Leukotriene D₄ receptors are not negatively coupled to adenylyl cyclase in guinea-pig lung parenchyma

¹Mark A. Giembycz, *Jack Diamond & **Ian W. Rodger

Department of Thoracic Medicine, Royal Brompton National Heart and Lung Institute, London SW3 6LY; *Division of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5 and **Merck-Frosst Centre for Therapeutic Research, Point Claire-Dorval, Quebec, Canada H9R 4P8

1 The possibility that receptors for the peptide-containing leukotrienes may be negatively coupled to adenylyl cyclase in guinea-pig lung parenchyma was investigated by comparing the effect of leukotriene D₄ (LTD₄) on the intracellular cyclic nucleotide (cyclic AMP and cyclic GMP) content and on the activity of cyclic AMP-dependent protein kinase (PKA). In addition, the potential association between changes in the cyclic nucleotide content and the ability of LTD₄ to increase lung parenchymal tone was also evaluated.

2 Non-cumulative challenge of parenchymal lung strips with LTD₄ elicited concentration-dependent contractions ($pD_2 = 8.23$) that were paralleled by concentration-related increases in the intracellular level of cyclic AMP and cyclic GMP, and in the activation state of PKA ($K_{act} = 33$ nM). Temporally, these biochemical effects of LTD₄ were transient, peaking after ~5 min drug contact thereafter decaying, despite the continued generation of tone. Both the biochemical and mechanical effects of LTD₄ were antagonized by the LTD₄-receptor blocking drug, ICI 198,615 (1 μ M for 60 min), indicating that they were receptor-mediated events.

3 Challenge of guinea-pig lung with LTD₄ (200 nM; EC_{100} for tension generation) stimulated a 150 and 70 fold increase in the elaboration of thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α}) respectively, relative to that generated spontaneously.

4 Pretreatment of lung strips for 60 min with an irreversible inhibitor of cyclo-oxygenase, flurbiprofen, at a concentration (8 μ M) that abolished both basal and LTD₄ (200 nM)-induced TXB₂ and 6-keto-PGF_{1 α} release, relaxed rapidly the spontaneous tone of the tissues, reduced the cyclic AMP content by ~50% and lowered the PKA activity ratio from 29% to 17%. In addition, flurbiprofen abolished the ability of LTD₄ (200 nM) to increase the cyclic AMP content and to activate PKA. Functionally, the magnitude of LTD₄ (200 nM)-induced tone and the increase in cyclic GMP content were attenuated by approximately 20% and 50% respectively in flurbiprofen-treated tissues.

5 In flurbiprofen-treated tissues, isoprenaline (10 μ M for 10 min) increased the cyclic AMP content (from 4 to 27 pmol mg⁻¹ protein) and activated PKA (from 15% to 26%). Preincubation (30 s or 5 min) of lung with LTD₄ (200 nM) did not inhibit (or enhance) these isoprenaline-induced effects.

6 Pretreatment of lung strips for 60 min with the thromboxane synthetase inhibitor, dazmegrel (10 μ M), relaxed the spontaneous tone of the tissues, abolished the LTD₄ (200 nM)-stimulated release of TXB₂ and significantly enhanced (~two fold) the elaboration of 6-keto-PGF_{1 α} . In addition, dazmegrel attenuated (by ~50%) LTD₄ (200 nM)-induced cyclic GMP accumulation but approximately doubled both the cyclic AMP content and PKA activity ratio. LTD₄-induced contractions, in contrast, were not affected by dazmegrel.

7 EP 092 (1 μ M for 60 min), a selective TP-receptor blocking drug, had no effect on spontaneous tone, eicosanoid formation or on the cyclic GMP content of guinea-pig lung parenchymal strips. Likewise, EP 092 exerted no significant mechanical effect in lung challenged with LTD₄ (200 nM) although it did potentiate, to a small extent, the ability of LTD₄ (200 nM) to increase the cyclic AMP content.

8 It is concluded that LTD₄ can increase the intracellular level of cyclic AMP in guinea-pig parenchyma and activate PKA by a leukotriene-receptor-mediated mechanism sensitive to ICI 198,615. However, these biochemical actions of LTD₄ are induced indirectly by an arachidonic acid-derived cyclo-oxygenase product(s) other than TXA₂. Thus, contrary to reports of other investigators, no evidence was found to corroborate the finding that stimulation of leukotriene receptors on guinea-pig lung parenchyma results in a rapid lowering of the cyclic AMP content even in cyclo-oxygenase-blocked tissues. These data, therefore, do not support the hypothesis that leukotriene-induced tension generation is dependent upon a prior reduction in the cyclic AMP content.

Keywords: Guinea-pig lung, effect of leukotriene D₄; excitation-contraction coupling; cyclic AMP; cyclic AMP-dependent protein kinase; cyclic GMP; signal transduction; leukotriene receptors, blockade by ICI 198,615; thromboxane receptors, blockade by EP 092; cyclo-oxygenase, inhibition by flurbiprofen; thromboxane synthetase, inhibition by dazmegrel

Introduction

Since the discovery that the peptide-containing leukotrienes account for the activity of slow reacting substance of ana-

phylaxis (SRS-A; Lewis *et al.*, 1980), many studies have been performed with the objective of understanding further the biochemical mechanism(s) by which leukotrienes elicit contraction of airways smooth muscle.

Evidence accrued from radioligand binding experiments indicates that leukotriene D₄ (LTD₄), the most potent of the peptido-leukotrienes, interacts with distinct receptors (Hoga-

¹ Author for correspondence at: Department of Thoracic Medicine, Royal Brompton National Heart and Lung Institute, Dovehouse Street, London SW3 6LY.

boom *et al.*, 1983; Mong *et al.*, 1984) that are located exclusively on the extracellular facing surface of the plasmalemma (Mong *et al.*, 1988). The binding of [³H]-LTD₄ to membranes prepared from guinea-pig lung is stereoselective and is displaced by LT-receptor agonists with a rank order of potency that correlates well with their ability to elicit smooth muscle contraction (Pong & DeHaven, 1982; Cheng & Townley, 1984; Cheng *et al.*, 1985; Mong *et al.*, 1985a). The interaction of LTC₄ and LTD₄ with their receptors is modulated by monovalent and divalent cations and by guanine nucleotides (Pong & DeHaven, 1982; Bruns *et al.*, 1983; Mong *et al.*, 1985b) in much the same way as agonists which have efficacy at α_2 -adrenoceptors, dopamine D₂-receptors, adenosine A₁-receptors, muscarinic cholinergic receptors, and receptors for angiotensin II, δ opioids and somatostatin (see Jakobs *et al.*, 1984). These latter observations prompted several investigators to postulate that the LT-receptor may be negatively coupled, through an inhibitory guanine nucleotide-binding protein (G_i, or variant thereof), to adenylyl cyclase (Hedman & Andersson, 1982a; Bruns *et al.*, 1983; Mong *et al.*, 1985b). Indeed, Andersson and his colleagues (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b) have found that LTC₄, LTD₄ and immunologically generated SRS-A produce a rapid reduction in the basal level of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in guinea-pig tracheae, that precedes the development of tension. Similar results have been reported by Barnes & Grandordy (1986) in guinea-pig lung. Further support for the involvement of a G_i-like GTP-binding protein in LT-receptor-effector coupling is the observation that pretreatment of guinea-pig minced lung with pertussis toxin attenuates the ability of LTD₄ to promote thromboxane (TX) and prostacyclin (PGI₂) biosynthesis (Mong *et al.*, 1986). Based upon these findings it has been proposed that a rapid reduction in the cyclic AMP content may be linked intimately to LTD₄-induced tension development (Andersson *et al.*, 1982).

In spite of these data, however, the effect of the peptido-leukotrienes on the cellular cyclic AMP content and on the activity of PKA has not been systematically studied in any tissue which express receptors for these lipid mediators. This may be a consequence, in part, of several more recent reports which provide compelling evidence that LTC₄ and LTD₄ can stimulate the hydrolysis of phosphatidylinositol (4,5)-bisphosphate by a receptor-mediated mechanism (e.g. Mong *et al.*, 1987; 1988; Sarau *et al.*, 1987) that, in some tissues, is sensitive to pertussis toxin (e.g. Howard *et al.*, 1992).

In view of the observations of Andersson *et al.* (1982, 1983) together with the other circumstantial evidence cited above, consideration should be given to the possibility that the peptido-leukotrienes can activate more than one signal transduction mechanism. Demonstration of such a phenomenon would be analogous to the effects of cholinomimetics on airways smooth muscle cells where they elicit an atropine-sensitive inhibition of adenylyl cyclase (Jones *et al.*, 1987), reduce the basal and isoprenaline-stimulated cyclic AMP content (Langlands *et al.*, 1987) and promote the reassociation (inactivation) of the regulatory and catalytic subunits of PKA (Langlands & Rodger, 1992), events associated temporally with tension generation. Over the same concentration-range these cholinomimetics also promote inositol polyphosphate accumulation, an intimate step in Ca²⁺-dependent force generation in smooth muscle (see Hall & Chilvers, 1989, for review).

In an attempt to reconcile the reported action of the peptido-leukotrienes on the cyclic AMP content in guinea-pig trachea and lung tissue (Andersson *et al.*, 1982a,b; Hedman & Andersson, 1982a,b; Barnes & Grandordy, 1986) with the currently accepted theory regarding LT-receptor-effector coupling (Mong *et al.*, 1987; 1988; Sarau *et al.*, 1987), the effect of LTD₄ on the cyclic nucleotide content and on the activity of PKA was examined in guinea-pig lung parenchyma and compared temporally to its established spasmogenic activity in this tissue.

Methods

Isolation, preparation and treatment of lung tissue

Male Camm-Hartley guinea-pigs (250–300 g) were used. Each animal was heparinised (140 units kg⁻¹), left for 10 min and then killed by cervical dislocation and exsanguinated. The thorax was opened and the lungs were perfused *in situ* with heparinised Krebs-Henseleit solution (KHS) through the right ventricle and pulmonary artery; the left atrium was removed from the heart to permit the outflow of the perfusion fluid. When the perfusate was free of blood the lungs were excised and placed in a petri dish containing KHS. The lower lung lobes were isolated from the major airways and blood vessels, and tissue showing significant haemorrhage was discarded. Sub-pleural parenchymal lung strips (~3 mm × 3 mm × 15 mm) were then cut and mounted vertically, under an initial stretching tension of 20 mN, in 10 ml tissue baths containing KHS maintained at 37°C. The KHS was bubbled gently with a gaseous mixture containing 95% O₂ and 5% CO₂ so as to produce a P_{O₂} = 70–73 kPa, P_{CO₂} = 5.1–5.3 kPa and a pH of 7.4. Each strip was left to equilibrate for 60 min before the addition of drugs. Cyclic nucleotide content and PKA activity was measured in tissues challenged with various concentrations of LTD₄ pretreated with either flurbiprofen, ICI 198,615, dazmegrel, EP 092 or their respective vehicles (as indicated in the text). In these studies LTD₄ was given as a bolus and at the peak of the induced mechanical response, or at pre-determined times (see text), tissues were freeze-clamped using tongs pre-cooled in liquid nitrogen and processed for cyclic nucleotide estimation and PKA activity as described below. Changes in tension resulting from drug treatment were measured isometrically by Grass FT03.c force-displacement transducers and recorded on a Grass 79D curvilinear, ink-writing polygraph. In some experiments the KHS bathing the lung strips was sampled for the estimation of TXB₂- and 6-keto-prostaglandin F_{1 α} (6-keto-PGF_{1 α})-like immunoreactivity (see below). The composition of the KHS was as follows (mM): NaCl 118, KCl 4.7, MgSO₄·7H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11.7, CaCl₂·6H₂O 2.5.

Assay of cyclic AMP, cyclic GMP, 6-keto-PGF_{1 α} and TXB₂

For cyclic nucleotide measurements each individual lung strip was weighed before being placed in a 2 ml capacity Teflon vial (pre-cooled in liquid nitrogen) containing 1 ml trichloroacetic acid (TCA, 6% w/v) and a 0.9 mm stainless steel grinding ball. Tissues were then disrupted (Braun Micro-Dismembrator II) for 30 s. The pulverised material was transferred to a 5 ml polypropylene centrifugation tube, residual material being removed from the teflon vial with a further 1 ml TCA. Each tube was then centrifuged at 11300 g_{max} for 15 min at 4°C. After centrifugation the supernatant was decanted into a large Pyrex tube and extracted six-times with 10 ml ice-cold water-saturated diethylether to ensure removal of the TCA and lipids in the extract. After the final extraction residual traces of ether were evaporated by heat in a water bath and the samples stored at -20°C. Before assaying for cyclic nucleotide content, samples were lyophilised and reconstituted in ice-cold Tris 20 mM/EDTA 2 mM buffer (pH 7.4). Tissue levels of cyclic AMP and guanosine 3':5'-cyclic monophosphate (cyclic GMP) were estimated with a commercially available competitive protein binding and radioimmunoassay kit respectively.

The concentrations of TXA₂ and PGI₂ released from guinea-pig lung into the bathing buffer was measured, as their stable non-enzymatic degradation products, TXB₂ and 6-keto-PGF_{1 α} respectively, by radioimmunoassay as described in detail previously (Giembycz & Rodger, 1987). It was assumed that the conversion of TXA₂ to TXB₂ and PGI₂ to 6-keto-PGF_{1 α} followed a 1:1 stoichiometry.

Preparation and assay of soluble PKA

Previous studies have demonstrated that guinea-pig lung contains primarily the Type II isoenzyme of PKA (Giembycz & Diamond, 1990a). To prevent the inactivation of this enzyme following drug treatment each frozen tissue was homogenized (Polytron PT 10) for 1 × 15 s burst at setting 8 in 20 vol (w/v) of ice-cold phosphate buffer (5 mM KH₂PO₄, 5 mM K₂HPO₄, pH 6.8) containing 10 mM EDTA, 10 mM DTT and 500 µM IBMX and supplemented with 20 mM NaCl and 140 mM KCl (Cherrington *et al.*, 1976). The resulting homogenate was centrifuged (Beckman J2 21) at 31000 *g*_{max} for 15 min at 4°C in a fixed arm JA 17 rotor (*r*_{av} = 9.6 cm) to form soluble (supernatant) and particulate (pellet) fractions. The soluble enzyme was diluted 16 fold in phosphate buffer immediately before use.

Soluble PKA activity was measured as described previously (Giembycz & Diamond, 1990b). One unit of enzyme activity is defined as that amount of PKA which catalysed the incorporation of 1 pmol of phosphate from ATP into the peptide substrate, Kemptide, in 1 min at 30°C. In some experiments PKA activity is expressed as an activity ratio which represents the specific activity of PKA in the absence of exogenous cyclic AMP divided by the specific activity of PKA in the presence of a concentration of cyclic AMP required maximally to activate the enzyme.

Measurement of haemoglobin content

The protein concentration attributable to haemoglobin in guinea-pig lung varied widely (6 to 24%) from tissue to tissue even after perfusion of >100 ml of heparinised KHS through the pulmonary circulation. The contribution made by this haemoglobin was thus determined for each tissue and subtracted from the total measured protein. Haemoglobin content was determined spectrophotometrically by measuring the absorbance, at 411 nm, of a suitably diluted aliquot of the soluble fraction of the original homogenate. The amount of protein corresponding to this absorbance was quantified by reference to a standard curve constructed using human haemoglobin.

Protein determination

The protein content of soluble fractions was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) used as standard. For quantifying the cyclic nucleotide data the protein in the pellet was first solubilised with 2% (w/v) sodium deoxycholate and then precipitated with TCA (24% w/v).

Chemical, drugs and analytical reagents

The following were purchased from the Sigma Chemical Company: ATP (free acid), bovine serum albumin (BSA; grade III) cyclic AMP, cyclic GMP, (±)-1,4-dithiothreitol (DTT), EDTA (free acid), human haemoglobin (fraction III), heparin, 3-isobutyl-1-methyl-xanthine (IBMX) and Kemptide. All other chemicals, drugs and analytical reagents were obtained from the following sources: [³²P]-ATP (20–40 Ci mmol⁻¹), [³H]-cyclic AMP competitive protein binding assay kit, TRK 432 and [³H]-cyclic GMP radioimmunoassay kit, TRK 500 (Amersham), dazmegrel (UK-38,485; 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole propanoic acid, Pfizer), EP 092 ((±)-5-endo-(6'-carboxyhex-2'-Z-enyl)-6-exo-([1'-(phenylthiocarbamoyl)-hydrozono]-ethyl)-bicyclo [2,2,1] heptane; University of Edinburgh), flurbiprofen Na⁺ (Boots), LTD₄ (Merck Frosst & Bayer), ICI 198,615 ([1-[2-methoxy-4-[[phenylsulphonyl] amino]carbonyl] phenyl] methyl]-1H-indazol-6-yl] carbamic acid cyclopentyl ester; ICI) and salts for buffers and constituents of the assay cocktail (BDH).

Stock solutions of all drugs and reagents were made up in distilled water with the following exceptions: ICI 198,615 and

dazmegrel were dissolved in 0.1 M NaOH to form their respective soluble sodium salts. IBMX was dissolved in 20% (v/v) ethanol.

LTD₄ was shipped to the laboratory in dry ice and stored at -80°C. Under these storage conditions the non-enzymatic conversion of LTD₄ to the inactive 11-*trans* isomer was minimized to a rate not exceeding 1.5% per month as assessed by reverse phase high performance liquid chromatography (h.p.l.c.). The molar concentration of stock solutions of LTD₄ was determined from its u.v. absorbance at 280 nm using its molar extinction coefficient of 4.03 × 10⁴ cm² mmol⁻¹.

Statistical analysis

Values in the text represent the mean ± s.e.mean of *n* independent determinations. All data were analysed parametrically with the statistical package 'Number Cruncher Statistical System'. Student's *t* test (two-tailed) was used to test the significance between paired and unpaired variates. When more than two groups of unpaired data were compared significance was tested by one-way analysis of variance (ANOVA) and assessed with the Newman-Keuls modified *t* test. The null hypothesis was rejected when *P* < 0.05.

Results

Effect of LTD₄ on tension, intracellular cyclic nucleotide content and on the elaboration of TXB₂ and 6-keto-PGF_{1α}

Figure 1 illustrates the non-cumulative effect of LTD₄ on tension and on the intracellular cyclic nucleotide (cyclic AMP and cyclic GMP) content of guinea-pig isolated lung parenchyma. In agreement with previous studies (e.g. Dahlen *et al.*, 1983), LTD₄ (200 pM to 200 µM) evoked concentration-

Figure 1 Concentration-response relationship for leukotriene D₄ (LTD₄)-induced tension generation (●), and cyclic AMP (solid columns) and cyclic GMP (open columns) accumulation in guinea-pig isolated lung parenchyma. Tissues were set up for isometric tension recording in KHS and after the equilibration period had elapsed challenged non-cumulatively with LTD₄ (0.2–200 nM). Changes in tension were subsequently measured at the peak of the resulting contracture. For cyclic nucleotide measurements, lung strips were incubated with LTD₄ (0.2–200 nM) for 5 min and then frozen rapidly by submersion in liquid nitrogen. Cyclic AMP and cyclic GMP were then extracted from tissues and their respective masses estimated as described in Methods. Data represent the mean ± s.e.mean (vertical lines) of six independent determinations. **P* < 0.05, significantly different from resting cyclic nucleotide levels as assessed by one-way ANOVA/Newman Keuls test.

dependent contractions of lung strips with a pD₂ and maximum force of 8.23 ± 0.09 ($n = 6$) and 4.3 ± 0.5 mN ($n = 6$) respectively. Pretreatment (60 min) of tissues with ICI 198,615 (1 μ M), a potent and highly selective LTD₄-receptor antagonist (Snyder *et al.*, 1987), had no effect on the spontaneous tone generated by the lung but markedly antagonized LTD₄ (200 nM)-induced tension generation (Figure 2a) indicating that this response was mediated through stimulation of LTD₄-receptors.

Associated with the development of force, LTD₄ (200 pM to 200 μ M) also promoted a concentration-dependent accumulation of both cyclic AMP and cyclic GMP (Figure 1), and enhanced over basal levels the elaboration of TXB₂ and 6-keto-PGF_{1 α} into the bathing buffer (Figure 3). At the concentration of LTD₄ (200 nM) that produced the maximum contracture, the intracellular content of both cyclic nucleotides was increased four to seven fold (Figures 1, 2; Table 1) while the concentration of immunoreactive TXB₂ and 6-keto-PGF_{1 α} present in the bathing buffer was increased ~150- and 70 fold respectively relative to time-matched controls (Figure 3).

Consistent with the effect of ICI 198,615 on LTD₄-induced tension generation, the increase in cyclic nucleotide content and the elaboration of TXB₂ and 6-keto-PGF_{1 α} evoked by LTD₄ was attenuated markedly following pretreatment (60 min) of lung with ICI 198,615 (1 μ M; Figure 2b,c; Figure 3a,b). ICI 198,615 did not, however, inhibit the spontaneous release of eicosanoids from the lung or alter the resting cyclic nucleotide content (data not shown).

The kinetics of the mechanical and cyclic nucleotide-elevating effects of LTD₄ on guinea-pig lung are shown in Figure 4 and in Table 1. LTD₄ (200 nM) produced slow contractures of lung strips that developed over the 15 min period of study and which persisted even after the tissue was washed free of drug. Temporally, the accumulation of both cyclic AMP and cyclic GMP occurred either in parallel to or, more often, lagged behind tension development (Figure 4; Table 1). Indeed, the mean data of six independent experiments show that LTD₄ promoted a significant increase in tone 30 s post-drug challenge without affecting the cyclic AMP content (Figure 4). The cyclic nucleotide-elevating effect of LTD₄ occurred only transiently peaking after 5 min drug contact thereafter decaying despite the continued generation of tone (Figure 4).

Figure 2 Effect of the leukotriene D₄ (LTD₄)-receptor antagonist ICI 198,615 on LTD₄-induced tension generation (a), cyclic AMP accumulation (b) and cyclic GMP accumulation (c) in guinea-pig isolated lung parenchyma. Lung strips were incubated with ICI 198,615 (1 μ M for 60 min) or its vehicle and then challenged with LTD₄ (200 nM). At the peak of the resulting contracture tissues were frozen rapidly by submersion in liquid nitrogen and cyclic nucleotides subsequently extracted and their concentration measured as described in Methods. (A) resting cyclic nucleotide levels; (B) responses induced by LTD₄; (C) responses induced by LTD₄ in ICI 198,615-pretreated tissues. Data represent the mean $\pm$ s.e.mean (vertical lines) of n independent observations shown at the top of each column. * $P < 0.05$, significant inhibition of LTD₄-induced responses as assessed by Student's unpaired t test.

Figure 3 Pharmacological modulation of the release of thromboxane B₂ (TXB₂) (a) and 6-keto-prostaglandin_{1 α} (6-keto-PGF_{1 α}) (b) by leukotriene D₄ (LTD₄) from guinea-pig isolated lung parenchyma. Lung strips were incubated for 60 min with either ICI 198,615 (1 μ M), flurbiprofen (8 μ M), dazmegrel, (10 μ M), EP 092 (1 μ M) or vehicle. Tissues were then challenged with LTD₄ (200 nM). Immediately before, and 15 min after LTD₄ the KHS was sampled and assayed for immunoreactive TXB₂ and 6-keto-PGF_{1 α} as described in Methods. The columns depict the total release (i.e. LTD₄ + spontaneous) of eicosanoids from the lung with respect to time-matched controls. (A) spontaneous; (B) LTD₄; (C) LTD₄ + ICI 198,615; (D) LTD₄ + flurbiprofen; (E) LTD₄ + dazmegrel; (F) LTD₄ + EP 092. Data represent the mean $\pm$ s.e.mean (vertical bars) of n independent observations shown at the top of each column. * $P < 0.001$ and *** $P < 0.0001$, significant inhibition of LTD₄-evoked eicosanoid release. *** $P < 0.05$, significant augmentation of LTD₄-induced 6-keto-PGF_{1 α} release. Data analysed by one-way ANOVA/Newman-Keuls test.

Effect of LTD₄ on PKA activity

Since receptor-mediated increases in the tissue cyclic AMP content are associated generally with smooth muscle relaxation (Diamond, 1978; Giembycz & Raeburn, 1991) studies were performed to determine if LTD₄-induced cyclic AMP accumulation was coupled to the activation of PKA, the enzyme through which all the actions of cyclic AMP are believed to be mediated (see Beebe & Corbin, 1986). The data in Figures 5 and 6 show the results of these experiments. In the absence of LTD₄, the specific activity of PKA extracted from the soluble fraction of lung homogenates was 1.93 ± 0.25 nmol min⁻¹ mg⁻¹ protein ($n = 6$) which was

Figure 4 Kinetics of leukotriene D₄ (LTD₄)-induced tension generation (●) and cyclic AMP accumulation (columns) in guinea-pig isolated lung parenchyma. Lung strips were challenged with LTD₄ (200 nM) and after 0, 0.5, 1, 2, 5 and 15 min frozen rapidly by submersion in liquid nitrogen. Cyclic nucleotides were then extracted from each tissue and the cyclic AMP content subsequently measured as described in Methods. Data represent the mean $\pm$ s.e.mean (vertical bars) of eight independent observations. * $P < 0.05$, significantly different from resting cyclic AMP levels as assessed by one-way ANOVA/Newman Keuls test.

Table 1 Effect of flurbiprofen, dazmegrel and EP 092 on leukotriene D₄ (LTD₄)-induced tension development and on cyclic GMP accumulation in guinea-pig lung parenchyma

Treatment	Contact Time (min)	Δ Tension (mN)	Cyclic GMP content (pmol mg ⁻¹ protein)
Control (n = 8)	- 60	-	3.9 $\pm$ 0.4
	0	0.3 $\pm$ 0.1	3.7 $\pm$ 0.7
	0.5	0.8 $\pm$ 0.2	6.3 $\pm$ 1.2
	1	1.1 $\pm$ 0.1	7.8 $\pm$ 2.5*
	2	1.5 $\pm$ 0.3	21.7 $\pm$ 5.3*
	5	2.0 $\pm$ 0.3	26.5 $\pm$ 2.8*
	15	3.3 $\pm$ 0.5	14.0 $\pm$ 2.8*
+ Flurbiprofen (8 μ M; n = 7)	- 60	-	6.1 $\pm$ 1.4
	0	1.4 $\pm$ 0.3	3.0 $\pm$ 0.3
	0.5	0.2 $\pm$ 0.1	7.3 $\pm$ 1.6
	1	0.6 $\pm$ 0.1	10.6 $\pm$ 0.9*
	2	0.8 $\pm$ 0.2	8.9 $\pm$ 1.6
	5	1.5 $\pm$ 0.4	10.2 $\pm$ 3.9*
	15	2.1 $\pm$ 0.4	11.0 $\pm$ 3.8*
+ Dazmegrel (10 μ M; n = 5)	- 60	-	2.3 $\pm$ 0.1
	0	1.2 $\pm$ 0.2	2.6 $\pm$ 0.3
	0.5	0.8 $\pm$ 0.1	4.9 $\pm$ 2.1
	1	0.9 $\pm$ 0.2	6.3 $\pm$ 1.4*
	2	1.7 $\pm$ 0.5	10.9 $\pm$ 0.7*
	5	2.3 $\pm$ 0.1	13.6 $\pm$ 0.7*
	15	2.8 $\pm$ 0.5	10.6 $\pm$ 2.4*
+ EP 092 (10 μ M; n = 5)	- 60	-	4.1 $\pm$ 0.7
	0	0.2 $\pm$ 0.1	3.5 $\pm$ 0.6
	0.5	0.7 $\pm$ 0.1	6.8 $\pm$ 1.1
	1	1.2 $\pm$ 0.4	11.7 $\pm$ 1.0*
	2	1.9 $\pm$ 0.2	14.7 $\pm$ 3.4*
	5	2.4 $\pm$ 0.4	19.1 $\pm$ 5.7*
	15	2.8 $\pm$ 0.5	17.4 $\pm$ 4.4*

Strips of guinea-pig lung were set up for isometric tension recording as described in Methods. After the initial equilibration period tissues were incubated for 60 min with either vehicle, flurbiprofen (8 μ M), dazmegrel (10 μ M) or EP 092 (10 μ M) before being challenged with LTD₄ (200 nM). At the time points indicated changes in tension were measured and tissues were snap frozen in liquid nitrogen for subsequent cyclic GMP determinations. Data represent the mean $\pm$ s.e.mean of *n* independent determinations indicated in parentheses.

* $P < 0.05$ with respect to '0 min' control values (one-way ANOVA/Newman-Keuls test).

Figure 5 Concentration-dependent activation of PKA in guinea-pig isolated lung parenchyma by leukotriene D₄ (LTD₄). Lung strips were pretreated for 60 min with 1 μ M ICI 198,615 (○) or its vehicle (●) and challenged non-cumulatively with LTD₄ (1–1000 nM). After 5 min drug contact, tissues were frozen rapidly by submersion in liquid nitrogen and PKA activity in the soluble fraction of lung homogenates measured as described previously (Giembycz & Diamond, 1990b). The bar indicates A-kinase activity in the absence of LTD₄ while the figures at each point refer to the percentage A-kinase present in an active form. Data represent the mean $\pm$ s.e.mean of six independent determinations measured in duplicate.

Figure 6 Kinetics of leukotriene D₄ (LTD₄)-induced PKA activation in guinea-pig isolated lung parenchyma and the effect of flurbiprofen. Lung strips were pretreated for 60 min with flurbiprofen (○) or its vehicle (●) and then challenged with LTD₄ (200 nM). At pre-determined times, indicated in the figure, tissues were frozen rapidly by submersion in liquid nitrogen and A-kinase activity in the soluble fraction of lung homogenates measured as described previously (Giembycz & Diamond, 1990b). Data represent the mean $\pm$ s.e.mean of six determinations at each time-point. * $P < 0.05$, significantly different from resting PKA activity ratio as assessed by one-way ANOVA/Newman-Keuls test.

equivalent to PKA being $\sim 26\%$ active (i.e. present as free catalytic subunits) under resting conditions. Incubation of guinea-pig lung for 5 min with LTD₄ (1 nM to 1 μ M) produced a concentration-dependent stimulation of PKA with a K_{act} of 32.6 ± 6.4 nM ($n = 6$) and maximum specific activity of 4.02 ± 0.31 nmol min⁻¹ mg⁻¹ protein ($n = 6$). Under these

conditions LTD₄ (1 μ M for 5 min) more than doubled (to $61 \pm 1.8\%$, $n = 6$) the proportion of PKA present in an active state (Figure 5). Kinetically, the profile of LTD₄ (200 nM)-induced PKA activation paralleled the changes in the cyclic AMP content described above peaking after 5 min contact then returning towards control activity (Figure 6). Moreover, no significant activation of PKA was detected at 30 s post-LTD₄ challenge despite a detectable increase in lung parenchymal tone (cf. Figure 4 with Figure 6).

Pre-incubation of guinea-pig lung with ICI 198,615 (1 μ M for 60 min) abolished the stimulation of PKA induced by LTD₄ (100 nM and 1 μ M) confirming that this effect of LTD₄ was receptor-mediated (Figure 5).

It is important to note that the observed activation of PKA was the result of an increase in the concentration of free (active) catalytic subunits since total cyclic AMP-dependent phosphotransferase activity was unchanged (data not shown).

Effect of inhibition of cyclo-oxygenase on the mechanical and biochemical actions of LTD₄

It is well documented that LTD₄ promotes the *de novo* biosynthesis and release of arachidonic acid-derived cyclo-oxygenase products in guinea-lung (e.g. Weichman *et al.*, 1982; Robinson *et al.*, 1984; Mong *et al.*, 1985a,b; Giembycz & Rodger, 1987). Since these eicosanoids exert marked biochemical and mechanical effects, themselves, their evoked release could mask effectively the direct actions of LTD₄. To examine the cyclo-oxygenase-independent actions of LTD₄, therefore, guinea-pig lung was pre-incubated (60 min) with the cyclo-oxygenase inhibitor, flurbiprofen (Rome & Lands, 1975), at a concentration (8 μ M), which abolished both the spontaneous and LTD₄ (200 nM)-stimulated release of TXB₂ and 6-keto-PGF_{1 α} (Figure 3).

Addition of flurbiprofen to guinea-pig lung strips resulted in a prompt reduction of spontaneous tone and an associated 47% decrease in the tissue cyclic AMP content (Figure 7a). Under these conditions, LTD₄ (200 nM) elicited contractures that were slower in onset and slightly less in magnitude than those seen in control tissues (cf. Figure 4 with Figure 7a) an observation consistent with the fact that LTD₄-induced tone is dependent, in part, upon the release of cyclo-oxygenase products (e.g. Weichman *et al.*, 1982; Giembycz & Rodger, 1987). Moreover, flurbiprofen abolished the ability of LTD₄ to elevate the cyclic AMP content at any time-point, and also suppressed (by ~68%) the increase in cyclic GMP (Table 1).

The effect of flurbiprofen on basal and LTD₄-stimulated PKA activity is shown in Figure 6. In agreement with the cyclic AMP results described above, flurbiprofen reduced the PKA activity ratio from ~29% at rest to 17% after 60 min pre-incubation with the cyclo-oxygenase inhibitor. Subsequent challenge of guinea-pig lung with LTD₄ (200 nM) neither increased nor decreased further the PKA activity ratio over the 15 min post-challenge period studied (Figure 6).

It is clear from the results described above that the effect of LTD₄ on cyclic AMP levels and PKA activity do not corroborate the observations of either Andersson and co-workers (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b) and Barnes & Grandordy (1986). In an attempt to explain this discrepancy it was reasoned that LTD₄ may only be able to reduce the cyclic AMP content and lower the PKA activity ratio in flurbiprofen-treated tissue in the presence of an activator of adenylyl cyclase. To examine this possibility, the ability of LTD₄ to inhibit isoprenaline-stimulated cyclic AMP accumulation and PKA activity was evaluated. Consistent with our previous report (Giembycz & Diamond, 1990b), isoprenaline (10 μ M for 10 min) elevated significantly the cyclic AMP content (from 3.9 ± 0.9 to 26.7 ± 5.1 pmol mg⁻¹ protein, $n = 4$) and almost doubled the PKA activity ratio (from $14.6 \pm 2.9\%$ to $26.3 \pm 5.5\%$, $n = 5$). The addition of LTD₄ (1 μ M) to lung strips either 30 s or

Figure 7 Kinetics of leukotriene D₄ (LTD₄)-induced tension generation and cyclic AMP accumulation in guinea-pig isolated lung parenchyma pretreated for 60 min with flurbiprofen (8 μ M; a), dazmegrel (10 μ M; b) and EP 092 (1 μ M; c). See legend to Figure 4 and text for further details. Data represent the mean $\pm$ s.e. mean of seven, five and five independent observations at each time-point for flurbiprofen-, dazmegrel- and EP 092-pretreated tissues respectively. * $P < 0.05$, significantly different from resting cyclic AMP levels as assessed by one-way ANOVA/Newman-Keuls test.

5 min prior to isoprenaline, however, failed to inhibit cyclic AMP accumulation or PKA activation (data not shown).

Effect of inhibition of thromboxane synthetase and of TP-receptor blockade on the mechanical and biochemical actions of LTD₄

The results obtained from the experiments with flurbiprofen, described above, suggested that LTD₄-induced cyclic nucleotide accumulation and PKA activation were dependent upon the generation of a prostaglandin(s) and/or TXA₂. A number of studies were, therefore, performed to gain further information on the identity of the cyclo-oxygenase product(s) responsible for these effects.

Pretreatment of guinea-pig lung with dazmegrel (10 μ M for 60 min), a selective inhibitor of thromboxane synthetase (Parry *et al.*, 1982) relaxed spontaneous tone of the tissue but, unlike flurbiprofen, did not affect either the basal cyclic

nucleotide content (Table 1; Figure 7b) or the PKA activity ratio (data not shown). LTD₄-induced tension development was similarly unaffected in dazmegrel-pretreated lung strips. In contrast, dazmegrel attenuated (by ~50%) the ability of LTD₄ to promote the accumulation of cyclic GMP, whereas the increase in the cyclic AMP content 5 min after challenge with LTD₄ was potentiated approximately two fold (Table 1; Figure 7b). Similarly, dazmegrel potentiated LTD₄-induced PKA activation (from $31.3 \pm 3.4\%$ to $74.1 \pm 7.9\%$ 5 min post LTD₄ challenge, $n = 5$).

To ensure that dazmegrel had, indeed, inhibited thromboxane synthetase in guinea-pig lung, the concentration of immunoreactive TXB₂ and 6-keto-PGF_{1 α} was measured in the bathing buffer immediately before and after challenge of tissues with LTD₄ (200 nM). As shown in Figure 3, dazmegrel (10 μ M for 60 min) reduced, to levels achieved spontaneously, the elaboration of TXB₂ from the lung but augmented (~2.5-fold) the LTD₄-stimulated release of 6-keto-PGF_{1 α} (Figure 3b).

Pretreatment of guinea-pig lung with EP 092 (1 μ M for 60 min), a potent and selective TP-receptor antagonist (Jones *et al.*, 1984), had no effect on spontaneous tone, the elaboration of TXB₂ and 6-keto-PGF_{1 α} or on the basal level of cyclic GMP (Table 1; Figures 3 and 7c). Similarly, LTD₄-induced tension generation was unaffected by EP 092 although there was a small potentiation of LTD₄-induced cyclic AMP accumulation at all time-points where measurements were made except at 2 min (Figure 7c).

Discussion

The results of these studies demonstrate that under equilibrium conditions LTD₄-induced tension development of guinea-pig isolated lung parenchyma is associated with, but divorced temporally from, a cyclo-oxygenase-dependent increase in the cyclic AMP content and in the activation state of PKA. Moreover, under conditions where the elaboration of cyclo-oxygenase products is prevented, LTD₄ affects neither of these biochemical parameters irrespective of whether the experiment is performed in the absence or presence of the β -adrenoceptor agonist, isoprenaline. It is clear, therefore, that the results obtained herein with LTD₄ in flurbiprofen-pretreated tissues contradict data published previously where LTC₄, LTD₄ and immunologically-generated SRS-A are reported to reduce rapidly the intracellular cyclic AMP content in guinea-pig tracheae and lung parenchyma (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b; Barnes & Grandordy, 1986) and, in the case of SRS-A, lower the PKA activity ratio (Hedman & Andersson, 1982b). These data, therefore, do not support the suggestion that receptors for the peptido-leukotrienes can couple directly to adenylyl cyclase in guinea-pig lung and thus argue against the assertion that a reduction in intracellular cyclic AMP is related to LTD₄-induced tension generation in these tissues (Andersson *et al.*, 1982; 1983; Hedman & Andersson, 1982a,b; Barnes & Grandordy, 1986). This conclusion is supported by preliminary data from Langlands *et al.* (1987), who also failed to detect any rapid decrease in the cyclic AMP content and PKA activity ratio in guinea-pig tracheae challenged with LTD₄. Moreover, since submission of this manuscript, Howard *et al.* (1992) reported that LTD₄ produced no demonstrable effect on the cyclic AMP content of guinea-pig cultured tracheal smooth muscle cells either in the absence or presence of the adenylyl cyclase activator forskolin.

It was reasoned that one explanation for the inability of LTD₄ to lower the cyclic AMP content in flurbiprofen-pretreated lung may relate to a low basal adenylyl cyclase activity such that inhibition of this enzyme would not result in a detectable decrement in the cyclic AMP content when whole cell cyclic nucleotide measurements were made. This reasoning was seemingly incorrect since LTD₄ failed also to

attenuate isoprenaline-induced cyclic AMP accumulation and PKA activation in this tissue.

Another explanation given consideration was derived from the knowledge that distinct receptors for LTC₄ and LTD₄ are expressed by guinea-pig lung (e.g. Hogaboam *et al.*, 1983; Mong *et al.*, 1985a) and trachea (e.g. Krell *et al.*, 1983; Weichman & Tucker, 1985). Conceivably a reduction in cyclic AMP levels may be affected only following activation of the LTC₄ receptor. Although a tenable hypothesis, this is unlikely (unless the LTC₄ and LTD₄ receptors in the lung are different from those expressed by guinea-pig trachea) since both LTC₄ and LTD₄ reduce rapidly the cyclic AMP content in guinea-pig tracheae (Andersson *et al.*, 1982, 1983). At the present time, therefore, we can provide no satisfactory explanation for the discrepancy between the data presented in this study and results reported previously (see above).

The results obtained with flurbiprofen indicate that the LTD₄-stimulated increase in cyclic AMP mass and in the activity of PKA is mediated indirectly via the *de novo* biosynthesis and subsequent release of one or more prostanoids catalysed by the cyclo-oxygenation of arachidonic acid. The observation that pretreatment of lung tissue with the TP-receptor blocking drug, EP 092, did not inhibit the increase in cyclic AMP induced by LTD₄ suggests that TXA₂ does not mediate these biochemical effects. This conclusion is corroborated by the results obtained with the thromboxane synthetase inhibitor, dazmegrel, which enhanced (rather than inhibited) LTD₄-induced cyclic AMP accumulation and activation of PKA. These data thus suggest that pretreatment of lung with dazmegrel leads to the accumulation and subsequent diversion of the prostaglandin endoperoxide intermediates to the synthesis of cyclic AMP-generating cyclo-oxygenase products in response to LTD₄. Further support for this proposal was the finding that dazmegrel augmented more than two fold the ability of LTD₄ to release the prostacyclin metabolite, 6-keto-PGF_{1 α} , from the lung.

Despite the fact that LTD₄ enhanced the generation of prostacyclin (this study), and prostaglandins E₂ and F_{2 α} from guinea-pig lung (Robinson *et al.*, 1984), the identity of the cyclic AMP-generating prostanoid(s) released is not clear and was outside the objectives of this study. However, the finding of Gardiner (1986) that butaprost, a highly selective EP₂-receptor agonist (which, unlike the naturally occurring prostaglandins, does not evoke TP-receptor-mediated contraction of guinea-pig lung) inhibits LTD₄-induced tension generation in this tissue indicates that this response may be mediated, at least in part, by prostaglandins which stimulate inhibitory EP₂-receptors which are reported to be positively coupled to adenylyl cyclase (Jumblatt & Paterson, 1991; Nakahata, 1992). It is noteworthy, however, that since lung parenchyma is a mixture of more than 40 different cell types (Stahlman & Gray, 1978) other adenylyl cyclase-coupled prostanoid receptors on non-contractile tissues may contribute significantly to the cyclic AMP-elevating action of LTD₄.

In addition to increasing the concentration of cyclic AMP in guinea-pig lung, LTD₄ also elevated the cyclic GMP content in a concentration-dependent manner which is consistent with the fact that many smooth muscle spasmogens promote cyclic GMP accumulation (Kuo & Kuo, 1973; Diamond, 1978). The precise biochemical events which culminate in an increase in cyclic GMP mass are uncertain though in many tissues an elevation in the cytosolic free Ca²⁺ concentration will stimulate soluble guanylyl cyclase (Schultz *et al.*, 1973; Spies *et al.*, 1980). Furthermore, a variety of long chain poly-unsaturated fatty acids, including arachidonate, can stimulate cyclic GMP biosynthesis (Gruetter & Ignarro, 1979; Lewicki *et al.*, 1983; Gerzer *et al.*, 1983) which has led to the proposal that arachidonic acid or one of its metabolites can function as an endogenous regulator of soluble guanylyl cyclase (e.g. Gerzer *et al.*, 1983). The results reported herein are consistent with this latter hypothesis given the observation that the elevation in cyclic GMP seen in response to LTD₄ was attenuated significantly in tissues pretreated with

the cyclo-oxygenase inhibitor, flurbiprofen. This result is comparable to the study of Gruetter *et al.* (1983) who reported that the activation of soluble guanylyl cyclase isolated from guinea-pig lung by arachidonate was dependent upon active cyclo-oxygenase.

Although the identity of the lipid species responsible for stimulating cyclic GMP biosynthesis is unknown, the observation that dazmegrel attenuated the LTD₄-induced increase in this cyclic nucleotide to a similar degree as flurbiprofen suggests that TXA₂ or one of its metabolites is a principal regulator of soluble guanylyl cyclase in guinea-pig lung. Further support for this idea derives from the finding that the TP-receptor antagonist, EP 092, which did not affect the cyclo-oxygenation of arachidonate as assessed by the elaboration of TXB₂, exerted little effect on the time course or magnitude of LTD₄-induced cyclic GMP accumulation.

In conclusion, the results presented herein do not confirm previous studies which document a cyclic AMP lowering

action of peptido-LT's on guinea-pig tracheae and lung parenchyma. As such, these data do not support the hypothesis that LTD₄-induced tension generation of these tissues requires a decrement in the cyclic AMP content.

M.A.G. was supported by the National Asthma Campaign (formerly the Asthma Research Council) and by the Canadian Heart Foundation and was a recipient of a Wellcome Foundation Travel Grant. Financial support was provided by the Wellcome Trust, the Smith-Kline Foundation, the British Columbia Heart Foundation and the British Columbia Health Care Research Foundation. We would like to thank Pfizer Central Research, Sandwich, Kent, Dr R.L. Jones, Department of Pharmacology, University of Edinburgh, and ICI Americas Inc, Wilmington, Delaware, U.S.A. for the gifts of dazmegrel, EP 092 and ICI 198,615 respectively. The generous supply of LTD₄ from Merck Frosst, Point-Claire, Dorval, Quebec, Canada and Bayer U.K., Ltd., Stoke-Poges, Slough is gratefully acknowledged.

References

- ANDERSSON, R.G.G., GUSTAFSSON, L.E., HEDMAN, S.E., HEDQVIST, P. & SAMUELSSON, B. (1982). Leukotriene C₄-induced cyclic nucleotide changes and contractile responses in guinea-pig trachea. *Acta Physiol Scand.*, **116**, 97–99.
- ANDERSSON, R.G.G., HEDMAN, S.E. & HULTGREN, A. (1983). Leukotrienes, leukotriene-receptive substances and the cyclic AMP system of guinea-pig airways. *Adv. Cyclic Nucl. Protein. Phosphorylation. Res.*, **17A**, 117.
- BARNES, P.J. & GRANDORDY, B. (1986). Leukotriene C₄ elicits inositol phosphate formation and inhibits adenylate cyclase in guinea-pig lung. *Br. J. Pharmacol.*, **89**, 783P.
- BEEBE, S.J. & CORBIN, J.D. (1986). Cyclic nucleotide-dependent protein kinases. *The Enzymes*, **17**, 43–111.
- BRUNS, R., THOMSEN, W.J. & PUGSLEY, T.A. (1983). Binding of leukotriene C₄ and D₄ to membranes from guinea-pig lung: regulation by ions and nucleotides. *Life Sci.*, **33**, 645–653.
- CHENG, J.B., LANG, D., BENTRA, A. & TOWNLEY, R.G. (1985). Tissue distribution and functional correlation of [³H] leukotriene C₄ and [³H] leukotriene D₄ binding sites in guinea-pig uterus and lung preparations. *J. Pharmacol. Exp. Ther.*, **232**, 80–87.
- CHENG, J.B. & TOWNLEY, R.G. (1984). Identification of leukotriene D₄ receptor binding sites in guinea-pig lung homogenates using [³H] leukotriene D₄. *Biochem. Biophys. Res. Commun.*, **118**, 20–26.
- CHERRINGTON, A.D., ASSIMACOPOULOS, F.D., HARPER, S.C., CORBIN, J.D., PARK, C.R. & EXTON, J.H. (1976). Studies on the α -adrenergic activation of hepatic glucose output. II. Investigation of the roles of adenosine cyclic 3',5'-monophosphate and guanosine cyclic 3',5'-monophosphate-dependent protein kinase in the actions of phenylephrine in isolated hepatocytes. *J. Biol. Chem.*, **251**, 5209–5218.
- DAHLEN, S.-E., HEDQVIST, P., WESTLUND, P., GRANDSTROM, E., HAMMARSTROM, S., LINDGREN, J.A. & RADMARK, O. (1983). Mechanisms of leukotriene-induced contractions of guinea-pig airways: leukotriene C₄ has a potent direct action whereas leukotriene B₄ acts indirectly. *Acta Physiol. Scand.*, **118**, 393–403.
- DIAMOND, J. (1978). Role of cyclic nucleotides in control of smooth muscle contraction. *Adv. Cyclic Nucl. Res.*, **9**, 327–340.
- GARDINER, P.J. (1986). Characterization of prostanoid relaxant/inhibitory receptors (ψ) using a highly selective agonist, TR4979. *Br. J. Pharmacol.*, **87**, 45–56.
- GERZER, R., HAMET, P., ROSS, A.H., LAWSON, J.A. & HARDMAN, J.G. (1983). Calcium-induced release from platelets of fatty acids that modulate soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **226**, 180–186.
- GIEMBYCZ, M.A. & DIAMOND, J. (1990a). Characterisation of cyclic AMP-dependent protein kinases in guinea-pig lung employing the novel synthetic substrate, Kemptide: In vitro sensitivity of the soluble enzyme to isoprenaline, forskolin, methacholine and leukotriene D₄. *Biochem. Pharmacol.*, **39**, 1297–1312.
- GIEMBYCZ, M.A. & DIAMOND, J. (1990b). Evaluation of Kemptide, a serine-containing heptapeptide, for the estimation of cyclic AMP-dependent protein kinase activity in respiratory tissues. *Biochem. Pharmacol.*, **39**, 271–283.
- GIEMBYCZ, M.A. & RAEBURN, D. (1991). Putative substrates for cyclic nucleotide-dependent protein kinases and the control of airway smooth muscle tone. *J. Autonomic Pharmacol.*, **11**, 365–398.
- GIEMBYCZ, M.A. & RODGER, I.W. (1987). Does thromboxane mediate the indirect contractile action of leukotriene D₄ in guinea-pig isolated lung parenchyma under equilibrium conditions? *Can. J. Physiol. Pharmacol.*, **65**, 1467–1477.
- GRUETTER, D.Y. & IGNARRO, L.J. (1983). Arachidonic acid activation of guinea-pig lung guanylate cyclase by two independent mechanisms. *Prostaglandins*, **18**, 541–556.
- HALL, I.P. & CHILVERS, E.R. (1989). Inositol phosphates and airway smooth muscle. *Pulm. Pharmacol.*, **2**, 113–120.
- HEDMAN, S.E. & ANDERSSON, R.G.G. (1982a). Effect of SRS (slow reacting substance) on cyclic nucleotides in guinea-pig tracheal smooth muscle. *Acta Pharmacol. Toxicol.*, **50**, 30–34.
- HEDMAN, S.E. & ANDERSSON, R.G.G. (1982b). The cyclic AMP system in sensitized and desensitized guinea-pig tracheal smooth muscle. *Eur. J. Pharmacol.*, **83**, 107–112.
- HOGABOOM, G.K., MONG, S., WU, H.-L. & CROOKE, S.T. (1983). Peptidoleukotrienes: distinct receptors of leukotriene C₄ and D₄ in the guinea-pig lung. *Biochem. Biophys. Res. Commun.*, **116**, 1136–1143.
- HOWARD, S., CHAN-YEUNG, M., MARTIN, L., PHANEUF, S. & SALARI, H. (1992). Polyphosphoinositide hydrolysis and protein kinase C activation in guinea-pig tracheal smooth muscle cells in culture by leukotriene D₄ involve a pertussis toxin sensitive G-protein. *Eur. J. Pharmacol. – Mol. Pharmacol. Section.*, **227**, 123–129.
- JAKOBS, K.H., AKTORIES, K. & SCHULTZ, G. (1984). Mechanisms and components involved in adenylate cyclase inhibition by hormones. *Adv. Cyclic Nucl. Protein. Phosphorylation. Res.*, **17**, 135–143.
- JONES, C.A., MADISON, J.M., TOM-MOY, M. & BROWN, J.K. (1987). Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. *Am. J. Physiol.*, **253**, C97–C104.
- JONES, R.L., SMITH, G.M. & WILSON, N.H. (1984). The effect of synthetic prostanoid EP 092 on intravascular platelet aggregation and bronchoconstriction. *Br. J. Pharmacol.*, **81**, 100P.
- JUMBLATT, M.M. & PATERSON, C.A. (1991). Prostaglandin E₂ effects on corneal cyclic adenosine monophosphate synthesis and cell shape change are mediated by a receptor of the EP₂ subtype. *Invest. Ophthalmol. Visual. Sci.*, **32**, 360–365.
- KRELL, R.D., TSAI, B.S., BERDOULAY, A., BARONE, M. & GILES, R.E. (1983). Heterogeneity of leukotriene receptors in guinea-pig trachea. *Prostaglandins*, **25**, 171–178.
- KUO, J.F. & KUO, W.-N. (1973). Regulation by β -adrenergic receptor and muscarinic cholinergic receptor activation of intracellular cyclic AMP and cyclic GMP levels in rat lung slices. *Biochem. Biophys. Res. Commun.*, **55**, 660–665.
- LANGLANDS, J.M. & RODGER, I.W. (1992). The effect of methacholine and histamine on cyclic AMP-dependent protein kinase activity in the guinea-pig isolated trachea. *Can. J. Physiol. Pharmacol.*, **70**, 344–348.

- LANGLANDS, J.M., RODGER, I.W. & WATSON, J. (1987). Effect of methacholine and leukotriene D₄ on cyclic AMP content and cyclic AMP-dependent protein kinase activity in airway smooth muscle. *Br. J. Pharmacol.*, **92**, 633P.
- LEWICKI, J.A., WALDMAN, S.A., CHANG, B. & MURAD, F. (1983). Membrane-dependent regulation of soluble guanylate cyclase by snake venom phospholipase A₂. *Fed. Proc.*, **42**, 902.
- LEWIS, R.A., AUSTEN, K.F., DRAZEN, J.M., CLARCK, D.M., MAREFAT, A. & COREY, E.J. (1980). Slow reacting substance of anaphylaxis: identification of leukotriene C-1 and D from human rat sources. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 3710–3714.
- LOWRY, H.O., ROSEBROUGH, M.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin-phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- MONG, S., CHI-ROSSO, G., HAY, D.W.P. & CROOKE, S.T. (1988). Subcellular localization of leukotriene D₄ receptors in sheep tracheal smooth muscle. *Mol. Pharmacol.*, **34**, 590–596.
- MONG, S., MILLER, J., WU, H.-L. & CROOKE, S.T. (1987). Leukotriene D₄ receptor-mediated hydrolysis of phosphoinositide and mobilization of calcium in sheep tracheal smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **244**, 508–515.
- MONG, S., WU, H.-L., CLARK, M.A., GLEASON, J.G. & CROOKE, S.T. (1986). Leukotriene D₄ receptor-mediated synthesis and release of arachidonic acid metabolites in guinea-pig lung: induction of thromboxane and prostacyclin biosynthesis by leukotriene D₄. *J. Pharmacol. Exp. Ther.*, **239**, 63–70.
- MONG, S., WU, H.-L., HOGABOOM, K.H., CLARK, M.A. & CROOKE, S.T. (1984). Characterization of the leukotriene D₄ receptor in guinea-pig lung. *Eur. J. Pharmacol.*, **102**, 1–11.
- MONG, S., WU, H.-L., HOGABOOM, K.G., CLARK, M.A., STADEL, J.M. & CROOKE, S.T. (1985b). Regulation of ligand binding to leukotriene D₄ receptors: effects of cations and guanine nucleotides. *Eur. J. Pharmacol.*, **106**, 241–253.
- MONG, S., WU, H.-L., MILLER, J., HALL, R.F., GLEASON, J.G. & CROOKE, S.T. (1988). SKF 104353, a high affinity antagonist for human and guinea-pig lung leukotriene D₄ receptor, blocked phosphatidylinositol metabolism and thromboxane synthesis induced by leukotriene D₄. *Mol. Pharmacol.*, **32**, 223–229.
- MONG, S., WU, H.-L., SCOTT, M.O., LEWIS, M.A., CLARK, M.A., WEICHMAN, B.M., KINZIG, C.M., GLEASON, J.G. & CROOKE, S.T. (1985a). Molecular heterogeneity of leukotriene receptors: correlation of smooth muscle contraction and radioligand binding in guinea-pig lung. *J. Pharmacol. Exp. Ther.*, **234**, 330–335.
- NAKAHATA, N. (1992). Signal transduction of prostaglandin E₂ and thromboxane A₂. *Folia Pharmacol. Jap.*, **99**, 1–11.
- PARRY, M.J., RANDALL, M.J., HAWKSWOOD, E., CROSS, P.E. & DICKINSON, R.P. (1982). Enhanced production of prostacyclin in blood after treatment with selective thromboxane synthetase inhibitor, UK-38,485. *Br. J. Pharmacol.*, **77**, 547P.
- PONG, S.S. & DE HAVEN, R.N. (1983). Characterization of a leukotriene D₄ receptor in guinea-pig lung. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 7415–7420.
- ROBINSON, C., HOULT, J.R.S., WADDELL, K.A., BLAIR, J.A. & DOLLERY, C.T. (1984). Total profiling by GC/NICIMS of the major cyclo-oxygenase products from antigen and leukotriene-challenged guinea-pig lung. *Biochem. Pharmacol.*, **33**, 395–400.
- ROME, L.H. & LANDS, W.E.M. (1975). Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory agents. *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4863–4865.
- SARAU, H.M., MONG, S., FOLEY, J.J., WU, H.-L. & CROOKE, S.T. (1987). Identification and characterization of leukotriene D₄ receptors and signal transduction processes in rat basophilic leukemia cells. *J. Biol. Chem.*, **262**, 4034–4041.
- SCHULTZ, G., HARDMAN, J.G., SCHULTZ, K., BAIRD, C.E. & SUTHERLAND, E.W. (1973). The importance of calcium ions for the regulation of guanosine 3',5'-monophosphate levels. *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 3889–3893.
- SNYDER, D.W., GILES, R.E., KEITH, R.A., YEE, Y.K. & KRELL, R.D. (1987). *In vitro* pharmacology of ICI 198,615: a novel, potent and selective leukotriene antagonist. *J. Pharmacol. Exp. Ther.*, **243**, 548–556.
- SPIES, C., SCHULTZ, K.-D. & SCHULTZ, G. (1980). Inhibitory effects of mepacrine and eicosatetraynoic acid on cyclic GMP elevations caused by calcium and hormonal factors in rat ductus deferens. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **311**, 71–77.
- STAHLMAN, M.T. & GRAY, M.E. (1978). Anatomical development and maturation of the lung. *Clin. Perinatol.*, **5**, 225–236.
- WEICHMAN, B.M., MUCCITELLI, R.M., OSBORNE, R.R., HOLDEN, D.A., GLEASON, J.G. & WASSERMAN, M.A. (1982). *In vitro* and *in vivo* mechanisms of leukotriene-mediated bronchoconstriction in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **222**, 202–208.
- WEICHMAN, B.M. & TUCKER, S.S. (1985). Differentiation of the mechanisms by which leukotrienes C₄ and D₄ elicit contraction of guinea-pig trachea. *Prostaglandins*, **29**, 694–699.

(Received July 25, 1992

Revised November 10, 1992

Accepted November 12, 1992)