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Metabolism of leukotrienes by L-γ-glutamyl-transpeptidase and dipeptidase from human polymorphonuclear granulocytes

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Summary. Stimulation of human polymorphonuclear granulocytes with the calcium-ionophore A23187 and opsonized zymosan leads to the release of leukotrienes. The cell-free supernatants of the stimulated cells revealed γ-glutamyl-transpeptidase and dipeptidase activity which induced the metabolism of exogeneously added LTC_4 and LTD_4 respectively. No glutathione-S-transferase activity was present in the supernatant. In the absence of calcium, no leukotrienes were generated; dipeptidase activity was slowly released and γ-glutamyl-transpeptidase activity was not detected. By subcellular fractionation, glutathione-S-transferase activity was present in the microsomal and cytosol fractions, and γ-glutamyl-transpeptidase and dipeptidase were recovered from the granular and microsomal fractions. By equilibrium density-gradient centrifugation, highest dipeptidase activity eluted in the range between 1·18 and 1·22 g/ml; γ-glutamyl-transpeptidase was present in the range from 1·13 to 1·18 g/ml and 1·20 to 1·22 g/ml; glutathione-S-transferase did not enter the gradient under these conditions. Solubilization of the 100,000 *g* pellet of homogenized cells with Triton X-100 led to the release of soluble γ-glutamyl-transpeptidase and dipeptidase enzymes into the supernatant.

Abbreviations: BSA, bovine serum albumin; CDNB, 1 chloro-2,4 dinitrobenzene; GST, glutathione-S-transferase; γ-GT, gamma-glutamyl-transpeptidase; LDH, lactate dehydrogenase; LT, leukotriene; MW, molecular weight; PMN, polymorphonuclear granulocyte; RIA, radioimmunoassay; RP-HPLC, reversed-phase high-pressure liquid chromatography.

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INTRODUCTION

The biosynthesis of leukotrienes is initiated in a lipoxygenase-type reaction leading to the 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This product is the precursor of 5-HETE and leukotriene A_4 (Hammarström, 1983). Leukotriene A_4, the instable allylic epoxide, is the key compound in the leukotriene pathway. Leukotriene A_4 can be hydrolysed to the chemotactic and chemokinetic active compound LTB_4 by a hydrolase (Rådmark *et al.*, 1980; Borgeat & Samuelsson, 1979), and non-enzymatically to other dihydroxy acids (LTB_4-isomers, 5(S)12(R)-6-trans-LTB_4 and 5(S)-12(S)-6-trans-LTB_4). Omega oxidation of LTB_4 and its isomers leads to the formation of 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (Feinmark *et al.*, 1981; Powell, 1984). Alternatively, LTA_4 can conjugate with glutathione to produce LTC_4 catalysed by glutathione-S-transferase. LTC_4 (=5(S) hydroxy-6(R)S-glutathionyl-7,9, trans 11,14 cis eicosatetraenoic acid) can be transformed to 5(S)hydroxy-6(S)S-cysteinyl-glycyl-7,9-trans 11,14 cis-eicosatetraenoic acid = LTD_4 by removal of the γ-glutamyl moiety with the γ-glutamyl-transpeptidase. This enzyme catalyses the initial

step in the metabolism of glutathione, glutathione disulphide and various glutathione conjugates (Meister & Tate, 1976).

Enzymatic conversion of LTC_4 to LTD_4 has been shown with purified γ-glutamyl-transpeptidase from kidney (Sok *et al.*, 1980; Örning & Hammarström, 1982; Hammarström, 1981), with lung- (Sirois & Brosseau, 1983), with RBL-cell- (Jakschik, Harper & Murphy, 1982) and with PMN-homogenate (Brom *et al.*, 1984). The last step in the metabolism is the cleavage of the carboxyl-terminal glycine from LTD_4 to form LTE_4 by LTD_4-dipeptidase. LTC_4, LTD_4 and LTE_4 were shown to be the bioactive compounds referred to for 40 years as slow-reacting substances of anaphylaxis (SRS-A).

In the past, we presented evidence to suggest that the stimulation of human neutrophils with zymosan, melittin (Kroegel *et al.*, 1981), bacterial exotoxins and endotoxins (Bremm *et al.*, 1983) and the calcium ionophore (Czarnetzki, König & Lichtenstein, 1975) induces the generation of the eosinophil chemotactic factor (EFC) which is identical with LTB_4 and its isomers (König, Kunau & Borgeat, 1982). It is also well known that ionophore A23187 causes release of granule-associated enzymes (Zabucchi *et al.*, 1975; Smith & Ignarro, 1975; Lee *et al.*, 1983), slow reacting substances of anaphylaxis (Conroy, Orange & Lichtenstein, 1976) and the production of oxygen-derived free radicals (Serhan, Korchak & Weissman, 1980) from neutrophils.

Generation of SRS by stimulation with the Ca-ionophore A23187 was also described for eosinophils (Jörg *et al.*, 1982). The purpose of the present investigation was to determine the release of leukotrienes and leukotriene-transforming enzymes (glutathione-S-transferase, L-γ-glutamyl-transpeptidase and LTD_4-dipeptidase) from human neutrophils stimulated with the Ca-ionophore A23187 and to functionally characterize the solubilized enzymes.

MATERIALS AND METHODS

Commercial source of reagents

Ca-ionophore A23187, zymosan A, *Micrococcus lysodeicticus*, phenolphthalein glucuronidate and heparin were obtained from Sigma Chemical Co., Munich, FRG; Ficoll 400 was from Pharmacia, Uppsala, Sweden; Dextran-Macrodex (6%) was from Knoll, Ludwigshafen, FRG. [^{3}H]LTC_4 (specific activity 35·7 Ci/mM) was from New England Nuclear, Dreieich, FRG.

Preparation of cells

This has been described elsewhere (Brom *et al.*, 1984).

Buffers

The medium used for washing the cells and for mediator release, unless stated otherwise, was a Tris-buffer (pH 7·4, 25 mM) with NaCl (120 mM), KCl (4 mM), $CaCl_2$ (0·6 mM) and $MgCl_2$ (1 mM) (referred to as TCM). In cases where PBS (pH 7·4) was used, it was supplemented with 1 mM $CaCl_2$ before the addition of the stimulus.

Cell breakage and subcellular fractionation

This has been described elsewhere (Brom *et al.*, 1984).

Stimulation of neutrophils

PMN (2×10^7/ml) were incubated with the ionophore A23187 at concentrations from 17·3 to $0{\cdot}17 \times 10^{-6}$ M for 20 min at 37°, or at different times for kinetic experiments. In some experiments, the cells were stimulated in the presence or absence of 1 mM Ca^{2+}. The incubation was stopped by centrifugation in the cold for 10 min at 400 ***g***. Alternatively, cells were incubated with zymosan coated with complement at a final concentration of 2 mg/ml for different times.

Analysis of the leukotriene release

For analysis of leukotriene release, the supernatants of the stimulated cells were deproteinized by the addition of two-volume acidified methanol (methanol:acetic acid = 1000:1) overlayered with argon and frozen at −70° for 12 hr. After centrifugation at 3000 ***g***, the supernatants were evaporated to dryness under a stream of nitrogen and resuspended in 400 μl methanol:1 water = 30:70 for reversed-phase HPLC; HPLC-analysis was performed using a Nucleosil C 18 column (5 μm, 4 × 200 mm, Macherey Nagel, Düren, FRG) with methanol:water:acetic acid (64:36:0·08, pH 5·9, titrated with ammonia) as eluent.

Under these conditions, the retention times of LTB_4 amounted to 19·60–20·00 min, LTC_4 11·30–11·70 min; LTD_4 17·20–17·60 min, and LTE_4 20·60–21·50 min, respectively. Identification of leukotrienes was assessed by the determination of the retention times and the comparison with external standards of synthetic leukotrienes (gift from Dr Rokach, Merck Frosst, Canada). The area integration of the absorption peaks allows the quantitative analysis of the substances.

With the described extraction procedure, the recovery rates of leukotrienes from 250 μl of cell

supernatants or 100 μl of subcellular fractions were 80–85% for leukotrienes C_4, D_4 and E_4 and 90–95% for leukotriene B_4. The overall recovery was obtained from subcellular fractions adding the respective leukotriene to aliquots of the fraction, subsequently followed by addition of acidified methanol to avoid the leukotriene conversion. Standard curves of the individual leukotrienes were obtained with five different concentrations (5–125 ng) and showed the following correlation: LTC_4—0·985; LTD_4—0·995; LTE_4—0·985; LTB_4—0·999. The detectable minimum quantities were: LTC_4—5 ng, LTD_4—2 ng, LTE_4—2 ng and LTB_4—1 ng.

Analysis of the leukotriene-transforming enzyme release

For analysis of the leukotriene-transforming enzymes (γ-glutamyl-transpeptidase by LTC_4-conversion and LTD_4-dipeptidase by LTD_4-conversion), 250-μl aliquots of the stimulated cell supernatants were incubated with 90 ng synthetic LTC_4 or 80 ng synthetic LTD_4 at 37°. The incubation times for LTC_4-conversion were 30 and 60 min, and for LTD_4-conversion were 15, 30 and 60 min. The incubation was stopped by addition of 1 ml acidified methanol; the extraction for reversed-phase HPLC was performed as above. L-γ-glutamyl-transpeptidase and LTD_4-dipeptidase activity were expressed as percentages of converted LTC_4 and LTD_4 respectively.

Conversion of leukotrienes by subcellular fractions

The subcellular and the density-gradient fractions obtained after differential and density-gradient centrifugation were either incubated in the presence of 0·01% Triton X-100 with synthetic LTC_4 or LTD_4 (each 90 ng) for 120 min at 37°. The fractions were extracted as described above and analysed by reversed-phase HPLC. These fractions were also incubated with 200 ng LTA_4 in the presence of 1 mM glutathione, 1 mM calcium and serine-borate (200 μM L-serine and 10 mM sodium borate) (Tate & Meister, 1978; Örning & Hammarström, 1980). The samples were deproteinized with acidified methanol, evaporated to dryness and resuspended in 200 μl Tris-HCl buffer (pH 7·4, 0·01 M containing 0·14 M NaCl and 0·1% gelatine). The radioimmunoassay for LTC_4 was performed as has been described. An LTC_4-BSA conjugate served as an immunogen using glutaraldehyde as coupling reagent. The coupling as well as immunization procedure was performed as has been described (Aehringhaus *et al.*, 1982).

Biochemical assays

(a) Determination of marker-enzymes. The supernatants and the fractions were assayed for the contents of LDH, lysozyme, peroxidase, β-glucuronidase, alkaline and acid pnp-phosphatase (tests have been described by Frickhofen & König, 1979; Bretz & Baggiolini, 1974) and the protein. The extracellular marker-enzyme release was calculated a percentage of the total enzyme activity available after sonication of an unstimulated neutrophil suspension (2×10^7/ml).

(b) Photometric determination of the leukotriene-transforming enzymes. The analysis of the glutathione-S-transferase activity was performed according to Carmagnol *et al.* (1981), the L-γ-glutamyl-transpeptidase (EC2.3.2.2) was estimated by a fluorometric assay according to Smith, Ding & Peters (1979), and the photometric assay for dipeptidase was described by Patterson (1976).

Radioimmunoassay for LTC_4

For radioimmunoassay, an appropriate anti-plasma dilution, as well as either standard LTC_4 (10 ng–25 pg) or unknown samples, were added to tubes containing [^{3}H]LTC_4 (15,000 d.p.m.) in a total volume of 0·6 ml. All dilutions were made in a Tris-HCl buffer (pH 7·4, 0·01 M with 0·14 M NaCl and 0·1% gelatine). After incubation at 4° overnight or for 2 hr at 37°, antibody-bound and free ligands were separated using 0·5 ml charcoal suspension (20 mg/ml). After charcoal precipitation by centrifugation, 0·9 ml of the supernatants was added to 9 ml Szintigel (Roth, Karlsruhe, FRG). The radioactivity was determined in a liquid scintillation counter.

Enzyme solubilization

PMNs (5×10^7 ml) were homogenized in a Potter-Elvehjem homogenizer; for cell breakage, human neutrophils (final concentration 5×10^7/ml) were suspended in hypotonic sucrose medium (0·1 M sucrose in Tris-HCl, 5 mM, 7·4) and homogenized in a Potter-Elvehjem teflon glass homogenizer (Braun Melsungen, 20 ml, clearance 0·095–0·0115 mm) driven by a drilling machine. After homogenization, the suspension was adjusted to isotonicity by the addition of the same volume of 0·58 M sucrose medium in 5 mM Tris HCl, pH 7·4. During homogenization, the cells were kept in ice and the degree of cell breakage was followed by light microscopy using toluidine blue staining; the suspension was centrifuged at 400 ***g*** and 3000 ***g*** for 15 min to obtain two low-speed pellets (400 ***g*** pellet=

Fraction 1, 3000 *g* pellet = Fraction 2); the 3000 *g* supernatant was further separated by centrifugation (100,000 *g*, 30 min) into a high-speed pellet and a high-speed supernatant (100,000 *g* supernatant = Fraction 3). The high-speed pellet was resuspended in 0·34 M sucrose buffer which contained Triton X-100 at a concentration of 0·3% and was incubated for 30 min at 4°. Repeated centrifugation of the suspension at 100,000 *g* for 30 min led to a 100,000 *g* pellet (Fraction 4) and 100,000 *g* supernatant (Fraction 5). Enzyme activities as well as leukotriene metabolizing enzymes were analysed as has been described.

The experiments described here were repeated at least five times.

RESULTS

Human polymorphonuclear leucocytes (1×10^7/ml) were stimulated with different concentrations of calcium-ionophore A23187. The released leukotrienes were detected by reversed-phase HPLC or radioimmunoassay. As is apparent, LTB_4 and LTC_4 release occurred at early times of incubation. A maximum was released after 20 min of incubation. The quantities of LTD_4 and LTE_4 in this experiment were too minute to be detected by HPLC. Leukotriene release was obtained with a concentration of 1×10^{-6}/ M (Fig. 1a). With regard to LTB_4 release, a donor-dependent pattern was observed. In several cases, a marked decrease in LTB_4 release was obtained after 20 min of incubation. Depending on the ionophore concentration, an increase in lysozyme release was detected (Fig. 1c). LDH was released by 15% at a concentration of $17{\cdot}6 \times 10^{-6}$ M ionophore. Thus, the ionophore-induced liberation of leukotrienes, as demonstrated in all experiments, occurred under non-cytotoxic conditions. The data suggest the secretion of the specific, but not of azurophilic granules by the calcium-ionophore.

Experiments were then carried out to analyse the leukotriene-transforming enzymes (γ-glutamyl-transpeptidase, LTD_4-dipeptidase) from stimulated cells. For this purpose, the cell-free supernatants were incubated with synthetic LTC_4 and LTD_4, respectively.

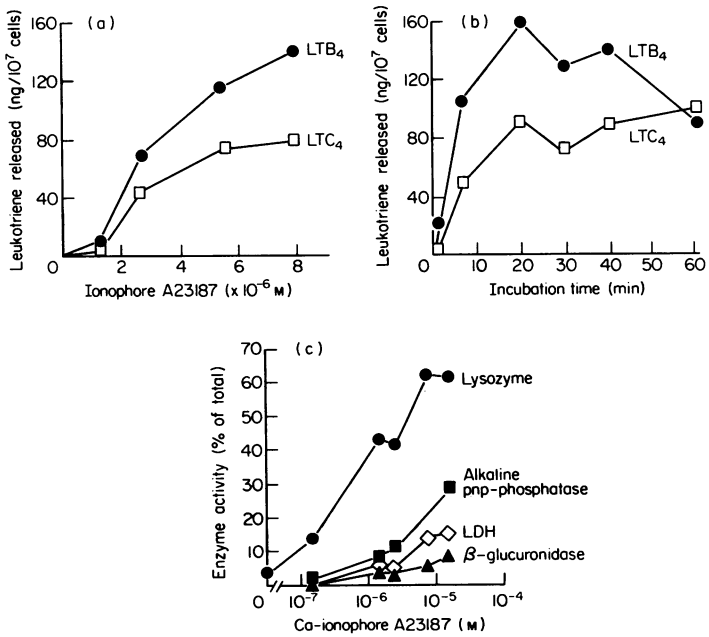

Figure 1. Leukotriene release from human PMN after stimulation with the calcium-ionophore A23187. Leukotrienes were analysed by HPLC. (a) Dose-response studies: incubation of the cells with different concentrations of the ionophore. (b) Time-dependent release of leukotrienes (calcium-ionophore concentration: $7{\cdot}9 \times 10^{-6}$M). (c) Release of granular and cytosolic enzymes from ionophore-stimulated cells. The total enzyme activities were determined from sonicated non-stimulated cells. The data represent a characteristic experiment; the donor-specific variation of leukotriene release was ±30% for six experiments.

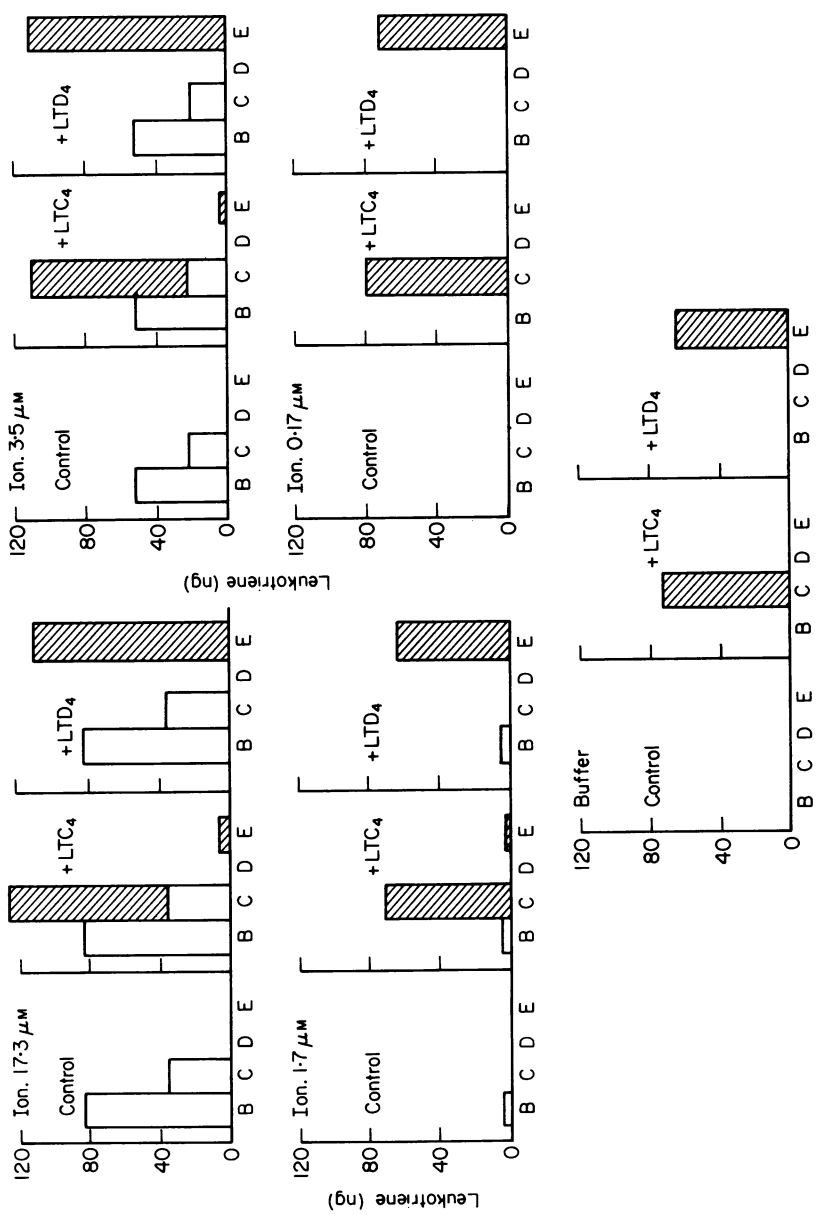

Figure 2. Determination of leukotriene-transforming enzymes (γ-glutamyl-transpeptidase, LTD_4-dipeptidase) in the supernatant. Neutrophils were stimulated with various concentrations of the calcium-ionophore A23187. After stimulation, aliquots of the cell supernatants were incubated with LTC_4 or LTD_4 for 60 min at 37°. Conversion of leukotrienes was analysed by RP-HPLC: (□) leukotriene content in the appropriate cell supernatant; (▩) turnover of synthetic LTC_4/LTD_4 into LTE_4; (B) LTB_4; (C) LTC_4; (D) LTD_4; (E) LTE_4. The data represent a typical experiment; the donor-specific variation of leukotriene-transforming enzymes ± 15% is measured from three experiments.

Leukotriene metabolites were analysed by reversed-phase HPLC. Figure 2 shows the quantities of leukotrienes (B_4, C_4) in the cell supernatants. Our data demonstrate that less than 10% of the added LTC_4 was metabolized as was calculated from the formation of LTE_4 with cell supernatants generated with 17·3–1·7 μM ionophore. In contrast, LTD_4 was transformed into LTE_4 with each supernatant obtained after ionophore stimulation (17·3–0·17 μM), as well as with the supernatant from unstimulated cells. Kinetic studies were then performed to analyse the leukotriene metabolism more precisely. The supernatant of ionophore-stimulated cells was incubated with LTD_4 and the time-dependent metabolism studied. It is apparent that transformation of LTD_4 had already started after 1 min of incubation and was completely finished after 10 min. The supernatant of non-stimulated cells also revealed LTD_4 transforming activity which was less pronounced; after 10 min of incubation, about 40% of LTD_4 was still recovered; after 30 min, LTD_4 was completely transformed into LTE_4 (Fig. 3).

We then studied the role of calcium with regard to the ionophore-induced leukotriene release, the liberation of leukotriene-transforming enzymes and the release of marker enzymes (Fig. 4). Granulocytes were suspended in PBS instead of TCM-buffer. The cells were then stimulated with the calcium-ionophore in the presence of Ca^{2+} or in its absence. Without the addition of calcium, no leukotrienes were detected in the supernatant. Lysozyme release slowly increased during prolonged incubation. The release of LTD_4 metabolizing activity showed a plateau after 20 min of incubation. These data differ remarkably from those obtained in the presence of calcium. There were no significant differences in enzyme release from non-stimulated cells and cells stimulated with the calcium-ionophore A23187 in the absence of calcium.

Under these conditions, leukotrienes were released as has been described; the maximal lysozyme release occurred after 30 min of incubation. Maximal LTD_4-converting activity (~85%) was generated from the cells within the first 20 min of incubation. LTC_4-metabolizing activity was detected after 30 min of incubation and steadily increased. Glutathione-S-transferase activity was not recovered from the cell-free supernatants. For comparison, human neutrophils were stimulated with opsonized zymosan. The cell-free supernatant was analysed for the release of marker enzymes as well as LT metabolizing activity (Fig. 5). As was shown, stimulation with opsonized zymosan led to the secretion of specific granules: e.g. lysozyme activity increased in the supernatant in the absence of β-glucuronidase and peroxidase activity (data not shown). LTD_4 converting activity increased within the first 10 min of cell stimulation, while LTC_4-metabolizing activity revealed a plateau after 30 min of incubation. As is apparent, the exogeneously added LTC_4 was completely metabolized.

A more detailed analysis was carried out with PMNs which had been subjected to subcellular fractionation. Human PMNs were homogenized and further processed by differential centrifugation at 400 *g*, 3000 *g*, 20,000 *g*, and 200,000 *g*, with a final 200,000 *g* supernatant (Fig. 6a). Our data were based on 10 different experiments and confirmed that Fraction 1 (400 *g* pellet) mainly contained intact cells and large fragments; Fractions 2 and 3 (3000 *g* and 20,000 *g* pellets) azurophilic granules; Fraction 4 (200,000 *g*)

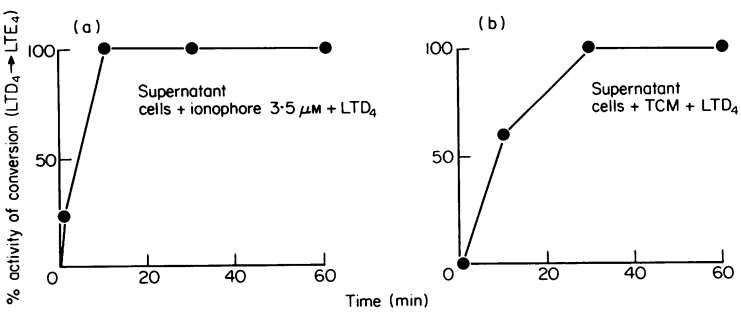

Figure 3. Kinetic analysis of LTD_4-turnover. PMNs (1×10^7) were stimulated with (a) the calcium-ionophore (3·5 μM) or (b) TCM-buffer for 15 min at 37°. The cell supernatants were then incubated with synthetic LTD_4 (80 ng) for various time intervals and the conversion rate to LTE_4 was analysed by reversed-phase HPLC. Values are expressed as percentage conversion of LTD_4 into LTE_4. This figure shows a representative experiment; donor-specific variation of LTD_4-dipeptidase release was ±15% measured from three experiments.

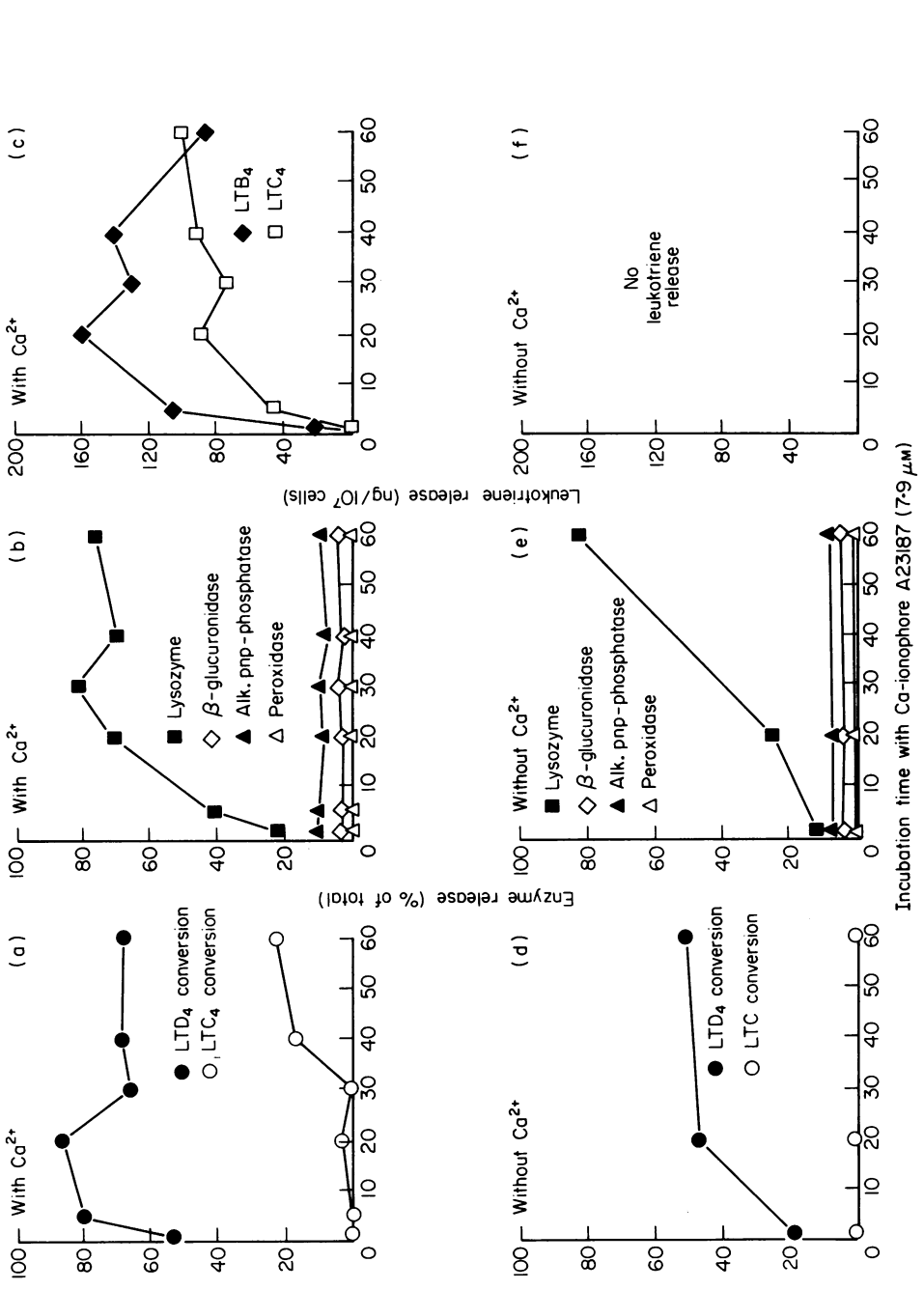

Figure 4. Stimulation of intact human neutrophils with calcium-ionophore A23187 (7·9 μM) in the presence (top row) or absence (bottom row) of 1 mM Ca^{2+} at various times. (a)+(d), cell supernatants were analysed for γ-glutamyl-transpeptidase and LTD_4-dipeptidase activity as was detected by conversion of exogeneously added synthetic leukotrienes (incubation time for LTC_4 and LTD_4 amounted to 30 min and 15 min, respectively); (b)+(e), release of granular and microsomal marker enzymes; (c)+(f), leukotriene release from cells as was detected by RP-HPLC in the presence and absence of calcium. Data represent a typical experiment out of three individual experiments.

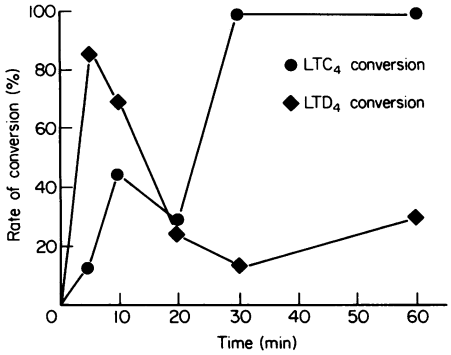

Figure 5. Analysis of γ-glutamyl-transpeptidase and LTD_4-dipeptidase activity in the cell supernatants of granulocytes stimulated with opsonized zymosan (2 mg/2×10^7 PMNs) obtained at various times. The cell-free supernatant was incubated with either synthetic LTC_4 or LTD_4. After 120 min, the conversion rates of exogeneously added LTC_4 and LTD_4 were analysed by HPLC. This figure represents a typical experiment; with zymosan-stimulation, the donor-specific variation was ±20% ($n=3$ experiments).

specific granules and microsomes, and Fraction 5 (200,000 *g* supernatant) cytoplasm. Glutathione-S-transferase activity was studied by photometric analysis with CDNB as substrate and by the generation of LTC_4 by incubating the fractions with LTA_4 (200 ng) in the presence of Ca^{2+}, glutathione and serine-borate. LTC_4 was measured by radioimmunoassay. With both methods, GST-activity was most pronounced in the cytoplasm (Fraction 5). Significant activity of GST was also observed in Fraction 4 after incubation with LTA_4 (Fig. 6b). γ-GT-activity was further studied by the fluorometric assay or the capability to convert LTC_4. In the former studies, the highest activity was present in Fraction 4, less activity in Fraction 3; LTC_4-metabolizing activity was equally distributed in Fractions 3 and 4, and was significantly present in Fraction 2. No γ-GT-activity was observed in the cytoplasmic fraction, When dipeptidase activity was analysed photometrically, peak activity resided in Fractions 3 and 4; however, fractions 2 and 5 also contained considerable activity. In contrast, the conversion of LTD_4 into LTE_4 was most pronounced in Fractions 3 and 4 (Fig. 6b).

A further analysis of enzymatic activity was carried out by subjecting the 400 *g* postnuclear supernatant of a PMN homogenate to equilibrium density centrifugation (Fig. 7). By biochemical analysis, the marker enzymes for the azurophilic granules distributed between 1·20 and 1·23 g/ml; lysozyme without peroxidase activity and minute amounts of β-glucuronidase were present in the range of 1·15–1·19 g/ml; glutathione-S-transferase activity coeluted with LDH on top of the gradient, γ-GT-activity ranged between 1·13–1·18 g/ml and 1·20–1·22 g/ml, as studied by LTC_4 turnover. Highest LTD_4-dipeptidase activity was obtained in the range between 1·18 and 1·22 g/ml.

A further analysis of the leukotriene-metabolizing enzymes was obtained by solubilization with Triton X-100. Homogenized granulocytes were pelleted at 400 *g*, 3000 *g* and 100,000 *g*, respectively. The latter pellet was treated with Triton X-100 for 30 min; subsequently, the fractions were centrifuged again; a 100,000 *g* pellet and supernatant were obtained. The various fractions were assayed for protein and enzyme

Table 1. Solubilization of γ-glutamyl-transpeptidase and dipeptidase

Fraction	LTC_4 conversion (%)	LTD_4 conversion (%)	β-glucuronidase (%)	Lysozyme (%)	LDH (%)
400 *g* pellet	0	10±4·3	15·2±3·6	6·4±3·5	26·4±6·2
3000 *g* pellet	22·4±7·5	22·5±5·0	31·8±6·0	20·0±6·0	15·0±4·9
100,000 *g* supernatant	9·2±2·8	14·5±3·3	7·9±3·0	3·5±3·2	42·2±5·5
Pellet + 0·3% Triton X-100 30 min, +4°					
100,000 *g* pellet	0	5·5±4·0	9·3±4·5	35·4±6·5	3·5±1·9
100,000 *g* supernatant	68·5±10·2	47·5±6·3	35·7±4·9	34·7±5·5	12·9±4·2

Solubilization of subcellular fractions with 0·3% Triton X-100: synthetic LTC_4 or LTD_4 was added to the various fractions and incubation proceeded for 60 min. The turnover was analysed by reversed-phase HPLC. Buffer with 0·3% Triton X-100 served as control. Marker enzymes were detected as described in the Materials and Methods. Enzyme activities were referred to the same protein content; total enzyme activity of the five fractions is expressed as 100%. Each value represents the mean ± SD of three experiments.

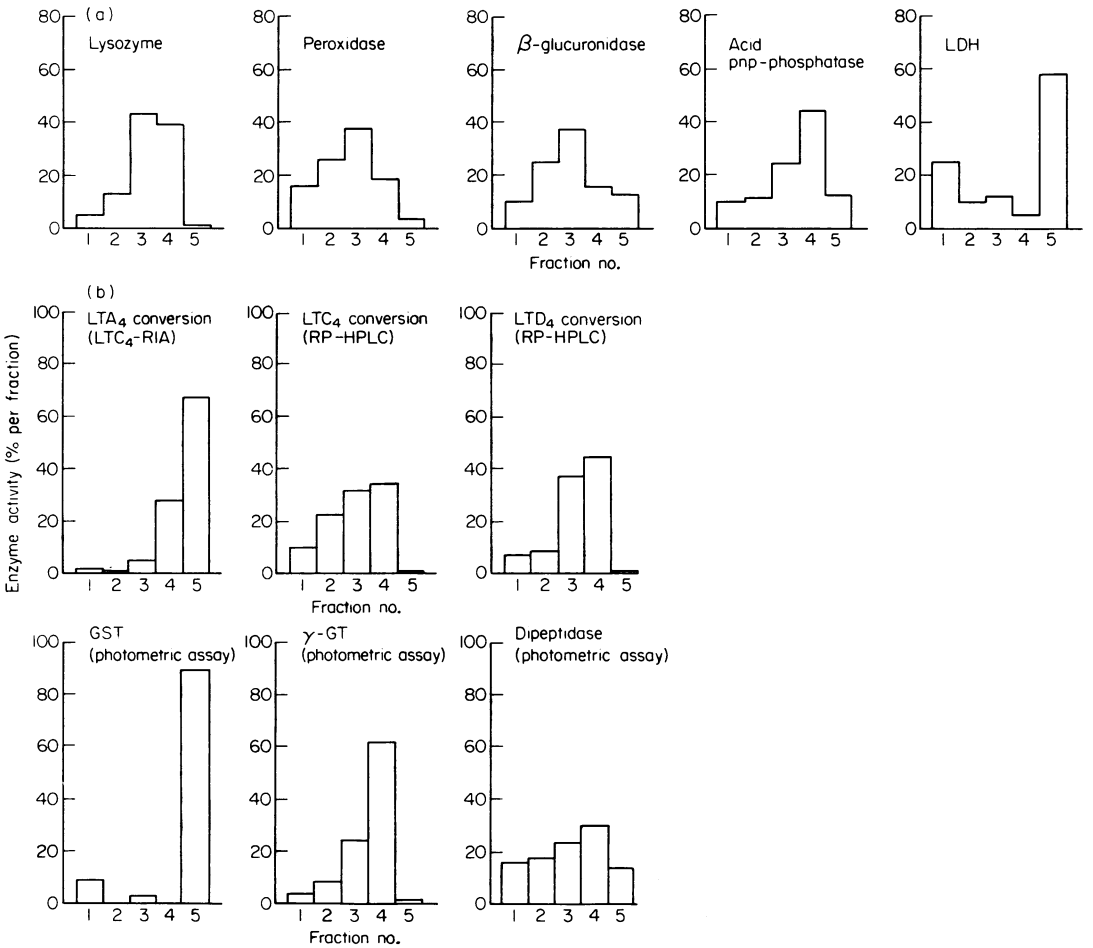

Figure 6. Subcellular fractionation of human PMNs (5×10^7/ml). Steps indicate the five fractions (Fraction 1 = 400 *g* pellet, Fraction 2 = 3000 *g* pellet, Fraction 3 = 20,000 *g* pellet, Fraction 4 = 200,000 *g* pellet and Fraction 5 = 200,000 *g* supernatant) obtained from PMN homogenate after differential centrifugation at 400, 3000, 20,000 and 200,000 *g*. Enzyme activities were referred to the same protein content; total enzyme activity of the five fractions is expressed as 100%. Data represent a typical enzyme distribution from $n = 10$ experiments with a variation of $\pm 7\%$. (a) distribution of marker enzymes (granular and cytosolic); (b) Localization of glutathione-S-transferase as was determined by conversion of LTA_4 with a specific LTC_4-RIA or according to the method of Carmagnol *et al.* (1981). Conversion of LTC_4 or LTD_4: comparison of turnover rates for synthetic leukotrienes with the photometric analysis for γ-glutamyl-transpeptidase and dipeptidase.

content. In addition, the LTC_4- and LTD_4-metabolizing activities were studied. It is apparent that the treatment of the fractions with Triton X-100 led to a remarkable solubilization of the leukotriene-metabolizing enzymes (Table 1). Further studies are currently in progress to analyse these solubilized enzymes.

DISCUSSION

Our data demonstrate the dose- and time-related release of LTB_4 and LTC_4 from human PMNs on stimulation with the calcium-ionophore A23187. Although donor-dependent variations of leukotriene

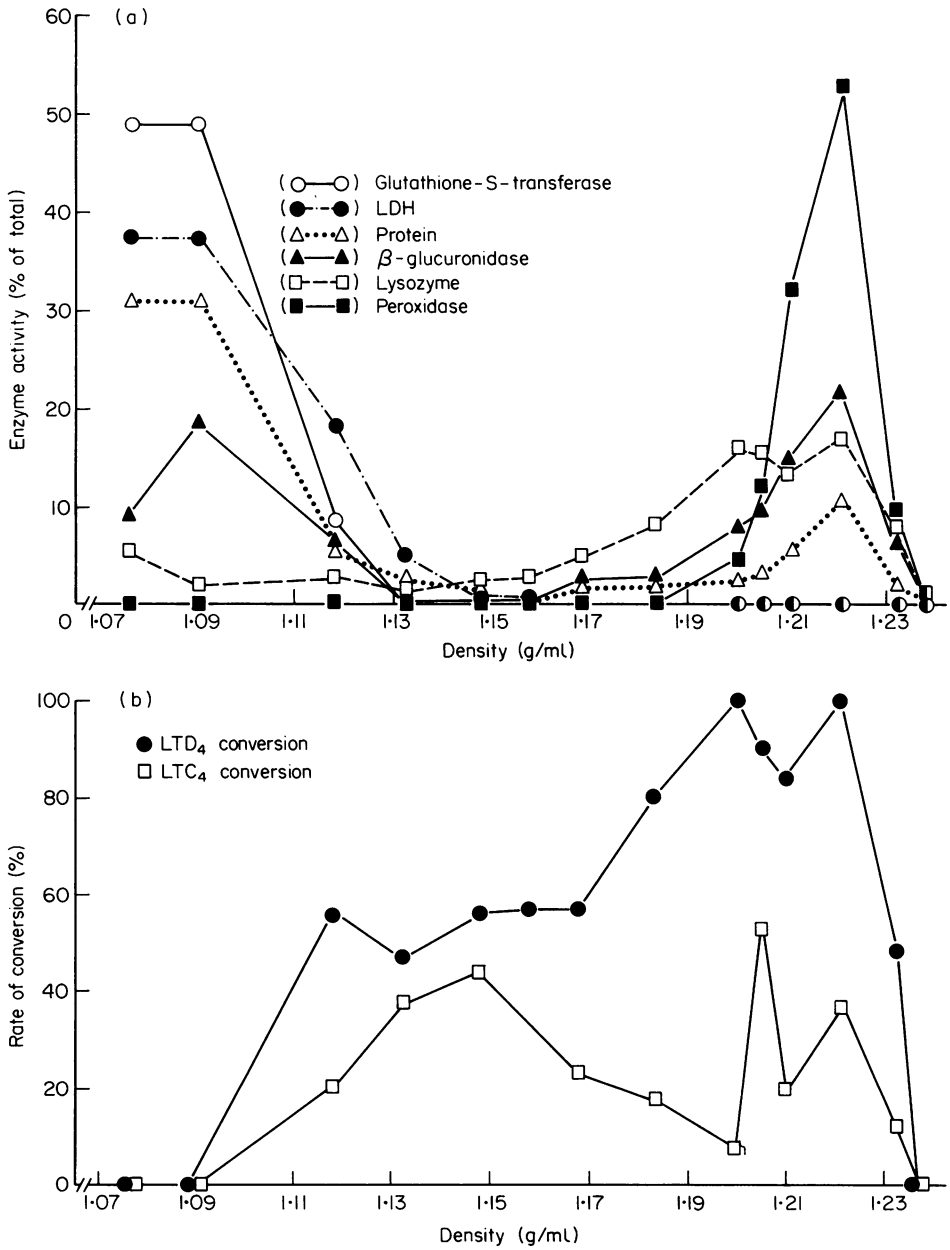

Figure 7. Separation of a postnuclear supernatant by equilibrium density centrifugation using a linear sucrose gradient from 19 to 54% w/v: (a) distribution of enzyme markers; (b) analysis of LTC_4 and LTD_4 converting activities; synthetic LTC_4 and LTD_4 were added to the various fractions and incubated for 120 min at 37°. The rate of conversion was detected by RP-HPLC.

release were observed, the release profiles of different experiments were very similar, in that a rapid release occurred which, in some cases, was followed by a decrease at later times of incubation. The liberation of leukotrienes occurred under non-cytotoxic conditions. Palmer & Salmon (1983) described the time-dependent release of LTB_4 after ionophore stimulation. In these experiments, 10^6 cells released 5–10 ng LTB_4 as was measured by radioimmunoassay. Our data obtained by HPLC-analysis correlate well with these results. By HPLC-analysis as well as RIA, LTC_4 activity was also detected in the supernatant. As is apparent from our data, the enzymes which induce and metabolize the peptido-leukotrienes are localized in different cellular compartments. The main glutathione-S-transferase activity was present within the cytosol and microsomal fraction, γ-glutamyl-transpeptidase in the granular and microsomal fractions, and the dipeptidase was correlated with the specific granules. Glutathione-S-transferase has been purified from human and rat liver with a MW of 45,000–49,000 (Habig & Jakoby, 1981b). γ-glutamyl-transpeptidase was solubilized by a proteinase from rat kidney with a MW of 68,000; it contained two subunits with MWs of 46,000 and 22,000, respectively (Meister, Tate & Griffith, 1981); the dipeptidase has been characterized using rat renal enzyme (Kozak & Tate, 1982), and samples from the human pancreas (Ito, Sugiura & Sawaki, 1983). Molecular weights of 105,000 for the former and 135,000 for the latter have been calculated, with subunits of 50,000 and 68,000. The major activity of glutathione-S-transferase obtained with LTA_4 and CDNB as substrates was localized in the 200,000 *g* supernatant. With LTA_4 as substrate, about 1/3 of total activity was also detected in the 200,000 *g* pellet.

Jakschik & Kuo (1983) described the glutathione-S-transferase enzyme in RBL-1 cells as a structurally bound enzyme present in the 10,000 *g* pellet. By further separating the plasma membranes, these results were not confirmed. GST activity was neither associated with the plasma nor the microsomal membranes. Quite recently, Bach, Brashler & Morton (1984) presented evidence to suggest that a particulate (LTC_4-synthetase), as well as soluble, glutathione-S-transferase was present in RBL-1 cells. A soluble glutathione-S-transferase was described for erythrocytes (Carmagnol *et al.*, 1981) and rat liver (Friedberg *et al.*, 1983). The various forms of the enzyme revealed a discrete substrate specificity (Habig & Jokoby, 1981a). One has to consider that the enzymatic activity which can be detected by photometric analysis does not necessarily correlate with the enzyme specific for the peptide-leukotrienes (Morris *et al.*, 1982). Chi-Yu & McKinney (1982) suggested that the presence of GST in microsomes is the result of specific and non-specific association between the microsomal membrane and soluble liver transferases. In our experiments, stimulated PMNs did not release GST activity into the supernatant. The major activities of γ-GT resided in the 20,000 *g* and 200,000 *g* pellets of the cell homogenate. The enzyme is a particulate enzyme, as has been described for different tissues and cell types (Jakschik *et al.*, 1982; Tate, Thompson & Meister, 1976; Anderson, Allison & Meister, 1982).

By equilibrium density-gradient centrifugation, the major activity appeared in the range of 1·13–1·18 and 1·20–1·22 g/ml.

Rosalki (1975) suggested that this enzyme is present in various tissues in a soluble and a structurally bound form; a conversion of insoluble to a soluble form might occur. In ionophore-treated cells, significant γ-GT activity appeared at later times (30 min) of stimulation in the supernatant. The simultaneous presence of Ca^{2+} and the ionophore is obligatory for its release; with opsonized zymosan as stimulus, γ-GT was released at early times of incubation and reached a plateau after 30 min. Our results confirm the data by Lee *et al.* (1983) that LTD_4-dipeptidase is associated with the specific granules as was indicated by the simultaneous release of lysozyme and subcellular fractionation data. The release of LTD_4-dipeptidase appears to be qualitatively independent from the stimuli used for cell triggering, since the calcium ionophore, melittin and oponized zymosan were effective. Incubation of cells with calcium (< 1 mM) led to the release of LTD_4-dipeptidase and of lysozyme. These data are supported by observations of Goldstein *et al.* (1974) who described a calcium-dependent lysozyme secretion. Quantitatively, LTD_4-dipeptidase activity depends on the presence of calcium and the stimulus. Jakschik & Kuo (1983) fractionated a 10,000 *g* pellet of homogenized RBL-1 cells by sucrose gradient centrifugation; they obtained a plasma membrane preparation which efficiently transformed LTC_4 to LTD_4 and LTE_4. In contrast to the release of γ-GT, the dipeptidase appears rapidly in the supernatant of stimulated cells. A pronounced decrease in enzyme activity is obtained at later times of incubation. Whether these results reflect a decrease in the release of enzyme activity, an inactivation or inhibition has to await further studies. Solubilization of a subcellular fraction (100,000 *g* pellet) with Triton X-100 led to a

significant recovery for γ-GT and dipeptidase activities. Further purification procedures are carried out to characterize these emzymes.

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