Metabolism of Arachidonic Acid in Ionophore-stimulated Neutrophils

ESTERIFICATION OF A HYDROXYLATED METABOLITE INTO PHOSPHOLIPIDS

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ABSTRACT [^{14}C]Arachidonic acid incubated with human neutrophils was esterified into phospholipids and triglycerides. Stimulation of these labeled neutrophils with ionophore A23187 (2 μM) results in release of [^{14}C]arachidonate from phospholipid and its metabolism to prostaglandin E_2 and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), a lipoxygenase product. The released arachidonate is also metabolized to a polar lipid of unknown composition here disignated compound A. 5-HETE was found to be released into the medium and then taken up again by the cells. To determine its metabolic fate, [^{14}C]5-HETE was prepared biosynthetically, purified, and incubated with stimulated, unlabeled neutrophils. Most of the radioactivity entered the cells and was esterified into phospholipids and triglycerides. The radiolabeled complex lipids were saponified, and the released fatty acids cochromatographed with authentic 5-HETE. The esterification of 5-HETE, a hydroxylated fatty acid, into membrane phospholipids may be an example of a more generalized mechanism for altering membrane characteristics.

INTRODUCTION

Although the metabolism of arachidonic acid has been less thoroughly studied in neutrophils than in many other cell types, there is evidence that it is metabolized through both the cyclooxygenase and lipoxygenase pathways. Both prostaglandin E_2 (PGE_2)[1] and thromboxane B_2 (TXB_2) have been reported to be formed in animal and human neutrophils (1–3); 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), a presumed product of the lipoxygenase pathway, has been identified in rabbit neutrophils (4), but evidence that its formation can be modulated by physiologic and pharmacologic stimuli of neutrophil function has not been presented, and its functional role is presently undefined. In a recent abstract we reported (5) that several soluble or insoluble stimuli of oxidative metabolism in human neutrophils (A23187, the divalent cation ionophore, fluoride, zymosan particles) stimulated the conversion of radioactive arachidonic acid to a metabolite cochromatographing with 5-HETE in these cells. In this paper we characterize the response to the most effective of these 5-HETE generating agents, A23187. In addition, evidence is presented that newly synthesized 5-HETE is incorporated into phospholipids and triglycerides, as well as released into the medium.

METHODS

Sources of materials. [^{14}C]arachidonic acid, specific activity 50–60 mCi/mmol, Amersham Corp., Arlington Heights, Ill.; [^{3}H]arachidonic acid, specific activity 55 Ci/mmol, New England Nuclear, Boston, Mass. A23187, a gift of Dr. R. Hamill, Eli Lilly & Co., Indianapolis, Ind.; silica gel sil G-25 thin-layer chromatography (TLC) plates, Brinkmann

This work was presented in part to the Federation of American Societies for Experimental Biology, Atlanta, Ga., April 1978.

Received for publication 13 April 1979 and in revised form 2 July 1979.

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-*l*-hydroxy-5,8,10,14-eicosatetraenoic acid; MCM, mast cell medium, a balanced salt medium; PGE_2, prostaglandin E_2; TLC, thin-layer chromatography; TXB_2, thromboxane B_2.

Instruments, Inc., Westbury, N. Y.; bovine serum albumin (BSA), Sigma Chemical Co., St. Louis, Mo.

Purification of cells. Purified human neutrophils and platelets were prepared from heparinized blood by dextran sedimentation followed by isopycnic centrifugation using a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Piscataway, N. J. [6]). Blood donors had taken no medication for at least 2 wk. Neutrophil preparations were monitored by phase microscopy and Giemsa staining and contained <1 platelet/neutrophil. More than 98% of the nucleated cells were neutrophils. In selected experiments platelets were further depleted by defibrinating the blood (7). In this case the purified neutrophil preparation had a neutrophil to platelet ratio of 10:1 to 100:1. Defibrinated blood was used only in selected experiments because of the great loss of neutrophils when this technique is used. To prepare purified platelets, platelets were separated from nucleated cells during the routine Ficoll-Hypaque purification of lymphocytes by serial low-speed centrifugation (6). Platelet preparations contained <1 nucleated cell/100 platelets. Rabbit peritoneal neutrophils were prepared by the method of Borgeat et al. (4) from glycogen-injected animals. More than 95% of the recovered peritoneal cells were neutrophils.

Incubation conditions. With the exception of a portion of the experiments evaluating platelet contamination, all experiments were performed using cells labeled with radioactive arachidonic acid. Neutrophils (1×10^7/ml) were incubated in mast cell medium (MCM), a balanced salt medium, for 1 h in the presence of [1-^{14}C]arachidonic acid (1 μM) (1.1×10^5 cpm/ml). This medium has been used previously in our laboratory in studies of mast cells (8). It contains 0.9 mM Ca^{2+} and is buffered at pH 7.4 with 6.5 mM phosphate. Experiments were carried out with and without 0.1% BSA in the medium. The [1-^{14}C]arachidonic acid concentration used does not metabolically activate the cells as evidenced by failure to cause production of PGE_2, 5-HETE, or other arachidonate metabolites when incubated with unstimulated cells. It nonetheless provides adequate incorporation of ^{14}C radioactivity as evidenced by the fact that essentially all of the [1-^{14}C]arachidonic acid is incorporated into phospholipids and triglycerides, The labeled cells were washed twice with MCM to remove any free arachidonic acid from the medium. The cells were suspended at 1×10^7/ml in MCM (with or without 0.1% BSA as indicated) and incubated in the presence or absence of A23187 at 37°C. Platelets were labeled with [1-^{14}C]arachidonate in the same way except that 1×10^8/ml platelet suspension in MCM without calcium was used.

A23187 was dissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml. Dilutions of ionophore were made directly in MCM with rapid mixing to minimize the concentration of DMSO used with cells. This procedure results in a final concentration of DMSO of only 0.02% (vol/vol) in incubation mixtures containing 2 μM A23187. Effects of DMSO alone were evaluated, and no effect on arachidonic acid metabolism was observed. Cell viability was determined by trypan blue exclusion and by comparing the ability of A23187-treated and control cells to phagocytose serum-coated zymosan particles at a 20:1 particle to cell ratio over various time periods. More than 95% of neutrophils remained viable under all experimental conditions, with no detectable change in viability from control cells.

Extraction of lipid and TLC. After incubation with A23187, the reaction was terminated either by adding cold ethanol directly to cell suspensions or by centrifuging the incubation mixture in a Beckman Microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for a few seconds to separate the cells from the medium before adding ethanol. To extract the lipids, 4 vol of cold ethanol were added to the cell suspension, supernate, or pellet (4). The mixture was kept at 4°C for 20 min and then centrifuged for 30 min at 41,000 *g* to remove precipitated material. The supernate containing the extracted lipids was dried under vacuum, redissolved in methanol, applied to TLC plates, and developed in the following solvent systems:

I. Benzene:diethylether:ethanol:glacial acetic acid (50:40:2:0:2) 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) R_f = 0.57, 5-HETE R_f = 0.45 (9).

II. Benzene:dioxane:glacial acetic acid (60:30:3) PGE_2 R_f = 0.39, TXB_2 R_f = 0.52, 5-HETE, and 12-HETE R_f = 0.90 (10).

III. Organic phase of ethyl acetate:glacial acetic acid:2,2,4 trimethylpentane:water (45:10:25:50) 12-HETE R_f = 0.75, 5-HETE R_f = 0.63 (11).

IV. Organic phase of ethyl acetate:2,2,4 trimethylpentane:water (75:150:150) 5-HETE methyl ester R_f = 0.29, 12-HETE methyl ester R_f = 0.38 (4).

After drying, areas of radioactivity in the TLC plates were detected by autoradiography. Bands were identified by comparison with known chromatographic standards. Standards for TXB_2, PGE_2, and 6-ketoprostaglandin $F_{1\alpha}$ were a gift from Dr. J. Pike, Upjohn Co., Kalamazoo, Mich. 12-HETE standard was prepared from human platelets by the method of Hamberg (12). 5-HETE standard prepared from rabbit peritoneal neutrophils comigrated with an apparently identical product in human neutrophil in solvent systems I, II, and III. Methyl esters of the rabbit and human 5-HETE were prepared with diazomethane; the methyl esters comigrated in solvent system IV. 5-HETE was further identified by gas-liquid chromatography—mass spectroscopy of the trimethylsilyl ether of the methyl ester. Analysis was done on a Finnegan 300 (Finnegan Corp., Sunnyvale, Calif.) system using an OV-101 column. Prominent peaks were found at methyl ester 255, 305, and 406, corresponding to the known fragmentation pattern of the trimethylsilyl ether derivative of 5-HETE methyl ester (4). The help of Dr. Michael Hoffman of the Department of Biochemistry and Mass Spectroscopy Center at Washington University School of Medicine in these studies is gratefully acknowledged. The mass spectrum obtained was identical to that reported by Borgeat (4). Triglycerides were further separated from other neutral lipids by TLC in another solvent system (hexane:ether:glacial acetic acid, 30:70:1) (13). In this system, radiolabeled triglycerides comigrated with a triglyceride standard at an R_f of 0.63.

After identification, TLC areas containing radioactivity were scraped and eluted. Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) was added, and the radioactivity was determined in a Searle 6880 liquid scintillation counter (Searle Radiographics, Inc., Des Plaines, Ill.).

Incubation studies with purified 5-HETE. Radioactive 5-HETE was prepared by incubating human neutrophils with [1-^{14}C]arachidonate in MCM in the presence of A23187 (2 μM) for 15 min. The incubation medium was extracted as described above, and the lipids were separated by TLC in solvent system III. The labeled 5-HETE band was again identified by autoradiography, scraped, and eluted. The purified 5-HETE recovered contained a minimal quantity of 0.6 ng and an overall recovery of 4.1% of the radioactivity in the [^{14}C]arachidonic acid present in the original incubation mixture with cells. The preparation was radiochemically homogeneous as indicated by TLC and high-pressure liquid chromatography. High-pressure liquid chromatography was performed on a Varian model 8550 instrument (Varian Associates, Palo Alto, Calif.) using a Knauer-Unimetrics C-18 column (Unimetrics Corp., Anaheim, Calif.) and a linear water/acetonitrile gradient (solvent A [water with 0.1% glacial acetic acid]; solvent B

[acetonitrile with 0.2% benzene]). 5-HETE, 12-HETE, and arachidonic acid eluted at 71, 76, and 80% acetonitrile, respectively, in this system.

Purified [1-^{14}C]5-HETE, 5×10^5 cpm/ml (a minimal concentration of 0.3 μM based on the specific activity of the [^{14}C]arachidonic acid used as a precursor and neglecting dilution by endogenous, unlabeled arachidonic acid) was incubated with 6×10^7 neutrophils in 6 ml of MCM in the presence of 2 μM A23187 at 37°C for 30 min. After centrifugation of the incubation mixtures in a Beckman Microfuge for 10 s, the supernate and cell pellet were separated and extracted by the usual method. The lipids recovered were then chromatographed in solvent system I and in a two-dimensional TLC system (14) to separate the individual phospholipid classes (first dimension—chloroform:methanol:ammonium hydroxide:water [65:35:2.5:2.5]; second dimension—chloroform:acetone:methanol:acetic acid:water [50:22:9:10:5]). The radiolabeled products were identified by autoradiography and compared with unlabeled standards visualized with I_2 vapor. To determine whether the radiolabel in phospholipid and triglycerides was esterified 5-HETE, the phospholipid and triglyceride bands were scraped and eluted, and the lipids recovered were subjected to transesterification in anhydrous methanol containing 0.2 N NaOH (15). The liberated fatty acid methyl esters were then chromatographed using solvent system IV and compared with known standards.

RESULTS

Labeling of cells. The effects of A23187 on the metabolism of arachidonic acid were studied in previously labeled neutrophils. To obtain labeled cells, neutrophils were incubated with [1-^{14}C]arachidonate (1 μM) for 1 h and then washed repeatedly with medium. In a representative experiment, extraction and chromatography of these cells showed that 57% of the incorporated radiolabel was in triglyceride and 43% was in phospholipid. In this and other experiments, essentially none of the radiolabel remained as free arachidonate or was metabolized to 5-HETE or the other metabolites produced when A23187 was present. To demonstrate that the radiolabel in phospholipid and triglyceride was in fact esterified arachidonate, the phospholipid and triglyceride bands were scraped, eluted, and subjected to transesterification (15). Virtually all of the released radiolabeled fatty acid chromatographed as fatty acid methyl esters in several chromatographic systems, and therefore presumably represents free arachidonic acid.

Characterization of metabolites in neutrophils stimulated with A23187. In prelabeled neutrophils prepared in the usual way and therefore significantly contaminated with platelets, A23187 (2 μM, 15 min at 37°C) stimulated formation of at least five known metabolites of arachidonic acid not seen in its absence. The identification of these products was based on their identical behavior to known chromatographic standards in the four solvent systems described above. These metabolites were determined to be 5-HETE, 12-HETE, TXB_2, PGE_2, and 12-L-hydroxy-5,8,10-heptadecatrienoic acid. In addition, an unknown metabolite was formed which we designate as compound A.[2] Compound A is chromatographically easily distinguishable from 5-HETE, PGE_2, TBX_2, prostaglandin D_2, prostaglandin $F_{2\alpha}$, 12-HETE, 12-L-hydroxy-5,8,10-heptadecatrienoic acid, and 6-ketoprostaglandin $F_{1\alpha}$. It is more polar than 5-HETE in each of the four solvent systems, migrating, with an R_f of 0.15 in I, 0.67 in II, 0.5 in III, and 0.09 in IV (after treatment with diazomethane).

Assessment of the role of platelet contamination. Because a number of these products are formed in platelets, and because platelets metabolize arachidonic acid rapidly, the role of platelet contamination of our neutrophil preparations was assessed. Isolated platelets, ordinary neutrophil preparations, and neutrophils purified further by defibrination containing as few as 1 platelet/100 neutrophils were studied in the presence and absence of A23187 (Table I). Because of differences in prelabeling efficiency between platelets and neutrophils, most of these experiments involved unlabeled cells incubated simultaneously with exogenous [^{14}C]-arachidonic acid and either buffer (with DMSO) or A23187. However, in three experiments with prelabeled platelets and neutrophils, much the same results were obtained. Similar conclusions were drawn from studies with defined neutrophil/platelet mixtures prepared by adding various numbers of platelets back to platelet-poor neutrophils. Although both platelets and neutrophils make PGE_2, it appeared that under our ordinary labeling conditions (with neutrophils prepared in the usual way) >90% of the labeled PGE_2 was from neutrophils. All of the 5-HETE and compound A synthesis could be attributed to neutrophils. Neutrophils also appeared to make very small quantities of TXB_2 (and therefore presumably thromboxane A_2), agreeing with a recent report (3). Even at relatively low platelet:neutrophil ratios, however, most of the TXB_2 probably comes from the platelets. Because TXB_2 is such a quantitatively minor arachidonate metabolite in human neutrophils, and because of possible problems in interpretation in attempting to distinguish platelet-derived TXB_2, we have not included it in the tubular presentation of our data.

Further characterization of the A23187 response in the presence and absence of BSA. Because BSA binds fatty acids with relatively high affinity, it serves as a trap to diminish the reuptake of released arachidonic

[2] After this manuscript was submitted for publication, Borgeat and Samuelsson (1979. *J. Biol. Chem.* **254**: 2643–2646.) reported the metabolism of arachidonic acid to 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid in rabbit peritoneal neutrophils. Compound A, reported in this paper, has the same ultraviolet absorption spectrum and the same migration characteristics on thin-layer and reverse-phase, high-pressure liquid chromatography as does 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid. This evidence strongly suggests that compound A is 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid.

TABLE I
Percentage of Total Radioactivity Recovered

	Platelets	Typical neutrophil preparations	Neutrophils from Defribrinated blood
Overall cell composition			
Platelets, *ml*	1×10^8	1×10^7	1×10^6
Neutrophils, *ml*	1×10^6	1×10^7	1×10^7
Products formed			
5-HETE	0.1±0.05	6.8±0.46	7.0±0.57
12-HETE	48.4±2.31	4.7±0.49	0.5±0.10
Compound A	0.1±0.05	3.6±0.39	3.2±0.42
PGE_2	0.5±0.11	4.5±0.58	4.0±0.44
TXB_2	19.6±1.72	2.5±0.33	0.8±0.16
HHT*	12.1±0.11	1.0±0.20	0.1±0.05

Percent of total recovered radiolabel in arachidonate metabolites in different cell preparations. Cells were prepared as described in Methods. Neutrophils (1×10^7/ml) or platelets (1×10^8/ml) were suspended in MCM with 1 mg/ml BSA and incubated 15 min at 37°C with A23187 (2 μM) and [1-^{14}C]arachidonate (1 μM). Extraction, chromatography, and product identification are as in Methods. Solvent systems I and II were used. The radioactivity not accounted for in the table was in phospholipid, triglycerides, or free arachidonate. 80% of added radioactivity was recovered. Mean of three experiments±SEM.
* HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid.

acid and certain of its metabolites (see below) by cells. For this reason, results obtained in the presence and absence of albumin will be discussed separately. Fig. 1A and B show the time-course for the fate of endogenous [1-^{14}C]arachidonate during stimulation with A23187 (2 μM) in MCM with 0.1% BSA. In the cell pellet (Fig. 1A), the percent of the total radiolabel in triglycerides declined very slightly during stimulation, whereas the percent in phospholipid declined markedly, indicating release of free arachidonate. The released arachidonate diffused into the medium and was trapped or was metabolized intracellularly to metabolites which subsequently entered the medium.

After 30 min of stimulation, the supernates contained ≅11% of the original radiolabel as arachidonate, 6% as PGE_2, 3% as 5-HETE, and 1.4% as compound A (Fig. 1B). All of these products accumulated rapidly for the first 5 min and more slowly thereafter.

When there was no albumin in the medium, significant amounts of arachidonate, 5-HETE, and compound A were apparently associated with the cells (Fig. 1C). On the other hand, all of the PGE_2 was released into the medium whether albumin was present or not (Fig. 1D). With no albumin in the medium, the levels of 5-HETE in the supernate rose for 2 min and then fell sharply. By contrast, when albumin was present in the medium, the levels of 5-HETE rose for 15 min and then leveled off. Despite the decline in 5-HETE in the albumin-free medium, there was no corresponding rise in the level of intracellular 5-HETE. However, as is shown below, the fall in 5-HETE can be explained by its subsequent incorporation into intracellular lipids.

Without albumin in the medium, the percent of total radiolabel in phospholipid remained higher during stimulation than when albumin was present, presumably because the released fatty acid was more readily available for reincorporation into cellular lipids (see below).

Dose-response curves for the stimulation of the incorporation of radiolabel into 5-HETE by A23187 are shown in Fig. 2. As the level of radioactivity in 5-HETE reached a peak at 2 min in the absence of BSA and at 15 min in the presence of BSA, these incubation times are shown. The two curves were almost identical, with maximal stimulation at 2 μM A23187. The ionophore dose-response curves for stimulation of compound A and PGE_2 formation were almost identical to the curve for 5-HETE (not shown) as might be expected if the rate-limiting step for all three reactions is the availability of free arachidonate. Under physiologic conditions, the level of intracellular and extracellular nonesterified arachidonic acid is extremely low, and the rate-limiting step in the synthesis of prostaglandins and other metabolites is generally thought to be the release of arachidonic acid from membrane lipids (16). In accordance with this postulated mechanism, a similar dose-response curve was seen for release of arachidonate radioactivity from cellular phospholipid (Fig. 2).

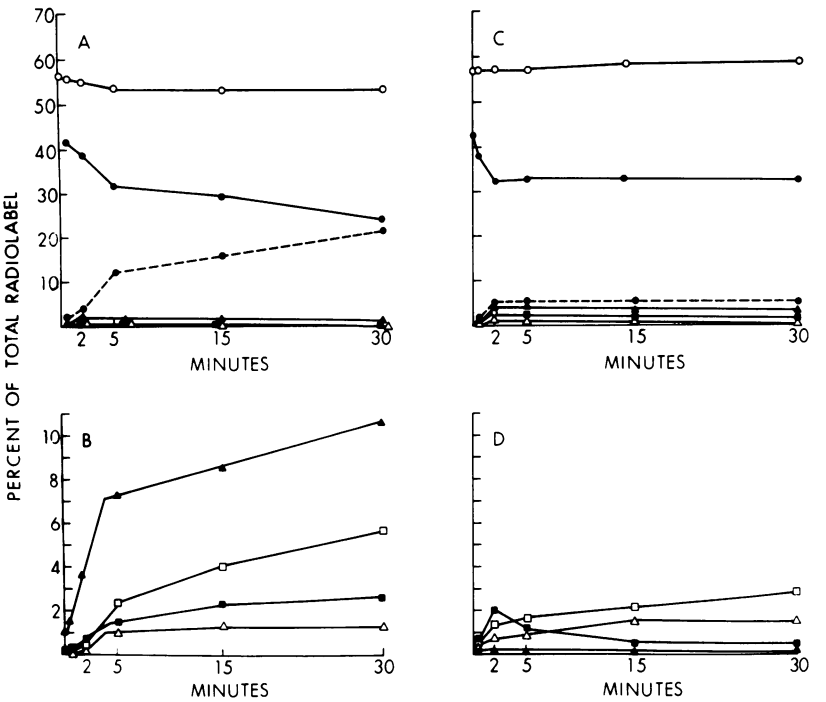

FIGURE 1 Time-course for the fate of radiolabeled "endogenous" arachidonic acid in neutrophils stimulated with A23187. Incubations were done in MCM with and without 1 mg/ml BSA. Neutrophils were incubated with [1-^{14}C]arachidonic acid for 1 h (see Materials and Methods), washed twice with MCM, and suspended at 1×10^7 cells/ml in MCM with and without BSA. The neutrophils were incubated at 37°C in the presence of A23187 (2 μM) for varying periods as shown in the figure. The cells and media were separated by 10-s centrifugation in a Beckman Microfuge. The cell pellets and the supernates were extracted and chromatographed separately. The data presented are the mean of five experiments. In each case the SEM is <10% of the mean. (A) Chromatographic analysis of the radiolabeled compounds found in the cell pellets. Incubation in MCM with BSA. Results are expressed as percent of total recovered radioactivity in cells plus medium. For comparison, total counts in the supernate are also shown. (B) Same experiment as A showing chromatographic analysis of radiolabeled compounds found in the supernate. (C and D) The same as A and B except that the medium contains no BSA. ●, phospholipid; ○, triglyceride; □, PGE_2; ■, 5-HETE; △, compound A; ▲, arachidonic acid; – – –, total counts in supernate.

Fig. 3 depicts the effect of calcium in the medium on release of arachidonate from phospholipid and on 5-HETE production. With no calcium in the medium, there was no release of arachidonate from phospholipid, and no 5-HETE was produced. 5-HETE production rose rapidly with increases in Ca^{2+} concentration up to 0.5 mM Ca^{2+} and then leveled off. The Ca^{2+} concentration for arachidonic acid release was very similar, although not identical, to a maximal response at 1 mM Ca^{2+}. In the absence of A23187, use of Ca^{2+} concentrations as high as 1.5 mM had no effect on either process.

Metabolism of exogenous 5-HETE by neutrophils. In the experiments shown in Fig. 1B and D, different time-courses for 5-HETE accumulation were seen. One possible explanation is that albumin traps 5-HETE just as it does arachidonic acid, and that when no albumin is present, 5-HETE reenters the cell and is metabolized. To investigate this possibility, purified [1-^{14}C]5-HETE was incubated with neutrophils in albumin-free medium, with and without A23187. The cells and medium were extracted and chromatographed separately (Table II). Most of the [1-^{14}C]5-HETE was taken up by the cells both in the presence and absence of A23187. In the unstimulated cells most of the label comigrated with triglycerides; in stimulated cells most comigrated with phospholipids. Although the level of available radioactivity in phosphatidic acid was substantially lower, incorporation of radioactivity into phosphatidic acid could also be demonstrated.

To determine whether the radiolabel in triglycerides and phospholipids represented esterified 5-HETE itself or a metabolite of 5-HETE such as L-6,8,11,14-eicosatetraenoic acid (which would be formed if the hy-

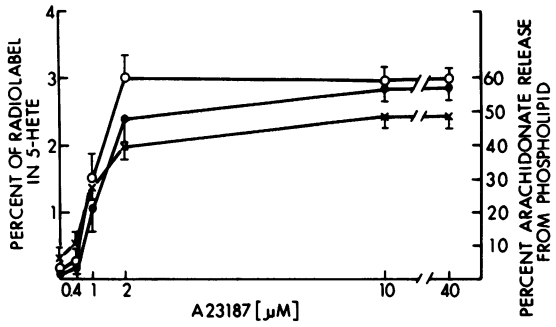

FIGURE 2 Dose-response curve for A23187 stimulation of incorporation of radiolabel in 5-HETE using "endogenous" arachidonic acid. Incubation conditions as in Fig. 1A and B. ×, percent of phospholipid-esterified [1-^{14}C]arachidonate released after incubation for 15 min, calculated as sum of radiolabel in free arachidonate and metabolites/sum of radiolabel in phospholipids plus free arachidonate plus arachidonate metabolites. ●, incorporation of radiolabel into 5-HETE with BSA in the medium, incubation 15 min. ○, incorporation of radiolabel in 5-HETE without BSA in the medium, incubation 2 min. 5-HETE represented here is the sum of that in the cell pellet and that in the medium. Mean of three experiments, ±SEM.

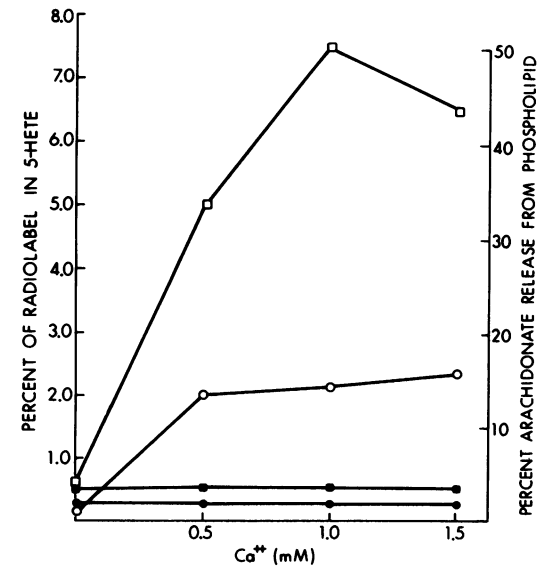

FIGURE 3 Effects of calcium on A23187-stimulated release of [1-^{14}C]arachidonate from phospholipid and on A23187-stimulated incorporation of radiolabel into 5-HETE. Neutrophils (1 × 10^7/ml) that had been prelabeled with [1-^{14}C]arachidonate were incubated at 37°C for 15 min in the presence and absence of arachidonate (2 μM) in MCM without calcium or BSA; enough 50 mM $CaCl_2$ was added to give the calcium concentrations shown in the figure. □, percent of phospholipid-esterified [1-^{14}C]arachidonate released after incubation with A23187 (2 μM); ■, percent of phospholipid esterified [1-^{14}C]-arachidonate released after incubation in the absence of A23187; ○, percent of radiolabel in 5-HETE after incubation with A23187 (2 μM); ●, percent of radiolabel in 5-HETE after incubation in the absence of A23187. Data are expressed as the mean of three experiments; SEM is <10% of the mean for all conditions.

TABLE II
Metabolism of Exogenous [1-^{14}C]5-HETE by Neutrophils

Product	Percentage of total recovered radioactivity: Resting neutrophils	Percentage of total recovered radioactivity: Neutrophils stimulated with A23187 (2 μM)
Cells		
Phosphatidylinositol	7.82	3.3
Phosphatidylcholine	7.37	17.8
Phosphatidylethanolamine	4.25	8.0
Phosphatidic acid	0.85	4.2
5-HETE	2.1	5.0
Triglycerides	62.6	40.3
Unknown	3.4	7.0
Supernate		
5-HETE	3.4	7.0
Unknown	9.6	9.8

Purified [1-^{14}C]5-HETE (0.3 μM) was incubated with neutrophils (1 × 10^7/ml) in the presence and absence of A23187 (2 μM) in a final volume of 6 ml MCM without BSA for 15 min at 37°C. The incubation mixture was centrifuged in a Beckman Microfuge for 10 s. The cells and supernate were extracted separately in 80% cold ethanol and chromatographed in solvent system I and in the two-dimensional TLC system. The TLC plates were autoradiographed and the radiolabeled bands identified using known standards. Results are expressed as percent of total recovered radioactivity; 85% of the radioactivity added to the medium was recovered. Mean of two similar experiments.

droxyl group were enzymatically removed from 5-HETE without steric rearrangement), the triglycerides and phospholipids were isolated, transesterified, and the products rechromatographed in solvent system IV. In each case the transesterification was at least 80% complete, and 95% of the released radiolabel rechromatographed with 5-HETE in striking contrast to the transesterified lipids from cells prelabeled with [^{14}C]arachidonic acid. Thus, lipid recovered by transesterification apparently retains its original 5-hydroxyl group, suggesting strongly that 5-HETE is being incorporated as such into cellular lipids. However, because the steric configuration of the double bonds in the recovered lipid has not yet been evaluated, the possibility of isomerization cannot be excluded.

Cytotoxicity studies. To evaluate the possible role of cytotoxicity in the response of neutrophils to A23187, the cells were exposed to 0.4, 1, 2, and 10 μM A23187 for 10 min at 37°C in the presence and absence of BSA (the same conditions as used for the experiments in Fig. 3; see above). Under all conditions 98% of the cells continued to exclude trypan blue (the same percent as in the controls), and phagocytosis of zymosan particles was not altered.

DISCUSSION

This study demonstrates that when labeled human neutrophils are stimulated with A23187, they transform arachidonic acid into 5-HETE, a hydroxylated fatty acid produced by neutrophils but not by platelets. Transformation of arachidonate into PGE_2 and a relatively polar lipid of unknown composition, here designated compound A, is also observed. 5-HETE was originally reported by Borgeat et al. (4) to be a major arachidonate metabolite in rabbit peritoneal neutrophils. These cells were obtained from the peritoneal cavity of rabbits injected in the same site with glycogen to promote the accumulation of a cellular exudate. Even though we also were able to demonstrate the conversion of labeled arachidonic acid to 5-HETE in rabbit neutrophils (also obtained using the glycogen procedure), 5-HETE was not labeled to a significant extent by resting human peripheral blood neutrophils under identical incubation conditions. Moreover, no labeling occurred during the 1-h preincubation with [^{14}C]arachidonic acid, which was routinely used in labeling the cells. By contrast, in the presence of 2 μM A23187 4% of the original radioactivity was released as 5-HETE over a period of 15 min (Fig. 1B). Thus, even though both rabbit and human neutrophils make 5-HETE, human neutrophils only express this capability when an exogenous stimulant such as A23187 is present. Although variations between species might account for these differences, it seems more likely that the 5-HETE production by rabbit neutrophils is a reflection of the in vivo stimulation by glycogen, and that, as in human neutrophils, 5-HETE is not normally produced unless the cells have first been metabolically activated.

In considering the possible mechanism of the A23187 stimulation, it is noteworthy that A23187 has a similar dose-response curve for stimulation of 5-HETE, PGE_2, free arachidonate, and compound A formation, and that all of these responses are Ca^{2+} dependent. Taken together with the rapid fall in radioactivity in phospholipids after A23187 is added, this suggests that A23187 is exerting its primary action by releasing arachidonic acid esterified to phospholipids. The availability of free arachidonate is normally regarded as the rate-limiting step in prostaglandin and hydroxylated fatty acid biosynthesis (15). There is an apparent parallel with recent results in platelets prelabeled with arachidonic acid in which the divalent cation ionophore A23187 also releases arachidonic acid from phospholipid, and in which thromboxane and 12-HETE labeling are concurrently increased (17).

Although the significance of the increases in arachidonate metabolism is not clear, it should be noted that A23187 mimics many of the effects of physiologic stimuli such as serum-coated zymosan particles in human neutrophils. Both ionophore and zymosan particles activate the respiratory burst, producing increases in oxygen consumption and hexose monophosphate shunt activity (18, 19). Moreover, work in progress indicates that zymosan particles produce effects apparently identical to those of A23187 on arachidonate metabolism in these cells with increased labeling of 5-HETE, PGE_2, and compound A (5) (manuscript in preparation), and Zurier has previously reported that PGE_2 synthesis is stimulated in human neutrophils by zymosan (1). Because the mechanism by which the respiratory burst is activated is not known, it may be significant that 5-hydroperoxy-eicosatetraenoic acid, the presumed intermediate in the conversion of arachidonic acid to 5-HETE by the lipoxygenase, is an alkyl hydroperoxide (4). Even though 5-hydroperoxy-arachidonic acid has not yet been directly demonstrated in plant or animal cells, all of the lipoxygenases that have been carefully studied to date produce hydroperoxy fatty acids as their primary products (20), and the presence of the corresponding hydroxy fatty acids is indicative of further metabolic conversion. Long chain alkyl peroxides are known substrates for glutathione peroxidase, an enzyme reported to be present in both animal and human neutrophils (21, 22). The availability of 5-hydroperoxy-arachidonic acid provides another possible substrate for this enzyme and could account for part of the increase in shunt activity. These findings in regard to lipoxygenase activation may also have significance for slow-reacting substance and lysosomal enzyme release and chemiluminescence in activated neutrophils.

The incorporation of 5-HETE into cell membranes was an unexpected finding. It appears that a series of metabolic conversions is occurring in which: (*a*) arachidonic acid and other fatty acids are released from phospholipid, presumably at least in part by activation of phospholipase A_2; (*b*) arachidonic acid is converted to 5-HETE by lipoxygenase; (*c*) 5-HETE is incorporated into membrane phospholipids and triglycerides (Fig. 4). In this connection Smolen and Shohet (23) reported 5 yr ago that neutrophil phagocytic vesicles contained much less arachidonic acid in their phospholipids than either the lysosomes or plasma membranes from which they were presumably derived. At the time of their study, 5-HETE had not yet been described. Substantial amounts of triglyceride and phosphatidic acid are also labeled in A23187-stimulated leukocytes, and the amounts of label are considerably higher in phosphatidylethanolamine and phosphatidylcholine than in phosphatidylinoistol. This suggests that most of the incorporation involves more than a simple exchange of fatty acid molecules in phospholipids, and that at least some *de novo* synthesis of phosphatidic acid and modification of head groups is occurring. Because the incorporation of 5-HETE into lipid is much greater when albumin is absent from the medium, its physiologic

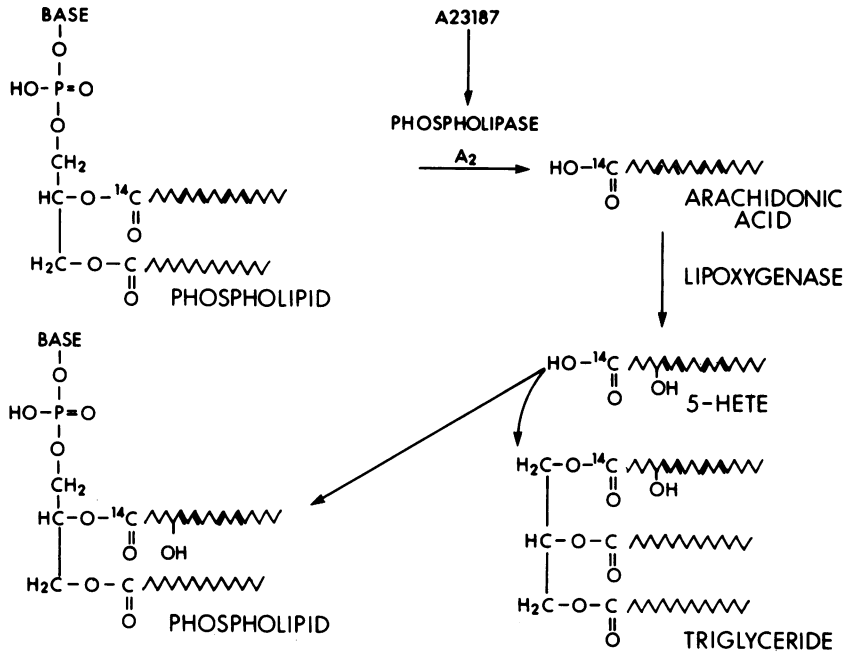

FIGURE 4 Scheme for release of arachidonate from phospholipid by phospholipase A_2; conversion of arachidonate to 5-HETE by lipoxygenase and esterification of 5-HETE into phospholipid and triglyceride.

significance is uncertain. Nonetheless, it seems likely that membranes containing substantial amounts of 5-HETE would be altered in their functional properties, and further study is indicated.

ACKNOWLEDGMENTS

The technical assistance of Ms. Lisa Goldfien is gratefully acknowledged.

Dr. Stenson is supported by training grant 1 T32 AI 00112 SRC from the National Institutes of Health.

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