Characterization of a leukotriene C_4 export mechanism in human platelets: possible involvement of multidrug resistance-associated protein 1

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Abstract Platelets express leukotriene (LT) C_4 synthase and can thus participate in the formation of bioactive LTC_4. To further elucidate the relevance of this capability, we have now determined the capacity of human platelets to export LTC_4. Endogenously formed LTC_4 was efficiently released from human platelets after incubation with LTA_4 at 37°C, whereas only 15% of produced LTC_4 was exported when the cells were incubated at 0°C. The activation energy of the process was calculated to 49.9 ± 7.7 kJ/mol, indicating carrier-mediated LTC_4 export. This was also supported by the finding that the transport was saturable, reaching a maximal export rate of 470 ± 147 pmol LTC_4/min × 10^9 platelets. Furthermore, markedly suppressed LTC_4 transport was induced by a combination of the metabolic inhibitors antimycin A and 2-deoxyglucose, suggesting energy-dependent export. The presence in platelets of multidrug resistance-associated protein 1 (MRP1), a protein described to be an energy-dependent LTC_4 transporter in various cell types, was demonstrated at the mRNA and protein level. Additional support for a role of MRP1 in platelet LTC_4 export was obtained by the findings that the process was inhibited by probenecid and the 5-lipoxygenase-activating protein (FLAP) inhibitor, MK-886. The present findings further support the physiological relevance of platelet LTC_4 production.—Sjölinder, M., S. Tornhamre, H-E. Claesson, J. Hydman, and J. Å. Lindgren. **Characterization of a leukotrine C_4 export mechanism in human platelets: possible involvement of multidrug resistance-associated protein.** ***J. Lipid Res.*** **1999.** 40: **439–446.**

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The cysteinyl leukotrienes, LTC_4 and its metabolites, LTD_4 and LTE_4, are powerful inflammatory agents involved in allergy and hypersensitivity (1), in particular bronchial asthma (2, 3). Leukotriene formation, predominantly occurring in myeloid cells, is provoked by calcium-dependent cellular activation, which leads to phospholipase A_2-catalyzed liberation of arachidonic acid from membrane phospholipids (4, 5), and translocation of arachidonate 5-lipoxygenase to the nuclear membrane (6, 7), where it co-localizes with a 5-lipoxygenase activating protein (FLAP) (6, 8). Subsequently, the activated 5-lipoxygenase catalyze a two-step conversion of arachidonic acid into the unstable epoxide, LTA_4 (9, 10). This intermediate may either be released (11) or metabolized intracellularly by two different enzymes; the cytosolic LTA_4 hydrolase, which produces LTB_4, a leukocyte-activating dihydroxy acid (1), or the membrane-bound LTC_4 synthase, which catalyzes specific conjugation of LTA_4 with glutathione, to yield LTC_4 (12, 13). Recently, cDNA and genomic cloning of LTC_4 synthase has been reported (14–16).

Eosinophils, basophils/mast cells, and monocytes/macrophages have been demonstrated to express both 5-lipoxygenase/FLAP and LTC_4 synthase, and are thus capable of producing LTC_4 from endogenous arachidonic acid. In contrast, platelets lack 5-lipoxygenase activity but contain LTC_4 synthase (17) and can produce LTC_4 when supplied with LTA_4 via transcellular mechanisms (18). Thus, platelets have been demonstrated to produce LTC_4 when coincubated with granulocytes that were stimulated to produce LTA_4 by ionophore treatment (19) or physiological stimuli (20). The LTC_4 synthase activity in human platelets was recently reported to be regulated by receptor-dependent mechanisms (21).

Upon synthesis, LTB_4 and LTC_4 are released to the extracellular space where these compounds can interact with specific receptors. Also, LTC_4 is transformed to the likewise biologically active LTD_4 and LTE_4 (1). The release as well as the removal of leukotrienes from the blood circulation has been reported to be controlled by specific transport mechanisms (for review see ref. 22). Carrier-mediated export of LTC_4 was first described in intact human eosinophils and human myeloid leukemic cell lines (23, 24). The mechanism of LTC_4 transport has been studied in plasma membrane vesicles prepared from murine mas-

Abbreviations: LT, leukotriene; MRP, multidrug resistance-associated protein; FLAP, 5-lipoxygenase activating protein.

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tocytoma cells and the export has been characterized as a specific ATP-dependent process (25).

More recently, multidrug resistance-associated protein 1 (MRP1) has been identified as an ATP-dependent transporter of LTC_4 and other glutathione S-conjugates (26, 27). This membrane glycoprotein is a 190 kDa member of the ATP-binding cassette transmembrane transporter superfamily. MRP1 was discovered because of its overexpression in a number of drug-resistant human tumor cell lines and cytostatic drugs have been demonstrated to function as substrates for MRP1 transport (for review, see ref. 28). Interestingly, LTC_4 binds to MRP1 with much higher affinity than any other known substrate, suggesting that the leukotriene may be an endogenous ligand for this transporter (26).

Analysis of the subcellular distribution of MRP1 in adriamycin-resistant HL60 cells has demonstrated that this protein is present predominantly in the endoplasmic reticulum, but also in the plasma membrane (29). Protein and mRNA expression of MRP1 has been described in several cell-types, including LTC_4-producing cells (30). Expression of MRP1 and its isoform cMRP or MRP2 in liver and kidney suggest a role for MRP1 and MRP2 in the removal of LTC_4 from the blood circulation (31). Additional members of the MRP family may exist, as a number of genes homologous to MRP1, namely MRP3-6, have been described (32). Overexpression of the gene coding for MRP1 has been demonstrated to result in elevated ATP-dependent transport of LTC_4 (28). In contrast, overexpression of another ATP-dependent transporter, P-glycoprotein did not confer increased LTC_4 excretion (28). Furthermore, it has been demonstrated that transport of LTC_4 can be inhibited by MRP1 antibodies (28). This protein has also been demonstrated to bind LTC_4 in photoaffinity labeling experiments (27). In addition, it was recently reported that MRP1 knockout mice displayed reduced inflammatory responses which was attributed to decreased excretion of LTC_4 from LT-producing cells (33). Cellular uptake of LTC_4 has not been extensively studied. However, a recent report indicates that the protein oatp1 (Organic Anion Transporter Polypeptide) can mediate glutathione exchange-dependent uptake of LTC_4 (34).

The occurrence of LTC_4 synthase in platelets suggests that these cells may play a physiological role in the biosynthesis of cysteinyl leukotrienes. To obtain further evidence for such a role, it was of interest to investigate whether the capability of platelets to produce LTC_4 was accompanied by an ability to export this compound via active transport.

MATERIALS AND METHODS

Leukotriene C_4, prostaglandin B_2, and L-665,238 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). MK-886 and BAY X1005 were kind gifts from Dr. A. W. Ford-Hutchinson, Merck Frosst Centre for Therapeutic Research, Quebec, Canada, and Dr. R. Müller-Peddinghaus, Bayer AG, Wuppertal, Germany, respectively. Leukotriene A_4-methyl ester was generously provided by Dr. R. Zipkin, Biomol Research Laboratories, and was saponified as described (21). Probenecid, antimycin A, and 2-deoxyglucose were purchased from Sigma (St. Louis, MO). Ultraspec II was from Biotecx (Houston, TX) and 1st strand cDNA synthesis kit, PCR Master amplification mixture, and TAQ DNA sequencing kit were from Boehringer-Mannheim (Mannheim, Germany). Advantage PCR cloning kit and β-actin PCR primers were from Clontech (Palo Alto, CA). PCR primers for MRP1 were from Scandinavian Gene Synthesis (Köping, Sweden). Temperature cycling was performed on a Mastercycler 5330 from Eppendorf (Hamburg, Germany). MRP1 monoclonal antibody QCRL-1 was from Centocor (Malvern, PA). MRP2 antiserum EAG5 was kindly provided by Pr. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Horseradish peroxidase-coupled anti-mouse and anti-rabbit secondary antibodies were from Amersham (Buckinghamshire, UK). Nitrocellulose membranes, precast polyacrylamide gels, and protein standards were from Novex (San Diego, CA).

Preparation of platelet suspensions

Peripheral blood was collected (using 7.5% (v/v) 77 mm EDTA as anticoagulant) from healthy volunteers who had not taken any medication for at least 10 days. After centrifugation at 200 *g* for 15 min, the platelet-rich plasma was carefully removed in order to avoid contamination with leukocytes or erythrocytes, and further centrifuged at 650 *g* for 20 min. The platelet-containing pellet was resuspended and washed twice in Tris-buffered saline containing 1.5 mm EDTA. Platelets used for RT-PCR analysis were subjected to hypotonic ammoniumchloride lysis to avoid erythrocyte contamination. Finally, the platelets were suspended in calcium-free Krebs buffer (containing 10 mm glucose) or calcium-free phosphate-buffered saline (PBS) to a final concentration of 400×10^6 platelets/ml. The platelet suspensions contained less than 1 leukocyte/2×10^4 platelets, as judged by light microscopy.

Studies of LTC_4 synthesis and release

Platelet suspensions (0.5 ml) were supplemented with $CaCl_2$ (final conc. 0.9 mm) and equilibrated at the indicated temperature for 5 min. Thereafter, the cells were incubated as described and centrifuged at 1000 *g* for 2 min at 4°C. Subsequently, the supernatants were rapidly removed and added to three volumes of ethanol (containing prostaglandin B_2 as internal standard) prior to LTC_4 analysis. Pellets were extracted with 1.5 ml of 80% ethanol (containing prostaglandin B_2) and analyzed for intracellular LTC_4 content. Total LTC_4 synthesis was measured after direct addition of three volumes of ethanol to the cell suspension without prior centrifugation. Leukotriene analyses were performed by reversed-phase HPLC as described (35), using a Nova-Pak C_{18} column (3.9 × 150 mm, Waters Associates, Milford, MA), eluted with acetonitrile–methanol–water–acetic acid 27:18:54:0.8 (v/v), apparent pH 5.6).

RNA isolation and cDNA synthesis

Total RNA was extracted from human platelets or leukocytes (the mixed leukocyte fraction was isolated by dextran sedimentation and ammonium chloride lysis) by the guanidinium–phenol–chloroform extraction technique (36) using the Ultraspec II kit. The yield and purity of RNA was estimated by measurements of UV absorbance at 260 and 280 nm. For the preparation of cDNA, 1 μg of RNA was used in a 20-μl cDNA synthesis reaction with 20 units AMV (Avian Myeloblastosis Virus) reverse transcriptase, 3.2 μg random hexamer primers, 10 mm Tris, 50 mm KCl, 5 mm $MgCl_2$, deoxynucleotide mix (1 mm each), and 50 units RNase inhibitor. The reaction mixture was incubated for 10 min at 25°C (primer annealing), 60 min at 42°C (transcription), and 5 min at 99°C (inactivation of the reverse transcriptase).

Polymerase chain reaction (PCR)

To 25 μl of PCR Master amplification mixture, containing 1.25 units TAQ polymerase, 20 mm Tris-HCl, 100 mm KCl, 3 mm $MgCl_2$, and dNTP mix (0.4 mm), was added 1 μl of the cDNA synthesis reaction mixture. Finally, the PCR primers were added to a final concentration of 0.3 μm and the volume was adjusted to 50 μl by addition of water. The following primers were used: β-actin: 5′ GAGGAGCACCCCGTGCTGCTGA 3′ and 5′ CTAGAAGCAT TTGCGGTGG 3′; MRP1: 5′ GGACCTGGACTTCGTTCTCA 3′ and 5′ CGTCCAGATTCCTTCATCCG 3′. The expected sizes of DNA fragments amplified with these primers were 784 bp for β-actin, and 291 bp for MRP1. Temperature cycling was as follows: first cycle, denaturation at 94°C for 60 s, annealing at 55°C for 15 s, and extension at 72°C for 15 s. Subsequent cycles, denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 15 s. In total, 35 cycles were performed for the MRP1 amplifications and 30 cycles for β-actin. Amplification products were separated on ethidium bromide-stained agarose gels (1.5%) and photographed under UV light. To verify the identity of the MRP1 amplification product, the PCR product was ligated into a plasmid vector using the Advantage PCR cloning kit (Clontech), following the instructions of the manufacturer. Bacteria were transformed, double-stranded plasmid DNA was isolated from recombinant clones and sequenced according to standard protocols.

Immunoblotting

Fifty mg of platelets or erythrocytes was homogenized in homogenizing buffer (25 mm Tris, 2.5 mm DTT, 1 × Complete™ protease inhibitor cocktail, pH 7.4) and protein levels were determined. Samples were mixed with one volume 2 × loading buffer (125 mm Tris (pH 6.8), 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.002% bromophenol blue) and 30 μg of protein was separated on 8–16% SDS-PAGE. Proteins were electroblotted onto a nitrocellulose membrane followed by blocking of the membrane with 5% milk powder in Tris-buffered saline (TBS) (50 mm Tris, pH 7.5, 0.1 m NaCl) for 60 min. After blocking, the membranes were incubated for 60 min with MRP1 (QCRL-1, 1:500 dilution) or MRP2 (EAG5, 1:10000 dilution) antibody in TBS with 0.1% Tween. Incubation with secondary anti-mouse or anti-rabbit antibody (1:10000 in TBS with 0.1% Tween) was for 60 min. Enhanced chemiluminescence, ECL Plus™ was used for detection as described by the manufacturer (Amersham, UK).

RESULTS

Effect of incubation temperature and time on LTC_4 export

The production and release of LTC_4 by human platelets at 0° and 37 °C were studied. After temperature equilibration, platelet suspensions were incubated with LTA_4 (10 μm) for 5 min, then immediately put on ice and centrifuged at 4°C. The amounts of LTC_4 in the supernatant and the cell pellet were analyzed in order to determine the proportions of released and intracellularly retained LTC_4 (**Fig. 1**). The total LTC_4 synthesis was calculated as the sum of intracellular and extracellular LTC_4 levels. As expected, the conversion of LTA_4 to LTC_4 was temperature-dependent. Thus, platelets incubated at 37°C synthesized 2150 ± 790 pmol $LTC_4/10^9$ platelets (mean ± SD, n = 4), as compared to the cells incubated at 0°C, which produced 778 ± 488 pmol/10^9 platelets (mean ± SD, n = 4). When platelet suspensions were incubated at 37°C, more than 85% of synthesised LTC_4 was detected extracellularly, demonstrating that LTC_4 formation was followed by efficient export at this temperature. In contrast, only approximately 15% of the total amount of LTC_4 could be detected in the extracellular fluid after incubation of the platelets at 0°C. The data indicate that LTC_4 was released via temperature-dependent transport mechanisms.

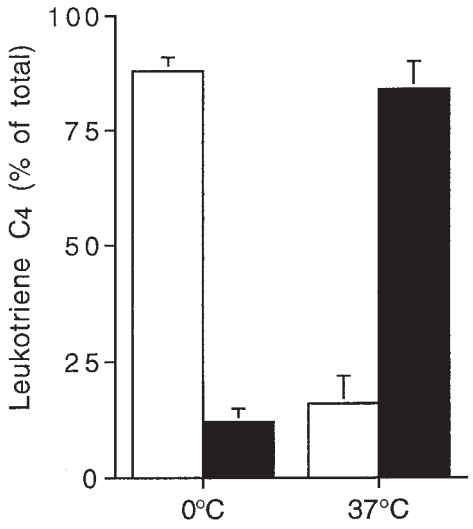

Fig. 1. Intracellular (open bars) and extracellular (filled bars) levels of LTC_4 in human platelet suspensions after incubation with LTA_4. Platelets (400×10^6 cells/ml) were incubated in Krebs buffer with LTA_4 (10 μm, 5 min) at 0° or 37°C prior to determination of LTC_4 levels. The values represent mean ± SD of four duplicate experiments.

The temperature-dependence was further investigated in experiments where platelets were preloaded with LTC_4 by incubation with LTA_4 (10 μm) for 10 min at 0°C. Thereafter, the incubation medium was rapidly removed by centrifugation at 4°C and the platelets were suspended in fresh Krebs buffer, which had been prewarmed to various temperatures (25°–40°C). Finally, the platelet suspensions were incubated at these temperatures for 1 min and the release of LTC_4 to the extra cellular fluid was determined. The results demonstrated highly temperature-dependent LTC_4 export in this temperature interval, with an average increase in export rate of approximately 60 pmol LTC_4/°C × min x 10^9 platelets (**Fig. 2A**). Using data obtained in these experiments, the activation energy (Ea) for LTC_4 export from human platelets was calculated by linear least-squares regression analysis of a plot of log k against 1/T (Fig. 2B), according to the Arrhenius equation: $\log k = \log A - Ea/2.303RT$ (k = export rate (mol LTC_4/°K × min × 10^9 platelets), A = frequency factor, R = the universal gas constant, and T = temperature in degrees Kelvin). The calculated value, 49.9 ± 7.7 kJ/mol (mean ± SD, n = 3) is in accordance with carrier-mediated transport (37).

The time-dependence of platelet LTC_4 export was investigated in experiments where the cells were preloaded with LTC_4, as described above. After centrifugation and resus-

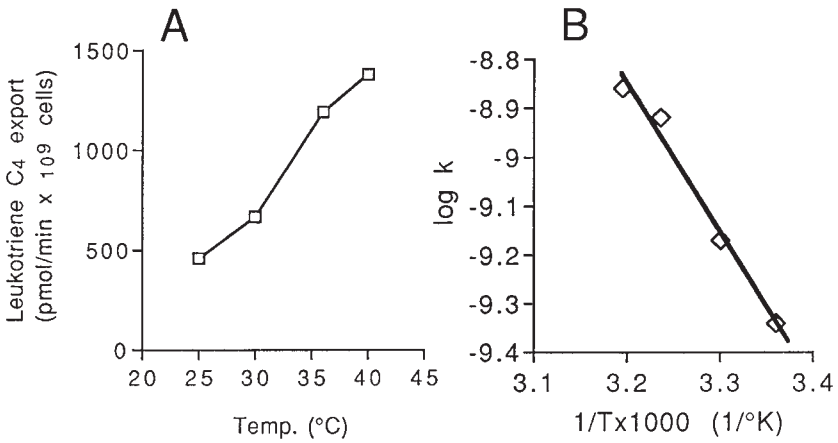

Fig. 2. Temperature sensitivity of LTC_4 release from human platelets. Platelet suspensions (400×10^6 cells/ml) in Krebs buffer were incubated with LTA_4 (10 μm,10 min) at 0°C. Thereafter, the platelets were centrifuged (650 *g*, 5 min 0°C) and release of LTC_4 was initiated by resuspending the cells in prewarmed Krebs buffer (25–40°C). Cells were incubated at various temperatures for 1 min prior to quantification of extracellular LTC_4. The values represent one representative duplicate experiment out of three. Panel A demonstrates LTC_4 export rate at various temperatures. In panel B the logarithm of the export velocity (k) was plotted against the inverse temperature in degrees Kelvin.

pension in fresh Krebs buffer, which had been prewarmed to 37°C, the platelets were incubated at this temperature for various time intervals and the release of LTC_4 was analyzed. The results demonstrated a time-dependent, rapid export of LTC_4 (**Fig. 3**), with approximately 50 and 80% of synthesized LTC_4 released after 2.5 and 10 min, respectively.

Effects of intracellular LTC_4 levels on the export of LTC_4 from human platelets

The rate of LTC_4 export at various intracellular concentrations of LTC_4 was studied. The cells were incubated with various concentrations of LTA_4 (1–40 μm) at 0°C for 15 min in order to preload platelets with increasing amounts of intracellular LTC_4. Thereafter, the leukotriene release during 2 min at 37°C was determined (**Fig. 4**). The total synthesis of LTC_4 increased with escalating substrate concentrations up to 20–40 μm LTA_4. In contrast, the export of LTC_4 reached a plateau already at a substrate concentration of approximately 5 μm. Accordingly, increasing amounts of LTC_4 were retained intracellularly, as the substrate concentration was elevated. The data demonstrate saturability of the LTC_4 export step. The export rate of LTC_4 under saturating conditions was calculated to be 470 ± 147 pmol LTC_4/min × 10^9 cells (mean ± SD, n = 3).

Effects of inhibitors of transport and metabolism on LTC_4 export

The release of LTC_4 from human platelets preloaded with LTC_4 by incubation with LTA_4 (10 μm, 5 min, 0°C) was measured at 37°C in the presence of increasing amounts of

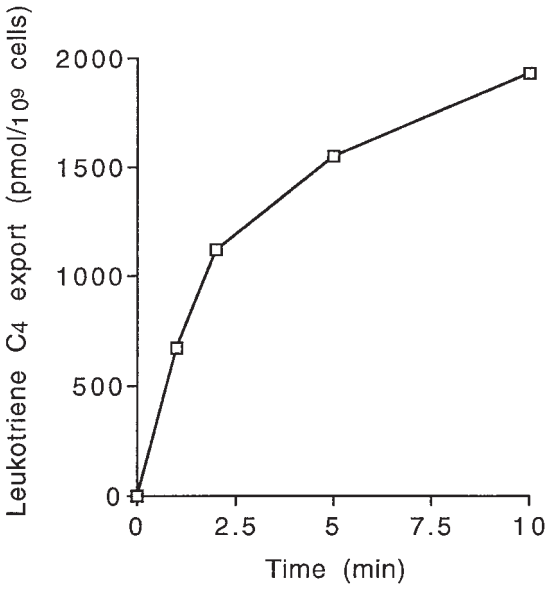

Fig. 3. Time-course of LTC_4 release from human platelets. Platelet suspensions (400×10^6 cells/ml) in Krebs buffer were incubated with LTA_4 (10 μm, 10 min) at 0°C. After centrifugation of the cells (650 *g*, 5 min, 0°C) the release of LTC_4 was initiated by resuspending the cells in 37°C Krebs buffer. Cells were incubated for various times at 37°C followed by quantification of extracellular LTC_4. The values represent one representative duplicate experiment out of three.

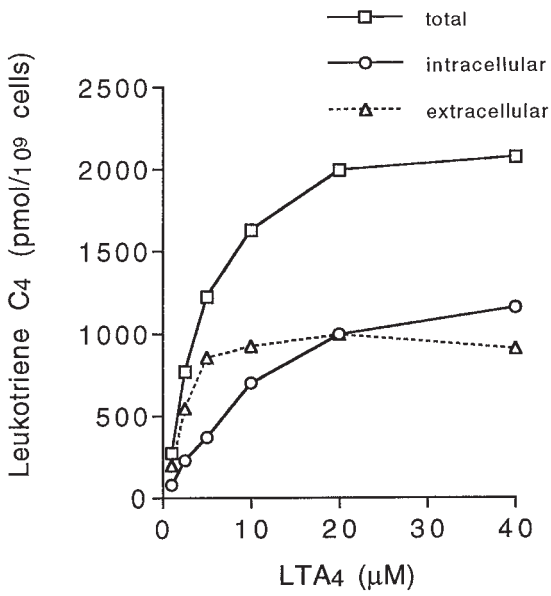

Fig. 4. Kinetics of LTC_4 release from human platelets. Platelet suspensions (400×10^6 cells/ml) in Krebs buffer were incubated with LTA_4 (1–40 μm, 15 min) at 0°C. Thereafter, the cells were incubated at 37°C for 2 min. Intracellular and extracellular levels of LTC_4 were determined and the total synthesis of LTC_4 was calculated as the sum of intracellular and extracellular levels. The values represent the mean ± SD of three duplicate experiments.

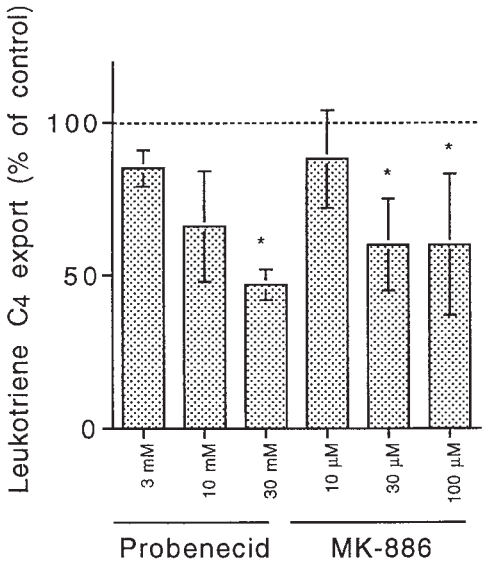

Fig. 5. Inhibition of LTC_4 export from human platelets. Platelet suspensions (400×10^6 cells/ml) in Krebs buffer were incubated with LTA_4 (10 μm, 5 min) at 0°C. Thereafter, the cells were treated with or without (control) transport inhibitors for 10 min at 0°C. Finally, the cells were incubated at 37°C for 2 min. and the extracellular levels of LTC_4 were determined. The values represent mean ± SD of three duplicate experiments. Statistical analyses were performed using Student's *t*-test for paired samples. $*P < 0.01$.

the organic anion transport inhibitor probenecid (**Fig. 5**) or the leukotriene biosynthesis inhibitors MK-886, BAY X1005, and L-655,238. The inhibitors were added subsequent to the LTA_4 incubation, to avoid effects of these drugs on the synthesis of LTC_4. Thereafter the platelets were incubated with or without (control) the inhibitors for 10 min at 0°C. Control experiments revealed that the levels of LTC_4 were not significantly altered in platelet suspensions treated with inhibitors (results not shown). Finally, the cells were incubated at 37°C for 2 min and the extracellular levels of LTC_4 were determined. As demonstrated in Fig. 5, probenecid and MK-886 dose-dependently inhibited LTC_4 release, whereas BAY X1005 and L-655,238 were ineffective at concentrations of 10 and 100 μm (results not shown).

To investigate whether the energy state of the cells could influence the ability of platelets to export LTC_4, the effects of metabolic inhibitors on LTC_4 transport were determined. Pretreatment of platelets with a combination of antimycin A and 2-deoxyglucose, which inhibits the electron transport chain and glycolysis, respectively, (38) was found to provoke approximately 90% inhibition of the release of LTC_4 from human platelets. In contrast, the effect of these metabolic inhibitors on the total synthesis of LTC_4 was significantly smaller (**Table 1**).

TABLE 1. Effect of the metabolic inhibitors antimycin A and 2-deoxyglucose on the synthesis and export of LTC_4 from human platelets

	% of Control
Synthesis of LTC_4	69 ± 3^a
Export of LTC_4	13 ± 4^b

Platelet suspensions (400×10^6 cells/ml) in PBS buffer were treated with or without (control) a combination of the metabolic inhibitors antimycin A (6 mg/ml) and 2-deoxyglucose (50 mM) for 15 min at 37 °C. After cooling the platelets for 5 min at 0°C, the cells were incubated with LTA_4 (10 μm) for 5 min at 0°C. Thereafter the cells were centrifuged at 650 *g* for 10 min at 4°C and resuspended in ice-cold PBS. Finally, the cells were incubated at 37°C for 1 min prior to determination of extra cellular and total LTC_4 levels. The values represent mean ± SD of four duplicate experiments. Statistical analyses were performed using Student's *t*-test for paired samples (treated samples versus control).

[a] $P < 0.01$.

[b] $P < 0.001$.

Detection of multidrug resistance-associated protein and mRNA in human platelets

The presence of mRNA transcripts encoding the LTC_4 transporter MRP1 was investigated by RT-PCR. cDNA prepared from total RNA isolated from human platelets was used as template in the PCR reaction. Using the primers specific for MRP1, a DNA fragment of the expected size (291 bp) could be detected (**Fig. 6**). The specific amplification of MRP1 cDNA was verified by cloning and sequencing of the amplified fragment. Amplification of a β-actin fragment was also performed as a positive control for the PCR reaction (Fig. 6). To exclude the possibility that contaminating leukocytes influenced the result of the PCR analysis, a control reaction was performed. Thus, cDNA from human leukocytes was used as template in a PCR reaction using the MRP1 primers. The amount of leukocyte cDNA used in the reaction corresponded to the amount of contaminating leukocytes in the platelet preparation (see Materials and Methods). No detectable PCR fragments were observed in this reaction (results not shown). To determine whether the presence of MRP1 mRNA in human platelets was accompanied by expression of MRP1, immunoblot analysis was performed. Using a monoclonal antibody specific for MRP1, a band of approximately 190 kDa was detected in protein extracts from human platelets as well as in the positive control obtained from human erythrocytes (**Fig. 7**), which have recently been demonstrated to contain MRP1 (39). In contrast, we failed to de-

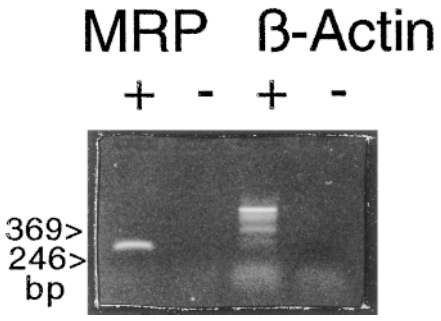

Fig. 6. Detection of mRNA encoding MRP1 in human platelets by RT-PCR. cDNA was synthesized from human platelet total RNA and used as template in the PCR reaction (see Materials and Methods). Lanes marked '−' are negative controls without cDNA. β-Actin was used as a positive control for the PCR reaction. Arrows indicate the migration of size markers from a 123 bp ladder.

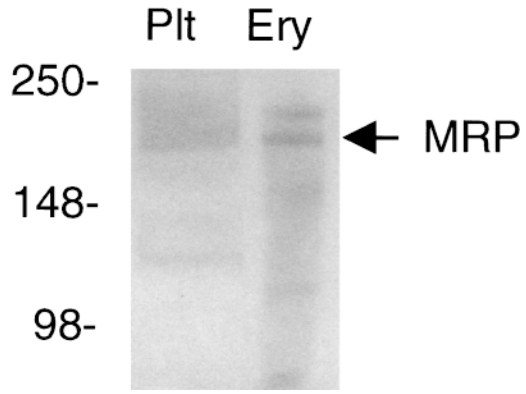

Fig. 7. Detection of MRP1 in human platelets by immunoblot analysis. Protein extracts were prepared from platelets and a positive control (erythrocytes) and analyzed as described in Materials and Methods. The migration of a size-marker and the bands corresponding with the size of MRP1 is indicated.

tect any immunoreactivity when using an antibody against human MRP2 (results not shown), which has been detected only in the liver canalicular membrane and the apical membrane of kidney proximal tubules (31).

DISCUSSION

The present study demonstrates that human platelets possess a mechanism whereby LTC_4 can be actively exported from the cells. Thus, incubation with LTA_4 was found to initiate time- and temperature-dependent release of LTC_4 to the extracellular fluid. Furthermore, the transport in human platelets was saturable, reaching a maximal export rate at approximately 500 pmol $LTC_4/10^9$ cells $\times$ min. Analysis of the temperature-dependence of LTC_4 release revealed an activation energy (Ea) of approximately 50 kJ/mol, a value exceeding that anticipated for passive diffusion ($<$16.8 kJ/mol) and clearly within the range commonly observed for carrier-mediated processes (29.4 to 105.0 kJ/mol) (37). In comparison, the activation energy for the release of LTC_4 from human eosinophils and two human myeloid leukemia cell lines has been reported to be in the range of 96 to 118 kJ/mol (24).

Pretreatment of platelets with probenecid, a known inhibitor of anion transport which has earlier been demonstrated to inhibit carrier-mediated release of LTC_4 from human eosinophils (23), led to approx. 50% inhibition of LTC_4 release. Furthermore, the FLAP-inhibitor MK-886 was found to suppress the release of LTC_4 from human platelets. This is in line with a previous finding, demonstrating that MK-886 inhibited export of cysteinyl leukotrienes from mastocytoma cells (25). The inhibitory effect of MK-886 on FLAP has been demonstrated to be due to competitive inhibition of arachidonic acid binding (40). Thus, it can be speculated that MK-886 inhibited LTC_4 export by binding to a similar substrate-binding domain in a putative carrier protein. However, two other competitive FLAP-inhibitors, L-655,238 and BAY X1005, were without effect on the export of LTC_4 from human platelets. The reason for this discrepancy is presently unknown but may be related to the fact that L-655,238 and Bay X1005 belong to a structurally different class of FLAP inhibitors than MK-886.

In order to examine the correlation between the cellular energy status and the capability to release LTC_4, platelets were treated with a combination of 2-deoxyglucose and antimycin A. These drugs, which inhibit the electron transport chain and glycolysis, respectively (38), have been utilized to suppress energy-dependent transport processes via inhibition of cellular energy generation (41, 42). In the presence of these inhibitors the capability of the platelets to export LTC_4 was markedly decreased, whereas the LTC_4 synthase activity was less effectively suppressed. These findings further suggest that the release of LTC_4 from human platelets is a carrier-mediated, energy-dependent process. In accordance, the requirement of ATP for the uptake of LTC_4 into plasma membrane vesicles prepared from murine mastocytoma cells (25) has been demonstrated.

Much evidence indicates that MRP1 is the principal transporter for LTC_4 (26–28, 33), although a recent report, describing MRP-independent LTC_4 transport in a murine mast cell line, indicates alternative mechanisms for the release of LTC_4 (43). It was therefore of interest to investigate whether MRP1 was expressed in human platelets. The presence and function of this protein in platelets or megakaryocytes has not been investigated previously. In the present study, however, we demonstrate that human platelets carry both MRP1 mRNA and protein. These findings indicate the probable importance of MRP1 in the export of LTC_4 from the platelets. Our findings, indicating that the transport is carrier-mediated and energy-dependent, is in accordance with this hypothesis. Furthermore, we demonstrate that probenecid, a known inhibitor of MRP1 (44), partially inhibits platelet LTC_4 export. However, more detailed studies are needed to further establish a role for MRP1 in platelet LTC_4 export.

The LTC_4-producing capacity of platelets may be of interest, as platelets have been suggested to be involved in the pathophysiology of bronchial asthma (45), in which a role for cysteinyl leukotrienes is well established (2). The importance of platelet-dependent LTC_4 formation can be questioned, as formation of LTC_4 in platelets depends on transfer of LTA_4 from neighboring cells, such as activated granulocytes. However, it has recently been demonstrated that the majority of the LTA_4 formed by neutrophils is released extracellularly after stimulation with calcium ionophore A23187(11) or physiological stimuli (46). This indicates the relevance of transcellular LT metabolism (18) and suggests that platelets may be of importance in in vivo production of cysteinyl leukotrienes. Furthermore, the capability of platelets to produce LTC_4 has recently been demonstrated to be conserved in several mammalian species (17).

The present results demonstrate that the formation of LTC_4 in human platelets is associated with a rapid and carrier-mediated active export of LTC_4 into the extracellular space and indicate the involvement of an energy-dependent and

probenecide-sensitive transporter, possibly MRP1. The finding that platelets possess not only specific LTC_4 synthase (17) but also have capability to efficiently release LTC_4 further suggests that these cells may contribute to the in vivo formation of cysteinyl leukotrienes.

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