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Biochemistry

Arachidonic acid metabolism in polymorphonuclear leukocytes*: Effects of ionophore A23187

(monohydroxy acids/dihydroxy acid/prostaglandins/human neutrophils)

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ABSTRACT **Addition of arachidonic acid and the divalent cation ionophore A23187 to a suspension of human peripheral blood polymorphonuclear leukocytes led to the formation of (5*S*)-hydroxy-6,8,11,14-icosatetraenoic acid, (15*S*)-hydroxy-5,8,11,13-icosatetraenoic acid, and (5*S*,12*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid. A method based on high-pressure liquid chromatography has been developed for assay of these metabolites. The addition of arachidonic acid to human polymorphonuclear leukocytes always resulted in formation of the isomeric monohydroxy acids. However, cells prepared from blood of different subjects were found to vary with respect to formation of the 5,12-dihydroxy acid. Addition of the ionophore alone strongly stimulated the formation of the 5-monohydroxy acid and more specifically the 5,12-dihydroxy acid from endogenous arachidonic acid. In all experiments performed the formation of the 5-hydroxy acid and the 5,12-dihydroxy acid was maximally stimulated when both arachidonic acid and the ionophore were added to the incubation mixture. Under these conditions, stimulation of 40-fold or more of the formation of both compounds was observed. The data demonstrate that, in addition to causing release of endogenous substrate, the ionophore also activated the enzymatic system involved in the further transformations of arachidonic acid. This finding raises the possibility that this pathway of arachidonic acid metabolism is involved in the biological response (e.g., release of lysosomal enzymes, the slow reacting substance of anaphylaxis, and chemotactic factors) of leukocytes to A23187 and other stimuli.**

Recent studies have indicated that polyunsaturated fatty acids and their metabolites are involved in the functions of leukocytes (1, 2). It was thus reported that two hydroxy acids formed from arachidonic acid—namely, 12-hydroxy-5,8,10,14-icosatetraenoic acid and 12-hydroxy-5,8,10-heptadecatrienoic acid—induced chemotaxis by polymorphonuclear leukocytes (PMNL) (3–5). It was also recently shown that unsaturated fatty acid peroxides, such as arachidonic acid peroxides, stimulate platelet guanyl cyclase activity (6, 7). This last finding might be particularly significant in view of the effects of 3′,5′-guanosine monophosphate on lysosomal enzyme release (8, 9) and leukocyte locomotion (10–12). Several studies have also demonstrated a stimulatory effect of ionophore A23187 on the formation of thromboxane B_2 (13, 14) and prostaglandins (13–15) in leukocytes. It is of interest in this context that ionophore A23187 has also been found to stimulate some phagocytosis-associated responses of leukocytes—i.e., oxidative metabolism (16, 17), chemiluminescence (14), and secretion of lysosomal enzymes (18, 19).

We have recently demonstrated the formation of two metabolites of arachidonic acid in rabbit peritoneal PMNL—namely, (5*S*)-hydroxy-6,8,11,14-icosatetraenoic acid (20) and (5*S*.12*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid (21). It was therefore of interest to extend our studies to the metabolism of arachidonic acid in human PMNL. In addition, the effect of ionophore A23187 on the formation of the metabolites has been studied. Our findings indicate that these arachidonic acid metabolites might be important in leukocyte functions.

MATERIALS AND METHODS

The materials used were described (20, 21). Ionophore A23187 was a gift from R. L. Hamill (Eli Lilly). Prostaglandin B_2 was a gift from John Pike (Upjohn). The menthoxycarbonyl methyl esters of the 2-hydroxy acids were prepared as described (22).

Suspensions of Human Polymorphonuclear Leukocytes. Suspensions of human PMNL were prepared as described (23). Red cells and platelets were virtually eliminated by NH_4Cl lysis (24) and centrifugation over Lymphoprep (Lymphoprep, Nyegaard and Co., Oslo, Norway), respectively. The cell pellets were suspended in Dulbecco's phosphate-buffered saline (25) (35×10^6 cells/ml). Observations on wet chamber preparations and smears (Giemsa stain) showed that PMNL accounted for more than 95% of the total leukocyte content of the purified preparation. Between 300 and 900×10^6 cells were obtained from 500 ml of blood. The viability of the cells as measured by the trypan blue exclusion test (26) was greater than 95%.

Incubation and Extraction Procedures. The cells were incubated in phosphate-buffered saline (0.87 mM $CaCl_2$) with [1-^{14}C]arachidonic acid [4000 dpm/μg (1 dpm = 16.7 mBq)] as indicated in the legend to Fig. 1. Arachidonic acid and ionophore A23187 were added to the cells in solution in ethanol (the final concentration of ethanol in the incubation buffer was 0.7%). The incubations were performed at 37°C for 4 min under a normal atmosphere and terminated by addition of 1.5 vol of methanol. Extractions were performed as described (20).

Column Chromatography. Silicic acid column chromatography was performed in 5-mm-diameter glass columns packed with 1 g of silicic acid (Mallinckrodt, Silicar CC-4, not further activated) as follows: The dry residue from ether extraction was dissolved in 0.5 ml of diethyl ether/hexane, 20:80 (vol/vol), and applied to the column. Two fractions of the sample were collected by using 30 ml of the two following solvents in order: (*i*) diethyl ether/hexane, 20:80 (vol/vol) eluting unreacted arachidonic acid and (*ii*) ethyl acetate, eluting mono- and dihydroxy acids.

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Abbreviations: PMNL, polymorphonuclear leukocytes; RP-HPLC, reversed-phase, high-pressure liquid chromatography; Me_3Si, trimethyl silyl.

* This is the third paper on this subject. The first and second papers are refs. 20 and 21, respectively.

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Reversed-Phase High-Pressure Liquid Chromatography (RP-HPLC). RP-HPLC was performed on a column (4.6 × 250 mm) packed with Nucleosil C_{18} (5 μm particles, purchased from Macherey Nagel Co., Düran, Germany). A variable wavelength, ultraviolet-visible spectrophotometer was used as detector. The solvent used was a mixture of methanol/water, 75:25 (vol/vol), plus acetic acid to 0.01%. The analyses were performed at a solvent flow of 1 ml/min [1100 pounds per square inch (psi) (1 psi = 6895 Pa)] and 25°C. Samples eluted in the ethyl acetate fraction of the silicic acid column chromatography (see above) were dissolved in 20 μl of methanol and directly injected (as free acids), without further purification or derivatization, into the chromatograph in 5-μl aliquots. The elution of prostaglandin B_2 and of the 5,12-dihydroxy acid (compound IV) was monitored at 280 nm during the initial 20–22 min, whereas the elution of monohydroxy acids (compounds I, II, and III) during the following 15 min was monitored at 232 nm. Prostaglandin B_2 was added to samples (in the methanol used to terminate incubations) prior to any purification procedure, as an internal standard. The amount of each metabolite was determined as follows: The surface areas of the peaks on the chromatograms were calculated and compared to the prostaglandin B_2 internal standard after correction for differences in attenuation settings and absorbtion coefficients (ϵ) of the different compounds. The ϵ values used for calculations were, respectively, 30,500, 39,500, and 26,800 for the monohydroxy acids, the 5,12-dihydroxy acid, and prostaglandin B_2 [these are ϵ values reported, respectively, for methyl 5-hydroxy-6,8,11,14-icosatetraenoate at $\lambda^{MeOH}_{235\ nm}$ (20) or methyl 12-hydroxy-5,8,10,14-icosatetraenoate at $\lambda^{EtOH}_{237\ nm}$ (27), for methyl 5,12-dihydroxy-6,8,10,14-icosatetraenoate at $\lambda^{MeOH}_{281\ nm}$ (21), and for prostaglandin B_2 at $\lambda^{EtOH}_{278\ nm}$ (28)].

Catalytic Hydrogenation and Gas Chromatography–Mass Spectrometry. The procedures were as described (21).

Steric Analysis of Alcohol Groups. Samples of compounds I, II, III, and IV for steric analysis of alcohols were prepared as follows: The ether extract of the incubation mixture of 1×10^9 human PMNL with arachidonic acid was fractionated by silicic acid column chromatography as described above except that silicic acid (Mallinckrodt, 100 mesh activated at 120°C) was the support and diethyl ether/hexane, 20:80 or 60:40 (vol/vol), and ethyl acetate were the solvents. The material eluted in the second fraction was treated with diazomethane and further purified by thin-layer chromatography [solvent system: diethyl ether/hexane, 25:65 (vol/vol), three successive developments]. The thin-layer radiochromatogram showed two zones of radioactivity. The more polar substance was found (by gas chromatography–mass spectrometry) to be compound I and the least polar, a mixture of compounds II and III (methyl esters). Steric analysis of these monohydroxy acids was carried out as described (20). Compound IV was eluted in the ethyl acetate fraction. The purification and steric analysis were done as described (21).

RESULTS

Metabolism of arachidonic acid in human PMNL

Human PMNL (140×10^6 cells) were incubated with [^{14}C]-arachidonic acid in the presence of ionophore A23187. The ether extract was fractionated by silicic acid column chromatography, and the material eluted in the ethyl acetate fraction was analyzed by RP-HPLC. Fig. 1*C* shows the ultraviolet absorbance pattern of the products obtained. Compounds I, II, III, and IV were collected for structural analysis. All the compounds detected were ^{14}C labeled. Details on the percentage of conversion of the substrate into the major compounds are given in the next section.

The ultraviolet spectra of the methyl esters of compounds I, II, and III showed an absorption band with $\lambda^{MeOH}_{max} = 235$ nm, indicating two conjugated double bonds. Gas chromatographic analysis of the trimethylsilyl (Me_3Si) derivatives of the methyl esters of compounds I, II, and III showed single peaks with equivalent chain length C-21.5 (OV-210, 3%). The mass spectra of these derivatives of compounds I, II, and III were identical to the mass spectra of the same derivatives of 5-hydroxy-6,8,11,14-icosatetraenoic acid (20), 12-hydroxy-5,8,10,14-icosatetraenoic acid (27), and 15-hydroxy-5,8,11,13-icosatetraenoic acid (29), respectively. That compounds I, II, and III were in-

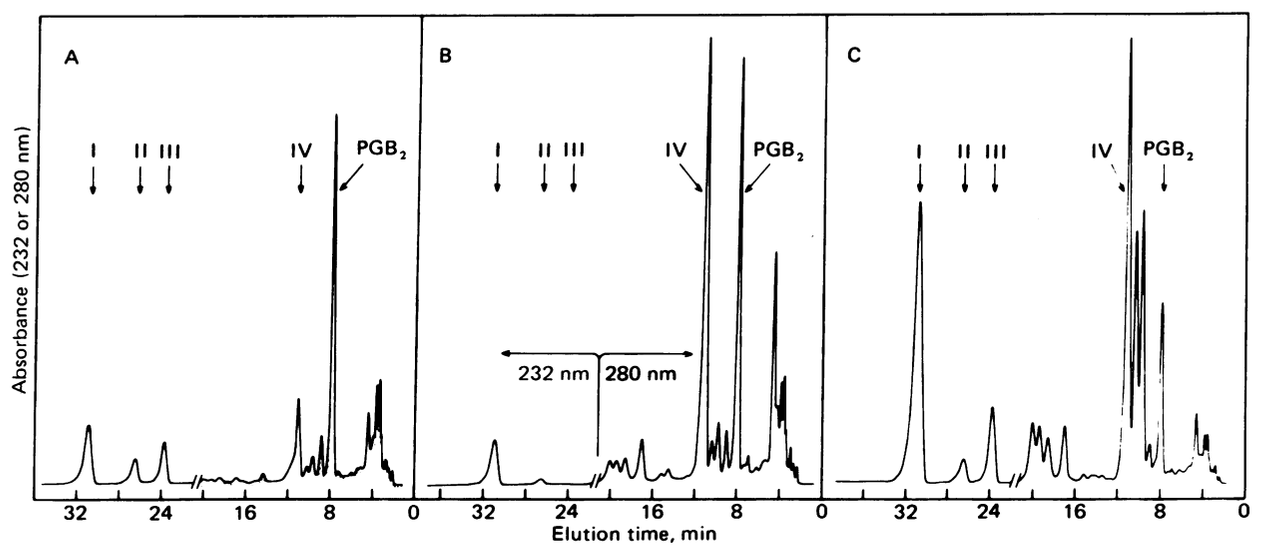

FIG. 1. RP-HPLC chromatogram of products (compounds I, II, III, and IV) obtained in the ethyl acetate fraction of silicic acid column chromatography of an ether extract. Human PMNL (140×10^6 cells/4 ml) were incubated 4 min with: (*A*) arachidonic acid (0.11 mM), (*B*) A23187 ionophore (20 μM), and (*C*) arachidonic acid (0.11 mM) and A23187 ionophore (20 μM). The dotted line in *A* is the control—i.e., addition of ethanol to the cells. The trace shows the ultraviolet absorbance of the sample at 280 nm (0–22 min) and 232 nm (22–36 min). The attenuation setting of the spectrophotometer was 4 times higher at 232 nm than at 280 nm. Prostaglandin B_2 (PGB_2) (2 μg) was added before ether extraction, as internal standard. The same preparation of PMNL was used for the experiments shown. The formation of the compounds under the different experimental conditions can thus be compared directly by taking into account the amount of internal standard present in each sample.

deed these three monohydroxy tetraunsaturated C_{20} fatty acid isomers was further confirmed by the mass spectra of the Me_3Si derivatives of the methyl esters of hydrogenated compounds I, II, and III.

About 15- to 20-μg quantities of the menthoxycarbonyl derivatives of the methyl esters of compounds I, II, and III were subjected to oxidative ozonolysis (30). The products were analyzed by gas chromatography–mass spectrometry. From the sample initially containing compound I, peaks corresponding to the menthoxycarbonyl derivatives of dimethyl-2*L*-hydroxyadipate (90%) and dimethyl-2*D*-hydroxyadipate (10%) were observed. From the sample initially containing a mixture of compounds II and III, peaks corresponding to the menthoxycarbonyl derivatives of dimethyl L-malate (80%), dimethyl D-malate (20%), methyl-2*L*-hydroxyheptanoate (70%), and methyl-2*D*-hydroxyheptanoate (30%) were observed.

The structures of compounds I, II, and III as described above are thus respectively, (5*S*)-hydroxy-6,8,11,14-icosatetraenoic acid, (12*S*)-hydroxy-5,8,10,14-icosatetraenoic acid, and (15*S*)-hydroxy-5,8,11,13-icosatetraenoic acid, three metabolites of arachidonic acid previously described (20, 27, 29). The reason for the lack of stereochemical purity of compounds II and III are not understood.

Compound IV showed an ultraviolet spectrum with λ_{max}^{MeOH} = 270 nm and two other bands at 260 and 281 ± 1 nm, which indicates three conjugated double bonds (31). Gas chromatographic analysis of the Me_3Si derivative (obtained by treatment with 30 μl of *N,O*-bis-Me_3Si-trifluoroacetamide and 30 μl of pyridine for 30 min at room temperature) of compound IV showed a single peak with an equivalent chain length C-24.5 (OV-210, 3%). The mass spectrum showed ions of high intensity at mass to charge ratios (*m/e*) 552 (M, ion intensity), 537 (M − 15), 462 (M − 90, loss of trimethylsilanol), 447 [M − (90 + 15)], 441 [M − 111, loss of $\cdot CH_2—CH{=}CH—(CH_2)_4—CH_3$], 385, 375, 351 [M − (111 + 90)], 325, 299, 287 [$\cdot CH{=}CH—CH(OSiMe_3)—(CH_2)_3—COOSiMe_3]^+$, 261 [M − (111 + 180)] and [$Me_3SiO^+{=}CH—(CH_2)_3—COOSiMe_3$], 219, 217 [probably ($Me_3SiO—CH{=}CH—CH{=}O^+SiMe_3$) and $H_2C{=}CH—C(OSiMe_3){=}O^+SiMe_3$, from rearrangements], 191, 189, 171 (261 − 90), 167, and 129.

This mass spectrum was identical to the mass spectrum of the Me_3Si derivative of (5*S*,12*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid, a compound recently identified in rabbit peritoneal PMNL (21). Further evidence for the identity of compound IV as the 5,12-dihydroxy acid mentioned above came from mass spectra (not shown) of the Me_3Si ether derivatives of methyl esters of compound IV and of hydrogenated compound IV. Oxidative ozonolysis and steric analysis of the alcohol groups indicated that compound IV had the "*S*" configuration at C-5 and the "*R*" configuration at C-12, and confirmed the presence of double bonds at Δ^6, Δ^8, Δ^{10}, and Δ^{14}. The chromatographic behavior (RP-HPLC, gas chromatography, and thin-layer chromatography) of compound IV was identical to that of the 5,12-dihydroxy acid (data not shown). The structure of compound IV is thus (5*S*,12*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid.[§]

Effect of ionophore A23187 on the metabolism of arachidonic acid in human PMNL

We have compared, by using RP-HPLC, the effects of addition of ionophore A23187 on the formation of compounds I, III, and IV, in the presence and absence of exogenous arachidonic acid. Fig. 1 and Table 1 show the results obtained with PMNL preparations from three human subjects. The dotted line in Fig. 1*A* indicates a control incubation in which only ethanol was added to the cells; under these basal conditions only small amounts of compounds I and III could be detected and compound IV was not measurable (using the technique described in this study). The small peak next to prostaglandin B_2 (internal standard) is a contaminant of the prostaglandin B_2 preparation.

Addition of arachidonic acid to suspensions of human PMNL led to variable results when cells from several subjects were compared. Some cell preparations showed little activity with respect to transformation of added arachidonic acid and only a slight increase in the formation of compounds I, III, and IV was observed (compound IV often remained undetectable). In these cases, addition of the ionophore together with arachidonic acid produced a strong stimulation of the transformation of the fatty acid into compounds I and IV (stronger than addition of ionophore alone); this is illustrated in Fig. 1 and Table 1 (data from two cell preparations). On the other hand, some cell preparations were very active, and the transformation of added arachidonic acid into compounds I, III, and IV (data not shown) was equivalent to the transformation observed in the presence of the ionophore and arachidonic acid—i.e., under optimal incubation conditions (see below).

[§] Ultraviolet and infrared spectrophotometric analysis of the compound isolated from rabbit PMNL indicated the presence of one *cis* and two *trans* double bonds in the conjugated triene (Δ^6, Δ^8, Δ^{10}), but the exact geometry of the triene structure is not known; it is however likely that the double bond at Δ^{14} has retained the *cis* geometry.

Table 1. Effect of ionophore A23187 on the metabolism of arachidonic acid in human PMNL

PMNL preparation	Additions	Compounds formed, nmol: 5-Hydroxy-acid (I)	15-Hydroxy-acid (III)	5,12-Dihydroxy-acid (IV)
A	None	0.6	<0.15	<0.15
	A23187	11.2	<0.22	4.2
	Arachidonic acid	2.2	4.1	<0.15
	A23187+ arachidonic acid	42.8	2.2	7.14
B	None	1.9	<0.15	<0.15
	A23187	10.3	<0.15	3.3
	Arachidonic acid	4.1	5.00	<0.15
	A23187+ arachidonic acid	81.6	4.68	7.44

Human PMNL (70×10^6 cells/2 ml) were incubated 4 min at 37°C in the presence or absence of ionophore A23187 (20 μM) and of arachidonic acid (0.11 mM), as indicated. Incubations were stopped by addition of 3 ml of methanol containing 1 μg of prostaglandin B_2 as internal standard. The amount of compounds formed was measured by RP-HPLC.

Out of 18 preparations of human PMNL (all from different subjects) tested, 12 were found to have weak spontaneous capacity to transform exogenous arachidonic acid into compounds I and IV, whereas 6 were active in this respect and were not further stimulated by simultaneous addition of ionophore.

Addition of ionophore A23187 to suspensions of human PMNL led to strong stimulation of the biosynthesis of compound I and especially of compound IV (see Fig. 1 *A* and *B* and Table 1) from endogenous arachidonic acid. It is noteworthy that the ionophore had no effect on the formation of compound III. The stimulatory effect of the ionophore on the formation of compound I varied between 5 and 20 fold in several experiments; this effect was even more pronounced on the formation of compound IV, because the compound was not measurable in unstimulated cells (see paragraph above).

Addition of ionophore A23187 together with arachidonic acid to suspensions of human PMNL (Fig. 1*C*) led to the strongest stimulation of the formation of compounds I and IV observed in these studies. Table 1 shows the amount of metabolites formed under various incubation conditions in two separate experiments. Under conditions of maximal stimulation by simultaneous addition of substrate and ionophore, a total transformation of about 20% of the added substrate was observed in the experiment shown in Fig. 1*C*, compounds I, II, III, and IV affording, respectively, 50, 5, 12, and 7% of the conversion. It is noteworthy that the other compounds appearing on the RP-HPLC chromatogram (Fig. 1*C*) were also labeled by ^{14}C and thus were metabolites of arachidonic acid (two compounds between prostaglandin B_2 and compound IV and four compounds between compound IV and compound III).

DISCUSSION

In this paper we report studies on the metabolism of arachidonic acid in human peripheral blood PMNL (see Fig. 2). (5*S*)-Hydroxy-6,8,11,14-icosatetraenoic acid, a compound already described in rabbit cells (20) was identified in human cells together with two other monohydroxy acids, (12*S*)-hydroxy-5,8,10,14-icosatetraenoic acid and (15*S*)-hydroxy-5,8,11,13-icosatetraenoic acid.¶ The two latter compounds were previously found to be metabolites of arachidonic acid in human platelets (27) and byproducts in the biosynthesis of prostaglandins (30), respectively. In addition, a metabolite of arachidonic acid, (5*S*,12*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid, recently identified in rabbit peritoneal PMNL (21) has been found in human PMNL. The mechanism of formation of these metabolites has not been investigated in the present study. However, it is likely that the monohydroxy acids are formed in a lipoxygenase-type reaction, involving a hydroperoxy acid intermediate as shown in the platelets (27) for the (12*S*)-hydroxy acid.

In a control experiment washed human platelets were incubated with arachidonic acid (unpublished data). The 5-hydroxy acid and 5,12-dihydroxy acid accounted together for less than 0.5% of the total conversion of arachidonic acid (in the presence or absence of ionophore A23187). The ratio of the 12-hydroxy acid to the 5-hydroxy acid was approximately 100:1. Thus with the exception of compound II (12-hydroxy acid), which is likely to be formed by contaminating platelets (see ref. 27), there was no doubt that the transformations described in this study are specific to PMNL.

In the present work the effects of ionophore A23187 on the metabolism of arachidonic acid in human PMNL have been studied. The major finding was the strong stimulatory effects

¶ This compound is also formed by rabbit peritoneal PMNL (unpublished data).

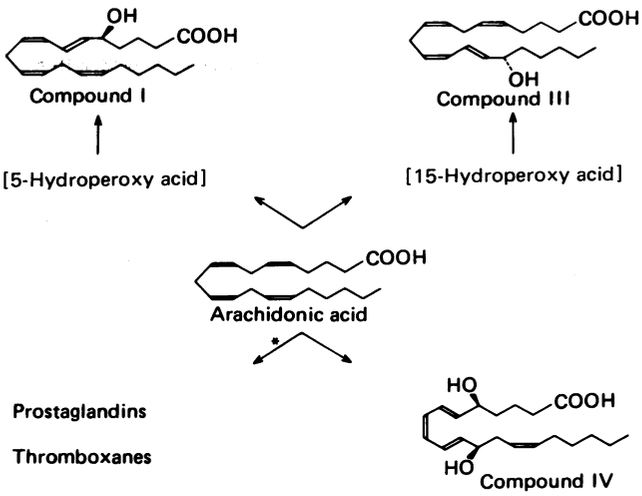

FIG. 2. Transformation of arachidonic acid in human PMNL. Compounds between brackets have not been isolated in this study. (12*S*)-Hydroxy-5,8,10,14-icosatetraenoic acid (compound II) was not included in this figure (see *Discussion*). Compounds derived from the cyclooxygenase pathway (*) have not been analyzed in these experiments.

of the ionophore on the synthesis of the 5-monohydroxy acid (compound I) and particularly of the 5,12-dihydroxy acid (compound IV) from endogenous substrate.

In the course of these studies, we also observed striking differences between PMNL preparations obtained from several human subjects. Indeed some cell preparations produced only small amounts of the 5-hydroxy acid, 15-hydroxy acid, and 5,12-dihydroxy acid upon addition of arachidonic acid. In these cases the transformation of exogenous substrate was strongly stimulated when incubations were performed in the presence of the ionophore (Fig. 1, Table 1). Some other preparations of human PMNL produced as much of the three metabolites upon addition of only arachidonic acid as upon addition of both arachidonic acid and the ionophore (the conditions found to produce the maximal amount of compounds I, III, and IV in all PMNL preparations tested). The reasons for this discrepancy in the capacity to transform exogenous arachidonic acid in different preparations of human PMNL are not understood, and this interesting point remains open for additional investigations.

It was not the purpose of this study to investigate the mechanism of action of the divalent cation ionophore A23187 on the stimulation of unsaturated fatty acid metabolism. However, our data deserve comments relative to this question. It is currently believed that the rate-limiting step in biotransformation of C_{20} polyunsaturated fatty acids is the release of these compounds from phospholipids (32, 33). Recently it has been suggested that the increase of cytoplasmic Ca^{2+} concentration induced by the ionophores activates Ca^{2+}-dependent phospholipase (34) and stimulates prostaglandin (and other compounds) synthesis through an increase in free arachidonic acid (13, 35, 36). Our data are in agreement with this hypothesis, because we also found that ionophore A23187 induced the biotransformation of endogenous arachidonic acid in human PMNL (see Fig. 1 and Table 1). However, our findings that some cell preparations, almost inactive in transforming exogenous arachidonic acid into compounds I, III, and particularly IV, become very active upon incubation with the ionophore strongly suggest that, in addition to its proposed effect on the phospholipase, the ionophore might also directly activate the enzymatic system (or unmask its activity) involved in the for-

mation of these compounds. However, more experiments will be required to understand the mode of action of the ionophore in this system.

Ionophore A23187 causes release of lysosomal enzymes (18, 19), slow reacting substance of anaphylaxis (37), and chemotactic factors (38) from leukocytes. The detailed mechanism of action of the ionophore, however, is not known. The finding that the pathways of arachidonic acid metabolism described in this paper are also stimulated by the ionophore raises the question of a relationship between mediator release and transformation of arachidonic acid in leukocytes.

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