British Journal of Pharmacology (1996) 118, 1397–1402

The induction of a biphasic bronchospasm by the ET_B agonist, IRL 1620, due to thromboxane A_2 generation and endothelin-1 release in guinea-pigs

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1 IRL 1620 (0.01–0.1 mg kg^{-1}, i.v.), a selective endothelin B (ET_B) receptor agonist, induced a dose-dependent biphasic increase in total lung resistance and a decrease in dynamic compliance in anaesthetized and artificially ventilated guinea-pigs. After intravenous injection of IRL 1620 (0.03 mg kg^{-1}), the first phase was observed within 2 min whereas the second phase started between 5 and 10 min after injection and was long lasting.

2 In order to characterize which endothelin receptors are involved in both phases of bronchoconstriction, we studied the effect of ET_A and ET_B receptor antagonists (BQ 123 and BQ 788, respectively). BQ 788 (0.1–1 mg kg^{-1}, i.v.) inhibited, in a dose-dependent manner, both phases of bronchoconstriction. BQ 123 (3 mg kg^{-1}, i.v.) markedly inhibited (by 76%) the second phase of bronchoconstriction but had no effect on the early component of the response.

3 The effect of atropine, neurokinin-1 (NK_1) and neurokinin-2 (NK_2) receptor antagonists (SR140333 and SR48968, respectively) were tested to investigate the possible involvement of cholinergic and sensory nerve activation, respectively, in the response to IRL 1620. Likewise, the role of arachidonic acid metabolites (leukotriene D_4 antagonist, ONO-1078 and thromboxane A_2 (TXA_2) inhibitor, OKY-046) in this response was also investigated. OKY-046 (1 mg kg^{-1}, i.v.) and atropine (1 mg kg^{-1}, i.v.) partially inhibited the first phase (by 80% and 20%, respectively) without affecting the late phase of bronchoconstriction. Neither ONO-1078 (1 mg kg^{-1}, i.v.) nor the combination of SR140333 (0.2 mg kg^{-1}, i.v.) and SR 48968 (0.2 mg kg^{-1}, i.v.) modified IRL 1620-induced bronchoconstriction.

4 A low dose of IRL 1620 (0.005 mg kg^{-1}, i.v.) induced a monophasic bronchoconstriction. Pretreatment by phosphoramidon (100 μmol kg^{-1}, i.v.) restored the second phase of bronchoconstriction. In this condition, BQ 123 (3 mg kg^{-1}, i.v.) was able to inhibit partially the second phase of bronchoconstriction.

5 These results suggest that both phases of bronchoconstriction induced by IRL 1620 were mediated primarily by ET_B receptor activation, the first phase being a consequence of TXA_2 and acetylcholine release. The inhibition by an ET_A receptor antagonist and the restoration by a neutral endopeptidase (NEP) inhibitor of the second phase of bronchoconstriction suggests that primary activation of ET_B receptors leads to autocrine/paracrine endothelin-1 (ET-1) release that would subsequently cause profound bronchoconstriction through both ET_A and ET_B receptor activation.

Keywords: IRL 1620; ET_B selective agonist; BQ 123; BQ 788; bronchoconstriction; thromboxane A_2 (TXA_2); autocrine release of ET-1; phosphoramidon

Introduction

Endothelin (ET) consists of 21 amino acids and contains two disulphide bridges (Yanagisawa *et al.*, 1988). Three different ET isoforms, ET-1, ET-2 (two amino acids substitution from ET-1) and ET-3 (six amino acids substitution from ET-1), which are encoded by three separate genes in man (Inoue *et al.*, 1989), have been identified. These ETs exert diverse biological responses which are thought to act primarily *via* the activation of two receptor subtypes (ET_A and ET_B). Since their discovery, many researchers have focused on the cardiovascular effects of ET presumably due to the fact that ET-1 originated from the endothelium and had an extremely potent vasoconstricting action in a variety of vasculature beds in different animal species. However, these peptides also have a wide array of effects in non-cardiovascular systems including the airways (Rubanyi & Polokoff, 1994; Rae *et al.*, 1995). Moreover, increased levels of ET-1-like immunoreactivity have been detected in bronchoalveolar lavage from asthmatic patients (Nomura *et al.*, 1989; Redington *et al.*, 1995) and increased ET-1-like immunostaining was detected within the airway epithelium in patients with atopic asthma (Springall *et al.*, 1991). Taken together, these observations suggest that ETs may be involved in the pathophysiology of pulmonary diseases such as asthma (Mattoli *et al.*, 1991; Barnes, 1994).

In vitro, ET-1 induced contraction of human bronchi (Advenier *et al.*, 1990; Henry *et al.*, 1990; Hay *et al.*, 1993b). The majority of ET receptors in human airway smooth muscle from asthmatic and non-asthmatic patients have been identified as ET_B receptor subtype (Knott *et al.*, 1995). *In vivo*, i.v. administration of ETs induces marked bronchoconstriction in guinea-pigs (Pons *et al.*, 1991a), which is not inhibited by ET_A receptor antagonists, BQ 123 (Noguchi *et al.*, 1993) and FR 139317 (Sogabe *et al.*, 1993), suggesting an ET_B receptor-mediated action. However, in view of the lack of selectivity of ETs for ET_A and ET_B receptor subtypes, it still remains unknown whether ETs mediated bronchoconstriction *via* ET_A and/or ET_B receptors. Moreover, a recent study suggests the involvement of both receptors in ET-1 induced bronchospasm in guinea-pigs (Nagase *et al.*, 1995).

In the present study, we have used the highly selective ET_B agonist, IRL 1620 (Takai *et al.*, 1992) to study the consequence

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of activation of this receptor on bronchoconstriction in guinea-pig airways. Moreover, we have used different pharmacological tools to explore the possible mediators involved in IRL 1620-induced bronchospasm.

Methods

Measurement of pulmonary functions

Hartley guinea-pigs (450–650 g, of either sex) were anaesthetized with 14% urethane (10 ml kg^{-1}. i.p.). The jugular vein, carotid artery and trachea were cannulated for drug administration, blood pressure monitoring and artificial ventilation, respectively. Animals were placed in a plethysmograph box and transpulmonary pressure (defined as the pressure difference between the box pressure and tracheal pressure) was measured with a differential pressure transducer (DP 45-28, Valydine Engineering). Airflow was monitored with a pneumotachometer (model A, Fleisch 0000) connected to a differential pressure transducer (DP 45-24, Valydine Engineering). Output signals representing transpulmonary pressure and airflow were amplified (MA6, Modular Instruments, UK) connected to an analog-digital converter. Airway resistance (R_L) and dynamic compliance C_{dyn} were calculated with Mi^2 Bio Report software (Modular Instruments). Pavulon (0.5 mg kg^{-1}, i.v.) was given to suppress spontaneous breathing.

Pharmacological characterization of bronchoconstriction

ET_A antagonist, BQ 123 and ET_B antagonist, BQ 788 (2 and 5 min before IRL 1620 injection, respectively), tachykinin antagonists (combination of NK_1 antagonist, SR 140333 and NK_2 antagonist, 30 min before IRL 1620 injection), atropine (15 min before IRL 1620), TXA_2 inhibitor, OKY-046 (15 min before IRL 1620) and leukotriene D_4 (LTD_4) antagonist, ONO-1078 (5 min before IRL 1620) were given intravenously. In another set of experiments, phosphoramidon (30 min before IRL 1620) and BQ 123 (2 min before IRL 1620) were given intravenously.

Changes in airway resistance and dynamic compliance were monitored for 30 min after IRL 1620 injection. To evaluate the effects of these drugs, first and second peak in airway resistance were measured as Max 1 and Max 2, respectively. Corresponding decreases in dynamic compliance were termed Min 1 and Min 2, respectively.

Materials

All drugs and peptides were made up fresh each day and stored on ice during the experiment. IRL 1620, (Suc-[Glu^9, $Ala^{11,15}$]endothelin-1-(8-21)), and BQ 788, (N-cis-2,6-dimethylpiperidinocarbonyl-L-gMeleu-D-Trp-(COOMe)-D-Nle-ONa), were synthesized in the International Research Laboratories (Ciba-Geigy Ltd., Takarazuka, Japan). ONO-1078, (4-oxo-8-[4-(phenylbutoxy)-benzoylamino]-2-(tetrazol-5-yl)-4H-1-benzopyran) hemihydrate, SR 140333, (S)1-{2-[3(3,4-dichlorophenyl)-1-(3-iso-propoxyphenlacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octone, hydrochloride, and SR 48968, (S)-N-methyl-N[4-(4-acetylamino-4-phenyl piperidino)-2-(3,4-dichlorophenyl)butyl]benzamide, were synthesized in house (Ciba-Geigy Ltd., Basel, Switzerland). BQ 123, (cycro(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu), was purchased from Bachem (Zurich, Switzerland).

OKY-046, (E)-3-[p-(1H-imidazol-l-ylmethyl)phenyl]-2-propenoic acid, was a kind gift from Kissei Pharmaceutical Co. Ltd. (Japan). Phosphoramidon was purchased from Peptide Institute Inc. (Osaka, Japan). All drugs except for OKY-046 and BQ 123 were dissolved in minimum volume of dimethylsulphoxide (DMSO) and diluted with cremophore-saline solution [2:18]. OKY-046 and BQ 123 were dissolved in saline.

Statistical analysis

Values are expressed as mean ± s.e.mean of 6 to 8 animals in each group. Statistical comparisons were performed by a one-way analysis of variance and a Dunnet test for multiple comparison or a bilateral Student's t test, when appropriate. In all cases, $P<0.05$ was considered significant.

Results

Effect of IRL 1620 on airway functions

Intravenous administration of IRL 1620 (0.03 mg kg^{-1}) induced a biphasic bronchoconstriction in anaesthetized guinea-pigs (Figure 1). The peak of the first phase (Max 1) was observed within 2 min after i.v. injection of the agonist and partially resolved in less than 3 min. The second phase of bronchoconstriction (Max 2) started between 5 and 10 min after injection. At a dose of 0.03 ml kg^{-1}, IRL 1620 induced a first phase of bronchoconstriction with R_L value of 0.85 ± 0.09 compared to baseline value of 0.21 ± 0.01 cmH_2O ml^{-1} s^{-1}. The corresponding C_{dyn} values (Min 1) were 0.14 ± 0.02 and 0.59 ± 0.02 ml cmH_2O^{-1}, respectively. The second phase of bronchoconstriction was higher (Max 2: $R_L = 1.85 \pm 10$ - cmH_2O ml^{-1} s^{-1} vs baseline; Min 2; $C_{dyn} = 0.08 \pm 0.00$ - ml cmH_2O^{-1} vs baseline, $n=6$) and sustained for at least 30 min. The effect of IRL 1620 was dose-dependent (0.01–0.1 mg kg^{-1}) for both phases of the bronchospasm (Figures 2a and b).

Pharmacological characterization of IRL 1620-induced bronchoconstriction

Firstly, we characterized the ET receptor involved in the biphasic bronchoconstriction. BQ 788, a selective ET_B receptor antagonist (0.01–1 mg kg^{-1}, i.v.) inhibited both phases of bronchoconstriction in a dose-dependent manner (Figure 3a). At 1 mg kg^{-1}, BQ 788 completely inhibited changes in airway resistance and dynamic compliance following IRL 1620 administration. BQ 123, a selective ET_A receptor antagonist (3 mg kg^{-1}, i.v.) markedly inhibited the second phase (by 76%) without affecting the first phase of contraction, suggesting the secondary involvement of ET_A receptors in the delayed bronchospasm (Figure 3b).

Secondly, we investigated the possible involvement of other mediators in the bronchoconstriction induced by IRL 1620. OKY-046 (1 mg kg^{-1}, i.v.) and atropine (1 mg kg^{-1}, i.v.) inhibited the first peak of bronchoconstriction by 80% and 20%, respectively, suggesting the involvement of TXA_2 and acetylcholine in the first phase of the response (Figure 4a and b). In contrast, these drugs had no effect on the second phase of the bronchospasm. Finally, a combination of tachykinin receptor antagonists (NK_1 antagonist, SR 140333, 0.2 mg kg^{-1} and

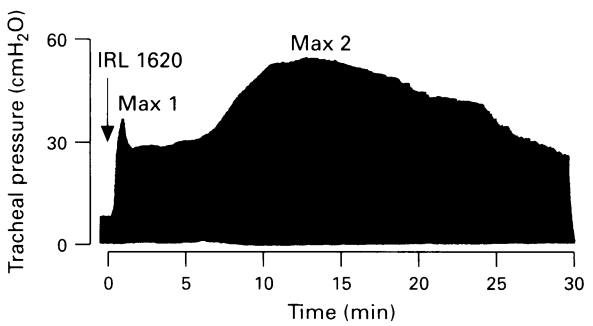

Figure 1 Typical tracing showing the biphasic increase in tracheal pressure (cmH_2O) produced by intravenous injection of the ET_B selective agonist, IRL 1620 (0.03 mg kg^{-1}), in anaesthetized guinea-pigs. The two peaks of bronchoconstriction were denoted Max 1 and Max 2.

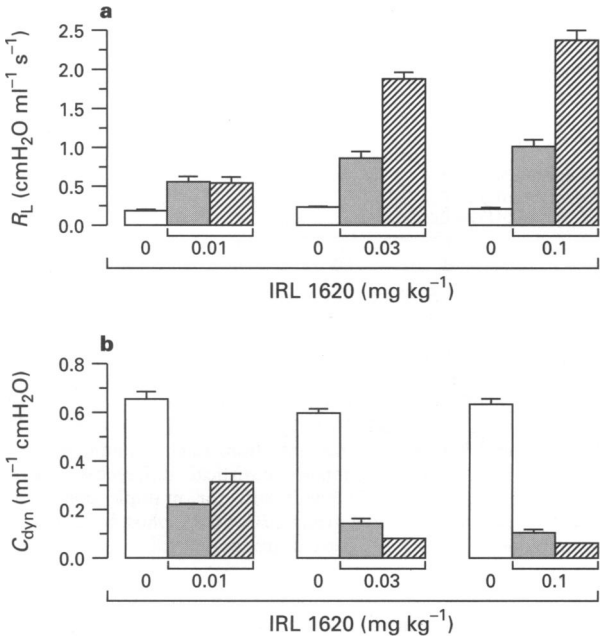

Figure 2 Effect of different doses of IRL 1620 on lung resistance (R_L; a) and dynamic compliance (C_{dyn}; b) in anaesthetized guinea-pigs. R_L was measured at Max 1 (stippled columns) and Max 2 (hatched columns). C_{dyn} was measured at Min 1 (stippled columns) and Min 2 (hatched columns). Baseline values for R_L and C_{dyn} (open columns) were measured immediately before IRL 1620 injection. Each column represents mean ± s.e.mean of 6–8 animals.

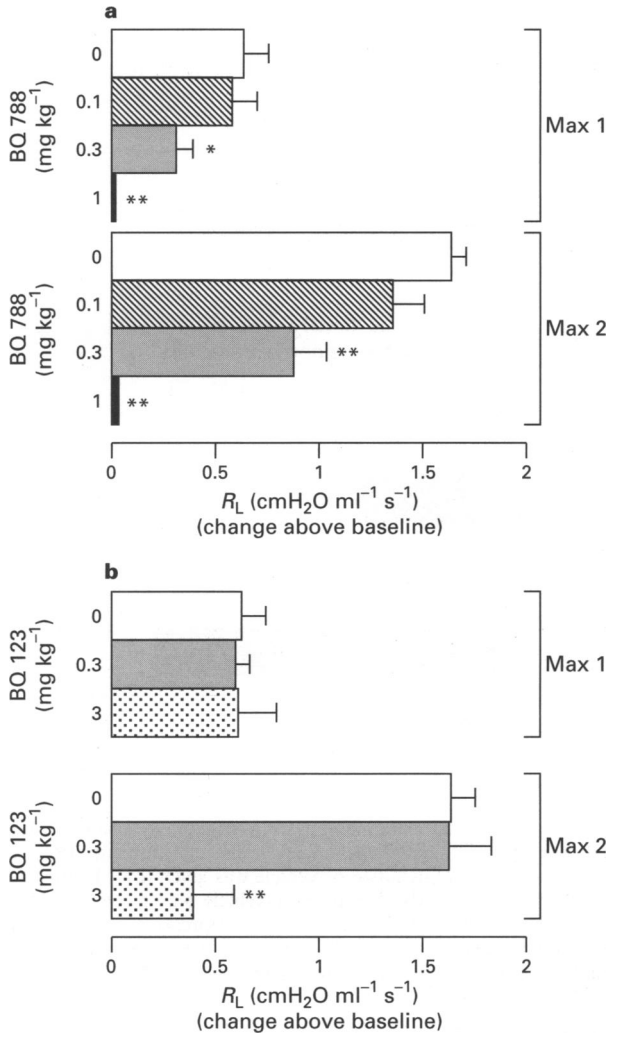

Figure 3 Effects of BQ123 and BQ 788 on R_L changes (Max 1 and Max 2) following the intravenous injection of IRL 1620 (0.03 mg kg^{-1}). (a) BQ 788 or vehicle was given (i.v.) 5 min before IRL 1620 injection. (b) BQ 123 or vehicle was given (i.v.) 2 min before IRL 1620 injection. Each column represents mean ± s.e.mean of 6–8 animals. Statistical differences between control and drug treated groups were analysed by Dunnet's test for multiple comparison (*$P<0.05$, **$P<0.01$).

NK_2 antagonist, SR 48968, 0.2 mg kg^{-1}) or LTD_4 antagonist (ONO-1078, 1 mg kg^{-1}) had no effect on either phase of bronchoconstriction induced by IRL 1620 (0.03 mg kg^{-1}). R_L values for Max 1 were 0.63 ± 0.03 after neurokinin antagonist pretreatment and 0.63 ± 0.02 after leukotriene antagonist pretreatment compared to control 0.64 ± 0.05 cmH$_2$O ml^{-1} s^{-1}. R_L values for Max 2 were 1.56 ± 0.12 after neurokinin antagonist pretreatment and 1.62 ± 0.08 after leukotriene antagonist pretreatment compared to control 1.64 ± 0.08 cmH$_2$O ml^{-1} s^{-1}.

Effect of phosphoramidon on IRL 1620 (low dose) induced bronchoconstriction

In another set of experiments, intravenous administration of IRL 1620 (0.005 mg kg^{-1}) induced a monophasic increase in R_L, having the same kinetic as the first peak of bronchoconstriction (Max 1) observed with higher doses of IRL 1620 (Figure 5). NEP has previously been shown to inactivate ET-1 enzymatically and inhibition of NEP by phosphoramidon results in a potentiation of ET-1-induced responses (Vijayaraghavan *et al.*, 1990; Tschirhart *et al.*, 1991). Pretreatment with phosphoramidon (100 μmol kg^{-1}, i.v.) for 30 min resulted in a biphasic bronchoconstriction in response to low dose IRL 1620. Moreover, under similar conditions, BQ 123 (3 mg kg^{-1}, i.v.) inhibited significantly (by 30%) the second phase of bronchoconstriction (Figure 5).

Discussion

In the present study, we have demonstrated that intravenous administration of IRL 1620, a selective ET_B agonist, results in a biphasic bronchoconstriction in guinea-pigs. The first phase was observed within 2 min after i.v. injection of the agonist. The second phase started between 5 and 10 min after injection of IRL 1620 and was long-lasting. The shape and the time-course of this response leads us to hypothesize that several components may be involved after an ET_B receptor stimulation as a first trigger.

To characterize the receptors and the possible mediators involved in the biphasic bronchoconstriction induced by IRL 1620, the effect of ET receptor antagonists (ET_A antagonist, BQ 123 and ET_B antagonist, BQ 788), muscarinic (atropine) and neurokinin antagonists (NK_1 and NK_2 antagonists, SR 140333 and SR 48968, respectively), arachidonic acid metabolite antagonist and inhibitor (LTD_4 antagonist, ONO-1078 and TXA_2 inhibitor, OKY-046) were investigated. BQ 788 completely inhibited both phases, indicating that the bronchoconstriction is mediated primarily by ET_B receptor activation. Then, the involvement of TXA_2 and acetylcholine (ACh) (first phase) and most probably ET-1, leading to ET_A receptor activation (second phase), were demonstrated.

The early phase of contraction is due mainly to indirect mechanisms following ET_B receptor activation. Firstly, our results with OKY-046 support previous observations by Noguchi and colleagues (1993), who have suggested that the bronchoconstrictor properties of ET-1 in guinea-pig *in vivo* are mediated predominantly by TXA_2 released following the activation of ET_B receptors. Furthermore, IRL 1620 has been shown to induce the release of TXA_2 from guinea-pig perfused lungs (D'Orleans-Juste *et al.*, 1994). Our observations that the first phase of bronchoconstriction was completely blocked by BQ 788 but not by BQ 123 further supports the predominant

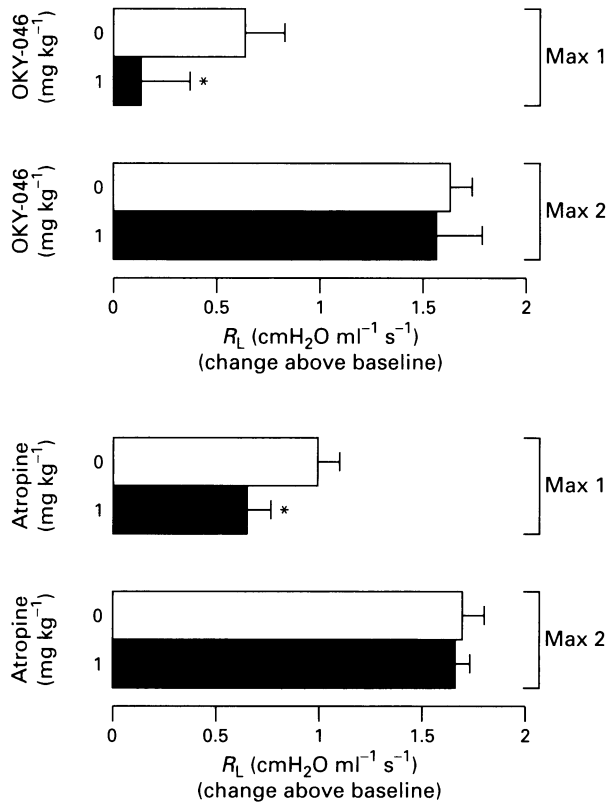

Figure 4 Effects of atropine and OKY-046 on R_L changes (Max 1 and Max 2) following the intravenous injection of IRL 1620 (0.03 mg kg^{-1}). (a) OKY-046 or vehicle was given (i.v.) 15 min before IRL 1620 injection. (b) Atropine or vehicle was given (i.v.) 15 min before IRL 1620 injection. Each column represents mean ± s.e.mean of 6–8 animals. Statistical differences between control and drug-treated groups were analysed by Student's *t* test (*$P<0.05$).

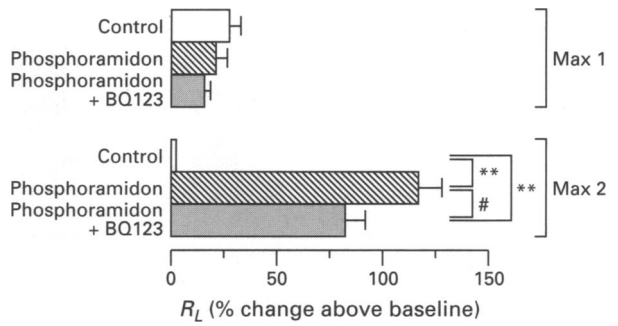

Figure 5 Effects of phosphoramidon with or without BQ 123 on R_L changes (Max 1 and Max 2) following the intravenous injection of IRL 1620 (0.005 mg kg^{-1}). Phosphoramidon (100 μmol kg^{-1}, i.v.) was given 30 min before IRL 1620 injection. BQ 123 (3 mg kg^{-1}, i.v.) was given 5 min before IRL 1620 injection. Each column represents mean ± s.e.mean of 6–8 animals. Statistical differences between groups were analysed by Dunnet's test for multiple comparison (**$P<0.01$, control vs treated groups; #$P<0.05$, phosphoramidon vs phosphoramidon + BQ 123-treated groups).

contribution of ET_B receptor activation in the release of TXA_2 *in vivo*. The cells involved in the ET_B receptor-mediated release of TXA_2 remain to be identified fully although vascular endothelial cells may be one possible cellular source (Pons *et al.*, 1991b). In addition, resident inflammatory cells may also participate significantly in this process, as leucocytes not only produce ET-1 (Ehrenreich *et al.*, 1990) but are also activated by this peptide to release several mediators including prostanoids (Ninomiya *et al.*, 1992).

Pretreatment of the animals with atropine also decreased the first phase of bronchoconstriction by 20%, suggesting the involvement of acetylcholine. This finding is consistent with recent results demonstrating that stimulation of ET_B receptors, presumably located at a prejunctional site, potentiates cholinergic nerve-mediated contractions in rabbit and mouse isolated airways (McKay *et al.*, 1993; Henry & Goldie, 1995). Furthermore, bronchoconstriction induced by ET-1 in guinea-pigs is modulated by hexamethonium and propranolol suggesting the involvement of the autonomic nervous system in this response (Macquin-Mavier *et al.*, 1989). Autoradiographical studies have detected ET receptors on the cell bodies, processes and varicosities of the autonomic parasympathetic intramural neurones in primary cultures of tracheal smooth muscle, and their stimulation leads to contraction of adjacent smooth muscle cells (Takimoto *et al.*, 1993). In contrast, neurokinin antagonists were inactive in our model confirming previous work showing that this treatment failed to inhibit ET-1-induced contraction of isolated guinea-pig trachea (Hay *et al.*, 1993a).

The second phase of bronchoconstriction is clearly due to the primary activation of the ET_B receptor subtype since pretreatment of the animals with BQ 788 inhibited the response. Then we postulated that ET_B receptor activation may induce, as a secondary event, ET-1 release. This hypothesis is supported by the demonstration that blockade of the ET_A receptors by BQ 123 markedly reduced the IRL 1620-induced late phase of bronchoconstriction without affecting the early phase at the same dose. Interestingly, the biphasic bronchoconstriction was observed only for a dose of IRL 1620 exceeding 0.01 mg kg^{-1}. A lower dose of the agonist, e.g. 0.005 mg kg^{-1}, induced a monophasic contraction. These results suggest that there is a threshold dose required to induce autocrine/paracrine ET-1 which is higher than the threshold dose needed to induce the first phase of bronchoconstriction. On the other hand, *in vivo*, the level of endogenous ET-1 measured in biological samples is thought to be the result of a balance between synthesis by endothelin converting enzyme (ECE) from big ET-1 and degradation of this peptide by neutral endopeptidase (NEP). Endogenous ET-1 is a good substrate for NEP (Vijayaraghavan *et al.*, 1990) and its inhibition results in the potentiation of the action of ET-1 (Boichot *et al.*, 1991; Tschirhart *et al.*, 1991). Therefore, in the present study, the effect of phosphoramidon, a potent NEP inhibitor with weak activity at inhibition of (ECE) was examined to clarify involvement of autocrine/paracrine ET-1 after ET_B receptor stimulation. Pretreatment with phosphoramidon (100 μmol kg^{-1}, i.v.) for 30 min induced a biphasic bronchoconstriction after injection of a low dose of IRL 1620 (0.005 mg kg^{-1}). Recently, it has been shown that the increase in mean arterial blood pressure induced by ET-1 is significantly inhibited by pretreatment of the rats with phosphoramidon, suggesting that, in their system, phosphoramidon is acting as an ECE inhibitor rather than a NEP inhibitor (Bird & Waldron, 1995). Although this hypothesis cannot be excluded in our model, the fact that we obtained a potentiation of the bronchoconstriction after phosphoramidon pretreatment at the same dose used by Bird and Waldron suggests the greater functional influence of this drug on NEP than on ECE. The sources of ET-1 could be other organs such as the kidney or local structures such as airway epithelial cells and/or endothelial cells from vessels of the pulmonary circulation. However, the presence of ET_B receptors on endothelial cells (Ogawa *et al.*, 1992) and the strong commitment of these cells to produce ET-1 suggests that the endothelium may be the main source of ET-1 following activation of the ET_B receptor by IRL 1620.

However, while spasmogenic prostanoid release following ET_A receptor activation has been demonstrated in human isolated bronchi (Hay *et al.*, 1993b), OKY-046 was inactive on the second phase of bronchoconstriction suggesting a direct effect of ET-1 on smooth muscle cells. In this regard, the presence of both ET_A and ET_B receptors has been suggested on the smooth muscle cells in human and guinea-pig airway preparations (Hay *et al.*, 1993b) and could cooperate in mediating ET-1-induced contraction (Inui *et al.*, 1994).

In conclusion, these results demonstrate that selective ET_B

receptor stimulation causes a potent biphasic bronchoconstriction in guinea-pigs, the first phase being mainly mediated by TXA_2 and ACh. The partial inhibition of the second phase by an ET_A antagonist suggests that the primary activation of ET_B receptors leads to ET-1 autocrine/paracrine release that would subsequently activate both ET_A and ET_B receptors. These data are further supported by the observation that a concentration of IRL 1620 that induces an acute monophasic response can be converted to a biphasic response after pretreatment with phosphoramidon. Thus it can be predicted that, if NEP activity is decreased in a disease process (Jacoby *et al.*, 1988; Piedimonte *et al.*, 1990), endothelin would be less rapidly inactivated, contributing to the exaggerated pulmonary responses. If a similar mechanism exists in man, both ET_A and ET_B receptor antagonistic activities may be required for optimal therapeutic value in airway disease.

The authors wish to thank Drs A.J. Coyle and G.P. Anderson for advice and helpful discussion. We are also grateful to Ms M. Erard, M. Hayakawa, M. Makatani and Mr D. Wyss for their skillful technical assistance.

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(Received February 14, 1996
Revised March 19, 1996
Accepted April 1, 1996)