Biochemical Pharmacology, Vol. 34, No. 22, pp. 3943–3950, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

LOCALIZATION OF 5-LIPOXYGENASE WITHIN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

MICHAEL STÜNING*, MONIKA RAULF and WOLFGANG KÖNIG†
Lehrstuhl Med. Mikrobiologie und Immunologie, Arbeitsgruppe für Infektabwehrmechanismen, Ruhr-Universität Bochum, Federal Republic of Germany

(*Received* 27 *February* 1985; *accepted* 28 *May* 1985)

Abstract—Human polymorphonuclear leukocytes (PMN) stimulated with the Ca-ionophore A23187 or opsonized zymosan not only release marker enzymes of specific granules but secrete 5-lipoxygenase activity as well. In the presence of BSA cells incubated with [^{14}C]AA were able to synthetize 5-HPETE but failed to produce 5-HETE, LTB_4, and its ω-oxidation metabolites. Subcellular fractionation studies by differential and isopycnic equilibrium density centrifugation demonstrated main lipoxygenase activity in particulate fractions consisting of specific granules, but not in cytosolic fractions. These results suggest the association of 5-lipoxygenase with specific granules. 5-lipoxygenase released from the cells upon appropriate stimulation reached its peak activity after 10 min and was then rapidly inactivated. It appears that the intermediate 5-HPETE may be generated extracellularly but has to re-enter the intracellular space for further metabolization.

The highly complex metabolism of arachidonic acid‡ (AA) leads to a variety of biologically active compounds, such as prostaglandins and thromboxanes via the cyclooxygenase pathway [1, 2], and hydroxy-eicosatetraenoic acids (HETEs) and leukotrienes (LTs) via the lipoxygenase enzymes [3–5]. Upon appropriate stimulation free arachidonic acid is released from membrane phospholipids [6] and may be oxygenated at C_5-position by a 5-lipoxygenase leading to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is either converted by a dehydrase to leukotriene A_4 (LTA_4) or by a glutathione peroxidase to the corresponding mono-hydroxy-eicosatetraenoic acid 5-HETE. LTA_4 can be transformed by an epoxide hydrolase to leukotriene B_4 (LTB_4), or via a glutathione *S*-transferase catalyzed reaction to leukotriene C_4, which is further metabolized to the leukotrienes D_4 and E_4 by a γ-glutamyl-transpeptidase and a dipeptidase respectively. ω-oxidation of LTB_4 leads to 20-hydroxy- and 20-carboxy-LTB_4. LTB_4 is a potent chemotactic and chemokinetic agent towards polymorphonuclear neutrophil granulocytes (PMN), monocytes and macrophages [7–10].

It stimulates leukocyte adhesion to vascular endothelium and increases vascular permeability [11, 12]. LTC_4, LTD_4 and LTE_4, known as slow reacting substance of anaphylaxis (SRS), cause broncho-constriction and cardiovascular effects [12]. Though the AA-metabolism of many cell types from various species has been studied thoroughly, little is known about the location of the enzymatic reactions leading from free AA to the leukotrienes. There is also little information about cell–cell interactions during leukotriene biosynthesis. In previous publications [13–14] we presented evidence that γ-glutamyl-transpeptidase and dipeptidase activity were associated with microsomes and specific granules, whereas glutathione *S*-transferase activity was localized within the cytoplasm. It was the purpose of the present investigation to localize the 5-lipoxygenase from human PMN on a subcellular level and to characterize the distribution of the enzymatic reactions initiating the leukotriene pathway.

MATERIALS AND METHODS

Reagents. Reagents used were from the following sources: [^{14}C]AA (sp. act. 60 mCi/mmol), [^{3}H]12-HETE (sp. act. 120 Ci/mmol), Radiochemical Center, Amersham, England; [^{3}H]LTB_4 (sp. act. 150 Ci/mmol), [^{3}H]5-HETE (sp. act. 150 Ci/mmol), New England Nuclear, Dreicich, FRG; phosphatidylcholine, phosphatidyl-ethanolamine, phosphatidylinositol, zymosan A, melittin, Ca-ionophore A23187, esculetin, caffeic acid, nor-dihydroguaiaretic acid (NDGA), indomethacin, quinacrine, ATP, Micrococcus lysodeicticus, phenolphtalein glucuronic acid, o-tolidin, NADH, pyruvate, heparin, Sigma, Taufkirchen, FRG; BSA, Serva Heidelberg, FRG; Macrodex 6% (w/v), Knoll, Ludwigshafen, FRG; sodium metrizoate solution 75% (w/v),

* In partial fulfilment of M. S.'s Ph.D. thesis.

† Author to whom correspondence should be addressed: Prof. Dr. W. König, Lehrstuhl für Med. Mikrobiologie und Immunologie, Arbeitsgruppe für Infektabwehrmechanismen, RUHR-Universität Bochum, Postfach 10 21 48, 4630 Bochum 1, F.R.G.

‡ Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; LTB_4, 5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid; NDGA, nor-dihydroguaiaretic acid; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMN, polymorphonuclear neutrophil leukocyte; TLC, thin layer chromatography.

Nyegaard, Oslo, Norway; BW755C was a gift obtained from Dr. Peskar, Ruhr-Universität Bochum, FRG.

Buffer. Unless stated otherwise the buffer used throughout all experiments consisted of 0.12M-NaCl/0.01M-Na_2HPO_4/0.003M-KH_2PO_4 pH 7.4 (referred to as PBS).

Preparation of PMN. Heparinized blood (3000 U/ml) of healthy donors was separated on a Ficoll-metrizoate cushion (45 min at 4° and 400 *g*) followed by dextran sedimentation [15]. After removal of platelets by repeated washing with PBS, erythrocytes were lysed by hypotonic exposure of the cell suspension to 0.3% (w/w) NaCl. Isotonicity was readjusted with 1.5% (w/w) NaCl. The cells were washed twice with PBS to remove the NaCl. Cell viability (exclusion of trypan blue) and purity were checked routinely and accounted for more than 97% pure and intact PMN.

Preparation of opsonized zymosan. Zymosan A (2 mg/sample) was suspended in 5 ml PBS and boiled for a few seconds. 15 ml PBS were added and the suspension was centrifuged for 10 min at 4° and 3000 rpm. The pellet was washed twice with PBS, suspended in 5 ml of human serum and incubated at 37° for 30 min. After centrifugation the pellet was washed twice with PBS.

Determination of marker enzymes and protein. Lysozyme (EC 3.2.1.17), β-glucuronidase (EC 3.2.1.31), myeloperoxidase (EC 1.11.1.7), lactate dehydrogenase (LDH, EC 1.1.1.27), alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) *p*-nitrophenylphosphatases, and protein were determined as was previously described in detail [16].

Lipoxygenase assay. The reaction mixture for intact cells contained 1×10^7 PMN, indomethacin (0.1 μM), 2 mM $CaCl_2$ and labelled AA (2.8 μM) in a total volume of 600 μl PBS according to the various procedures described under results. Prior to stimulation the cells were preloaded with labelled AA for 30 min at room temperature; non-incorporated AA was removed by repeated washing with PBS. Alternatively labelled AA and stimulus were added simultaneously. If cellular fractions or cell-free supernatants were assayed, the reaction mixtures were supplemented with 1 mM ATP [17]. For entrapping of fatty acids and fatty acid derivatives within the extracellular space BSA (4 mg/ml) was added to the reaction mixture. The reaction was performed at 37° and terminated by adding 2 ml of acidified methanol (0.01% acetic acid, v/v). Denaturated protein and cell debris were removed by centrifugation, and the clear supernatants were extracted twice with 3.5 ml of chloroform. To minimize losses of radioactive material that was poorly extractable with methanol, the pellet was additionally extracted with 1 ml of chloroform/methanol 2:1 (v/v) [18]. The losses of radioactivity within the final pellet and the aqueous phase determined by liquid scintillation counting accounted for 4–5% (Packard Tri-Carb Liquid Scintillation Spectrometer 3255, external standardization). The pooled extracts were evaporated to dryness under nitrogen, the residues were dissolved in 80 μl of chloroform and spotted on a silica gel thin layer plate (250 μm, Kieselgel 60, Merck, Darmstadt, FRG). AA, 5-HETE, LTB_4, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) served as references. The plates were developed once with solvent system 1 (ethylacetate/2,2,4-trimethylpentane/acetic acid/water, 55:25:10:50, v/v, organic phase) [19]. Solvent system 2 (chloroform/methanol/acetic acid/water, 90:10:1:0.65, v/v) was used for re-chromatography of LTB_4 and 5-HETE. Solvent systems 3 (chloroform/methanol, 100:2, v/v) and 4 (chloroform/methanol/acetic acid/water, 100:60:16:7, v/v) were used combined for one-dimensional separation of the phospholipid fraction [20]. Non-labelled phospholipids were visualized by staining with Coomassie blue [21]. Radioactivity was detected with an ISOMESS Radio-Dünnschicht-Analysator IM3000 (Isotopenmeßgeräte GmbH, Straubenhardt, FRG).

Identification of [^{14}C] *AA products.* The synthesis of [^{14}C] AA metabolites identified as 5-HETE, 5-HPETE, LTB_4-isomers, and ω-oxidation products was completely inhibited by known inhibitors of lipoxygenase activity, such as BW755C (30 μg/ml), esculetin (0.5 mM), caffeic acid (0.1 mM) and NDGA (0.1 mM). The cyclooxygenase inhibitor indomethacin (0.1 μM) did not show any effect. ID_{50} values obtained by dose–response curves correlated well with those published elsewhere [22–24].

5-HETE and LTB_4 were further characterized by co-chromatography with authentic [^{3}H]5-HETE and [^{3}H]LTB_4 with solvent system 1 and re-chromatography with solvent system 2. Incubation of stimulated PMN with synthetic [^{3}H]LTB_4 showed a single product by TLC analysis with a r_f that was indistinguishable from the r_f of the [^{14}C]AA product identified as ω-oxidation products. No attempts were made to separate 20-hydroxy- and 20-carboxy-LTB_4 by TLC. Although TLC was not suitable for the separation of LTB_4 from its isomers, this analytic system allowed the separation and quantitation of free AA, HPETE, mono- and di-HETEs, ω-oxidation products of LTB_4, and phospholipids in a single step procedure. For identification of 5-HPETE PMN labelled with [^{14}C]AA were stimulated with the Ca-ionophore in the presence of 0.02% (w/v) $NaBH_4$. By reduction with $NaBH_4$ the [^{14}C]AA product identified as 5-HPETE was shifted to co-chromatography with 5-HETE [17]. 5-HPETE ($r_f = 0.78 \pm 0.01$ S.E.M., N = 8) chromatographed clearly separated from [^{3}H]12-HETE ($r_f = 0.74 \pm 0.01$ S.E.M., N = 8; solvent system 1).

The [^{14}C]AA products identified as phospholipids could not be abolished by inhibitors of lipoxygenase but of phospholipase A_2 activity (quinacrine 1 mM). After TLC analysis with solvent system 1 radioactivity corresponding with the assumed phospholipid fraction was scraped off the plates and eluted with chloroform/methanol/water (65:40:9.5 v/v) [25]. One dimensional re-chromatography with solvent systems 3 and 4 revealed three principal products co-chromatographing with authentic phosphatidylcholine ($r_f = 0.31$; up to 37% of the total phospholipid labelled after 50 min time of incubation), phosphatidylethanolamine ($r_f = 0.75$; 22%), and phosphatidylinositol ($r_f = 0.52$; 40%). Saponification of the assumed phospholipid fraction with methanolic KOH (1 mM) at −20° overnight

revealed two labelled products co-chromatographing with authentic AA and 5-HPETE by TLC with solvent system 1.

Enzyme release. PMN (1×10^7 per sample suspended in PBS (final volume 600 μl) supplemented with 2 mM $CaCl_2$ were incubated at 37° with the Ca-ionophore, or opsonized zymosan (2 mg) according to the various procedures described under results. After incubation the cells were removed by centrifuging at high speed for 5 sec (Eppendorf centrifuge 5414). The clear supernatants were assayed for enzyme activities. Unless stated otherwise the amount of enzyme released is given as percentage of the enzyme content related to an equal number of sonicated cells. Because sonicated PMN exhibited less lipoxygenase activity than the supernatants obtained with non-cytotoxic degranulation, the release of lipoxygenase activity is expressed as total extracellular activity and not as percentage release.

Subcellular fractionation. 1×10^9 PMN were homogenized in a cooled Potter Elvehjem teflon homogenizer (Braun Melsungen, Melsungen, FRG, 20 ml, clearance 0.095–0.115 mm) driven by a drilling machine at 200 rpm. Cell breakage was controlled by staining aliquots with toluidine blue. 80–100 strokes were sufficient to break about 95% of the cells. Prior to homogenization the cells were suspended in a hypotonic medium (0.1 M sucrose in Tris–HCl, 5 mM, pH 7.4). Isotonicity was readjusted with 0.54 M sucrose after homogenization.

Cell fractionation by differential centrifugation was carried out by centrifuging the whole homogenate at 4° and 400 *g*, 3000 *g*, 20,000 *g* for 15 min, and 200,000 *g* for 60 min in a Christ centrifuge (Christ, Osterode, FRG), and a Beckman ultracentrifuge model L8-70 (Beckman Instruments, Palo Alto, U.S.A.) using a SW40 rotor. Four particulate fractions [1–4], and one final supernatant [5] were obtained. For isopycnic equilibrium density centrifugation a 400 *g* supernatant was layered on top of a linear sucrose gradient (19–54%, w/v) and centrifuged at 4° and 100,000 *g* for 12 hr, using a Beckman ultracentrifuge and a SW40 rotor. The enzyme activities of the subcellular fractions were calculated from specific activity; the sum of enzyme activities within all fractions of a homogenate was taken as 100% [16].

RESULTS

Metabolization of [^{14}C]*AA: effect of BSA*

Human PMN stimulated with the Ca-ionophore A23187 (5.7 μM) for 10 min at 37° in the presence of indomethacin (0.1 μM) converted exogenously added [^{14}C]AA to at least five isotopically labelled products as could be detected by TLC. These products were identified as phospholipids (PI, PC, PE, $r_f = 0.0$), 20-hydroxy- and 20-carboxy-LTB_4 ($r_f = 0.24$), LTB_4 and its isomers ($r = 0.57$), 5-HETE ($r_f = 0.72$) and 5-HPETE ($r_f = 0.78$). In the absence of

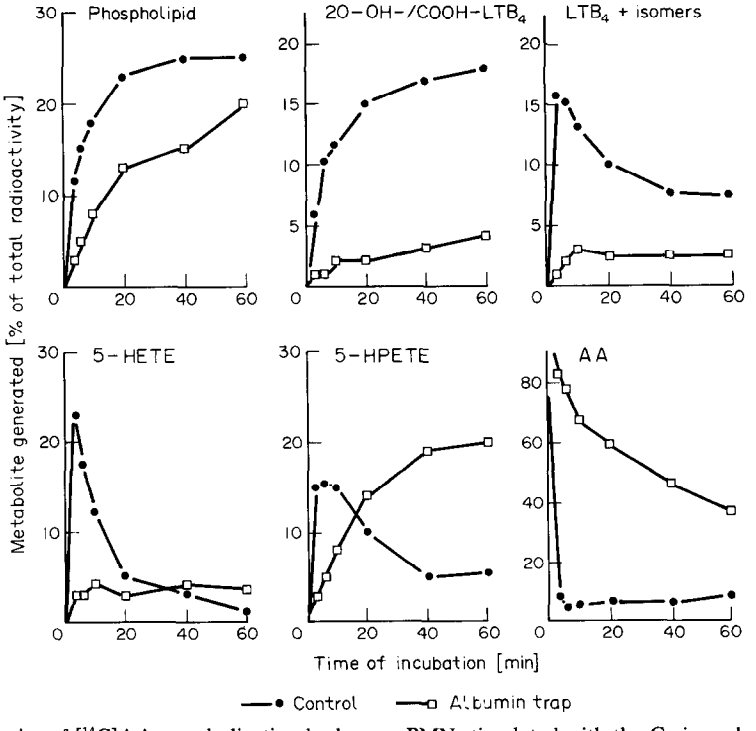

Fig. 1. Kinetics of [^{14}C]AA metabolization by human PMN stimulated with the Ca-ionophore A23187 (5.7 μM). The cells were preloaded with [^{14}C]AA for 30 min at room temperature. Open squares: the reaction mixture was supplemented with BSA (4 mg/ml) for trapping of fatty acid derivatives within the extracellular space; closed circles: control without BSA. Each dot represents the mean value of three experiments with S.E.M. ranging from 4 to 8%.

indomethacin no additional products were generated. Thus under the chosen conditions no cyclooxygenase transformation of [^{14}C]AA had occurred. In contrast to human PMN, mixed suspensions of human lymphocytes, monocytes and basophilic granuloctyes, or rat peritoneal cells synthetized cyclooxygenase products in good yields when stimulated under the same conditions (unpublished results). PMN as a poor source for cyclooxygenase products were already described [26]. Kinetic experiments demonstrated a rapid conversion of [^{14}C]AA. 20-hydroxy- and 20-carboxy-LTB_4 accumulated as the only products, together with an increase of radioactivity in the phospholipid fraction (Fig. 1). The time-dependent decrease in LTB_4/LTB_4-isomers and 5-HPETE was most likely to be due to the transformation to ω-oxidation-products and LTB_4/5-HETE, respectively. 5-HETE, but not LTB_4 or one of its isomers underwent reesterification into phospholipids, as was demonstrated by hydrolysis of the phospholipid fraction. Reesterification of 5-HETE was already described [27, 28]. 75–90% of labelled metabolites appeared in the supernatants of stimulated cells; labelled phospholipids remained with the pellet. PMN preloaded with [^{14}C]AA prior to stimulation generated the same metabolic pattern (qualitatively as well as quantitatively) as compared to cells receiving labelled AA and stimulus simultaneously (data not shown). More dramatic changes were observed when AA and its metabolites were immobilized in the extracellular space using bovine serum albumin (BSA, 4 mg/ml) as albumin trap [29] (Fig. 1). In the presence of albumin the synthesis of 5-HETE, LTB_4, its isomers and ω-oxidation products was significantly reduced, whereas 5-HPETE accumulated in the extracellular space. Again preloading of cells with labelled AA and simultaneous addition of substrate and stimulus led to the same metabolic pattern (data not shown). Since the incorporation of exogeneously added [^{14}C]AA into phospholipids was reduced only by 80% in the presence of BSA (Table 1), it was difficult to exclude that in spite of the albumin trap a part of the [^{14}C]-AA had entered the cells for metabolization. Thus these results suggest that either the precursor 5-HPETE or AA itself was released from the cells upon stimulation. The second possibility implicates extracellular lipoxygenation of AA, a process that would require secretion of 5-lipoxygenase from stimulated cells.

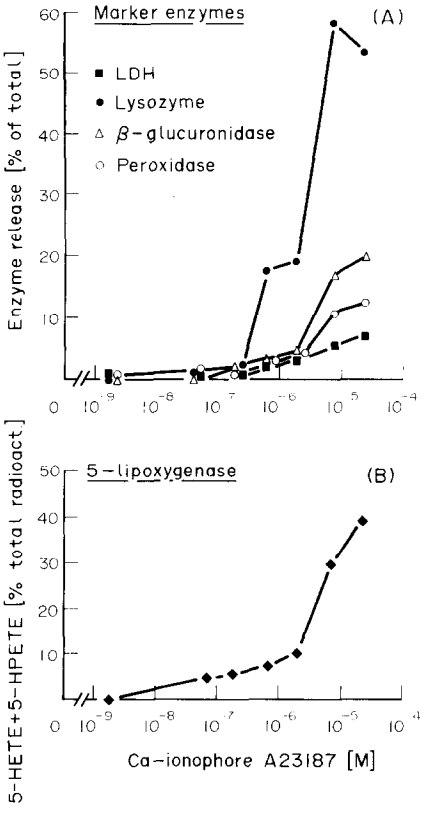

Fig. 2. Demonstration of 5-lipoxygenase activity within cell-free supernatants obtained from human PMN stimulated with various concentrations of the Ca-ionophore A23187 for 20 min. (A) marker enzymes of specific (lysozyme), azurophilic granules (lysozyme, β-glucuronidase), and cytoplasm (LDH); (B) 5-lipoxygenase activity as was represented by the amounts of 5-HPETE and 5-HETE synthetized from added [^{14}C]AA. For elimination of the endogenous AA-release, the cells were pre-incubated with the phospholipase inhibitor quinacrine for 10 min at 0° prior to stimulation. For details as to the calculations see under Material and Methods. Each dot represents the mean value of four experiments with S.E.M. ranging (A) from 6 to 9% and (B) from 8 to 12%.

Table 1. Incorporation of exogenously added [^{14}C]AA into phospholipids by non-stimulated PMN in the presence and absence of BSA (4 mg/ml). Radioactivity was determined by liquid scintillation counting, the amounts of labelled phospholipid by TLC. The authenticity of the label with [^{14}C]AA was confirmed by hydrolysis with methanolic KOH followed by TLC analysis as described under Materials and Methods. Data are given as % of the total radioactivity that was present in the reaction mixture

Time of incubation (min)	Radioactivity recovered from the cell pellet (phospholipid fraction) % of total radioactivity with BSA	without BSA
5	1.5	11.8
10	2.6	18.2
15	3.0	20.3
25	4.1	21.4
35	4.9	19.6
45	5.0	20.1

Granular enzyme release

During subsequent experiments supernatants of PMN stimulated with opsonized zymosan (2 mg/ml) or the Ca-ionophore A23187 were assayed for lipoxygenase and marker enzyme activities. Depending on the concentration of stimulus and the duration of incubation PMN release the granular marker enzyme lysozyme. Peroxidase and β-glucuronidase were hardly detectable (Figs. 2 and 3), suggesting the secretion of specific, but not of azurophilic granules.

Release of granular enzymes was non-cytotoxic as

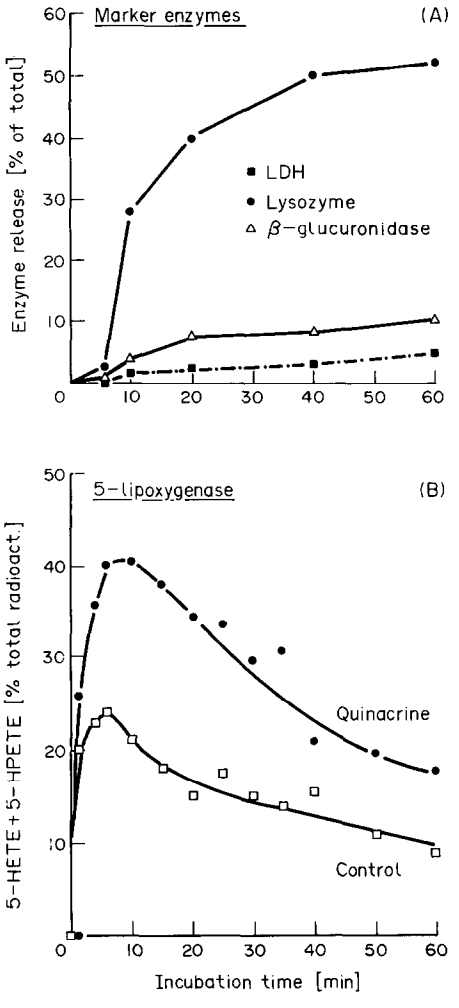

Fig. 3. Time dependent release of enzymes from human PMN by stimulation with opsonized zymosan (2 mg/600 μl). (A) marker enzymes of specific (lysozyme), azurophilic granules (lysozyme, peroxidase, β-glucuronidase) and cytoplasm (LDH); (B) 5-lipoxygenase activity as was represented by the amounts of 5-HPETE and 5-HETE synthetized from added [^{14}C]AA. Each dot represents the mean value of three experiments with S.E.M. ranging from 5 to 10%.

indicated by low levels of the cytoplasmic marker enzyme LDH within the supernatants. The increase in lysozyme activity correlated with an increase in 5-lipoxygenase activity as was indicated by the conversion of added [^{14}C]AA to 5-HPETE as principal product and minor amounts of 5-HETE. LTB_4 and its ω-oxidation metabolites were hardly detectable. The conversion of [^{14}C]AA by cell supernatants was completely inhibited by known lipoxygenase inhibitors at the given concentrations (BW755C 30 μg/ml, esculetin 0.5 mM, caffeic acid 0.1 mM and NDGA 0.1 mM). No effects were expressed by the cyclooxygenase-inhibitor indomethacin (0.1 μM). Kinetic studies with the Ca-ionophore or opsonized zymosan as stimuli demonstrated a maximum of 5-lipoxygenase within the supernatants after 10 min followed by a steady decrease at later times. This pattern strongly suggested a rapid inactivation of the 5-lipoxygenase soon after it was released from the cells. Autocatalytic inactivation of lipoxygenase by its hydroperoxide product was previously reported by Rapoport *et al.* [30]. We therefore assumed that blocking of the endogenous AA-metabolism by inhibiting AA-release from phospholipids might increase the 5-lipoxygenase activity detectable in the supernatants. Pre-incubation of PMN with the phospholipase inhibitor quinacrine (0.5 mM) for 10 min at 0° prior to stimulation with the Ca-ionophore led to a twofold increase in lipoxygenase activity within the supernatants as compared to the control (Fig. 3). The inactivation of the 5-lipoxygenase was neither abolished nor was its peak actively shifted.

Subcellular fractionation studies

The data presented so far suggest the localization of 5-lipoxygenase within a secretory compartment. Subcellular fractionation studies were carried out for a more detailed analysis. Human PMN were homogenized and subjected to differential centrifugation (see Materials and Methods). Biochemical analysis revealed that fraction 1 (400 *g* pellet) mainly contained intact cells and large cell fragments, fraction 2 (3000 *g* pellet), and fraction 3 (20,000 *g* pellet) azurophilic granules, fraction 4 (200,000 *g* pellet) specific granules and microsomes and fraction 5 (200,000 *g* supernatant) cytoplasm. The highest amount of lipoxygenase activity was detected within the fraction containing specific granules and microsomes (200,000 *g* pellet) (Fig. 4). Low amounts of lipoxygenase activity were detectable within the cytoplasmic fraction. By isopycnic equilibrium density centrifugation of a 400 *g* supernatant main lipoxygenase activity was recovered from fractions with a density ranging from 1.13–1.18 g/ml (Fig. 5). In accordance with Bretz and Baggiolini [31] the biochemical analysis revealed that these fractions mainly contained specific granules.

DISCUSSION

The accumulation of polymorphonuclear leukocytes at the site of inflammation is a characteristic feature for most tissue injuries. The active contribution of PMN to the inflammatory response is mediated by the secretion of granular bound enzymes, by generation of superoxide anions, and by the synthesis of lipid mediators of inflammation derived from AA via the lipoxygenase pathway (for reviews see [32, 33]). It has been proposed by numerous authors that discrete elements of the inflammatory response are interdependently related to each other. In the past a direct evaluation of the interrelationship between degranulation and leukotriene biosynthesis was difficult since precise informations about the subcellular localization of the enzymes for leukotriene biosynthesis were lacking.

Our data suggest that the 5-lipoxygenase of human PMN is associated with a secretory compartment. In the presence of BSA human PMN stimulated with the Ca-ionophore or opsonized zymosan converted labelled AA to 5-HPETE but failed to synthetize LTB_4, its isomers and ω-oxidation metabolites, even

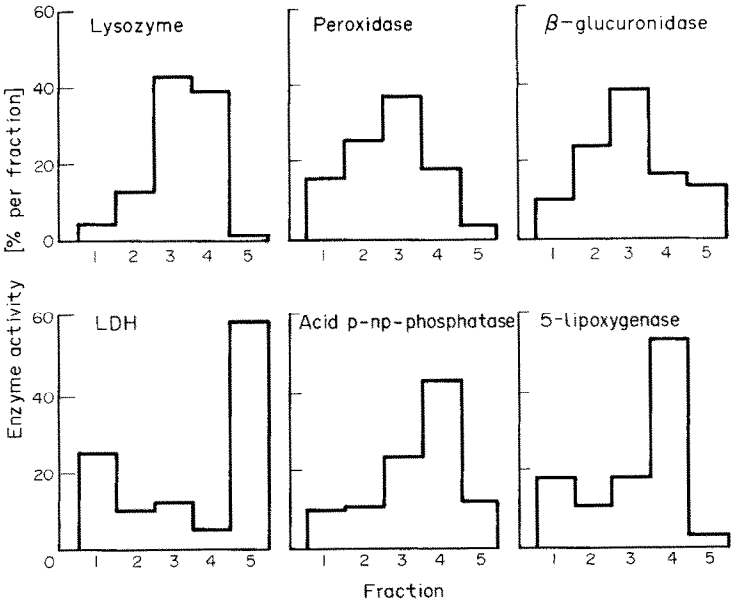

Fig. 4. Subcellular fractionation of human PMN by differential centrifugation. Enzyme activity per fraction was calculated from specific activity with respect to the protein content. The sum of enzyme activities from fractions 1–5 was taken as 100%. Data are given as mean values from five homogenizations with S.E.M. calculated on the basis of each individual experiment ranging from 9 to 14% due to differences in the degree of homogenization.

if the cells were preloaded with labelled AA. The possibility of a direct interference between BSA and the stimulus seems to be most unlikely. If the concentration of the stimulus is decreased, the amounts of all metabolites derived from added [^{14}C]AA were reduced at the same rate, whereas 5-HPETE alone accumulated in the presence of BSA. Accumulation of the intermediate 5-HPETE within the extracellular space by entrapping with BSA suggests that either 5-HPETE has been synthetized intracellularly with subsequent secretion, or lipoxygenation of AA has taken place extracellularly.

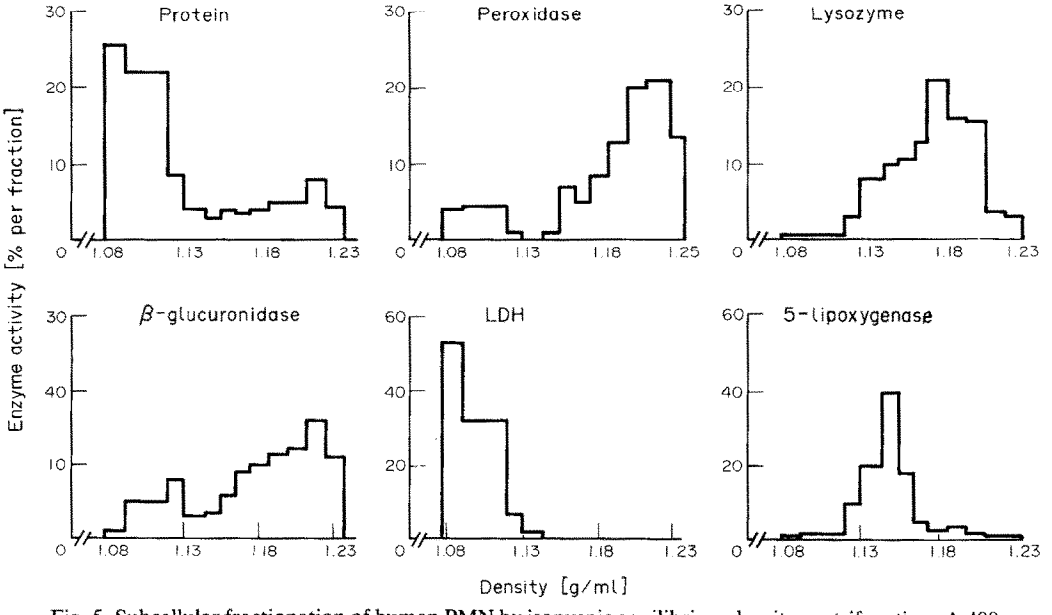

Fig. 5. Subcellular fractionation of human PMN by isopycnic equilibrium density centrifugation. A 400 *g* supernatant was layered on top of a sucrose gradient (19–54%, w/v). For calculation see Fig. 4.

As was previously reported by Goldstein *et al.* [34] and Gallin *et al.* [35], human PMN release the enzyme content of their specific, but not of azurophilic granules upon stimulation with the Ca-ionophore A23187 or opsonized zymosan. Our data demonstrated that secretion of the specific granular content was paralleled by a release of 5-lipoxygenase activity. Conversion of [^{14}C]AA by cell-free supernatants correlated well with the concentration of the stimulus and the duration of incubation. The lack of temporal correlation between lysozyme release and the appearance of 5-lipoxygenase at early times of stimulation was most likely to be due to a higher sensitiveness of the lipoxygenase assay. Low levels of LDH activity indicated that the release of enzyme activity was due to secretion and not to cell damage. Cell-free supernatants obtained from stimulated cells converted [^{14}C]AA to one principal product that appeared to be 5-HPETE. Evidence was presented as such that: (1) inhibitors of lipoxygenase, but not of cyclooxygenase activity inhibited conversion of labelled AA by supernatants, (2) in the presence of $NaBH_4$ supernatants converted AA to a product cochromatographing with 5-HETE and (3) 5-HPETE accumulated as principal product when [^{14}C]AA was converted by cell free supernatants as well as by intact cells in the presence of BSA. Thus, it can be concluded that lipoxygenation of AA released from stimulated cells may occur extracellularly. It appeared that for metabolization of 5-HPETE into LTB_4 the intermediate has to re-enter the intracellular space.

The Ca-ionophore A23187 is not as specific for the release of specific granules as other stimuli. However, our assumption that the 5-lipoxygenase may be associated with specific granules is strongly supported by subcellular fractionation studies. Differential and equilibrium density centrifugation of cell homogenates strongly suggested the association of 5-lipoxygenase activity with particulate fractions which consisted mainly of specific granules, as was indicated by the presence of the marker enzyme lysozyme in the absence of peroxidase and β-glucuronidase. Hardly any lipoxygenase activity was detectable within the cytoplasmic fractions. These results are in contrast to those published by other authors. Unfortunately the various results are difficult to compare to each other since the cell sources and methods chosen for cell breakage and fractionation vary from author to author. Ochi *et al.* [17] and Hamasaki and Tai [36] localized lipoxygenase activity within the cytosolic fraction of guinea pig polymorphonuclear granuloctyes and RBL-1 cells respectively. The cell breakage was carried out by sonication; the 105,000 *g* supernatant was defined as cytosolic fraction. A bimodal distribution of 12-lipoxygenase within the cytosol (105,000 *g* supernatant) and microsomes was reported by Yokoyama *et al.* [37] for the rat lung, and for human platelets by Nugteren [38], Ho *et al.* [39], and Lagarde *et al.* [40]. Chang *et al.* [41] and Yoshimoto *et al.* [42] demonstrated 12-lipoxygenase activity in the cytosol (105,000 *g* supernatant) of rat platelets and porcine PMN, respectively. Contradictory results might arise from the fact that sonication is often used for cell breakage. This method is not as suitable for the preparation of intact granules as compared to the Potter–Elvehjem homogenization procedure. In previous papers [13, 14] we demonstrated that γ-glutamyltranspeptidase and dipeptidase activity were also associated with microsomes and specific granules, whereas glutathione *S*-transferase was localized in the cytoplasm. The physiological significance of enzyme release upon stimulation remains still unclear at the present state of investigation. One function of 5-lipoxygenase release could be the cooperation with other cell types. Cell–cell interactions during AA metabolism were already proposed by several authors, e.g. metabolization of platelet-derived AA and 12-HETE to LTB_4, 5-HETE and 5,12-diHETE, respectively by human PMN [43, 44], activation of human PMN 5-lipoxygenase by platelet-derived 12-HPETE [45], synthesis of prostacyclin from platelet-derived endoperoxides by human endothelial cells [46] and conversion of PMN-derived LTA_4 into LTB_4 by human erythrocytes [47]. One might suggest that by extracellular lipoxygenation of AA the precursor 5-HPETE is provided to cell types lacking 5-lipoxygenase activity.

Another aspect of enzyme secretion could be the control of the enzymatic activity itself. Our data indicate that the lipoxygenase is rapidly inactivated after release from the cells. Autocatalytic inactivation by its hydroperoxide product alone [30] might not account for this phenomenon, because elimination of the endogenous AA-release by a phospholipase inhibitor did not prevent the enzyme from inactivation. Alternatively, Rapoport *et al.* [30] suggested that the lipoxygenase is highly susceptible to aerobic inactivation at 37° even without the presence of a hydroperoxide. Thus, once the enzyme is released it may become accessible for oxygen attack restricting its time dependent activity.

Acknowledgement—This work was supported by the Deutsche Forschungsgemeinschaft (Kö. 427/7-2) and in part by the Deutsche Psoriasis-Bund.

REFERENCES

1. G. A. Higgs and L. J. F. Youlton, *Br. J. Pharmac.* **44**, 330 (1972).
2. G. A. Higgs, S. Bunting, S. Moncada and J. R. Vane, *Prostaglandins* **12**, 749 (1976).
3. P. Borgeat, M. Hamberg and B. Samuelsson, *J. biol. Chem.* **251**, 7816 (1976).
4. E. J. Goetzl and F. F. Sun, *J. exp. Med.* **150**, 406 (1979).
5. P. Borgeat and B. Samuelsson, *Proc. natl. Acad. Sci. U.S.A.* **76**, 2148 (1979).
6. C. C. Walsh, B. M. Waite, M. J. Thomas and L. R. deChatelet, *J. biol. Chem.* **256**, 7228 (1981).
7. E. J. Goetzl and W. C. Pickett, *J. Immunol.* **125**, 1789 (1980).
8. A. W. Ford-Hutchinson, M. A. Bray, M. N. Doig, E. Shipley and M. J. H. Smith, *Nature, Lond.* **286**, 264 (1980).
9. P. Bhattacherjee, V. Hammond, J. A. Salmon, R. Stepney and K. E. Eakins, *Eur. J. Pharmac.* **73**, 24 (1981).
10. A. W. Ford-Hutchinson, M. A. Bray, F. M. Cunningham, E. M. Davidson and M. J. Smith, *Prostaglandins* **21**, 143 (1981).

11. M. A. Bray, F. M. Cunningham, A. W. Ford-Hutchinson and M. J. H. Smith, *Br. J. Pharmac.* **72**, 483 (1981).
12. P. J. Piper, *Phys. Reviews* **64**, 744 (1984).
13. M. Raulf, M. Stüning and W. König, *Immunol.* **55**, 135 (1985).
14. J. Brom, M. Raulf, M. Stüning, P. Spur, A. Crea, K.-D. and W. König, *Immunol.* **51**, 571 (1984).
15. A. Böyum, *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97), 77 (1968).
16. N. Frickhofen and W. König, *Immunol.* **37**, 111 (1979).
17. K. Ochi, T. Yoshimoto, S. Yamamoto, K. Taniguchi and T. Miyamoto, *J. biol. Chem.* **258**, 5754 (1983).
18. J. Folch, M. Lees and G. H. S. Stanley, *J. biol. Chem.* **226**, 497 (1957).
19. M. Claeys, M.-C. Coene, A. G. Herman and G. H. Jouvenaz, *Biochim. biophys. Acta* **713**, 160 (1982).
20. M. Guarnieri, *Lipids* **10**, 294 (1975).
21. K. Nakamura and S. Handa, *Anal. Biochem.* **142**, 406 (1984).
22. Y. Koshihara, T. Neichi, S.-I. Murota, A.-N. Lao, Y. Fujimoto and T. Tatsuno, *Biochim. biophys. Acta* **792**, 92 (1984).
23. H. Salari, P. Braquet, P. Borgeat, *Prostaglandins, Leukotrienes and Medicine* **13**, 53 (1984).
24. K. Sekiya, H. Okuda and S. Arichi, *Biochim. biophys. Acta* **713**, 68 (1982).
25. E. E. Kröner, B. A. Peskar, H. Fischer and E. Ferber, *J. biol. Chem.* **256**, 3690 (1981).
26. R. J. Smith, F. F. Sun, B. S. Iden, B. J. Bowman, H. Sprecher and J. C. McGuire, *Clin. Immunol. Immunopathol.* **20**, 157 (1981).
27. R. W. Bonser, M. Siegel, S. M. Chung, R. T. McConnel and P. Cuatrecasas, *Biochem.* **20**, 5297 (1981).
28. W. F. Stenson and C. W. Parker, *J. clin. Invest.* **64**, 1457 (1979).
29. C. W. Parker, J. P. Kelly, S. F. Falkenheim and M. G. Huber, *J. exp. Med.* **149**, 1487 (1979).
30. S. Rapoport, B. Härtel and G. Hausdorf, *Eur. J. Biochem.* **139**, 573 (1984).
31. U. Bretz and M. Baggiolini, *J. cell. Biol.* **63**, 251 (1974).
32. S. T. Hoffstein, in: *The Cell Biology of Inflammation*, (Ed. Weissman, G.), p. 387. Elsevier, Amsterdam (1980).
33. H. M. Korchak, S. T. Hoffstein and G. Weissman, in: *The Secretory Granule* (Eds. Poisner, A. M. and Trifaro, J. M.), p. 317. Elsevier, Amsterdam (1982).
34. I. M. Goldstein, J. K. Horn, H. B. Kaplan and G. Weissmann, *Biochem. biophys. Res. Comm.* **60**, 807 (1974).
35. J. I. Gallin, J. A. Sandler, D. G. Wright, V. A. Manganiello and M. Vaughan, *Fedn Proc.* **35**, 492 (1976).
36. Y. Hamasaki and H.-H. Tai, *Biochim. biophys. Acta* **793**, 393 (1984).
37. C. Yokoyama, K. Mizuno, H. Mitachi, T. Yoshimoto, S. Yamamoto and C. R. Pace-Asciak, *Biochim. biophys. Acta* **750**, 237 (1983).
38. D. H. Nugteren, *Biochim. biophys. Acta* **380**, 299 (1975).
39. P. P. K. Ho, P. Walters and H. R. Sullivan, *Biochem. biophys. Res. Comm.* **76**, 398 (1977).
40. M. Lagarde, M. Croset, K. S. Authi and N. Crawford, *Biochem. J.* **222**, 495 (1984).
41. W. C. Chang, J. Nakao, H. Orimo and S. Murota, *Biochem. J.* **202**, 771 (1982).
42. T. Yoshimoto, Y. Miyamoto, K. Ochi and S. Yamamoto, *Biochim. biophys. Acta* **713**, 638 (1983).
43. A. J. Marcus, L. B. Safier, J. Broekman, H. L. Ullman, N. Islam, T. C. Sorrell, C. N. Serhan, G. Weissman, T. D. Oglesby and R. R. Gorman, *J. Allergy Clin. Immunol.* **74**, 338 (1984).
44. A. J. Marcus, M. J. Broekman, L. B. Safier, H. L. Ullman and N. Islam, *Biochem. biophys. Res. Comm.* **109**, 130 (1982).
45. J. Maclouf, B. Fruteau de Laclos and P. Borgeat, *Proc. natn. Acad. Sci. U.S.A.* **79**, 6042 (1982).
46. A. J. Marcus, B. B. Weksler, E. A. Jaffe and M. J. Broekman, *J. clin. Invest.* **66**, 979 (1980).
47. F. Fitzpatrick, W. Liggett, J. McGee, S. Bunting, D. Morton and B. Samuelsson, *J. biol. Chem.* **259**, 11403 (1984).