefore, the arachidonic acid recovered contained radioactive arachidonic acid derived from exogenously supplied [1-^{14}C]arachidonic acid and undiluted by endogenous fatty acid. The retention time of this material on GC and the MS data agreed with those of authentic [1-^{14}C]arachidonic acid. Also, chromatography on Sil G-25 plates using solvent system I of the product of KOH hydrolysis in methanol gave an R_F identical with arachidonic acid methyl ester.

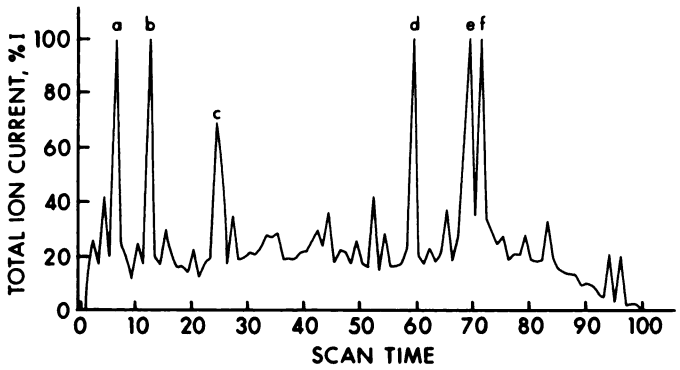

FIG. 5. *The GC-MS trace of the product of 1 after stepwise reaction with KOH, diazomethane, and BSTFA*

The column conditions used were as follows: a glass column (6 feet × 2 mm) containing 3% OV-1 programmed between 100° and 250° at 10°/min with a 2-min hold at injection. The reactions with KOH, diazomethane, and BSTFA are described under Materials and Methods. *a*, Unknown; *b*, methyl caproate standard, used as a marker; *c*, unknown; *d*, radiolabeled product; *e*, triphenyl phosphenoxide, used as a marker; *f*, dioctylphthalate.

The mobility of 1 with solvent systems I and II suggested that the unknown metabolite was actually a diglyceride. The following experiments supported the hypothesis that the arachidonic acid was contained in a diglyceride: Incubation of 1 with 0.1 M phenylisocyanate in ether gave rise to a new radioactive material with a R_F of 0.78 on silica TLC developed with solvent system I. This result indicated the presence of an hydroxyl-group on 1. When the ^{14}C-labeled neutrophil metabolite, 1, was incubated for 20 min at 37° with a horse platelet preparation containing diglyceride kinase (12) in 2.5 mM deoxycholate, 1 mM $MgCl_2$, 200 mM $CaCl_2$, and 1 mM ATP, a new radioactive compound appeared which chromatographed with authentic phosphatidic acid on silica TLC developed with solvent system II. In addition, treatment of the neutrophil metabolite, 1, with pancreatic lipase, as described above, followed by incubation with glycerokinase (EC 2.7.1.30; Sigma Chemical Company) for 1 hr at 37° in 0.86 M hydrazine (pH 9.8) containing 0.17 M glycine, 1.7 mM $MgCl_2$, and 1 mM [α-^{32}P]ATP (1 μCi/μmole; New England Nuclear Corporation) yielded a ^{32}P-labeled product indistinguishable from glycerol-phosphate on polyethyleneimine-cellulose thin-layer plates developed with ascending 1 M LiCl. Therefore, the results of chemical and enzymatic treatment further supported the conclusion that the unknown arachidonic acid-derived metabolite, 1, formed from exogenously added fatty acid by neutrophils isolated from the rat pleural cavity 3 hr after injection of carrageenan was an arachidonic acid-containing diglyceride. Since no fatty acids other than arachidonic acid were observed in the GC-MS experiments, it was likely that the product was a diarachidonic acid diglyceride.

Direct physical evidence to support a diglyceride structure was obtained by $^{252}CfPMDS$. The sample, prepared as described under Materials and Methods, exhibited ions $[M + H]^+$ at m/z 668 (intensity = 302 total counts) and $[M + Na]^+$ at m/z 691 (intensity = 1927 total counts). In addition, a small $[M + {}^{39}K]^+$ was observed at m/z 707, but it was too weak to detect the corresponding $[M + {}^{41}K]^+$. An enhanced view of the m/z 692 region is illustrated in Fig. 6. The molecular weight of 668.50 is calculated for a diglyceride molecule containing two ^{14}C atoms, one on each C-1 of arachidonic acid. The weight of molecule determined by substracting the mass of sodium from the mass of the $[M + Na]^+$ ion as determined by $^{252}CfPMDS$ was 668.45.

Effect of anti-inflammatory drugs. Nonsteroidal anti-inflammatory drugs inhibit the conversion of arachidonic acid to prostaglandins, HHT, 11-HETE, and 15-HETE by these rat neutrophils (4). During the course of these investigations, it was observed that the amount of arachidonic acid incorporated into diglyceride was increased by the presence of anti-inflammatory drugs. As illustrated in Fig. 7, aspirin, indomethacin, and BW 755C increase the incorporation of arachidonic acid into diglyceride in a concentration-dependent manner. The

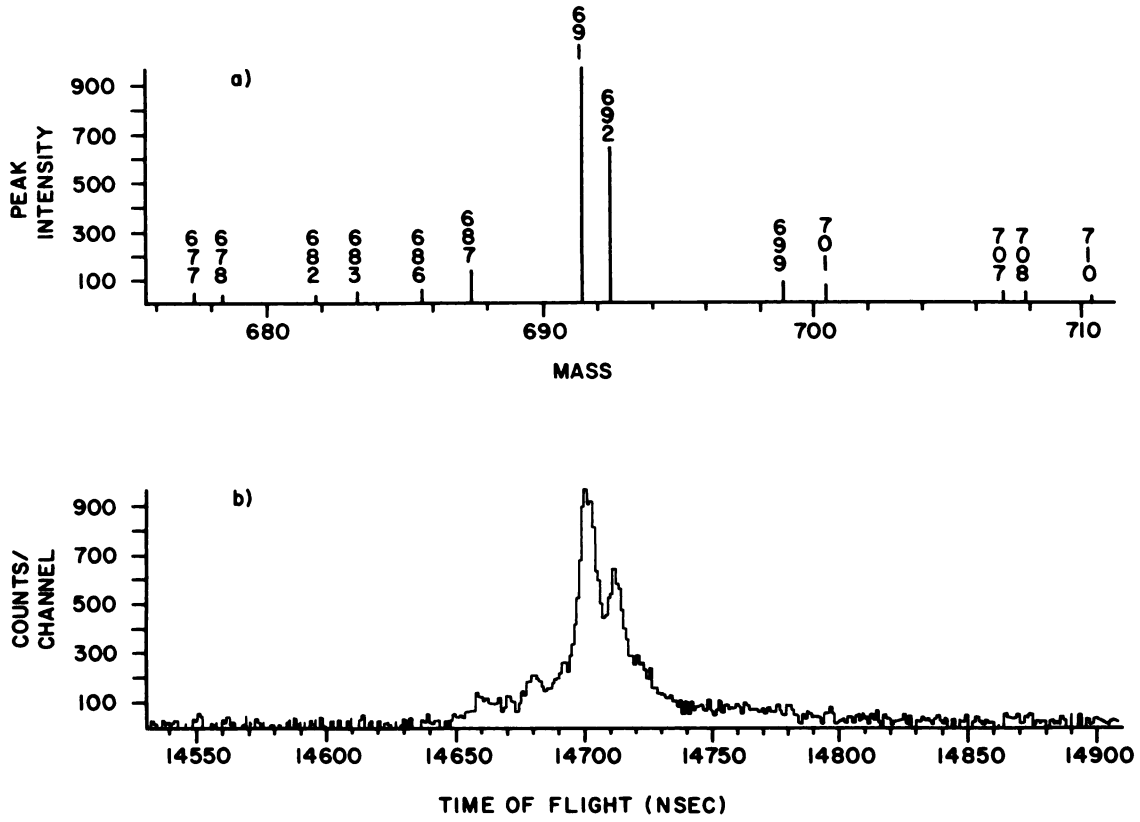

FIG. 6. *^{252}Cf plasma desorption-positive ion mass spectra of 1*

^{252}CfPDMS was performed as described under Materials and Methods.

a. The bar graph output from m/z 676 to m/z 710. The molecular weight of the $[M + Na]^+$ adduct containing one atom of ^{14}C in each arachidonic acid moiety is 691.

b. Enhanced view of the histogram of the m/z 691 to m/z 692 region of the spectrum.

more recently described compound, BW 755C, has been reported to be a potent anti-inflammatory agent (13). Moreover, this compound has been shown to be an inhibitor of both cyclooxygenase and lipoxygenase activities of various tissues (4, 13). The concentrations necessary for half-maximal stimulation of arachidonic acid incorporation into diglyceride (75 μM for aspirin, 8 μM for indomethacin, and 20 μM for BW 755C) appear to be similar to those previously reported for inhibition of cyclooxygenase in these same cells (4). Incubation of these cells with [1-^{14}C]palmitic acid gave rise to negligible incorporation of labeled fatty acid into a similarly chromatographing material. Moreover, anti-inflammatory drugs did not increase the incorporation of labeled palmitate into this material (data not shown).

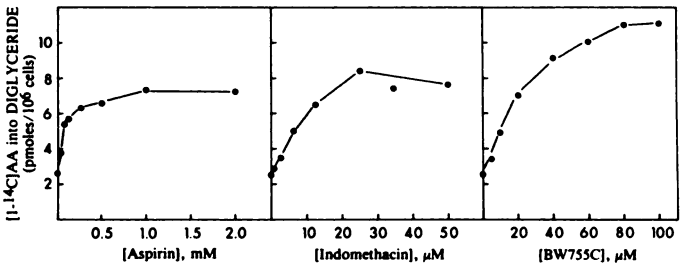

FIG. 7. *Effect of anti-inflammatory drugs on diglyceride product*

Neutrophils were assayed with 15 μM [1-^{14}C]arachidonic acid (*[1-^{14}C]AA*) for 1 min at 37° in the presence of various concentrations of aspirin, indomethacin, or BW 755C, and the products were analyzed as described in Fig. 1. These results are one of three replicate experiments, and values are ±10%.

DISCUSSION

The application of mass spectroscopic techniques has been critical in the identification of arachidonic acid metabolites (14). The analysis of one such arachidonic acid metabolite is described in this report.

One of the more interesting aspects of the identification of diarachidonyl diglyceride is the observation that the molecule appears to be formed exclusively from the exogenously supplied [1-^{14}C]arachidonic acid. The ^{14}C-labeled fatty acid purchased from Amersham and incubated with rat neutrophils contains more than 91% ^{14}C at position 1. After hydrolysis of the isolated diglyceride, the arachidonic acid methyl ester observed on GC/MS appears with a molecular ion at m/z 320 with a relative intensity of 1%. The m/z 318 cannot be distinguished from background. In addition, the ^{252}CfPDMS studies yielded ions consistent with a diglyceride containing only [^{14}C]arachidonic acid within the limits of detection. Therefore, the arachidonic acid supplied exogenously to these cells is not appreciably diluted with endogenous fatty acid during the apparently *de novo* synthesis of the diglyceride.

The amount of diarchidonyl diglyceride produced in these neutrophils appears to reach a steady state after 2 min (Fig. 1). This probably reflects the observed slow but

continuous production of [1-^{14}C]arachidonic acid-containing triglyceride. In fact, the labeled triglyceride accumulates linearly for at least 10 min (data not shown).

Anti-inflammatory drugs increase the incorporation of arachidonic acid into diglyceride (Fig. 7). Since this increase occurs at doses of these drugs which inhibit the ability of these cells to metabolize arachidonic acid via cyclooxygenase, the increased synthesis of diglyceride may be the result of increased substrate availability. Some of the effects of these drugs may be the result of their ability to increase the production of diarachidonyl diglyceride, a precursor for triglycerides and phospholipids. The formation of these unique molecules may perturb the membranes into which they are incorporated. For example, it has recently been reported (15) that a small amount of unsaturated diacylglycerol markedly increases the affinity of a cyclic nucleotide-independent protein kinase for Ca^{2+}, thereby stimulating its activity. In addition, an enzyme, protein kinase C, has been reported to require Ca^{2+} and phospholipids and/or diacylglycerol (16, 17). This enzyme may be involved in the regulation of arachidonic acid metabolism by phosphorylating lipomodulin, a regulator of phospholipases (18). Possibly even more important to the functioning of these neutrophils, the formation of this diarachidonyl diglyceride may greatly affect the composition of phosphatidic acid and the resultant phosphitidylinositol derived from it (19, 20).

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