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ATP-dependent leukotriene export from mastocytoma cells

Thomas Schaub, Toshihisa Ishikawa and Dietrich Keppler

Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Germany

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The biosynthesis of leukotrienes (LT) C_4 and B_4 is followed by an export of these mediators into the extracellular space. This transport was characterized using plasma membrane vesicles prepared from mastocytoma cells and identified as an ATP-dependent primary active process. The apparent K_m-values were 110 nM for LTC_4 and 48 μM for ATP. The transport rate was highest for LTC_4, whereas LTD_4, LTE_4, and *N*-acetyl-LTE_4 were transported with relative rates of 31, 12 and 8%, respectively, at a concentration of 10 nM. LTB_4 transport was also dependent on ATP. LTC_4 transport was inhibited by LTD_4 receptor antagonists (IC_{50} = 1.0 μM for MK-571 and 1.3 μM for LY245769) and by the inhibitor of leukotriene biosynthesis MK-886 (IC_{50} = 1.8 μM). The ATP-dependent export carrier for leukotrienes in leukotriene-synthesizing cells represents a novel member of the family of ATP-dependent exit pumps.

ATP-dependent transport; Mastocytoma cells; Leukotriene export

1. INTRODUCTION

The leukotrienes as members of the eicosanoid family of mediators are most biologically active substances eliciting inflammatory and allergic reactions [1–5]. In a limited number of cell types, e.g. mast cells, eosinophils, monocytes, neutrophils, and macrophages, the formation of leukotrienes is initiated by liberation of arachidonate from membrane phospholipids and by the synthesis of LTA_4 which is converted to LTB_4 or to the glutathione conjugate LTC_4. Most actions of the leukotrienes are exerted from the extracellular space. Leukotriene transport across the plasma membrane [6,7] is therefore a prerequisite for expression of their biological activities through the interaction with receptors [8].

The present study deals with the mechanism underlying the membrane transport of LTC_4 and LTB_4 in leukotriene-producing cells. The formation of leukotrienes is particularly active in mastocytoma cells, the source from which LTC_4 was first isolated [9]. Using plasma membrane vesicles from mouse mastocytoma cells we present evidence for the existence of an ATP-dependent export system responsible for the release of LTC_4 (Fig. 1) as well as LTB_4.

Correspondence address: D. Keppler, Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, Germany

Abbreviations: LT, leukotriene; LTE_4NAc, *N*-acetyl-leukotriene E_4; ω-OH-LTB_4, ω-hydroxy leukotriene B_4; ω-COOH-LTB_4, ω-carboxy leukotriene B_4; PG, prostaglandin; DNP-SG, *S*-(2,4-dinitrophenyl)-glutathione; MK-886, 3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethyl-propanoic acid; MK-571, 3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)(3-dimethyl amino-3-oxopropyl)thio)methyl)thio)propanoic acid; LY245769, (1S,2R)-5-(3-[1-hydroxy-15,15,15-trifluoro-2-(2-1H-tetrazol-5-ylethylthio)-pentadeca-3(E),5(Z)-dienyl]phenyl)-1H-tetrazole

2. MATERIALS AND METHODS

2.1. *Biochemicals and enzymes*

ATP, creatine phosphate, creatine kinase, and phenylmethylsulfonylfluoride were obtained from Boehringer Mannheim (Mannheim, Germany). [5,6,8,9,11,12,14,15-$^3H_8$]Arachidonic acid, 14,15-$^3H_2$-labeled LTB_4, ω-OH-LTB_4, LTC_4, LTD_4, and LTE_4 were purchased from DuPont-New England Nuclear (Boston, MA, USA). [^{3}H]ω-COOH-LTB_4 was synthesized from [^{3}H]ω-OH-LTB_4 and kindly provided by G. Jedlitschky from our laboratory [10].

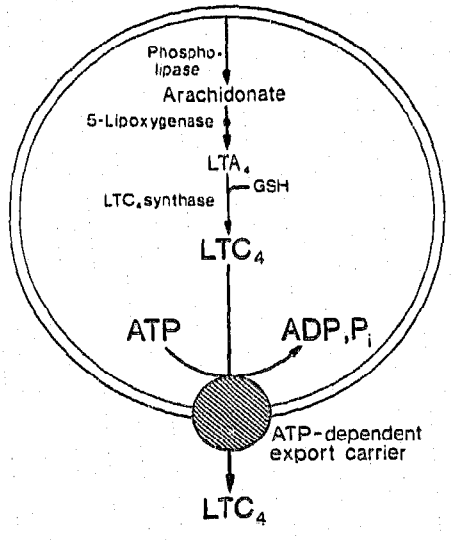

Fig. 1. Scheme of biosynthesis and ATP-dependent export of LTC_4 from leukotriene-generating cells.

[^{3}H]LTE$_4$NAc was prepared by chemical *N*-acetylation of [^{3}H]LTE$_4$ [11]. Unlabeled LTB$_4$ was from Amersham Buchler (Braunschweig, Germany); LTC$_4$, LTD$_4$, and LTE$_4$ were from Salford Ultrafine Chemicals and Research (Manchester, UK). DNP-SG was prepared as described previously [12]. The LTD$_4$ receptor antagonist MK-571 [(3-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][(3-dimethyl amino-3-oxo propyl)thio]methyl]thio]propanoic acid] ([13]) and the leukotriene biosynthesis inhibitor MK-886 [(3-[1-(4-chlorobenzyl)-3-*t*-butyl-thio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid ([14]) were from Dr A.W. Ford-Hutchinson, Merck-Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada). The LTD$_4$ receptor antagonist LY245769 [(1S,2R)-5-(3-[1-hydroxy-15,15,15-trifluoro-2-(2-1H-tetrazol-5-ylethylthio)-pentadeca-3(E),5(Z)-dienyl]phenyl)-1H-tetrazole] ([10]) was supplied by Dr J.R. Boot from Eli Lilly, Lilly Research Centre (Erl Wood Manor, Windlesham, UK). RPMI 1640 containing 2 mM L-glutamine, fetal calf serum (FCS), and penicillin/streptomycin (1000 IU/$^{-1}$ were from Gibco (Paisley, Scotland, UK). α-Thioglycerol was from Sigma Chemicals (St. Louis, MO, USA).

2.2. *Culture of mastocytoma cells*

The autonomous malignant mastocytoma subline L138C3-10a from BALB/c mouse-derived bone marrow mast cells [15] was kindly provided by Dr L. Hültner (München, Germany). The cells were maintained to grow in RPMI 1640 medium supplemented with 2 mM L-glutamine, 20% heat-inactivated FCS, 100 IU/ml penicillin/streptomycin, and 100 μM α-thioglycerol, in a humidified incubator (5% CO_2, 37°C) [15]. The doubling time of the cells was about 20 h. Production of LTC$_4$ by the mastocytoma cells after treatment with 5 μM calcium ionophore A23187 [9] was examined by a radioimmunoassay after HPLC separation [16].

2.3. *Preparation of plasma membrane vesicles from mastocytoma cells*

Mastocytoma cells (0.5-1 × 10^9 cells) were harvested from the cell culture by centrifugation (2500 × *g*, 10 min, 4°C). Cells were suspended in 50 ml of ice-cold phosphate-buffered saline (150 mM NaCl, 5 mM Na-phosphate, pH 7.4) and centrifuged at 2500 × *g* for 10 min at 4°C. The resulting pellet (about 2 ml) was diluted 40-fold with hypotonic buffer (0.5 mM Na-phosphate (pH 7.0), 0.1 mM EGTA, and 0.1 mM phenylmethylsulfonylfluoride) and gently stirred on ice for 3 h. The cell lysate was subsequently centrifuged at 100 000 × *g* for 40 min at 4°C. White fluffy material around the yellowish pellet was collected and suspended in the same hypotonic buffer. After homogenization with a Potter-Elvehjem homogenizer (20 strokes), the crude membrane fraction was layered over a 38% sucrose solution and centrifuged at 100 000 × *g* for 30 min at 4°C. The turbid layer at the interface was collected and diluted in 25 ml of 250 mM sucrose solution containing 10 mM Tris-HCl (pH 7.4). This solution was centrifuged at 100 000 × *g* for 30 min and the resulting pellet was suspended in a volume of about 0.5 ml with 250 mM sucrose and 10 mM Tris-HCl (pH 7.4). Vesicles were formed by passing the suspension through a 27-gauge needle with a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −70°C until use. Protein was determined according to Lowry et al. [17]. The resulting plasma membrane vesicles exhibited a Na$^+$,K$^+$-ATPase activity [18] of 170 nmol·min^{-1}·mg protein^{-1} with a purification factor of 30-fold as compared to the original cell homogenate.

2.4. *Measurement of leukotriene uptake by plasma membrane vesicles*

The standard incubation medium contained plasma membrane vesicles from mastocytoma cells (100 μg of protein), 10 nM [^{3}H]leukotriene, 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg/ml creatine kinase (80 U/ml) in a final volume of 110 μl. The reaction was carried out at 37°C. [^{3}H]Leukotriene uptake was measured by rapid filtration as described previously [12].

3. RESULTS

3.1. *Characterization of leukotriene transport into membrane vesicles from mastocytoma cells*

Fig. 2 shows the time course of [^{3}H]LTC$_4$ uptake into inside-out membrane vesicles in the presence or absence of 1 mM ATP; the incubation with ATP resulted in a significant accumulation of LTC$_4$ into the vesicles. The uptake was linear with incubation time for 5 min. Likewise, [^{3}H]LTB$_4$ was accumulated in vesicles in an ATP-dependent manner (Fig. 2).

Table I summarizes the ATP-dependent uptake of leukotrienes, prostaglandins E_2 and $F_{2\alpha}$, and arachidonate. LTC$_4$ was the best substrate among the compounds studied. LTD$_4$, LTE$_4$, and LTE$_4$NAc were transported ATP-dependently with relative transport rates of 31%, 12%, and 8%, respectively, as compared to LTC$_4$. LTB$_4$ was transported at a rate of 4% relative to LTC$_4$ uptake, whereas ω-COOH-LTB$_4$ transport was less than 1%. PGE_2, $PGF_{2\alpha}$, and arachidonate were not substrates for the ATP-dependent transport.

The transport of LTB$_4$ (10 nM) was inhibited by 10 μM LTC$_4$ and DNP-SG by 92% and 42%, respectively. On the other hand, LTC$_4$ (10 nM) transport was inhibited by 10 μM LTB$_4$ by only 48%. This mutual competition indicates that transport of LTB$_4$ and LTC$_4$ may be mediated by a common carrier. Fig. 3 shows the Lineweaver-Burk diagrams for LTC$_4$ uptake into plasma membrane vesicles with apparent K_m values of 110 nM for LTC$_4$ (A) and 48 μM for ATP (B).

3.2. *Inhibition of LTC$_4$ transport into vesicles*

Fig. 4 shows the inhibitory effect of two potent LTD$_4$-receptor antagonists (MK-571 and LY245769),

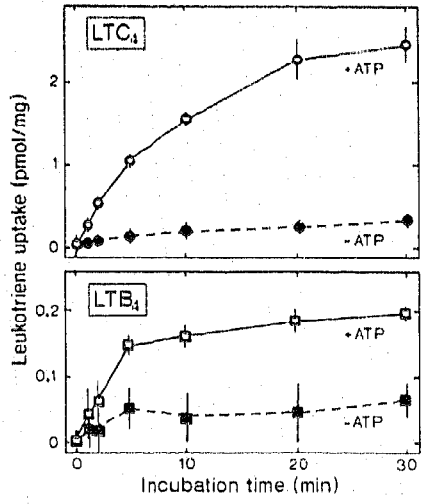

Fig. 2. Time course of LTC$_4$ and LTB$_4$ uptake into plasma membrane vesicles from mastocytoma cells. Membrane vesicles (100 μg of protein) were incubated with 10 nM [^{3}H]LTC$_4$ or [^{3}H]LTB$_4$ at 37°C in the absence or presence of 1 mM ATP. Data are presented as the means ± SD (n = 3).

Table I

ATP-dependent transport of leukotrienes and other eicosanoids into plasma membrane vesicles from mastocytoma cells

	ATP-dependent uptake	
	pmol × mg protein^{-1}	%
LTC_4	1.64 ± 0.03	100
LTD_4	0.51 ± 0.07	31
LTE_4	0.20 ± 0.09	12
LTE_4NAc	0.13 ± 0.01	8
LTB_4	0.07 ± 0.02	4
ω-OH-LTB_4	0.07 ± 0.05	4
ω-COOH-LTB_4	≤0.001	≤1
PGE_2	≤0.01	≤1
$PGF_{2\alpha}$	≤0.01	≤1
Arachidonate	≤0.01	≤1

Plasma membrane vesicles from mastocytoma cells (100 μg of protein) were incubated with the ^{3}H-labeled compounds (10 nM) at 37°C for 5 min and radioactivity incorporated into the vesicles was measured as described in section 2. Mean values from triplicate experiments ± SD

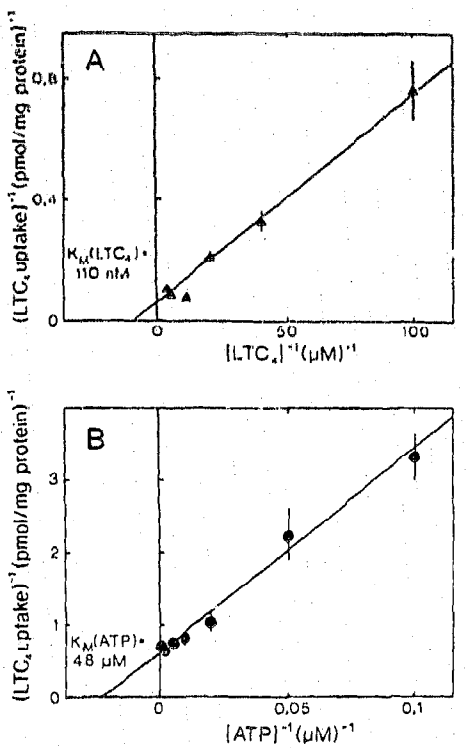

Fig. 3. Lineweaver-Burk diagrams of ATP-stimulated LTC_4 uptake into plasma membrane vesicles at varying concentrations of LTC_4 (A) and ATP (B). (A) Membrane vesicles were incubated with [^{3}H]LTC_4 at 37°C for 5 min in the presence of 1 mM ATP. (B) Membrane vesicles were incubated with 10 nM [^{3}H]LTC_4 in the presence of varying concentrations of ATP. ATP-stimulated LTC_4 uptake was calculated from the difference in radioactivities incorporated into vesicles in the presence or absence of ATP. Data are presented as the means ± SD ($n = 3$).

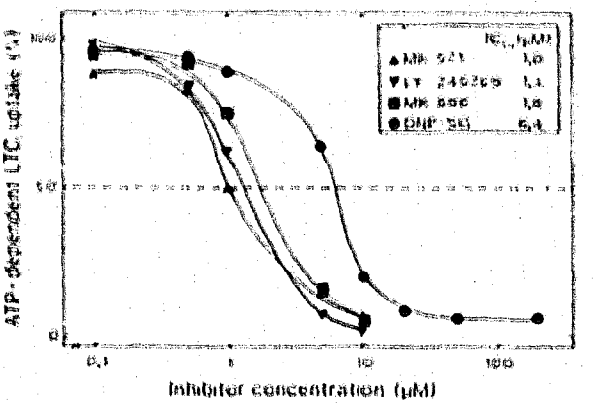

Fig. 4. Inhibition of ATP-dependent LTC_4 uptake into plasma membrane vesicles by LTD_4 receptor antagonists (MK-571 and LY245769), the leukotriene biosynthesis inhibitor MK-886, and the glutathione *S*-conjugate DNP-SG. Membrane vesicles were incubated with 10 nM [^{3}H]LTC_4 in the presence of the inhibitors (0.1–200 μM) at 37°C for 5 min as described in section 2. Inhibition curves with IC_{50} values represent the mean values from triplicate measurements.

the leukotriene biosynthesis inhibitor MK-886, and the glutathione *S*-conjugate DNP-SG on ATP-dependent LTC_4 uptake into membrane vesicles. MK-571, LY245769, and MK-886 inhibited the uptake in a dose-dependent manner with IC_{50} values of less than 2 μM. The inhibition by DNP-SG was less effective ($IC_{50} = 6.4$ μM).

4. DISCUSSION

An export of LTC_4 from human eosinophils has recently been characterized [6]. However, the underlying mechanism of transport was not identified. ATP-dependent transport of LTC_4 across plasma membranes was originally reported by Ishikawa et al. using rat heart sarcolemma and liver plasma membrane vesicles [12,19,20], and most recently an ATP-dependent transport system responsible for the hepatobiliary export of LTC_4 and its metabolites has been identified in liver canalicular membrane [21]. Based on these studies it was hypothesized that an ATP-dependent carrier system may exist in LTC_4-producing cells in order to mediate the release of LTC_4 from the cells. We have examined this hypothesis using a murine mastocytoma cell line. LTC_4 as well as LTB_4 are generated by mastocytoma cells after the stimulation with calcium ionophore A23187 [8,9]. As demonstrated in Fig. 2, plasma membrane vesicles prepared from mastocytoma cells exhibited ATP-dependent transport of LTC_4 and LTB_4. This transport is relatively specific for LTC_4 as compared to other cysteinyl leukotrienes (Table I). The apparent K_m value for LTC_4 (110 nM) is similar to that in rat heart plasma membranes (150 nM) [20] and about 2-fold lower than that in rat liver canalicular membranes (250 nM) [21].

Similar as in plasma membrane vesicles from rat heart and liver [20,21], LTC_4 transport into mastocytoma vesicles was inhibited by the glutathione *S*-conjugate DNP-SG ($IC_{50} \approx 6.4\ \mu M$). It is noteworthy that the LTD_4 receptor antagonists MK-571 and LY-245769 as well as the leukotriene biosynthesis inhibitor MK-886 inhibited the ATP-dependent transport of LTC_4 more effectively than DNP-SG (Fig. 4). These inhibitors and the cysteinyl leukotrienes share the property of amphiphilic anions. These results raise the question whether some of these compounds may exert their anti-inflammatory actions not only by antagonizing LTD_4 receptors or by inhibiting leukotriene biosynthesis but, in addition, by inhibiting leukotriene export from cells such as mast cells, eosinophils, and monocytes. The ATP-dependent export carrier in mastocytoma cells represents a novel member of the family of ATP-dependent exit pumps [22,23]. The molecular structure and properties of leukotriene transport systems in different tissues are currently under study in this laboratory.

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REFERENCES

[1] Samuelsson, B., Dahlén, S.-E., Lindgren, J.A., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171-1176.
[2] Piper, P.J. (1984) Physiol. Rev. 64, 744-761.
[3] Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R. and Lefkowith, J.B. (1986) Annu. Rev. Biochem. 55, 69-102.
[4] Denzlinger, C., Rapp, S., Hagmann, W. and Keppler, D. (1985) Science 230, 330-332.
[5] Decker, K. (1990) Eur. J. Biochem. 192, 245-261.
[6] Lam, B.K., Owen, W.F., Austen, K.F. and Soberman, R.J. (1989) J. Biol. Chem. 264, 12885-12889.
[7] Lam, B.K., Gagnon, L., Austen, K.F. and Soberman, R.J. (1990) J. Biol. Chem. 265, 13438-13441.
[8] Rokach, J. (ed.) (1989) Leukotrienes and Lipoxygenases, Elsevier, Amsterdam.
[9] Murphy, R.C., Hammarström, S. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76, 4275-4279.
[10] Jedlitschky, G., Leier, I., Huber, M., Mayer, D. and Keppler, D. (1990) Arch. Biochem. Biophys. 282, 333-339.
[11] Hagmann, W., Denzlinger, C., Rapp, S., Weckbecker, G. and Keppler, D. (1986) Prostaglandins 31, 239-251.
[12] Ishikawa, T. (1989) J. Biol. Chem. 264, 17343-17348.
[13] Jones, T.R., Zamboni, R., Belley, M., Champion, E., Charette, L., Ford-Hutchinson, A.W., Frenette, R., Gauthier, J.-Y., Leger, S., Masson, P., McFarlane, S., Piechuta, H., Rokach, J., Williams, H., Young, R.N., DeHaven, R.N. and Pong, S.S. (1989) Can. J. Physiol. Pharmacol. 67, 17-28.
[14] Gillard, J., Ford-Hutchinson, A.W., Chan, C., Charleson, S., Denis, D., Foster, A., Fortin, R., Leger, S., McFarlane, C.S., Morton, H., Piechuta, H., Riendeau, D., Rouzer, C.A., Rockach, J., Young, R., MacIntyre, D.E., Peterson, L., Bach, T., Elermann, G., Hopple, S., Humes, J., Hupe, L., Luell, S., Metzger, J., Meurer, R., Miller, D.K., Opas, E. and Pacholok, S. (1989) Can. J. Physiol. Pharmacol. 67, 456-464.
[15] Hültner, L., Szöts, H., Welle, M., van Snick, J., Moeller, J. and Dörmer, P (1989) Immunology 67, 408-413.
[16] Guhlmann, A., Keppler, A., Kästner, S., Krieter, H., Brückner, U.B., Messmer, K. and Keppler, D. (1989) J. Exp. Med. 170, 1905-1918.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
[18] Jones, L.R., Besch, H.R.Jr, Flemming, J.W., McConnaughey, M.M. and Watanabe, A.M. (1979) J. Biol. Chem. 254, 530-539.
[19] Ishikawa, T. (1989) FEBS Lett. 246, 177-180.
[20] Ishikawa, T., Kobayashi, K., Sogame, Y. and Hayashi, K. (1989) FEBS Lett. 259, 95-98.
[21] Ishikawa, T., Müller, M., Klünemann, C., Schaub, T. and Keppler, D. (1990) J. Biol. Chem. 265, 19279-19286.
[22] Juranka, P.F., Zaszawny, R.L. and Ling, V. (1989) FASEB J. 3, 2583-2592.
[23] Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E. and Higgins, C.F. (1990) Nature 346, 362-365