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Studies of Receptors and Modulatory Mechanisms in Functional Responses to Cysteinyl-leukotrienes in Smooth Muscle

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Abstract

Cysteinyl-leukotrienes, i.e. leukotriene (LT) C_4, D_4 and E_4, are inflammatory mediators and potent airway- and vasoconstrictors. Two different cysteinyl-leukotriene receptors have been cloned, $CysLT_1$ and $CysLT_2$. The aim of this thesis was to explore the effects of cysteinyl-leukotrienes in smooth muscle containing tissues of mainly vascular or airway origin. In order to characterise the CysLT receptors as well as the modulatory mechanisms involved in cysteinyl-leukotriene-induced responses, contractions of isolated tissue preparations were studied in organ baths and mediator release and leukotriene metabolism were analysed by enzyme immunoassay and RP-HPLC, respectively.

In the guinea-pig trachea, the $CysLT_1$ receptor antagonist ICI 198,615 partially inhibited the contractions induced by LTD_4, and abolished the LTE_4-induced contractions. The contractions induced by LTC_4 in the guinea-pig trachea and ileum were resistant to $CysLT_1$ receptor antagonism and competitively inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773, supporting that LTC_4 activates $CysLT_2$ receptors in these preparations. In contrast, the contractions of the human and porcine pulmonary arterial smooth muscles were resistant also to BAY u9773, suggesting the presence of another CysLT receptor subtype.

In human and porcine pulmonary arteries, cyclooxygenase products were the major modulators of cysteinyl-leukotriene responses. In the human pulmonary artery, LTC_4 and LTD_4 stimulated the release of prostacyclin and consequently, the contractions induced by the leukotrienes were significantly enhanced after cyclooxygense inhibition. In contrast, porcine pulmonary arterial preparations mainly generated contractile cyclooxygenase products in response to LTC_4. Nitric oxide synthase inhibition unmasked contractions to LTC_4 in porcine pulmonary veins, but had no effect on LTC_4-induced contractions in porcine pulmonary arteries. A preferential regulation by nitric oxide in porcine pulmonary veins compared with arteries was also observed with noradrenaline, acetylcholine and bradykinin.

The metabolism of LTC_4 into LTD_4 and subsequently into LTE_4 may modulate the responses to LTC_4 and LTD_4 in the guinea-pig trachea. In addition to this metabolism, it was discovered that the guinea-pig trachea also formed LTC_4 from LTD_4. The latter alternative metabolic pathway changed the LTD_4-induced $CysLT_1$ response into a $CysLT_2$ receptor response.

In conclusion, the present thesis suggests the existence of a previously unrecognised receptor for cysteinyl-leukotrienes. In addition, it was demonstrated that cysteinyl-leukotriene responses in pulmonary vessels were regulated by the release of modulatory factors, of which cyclooxygenase products dominated in the arteries and nitric oxide was the main modulator in porcine pulmonary veins. Moreover, in tissues with a heterogeneous CysLT receptor population, the interconversion between LTC_4 and LTD_4 may represent a major modulatory mechanism by deciding which CysLT receptor is activated by the cysteinyl-leukotrienes.

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Bäck, M., Wikström Jonsson, E. & Dahlén, S.E. 1996.
The cysteinyl-leukotriene antagonist BAY u9773 is a competitive antagonist of leukotriene C_4 in the guinea-pig ileum
Eur J Pharmacol, 317, 107-113

II. Bäck, M., Norel, X., Walch, L., Gascard, J.P., Mazmanian, G., Dahlén, S.E. & Brink, C. 2000.
Antagonist resistant contractions of the porcine pulmonary artery by cysteinyl-leukotrienes
Eur J Pharmacol, 401, 381-388

III. Bäck, M., Norel, X., Walch, L., Gascard, J.P., de Montpreville, V., Dahlén, S.E. & Brink, C. 2000.
Prostacyclin modulation of contractions of the human pulmonary artery by cysteinyl-leukotrienes
Eur J Pharmacol, 401, 389-395

IV. Bäck, M., Walch, L., Norel, X., Gascard, J.P., Mazmanian, G. & Brink, C. 2002.
Modulation of vascular tone and reactivity by nitric oxide in porcine pulmonary arteries and veins
Acta Physiol Scand, 174, 9-15

V. Bäck, M., Kumlin, M., Cotgreave, I.A. & Dahlén, S.E. 2001.
An alternative pathway for metabolism of leukotriene D_4: effects on contractions to cysteinyl-leukotrienes in the guinea-pig trachea
Br J Pharmacol, 133, 1134-1144

CONTENTS

Abbreviations

cAMP	cyclic adenosine 3',5'-monophosphate
COX	cyclooxygenase
cGMP	cyclic guanosine-3',5'-monophosphate
EGF	epidermal growth factor
E_{max}	maximal contraction
γ-GT	γ-glutamyl transpeptidase
GP	guinea-pig
GPNA	γ-glutamyl-*P*-nitoanilide
GSH	reduced glutathione
GSSG	oxidised glutathione
L-NOARG	N^{ω}-nitro-L-arginine
LT	leukotriene
IUPHAR	International Union of Pharmacology
PBS	phosphate buffered saline
PG	prostaglandin
PNA	*P*-nitoanilide
RP-HPLC	reverse phase high performance liquid chromatography
SRS-A	slow reacting substance of anaphylaxis
TX	thromboxane

1. BACKGROUND

1.1. Cysteinyl-leukotrienes

1.1.1. Introduction

Cysteinyl-leukotrienes, i.e. leukotriene (LT) C_4, D_4 and E_4 (Fig. 1), are inflammatory mediators belonging to the group of eicosanoids. The name leukotriene originates in that these compounds were first isolated from leukocytes and that the molecular structure includes three conjugated double bonds, i.e. a triene (Samuelsson et al., 1987). Leukotriene C_4, D_4 and E_4 all contain cysteine (Fig. 1) and are therefore referred to as the cysteinyl-leukotrienes in order to distinguish them from LTB_4, which is a non-cysteine containing dihydroxy-leukotriene formed by the action of LTA_4 hydrolase (Samuelsson et al., 1987).

Before the structures of the cysteinyl-leukotrienes were known, a Slow Reacting Substance of Anaphylaxis (SRS-A; Brocklehurst, 1960) was shown to be released from animal lungs after challenge with a snake venom (Feldberg & Kellaway, 1938). This substance induced slow contractile responses of guinea-pig intestinal preparations and was later related to anaphylactic reactions and broncho-constriction (Brocklehurst, 1960; Chakravarty & Uvnäs, 1960). The identification of the cysteinyl-leukotrienes led to the conclusion that what had been referred to as SRS-A was actually a mixture of LTC_4, LTD_4 and LTE_4 (Samuelsson et al., 1987).

1.1.2. Formation and metabolism of cysteinyl-leukotrienes

1.1.2.1. Chemistry

The cysteinyl-leukotrienes are derived from arachidonic acid that is liberated from cell membrane phospholipids by the action of phospholipase A_2 (PLA_2). The next two steps in cysteinyl-leukotriene biosynthesis are catalysed by 5-lipoxygenase and result in the formation of LTA_4 via the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This reaction requires a 5-lipoxygenase activating protein (FLAP), which, in addition to the actual enzyme, is a target for inhibitors of cysteinyl-leukotriene formation. The unstable epoxide LTA_4 is conjugated with glutathione (GSH) by LTC_4 synthase, an enzyme belonging to the family of Membrane Associated Proteins in Eicosanoid and Glutathione Synthesis (MAPEG), and the reaction yields LTC_4. The biosynthesis of cysteinyl-leukotrienes has been reviewed by Samuelsson and co-workers (1987).

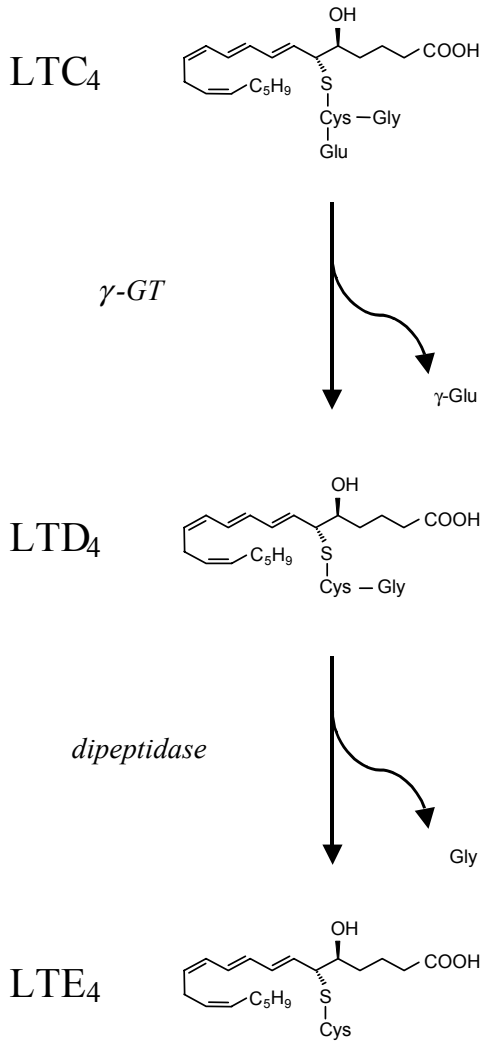

Fig. 1. Cysteinyl-leukotrienes (LTC_4, LTD_4 and LTE_4) and their metabolism by γ-glutamyl transpeptidase (γ-GT) and a Cys-Gly dipeptidase. Glu-Cys-Gly represents the glutathione (GSH, γ-glutamyl cysteinylglycine) side-chain of LTC_4.

LTC_4 is thus a glutathionyl eicosatetraenoic acid (Fig. 1) and shares its subsequent metabolism with GSH (Örning & Hammarström, 1980; Hammarström, 1981). The metabolism of LTC_4 is catalysed by γ-glutamyl transpeptidase (γ-GT), a membrane bound enzyme that cleaves off the γ-glutamyl group of the GSH side chain of LTC_4, thus yielding LTD_4 (Fig. 1; Hammarström, 1981). The enzyme γ-GT has two sites, a γ-glutamyl donor site and an acceptor site (Tate & Meister, 1985). The donor site has a broad specificity allowing most γ-glutamyl compounds to serve as donor substrates whereas the acceptor site has a restricted stereospecificity in that only L-amino acids or dipeptides with both amino acids in L-configuration can serve as acceptors (Tate & Meister, 1985). Examples of donors, in addition to GSH and LTC_4, are oxidised glutathione (GSSG), S-conjugated glutathiones such as S-hexyl GSH and S-decyl GSH (Fig. 2) as well as γ-glutamyl-*P*-nitroanilide (GPNA), a commonly used substrate in experimental characterisation of γ-GT (Aharony & Dobson, 1984; Pologe et al., 1984; Silber et al., 1986). In addition, isoforms of γ-GT that preferentially metabolise LTC_4 rather than GSH have been described (Carter et al., 1997; Heisterkamp et al., 1991) and will be discussed in Section 4.2.3. L-cysteine, L-glutamine, L-glycylglycine and L-cystine are examples of acceptors (Tate & Meister, 1985) and in addition, as will be shown in this thesis (Section 4.2.3.), also LTD_4 can act as an acceptor of the γ-glutamyl group, in this way yielding LTC_4 (Hammarström, 1981; Anderson et al., 1982; Paper V). Three different groups of γ-GT inhibitors will be addressed in the present thesis, namely the reversible inhibitor L-serine borate (Tate & Meister, 1978; Örning & Hammarström, 1980), the irreversible inhibitor acivicin (Stole et al., 1994) and the substrate competitors GSH (Hammarström, 1981) and S-hexyl GSH.

The metabolism of LTD_4 involves a dipeptidase that cleaves the peptide bond between the cysteinyl and glycyl residues in the LTD_4 side chain (Bernström & Hammarström, 1981; Hammarström, 1981; Sok et al., 1981). The product is thus a leukotriene with a cysteinyl group at carbon number 6 and is referred to as LTE_4 (Fig. 1; Samuelsson et al., 1987). The enzymatic conversion of LTD_4 into LTE_4 is inhibited by L-cysteine (Sok et al., 1981).

Cysteinyl-leukotriene metabolism takes place in a number of tissues including human lung (Kumlin & Dahlén, 1990) and in addition, the enzymes catalysing the formation of LTE_4 are present in human plasma (Köller et al., 1985). Elimination of cysteinyl-leukotrienes takes place both via the urinary and faecal routes (Örning et al., 1985; Maltby et al., 1990) and urinary LTE_4 can be used as a measure of whole body production of cysteinyl-leukotrienes (Maltby et al., 1990).

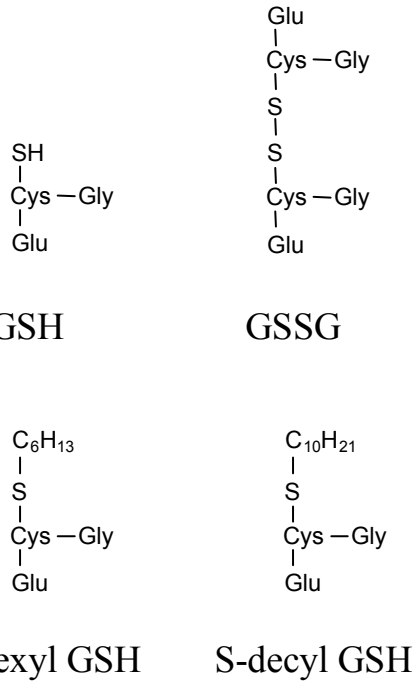

Fig. 2. Compounds used as γ-glutamyl donors in this thesis (Paper V): reduced glutathione (GSH), oxidised glutathione (GSSG), S-hexyl GSH and S-decyl GSH.

1.1.2.2. Source of cysteinyl-leukotrienes

The formation of LTA_4 via the 5-lipoxygenase pathway takes place in myeloid cells, e.g. leukocytes, macrophages, mast cells (Samuelsson et al., 1987). The next step in cysteinyl-leukotriene synthesis, conjugation of LTA_4 with GSH, can take place in endothelial cells, vascular smooth muscle cells, platelets, mast cells and macrophages as well as in eosinophilic granulocytes (Claesson & Haeggström, 1988; Maclouf & Murphy, 1988; Lindgren & Edenius, 1993). These differential activities mean that in some cells (e.g. eosinophils, macrophages and mast cells), LTC_4 can be formed from arachidonic acid, whereas in cells that have LTC_4 synthase but lack 5-lipoxygenase, LTA_4 is needed as a substrate. The LTA_4 formed in one cell type can be donated to another cell type in order to form LTC_4. This phenomenon is referred to as transcellular biosynthesis and has been reviewed by Lindgren and Edenius (1993). The biosynthesis of leukotrienes takes place in for example the human lung vasculature as shown by the increased formation of cysteinyl-leukotriene after stimulation of isolated perfused and ventilated human lungs with calcium ionophore (Kiss et al., 2000).

1.1.3. Cysteinyl-leukotriene receptors

1.1.3.1. Functional characteristics

The notion of cysteinyl-leukotriene receptors originates in the acetophenon FPL 55712, which was developed as an antagonist of SRS-A (Augstein et al., 1973). After the structure elucidation of SRS-A, the effects of the cysteinyl-leukotrienes were tested in different functional assays, for example guinea-pig trachea and ileum. In the initial studies of the guinea-pig trachea, it was found that FPL 55712 inhibited the contractions to all cysteinyl-leukotrienes (Krell et al., 1981b; Jones et al., 1983). However, when the metabolism of LTC_4 into LTD_4 (see above) was inhibited by either L-serine borate or GSH, the contractions to LTC_4 were no longer inhibited by FPL 55712 (Snyder et al., 1984; Snyder & Krell, 1984; Weichman & Tucker, 1985; Hand & Schwalm, 1987; Muccitelli et al., 1987).

Although FPL 55712 apparently antagonises the receptors for cysteinyl-leukotrienes, its effect has been reported not to be highly specific (Chasin & Scott, 1978; Krell et al., 1981a). However, a number of specific and potent cysteinyl-leukotriene receptor antagonists were subsequently developed, of which some are listed in Table 1. In the guinea-pig ileum and/or guinea-pig trachea these antagonists all share the characteristics of being potent inhibitors of the LTD_4- and LTE_4-induced contractions, whereas they either not at all, or only marginally, inhibit the LTC_4-induced contractions (see Table 4 in Section 4.1.). Despite these findings, the hypothesis of separate LTC_4 and LTD_4/LTE_4 receptors was not found generally applicable since in isolated human bronchi (Buckner et al., 1986; Buckner et al., 1990) as well as guinea-pig gall bladder (Falcone & Krell, 1992) and rat lung strip (Norman et al., 1994), the contractions to all three cysteinyl-leukotrienes are inhibited by this class of antagonists. Moreover, in some preparations, both LTC_4- and LTD_4-induced contractions are resistant to the antagonists (Snyder & Krell, 1986; Cuthbert et al., 1991a; Gardiner et al., 1993). In addition, in the guinea-pig trachea some results of studies of both contractions (Krell et al., 1983; Hand et al., 1989) and agonist binding (Aharony et al., 1988) suggest that LTD_4 may interact with more than one distinct receptor.

Based on the above-mentioned findings, the names $CysLT_1$ and $CysLT_2$ were introduced for the receptors being sensitive ($CysLT_1$) or resistant ($CysLT_2$) to the class of antagonists that had been developed (Table 1), which consequently are referred to as $CysLT_1$ receptor antagonists (Coleman et al., 1995). The major interest of antagonists of the $CysLT_1$ receptor is because of their

beneficial effects in the treatment of asthma (see Section 1.2.1.), and ICI 204,219

CODE NAME	GENERIC NAME	TRADE NAME	REFERENCE
ICI 198,615			Snyder et al., 1987
ICI 204,219	zafirlukast	Accolate™	Krell et al., 1990
L 649,923			Jones et al., 1986
MK 571/L 660,711			Jones et al., 1989
MK 0476/L 706,631	montelukast	Singulair™	Jones et al., 1995
LY 171,883	tomelukast		Fleisch et al., 1985
SKF 104,353	pobilukast		Hay et al., 1987
ONO 1078	pranlukast	Onon™	Obata et al., 1992

Table 1: $CysLT_1$ receptor antagonists.

(zafirlukast, Accolate™), MK 0476 (montelukast, Singulair™), and ONO 1078 (pranlukast, Onon™) have been clinically introduced for this purpose (Table 1; Drazen et al., 1999).

The first evidence for the existence of also $CysLT_2$ receptors in human tissues, were provided by Labat and co-workers (1992), who showed that the LTC_4- and LTD_4-induced contractions of the human pulmonary venous smooth muscle were resistant to the $CysLT_1$ receptor antagonists ICI 198,615, MK 571 and SKF 104,353. In addition, Ortiz and co-workers (1995) extended these observations by indicating the presence of both $CysLT_1$ and $CysLT_2$ receptors on the endothelium of human pulmonary veins.

By structural modification of LTE_4, two CysLT receptor antagonists were developed, BAY x7195 and BAY u9773 (Cuthbert et al., 1991b; Abram et al., 1993). BAY x7195 was shown to be a $CysLT_1$ receptor antagonist, inhibiting LTD_4-induced contractions in guinea-pig trachea and human bronchi but not in human pulmonary veins (Abram et al., 1993; Gorenne et al., 1995), whereas BAY u9773 had a broader activity than the previously described $CysLT_1$ receptor antagonists. In fact, BAY u9773 was shown to be a competitive antagonist of both LTC_4- and LTE_4-induced contractions of the guinea-pig trachea, i.e. a dual $CysLT_1$ and $CysLT_2$ receptor antagonist (Cuthbert et al., 1991b). Subsequent studies extended this observation by showing competitive antagonism by BAY u9773 of both $CysLT_1$ and $CysLT_2$ receptors in human bronchi and pulmonary veins (Labat et al., 1992) as well as in rat lung, ferret spleen and sheep bronchi (Tudhope et al., 1994).

1.1.3.2. Molecular characteristics

During the course of the project presented in this thesis, the human $CysLT_1$ and $CysLT_2$ receptors were cloned (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000). Binding of radiolabelled cysteinyl-leukotrienes to the first cloned human CysLT receptor was shown to be inhibited by antagonists such as zafirlukast, montelukast and pranlukast, and the receptor was thus classified as being the human $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999). This classification was further supported by results of *in situ* hybridisation showing receptor mRNA expression in human bronchial smooth muscle cells (Lynch et al., 1999; Sarau et al., 1999). About one year later a second CysLT receptor was cloned by three laboratories (Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000). This receptor exhibited similar characteristics as the functionally defined $CysLT_2$ receptor in that it was resistant to

$CysLT_1$ receptor antagonists (Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000) but inhibitable by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (Heise et al., 2000; Nothacker et al., 2000).

1.1.3.3. Transduction mechanisms

The CysLT receptors are 7-transmembrane G-protein coupled receptors (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000). The calcium mobilisation in $CysLT_1$ and $CysLT_2$ receptor transfected cells is not inhibited by pertussis toxin, suggesting that the G-protein involved is of the $G_{q/11}$ rather than the $G_{i/o}$ class (Sarau et al., 1999; Heise et al., 2000). However there may be a difference between transfected cells and cells with endogenously expressed receptors since in differentiated U937 cells, a human monocyte leukaemia cell line, the LTD_4-induced calcium increase is partially inhibited by pertussis toxin (Crooke et al., 1990). In support of the suggestion that both pertussis toxin sensitive and insensitive G proteins are involved in CysLT receptor transduction (Crooke et al., 1990), the LTD_4-induced reorganisation of the actin cytoskeleton in cultured human bronchial smooth muscle cells is sensitive to pertussis toxin (Saegusa et al., 2001).

The increased intracellular calcium induced by LTD_4 in $CysLT_1$ receptor transfected cells is minimally affected by removal of extracellular calcium, suggesting a release from intracellular stores (Sarau et al., 1999), whereas in U937 cells most of the calcium is extracellularly derived (Crooke et al., 1990). In isolated human bronchi, the contractions induced by LTD_4 are significantly suppressed by an inhibitor of receptor operated calcium channels (SKF 96,396; Gorenne et al., 1998) but not by diltiazem or nifedipine, which are inhibitors of voltage operated calcium channels (Bourdillat et al., 1987; Gorenne et al., 1998).

A recent report has demonstrated a small residual contraction induced by LTD_4 in human bronchi also in the absence of both intra- and extracellular calcium, and that LTD_4 induces contractions of cultured human airway smooth muscle cells without increasing intracellular calcium (Accomazzo et al., 2001). In the latter report, it was suggested that the LTD_4-induced contractions of human bronchi may consist of both a calcium-dependent phase, responsible for the onset of contraction, and a slower phase that involves the activation of a calcium-independent isoform of protein kinase C, responsible for the maintenance of contractile tone (Accomazzo et al., 2001). In the guinea-pig trachea, the LTD_4-induced contractions are only somewhat inhibited by nifedipinde or verapamil, but effectively inhibited by SKF 96,396 (Weichman & Tucker, 1985; Cuthbert et al., 1994), supporting the involvement of receptor operated calcium channels in smooth muscle contractions induced by cysteinyl-leukotrienes.

1.2. Effects of cysteinyl-leukotrienes

1.2.1. Airways

1.2.1.1. Asthma

Asthma is a chronic inflammatory disease of the airways causing wheezing, breathlessness and cough associated with airflow obstruction. The basic alterations in asthma are bronchoconstriction, increased mucus formation, oedema of the airway mucosa and increase in airway responsiveness to a variety of stimuli (Bousquet et al., 2000). The chronic inflammation of the airways in addition causes long-term effects, resulting in structural changes of the airway wall (Bousquet et al., 2000). Drugs modifying the cysteinyl-leukotriene pathway have beneficial effects in the treatment of asthma (Drazen et al., 1999) and the actions of these inflammatory mediators in this disease are described below.

1.2.1.2. Bronchoconstriction

The direct and potent contractile responses induced by cysteinyl-leukotrienes in isolated human bronchi was first demonstrated by Dahlén and co-workers (1980), and confirmed in subsequent studies (Hanna et al., 1981; Jones et al., 1982; Buckner et al., 1986). In addition, inhaled cysteinyl-leukotrienes were shown to induce bronchoconstriction (Holroyde et al., 1981), being up to 10,000 times more potent than histamine (Weiss et al., 1982). Furthermore, the clinically introduced leukotriene synthesis inhibitors and $CysLT_1$ receptor antagonists have been shown to significantly reduce bronchoconstriction in asthmatics (Dahlén et al., 1994; Drazen et al., 1999).

1.2.1.3. Mucus secretion

Cysteinyl-leukotrienes have been shown to increase bronchial mucus secretion in human airways *in vitro* using radiolabelled glucosamine as marker (Marom et al., 1982; Coles et al., 1983). However, a recent study measuring a specific mucin gene product (MUC5AC) from human bronchi *in vitro* did not observe an increased release after challenge with LTD_4 (Labat et al., 1999). Although the exact role of cysteinyl-leukotrienes in mucus secretion remains to be established, the notion of cysteinyl-leukotrienes being potent mucus secretagogues has received support from studies of cats and dogs *in vivo* (Peatfield et al., 1982; Johnson & McNee, 1983) as well as guinea-pig trachea *in vitro* (Liu et al., 1998). In the guinea-pig trachea, the increased mucus secretion induced by mucosal administration of either LTD_4 or antigen is inhibited by the $CysLT_1$ receptor antagonists zafirlukast and pranlukast (Liu et al., 1998).

1.2.1.4. Other airway effects

Oedema of the bronchial mucosa is another key finding in asthma, in addition to bronchoconstriction and increased mucus formation (Bousquet et al., 2000). Cysteinyl-leukotrienes have been shown to induce plasma exudation in guinea-pig airways (Hua et al., 1985) associated with an extravasation of macromolecules (Persson et al., 1986). These studies suggest a possible involvement of cysteinyl-leukotrienes in the development of bronchial oedema.

The increase in airway responsiveness to methacholine induced by allergen in sensitised rats is inhibited by the $CysLT_1$ receptor antagonist MK 571 (Wang et al., 1993), suggesting that the bronchial hyper-responsiveness observed in asthmatics may be induced by cysteinyl-leukotrienes. Prolonged exposure to LTE_4 has been reported to induce hyperresponsiveness to histamine in both guinea-pig and human airways *in vitro* via a mechanism proposed to involve a facilitation of cholinergic neurotransmission due to secondary released cyclooxygenase products (Lee et al., 1984; Jacques et al., 1991). In the porcine trachea, LTC_4 does not induce contractions, but enhances the contractions induced by charbachol or K^+ depolarisation by increasing the responsiveness of the contractile apparatus to calcium (Setoguchi et al., 2001).

In patients suffering from chronic asthma, changes in the structure of the airway wall, leading to a narrowing of the bronchial lumen, is a common feature referred to as airway remodelling (Bousquet et al., 2000). Experimental models have shown that 5-lipoxygenase inhibitors and $CysLT_1$ receptor antagonists inhibit the allergen-induced increase in airway smooth muscle in rats (Wang et al., 1993; Salmon et al., 1999). In cultured human airway smooth muscle cells, LTD_4 does not have any direct effects on mitogenesis, but it augments the DNA synthesis induced by epidermal growth factor (EGF) and induces reorganisation of the actin cytoskeleton, both of which is inhibited by the $CysLT_1$ receptor antagonist pranlukast (Panettieri et al., 1998; Saegusa et al., 2001). Taken together, these studies thus suggest a role of cysteinyl-leukotrienes also in airway

remodelling and that this effect may be an additional target for anti-leukotriene drugs in long-term treatment of asthma.

1.2.2. Cardiovascular system

1.2.2.1. Hemodynamic effects

Hypotension is a common feature in severe allergic reactions (Smith et al., 1980) and in addition, cardiac changes such as arrhythmias and ischemia have been observed during anaphylactic shock (Bernreiter, 1959). The identification of SRS-A as the cysteinyl-leukotrienes thus raised the suggestion that, in addition to the airways, also the cardiovascular system may be a target for these inflammatory mediators.

In humans, LTC_4 injected into the right atrium results in a transient increase in cardiac output followed by a prolonged phase with cardiac output below initial (Kaijser, 1982). These cardiac changes are associated with similar variations in heart rate and forearm blood flow as well as a slight decrease in pulmonary and systemic blood pressures (Kaijser, 1982). Also after intravenous injection, LTC_4 decreases the mean arterial pressure and in addition, intracoronary injection of LTD_4 causes an increase in coronary vascular resistance (Vigorito et al., 1997).

In guinea-pigs, both increase and decrease of mean systemic arterial pressure have been reported after injection of cysteinyl-leukotrienes (Drazen et al., 1980; Omini et al., 1981; Dahlén, 1983; Berkowitz et al., 1984; Hua et al., 1985;). Some of the apparent differences between those studies may refer to that the use of anaesthesia may affect the systemic cysteinyl-leukotriene-induced effects (Drazen et al., 1980). In addition, intravenously injected leukotrienes cause changes in pulmonary function in guinea-pigs (Weichman et al., 1982; Dahlén, 1983), which in turn may affect systemic responses if the animals are not artificially ventilated. Although the time-course of the blood pressure changes in guinea-pigs differs somewhat between the reports, the main conclusion is a biphasic pressor/depressor response with a sustained hypotension (Drazen et al., 1980; Omini et al., 1981; Dahlén, 1983; Hua et al., 1985). Likewise, in monkeys and rats, a transient rise in arterial pressure has been reported, followed by a long-lasting hypotensive period (Smedegård et al., 1982; Zukowska-Grojec et al., 1982; Iacopino et al., 1983) whereas in other species, such as sheep and pigs, cysteinyl-leukotrienes induce an increase in systemic blood pressure (Ahmed et al., 1985; Olson & Fleisher, 1989; Zellner et al., 1991).

Hemodynamic effects after systemic administration of cysteinyl-leukotriene are complex responses and may vary between different species and also depend on what doses of cysteinyl-leukotrienes that have been used in the studies. Moreover, the responses may be further complicated by secondary released factors. For example, the vascular responses to cysteinyl-leukotrienes are closely linked the cyclooxygenase and nitric oxide pathways (see Sections 1.2.4. and 4.3.) and in addition, cysteinyl-leukotrienes have been shown to increase the levels of circulating cathecolamines in both rats (Zukowska-Grojec et al., 1982) and humans (Vigorito et al., 1997).

Another difficulty is to interpret from what action of the cysteinyl-leukotrienes the effects on blood pressure originate. In general, the changes in systemic blood pressure induced by cysteinyl-leukotrienes reflect both a vasoconstriction (pressor response) and a depression of cardiac function (depressor response). In humans (Kaijser, 1982), monkeys (Smedegård et al., 1982), rats (Iacopino et al., 1983) and pigs (Leffler et al., 1984; Olson & Fleisher, 1989) a decreased cardiac function is observed after cysteinyl-leukotriene injection. The decrease in cardiac output induced by cysteinyl-leukotrienes may be a result of increased vascular resistance, dilatation of capacitance vessels leading to decreased venous return (Pawloski & Chapnick, 1993b) or an increased

extravasation of plasma leading to a decreased circulating blood volume (Hua et al., 1985). There are however observations supporting that cysteinyl-leukotrienes also have a direct effect on cardiac function.

1.2.2.2. Cardiac effects

In sheep and pigs, cysteinyl-leukotrienes induce coronary vasoconstriction, associated with ischemia and impaired left ventricular function (Michelassi et al., 1982; Fiedler et al., 1985). Increased coronary vascular resistance after intracoronary administration of LTD_4 has also been observed in humans (Vigorito et al., 1997), and cysteinyl-leukotrienes contract isolated atherosclerotic human coronary arteries *in vitro* (Allen et al., 1993). Likewise, in Langendorff-perfused guinea-pig hearts, cysteinyl-leukotrienes reduce coronary flow (Burke et al., 1982; Letts & Piper, 1982) and in addition, a direct effect on the myocardium has been suggested to be part of the resulting impaired cardiac function (Burke et al., 1982). However, studies of isolated guinea-pig cardiac muscle preparations have generated conflicting data concerning the direct effects of cysteinyl-leukotrienes, including no myocardial effects (Letts & Piper, 1982) as well as negative (Burke et al., 1982) and positive (Falcone et al., 1991) inotropic effects. The cardiac effects of cysteinyl-leukotrienes may in addition be dose-dependent since in Langendorff-perfused isolated rat hearts, low concentrations of either LTC_4 or LTD_4 induce positive inotropic effects whereas higher concentrations induce negative inotropic effects (Karmazyn & Moffat, 1990). In the study reporting negative inotropic effects of cysteinyl-leukotrienes on isolated guinea-pig myocardium, a reduced contractile force of human atrial myocardium was also observed (Burke et al., 1982).

Interestingly, the recently cloned human $CysLT_2$ receptor has been shown to be expressed in the heart (Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000), further supporting the possibility that circulating cysteinyl-leukotrienes directly may affect cardiac function and suggesting a possible role for specific $CysLT_2$ receptor antagonists and/or agonists in the treatment of cardiac diseases. In fact, 5-lipoxygenase inhibitors and CysLT receptor antagonists have been shown to reduce infact-size and reperfusion arrhythmias in animal models of myocardial ischemia (Lepran & Lefer, 1985; Hock et al., 1992)

1.2.2.3. Pulmonary circulation

In the pulmonary circulation, cysteinyl-leukotrienes have been reported to induce a pressor response in most species (Kadowitz & Hyman, 1984; Ahmed et al., 1985; Farrukh et al., 1986; Ohtaka et al., 1987; Olson & Fleisher, 1989; Zellner et al., 1991). However, in rats, LTD_4 induces a pulmonary hypotension (Iacopino et al., 1983) and in humans, a slight decrease in pulmonary artery pressure has been reported after LTC_4 injection into the right atria (Kaijser, 1982). In monkeys, the pulmonary artery pressure follows the changes in systemic blood pressure, i.e. a transient rise, associated with increased vascular resistance followed by a prolonged fall associated with a decrease in cardiac output (Smedegård et al., 1982) and a subsequent study showed that LTD_4 induces contractions of pulmonary arteries derived from monkeys (Berkowitz et al., 1984). Likewise, an increased pulmonary vascular resistance has been reported in guinea-pigs (Berkowitz et al., 1984), sheep (Ahmed et al., 1985; Kadowitz & Hyman, 1984) and pigs (Leffler et al., 1984; Ohtaka et al., 1987; Olson & Fleisher, 1989; Zellner et al., 1991). Since the pig has been extensively studied with respect to cysteinyl-leukotrienes in the cardiovascular system (Leffler et al., 1984; Fiedler et al., 1985; Galton & Piper, 1987; Ohtaka et al., 1987; Olson & Fleisher, 1989; Zellner et al., 1991) it was considered a suitable species for the exploration of the effects of cysteinyl-leukotrienes on the pulmonary vasculature in the present project. Interestingly, pulmonary venous resistance is increased by LTC_4 in pigs *in vivo*, whereas

isolated porcine pulmonary veins have been reported not to contract in response to LTC_4 *in vitro* (Ohtaka et al., 1987).

1.2.2.4. Isolated vessels

The first evidence of contractile effects of cysteinyl-leukotrienes on isolated human vessels was provided by Hanna and co-workers (1981). These findings in pulmonary vessels were examined in greater detail in subsequent studies (Schellenberg & Foster, 1984; Bourdillat et al., 1987), with the conclusion of a preferential vasoconstriction to cysteinyl-leukotrienes in human pulmonary veins compared with pulmonary arteries. It was also shown that the LTD_4-induced contractions of human pulmonary veins are modulated by both relaxant and contractile factors released from the endothelium (Ortiz et al., 1995). Cysteinyl-leukotriene-induced effects in the pulmonary vasculature have also been observed in studies of pulmonary vessels derived from different animals, for example guinea-pigs where cysteinyl-leukotrienes induce contractions of pulmonary arteries (Hand et al., 1981; Berkowitz et al., 1984; Fedyna et al., 1990) but only small contractions of pulmonary veins (Berkowitz et al., 1984). Also rodent and porcine pulmonary arteries have been reported to be more sensitive to cysteinyl-leukotrienes compared with pulmonary veins (Maddox et al., 1985; Ohtaka et al., 1987).

Human systemic vessels also contract in response to cysteinyl-leukotrienes *in vitro*, the saphenous vein and internal mammary artery being the most studied, due to their use in coronary graft bypass surgery. The human saphenous vein exhibits greater contractile responses to cysteinyl-leukotrienes than the internal mammary artery (Allen et al., 1994), suggesting that cysteinyl-leukotrienes preferentially contract systemic veins compared with arteries. This notion is supported by findings in the guinea-pig where cysteinyl-leukotrienes induce contractions of the inferior vena cava (Berkowitz et al., 1984; Rinkema et al., 1993) whereas the aorta is only slightly contracted by cysteinyl-leukotrienes (Berkowitz et al., 1984; Sakuma et al., 1987).

Cysteinyl-leukotrienes not only contract isolated vessels but have also been reported to induce endothelium-dependent relaxations, first shown in canine renal and mesenteric arteries (Secrest et al., 1985) and subsequently described in canine renal and splanchnic veins (Pawloski & Chapnick, 1991; Pawloski & Chapnick, 1993b) as well as in guinea-pig aorta and pulmonary artery (Sakuma et al., 1987; Fedyna et al., 1990). In addition, both systemic and pulmonary vessels from humans exhibit endothelium-dependent relaxations to cysteinyl-leukotrienes (Allen et al., 1992; Ortiz et al., 1995).

1.2.2.6. Microcirculation

In the hamster cheek pouch microcirculation, LTC_4 and LTD_4 are potent constrictors of arterioles and in addition, induce a dose-dependent plasma leakage and extravasation of macromolecules from postcapillary venules (Dahlén et al., 1981). Moreover, intravenous injection of cysteinyl-leukotrienes causes plasma extravasation throughout the body in guinea-pigs (Hua et al., 1985) and LTD_4 increases pulmonary vascular permeability in rabbits (Farrukh et al., 1986).

Intradermal injection of either LTC_4 or LTD_4 in humans leads to an increase in the microvascular cutaneous blood flow associated with a local erythema and an increased venular permeability causing wheal-formation (Bisgaard et al., 1982; Soter et al., 1983). The $CysLT_1$ receptor antagonist zafirlukast inhibits this cutaneous reaction to a lesser extent compared with its inhibition of bronchoconstriction (Dahlén et al., 1994).

Cysteinyl-leukotrienes may also be involved in the recruitment of leukocytes from the circulation into a tissue (Laitinen et al., 1993; Salmon et al., 1999). The mechanism behind this recruitment has been proposed to involve cysteinyl-leukotriene-

induced expression of the adhesion molecule P-selectin on the surface of endothelial cells (Pedersen et al., 1997), leading to increased adhesion of neutrophils. Interestingly, the surface expression of P-selectin induced by cysteinyl-leukotrienes in cultured human umbilical vein endothelial cells is not blocked by the $CysLT_1$ receptor antagonists SKF 104,353, pranlukast or zafirlukast (Pedersen et al., 1997).

1.2.3. Other targets

1.2.3.1. Central nervous system

Cysteinyl-leukotrienes are formed in brain tissue from rats (Lindgren et al., 1984) and humans (Simmet et al., 1988) and the human $CysLT_2$ receptor have been demonstrated to be expressed in the brain (Heise et al., 2000; Nothacker et al., 2000), suggesting a possible role of cysteinyl-leukotrienes in the central nervous system. A limited number of studies have investigated the effects of cysteinyl-leukotrienes on isolated human intracerebral arteries, reporting no effects (von Holst et al., 1982) or small contractions (Tagari et al., 1983a; Tagari et al., 1983b). In the rat brain, cysteinyl-leukotrienes have been demonstrated to stimulate release of luteinising hormone (LH) from pituitary cells (Hulting et al., 1985) and to induce a prolonged excitation of cerebellar purkinje neurons (Palmer et al., 1981).

In two reported cases of LTC_4 synthase deficiency, the patients presented neurological symptoms, including muscular hypotonia and psychomotor retardation, and died at the age of 6 months (Mayatepek & Flock, 1998; Mayatepek et al., 1999), suggesting that cysteinyl-leukotrienes may be involved in normal neurological development. However, no studies of DNA were performed in these studies and it cannot be excluded that the children, in addition to LTC_4 synthase deficiency, had a second disorder that led to the neurological problems. In fact, mice that lack cysteinyl-leukotriene formation after disruption of the 5-lipoxygenase-gene function (5-lipoxygenase knockout mice) have no apparent neurological abnormalities (Funk, 1996). However, in support of a link between cysteinyl-leukotrienes and human neurological function it has been reported that patients with GSH synthase defiency, who have low levels cysteinyl-leukotrienes due to decreased formation of LTC_4, also present progressive neurological symptoms (Pace-Asciak et al., 1986; Mayatepek et al., 1993; Mayatepek et al., 1994).

1.2.3.2. Gastrointestinal system

In addition to airway and vascular smooth muscle, also other tissues contract in response to cysteinyl-leukotrienes, for example isolated guinea-pig ileum and gall bladder (Dahlén et al., 1987; Gardiner et al., 1990; Falcone & Krell, 1992). Moreover, the human $CysLT_1$ receptor has been reported to be expressed in small intestine, colon, liver and pancreas (Sarau et al., 1999). Taken together, these findings suggest that cysteinyl-leukotrienes may be inflammatory mediators also in the gastrointestinal tract. However, trials using leukotriene synthesis inhibitors in the treatment of inflammatory bowel disease have not shown any significant beneficial effects (Hawkey et al., 1997).

1.2.3.3. Immune system

The human $CysLT_1$ and $CysLT_2$ receptors are expressed in spleen, lymph nodes and on peripheral blood leukocytes (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000; Takasaki et al., 2000; Nothacker et al., 2000). A recent report extended these observations by immunohistochemically showing the expression of the $CysLT_1$ receptor on B-lymphocytes, eosinophils and monocytes, but not on T-cells or neutrophils (Figueroa et al., 2001).

1.2.4. Secondary released factors

1.2.4.1. Cyclooxygenase products

In some cells, activation of CysLT receptors leads to the release of various mediators that contribute to the biological actions of the cysteinyl-leukotrienes. For example, some of the effects of cysteinyl-leukotrienes in both respiratory and cardiovascular systems have been shown to be mediated via release of products of the cyclooxygenase pathway of arachidonic acid metabolism (see Sections 4.2.2. and 4.3.3.). It has been reported that LTD_4 increases the release of arachidonic acid in isolated cells via a receptor driven mechanism involving RNA and protein synthesis, leading to the formation of various metabolites (Crooke et al., 1990). Depending on what metabolites are formed, the response of the smooth muscle may be either contractile or relaxant.

Prostaglandin endoperoxide synthase or PGH synthase (in this thesis referred to as cyclooxygenase) is a membrane bound enzyme that catalyses a cyclooxygenation of arachidonic acid leading to the formation of prostaglandin G_2 (PGG_2), which in turn is peroxidised into prostaglandin H_2 (PGH_2) by the same enzyme (DeWitt, 1999; Narumiya et al., 1999). Two iso-forms of cyclooxygenase have been identified, called COX-1 and COX-2 and specific inhibitors of the latter isoform have recently been introduced for clinical use (for review, see DeWitt, 1999). While COX-1 is constitutively expressed in most tissues and though to produce prostaglandins involved in the physiologic functions, COX-2 is in general not expressed in normal tissues (DeWitt, 1999). However, the COX-2 enzyme is upregulated after inflammatory stimuli, making it a suitable target for anti-inflammatory treatment without affecting the physiological functions of prostaglandins such as gastric mucosal protection (DeWitt, 1999). Indomethacin, one of the most often used cyclooxygenase inhibitors in experimental studies, non-selectively inhibits COX-1 and COX-2 (O'Neill et al., 1994) and was therefore used in all experiments evaluating the role of cyclooxygenase modulation of cysteinyl-leukotriene responses within the present thesis (Paper II, III, IV and Fig. 6).

The product of the cyclooxygenase enzyme is thus PGH_2, which is an unstable endoperoxide that can be converted into the different prostaglandins. The enzyme thromboxane synthase catalyses the formation of thromboxane A_2 (TXA_2), which activates a TP receptor (Narumiya et al., 1999) leading to constriction of airway and vascular muscle (McKenniff et al., 1988). However, TXA_2 is rapidly converted to the biologically inactive thromboxane B_2 (TXB_2), and concentrations of the latter compound can be measured in order to determine the release of TXA_2 in biological systems (see Paper II). The rapid breakdown of TXA_2 also makes it difficult to use in experimental studies, and therefore the stable TP receptor agonist U 46 619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$) was used in the present study (see Sections 4.1.5. and 4.3.3.). The synthesis of TXA_2 (measured as TXB_2) after cysteinyl-leukotriene challenge has been reported in a number of tissues and cells, for example guinea-pig lungs (Omini et al., 1981; Dahlén et al., 1983), pig heart (Fiedler et al., 1985) and rat peritoneal macrophages (Feuerstein et al., 1981).

The enzyme prostacyclin synthase converts PGH_2 into prostacyclin (prostaglandin I_2, PGI_2), which via the IP receptor activates adenylate cyclase leading to increased levels of cAMP and consequently, smooth muscle relaxation (Hadhazy et al., 1983; Haye-Legrand et al., 1987; Narumiya et al., 1999). Prostacyclin is rapidly metabolised into 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), and measured concentrations of this metabolite correspond to the release of prostacyclin (see Papers II and III). Cysteinyl-leukotrienes have been shown to induce formation of prostacyclin in cultured human umbilical vein endothelial cells (Cramer et al., 1983; Pologe et al., 1984) as well as in guinea-pig lungs (Omini et al., 1981).

Other prostaglandins that are formed from PGH_2 include prostaglandins E_2, D_2 and $F_{2\alpha}$ (PGE_2, PGD_2 and $PGF_{2\alpha}$). These prostaglandins activate receptors denoted EP (EP_1, EP_2, EP_3, EP_4), DP and FP, respectively (for review, see Narumiya et al., 1999).

1.2.4.2. NO

In endothelial cells, cysteinyl-leukotrienes may induce activation of the enzyme nitric oxide synthase leading to formation of nitric oxide (Ignarro, 1990; Allen et al., 1992; Pawloski & Chapnick, 1993b; Ortiz et al., 1995). The nitric oxide released by the endothelial cells diffuses to the underlying vascular muscle where it stimulates the enzyme soluble guanylate cyclase, which by increasing intracellular levels of cGMP leads to a relaxation of the vascular smooth muscle (Arnold et al., 1977).

The notion of endothelial nitric oxide as a regulator of vascular tone and reactivity originates in the finding that acetylcholine induced relaxations of isolated rabbit aortic preparations with an intact endothelium, but not in endothelium denuded preparations (Furchgott & Zawadzki, 1980). This "endothelium derived relaxing factor" (Furchgott & Zawadzki, 1980) was later shown to be associated with an increase in cGMP in rat aorta (Rapoport & Murad, 1983) and bovine pulmonary arteries and veins (Ignarro et al., 1987b), which led to the identification of this factor as nitric oxide (Ignarro et al., 1987a; Palmer et al., 1987; for review, see Ignarro, 1990).

2. AIMS OF THE THESIS

The aim of this thesis was to explore the effects of cysteinyl-leukotrienes in order to search for novel receptors or functions for cysteinyl-leukotrienes. Therefore, responses to cysteinyl-leukotrienes were characterised using tissue preparations that were set up in organ baths. When the work with this thesis was initiated, the molecular structure of the $CysLT_1$ and $CysLT_2$ receptors were not known, and one primary aim was therefore to further explore the functional characteristics of CysLT receptors.

In addition, pharmacological studies of whole tissue preparations allow correlating receptor characterisation with a physiological function. Such studies of CysLT receptors may hence help to understand the physiological or pathophysiological role of cysteinyl-leukotrienes in different tissues. This offers an essential advantage compared with studies using molecular pharmacological methods or studies of ligand binding.

In order to further explore the physiological significance of CysLT receptor function, also the modulatory mechanisms involved in the responses must be assessed. In this thesis, modulation of contractile responses induced by cysteinyl-leukotrienes was studied and classified as either interconversion between the agonists or release of modulatory factors, of which factors of the cyclooxygenase and nitric oxide pathways were particularly studied.

The following three classes of preparations were used: airways (guinea-pig trachea), pulmonary vessels (human and porcine pulmonary arteries and porcine pulmonary veins) and intestinal smooth muscle (guinea-pig ileum longitudinal muscle).

The guinea-pig trachea is one of the most commonly used preparations in airway pharmacology and although the functional CysLT receptors have been extensively studied in this preparation, no definitive conclusion as to the receptor activation by the individual cysteinyl-leukotrienes has been obtained. In addition, the guinea-pig trachea metabolises cysteinyl-leukotrienes (Snyder et al., 1984) and was therefore considered to be a suitable model for the study of metabolic modulation of cysteinyl-leukotriene-induced contractions.

In addition to airway effects, cysteinyl-leukotrienes also have potent cardiovascular effects and affect the pulmonary circulation in many species (see Section 1.2.2.). The responses to cysteinyl-leukotrienes in human pulmonary veins have been characterised (Labat et al., 1992; Ortiz et al., 1995), but also pulmonary arteries have been reported to contract to cysteinyl-leukotrienes (Hanna et al., 1981; Schellenberg & Foster, 1984; Maddox et al., 1985; Bourdillat et al., 1987). However, no previous studies have assessed either the receptors or the modulatory mechanism involved in the cysteinyl-leukotriene-induced contractions of human pulmonary arteries. Therefore, this was set up as one primary goal of this thesis. However, since human tissue may be difficult to obtain, a suitable model for the cysteinyl-leukotriene-induced contractions of the human pulmonary vessels would be useful. To address this matter, the porcine pulmonary vasculature was examined in order to evaluate if these preparations could be used as models for human pulmonary vessels.

The guinea-pig ileum was previously the main preparation used for bioassay of SRS-A and cysteinyl-leukotrienes (Dahlén et al., 1987). However, no previous studies have investigated the receptor involved in contractile responses induced by LTC_4, which are resistant to $CysLT_1$ receptor antagonism (Gardiner et al., 1990). Since the modulatory factors involved in regulating responses in the guinea-pig ileum previously have been reported to be associated with the epithelium (Krilis et al., 1983), the guinea-pig ileum longitudinal muscle preparation (which does not contain epithelium) was not further studied with regard to modulatory mechanisms.

3. METHODS

3.1. General

According to the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR), a functional assay is defined as "a pharmacological test system in which a response can be firmly attributed to the function of a defined receptor type or subtype" (Vanhoutte et al., 1998). In the present thesis, the responses studied are contractions and relaxations of smooth muscle containing preparations placed in organ baths in order to characterise the CysLT receptors as well as the mechanisms involved in these responses. In addition, the release of modulatory factors was measured using enzyme immunoassay and the metabolism of cysteinyl-leukotrienes was assessed by incubation of tissue with radiolabelled cysteinyl-leukotrienes that were separated on reverse phase high performance liquid chromatography (RP-HPLC).

3.2. Experiments

3.2.1. Organ bath experiments

The tissues were prepared as rings (vascular preparations), spirals (guinea-pig trachea, Constantine, 1965) or longitudinal muscle (guinea-pig ileum, Rang, 1964) and set up in 5- or 10-ml organ baths containing Tyrode's solution bubbled with carbogen gas (5-6.5% CO_2 in O_2) and kept at a constant temperature of 37 °C. Changes in smooth muscle tension, i.e. contractions and relaxations, were recorded via isometric force-displacement transducers connected to a polygraph and responses were either displayed via a chart-recorder or recorded with a computerised data acquisition software (IOX; EMKA Technologies, France). Calculations of tension changes were made manually from charts or, more recently, computer aided with a data analysis software (Datanalyst; EMKA Technologies, France).

Cysteinyl-leukotrienes and other agonists were added either as single concentrations or as cumulative administration of increasing concentrations according to the method of van Rossum (1963). Maximum contractions were performed with histamine (100 μM; guinea-pig ileum, Paper I), noradrenaline (10 μM; human and porcine pulmonary vessels, Paper II-IV) or the combination of histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM; guinea-pig trachea, Paper V) in order to obtain a reference contraction.

3.2.2. Enzyme immunoassay

In order to determine the release of the vasoactive cyclooxygenase products prostacyclin and thromboxane A_2, supernatants were collected from the organ baths after challenge of the vascular preparations with cysteinyl-leukotrienes (Paper II and III). Measurements of the stable metabolites 6-keto $PGF_{1\alpha}$ (prostacyclin) and TXB_2 (TXA_2) were performed using enzyme immunoassay (Stallergenes, France) as previously described in detail by Pradelles and co-workers (1985). Briefly, 50 μl samples of the supernatants were added to 96-well microtiter plates with mouse monoclonal anti-rabbit IgG attached to the wells. The quantification is based on the competition of free 6-keto $PGF_{1\alpha}$ or TXB_2 with the respective acetylcholinesterase-linked prostanoid for binding to specific polyclonal rabbit antisera, forming complexes that then bind to the antibody in the well. The addition of Ellman's reagent allows measuring the increase in absorbance at 412 nm, which is inversely proportional to the amount of 6-keto $PGF_{1\alpha}$ or TXB_2 in the samples.

3.2.3. RP-HPLC

In order to establish the metabolism of cysteinyl-leukotrienes in the guinea-pig trachea and to evaluate the effects of different conversion inhibitors (Paper V), guinea-pig tracheal tissue was incubated in 0.5 ml phosphate buffered saline (PBS-buffer) or PBS-buffer containing the different

conversion inhibitors. After a 30-min incubation period, [^{3}H]LTC_4, [^{3}H]LTD_4 or [^{3}H]LTE_4 was added (3.7 kBq, 1 pmol) and after another 30 min the reaction was stopped by the addition of 0.5 ml ethanol. The supernatants were injected onto an RP-HPLC column in order to separate the cysteinyl-leukotrienes as previously described in detail by Kumlin and Dahlén (1990). The retention-times for the individual cysteinyl-leukotrienes were determined with authentic standards and the distribution of radioactivity in the fractions was determined by liquid scintillation counting.

3.2.4. Measurements of γ-GT activity

Measurements of γ-GT activity were performed using the synthetic γ-GT substrate γ-glutamyl-*P*-nitroanilide (GPNA) according to the method described in detail by Silber and co-workers (1986). The experiments were carried out using purified porcine kidney γ-GT (100 U/l; from Sigma, MO, USA) dissolved in 0.5 ml PBS-buffer. After 30 min incubation in the absence (control) or presence of different concentrations of the conversion inhibitors, 30 µl of the supernatant was transferred to a 96-well microtiter plate. The reaction solution had the following composition: GPNA (6 mM), glycylglycine (80 mM) and Tris (120 mM; all from Sigma, MO, USA) and 280 µl was added to each well. The catalysis of GPNA by γ-GT involves the transfer of the γ-glutamyl group to an acceptor (glycylglycine), yielding *P*-nitroanilide (PNA). The formation of PNA produces a yellow colour and the increase in absorbance, measured at 405 nm after 90 min, is correlated to the amount of PNA formed. Results (Fig. 8) are expressed at percent of inhibition of enzymatic formation of PNA compared with control.

3.2.5. Experimental protocols

Descriptions of the experimental protocols, the drugs used and other details are indicated in the individual papers. When previously unpublished data are presented, all experiments were carried out as described in the reports of the related studies. Any additional information is indicated in the figure legends.

The receptor antagonists and enzyme inhibitors used and their actions are summarised in Table 2.

3.2.6. Ethical approval

The experiments in the present thesis were approved by Stockholms norra djurförsöks-etiska nämnd (N 343/95, N 317/98), Etiska kommittén at Karolinska Hospital (KS 87:176, KS 00:267) or Ministère des Affaires Sociales et de l'Intégration and Ministère de l'Agriculture et de la Forêt du Gouvernement Français (arrêté du 25 février 1992).

3.3. Data analysis

3.3.1. Contractions and relaxations

Contractions are expressed as percent of the specified reference contraction in each study (see above) and relaxations in percent of the precontraction before the relaxant agonist was added. The results of the cumulative dosings are expressed as concentration-response curves.

3.3.2. Pharmacological analysis

In the cases where the concentration-response curves reached a plateau, the maximal contraction (E_{max}), i.e. the contraction induced by the highest concentration of each agonist, was determined. The half-maximum effective concentrations (EC_{50}-values) were calculated by linear regression and the pD_2-value was calculated as the negative log of the EC_{50}-value. In the guinea-pig ileum, the half-maximum effective concentrations were estimated as the concentration of LTC_4 inducing a contraction that was 25% of the maximal histamine-induced contraction (EC_{25}-value), since LTC_4 induced an E_{max} of

about 50% of histamine maximum in this preparation (Paper I).

In order to characterise the results with the CysLT receptor antagonists, pK_B- and pA_2-values were calculated as described below. These values are estimates of the dissociation constant of the antagonist-receptor complex and represent an indirect measurement of the antagonist's affinity for its receptor (Tallarida et al., 1979). The pK_B- and pA_2-values will coincide if antagonism is competitive (MacKay, 1978; Jenkinson et al., 1995), which is assumed when the two values are compared in this thesis. If two agonists act on the same receptor, they can be expected to be antagonised to the same degree by the same antagonists, i.e. similar pK_B- or pA_2-values should be obtained. In contrast, if the values are different, the agonists probably act on different receptors. Moreover, different preparations with similar receptors would be expected to give the same pK_B- and pA_2-values for a particular antagonist (Tallarida et al., 1979).

A concentration ratio (CR) was determined as the ratio of EC_{50}- or EC_{25}-values in the presence and in the absence of antagonist. The pK_B-value was calculated as the negative logarithm of the following equation: [B]/(CR-1), where [B] is the concentration of the antagonist and CR the concentration ratio. The pA_2-value was determined by Schild-plot analysis as described in detail by Arunlakshana and Schild (1959). Briefly, the logarithm of (CR-1) was plotted against the negative logarithm of the antagonist concentrations and a regression line was fitted to all data points. The slope of this regression line must not be significantly different from 1 in order to confirm competitive antagonism and to allow estimation of the pA_2-value (Arunlakshana & Schild, 1959). An alternative method to determine if the antagonism is competitive, used in Paper V, is to compare the pK_B-values obtained for each antagonist concentration. If the pK_B-values are not significantly different depending on antagonist concentrations, the slope of the Schild-plot regression is not significantly different from 1 and antagonism consequently competitive (MacKay, 1978). In this case the pA_2 value can also be determined as the mean of all pK_B-values (MacKay, 1978; Paper V).

3.3.3. Statistics

Statistical evaluation was performed using either a Student's t-test or a one- or two-way analysis of variances (ANOVA) test followed by Dunnett's test (multiple comparisons). A *P*-value of less than 0.05 was considered significant.

RECEPTOR ANTAGONISTS

Name	Chemical name	Function	References
BAY u9773	6(*R*)-(4'-carboxyphenylthio)-5(*S*)-hydroxy-7(*E*),9(*E*),11(*Z*)14(*Z*)-eicosatetrenoic acid	$CysLT_1/CysLT_2$ receptor antagonist	Cuthbert et al. 1991b
ICI 198,615	(1-((2-methoxy-4-(((phenylsulfonyl)amino) carbonyl)-phenyl)methyl)-1H-indazol-6-yl) carbamic acid cyclopentyl ester	$CysLT_1$ receptor antagonist	Snyder et al., 1987
ICI 204,219 (zafirlukast)	4-(5-cyclopentyloxycarbonylamino-1-methyl indol-3-ylmethyl)-3-methoxy-N-*o*-tolylsulfonyl benzamide	$CysLT_1$ receptor antagonist	Krell et al., 1990
MK 571	(3-(-2(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-(dimethylamino-3-oxopropyl)thio)methyl)thio propanoic acid	$CysLT_1$ receptor antagonist	Jones et al. 1988
SKF 104,353 (pobilukast)	2(*S*)-hydroxy-3(*R*)-((2-carboxyethyl)thio)-3-(8-phenyloctyl)phenyl)-propanoic acid	$CysLT_1$ receptor antagonist	Hay et al., 1987

ENZYME INHIBITORS

Name	Abbreviation	Function	References
acivicin	-	irriversible γ-GT inhibitor	Stole et al., 1994
L-cysteine	L-cys	inhibitor of LTD_4 metabolism	Sok et al., 1981
indomethacin	INDO	cyclooxygenase (COX) inhibitor (COX-1/COX-2 unselective)	O'Neill et al., 1993
glutathione	GSH	inhibitor of LTC_4 metabolism (substrate competition at γ-GT)	Hammarström, 1981
S-hexyl glutathione	S-hexyl GSH	inhibitor of LTC_4 metabolism (substrate competition at γ-GT)	Paper V
N^{ω}–nitro-L-arginine	L-NOARG	nitric oxide synthase inhibitor	Bina et al., 1998
L-serine borate	SeBo	reversible γ-GT inhibitor	Tate & Meister, 1978

Table 2: Receptor antagonists and enzyme inhibitors used in pharmacological and biochemical studies in the present thesis

4. RESULTS & DISCUSSION

4.1. Cysteinyl-leukotriene receptors

4.1.1. Specific aims

At the start of the work with this thesis, the $CysLT_2$ receptor was characterised as a functional CysLT receptor resistant to $CysLT_1$ receptor antagonists (Coleman et al., 1995). However, BAY u9773 had recently been shown to inhibit $CysLT_1$ receptor antagonist resistant cysteinyl-leukotriene responses (Cuthbert et al., 1991b; Labat et al., 1992; Tudhope et al., 1994). The primary aim was therefore to study functional responses that were resistant to $CysLT_1$ receptor antagonists in order to examine if the $CysLT_2$ receptor represented a heterogeneous class of receptors. In this context, BAY u9773 was tested against LTC_4-induced contractions in the guinea-pig ileum and trachea (Paper I and V, respectively). In addition, since in the human lung, $CysLT_2$ receptors had been associated with vascular tissues (Labat et al., 1992; Ortiz et al., 1995), this thesis also included investigations of two pulmonary vascular preparations that had not previously been studied with respect to cysteinyl-leukotriene receptors, namely porcine and human pulmonary arteries (Paper II and III, respectively).

In the guinea-pig trachea, it had been proposed that LTD_4 interacted with more than one receptor subtype (Krell et al., 1983; Hand et al., 1989), but no definite conclusion as to these proposals had been obtained. Therefore, this part of the thesis also evaluates LTD_4-induced contractions of the guinea-pig trachea in order to examine the possibility of heterogeneity of the $CysLT_1$ receptor.

4.1.2. $CysLT_1$ receptors

4.1.2.1. LTD_4-induced contractions of the guinea-pig trachea

The notion that the LTD_4- and LTE_4-induced contractions of the guinea-pig trachea are mediated via a $CysLT_1$ receptor is supported by the results in Paper V. The contractions to LTD_4 were inhibited by the three $CysLT_1$ receptor antagonists ICI 198,615 (pA_2=9.3±0.2), MK 571 (pK_B=9.2±0.3) and SKF 104,353 (pK_B=8.0±0.4) and LTE_4 was inhibited by ICI 198,615 (pK_B=9.4±0.2). Krell and co-workers (1983) were the first to suggest the presence of different subtypes of LTD_4-receptors in the guinea-pig trachea, based on the finding of a bimodal distribution of pK_B-values for the inhibition of LTD_4-induced contractions by FPL 55712. Hand and co-workers (1989) extended this observation by demonstrating non-linear Schild-plots for a number of different $CysLT_1$ receptor antagonists against LTD_4-induced contractions of the guinea-pig trachea. However, the use of inhibitors of cysteinyl-leukotriene metabolism in some (Hand et al., 1989), but not in other (Krell et al., 1983; Snyder et al., 1987; Jones et al., 1989) studies of $CysLT_1$ receptor antagonists in this context makes the results difficult to compare and interpret. As will be discussed in Section 4.2.3. (Paper V), inhibiting the metabolism of LTD_4 is necessary when studying CysLT receptor pharmacology in the guinea-pig trachea. The suggestion of more than one receptor for LTD_4 is supported by the finding that [^{3}H]LTE_4 binds to a subset of LTD_4-receptors in guinea-pig lung membranes (Aharony et al., 1988).

The antagonism of LTD_4-induced contractions of guinea-pig trachea by ICI 198,615 was dose-dependent for antagonist-concentrations up to 10 nM (Paper V). However, at ICI 198,615-concentrations of 30 nM and higher, no further antagonism was observed, and a residual contraction to LTD_4 was unmasked. This finding was in contrast to the LTE_4-induced contractions, which could be abolished by ICI 198,615 (300 nM), supporting that there may be a difference between how LTD_4 and LTE_4 interact with the $CysLT_1$ receptor. Interestingly, the residual contraction to LTD_4 was inhibited by BAY u9773, suggesting that

LTD_4 activates both $CysLT_1$ and $CysLT_2$ receptors, whereas LTE_4-induced contractions were mediated solely by the $CysLT_1$ receptor. These results (Paper V) may offer an explanation of the above-mentioned studies (Krell et al., 1983; Aharony et al., 1988; Hand et al., 1989) by suggesting that, in the guinea-pig trachea, the $CysLT_1$ receptor represents a high affinity LTD_4-receptor and the $CysLT_2$ receptor a low affinity LTD_4-receptor.

4.1.2.2 Comparison between guinea-pig and human $CysLT_1$ receptor

The receptor activated by LTD_4 and LTE_4 in the guinea-pig trachea share the profile of the human bronchial CysLT receptor of being inhibitable by $CysLT_1$ receptor antagonists, but differs in that the $CysLT_1$ receptor in human bronchi is activated by all three cysteinyl-leukotrienes (Buckner et al., 1986; Buckner et al., 1990). The rank order of agonist potency at the $CysLT_1$ receptor in isolated human bronchial preparations varies somewhat between the limited numbers of studies that have addressed this issue, being either LTC_4=LTD_4>LTE_4 (Muccitelli et al., 1987; Labat et al., 1992; Björck, 1993) or LTC_4=LTD_4=LTE_4 (Buckner et al., 1986; Buckner et al., 1990). In addition, in studies of cells transfected with the human $CysLT_1$ receptor, the rank order of potency is LTD_4>LTC_4>LTE_4 for calcium mobilisation (Lynch et al., 1999; Sarau et al., 1999; Takasaki et al., 2000; Nothacker et al., 2000), with LTE_4 being a partial agonist (Sarau et al., 1999; Takasaki et al., 2000). An explanation for the differences in potency between LTC_4 and LTD_4 in $CysLT_1$ receptor transfected cells but not in human bronchi has not been proposed. It is possible that in preparations with a high $CysLT_1$ receptor reserve, LTC_4 may be equipotent with LTD_4 despite its lower affinity for the receptor. Anyhow, in contrast to results with the human $CysLT_1$ receptor on isolated bronchial preparations and on transfected cells, LTC_4-induced contractions of the guinea-pig trachea were resistant to $CysLT_1$ receptor antagonists (Paper V). The rank orders of agonist potency at the human and guinea-pig $CysLT_1$ receptors are summarised in Table 3.

Another difference between the human and guinea-pig $CysLT_1$ receptor is the sensitivity of the cysteinyl-leukotriene-induced contractions to $CysLT_1$ receptor antagonists. Although the pA_2 and pK_B values that were obtained for ICI 198,615 and MK 571 against LTD_4-induced contractions (Paper V) were similar to previous studies in the guinea-pig trachea (Snyder et al., 1987; Jones et al., 1989), they were about one log order lower than the values previously reported for the same antagonists in human airways (Table 4).

Rank order of agonist potency		
GPT $CysLT_1$	**Human bronchus**	**h$CysLT_1$ receptor**
LTD_4,LTE_4>>LTC_4*	LTC_4=LTD_4>LTE_4	LTD_4>LTC_4>LTE_4
GPT $CysLT_2$	**Human pulmonary vein**	**h$CysLT_2$ receptor**
LTC_4>LTD_4>>LTE_4=BAY u9773	LTC_4=LTD_4>LTE_4>BAY u9773	LTC_4= LTD_4>LTE_4=BAY u9773

Table 3: The rank order of agonist potency at the CysLT receptors was estimated in the guinea pig trachea (GPT; Paper V). *The exact rank order of potency at the guinea pig tracheal $CysLT_1$ receptor could not be determined since there are currently no selective $CysLT_2$ receptor antagonists available (see text). Results are compared with previously published reports studying contractions of isolated human bronchus and pulmonary vein (Labat et al., 1992) or calcium mobilisation in cells transfected with the human CysLT receptors (h$CysLT_1$ or h$CysLT_2$ receptor; Nothacker et al., 2000).

	FPL 55712			ICI 198,615			MK 571		
	LTC_4	**LTD_4**	**LTE_4**	**LTC_4**	**LTD_4**	**LTE_4**	**LTC_4**	**LTD_4**	**LTE_4**
GP trachea	**4.7 – 4.9** *(5, 8)*	**6.5 – 6.8** *(3, 4 ,5, 8)*	**6.2 – 7.2** *(4, 5, 8)*	**N.S. – 5** *(2, 9, 10)*	**8.7 – 9.5** *(2, 9, 10, 23)*	**9.4 – 10.1** *(2, 9, 10)*	**N.S. – 5.6** *(2, 11, 23)*	**8.2 – 9.4** *(2, 11, 23)*	**9.1** *(11)*
GP ileum (segment)		**6.9 – 7.3** *(3, 12, 14)*	**6.7 – 7.4** *(12)*	**N.S.** *(12)*	**9.2 – 11.5** *(12, 14)*			**10.5** *(11)*	**10.4** *(11)*
GP ileum (muscle)		**6.8** *(13)*		**N.S.** *(1)*	**10.3** *(19)*				
GP gall bladder	**6.1** *(21)*			**8.2 – 8.6** *(17)*	**8.3 – 8.7** *(17)*	**8.3 – 8.7** *(17)*			
Rat Lung				**7.6 – 7.9** *(22, 23)*	**7.5** *(22, 23)*	**7.4** *(22)*	**7.8** *(22, 23)*	**7.5 - 7.6** *(22, 23)*	**7.3** *(22)*
Human bronchus	**5.8 - 6.4** *(6)*	**5.8 - 6.3** *(6)*		**8.5 – 9.8** *(9, 18)*	**8.2 – 9.2** *(9, 18, 24)*		**8.3 - 8.6** *(18)*	**8.5 - 8.8** *(18)*	
Human pulm vein				**N.S.** *(18)*	**N.S.** *(18)*		**N.S.** *(18)*	**N.S.** *(18)*	
Ferret spleen	**N.S.** *(20)*	**N.S.** *(20)*		**N.S. – 5.6** *(20, 23)*	**N.S.** *(20, 23)*		**N.S. - 4.7** *(20, 23)*	**N.S.** *(20, 23)*	
Sheep bronchus				**N.S.** *(15, 23)*	**N.S.** *(15, 23)*		**N.S.** *(15, 23)*	**N.S.** *(15, 23)*	
Sheep trachea				**N.S.** *(15, 25)*	**N.S.** *(15, 25)*		**N.S.** *(15)*	**N.S.** *(15)*	

	SKF 104,353			BAY u9773		
	LTC_4	**LTD_4**	**LTE_4**	**LTC_4**	**LTD_4**	**LTE_4**
GP trachea	**N.S. - 6.3** *(2, 7, 23)*	**7.8 – 8.6** *(2, 7, 23)*	**>8.9** *(7)*	**6.8 – 7.3** *(2, 16, 23)*	**7.4** *(23)*	**7.7** *(16)*
GP ileum (segment)	**N.S.** *(12)*	**7.6 – 8.4** *(12, 14)*				
GP ileum (muscle)				**6.1** *(1)*		
GP gall bladder	**6.8 – 7.4** *(17)*	**7.6 – 7.9** *(17)*	**7.3 – 7.7** *(17)*			
Rat Lung	**8.0 – 8.3** *(21, 22, 23)*	**7.5 – 8.2** *(22, 23)*	**8.0** *(22)*	**7.2** *(23)*	**6.8** *(23)*	
Human bronchus	**8.4** *(7)*	**7.0 – 8.2** *(7, 18, 26)*	**>8.2** *(7)*	**5.4** *(18)*	**6.2 – 6.8** *(18)*	
HPV	**N.S.** *(18)*	**N.S.** *(18)*		**5.8 – 6.7** *(18)*	**6.5 – 6.8** *(18)*	
Ferret spleen	**N.S.** *(20)*	**N.S.** *(20)*		**6.9** *(23)*	**6.8** *(23)*	
Sheep bronchus	**N.S.** *(15, 23)*	**N.S.** *(15, 23)*		**7.6** *(23)*	**7.7** *(23)*	
Sheep trachea	**N.S.** *(15)*	**N.S.** *(15)*		**7.0** *(25)*	**6.8** *(25)*	

Table 4:
Antagonism of the contractile responses of different preparations in functional assays expressed as pA_2- or pK_B-values. N.S.= no significant inhibition.

Values are from the following reports (numbers correspond to those indicated in parenthesis under each value):

1: Paper I
2: Paper V
3: Fleisch et al., 1982
4: Krell et al., 1983
5: Weichman & Tucker, 1985
6: Buckner et al., 1986
7: Hay et al., 1987
8: Muccitelli et al., 1987
9: Snyder et al., 1987
10: Hand et al., 1989
11: Jones et al., 1989
12: Gardiner et al., 1990
13: Gieske et al., 1990
14: Norman et al., 1990
15: Cuthbert et al., 1991a
16: Cuthbert et al., 1991b
17: Falcone & Krell, 1992
18: Labat et al., 1992
19: Björck, 1993
20: Gardiner et al., 1993
21: Gardiner et al., 1994
22: Norman et al., 1994
23: Tudhope et al., 1994
24: Gorenne et al., 1995
25: Wikström Jonsson, 1997
26: Panettieri et al., 1998

A simple explanation to the differences in agonist and antagonist properties between human and guinea-pig $CysLT_1$ receptors would be to claim species-differences. However, in another guinea-pig preparation, namely the guinea-pig gall bladder, cysteinyl-leukotrienes induce contractions with a rank order of potency of LTC_4=LTD_4>LTE_4 (Falcone & Krell, 1992), i.e. similar to what most investigators have reported for human bronchi (Muccitelli et al., 1987; Labat et al., 1992; Björck, 1993). In addition, in the guinea-pig gall bladder, the contractions to all cysteinyl-leukotrienes are inhibited by ICI 198,615 and SKF 104,353 (Falcone & Krell, 1992), indicating that they all activate only $CysLT_1$ receptors in that preparation. Interestingly, the pK_B-values obtained for ICI 198,619 in the guinea-pig gall bladder are more close to those reported for human bronchi than those obtained in the guinea-pig trachea (Paper V; Table 4).

4.1.2.3. Subtypes of $CysLT_1$ receptors

The current IUPHAR classification of CysLT receptors thus support that the guinea-pig trachea, gallbladder and human bronchi all contain $CysLT_1$ receptors (Coleman et al., 1995). A possible explanation of the differences in rank order of agonist potency and the differences in sensitivity to $CysLT_1$ receptor antagonists between the preparations may be that the $CysLT_1$ receptor is a heterogeneous class of CysLT receptor subtypes and that further subdivision may be necessary. Although a number of structurally different $CysLT_1$ receptor antagonists equally inhibit cysteinyl-leukotriene binding to cells expressing the human $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999) as well as cysteinyl-leukotriene-induced contractions of human bronchi (Buckner et al., 1986; Buckner et al., 1990; Labat et al., 1992; Gorenne et al., 1995; Panettieri et al., 1998; Table 4), differential effects between $CysLT_1$ receptor antagonists have also been reported, supporting the notion of different $CysLT_1$ receptors.

The findings compiled in Table 4 indicate that there are differences in rank order of antagonist potency between the different preparations, as well as a difference in rank order of sensitivity to the different antagonists in each investigated tissue. For example, FPL 55712, ICI 198,615 and MK 571 inhibit $CysLT_1$ receptor mediated contractions with a rank order of sensitivity of guinea-pig ileum > guinea-pig trachea > guinea-pig gall bladder = human bronchus > rat lung, whereas for SKF 104,353, the values reported are not so different between these tissues (Table 4). In fact, the differences between the guinea-pig ileum and trachea in sensitivity to FPL 55712 (Table 4), have led to the suggestion that the $CysLT_1$ receptor in the guinea-pig ileum may be different from that of the trachea (Fleisch et al., 1982). Likewise, the rank orders of potency of the $CysLT_1$ receptor antagonists in inhibiting LTD_4-induced contractions (Table 4) are:

✓ **Guinea-pig trachea and ileum:**

ICI 198,615 = MK 571 > SKF 104,353

✓ **Human bronchi:**

ICI 198,615 = MK 571 = SKF 104,353

✓ **Rat lung:**

SKF 104,353 > ICI 198,615 = MK 571

These variations in rank order of antagonist potency may suggest that the $CysLT_1$ receptors are different between the preparations. Differential effects of $CysLT_1$ receptor antagonists have also been reported within the same preparation, hence arguing against that variations in $CysLT_1$ receptor antagonist sensitivity are solely due to tissue differences. For example, the endothelium-dependent relaxations induced by LTD_4 in canine renal arteries and veins are inhibited by ICI 198,615 but not by MK 571 (Pawloski

& Chapnick, 1993a). In the same study (Pawloski & Chapnick, 1993a), LY 171,883 inhibited LTD_4-induced relaxations of canine renal veins but not arteries, whereas L 649,923 inhibited arterial but not venous relaxations. In addition, a previous study has shown that the relaxations in canine renal veins are sensitive to pranlukast (Pawloski & Chapnick, 1991). Similar observations have been reported also in human tissues. In a study of human airway smooth muscle, the equal potency of zafirlukast, pranlukast and SKF 104,353 in inhibiting LTD_4-induced contractions was confirmed (Panettieri et al., 1998). However, in the same study the potentiating effect of LTD_4 on EGF-induced DNA synthesis in human airway smooth muscle cells was abolished by either pranlukast or a higher concentration of SKF 104,353, but unaffected by zafirlukast (Panettieri et al., 1998).

The notion that different $CysLT_1$ receptor antagonists may be used in order to distinguish different subtypes of human $CysLT_1$ receptors has also received support from radioligand binding studies of human lung parenchyma membranes using [^{3}H]-labelled leukotrienes as agonists (Capra et al., 1998b; Ravasi et al., 2000). In these preparations, [^{3}H]LTC_4 and [^{3}H]LTD_4 have been proposed to interact with both high and low affinity binding-sites (Rovati et al., 1992; Capra et al., 1998a; Capra et al., 1998b) with differential abilities of $CysLT_1$ receptor antagonists in displacing binding of either agonist. For example, (in the absence of S-decyl GSH, see Paper V), SKF 104,353 displaces both [^{3}H]LTC_4 and [^{3}H]LTD_4 binding whereas zafirlukast and pranlukast displace only [^{3}H]LTD_4 binding (Ravasi et al., 2000). Although it can not be excluded that binding sites for [^{3}H]-labelled leukotrienes, in addition to CysLT receptors, also may represent enzymes or transport proteins that binds cysteinyl-leukotrienes (Sun et al., 1986; Bannenberg et al., 1999), the results of the binding studies may suggest the presence of different $CysLT_1$ receptors in the human lung.

4.1.2.4. Heterogeneous $CysLT_1$/$CysLT_2$ receptor populations

While a general species differences does not seem to be a probable explanation for the varying properties of $CysLT_1$ receptors in different preparations, the findings discussed in the previous paragraph thus suggest that the $CysLT_1$ receptor may be a heterogenous class of receptor subtypes. Another possible hypothesis is that the expression of both $CysLT_1$ and $CysLT_2$ receptors may change how agonists and antagonists interact with the receptors. In some of the preparations studied in Table 4, both $CysLT_1$ and $CysLT_2$ receptors appear to be expressed, for example in the guinea-pig ileum and trachea (Paper I and V). In contrast, human bronchi (Buckner et al., 1986; Buckner et al., 1990) and guinea-pig gall bladder (Falcone & Krell, 1992) contain a homogenous $CysLT_1$ receptor population.

In fact, recent findings in CysLT receptor transfected cells, support that the human $CysLT_1$ receptor is the preferred target for LTD_4 and the human $CysLT_2$ receptor is the preferred target for LTC_4 (Nothacker et al., 2000). The latter observation may hence offer an explanation as to why LTC_4-induced contractions of the guinea-pig trachea were resistant to $CysLT_1$ receptor antagonists (Paper V) although LTC_4 would be expected to be an agonist at this receptor. It can therefore be anticipated that LTC_4 may activate the $CysLT_1$ receptor only in the absence of $CysLT_2$ receptors. Unfortunately, there are presently no pharmacological tools available to selectively block the $CysLT_2$ receptors in the guinea-pig trachea and this hypothesis could therefore not be tested in the present study.

It has been reported that in human saphenous veins the contractions induced by LTD_4 are inhibited by ICI 198,615, whereas the LTC_4-induced contractions (in the presence of acivicin in order to inhibit LTC_4 metabolism, see Section 4.2.3.) are resistant to this antagonist (Allen et al., 1994). The latter observation is thus similar to results in

the guinea-pig ileum (Paper I) and trachea (Paper V) and may support the hypothesis proposed above, i.e. that co-expression of $CysLT_1$ and $CysLT_2$ receptors changes the agonist preferences of the receptors. This assumption is reinforced by results of studies of cysteinyl-leukotriene-binding to human lung membranes that also have proposed human CysLT receptors that are preferentially activated by either LTC_4 or LTD_4 (Rovati et al., 1985; Rovati et al., 1992; Capra et al., 1998b; Ravasi et al., 2000).

4.1.3. $CysLT_2$ receptors

4.1.3.1. LTC_4-induced contractions of the guinea-pig ileum and trachea

In both guinea-pig ileum and trachea, the LTC_4-induced contractions were resistant to $CysLT_1$ receptor antagonism but inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773 (Paper I and V), supporting that LTC_4 activates a $CysLT_2$ receptor in these tissues. However, although the two different tissues were studied under similar conditions, the pA_2-values were somewhat different, 6.1 in the guinea-pig ileum and 6.8 in the guinea-pig trachea (Paper I and V). This is hence a reversed relationship compared with studies using $CysLT_1$ receptor antagonists, that have reported a higher potency of the antagonists in ileum compared with trachea (Table 4), and would suggest that also the $CysLT_2$ receptor may be different between ileal and tracheal preparations. However, it cannot be excluded that the variation in pA_2-values were due to methodological differences. For example, the concentration-response curves for LTC_4 were different in the ileum compared with the trachea, with pD_2 values of 8.1 (Paper I) and 8.7 (Paper V), respectively and E_{max} of around 90 % and 50 %, respectively. In addition, in the guinea-pig trachea, half-log steps were used when establishing the concentration-response curves as opposed to whole-log steps in the ileum. Anyhow, data from previous reports using BAY u9773 (Table 4) indicate that there are certain variations in results between studies using this antagonist. Since the results in both guinea-pig ileum (Paper I) and trachea (Paper V) are within the range of pA_2/pK_B-values described for BAY u9773 by other investigators (Table 4), it cannot be excluded that the $CysLT_2$ receptor has similar properties in the two preparations.

4.1.3.2. The mechanism of antagonism by BAY u9773

The findings in guinea-pig ileum (Paper I) and trachea (Paper V) suggest that BAY u9773 is a competitive antagonist at the $CysLT_2$ receptor. However, since BAY u9773 is structurally related to LTE_4 and a dual $CysLT_1$/$CysLT_2$ receptor antagonist, the question raised was if its antagonism at the $CysLT_2$ receptor was dependent on interaction with the $CysLT_1$ receptor. In the guinea-pig ileum, the combination of BAY u9773 with the $CysLT_1$ receptor antagonist ICI 198,615 did not alter the inhibition of the LTC_4-induced contraction by BAY u9773 (Paper I), suggesting that the antagonistic effect of BAY u9773 was unrelated to activation of $CysLT_1$ receptors.

BAY u9773 did not induce any significant contractions of either the guinea-pig ileum (Paper I) or trachea (Table 5, previously unpublished data). These findings are similar to a number of other functional smooth muscle assays (Tudhope et al., 1994; Wikström Jonsson, 1997) where BAY u9773 competitively inhibits $CysLT_1$ and $CysLT_2$ receptor responses without having an agonistic activity. However, Labat and co-workers (1992) described the antagonism of $CysLT_2$ responses by BAY u9773 as partial agonism. Likewise, in cells expressing the human $CysLT_2$ receptor, BAY u9773 has been shown to be a partial agonist (see below) and its antagonistic properties at this receptor proposed to be due to receptor desensitisation (Nothacker et al., 2000). In the latter study, BAY u9773 inhibited calcium mobilisation also in $CysLT_1$ receptor transfected cells, but without having any agonistic activity

(Nothacker et al., 2000). Taken together, these findings suggest a differential action of antagonism by BAY u9773, i.e. competitive antagonism without agonist activity at $CysLT_1$ and $CysLT_2$ receptors in for example guinea-pig ileum (Paper I) and trachea (Paper V) and at cloned human $CysLT_1$ receptors (Nothacker et al., 2000), as well as partial agonism, as in human pulmonary venous smooth muscle (Labat et al., 1992) and cloned human $CysLT_2$ receptors (Nothacker et al., 2000).

BAY u9773			
0.3 μM	**1 μM**	**3 μM**	**10 μM**
4.2±2 %	4.8±1 %	3.1±1 %	5.0±1 %
(*n*=8)	(*n*=5)	(*n*=7)	(*n*=4)

Table 5: Changes in basal tone of guinea-pig tracheal preparations during incubation with the $CysLT_1/CysLT_2$ receptor antagonist BAY u9773. Contractions (means±S.E.M) are expressed as percent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM; previously unpublished data).

4.1.3.3. Other $CysLT_2$ receptor antagonists

The unselective $CysLT_1/CysLT_2$ receptor antagonists BAY u9773 is presently the only pharmacological tool available for characterisation of $CysLT_2$ receptors. However, the use of BAY u9773 is limited since it is only commercially available at a very high price.

Some of the $CysLT_1$ receptor antagonists have been shown to inhibit also $CysLT_2$ responses, but only at high concentrations where their specificity may be questionable. For example, pranlukast antagonises the LTC_4-induced contractions of the guinea-pig trachea (in the presence of L-serine borate) with a 100 fold lower potency than against LTD_4-induced contractions (Obata et al., 1992) and competes for $[^3H]LTD_4$ binding at the cloned $CysLT_2$ receptor but with 1000-fold higher IC_{50} than at the $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999; Heise et al., 2000).

Gieske and co-workers (1990) have reported that the LTD_4-mimetic MDL 28,753 inhibits the LTC_4-induced contractions of the guinea-pig ileum longitudinal muscle. Since it was shown in Paper I that the LTC_4-induced contractions of this preparation were mediated by a $CysLT_2$ receptor, it can thus be anticipated that MDL 28,753 would be a $CysLT_2$ receptor antagonist. However, MDL 28,753 is a full LTD_4-mimetic in the guinea-pig ileum, and the inhibition of LTC_4-induced contractions was observed only in the presence of ICI 198,615 (Gieske et al., 1990). Interestingly, in the presence of ICI 198,615 (100 nM), a residual contraction to both MDL 28,753 and LTD_4 was reported (Gieske et al., 1990), which, for the latter agonist, is identical to results in the guinea-pig trachea (Paper V). In the guinea-pig trachea, the residual contraction was inhibited by BAY u9773, indicating that LTD_4 was a partial agonist at the $CysLT_2$ receptor in this preparation (Paper V, see below). If it is hypothesised that, in the guinea-pig ileum longitudinal muscle, LTD_4 and MDL 28,753 activate $CysLT_2$ receptors as well, one possible explanation for the observations by Gieske and co-workers (1990) may be that both LTD_4 and MDL 28,753 in the presence of ICI 198,615 inhibit LTC_4-induced contraction by partial agonism at the $CysLT_2$ receptor.

4.1.3.4. LTE_4 as a CysLT receptor antagonist

In a number of different tissues, LTE_4 have been described to inhibit contractions induced by LTC_4 or LTD_4 (Table 6). The mechanism of this antagonism has in some tissues been characterised as partial agonism (Gardiner et al., 1990; Labat et al., 1992), whereas in other tissues, LTE_4 inhibits LTC_4- and LTD_4-induced contractions although it induces no, or only very small, contractions of the

preparations (Snyder & Krell, 1986; Tomioka et al., 1991; Gardiner et al., 1993).

	LTC_4	LTD_4
GP ileum (Gardiner et al., 1990)	[minor shift] (10 μM)	**pK_P = 6.8** (10 μM)
Ferret trachea (Snyder & Krell, 1986)	**pK_B = 6.3-6.5** (10 & 1 μM)	**pK_B = 6.4-7.3** (10 & 1 μM)
Ferret spleen (Gardiner et al., 1993)	**pK_B = 5.8** (10 μM)	**pK_B = 5.5** (10 μM)
Sheep trachea (Tomioka et al., 1991)	[pK_B = 6.9] (1 μM)	**pK_B = 7.3** (0.1-1 μM)
Human pulm. vein (Labat et al., 1992)	**pK_P = 6.6** (1 μM)	**pK_P = 6.3** (1 μM)

Table 6: Previous reports demonstrating the inhibition of LTC_4- and LTD_4-induced contractions by LTE_4. Results are expressed as either pK_B- or pK_P-values according to the original reports, where a pK_P-value indicates inhibition by partial agonism. [ ] indicates that experiments were carried out in the absence of inhibitors of LTC_4 metabolism.

In the guinea-pig ileum, LTE_4 (10 μM) induces a rightward shift of the LTD_4 concentration-response curve due to partial agonism at the $CysLT_1$ receptor (Gardiner et al., 1990; Table 6). In contrast, in this thesis, the LTC_4-induced contractions were not inhibited by LTE_4 (30 nM-1 μM; Paper I). Since the LTC_4-induced contractions of the guinea-pig ileum were shown to be mediated via a $CysLT_2$ receptor, inhibition by partial agonism is not expected for LTE_4 (Paper I). However, in the study by Gardiner and co-workers (1990), also a shift of the concentration-response curve to LTC_4 was observed, although this was minor compared with that observed for LTD_4. A possible explanation as to the apparent differences in effects of LTE_4 on LTC_4-induced contractions between these two studies may be the use of inhibitors of LTC_4 metabolism in Paper I, but not in the study by Gardiner and co-workers (1990). It can thus not be excluded that the effects of LTE_4 against LTC_4-induced contractions in the latter study may have been due to an effect against LTD_4 being produced from the metabolism of LTC_4. Pre-treatment of guinea-pig ileum longitudinal muscle with the TP-receptor agonist U 46 619 mimicked the effect of LTE_4, i.e. induced a similar degree of contraction but did not produce a shift of the concentration-response curve for LTC_4 (Paper I), supporting that LTE_4 behaved like any non-$CysLT_2$ receptor agonist.

4.1.3.5. Rank order of agonist potency at the $CysLT_2$ receptor

At the $CysLT_2$ receptor on human pulmonary venous smooth muscle preparations, the rank order of agonist potency is LTC_4=LTD_4>LTE_4 (Labat et al., 1992). In ferret trachea and spleen, two other preparations with a homogenous $CysLT_2$ receptor population, a rank order of potency of LTC_4=LTD_4>>LTE_4 have been reported (Snyder & Krell, 1986; Gardiner et al., 1993). In the guinea-pig trachea, the rank order of potency was LTC_4=LTD_4>LTE_4 (Paper V), which is identical to previous reports in this tissue (Cuthbert et al., 1991b; Tudhope et al., 1994) and in the guinea-pig ileum (Dahlén et al., 1987; Gardiner et al., 1990). The latter preparations both contain a heterogeneous receptor population and the meaning of estimates of rank order of potency can thus be disputable. However, if it is hypothesised that ICI 198,615 (300 nM) selectively inhibited the whole population of $CysLT_1$ receptors in the guinea-pig trachea, the cysteinyl-leukotriene-induced contractions in the presence of this antagonist concentration would be mediated solely via the $CysLT_2$ receptor (Paper V). In this case, the rank order of potency at the $CysLT_2$-receptor in the guinea-pig trachea was LTC_4 (pD_2=9.0±0.1) > LTD_4 (pD_2=7.7±0.2) >> LTE_4 (no contractions at concentrations up to 1 μM) as is shown in Fig. 3 and Table 3. In addition, LTD_4 was a partial agonist at the $CysLT_2$ receptor (maximal contraction 48±4% compared with 86±6% for LTC_4, Fig. 3).

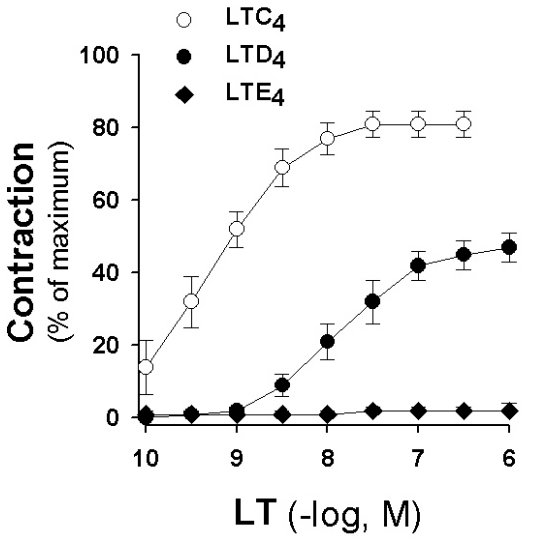

Fig. 3: Concentration response curves for LTC_4 (in the presence of 100 μM S-hexyl GSH and 5 mM L-cysteine), LTD_4 (in the presence of 5 mM L-cysteine) and LTE_4. All preparations were treated with the $CysLT_1$ receptor antagonist ICI 198,615 (300 nM). Contractions (means±S.E.M) are expressed as percent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM), *n*=4-9 (data from Paper V).

The rank order of agonist potency in cells transfected with the human $CysLT_2$ receptor is LTC_4=LTD_4>LTE_4, with LTE_4 being a partial agonist (Heise et al., 2000; Nothacker et al., 2000; Takasaki et al., 2000), thus supporting functional results in human pulmonary veins (Labat et al., 1992). Interestingly, Nothacker and co-workers (2000) recently described BAY u9773 as being a selective agonist at the human $CysLT_2$ receptor with the same potency and efficacy as LTE_4, but without any agonistic activity at the human $CysLT_1$ receptor. This is in contrast to the results within the present thesis using guinea-pig ileum (Paper I) and trachea (Table 5), where pharmacologically a $CysLT_2$ receptor was shown to be present, but no significant contractions to BAY u9773 were observed. Estimates of the rank orders of agonist potency at the human and guinea-pig $CysLT_2$ receptors are summarised in Table 3.

The guinea-pig lung parenchyma has previously been shown to contract to BAY u9773, but in that preparation the contractions are inhibited by the $CysLT_1$ receptor antagonist ICI 198,615 (Wikström Jonsson et al., 1998), suggesting that the agonist activity of BAY u9773 may vary between guinea-pig and human tissues. In addition, since BAY u9773 induced negligible contractions of both the guinea-pig ileum and trachea, differences may also exist between different tissues within the same species. In the context of BAY u9773 being a selective agonist at the human $CysLT_2$ receptor (Nothacker et al., 2000), it is noteworthy that Labat and co-workers (1992) have reported contractions to BAY u9773 in the human pulmonary vein. Moreover, those investigators also observed contractions to BAY u9773 in isolated human bronchi (Labat et al., 1992). Since BAY u9773 have been reported not to be an agonist at the cloned human $CysLT_1$ receptor (Nothacker et al., 2000), this may indicate the presence of also $CysLT_2$ receptors in human bronchi. However, it has also been reported that BAY u9773 may activate TP-receptors (Wikström Jonsson et al., 1998), and such receptors are present in human bronchi (McKenniff et al., 1988).

4.1.4. Other CysLT receptor subtypes?

4.1.4.1. Porcine pulmonary arteries

Ohtaka and co-workers (1987) were the first to report that LTC_4 and LTD_4 contract porcine pulmonary arteries, and those results were confirmed in Paper II. In endothelium intact porcine pulmonary arteries, LTC_4 was somewhat more potent than LTD_4 and the contractions to both agonists were only slightly inhibited by either the $CysLT_1$ receptor antagonist zafirlukast (ICI 204,219) or the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773. In addition, after endothelium denudation, LTC_4 and LTD_4 were equipotent and the LTC_4-induced contractions were resistant to BAY u9773. The resistance to both $CysLT_1$ and $CysLT_2$ receptor antagonism suggest that the receptor situated on the porcine pulmonary arterial smooth muscle was different from the above-

described $CysLT_1$ and $CysLT_2$ receptors (Paper II).

In the guinea-pig lung parenchyma a residual contraction has been observed after either $CysLT_1$ receptor antagonism or combined $CysLT_1/CysLT_2$ receptor antagonism by BAY u9773 (Tudhope et al., 1994; Wikström Jonsson et al., 1998). However, in the lung parenchymal strip, several different target tissues contribute to the contractile response (Brink et al., 1981) and responses to cysteinyl-leukotrienes are in part mediated by secondary released factors (Dahlén et al., 1983). Therefore, the role of different CysLT receptors in the guinea-pig lung parenchyma is difficult to assess and the porcine pulmonary arterial smooth muscle may represent a more appropriate model since in addition, as will be shown below, the results were similar to findings in the human lung.

The findings that both zafirlukast and BAY u9773 slightly inhibited the LTC_4- and LTD_4-induced contractions in endothelial intact preparations but were inactive in rubbed preparations also suggest that LTC_4 and LTD_4 activated CysLT receptors on the endothelium that enhanced the contractions to these agonists, probably by the release of contractile factors (see Section 4.3). In addition, these findings indicate that the endothelial receptors were of the $CysLT_1$ type. However, there was a differential effect between zafirlukast and BAY u9773 against LTC_4-induced contractions (Paper II). Whether this difference simply reflects the differences in concentration and potency of the antagonists or is due to the presence of also $CysLT_2$ receptors on the endothelium remains to be established. In support of the latter suggestion, it was observed that LTC_4 induced larger contractions than LTD_4 in endothelial intact preparations, whereas the two cysteinyl-leukotrienes were equipotent in rubbed preparations and that the difference between zafirlukast and BAY u9773 was observed only against LTC_4-induced contractions (Paper II). Taken together, the data suggest that LTC_4, but not LTD_4, activated endothelial $CysLT_2$ receptors, and a schematic figure of the distribution of CysLT receptors in porcine pulmonary arteries is proposed in Fig 4. As will be discussed in Section 4.3.8., these experiments were carried out in the absence of inhibitors of LTC_4 metabolism and it cannot be excluded that the activation of endothelial $CysLT_1$ receptors by LTC_4 may have been due to its metabolism into LTD_4 (Fig. 4).

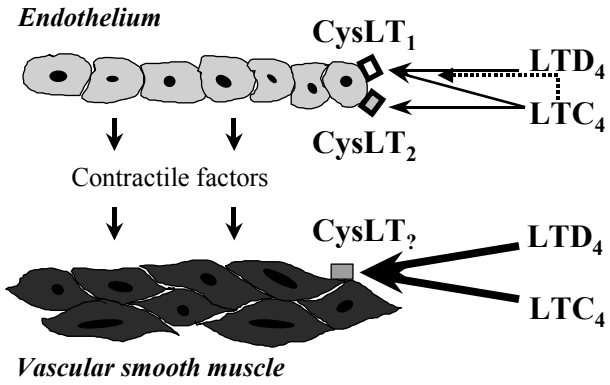

Fig. 4: Schematic presentation of CysLT receptors in the porcine porcine pulmonary artery and their activation by LTC_4 and LTD_4. Dotted line indicates possible alternative mechanism for $CysLT_1$ receptor activation by LTC_4. The scheme is based on data from Paper II.

Incubation of porcine pulmonary arteries with BAY u9773 (3 μM) did not induce changes in basal tone that were significantly different from controls in either endothelium-intact or rubbed preparations (Fig. 5a and b, previously unpublished data). Since BAY u9773 has been reported to be a selective agonist at the human $CysLT_2$ receptor (Nothacker et al., 2000) and to contract human pulmonary veins (Labat et al., 1992), these findings may support the notion of a non-$CysLT_2$ receptor on the porcine pulmonary arterial smooth muscle.

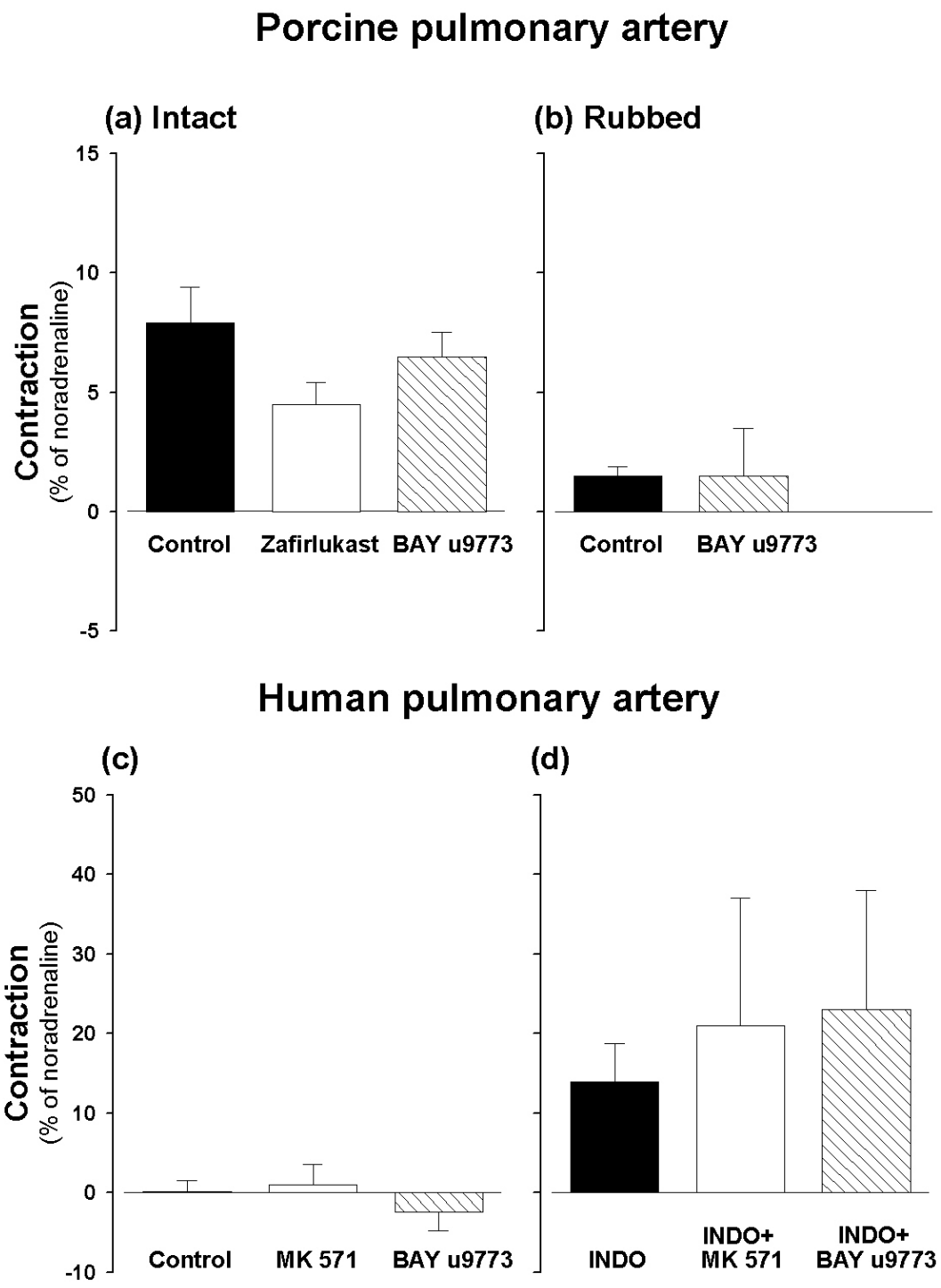

Fig. 5: Changes in basal tone of isolated porcine (panels a and b) and human (panels c and d) pulmonary arterial preparations during a 30 min incubation period. Porcine pulmonary arterial preparations with (a) or without (b) endothelium were incubated in the absence (Control) or presence of either the $CysLT_1$ receptor antagonist zafirlukast (ICI 204,619; 1 μM) or the $CysLT_1/CysLT_2$ receptor antagonist BAY u9773 (3 μM). Human pulmonary arterial preparations (endothelium intact, panels c and d) were incubated in the absence (Control) or presence of the cyclooxygenase inhibitor indomethacin (1.7 μM, INDO) and/or either the $CysLT_1$ receptor antagonist MK 571 (1 μM) or BAY u9773 (3 μM). Contractions (means±S.E.M) are expressed as percent of a reference contraction to noradrenaline (10 μM), *n*=4-12 (previously unpublished data).

The rank order of potency (LTC_4=LTD_4>>LTE_4; Paper II) in rubbed porcine pulmonary arteries is similar to results of previous studies of ferret spleen (Gardiner et al., 1993), ferret trachea (Snyder & Krell, 1986) and sheep trachea (Tomioka et al., 1991). However, although no or negligible contractions are observed after LTE_4 administration to the three latter preparations, LTE_4 has been demonstrated to be able to inhibit contractions induced by either LTC_4 or LTD_4 (Table 6), suggesting that LTE_4 can interfere with a CysLT receptor without inducing contractions. This is in contrast to the findings in the porcine pulmonary artery where pre-treatment of rubbed preparations with LTE_4 did not alter the LTC_4-induced contractions (Paper II). These findings suggest that LTE_4 does not interfere with the CysLT receptor present on the porcine pulmonary arterial smooth muscle neither as an agonist nor as an antagonist, further supporting a difference from previously described CysLT receptors.

Taken together, the findings in the porcine pulmonary artery (Paper II) indicate that the cysteinyl-leukotriene receptor mediating contractions of rubbed preparations is different from the previously described $CysLT_1$ and $CysLT_2$, based on the following observations:

- ✓ The rank order of potency was LTC_4=LTD_4, whereas neither LTE_4 (1 μM) nor BAY u9773 (3 μM) induced any contractions.
- ✓ The contractions induced by LTC_4 were resistant to both $CysLT_1$ and $CysLT_2$ receptor antagonism as well as to pre-treatment with LTE_4.

It is not probable that the differences between the CysLT receptor on the porcine pulmonary arterial smooth muscle and previously described CysLT receptors are due to species-differences. The findings that both zafirlukast and BAY u9773 inhibited the contractions in endothelial intact preparations (Paper II) suggest that there was heterogeneity of CysLT receptors also within this species. In addition, the latter findings confirm that the antagonists used indeed were active compounds and that the resistance of the contractions was not due to experimental factors.

4.1.4.2. Human pulmonary arteries

The findings in the porcine pulmonary artery thus suggest an additional subtype of CysLT receptors (Paper II). In order to examine if this observation had relevance for human tissue, the study was continued using human lung samples. The results (Paper III) interestingly indicate that the human pulmonary artery exhibited a similar profile as the porcine pulmonary artery.

In the human pulmonary artery, LTC_4 and LTD_4 induced similar contractions (Paper III), and Schellenberg and Foster (1984) have previously reported that LTE_4 is inactive as agonist in this preparation. The LTC_4-induced contractions of the human pulmonary artery were resistant to the $CysLT_1$ receptor antagonist MK 571 (Paper III). However, in contrast to human pulmonary veins (Labat et al., 1992), but in line with the findings in the porcine pulmonary artery (Paper II), the LTC_4-induced contractions were resistant also to BAY u9773 (Paper III). In addition, incubation of human pulmonary arteries with BAY u9773 (3 μM) did not induce changes in basal tone that were significantly different from untreated preparations both in absence and presence of indomethacin (Fig. 5c and d, previously unpublished data).

The concentration-response curves for LTC_4 were significantly enhanced when the LTC_4-induced prostacyclin-release was inhibited by indomethacin (see Section 4.3.3.). However, maximal contractions could not be established since this would have demanded higher concentrations of leukotriene, which were not available at the time the experiments were carried out. The non-sigmoidal shape of the concentration-response curves may limit the interpretation

of the results obtained with LTC_4 in the human pulmonary artery. Further experiments are needed in order to obtain the best conditions for studies of cysteinyl-leukotriene-induced contractions of the human pulmonary artery and to establish full concentration-response curves. However, the results obtained (Paper III) provide a first suggestion of a human CysLT receptor that may be different from $CysLT_1$ and $CysLT_2$ receptors.

4.1.5. Classification of CysLT receptors

According to the IUPHAR recommendations for nomenclature of receptors, a receptor type is defined as "nomenclature for a structurally and operationally distinct receptor in a given family" and a receptor subtype as "nomenclature for a receptor with strong structural homology to other types, but with distinct operational characteristics" (Vanhoutte et al., 1998). The CysLT receptors together with the receptor(s) for LTB_4 (BLT receptor) constitute the family of leukotriene receptors, and $CysLT_1$ and $CysLT_2$ are thus CysLT receptor subtypes (Coleman et al., 1995).

The application of the classification of CysLT receptors is generally that a functional response resistant to $CysLT_1$ receptor antagonists is referred to as a $CysLT_2$ receptor (Coleman et al., 1995). However, with the recent cloning of a second human CysLT receptor it is probable that the receptor described in those reports (Heise et al., 2000; Nothacker et al., 2000; Takasaki et al., 2000) represents a distinct $CysLT_2$ receptor. In addition, there is only a weak homology (31-38%) between the $CysLT_1$ and $CysLT_2$ receptors (Heise et al., 2000; Nothacker et al., 2000; Takasaki et al., 2000), suggesting that they may be distinct receptor types rather than subtypes.

The results obtained in porcine and human pulmonary arteries (Paper II and III) indicate that what is currently referred to as the $CysLT_2$ receptor (i.e. resistant to $CysLT_1$ receptor antagonists) may be a heterogeneous group of receptors. In addition, the findings presented in Table 4 indicate a further subdivision of also $CysLT_1$ receptors. In the initial preliminary report of the findings in Paper II, the name $CysLT_3$ was proposed for the receptor mediating contractions of the porcine pulmonary arterial smooth muscle (Bäck et al., 1999). The term $CysLT_3$ has also been used in reviews of cysteinyl-leukotriene receptors when referring to receptors mediating contractions resistant to BAY u9773 (Dahlén, 2000; Nicosia et al., 2000). Another possibility is a subdivision of $CysLT_2$ receptors into $CysLT_{2A}$ and $CysLT_{2B}$. Such classification however requires the knowledge of the molecular structures in order to clarify what can be referred to as receptor types and subtypes.

4.1.6. Summary: Cysteinyl-leukotriene receptors

✓ The finding that neither $CysLT_1$ nor $CysLT_2$ receptor antagonism inhibited the cysteinyl-leukotriene-induced contractions of human and porcine pulmonary arteries suggests that BAY u9773 does not inhibit all $CysLT_1$ receptor antagonist resistant responses and that what is presently referred to as the $CysLT_2$ receptor may in fact represent a heterogeneous group of receptors.

✓ In the guinea-pig ileum longitudinal muscle, the LTC_4-induced contractions were resistant to $CysLT_1$ receptor antagonism but inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773, indicating that LTC_4 activated $CysLT_2$ receptors in this preparation.

✓ In the guinea-pig trachea, the $CysLT_1$ receptor antagonist ICI 198,615 (300 nM) abolished the LTE_4-induced contractions and partially inhibited the LTD_4-induced contractions, whereas the LTC_4-induced contractions were unaltered by this treatment. These results suggest that LTC_4 activated $CysLT_2$ receptors and LTE_4 $CysLT_1$ receptors

and that LTD_4 activated both $CysLT_1$ and $CysLT_2$ receptors.

✓ The current classification of leukotriene receptors probably represents a simplification since the results indicate additional CysLT receptor subtypes or a further subdivision of the current CysLT receptor subtypes $CysLT_1$ and $CysLT_2$.

4.2. Modulation of responses to cysteinyl-leukotrienes in the guinea-pig trachea

4.2.1. Specific aims

The aim of this part of the thesis was to evaluate the modulatory mechanisms involved in the contractile responses to cysteinyl-leukotrienes in the guinea-pig trachea. Inhibitors of cysteinyl-leukotriene metabolism administered intravenously to guinea-pigs *in vivo* increase pulmonary insufflation pressure in response to antigen (Funayama et al., 1996), suggesting that the metabolic conversion of cysteinyl-leukotrienes may modulate their biological action. Therefore, the effects of cysteinyl-leukotriene metabolism on the contractile responses to LTC_4, LTD_4 and LTE_4 in isolated guinea-pig tracheal spiral preparations were studied.

In addition, the effects of factors of the cyclooxygenase pathway in modulating cysteinyl-leukotrienes-induced contractions of the guinea-pig trachea were examined. This question was primarily addressed as a pre-study in order to obtain optimal conditions for the study on the metabolic modulation of contractions to cysteinyl-leukotrienes (Paper V). Since most previous studies of the effects of cysteinyl-leukotrienes in isolated guinea-pig tracheal preparations have been performed in the presence of indomethacin (Jones et al., 1983; Snyder et al., 1984; Snyder & Krell, 1984; Jones et al., 1986; Hand & Schwalm, 1987; Snyder et al., 1987; Hand et al., 1989; Jones et al., 1989; Buckner et al., 1990; Krell et al., 1990; Cuthbert et al., 1991b; Obata et al., 1992; Jones et al., 1995), it was considered a principal issue to establish the effect of this treatment in the present experimental protocol.

4.2.2. Modulation by prostaglandins

4.2.2.1. Prostaglandins released from the guinea-pig trachea

Addition of indomethacin reduced basal tone of the guinea-pig tracheal preparations (data not shown). A decrease of basal tone in guinea-pig tracheal preparations after cyclooxygenase inhibition has been well established in previous reports (Orehek et al., 1973; Braunstein et al., 1988) and indicates that endogenously formed contractile cyclooxygenase products are involved in the regulation of basal tone. In addition, since changes in basal tone have been shown to alter the amplitude of contractile responses in the guinea-pig trachea (Braunstein et al., 1988), basal tone was mechanically adjusted throughout the experiments in the present study, ensuring that all preparations were studied under the same preload (10 mN). This correction of basal tone thus excludes any indomethacin-induced influence on the length-tension relationship of the preparations and allows adequate comparisons between controls and indomethacin-treated preparations.

Contractile responses in the guinea-pig trachea may be both inhibited and potentiated by cyclooxygenase products since either aspirin or indomethacin inhibits contractions induced by low concentrations of histamine or acetylcholine, whereas at higher concentrations of the agonists, the contractions are enhanced by the cyclooxygenase inhibitors (Orehek et al., 1973). One possible explanation for this observation is that the predominantly released prostaglandin in the guinea-pig trachea, PGE_2 (Grodzinska et al., 1975; Braunstein et al., 1988) can both relax and contract guinea-pig tracheal preparations under different conditions (Braunstein et al., 1988). Another possibility is a release of both contractile and relaxant cyclooxygenase products, which receives support from a report describing the rank order of both spontaneous and stimulated prostanoid release from the guinea-pig trachea as PGE_2>$PGF_{2\alpha}$>prostacyclin>TXA_2 (Burka et al., 1981).

Charette and co-workers (1995) have shown that in the guinea-pig trachea, the COX-2 inhibitor NS-398 decreased basal tone and enhanced histamine-induced contractions

to a similar degree as was observed with indomethacin, suggesting that these effects were mediated by COX-2. This suggestion was supported by the observation with western blot analysis that COX-2, but not COX-1, proteins were detected in tracheal muscle and cartilage (Charette et al., 1995). There was however no detection of either COX-1 or COX-2 in tissues analysed directly after removal from the animal (Charette et al., 1995), suggesting that dissection and organ bath experiments may induce COX-2 in the guinea-pig trachea.

4.2.2.2. Effects on cysteinyl-leukotriene contractions

The bronchoconstriction induced by intravenously injected cysteinyl-leukotrienes in guinea-pigs is inhibited by cyclooxygenase inhibition (Omini et al., 1981; Weichman et al., 1982; Dahlén, 1983), whereas after aerosol administration of cysteinyl-leukotrienes the bronchoconstrictor response is potentiated by cyclooxygenase inhibition (Weichman et al., 1982; Dahlén, 1983). Taken together, these studies indicate that in the guinea-pig, cysteinyl-leukotrienes stimulate the release of both bronchoconstrictor and bronchodilator cyclooxygenase products *in vivo*. However, the results presented in Fig. 6 (previously unpublished data) show that in the presence of indomethacin, the concentration-response curves for LTC_4 and LTD_4 in isolated guinea-pig tracheal preparations were not significantly different from those obtained in absence of this cyclooxygenase inhibitor (Table 7).

Krell and co-workers (1981b) have previously reported that indomethacin enhances the contractions of guinea-pig trachea induced by higher concentrations of LTC_4 whereas little or no effect on the lower portion of the LTC_4 concentration-response curves was observed. Likewise, another cyclooxygenase inhibitor, meclofenamic acid has been reported to enhance the maximal contractions to LTC_4, LTD_4 and LTE_4 in the guinea-pig trachea, without altering their EC_{50}-values (Weichman et al., 1982). There are however some differences between those studies and the present.

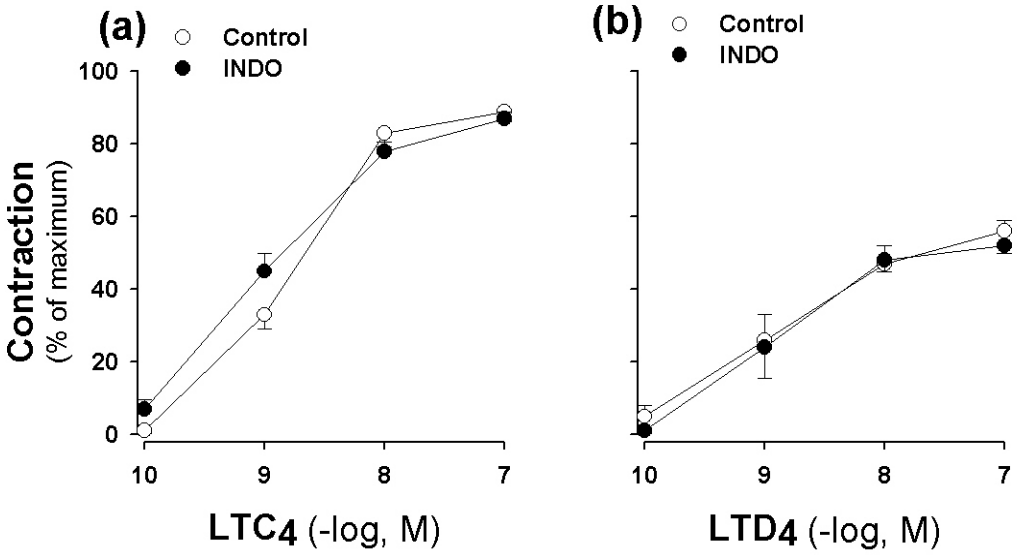

Fig. 6: Concentration response curves for LTC_4 (a; in the presence of S-hexyl GSH and L-cysteine) and LTD_4 (b; in the presence of L-cysteine) in the guinea-pig trachea in the absence (Control) or presence of the cyclooxygenase inhibitor indomethacin (10 μM, INDO). Experiments were performed according to the protocol described in detail in Paper V. Contractions (means±S.E.M) are expressed as percent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM), *n*=3-5 (previously unpublished data, see also Table 7).

		n	E_{max} (%)	E_{max} (g)	pD_2
LTC_4	**Control**	*4*	89 ± 3	1.2 ± 0.2	8.6 ± 0.1
	INDO	*5*	87 ± 2	1.2 ± 0.2	8.9 ± 0.1
LTD_4	**Control**	*3*	57 ± 3	0.64 ± 0.17	8.8 ± 0.2
	INDO	*3*	53 ± 1	0.85 ± 0.17	8.9 ± 0.3
Max	**Control**	*7*		1.2 ± 0.2	N.D.
	INDO	*8*		1.4 ± 0.2	N.D.

Table 7: Maximal contractions (E_{max}) and pD_2-values for LTC_4 (in the presence of S-hexyl GSH and L-cysteine) and LTD_4 (in the presence of L-cysteine) in the guinea-pig trachea in the absence (Control) or presence of the cyclooxygenase inhibitor indomethacin (10 μM, INDO). The maximal contraction induced by the combination of histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) is also shown (previously unpublished data, see also Fig. 6). There were no significant differences between controls and INDO in any of the parameters compared within the different agonists (Student's t-test). N.D.= not determined.

Firstly, in the previous reports, experiments were carried out in the absence of inhibitors of cysteinyl-leukotriene metabolism (Krell et al., 1981b; Weichman et al., 1982), whereas the results in Fig. 6 were obtained in the presence of either L-cysteine (LTD_4) or the combination of L-cysteine with S-hexyl GSH (LTC_4). Secondly, the results presented in Fig. 6 and Table 7 are expressed as percent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM) performed at the end of the experiment, which means that also the reference contraction was obtained in the presence of indomethacin in the treated preparations. This is in contrast to the report by Krell and co-workers (1981b) where responses are presented as percent of an initial charbachol-induced contraction in the absence of indomethacin. However, in the present study, there were no significant differences in absolute values between controls and indomethacin-treated preparations regarding LTC_4, LTD_4 or maximal contractions (Table 7), indicating that the lack of effect of indomethacin on contractions of the guinea-pig trachea (Fig. 6) was not due to an enhanced maximal contraction masking the enhancement of responses to LTC_4 or LTD_4. A third difference is the correction for the decrease in basal tone induced by indomethacin in the present study (see above), ensuring that differences in basal tone did not affect the contractile responses of the guinea-pig trachea.

Watts and Cohen (1993) have shown that in the guinea-pig trachea, contractions induced by serotonin and KCl are enhanced with time until about 2.5 hours. However, in the presence of cyclooxygenase inhibitors, the time to obtain optimal contractility in the guinea-pig trachea decreases since contractions induced by KCl are significantly enhanced by indomethacin at time-points up to 2 hours, whereas the KCl-induced contractions are unaffected by indomethacin at later time-points (Watts & Cohen, 1993). The results of that study thus suggest that the guinea-pig trachea releases relaxant cyclooxygenase products that during the initial part of the experiments functionally inhibit contractions and that this release decreases with time, which causes an improvement of contractile responses. When studying the effects of cysteinyl-leukotrienes in the guinea-pig trachea (Fig. 6; Paper V), a relatively long experimental protocol was therefore chosen. An initial 90-min equilibration-period was followed by histamine challenge (around 30 min) and then

another 60-min equilibration-period before the start of the 30-min treatment period (see Paper V for a detailed description of the protocol). This means that the concentration-response curves to cysteinyl-leukotrienes were commenced after a total incubation of about 3.5 hours, of which the indomethacin-treated preparations (Fig. 6) had been in contact with indomethacin for about 2 hours. At these time-points, the contractions to KCl in the study by Watts and Cohen (1993) were identical between controls and indomethacin-treated preparations.

The differences in effects of indomethacin on cysteinyl-leukotriene-induced contractions between previous studies (Krell et al., 1981b; Weichman et al., 1982) and the present (Fig. 6) may hence refer to experimental factors such as the use of inhibitors of cysteinyl-leukotriene metabolism, correction for indomethacin-induced decrease in basal tone or the time-point of cysteinyl-leukotriene administration. Anyhow, the findings indicate that under the present experimental conditions, indomethacin did not alter the contractile responses induced by either LTC_4 or LTD_4 in the guinea-pig trachea. Accordingly, the experiments within the study presented in Paper V were performed in the absence of indomethacin.

4.2.3. Metabolic modulation of contractions to cysteinyl-leukotrienes

4.2.3.1. Metabolism of cysteinyl-leukotrienes

The guinea-pig trachea metabolised exogenously added radiolabelled LTC_4 and LTD_4, whereas LTE_4 was not further metabolised (Fig. 7a; Paper V). These results support previous observations in the guinea-pig trachea (Snyder et al., 1984) and are similar to findings in the human lung (Kumlin & Dahlén, 1990). Under control conditions, LTC_4, D_4 and E_4 induced similar contractions of the guinea-pig trachea (Fig. 7b), but when metabolism was inhibited with the combination of L-serine borate and L-cysteine, each of the three cysteinyl-leukotrienes had a specific profile (Fig. 7c; Paper V).

In human bronchi, L-serine borate has been reported to slightly inhibit the LTC_4-induced contractions (Buckner et al., 1986; Yamaguchi et al., 1992), but also no effect has been reported (Muccitelli et al., 1987). Likewise, L-cysteine does not alter the concentration-response curves for LTD_4 in human bronchi (Buckner et al., 1986; Bourdillat et al., 1987). It is possible that the inhibition of LTC_4-induced contractions of human bronchi observed by some investigators (Buckner et al., 1986; Yamaguchi et al., 1992) might have been an unspecific effect of L-serine borate on the preparations, which has been reported for this inhibitor in the guinea-pig ileum (Gardiner et al., 1990). However, in contrast to findings in the guinea-pig trachea, L-serine borate does not significantly influence the ability of the $CysLT_1$ receptor antagonists FPL 55712 (Buckner et al., 1986; Muccitelli et al., 1987) or pranlukast (Yamaguchi et al., 1992) to antagonise the LTC_4-induced contractions of human bronchi. Since LTC_4 and LTD_4 are equipotent in contracting the human bronchus and apparently act on the same $CysLT_1$ receptor (see Sections 1.1.3. and 4.1.2.), interconversion would not be expected to alter their functional responses in human bronchi *in vitro* in the same way as in the guinea-pig trachea.

4.2.3.2. LTC_4 metabolism and contractions

The metabolism of LTC_4 caused a major change of the LTC_4-induced contractions of the guinea-pig trachea (Paper V). Inhibition of LTC_4 metabolism with L-serine borate, acivicin, GSH or S-hexyl GSH shifted the concentration-response curves almost one log order to the left and significantly enhanced the maximal contractions induced by LTC_4 (Paper V).

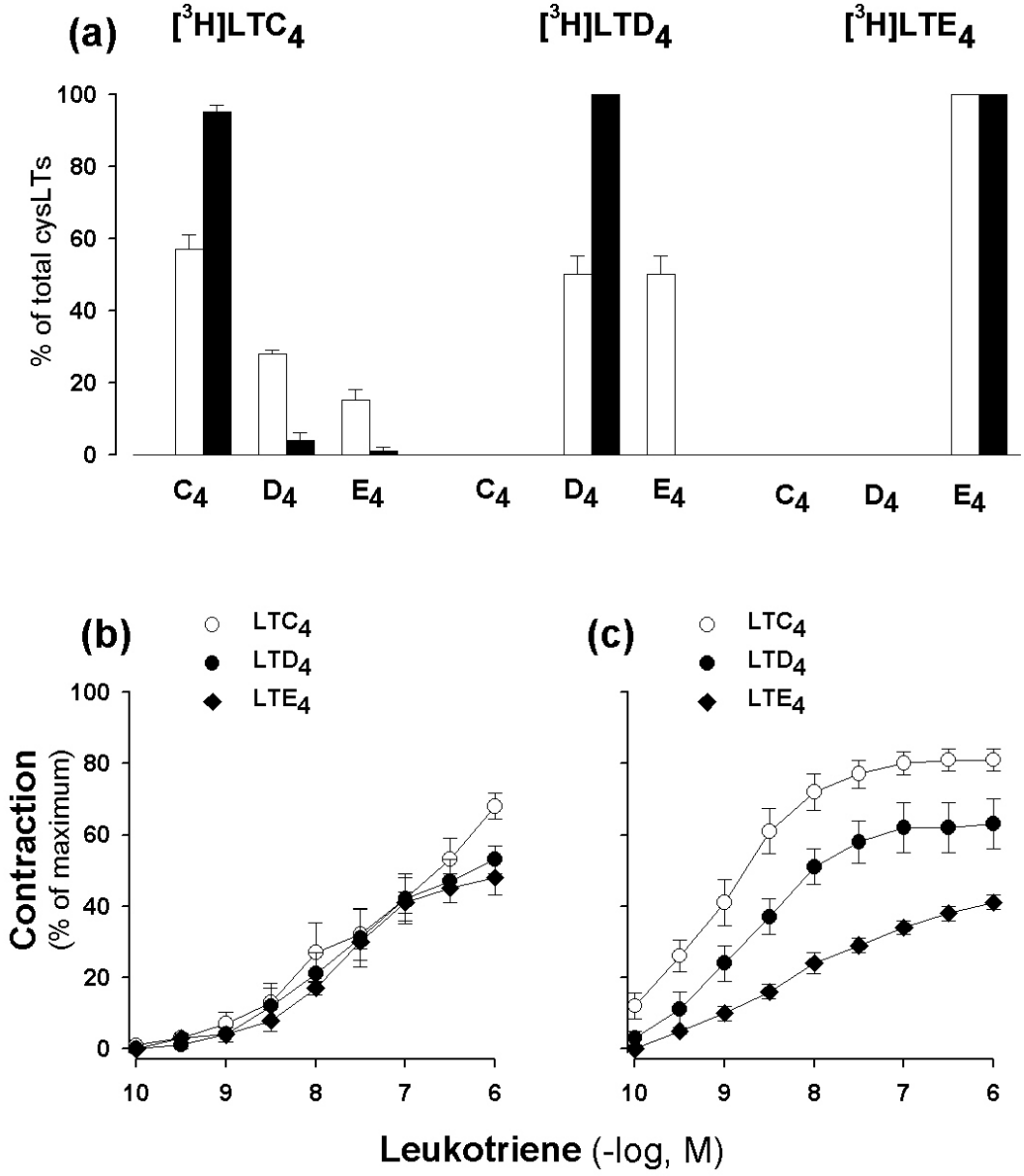

Fig. 7: The metabolism of radiolabelled cysteinyl-leukotrienes (a) and concentration response curves for LTC_4, LTD_4 and LTE_4 (b and c) in the guinea pig trachea. In panel a each bar is the mean+S.E.M. of 4 observations, representing the metabolism of $[^3H]LTC_4$, $[^3H]LTD_4$ and $[^3H]LTE_4$ in the absence (open bars) and presence (black bars) of the combination of L-serine borate (45 mM) with L-cysteine (5 mM). Contractions (means±S.E.M) induced by LTC_4, LTD_4 and LTE_4 were studied in the absence (b) or presence (c) of the combination of L-serine borate (45 mM) with L-cysteine (5 mM) and are expressed as percent of a maximal contraction to histamine (1 mM), acetylcholine (1 mM) and KCl (40 mM), *n*=4-12 (data from Paper V).

In addition to the potentiation and enhancement of the LTC_4-induced contractions (Paper V), inhibition of LTC_4 metabolism changes the character of the LTC_4-induced response in the guinea-pig trachea from $CysLT_1$ into $CysLT_2$. This notion is based on the findings that $CysLT_1$ receptor antagonists inhibit LTC_4-induced contractions of the guinea-pig trachea in the absence, but not in the presence of inhibitors of LTC_4 metabolism (see Section 1.1.3.). Accordingly, after treatment with either S-hexyl GSH or L-serine borate, the LTC_4-induced contractions were resistant to $CysLT_1$ receptor antagonism, thus confirming that the biochemically established inhibition of $[^3H]LTC_4$ metabolism was correlated to the functional results (Paper V).

Two LTC_4-preferential iso-forms of γ-GT have been described, γ-GT rel in humans (Heisterkamp et al., 1991) and γ-glutamyl leukotrienease in mice (Carter et al., 1997). These two isoforms share the profile of being able to metabolise LTC_4 but not synthetic γ-GT substrates such as γ-glutamyl-*P*-nitroanilide (GPNA; Heisterkamp et al., 1991; Carter et al., 1997). While γ-GT rel also metabolises GSH and GSSG (Heisterkamp et al., 1991), γ-glutamyl leukotrienease has been described not to metabolise either GSH or GSSG, but to be able to metabolise S-decyl GSH (Carter et al., 1997).

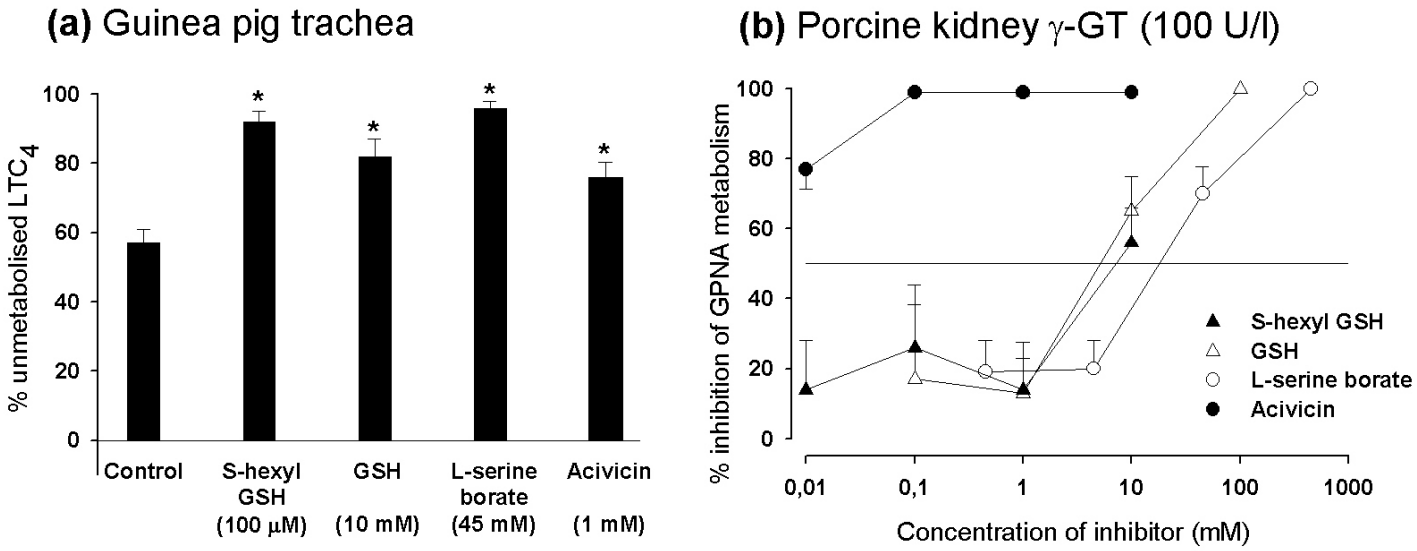

Fig. 8: The metabolism of LTC_4 in guinea pig trachea (a) and the metabolism of γ-glutamyl-*P*-nitroanilide (GPNA) by porcine kidney γ-GT (b). In panel a**,** each bar represents the percent of unmetabolised $[^3H]LTC_4$ after 30 min incubation in the absence (Control) or presence of inhibitors of LTC_4 metabolism, n=4-7 (data from Paper V). The data presented in panel b show the metabolism of the synthetic γ-GT substrate GPNA (6 mM) by porcine kidney γ-GT (100 U/l) in the presence of the acceptor molecule glycylglycine (80 mM), measured by increase in absorbence at 405 nm according to the method described in detail by Silber and co-workers (1986; see Methods). Each point is the mean of 3 measurements (previously unpublished data). * indicates a significant (P<0.05) difference compared with control.

In an attempt to characterise the γ-GT in the guinea-pig trachea according to the findings in humans and mice (Heisterkamp et al., 1991; Carter et al., 1997), the inhibition of LTC_4 metabolism in the guinea-pig trachea was compared with the inhibition of the metabolism of GPNA by purified porcine kidney γ-GT (previously unpublished data). GSH inhibited LTC_4 metabolism in the guinea-pig trachea at 10 mM (Fig. 8a) but not lower concentrations (data not shown), which is similar to its inhibition of GPNA metabolism by purified porcine kidney γ-GT (Fig. 8b). S-hexyl GSH was equipotent with GSH in inhibiting GPNA metabolism whereas S-hexyl GSH inhibited LTC_4 metabolism in the guinea-pig trachea at a 100-times lower concentration compared with GSH. Since S-hexyl GSH and GSH both inhibit LTC_4 metabolism by substrate competition, these findings suggest that the γ-GT in guinea-pig trachea may preferentially metabolise LTC_4 and other S-conjugates of GSH. However, a more direct investigation is required in order to establish the γ-GT iso-enzymes in the guinea-pig trachea and there are currently no tools available for such characterisation

In further support of the suggestion of a difference between enzymes metabolising GPNA and LTC_4, it was found that acivicin, previously described to be a potent and rapidly acting γ-GT inhibitor (Stole et al., 1994), potently inhibited the metabolism of GPNA by porcine kidney γ-GT (Fig. 8b) whereas a high concentration (1 mM) did not completely block [^{3}H]LTC_4 metabolism in the guinea-pig trachea (Fig. 8a, Paper V). These observations hence indicate that acivicin may be a less potent inhibitor of the LTC_4-preferential iso-enzyme compared with its ability to inhibit γ-GT. A discriminative effect of other γ-GT inhibitors on metabolism of GPNA and LTC_4 has previously been described in cultured human umbilical vein endothelial cells (Pologe et al., 1984) and in rat peritoneal cells (Aharony & Dobson, 1984).

4.2.3.3. LTD_4 metabolism and contractions

In the guinea-pig trachea, the metabolism of [^{3}H]LTD_4 into [^{3}H]LTE_4 was inhibited by L-cysteine, resulting in a potentiation of the concentration-response curves (Fig. 7; Paper V), which supports previous findings (Sok et al., 1981; Snyder et al., 1984). However, a formation of [^{3}H]LTC_4 from [^{3}H]LTD_4 was discovered in the presence of S-hexyl GSH (Paper V), a reaction not previously described in lung tissue nor in smooth muscle preparations. In addition to S-hexyl GSH, also other γ-glutamyl donors, such as GSH, GSSG and S-decyl GSH induced formation of LTC_4 from LTD_4 in the guinea-pig trachea (Paper V). These donors are metabolised by γ-GT and the γ-glutamyl group that is cleaved off by the enzyme is transferred to an acceptor (Tate & Meister, 1985) and if this acceptor is LTD_4, the reaction will yield LTC_4 (Hammarström, 1981) as is shown in Fig. 9.

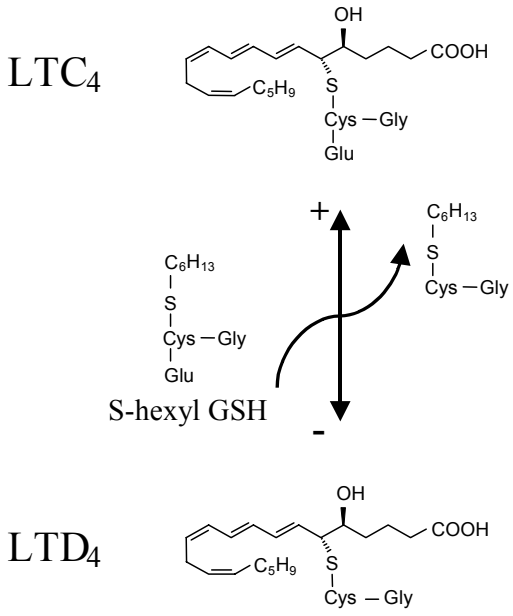

Fig. 9: The interconversion between LTC_4 and LTD_4 in the guinea pig trachea. S-hexyl GSH inhibited the metabolism of LTC_4 into LTD_4 and stimulated the formation of LTC_4 from LTD_4 (Paper V).

The formation of LTC_4 from LTD_4 changed the pharmacology of the LTD_4-induced contractions. In fact, in the presence of the γ-glutamyl donors, the responses to exogenously administered LTD_4 displayed the characteristics of the LTC_4-induced contractions (Paper V). Firstly, in the presence of L-cysteine, the LTD_4-induced contractions were inhibited by the $CysLT_1$ receptor antagonist ICI 198,615 (Paper V), but after pre-treatment with the combination of L-cysteine with one of the γ-glutamyl donors, the LTD_4-induced contractions were resistant to ICI 198,615 (Paper V). Secondly, the maximal contraction (E_{max}) induced by LTD_4 was increased after pre-treatment with the γ-glutamyl donors (Paper V) and thirdly, the LTD_4-induced contractions displayed a significantly slower time-course in the presence of γ-glutamyl donors (Paper V). These results were not significantly different from those obtained with LTC_4, thus supporting that the biochemically detected formation of LTC_4 from LTD_4 also changed the functional responses into an LTC_4-response.

This alternative pathway for metabolism of LTD_4 may modulate cysteinyl-leukotriene responses in any tissue expressing different CysLT receptors that are preferentially activated by either LTC_4 or LTD_4. Interestingly, LTC_4 and LTD_4 have been reported to activate separate receptors in isolated human saphenous veins and in human lung membranes (Allen et al., 1992; Rovati et al., 1992; Capra et al., 1998a; Capra et al., 1998b; Ravasi et al., 2000). Taken together, these reports suggest that the alternative metabolic pathway for LTD_4 described in the guinea-pig trachea (Paper V) may also modulate cysteinyl-leukotriene responses in human tissues.

4.2.3.4. LTE_4 metabolism and contractions

Previous *in vitro* studies have shown that γ-glutamyl LTE_4 (LTF_4) can be formed from LTE_4 after incubation with purified kidney γ-GT in the presence of GSH (Anderson et al., 1982; Bernström & Hammarström, 1982). However, the results in Paper V indicate that no such transpeptidation of LTE_4 occurred in the guinea-pig trachea, since there was no further metabolism of [^{3}H]LTE_4 under control conditions nor in the presence of the γ-glutamyl donor S-hexyl GSH. This is further supported by the finding that the LTE_4-induced contractions of the guinea-pig trachea were not altered in the presence of S-hexyl GSH (Paper V). If LTE_4 was metabolised into LTF_4, a leftward displacement of the concentration-response curve would be expected since LTF_4 previously have been described to be somewhat less potent than LTE_4 in inducing contractions of the guinea-pig trachea (Jones et al., 1983).

4.2.4. Summary: Modulation of cysteinyl-leukotriene responses in the guinea-pig trachea

✓ The cyclooxygenase inhibitor indomethacin decreased the basal tension of guinea-pig tracheal spiral preparations. In order to prevent an influence of this decrease in basal tone on subsequent responses, basal tone (10 mN) was re-established mechanically. Using an experimental protocol with a long incubation period before challenge with cysteinyl-leukotrienes, indomethacin did not alter the concentration-response curves for either LTC_4 or LTD_4 in the guinea-pig trachea (Fig. 6), suggesting that prostaglandins did not modulate the cysteinyl-leukotriene-induced contractions under these conditions. Therefore, subsequent experiments were performed in the absence of indomethacin.

✓ In untreated preparations, LTC_4, D_4 and E_4 induced similar contractions of the guinea-pig trachea, but in the presence of inhibitors of their metabolism, the contractions induced by the individual cysteinyl-leukotrienes had a specific profile (Paper V; Fig. 7).

✓ The guinea-pig trachea metabolised exogenously added radiolabelled LTC_4 and the results (Fig. 8) suggest that the guinea-pig trachea may have an isoform of γ-GT that is preferential for LTC_4 and other S-conjugates of GSH.

✓ Under control conditions, exogenously added LTD_4 was metabolised into LTE_4 by the guinea-pig trachea, whereas in the presence of γ-glutamyl donors, LTD_4 was converted into LTC_4 (Paper V). In fact, these two alternative pathways for metabolism of LTD_4 may determine what receptor is activated in the guinea-pig trachea since LTC_4 activated a $CysLT_2$ receptor, LTE_4 a $CysLT_1$ receptor and LTD_4 both $CysLT_1$ and $CysLT_2$ receptors (see Section 4.1 and Paper V). Accordingly, the metabolism of LTD_4 into LTE_4 will push the LTD_4-induced response towards a pure $CysLT_1$ receptor response (Fig. 10). In contrast, in the presence of a γ-glutamyl containing compound (e.g. GSH, S-hexyl GSH etc., see Paper V), LTC_4 will be formed from LTD_4, and the response will thus be pushed towards a $CysLT_2$ receptor response (Fig. 10).

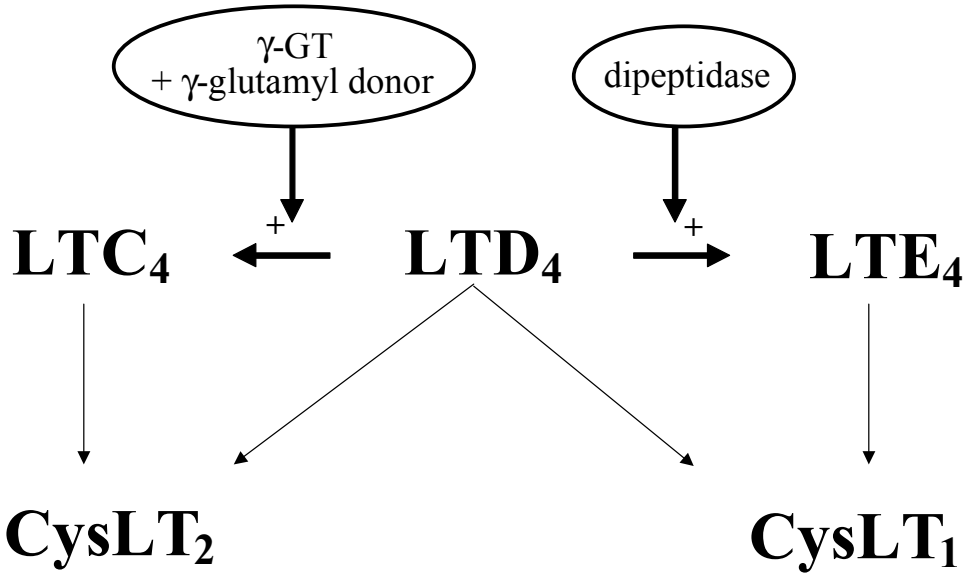

Fig. 10: The metabolism of LTD_4 and the activation of CysLT receptors in the guinea pig trachea.

4.3. Modulation of responses to cysteinyl-leukotrienes and other agonists in human and porcine pulmonary vessels

4.3.1. Specific aims

The aim of this part of the thesis was to examine the contractile effects of cysteinyl-leukotrienes in pulmonary vascular preparations. In order to compare the modulations of cysteinyl-leukotriene-induced responses with those of other agonists, also vascular responses to noradrenaline, acetylcholine and bradykinin were examined. In addition, as was found in the guinea-pig trachea (see Section 4.2.3. and Paper V), metabolism of cysteinyl-leukotrienes may modulate their responses. Therefore, the effects of inhibitors cysteinyl-leukotriene metabolism were assessed in preliminary experiments. Since a similarity between human and porcine pulmonary arteries was observed at the level of cysteinyl-leukotriene receptors (see Section 4.1.3.), this part of the thesis is focused on human and porcine pulmonary vessels.

4.3.2. Cysteinyl-leukotriene responses in the pulmonary vascular bed

4.3.2.1. Human pulmonary vessels

Previous reports have indicated that cysteinyl-leukotrienes are potent constrictors of human pulmonary veins, whereas human pulmonary arteries are only slightly contracted by either LTC_4 or LTD_4 (Schellenberg & Foster, 1984; Bourdillat et al., 1987). However, when the endothelium had been removed, the contractions LTC_4 and LTD_4 in the human pulmonary artery were enhanced (Paper III) to a level similar to what has previously been described for human pulmonary veins (Schellenberg & Foster, 1984; Bourdillat et al., 1987; Labat et al., 1992). These results suggest that the mechanism behind the apparent small contractions induced by cysteinyl-leukotrienes in human pulmonary arteries was due to release of inhibitory endothelial factors.

Also in isolated human systemic vessels, venous preparations have been reported to be more sensitive to cysteinyl-leukotrienes than arterial preparations (Allen et al., 1992; Allen et al., 1994; Cracowski et al., 1999; Stanke-Labesque et al., 2000). However, the preferential vasoconstriction of human saphenous veins compared with internal mammary, femoral and gastroepiploic arteries is not due to endothelial modulation since endothelium denudation (Allen et al., 1994; Cracowski et al., 1999; Stanke-Labesque et al., 2000) or pre-treatment with either indomethacin or a nitric oxide synthesis inhibitor (Allen et al., 1994) fails to significantly potentiate the cysteinyl-leukotriene-induced responses in these human systemic arterial preparations.

4.3.2.2. Porcine pulmonary vessels

In the pig, the relationship between pulmonary arteries and veins was the reversed compared with human pulmonary vessels, i.e. the pulmonary arteries were more sensitive to LTC_4 and LTD_4 than the pulmonary veins (Paper II and IV). In fact, the porcine pulmonary veins were practically insensitive to LTC_4 (Paper IV), which has also been observed in a previous study (Ohtaka et al., 1987). As will be discussed in greater detail below, significant contractions to LTC_4 were unmasked after the preparations were treated with a combination of cyclooxygense and nitric oxide synthesis inhibitors (Paper IV). When the modulatory factors had been removed, the contractions to LTC_4 were similar in porcine pulmonary arteries and veins (Paper IV) and, in addition, similar to contractions of human pulmonary arteries (Paper III).

4.3.3. Modulation of cysteinyl-leukotriene responses by prostaglandins

4.3.3.1. Isolated vessels

In untreated endothelium-intact pulmonary arterial preparations, LTC_4 induced significantly greater contractions of porcine preparations compared with human preparations (Fig. 11a; Paper II and III). However, the LTC_4-induced contractions of porcine pulmonary arteries were significantly inhibited by the cyclooxygenase inhibitor indomethacin (Paper II), whereas in human pulmonary arteries a significant enhancement was observed after indomethacin pre-treatment (Paper III). In fact, in the presence of indomethacin, LTC_4 induced similar contractions of porcine and human pulmonary arteries (Fig. 11b). Taken together, these findings indicate that LTC_4 stimulates the release of cyclooxygenase metabolites in both porcine and human pulmonary arteries, but that while in porcine pulmonary arteries vasoconstrictor prostanoids dominate, vasorelaxant prostanoids are released from human pulmonary arteries. These findings support a previous comparison of human and porcine pulmonary arteries using other agonists (Lawrence et al., 1998), suggesting that this difference in cyclooxygenase modulation may be general rather than LTC_4-specific.

The prostaglandin that was released after LTC_4 challenge of human pulmonary arteries was thus relaxant (Fig. 11) and, according to a previous report, human pulmonary arteries can produce prostacyclin (Schellenberg et al., 1986). The relaxant effects of prostacyclin on human pulmonary arteries are well known (Hadhazy et al., 1983; Haye-Legrand et al., 1987) and in fact, prostanoid-induced relaxations of human pulmonary arteries are mediated solely via an IP-receptor (Walch et al., 1999), i.e. the receptor for prostacyclin (Narumiya et al., 1999). Moreover, cysteinyl-leukotrienes have previously been reported to induce release of prostacyclin in cultured human umbilical vein endothelial cells (Cramer et al., 1983; Pologe et al., 1984).

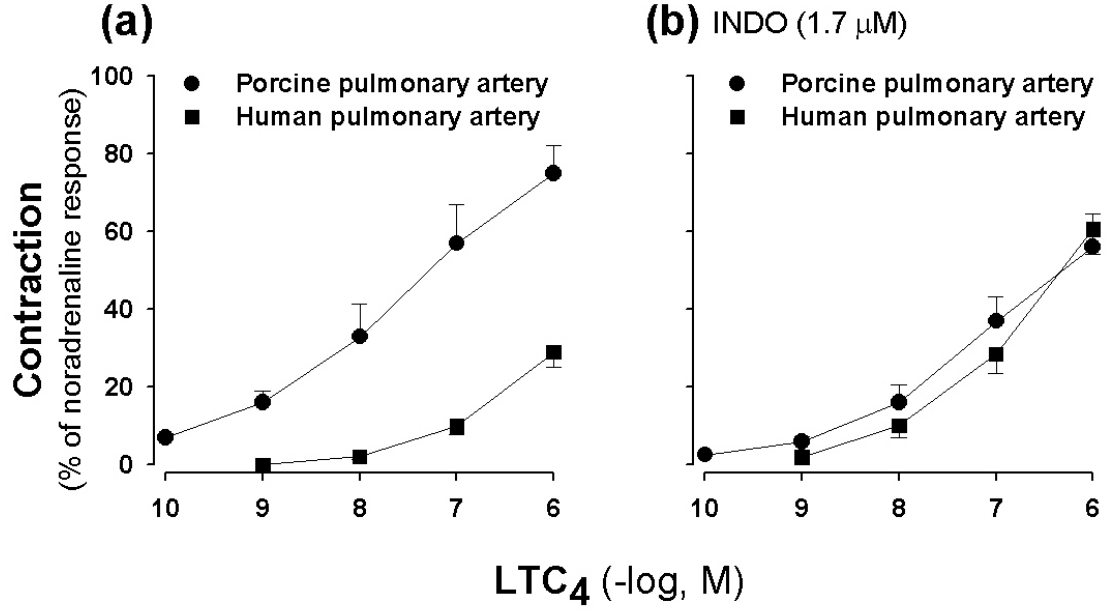

Fig. 11: Concentration response curves for LTC_4 in porcine and human pulmonary arterial preparations in the absence (a) or presence (b) of the cyclooxygenase inhibitor indomethacin (1.7 μM, INDO). Contractions (means±S.E.M) are expressed as percent of a reference contraction to noradrenaline (10 μM), *n*=5-10 (data from Paper II and III).

The release of prostacyclin in the human pulmonary artery was confirmed by measurements of the stable metabolite 6-keto $PGF_{1\alpha}$ and was found to correlate well with the functional tri-phasic response observed for both LTC_4 and LTD_4 (Paper III). After administration of either LTC_4 or LTD_4 (1 μM) an initial contractile response was observed followed by a relaxation with a maximum around 2 min the challenge. This relaxant phase correlated with an increased release of prostacyclin, which was significantly lower during the 18 following minutes of the LTC_4- and LTD_4-induced responses. When the prostacyclin release subsided, the contractions to LTC_4 and LTD_4 (which are long lasting responses) continued, which resulted in the third phase of the response, i.e. a contraction. However, the secreted prostacyclin was still enough to prevent a full cysteinyl-leukotriene contraction (Paper III).

On the basis of the observation that LTC_4-induced contractions of isolated porcine pulmonary arteries were somewhat more inhibited by a thromboxane synthesis inhibitor than by indomethacin, Ohtaka and co-workers (1987) suggested that thromboxane A_2 (TXA_2) may be the contractile factor which is released from porcine pulmonary arteries during challenge with LTC_4. However, the findings in Paper II raise a doubt as to this suggestion since measurements of thromboxane B_2 (TXB_2) showed that the increase in this stable TXA_2 metabolite was very small. Interestingly, porcine pulmonary arteries also released prostacyclin in response to both LTC_4 and LTD_4 (Paper II). Since prostacyclin is a vasorelaxant also in porcine pulmonary arterial preparations (Zellers et al., 1994), this finding suggests that a balance between different cyclooxygenase products, rather than one specific metabolite, regulates the contractions to cysteinyl-leukotrienes in porcine pulmonary arteries. However, the contractile prostanoid responsible for the inhibitory effects of indomethacin on the LTC_4-induced contractions remains to be established. For example, exogenously administered PGD_2 and $PGF_{2\alpha}$ have been reported to induce contractions of porcine pulmonary arteries (Greenberg et al., 1981).

Interestingly, in contrast to the results obtained with LTC_4, the LTD_4-induced contractions of porcine pulmonary arteries were not altered by indomethacin (Paper II). The latter finding is similar to a previous study of isolated human saphenous veins where indomethacin augments the contractions to LTC_4 whereas the LTD_4-induced contractions are unchanged by this treatment (Allen et al., 1992). Taken together, these findings thus suggest that there may be differences between how LTC_4 and LTD_4 activate the cyclooxygenase pathway.

In conclusion, cyclooxygenase products modulated the LTC_4-induced responses of both human and porcine pulmonary arteries. However, the composition of the products formed in response to LTC_4 may vary between the two preparations as is indicated in Fig. 12.

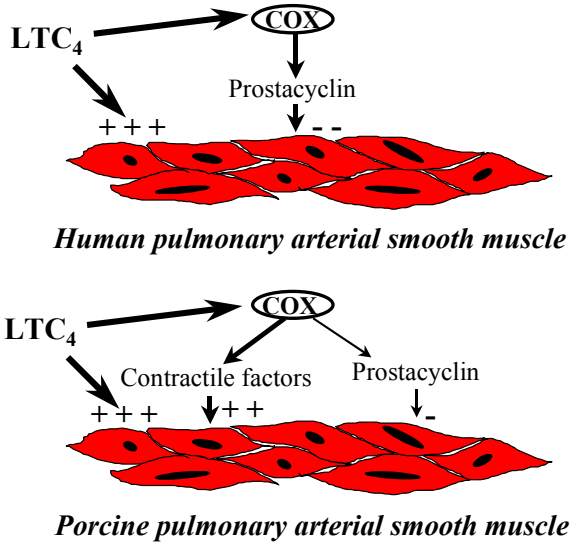

Fig. 12: The regulation of cysteinyl-leukotriene-induced contractions by cyclooxygenase (COX) in human and porcine pulmonary arteries. The figure is based on data from Paper II and III.

In porcine pulmonary veins, the negligible contractions induced by LTC_4 were not changed after pre-treatment with indomethacin (Paper IV) and Bourdillat and co-workers (1987) have previously reported that LTD_4-induced contractions of human pulmonary veins are not altered by cyclooxygenase inhibition. Taken together, these observations suggest that the cyclooxygenase pathway may preferentially modulate contractions to cysteinyl-leukotrienes in pulmonary arteries compared with pulmonary veins.

4.3.3.2. Comparison with in vivo studies

Also *in vivo* studies of cysteinyl-leukotrienes have indicated that their responses in the pulmonary vascular bed may be related to secondary released cyclooxygenase products. In the pig, the LTC_4-induced increase in pulmonary arterial pressure is inhibited by indomethacin (Olson & Fleisher, 1989). However, the finding that LTC_4 did not contract porcine pulmonary veins (Paper IV; Ohtaka et al., 1987) is opposed by the previously reported *in vivo* finding that in the pulmonary circulation of the pig, LTC_4 causes a 15-fold increase in venous resistance compared with a 2.5-fold increase in arterial resistance (Ohtaka et al., 1987). Ohtaka and co-workers (1987) suggested that TXA_2 from the porcine pulmonary artery may be transported downstream to the pulmonary veins and induce a venoconstriction. In support of that suggestion, the data presented in Fig. 13 (previously unpublished data) show that the TP-receptor agonist U 46 619 preferentially contracted porcine pulmonary veins compared with arteries. As discussed above, the low TXB_2-release detected from porcine pulmonary arterial preparations (see above, Paper II) raise a doubt as to how much TXA_2 that is actually produced by the porcine pulmonary arteries *in vitro*. However, it is possible that different cells account for the contribution of cyclooxygense modulation of vascular effects *in vitro* and *in vivo*. For example, LTC_4 is a potent aggregator of porcine platelets (Letts et al., 1985), and platelet aggregation elicits release of TXA_2 (Hourani & Cusack, 1991). Therefore, the differential findings may depend on the amount of platelets present during the investigations.

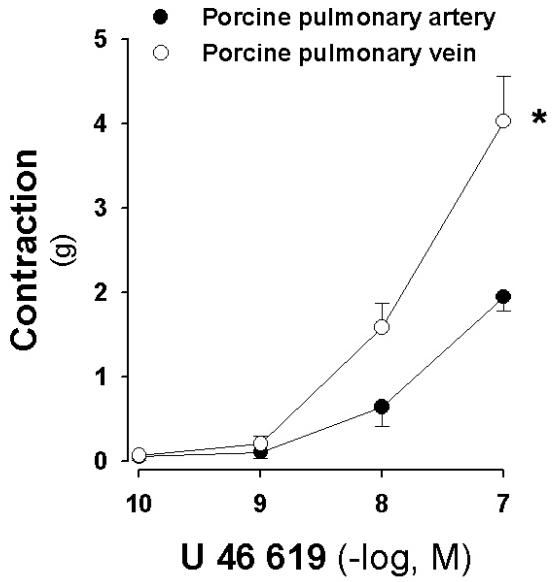

Fig. 13: Concentration response curves for the TP receptor agonist U 46 619 in porcine pulmonary arterial and venous preparations. Cumulative administration of the agonist was performed according to the experimental protocols described in detail in Paper II and IV. Contractions (means±S.E.M) are expressed in gram, n=4 (previously unpublished data). * indicates a significant difference (P<0.05, two way ANOVA) compared with porcine pulmonary artery.

The exact mechanisms of cysteinyl-leukotriene-induced responses in the pulmonary circulation and the relative importance of direct vascular effects in these responses remain to be established. In general, cyclooxygenase product seem to mediate cysteinyl-leukotriene pressor responses in the pulmonary circulation in pigs (Ohtaka et al., 1987; Olson & Fleisher, 1989) as well as in other species, for example guinea-pigs (Omini et al., 1981) and sheep (Kadowitz & Hyman, 1984), whereas in the cat, no effect of cyclooxygenase inhibition have been observed (Kadowitz & Hyman,

1984). In fact, in the study by Kadowitz and Hyman (1984), the remaining pressor activity of LTD_4 in the ovine pulmonary circulation after cyclooxygenase inhibition was very similar to that in the untreated cat, suggesting that without cyclooxygenase products, cysteinyl-leukotrienes have only moderate effects on the pulmonary circulation *in vivo*.

4.3.4. Modulation of cysteinyl-leukotriene responses by nitric oxide

Nitric oxide synthesis inhibition with L-NOARG in combination with cyclooxygenase inhibition with indomethacin did not modify the contractions induced by LTC_4 in porcine pulmonary arteries compared with results obtained with indomethacin alone (Paper IV). In contrast, treatment of porcine pulmonary veins with L-NOARG in combination with indomethacin unmasked a contractile response to LTC_4 (Paper IV). These results suggest that LTC_4 stimulated the release of nitric oxide from porcine pulmonary veins that functionally inhibited the contractile effect of the leukotriene, and that significant contractions were unmasked only after nitric oxide synthesis had been inhibited. Taken together, these observations indicate a preferential modulation of cysteinyl-leukotriene responses by nitric oxide in porcine pulmonary veins compared with arteries. The LTD_4-induced contractions of human pulmonary veins have previously been reported to be enhanced after L-NOARG treatment (Ortiz et al., 1995), suggesting that also in human pulmonary veins cysteinyl-leukotriene responses are regulated by nitric oxide. The effect of the combination of L-NOARG with indomethacin on the LTC_4-induced contractions of human pulmonary arteries was evaluated in a limited number of experiments, and the preliminary results suggested no further enhancement of the amplitude compared with indomethacin alone (Paper III). These results thus suggest a similarity with results in the porcine pulmonary artery, indicating that also in human pulmonary vessels, nitric oxide may preferentially regulate cysteinyl-leukotriene responses in pulmonary veins compared with arteries.

4.3.5. Modulation of responses to other agonists

4.3.5.1. Contractile agonists

In line with the findings that LTC_4 under control conditions contracted porcine pulmonary arteries but not pulmonary veins (Paper II and IV), noradrenaline exhibited a similar profile (Paper IV). The latter results confirm a previous report (Joiner et al., 1975), but no studies have been performed in order to elucidate the mechanisms involved in the lack of venous contractions to noradrenaline.

Indomethacin did not alter the noradrenaline-induced contractions in either arteries or veins, whereas L-NOARG significantly potentiated the contractile responses to noradrenaline in both vessels (Paper IV). However, after treatment with either L-NOARG or indomethacin+L-NOARG, the contractions to noradrenaline exhibited a markedly greater augmentation in venous (about 4-fold) compared with arterial (about 1.5-fold) preparations (Paper IV). These findings indicate that contractions induced by noradrenaline and LTC_4 are similarly regulated in porcine pulmonary vessels, and support the notion of a preferential regulation by nitric oxide in porcine pulmonary veins compared with arteries (Paper IV). This is further supported by a previous report that demonstrated that porcine pulmonary venous, but not arterial, contractions induced by endothelin-1 are potentiated by a nitric oxide synthesis inhibitor (Zellers et al., 1994). In contrast, contractions induced by noradrenaline and endothelin-1 in human pulmonary vessels may not be regulated by nitric oxide, since the combination of cyclooxygenase and nitric oxide synthase inhibition does not alter the maximal contractions induced by these agonists in isolated human pulmonary arteries

or veins (Pussard et al., 1995; Holm & Franco-Cereceda, 1996).

4.3.5.2. Endothelium-dependent relaxations

Although endothelium-dependent relaxations to cysteinyl-leukotrienes were not experimentally addressed in the present project, it warrants some discussion in the context of other agonists examined. In endothelium intact human pulmonary vessels, LTD_4 induces relaxations that are greater in arteries compared with veins (Ortiz et al., 1995). The exact mechanisms involved in these relaxations have not been investigated. It is likely that the prostacyclin released from human pulmonary arteries after challenge with either LTC_4 or LTD_4 (Paper III) contribute, but if also other mechanisms are involved remains to be established.

In human pulmonary vessels, also acetylcholine has been shown to induce greater relaxations of arteries compared with veins (Walch et al., 1997). However, the arterial relaxations induced by acetylcholine are markedly inhibited by indomethacin, whereas the venous relaxations are practically unaltered by this treatment (Norel et al., 1996; Walch et al., 1998). In addition, human pulmonary arterial preparations release more prostacyclin compared with venous preparations in response to acetylcholine (Walch et al., 1998), thus suggesting a similarity between responses induced by acetylcholine and cysteinyl-leukotrienes in the human pulmonary vasculature.

In porcine pulmonary vessels, venous preparations relaxed to a greater extent in response to acetylcholine than did arterial preparations (Paper IV). In addition, L-NOARG completely abolished the relaxations in both types of vessels, indicating that the acetylcholine-induced relaxations of porcine pulmonary vessels were mediated solely via release of nitric oxide (Paper IV). Consequently, the results with acetylcholine are in line with the results obtained with the contractile agonists LTC_4 and noradrenaline and support the notion of a dominant role of nitric oxide in porcine pulmonary veins.

In contrast to acetylcholine, bradykinin induced similar relaxations of porcine pulmonary arterial and venous preparations (Fig. 14, previously unpublished data). Nevertheless, also these results support the notion of a dominant role of nitric oxide in pulmonary veins, since bradykinin-induced relaxations in the veins were completely abolished by L-NOARG whereas the arterial relaxations were not blocked by nitric oxide synthesis inhibition and/or cyclooxygenase inhibition (Fig. 14). These results are in line with a previous report where bradykinin-induced relaxations of porcine pulmonary arteries were suggested to be mediated via an endothelium derived hyperpolarizing factor (Félétou et al., 1995). Although the nature of such hyperpolarizing factor has been debated, two different mechanisms are currently believed to be involved. The first possibility is the release of a factor from the endothelium that hyperpolarizes the smooth muscle and this factor has been identified as 11,12-epoxyeicosatrienoic acid, a cytochrome p450 metabolite of arachidonic acid (Fisslthaler et al., 1999). The other possibility is that an agonist hyperpolarizes the endothelial cells and that this hyperpolarization is spread via gap-junctions to the smooth muscle cells (Edwards et al., 2000).

In canine renal arteries, the LTD_4-induced endothelium-dependent relaxations are not attenuated by nitric oxide synthase inhibitors in combination with indomethacin (Pawloski & Chapnick, 1990), suggesting the possibility that an endothelium derived hyperpolarizing factor may be involved also in cysteinyl-leukotriene responses. Likewise, neither cyclooxygenase nor nitric oxide synthesis inhibitors block the endothelium-dependent relaxations induced by LTC_4 in human saphenous veins, although the effect of the combination of these to inhibitors was not examined in that study (Allen et al., 1992).

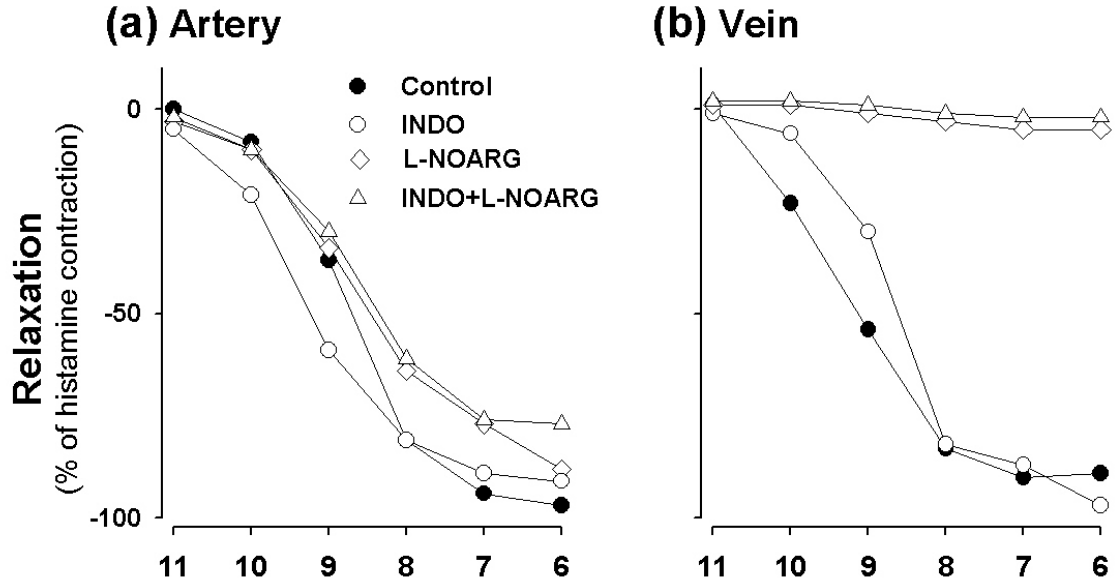

Fig. 14: Relaxations induced by bradykinin in endothelium intact porcine pulmonary arterial (a) and venous (b) preparations after a 30 min incubation period in the absence (Control) or presence of either indomethacin (1.7 μM, INDO), N^{ω}–nitro-L-arginine (100 μM; L-NOARG), or the combination INDO (1.7 μM) and L-NOARG (100 μM). Cumulative administration of the agonists was performed after a precontraction with histamine (10 μM) according to the experimental protocol described in detail in Paper IV. Relaxations are expressed as percent of the precontraction. Each point is the mean of n=2 (previously unpublished data).

4.3.6. Regulation of basal vascular tone

In both porcine pulmonary arteries and veins, basal tension was increased after addition of the nitric oxide synthesis inhibitor L-NOARG with the latter preparation exhibiting larger contractions (Paper IV). These findings support a previous study (Bina et al., 1998) and extend the observation by showing that indomethacin did not affect basal tone or further alter the effects of L-NOARG in either arteries or veins (Paper IV). Together these results suggest that in the porcine pulmonary vasculature, a basal release of nitric oxide, but not cyclooxygenase products, regulates basal tone. In contrast, in the human pulmonary artery, basal tone was significantly increased by indomethacin (Fig. 5 in Section 4.1.4.), which is similar to previous studies in the same preparation using either indomethacin (Hadhazy et al., 1983; Ortiz et al., 1992) or flurbiprofen (Lawrence et al., 1998).

4.3.7. Mechanisms involved in the differential modulation of vascular responses in porcine pulmonary arteries and veins

The results with LTC_4, noradrenaline, acetylcholine and bradykinin thus suggest a preferential role of nitric oxide regulation in porcine pulmonary veins compared with arteries, regulating relaxations and contractions as well as basal tone (Paper IV). The findings in porcine pulmonary arteries also indicate that LTC_4 stimulates the release of prostacyclin in this preparation (Paper II).

Since different enzymes and mechanisms are involved in the formation and action of nitric oxide, the question raised was from what level the observed differences in nitric oxide regulation between arteries and veins originated. The nitric oxide released from arteries and veins leads to the formation of cGMP via stimulation of soluble guanylate cyclase (see Section 1.2.4.2.). The nitric oxide donor sodium nitroprusside (SNP) induced

similar relaxations of porcine pulmonary arteries and veins, indicating that there was no difference in guanylate cyclase stimulation by nitric oxide between arteries and veins (Paper IV). Neither was there any difference between arteries and veins in sensitivity to the relaxant effect of 8-bromo-cGMP, a cell membrane permeable cGMP analogue (Paper IV). Together these findings suggest that the observed differences in nitric oxide regulation of basal tone, relaxations and contractions between arteries and veins were due to greater amounts of nitric oxide being released from the veins rather than a difference in sensitivity to either nitric oxide or cGMP. This is in concordance with a previous biochemical study (Bina et al., 1998) that reported both a greater nitric oxide release as well as a greater amount of constitutive nitric oxide synthase protein in porcine pulmonary veins than in arteries.

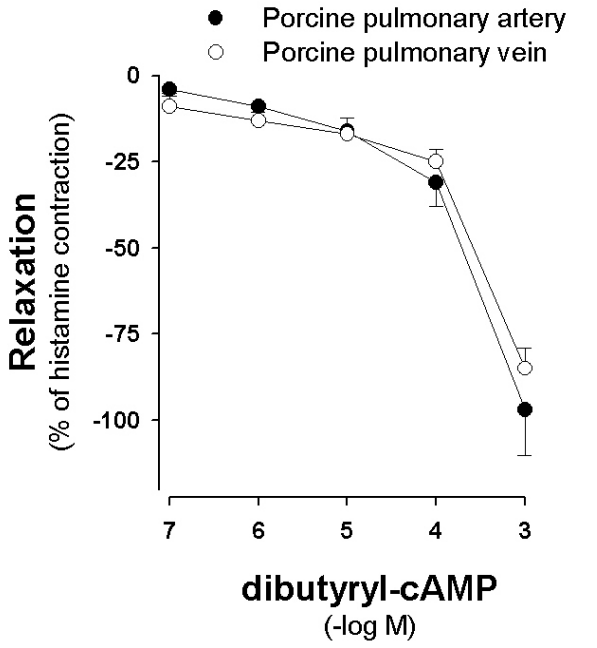

Fig. 15: Relaxations to dibutyryl-cAMP in porcine pulmonary arterial and venous preparations with the endothelium removed. Cumulative administration of the agonists was performed after a precontraction with histamine (10 μM) according to the experimental protocol described in detail in Paper IV. Relaxations (mean±S.E.M) are expressed as percent of the precontraction, *n*=4 (previously unpublished data).

Prostacyclin activates the IP receptor, which stimulates adenylate cyclase and increases levels of cAMP (Narumiya et al., 1999). Zellers and co-workers (1994) have previously reported similar maximal relaxations to prostacyclin in porcine pulmonary arteries and veins and the data presented in Fig. 15 (previously unpublished data) extend that observation by showing that the sensitivity to dibutyryl-cAMP was the same in arteries and veins. This latter observation is similar to what was observed with 8-bromo-cGMP (Paper IV) and further supports the notion that there may not be any difference between arteries and veins at the level of sensitivity of the smooth muscle cells to intracellular cyclic nucleotides.

4.3.8. Metabolism of cysteinyl-leukotrienes in vascular preparations

In the guinea-pig trachea, the interconversion between LTC_4 and LTD_4 and the metabolism of LTD_4 into LTE_4 represented major modulatory mechanisms (see Section 4.2.3. and Paper V). In the porcine pulmonary artery, LTC_4 and LTD_4 induced contractions whereas the preparations were insensitive to LTE_4 (Paper II), suggesting that the metabolism into LTE_4 would lead to an inactivation of the cysteinyl-leukotrienes in this preparation. However, Galton and Piper (1987) have previously reported a slower metabolism of LTD_4 compared with LTC_4 in chopped porcine pulmonary arteries and the present report extends those findings by incubating endothelium intact porcine pulmonary arterial preparations with radiolabelled LTD_4. The results presented in Fig. 16 (previously unpublished data) indicate that there was only marginal metabolism of LTD_4 when the tissues were studied as endothelium intact vascular preparations. These findings are further supported by the findings that treatment with L-cysteine, which is an inhibitor of LTD_4 metabolism (Paper V), did not alter the concentration-response curves to LTD_4 (Fig. 17; Paper II).

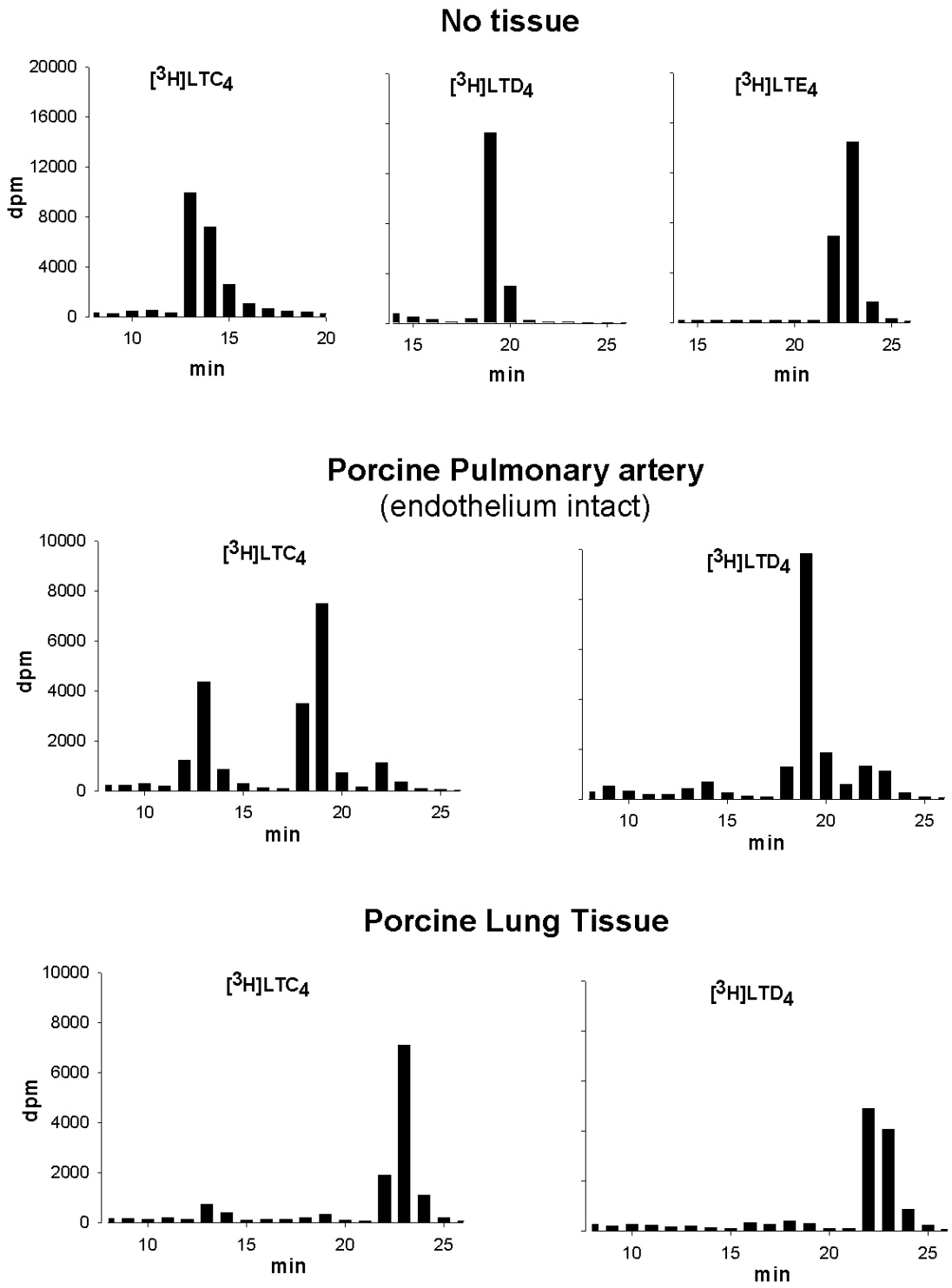

Fig. 16: The metabolism of [^{3}H]LTC$_4$ and [^{3}H]LTD$_4$ by porcine pulmonary artery and chopped porcine lung, examined according to the experimental protocol described in detail in Paper V. Top panels show the distribution of radioactivity for authentic standards of [^{3}H]LTC$_4$, [^{3}H]LTD$_4$ and [^{3}H]LTE$_4$. The results presented in the middle panels show that the endothelium intact porcine pulmonary artery metabolised [^{3}H]LTC$_4$ into [^{3}H]LTD$_4$ whereas exogenously added [^{3}H]LTD$_4$ showed less metabolic degradation. In contrast, after incubation of chopped porcine lung (lower panels) with either [^{3}H]LTC$_4$ or [^{3}H]LTD$_4$, the distribution of radioactivity was identical to that for [^{3}H]LTE$_4$, suggesting a rapid cysteinyl-leukotriene metabolism into LTE$_4$ in porcine lung tissue (each panel shows *n*=1, previously unpublished data).

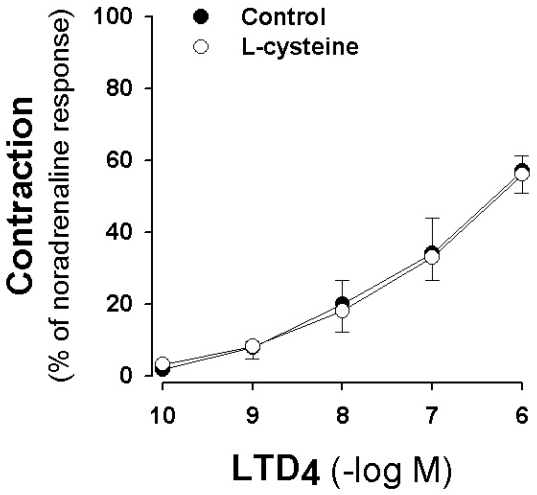

Fig. 17: Concentration response curves for LTD_4 in porcine pulmonary arterial preparations in the absence (Control) or presence of the inhibitor of LTD_4 metabolism, L-cysteine (5 mM). Experiments were performed according to the protocol described in detail in Paper II. Contractions (means±S.E.M) are expressed as percent of a reference contraction to noradrenaline (10 μM), *n*=3.

In fact, also in cultured human umbilical vein endothelial cell, the conversion of LTD_4 into LTE_4 is considerably less compared with the conversion of LTC_4 into LTD_4 (Pologe et al., 1984; Claesson & Haeggström, 1988), suggesting that vascular tissues may exhibit a minor metabolism of LTD_4. This suggestion is further supported by similar findings in the guinea-pig, using pulmonary artery (Fedyna et al., 1990), inferior vena cava (Rinkema et al., 1993) or cardiac tissue (Falcone et al., 1991).

In contrast the porcine pulmonary artery, the results obtained in chopped porcine lung tissue indicate a rapid metabolism of both $[^3H]LTC_4$ and $[^3H]LTD_4$ in this tissue, since only $[^3H]LTE_4$ was detected after 30 min incubation (Fig 16). These results thus suggest that other cells in the porcine lung than those associated with the vasculature are responsible for the metabolism of LTD_4.

Although the porcine pulmonary artery exhibited some metabolism of $[^3H]LTC_4$ (Fig. 16), the γ-GT inhibitor L-serine borate (Paper V) did not cause any apparent changes of the concentration-response curves for LTC_4 in this preparation (Paper II). However, L-serine borate have been described to have unspecific effects (Gardiner et al., 1990) and it cannot be excluded such effects may have masked a possible potentiation of the LTC_4-induced contractions due to an inhibition of LTC_4 metabolism. In any case, the data (Paper II) suggest that LTC_4 and LTD_4 activate the same receptor on the vascular smooth muscle and that this receptor mediates the major part of the contractions to LTC_4 and LTD_4. Therefore, an interconversion between LTC_4 and LTD_4 would not be expected to cause any major changes in the overall responses to these cysteinyl-leukotrienes in the porcine pulmonary artery *in vitro*.

Bourdillat and co-workers (1987) have previously reported that the LTD_4-induced contractions of human pulmonary arteries and veins are not altered by pre-treatment with L-cysteine and moreover, LTC_4-induced contractions of human pulmonary veins are not altered by L-serine borate (Labat et al., 1992). Preliminary experiments using S-hexyl GSH as an inhibitor of LTC_4-metabolism demonstrated no apparent effects of this treatment on the LTC_4-induced contractions of the human pulmonary artery (data not shown). However, in line with the findings in chopped porcine lung, human lung tissue metabolises cysteinyl-leukotrienes (Kumlin & Dahlén, 1990) and in addition, cysteinyl-leukotriene metabolising enzymes are present in human plasma (Köller et al., 1985).

Taken together these results indicate that cysteinyl-leukotriene metabolism may be an additional way of modulating responses to cysteinyl-leukotrienes in the pulmonary circulation, but that this modulatory mechanism seems to be difficult to study using isolated vessels.

4.3.9. Pulmonary hypertension

Stenmark and co-workers (1983) detected elevated levels of cysteinyl-leukotrienes in

bronchoalveolar lavage fluid from infants with pulmonary hypertension, leading to the suggestion that cysteinyl-leukotrienes may be involved in this disease, and a recent study demonstrated that cysteinyl-leukotriene are formed in the human pulmonary circulation (Kiss et al., 2000). However, based on the present *in vitro* findings in the human pulmonary artery (Paper III), the question can be raised as to what role cysteinyl-leukotrienes play in a disease like pulmonary hypertension. Isolated human pulmonary arteries were contracted by LTC_4 and LTD_4 (Paper III), which supports the hypothesis that cysteinyl-leukotrienes may increase pulmonary arterial pressure in humans. On the other hand, LTC_4 challenge led to the release of prostacyclin at concentrations sufficient to functionally antagonise the pulmonary arterial contractions (Paper III). Prostacyclin's relaxant effect has a beneficial role in pulmonary hypertension and is used as treatment of this disease (Gaine & Rubin, 1998). In addition, LTC_4 injection into humans leads to a slight decrease in pulmonary artery pressure (Kaijser, 1982). While a definitive role for cysteinyl-leukotrienes (constriction and/or dilatation) in pulmonary vessels under physiological conditions is difficult to establish, the data presented in Paper III suggest that the degree of cyclooxygenase activity and endothelial function may have modulatory influences on the LTC_4 responses *in vivo*.

4.3.10. Summary: Modulation of cysteinyl-leukotriene responses in the pulmonary vasculature

✓ The results obtained in porcine pulmonary vessels (Paper II and IV) and human pulmonary arteries (Paper II), taken together with previous studies of human pulmonary veins (Bourdillat et al., 1987; Ortiz et al., 1995), suggest that nitric oxide preferentially modulates cysteinyl-leukotriene-induced responses in pulmonary veins. A similar pattern was observed with noradrenaline, acetylcholine and bradykinin in porcine pulmonary vessels, further supporting the notion of a dominant role of nitric oxide in porcine pulmonary veins compared with arteries.

✓ In both human and porcine pulmonary arteries, cyclooxygenase products were the major modulators of cysteinyl-leukotriene responses. However, while the contraction of the human pulmonary artery by LTC_4 was functionally inhibited by the release of prostacyclin, porcine pulmonary arterial preparations mainly generated contractile cyclooxygenase products in response to LTC_4.

✓ The insensitivity of the porcine pulmonary veins to LTC_4 (Paper II) is opposed by the increase in venous resistance that has been reported for LTC_4 *in vivo* (Ohtaka et al., 1987). Taken together, the results (Paper IV; Fig. 13) indicate that these contradictory findings may relate to release of nitric oxide from porcine pulmonary veins *in vitro* and/or to secondary release of TXA_2 *in vivo*.

✓ The relaxant responses to bradykinin in porcine pulmonary arteries were not blocked by indomethacin and/or L-NOARG, suggesting the involvement of an endothelium-dependent hyperpolarizing factor. However, the role of this/these mediator(s) in regulation of cysteinyl-leukotriene-induced responses remains to be established.

✓ In line with the findings in the guinea-pig trachea (Paper V), metabolising enzymes may alter responses to cysteinyl-leukotrienes also in the porcine and human pulmonary circulations, but do not seem to significantly alter the contractions induced by these agonists in isolated pulmonary vessels.

✓ The results suggest that studying modulatory and indirect factors may be pertinent in order to elucidate the pathological and/or physiological role of cysteinyl-leukotrienes in the cardiovascular system.

5. CONCLUSIONS

5.1 CysLT receptors

The results presented in this thesis indicate that the current classification of cysteinyl-leukotriene receptors as $CysLT_1$ and $CysLT_2$, based on the sensitivity or resistance to $CysLT_1$ receptor antagonists, probably represents a simplification. The resistance of cysteinyl-leukotriene-induced contractions of human and porcine pulmonary arteries to both $CysLT_1$ and $CysLT_2$ receptor antagonism suggest the presence of additional receptors for cysteinyl-leukotrienes.

In the guinea-pig ileum and trachea, the contractions induced by LTC_4 were resistant to $CysLT_1$ receptor antagonism but inhibited by the dual $CysLT_1$/$CysLT_2$ receptor antagonist BAY u9773, supporting that LTC_4 activates a $CysLT_2$ receptor in these preparations.

The contractions induced by LTD_4 and LTE_4 in the guinea-pig trachea were inhibited by $CysLT_1$ receptor antagonists, supporting that both these leukotrienes activate $CysLT_1$ receptors in this preparation. However, systematic comparisons of these results with previous studies of $CysLT_1$ receptor antagonists in different tissues revealed a pattern that may suggest that what is now referred to as the $CysLT_1$ receptor, may in fact represent a heterogeneous group of receptor subtypes.

In addition, in the presence of the $CysLT_1$ receptor antagonist ICI 198,615, LTD_4, but not LTE_4 activated $CysLT_2$ receptors, suggesting that the expression of multiple CysLT receptors in a preparation may affect the receptor preference of the cysteinyl-leukotrienes.

5.2 Modulatory mechanisms

5.2.1. Release of modulatory factors

5.2.1.1. Cyclooxygenase products

In both human and porcine pulmonary arteries, the contractions induced by the cysteinyl-leukotrienes were modulated by cyclooxygenase products. However, while contractile prostanoids dominated in porcine pulmonary arteries, release of the relaxant prostanoid prostacyclin represented a major modulatory mechanism in human pulmonary arteries. In contrast, in the porcine pulmonary vein and guinea-pig trachea, contractions induced by the cysteinyl-leukotrienes examined were not altered by indomethacin.

5.2.1.2. Nitric oxide

Nitric oxide was a preferential regulator of basal vascular tone as well as of contractions and relaxations in porcine pulmonary veins compared with arteries. In fact, contractions of porcine pulmonary veins in response to LTC_4 were uncovered only after inhibition of nitric oxide synthesis.

5.2.2. Interconversion between the cysteinyl-leukotrienes

In the guinea-pig trachea, LTD_4 was shown to have two alternative pathways of metabolism. Metabolism of LTD_4 by a dipeptidase yielded LTE_4 and consequently activation of the $CysLT_1$ receptor. In the presence of a γ-glutamyl donor, LTD_4 was converted into LTC_4 via the action of γ-GT, leading to the formation of LTC_4 and hence, activation of the $CysLT_2$ receptor. In tissues with a heterogeneous CysLT receptor population with preferential activation by either LTC_4 or LTD_4 the metabolic interconversion between these agonists may represent a major modulatory mechanism since it may decide which CysLT receptor is activated by the cysteinyl-leukotrienes.

5.3. Current issues

The results of this thesis thus raise the possibility of additional CysLT receptors. However, further characterisation of this receptor is needed and identification of its molecular structure or the development of

selective $CysLT_2$ receptor antagonists would facilitate such studies.

The potent vasoconstrictor actions of the cysteinyl-leukotrienes in human pulmonary arteries, characterised for the first time in the present thesis, warrant further exploration of the role of cysteinyl-leukotrienes in the pulmonary circulation. The finding that the CysLT receptors on human pulmonary arteries and veins may be different opens up for possibilities to pharmacologically target specifically the arterial or venous circulations. In the search for selective antagonists at the respective receptors, the porcine pulmonary artery may serve as a suitable model for the corresponding human vascular segment.

The recent molecular characterisation of the human CysLT receptors will offer further possibilities for studies of CysLT receptors. However, the initial molecular studies have revealed discrepancies between studies of cells transfected with the human CysLT receptors compared with functional studies of human tissues. These discrepancies draw attention to the need of functional studies using whole tissues, where the characterisation of response to cysteinyl-leukotrienes can be studied taking into account receptor function as well as modulatory mechanisms.

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