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European Journal of Pharmacology 406 (2000) 139–147

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Effect of a novel bifunctional endothelin receptor antagonist, IRL 3630A, on guinea pig respiratory mechanics

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Received 21 July 2000; received in revised form 15 August 2000; accepted 18 August 2000

Abstract

This study characterized the in vitro pharmacological properties of a newly developed endothelin receptor antagonist, *N*-butanesulfonyl-[*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)-phenyl]-(D)-alanyl]-(L)-valineamide sodium salt (IRL 3630A), and its in vivo effects on respiratory mechanics were determined. IRL 3630A showed highly balanced affinities to human endothelin ET_A and ET_B receptors, giving apparent K_i values of 1.5 and 1.2 nM, respectively. This compound also potently antagonized the endothelin-1-induced intracellular Ca^{2+} increases in both embryonic bovine tracheal (EBTr) cells expressing endothelin ET_A receptors and human Girardi heart (hGH) cells expressing endothelin ET_B receptors. In guinea pig isolated tracheas having both endothelin ET_A and ET_B receptors, IRL 3630A greatly inhibited endothelin-1-induced contraction ($pA_2 = 7.1$), which was partially or scarcely suppressed by the endothelin ET_A receptor antagonist *cyclo*[-(D)-Trp-(D)-Asp-(L)-Pro-(D)-Val-(L)-Leu-] (BQ-123) or the endothelin ET_B receptor antagonist *N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-(4-phenyl)-(D)-phenylalanyl-(L)-tryptophan (IRL 2500), respectively. Bolus i.v. injections of IRL 3630A administered into anaesthetized guinea pigs at 10 and 30 μg/kg inhibited endothelin-1 (1.3 μg/kg)-induced changes in respiratory resistance and compliance in a dose dependent manner, whereas both sodium 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enoate (an endothelin ET_A receptor antagonist: PD 156707) and IRL 2500 at doses of up to 30 μg/kg did not affect endothelin-1-induced changes in respiratory mechanics, reflecting the in vitro results. IRL 3630A is thus an effective bifunctional endothelin receptor antagonist, and will be useful in clarifying the role of endothelin in pulmonary diseases such as bronchial asthma.

Keywords: Endothelin; Endothelin ET_A/ET_B receptor antagonist bifunctional; IRL 3630A; Respiratory mechanics

1. Introduction

Endothelin-1, initially identified as a potent vasoconstrictor peptide produced by endothelial cells (Yanagisawa et al., 1988), is one of a family of three isopeptides, endothelin-1, endothelin-2 and endothelin-3, collectively termed endothelins (Inoue et al., 1989). Endothelins have increasingly shown to have many effects on various tissues besides the vascular system (Masaki et al., 1992). The functions of endothelins are mediated by at least two distinct subtypes of endothelin receptors, ET_A and ET_B receptors (Arai et al., 1990; Sakurai et al., 1990). The endothelin ET_A receptor has a higher affinity for endothelin-1 and endothelin-2 than for endothelin-3 (Arai et al., 1990), whereas the endothelin ET_B receptor shows an almost equal affinity for the three isopeptides (Sakurai et al., 1990). Of the significant actions of endothelins in non-vascular tissues, endothelins are well known to elicit a

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00652-X

potent airway constrictor response (Hay, 1995). Endothelin-1 and endothelin-3 was also shown to be produced by airway epithelial cells (Black et al., 1989). Increased endothelin-like immunoreactivity has been observed in the airway epithelium (Springall et al., 1991) and bronchoalveolar lavage fluid (Redington et al., 1995) of asthmatic patients. Furthermore, inhaled endothelin-1 has been reported to cause potent bronchoconstriction in asthmatic subjects, with a potency comparable to 100 times that of methacholine on a molar basis (Chalmers et al., 1997). Endothelins thus appear to play a pathophysiological role in asthma.

Our previous study (Inui et al., 1994) found that endothelin ET_A and ET_B receptors coexist in the major population of single smooth muscle cells isolated from the guinea pig trachea, and both mediate intense contraction of the tracheal smooth muscle. We further revealed that endothelin-1 activates distinct excitation–contraction coupling pathways via endothelin ET_A and ET_B receptors in guinea pig tracheal smooth muscle (Inui et al., 1999). Consequently, endothelin-1 is thought to induce tracheal contraction by adroitly utilizing these two distinct signaling pathways specialized for the two receptor subtypes. Potent antagonists, with balanced affinities to endothelin ET_A and ET_B receptors, will therefore be required to effectively inhibit endothelin-1-induced tracheal contraction.

We previously reported the discovery of an endothelin ET_B receptor-selective antagonist, *N*-(3,5-dimethylenzoyl)-*N*-methyl-3-(4-phenyl)-(D)-phenylalanyl-(L)-tryptohan (IRL 2500), found via a rational approach combining a sequence study of endothelin-1 analogs and a homology study of the rhodopsin superfamily of seven transmembrane receptors (Früh et al., 1996). Recently, we also identified *N*-butanesulfonyl-[*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)-phenyl]-(D)-alanyl]-(L)-valineamide (IRL 3630), an antagonist with binding affinities to both endothelin ET_A and ET_B receptors, which was discovered through the structural modification of IRL 2500 (Sakaki et al., 1998a, b). Furthermore, a sodium salt form of IRL 3630, *N*-butanesulfonyl-[*N*-(3,5-dimethylbenzoyl)-*N*-methyl-3-[4-(5-isoxazolyl)-phenyl]-(D)-alanyl]-(L)-valineamide sodium salt (IRL 3630A), was made to improve its water solubility.

In the present study, we have characterized the bifunctional antagonism of IRL 3630A on endothelin ET_A and ET_B receptors using in vitro experiments: receptor-binding assays, intracellular Ca^{2+} measurements, and contraction assays using guinea pig isolated tracheas. We further assessed the in vivo ability of IRL 3630A to inhibit the respiratory actions of endothelin-1 in the anaesthetized guinea pig. There have been no previous reports concerning the in vivo effect of bifunctional endothelin ET_A/ET_B receptor antagonists on respiratory mechanics. This paper highlights the high efficacy of IRL 3630A as a suppressor of the respiratory mechanical response to endothelin-1.

2. Methods

2.1. Materials and cells

Endothelin-1 was purchased from the Peptide Institute (Osaka, Japan) and *cyclo*[-(D)-Trp-(D)-Asp-(L)-Pro-(D)-Val-(L)-Leu-] (BQ-123) was obtained from Peninsula Laboratories (Belmont, MA, USA). IRL 3630A, IRL 2500 and sodium 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enoate (PD 156707) were synthesized in our laboratory. Fura-2-acetoxymethyl ester (fura-2/AM) was obtained from Dojin (Kumamoto, Japan). ^{125}I-endothelin-1 (74 TBq/mmol) was purchased from Amersham International (Bucks, UK). Carbachol and cremophor-EL (a derivative of castor oil and ethylene oxide) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

Chinese hamster ovary (CHO) cells expressing human endothelin ET_A or ET_B receptors were a kind gift from Prof. Masaki of Kyoto University (Kyoto, Japan). Embryonic bovine tracheal (EBTr) cells and human Girardi heart (hGH) cells were purchased from the American Type Cell Culture Collection (ATCC CCL44) and Dainippon Pharma, respectively.

2.2. Preparation of compound solutions

Endothelin-1 was dissolved in 0.1% acetic acid/H_2O at a concentration of 10^{-5} M and stored at −40°C until it is used. Endothelin-1 solutions used for the in vitro tracheal contraction assays were prepared by diluting the stock solution with Krebs–Henseleit solution (pH 7.4) containing the following components (mM): 113.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 5.5 D-glucose, 0.01 EDTA, and 0.01% bovine serum albumin. For the Ca^{2+} assays, stock solutions were diluted with 20-mM HEPES (pH 7.4)-buffered salt solution (HBSS) with the following composition (mM): 140.0 NaCl, 4.0 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 10.0 D-glucose and 0.1% bovine serum albumin. For in vivo experiments, the endothelin-1 stock solution was diluted with 0.01% bovine serum albumin/saline. Fura-2/AM was dissolved in dimethyl sulfoxide (DMSO).

2.3. Animals

Male Hartley guinea pigs (350–500 g body weight) were purchased from SLC (Shizuoka, Japan). The animals were housed in a temperature (25 ± 2°C)- and moisture (50%)-controlled room with a 12-h light/dark cycle (light turned on at 8 AM and turned off at 8 PM). Animals were given standard guinea pig chow (CLEA Japan, Osaka) and tap water ad libitum. All experimental procedures were approved by the Ciba-Geigy Animal Welfare Meeting.

2.4. Receptor binding assays

For competitive binding studies, cellular membranes of CHO cells expressing either human endothelin ET_A or ET_B receptors were prepared as described previously (Sasaki et al., 1998). Membranes (0.05–0.3 μg of protein) were incubated at 37°C for 1 h with 10-pM ^{125}I-endothelin-1 in the presence of various concentrations of a given endothelin receptor antagonist in a 20-mM HEPES (pH 7.4) solution with the following composition (mM): 145.0 NaCl, 4.0 KCl, 1.2 $MgCl_2$, 1.0 EGTA, and 0.1% bovine serum albumin and 0.02% bacitracin. Binding was terminated by centrifugation at $20{,}000 \times g$ for 20 min at 4°C. Membrane-associated radioactivity was measured with a Wallac-1470 Wizard auto-gamma counter (Pharmacia). Non-specific binding was defined as the radioactivity measured at the saturating concentration (100 nM) of unlabeled endothelin-1. Specific binding was determined by total binding minus non-specific binding. Total binding was always $< 10\%$ of the total radioactivity added.

Scatchard analysis using ^{125}I-endothelin-1 was performed as described previously (Sasaki et al., 1998). The endothelin ET_A and ET_B receptors showed apparent K_d values (mean ± s.d.) of 5.9 ± 0.7 and 3.5 ± 0.2 pM, respectively. From these apparent K_d values and the inhibition curves for the binding of ^{125}I-endothelin-1, apparent K_i values were calculated as parameters representing the affinities of antagonists to the endothelin ET_A and ET_B receptors.

2.5. Measurement of intracellular Ca^{2+} levels

Intracellular Ca^{2+} levels of EBTr and hGH cells were measured according to the method previously described (Oda et al., 1992) with some modifications. In brief, EBTr and hGH cells in 100-mm culture dishes were dispersed by 0.025% trypsin treatment, then loaded with fura-2 by incubation at 20°C for 35 min in HBSS containing 5.0-μM fura-2/AM. Fura-2 loaded cells were then washed twice, re-suspended in HBSS without fura-2/AM and kept at 20°C. Approximately 10^6 cells/ml were used in each set of experiments. The fluorescence of fura-2 loaded cells was measured with emission at 500 nm for excitation at 340 and 380 nm. Intracellular Ca^{2+} levels were represented by the ratio of fluorescence intensity for excitation at 340 nm to that for excitation at 380 nm. Fluorescence was monitored with a spectrofluorometer (CAF-100, Japan Spectroscopy, Tokyo). Intracellular Ca^{2+} levels are denoted as $[Ca^{2+}]_i$ in this paper for convenience.

2.6. In vitro tracheal contraction assay

Guinea pig tracheas were removed quickly and placed in oxygenated (95% O_2/5% CO_2) Krebs–Henseleit solution at 37°C. After the removal of adherent fat and connective tissue, tracheas were cut into rings of approximately 2 mm in width. Epithelia were removed by gently turning the rings on the shaft of a pair of watchmaker's forceps. Rings were equilibrated for at least 1 h in oxygenated Krebs–Henseleit solution at 37°C, and placed in a 4.0-ml organ bath with a water-jacket (Iwashiya UFER, Kyoto, Japan) under a constant resting tension of 1 g. Responses were measured isometrically with a force-displacement transducer (Nihon Kohden TB-612T, Tokyo, Japan). Each preparation was stimulated at least three times with 10-μM carbachol and the contraction intensity due to the final stimulation was used as a reference standard. Tissues were washed three times at 15-min intervals before the application of endothelin-1. Concentration-dependence was tested by the cumulative addition of endothelin-1 in logarithmic increments.

IRL 3630A/0.01% bovine serum albumin/H_2O or BQ-123/H_2O solutions were added to the organ baths 30 or 20 min prior to endothelin-1 stimulation, respectively. IRL 2500/DMSO was diluted to 1/1000 with Krebs–Henseleit solution, and then the whole volume of the Krebs–Henseleit solution in the organ bath was replaced with the solution containing IRL 2500 30 min prior to endothelin-1 stimulation.

2.7. In vivo measurements of respiratory mechanics

Guinea pigs were anaesthetized by i.p. injections of 0.5 g/kg urethane supplemented with 25 mg/kg pentobarbitone. Bolus i.v. administration of drugs was conducted through a polyethylene catheter inserted into the right jugular vein. Guinea pigs were connected to a ventilator via a tracheal cannula. The respiratory frequency and tidal volume were adjusted at 60 strokes/min and 8 ml/kg, respectively. The animals were paralyzed by i.v. injections of 2 mg/kg gallamine triethiodide, then set in a plethysmograph box (BUXCO, USA). Respiratory system resistance and compliance of the guinea pigs were measured by a respiratory mechanics analyzer (BUXCO model 6). Drugs at a volume of 1 ml/kg were intravenously administrated 5 min prior to i.v. injection of endothelin-1 at 1.3 μg/kg. A solution containing 0.1% (v/v) DMSO and 0.1% (v/v) cremophor-EL in saline was used as a vehicle control.

Table 1
Apparent K_i values of endothelin receptor antagonists used in this study determined using the membranes of CHO cells expressing either human endothelin ET_A or ET_B receptors

	ET_A (nM)	ET_B (nM)
IRL 3630A	1.5	1.2
BQ-123	1.4	1500
PD 156707	0.14	130
IRL 2500	450	0.75

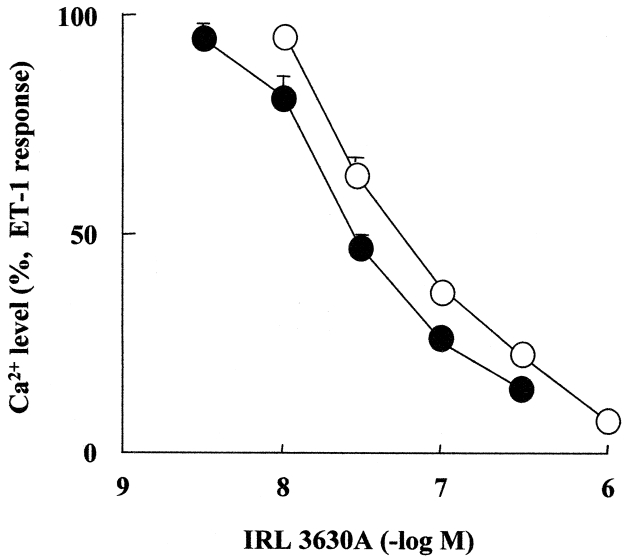

Fig. 1. Inhibitory effects of IRL 3630A on the increase in intracellular Ca^{2+} levels induced by endothelin-1 in EBTr (○) and hGH cells (●). Results are expressed as a percentage of the response to 3 (○) or 1 nM (●) of endothelin-1 and are given as the mean ± s.d. of 3–4 experiments. ET-1: endothelin-1.

2.8. Statistical analysis

Values are expressed as mean ± SEM or mean ± s.d. The Dunnett's test for multiple comparison was used to assess the statistical significance of difference after one-way analysis of variance. Differences were considered to be significant where $P < 0.05$.

3. Results

3.1. Binding affinities of IRL 3630A for the human endothelin ET_A and ET_B receptors

IRL 3630A competitively and potently inhibited the binding of 10-pM ^{125}I-endothelin-1 to both cloned human endothelin ET_A and ET_B receptors, displaying almost identical binding affinities for both receptors (Table 1). For comparison, receptor affinities of the other endothelin antagonists used in this study, the endothelin ET_A receptor-selective antagonists BQ-123 (Ihara et al., 1992) and PD 156707 (Reynolds et al., 1995), and the endothelin ET_B receptor-selective antagonist IRL 2500, were also investigated with this competitive binding assay system. The apparent K_i values of these antagonists are summarized along with those of IRL 3630A in Table 1. BQ-123 and PD 156707 showed approximately 1000 times greater affinity for the endothelin ET_A receptor than for the endothelin ET_B receptor, whereas IRL 2500 had 600 times greater affinity for the endothelin ET_B receptor than for the endothelin ET_A receptor.

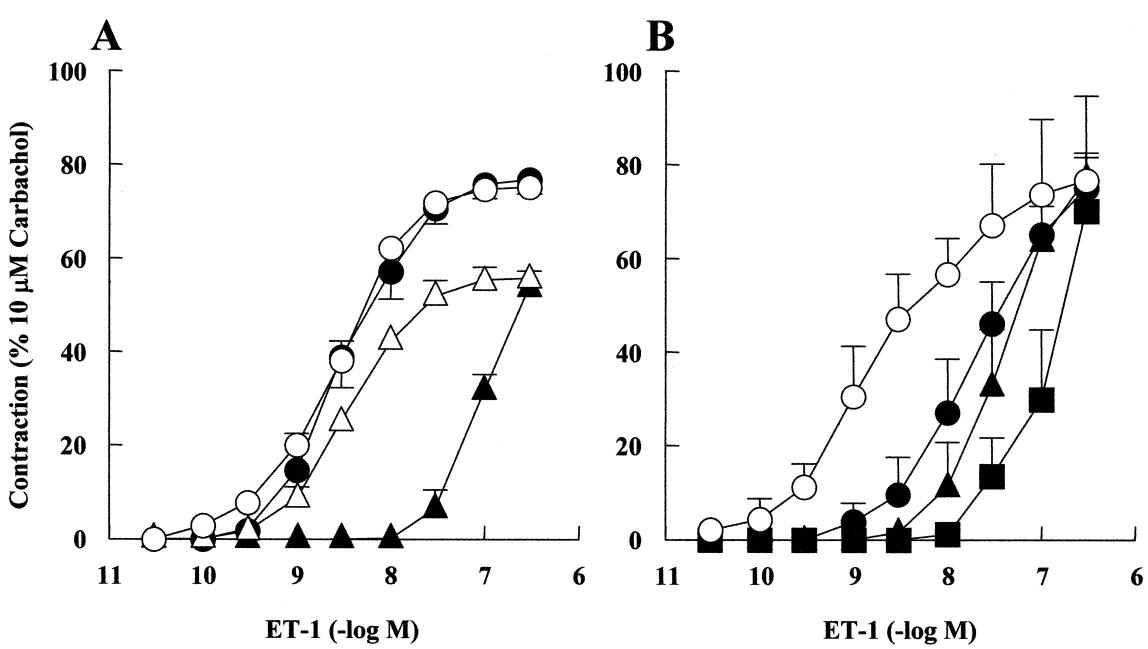

Fig. 2. Effects of IRL 2500 , BQ-123, a combination of IRL 2500 and BQ-123, and IRL 3630A on endothelin-1-induced contraction of epithelium-denuded tracheal rings isolated from guinea pigs. A: control (○), IRL 2500 (10 μM, ●), BQ-123 (10 μM, △), combination of IRL 2500 and BQ-123 (10 μM each, ▲). B: control (○); IRL 3630A (1, ●; 3, ▲; 10 μM, ■). Results are expressed as a percentage of the response to 10-μM carbachol and are given as the mean ± SEM of 3–5 (A) or 4 (B) experiments.

3.2. In vitro functional assay (1): the inhibition of endothelin-1-induced increases in intracellular Ca^{2+} levels by IRL 3630A

EBTr (Oda et al., 1992) and hGH cells (Mihara and Fujimoto, 1992) predominantly express endothelin ET_A and ET_B receptors, respectively. Both cells respond to endothelin-1, resulting in an increased $[Ca^{2+}]_i$ depending on the endothelin-1 concentration in a biphasic manner (a rapid and transient increase followed by a lower and sustained increase). IRL 3630A concentration-dependently attenuated the increases in $[Ca^{2+}]_i$ induced by 3-nM endothelin-1 for EBTr cells and 1-nM endothelin-1 for hGH cells (Fig. 1). These endothelin-1 concentrations led to an increase in $[Ca^{2+}]_i$ of about 70% that of the maximum increase found in these cell types. IRL 3630A inhibited both the transient and sustained phases. The half-maximal inhibition concentrations (IC_{50}) were 51 and 28 nM for EBTr and hGH cells, respectively.

3.3. In vitro functional assay (2): the inhibition of endothelin-1-induced contraction of guinea pig isolated tracheas

Endothelin-1 caused long-lasting contractions in epithelium-denuded tracheal rings isolated from guinea pigs. The half-maximal effective concentration (EC_{50}) was estimated at 2.9 nM from the concentration–response curve for endothelin-1 (Fig. 2A). The maximal contraction (T_{max}) was 75% that of the 10-μM carbachol-induced contraction. The endothelin ET_B receptor-selective antagonist IRL 2500 at a concentration of 10 μM scarcely affected the endothelin-1-induced contraction (Fig. 2A). On the other hand, the endothelin ET_A receptor-selective antagonist BQ-123 at 10 μM shifted the endothelin-1-response curve slightly to the

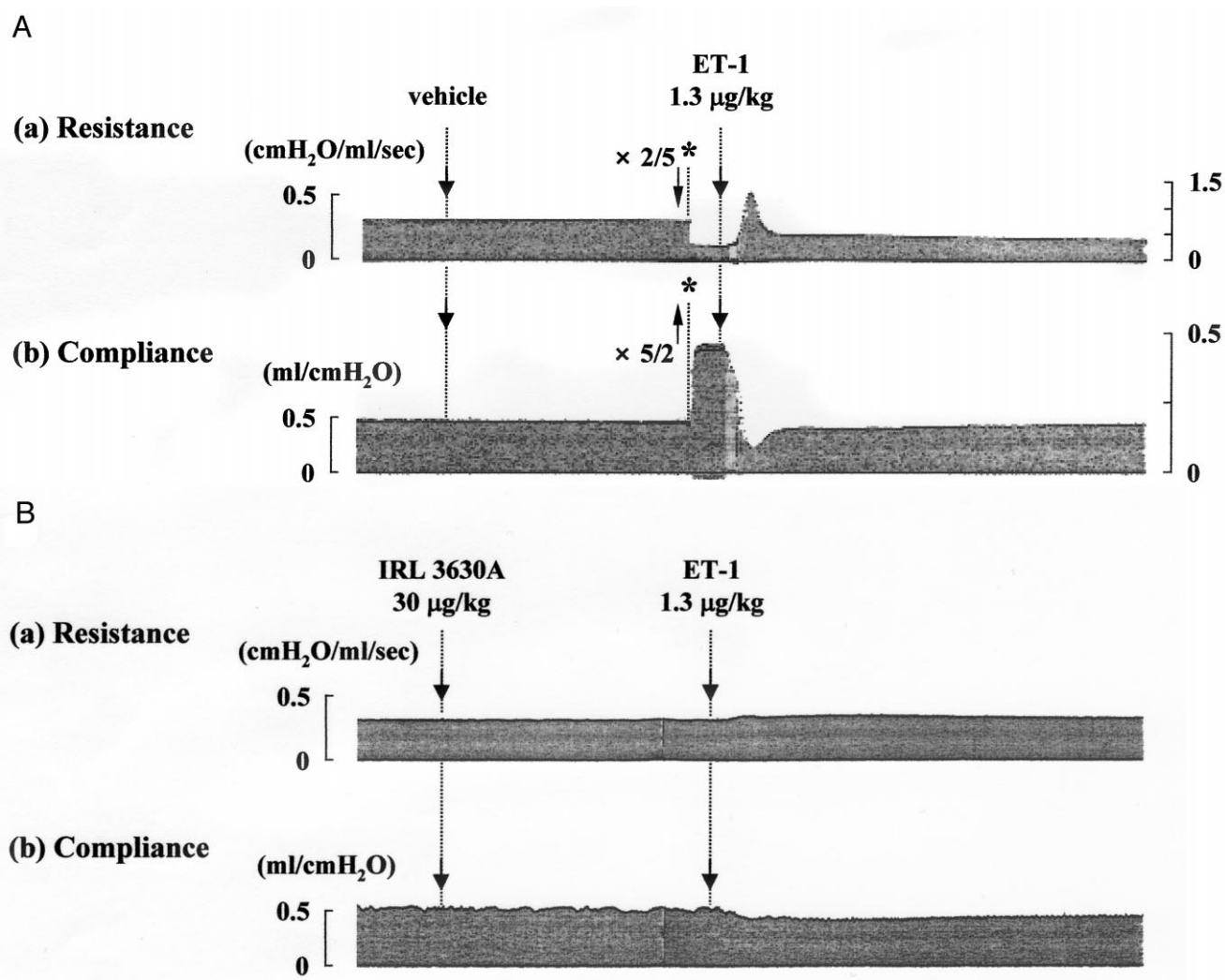

Fig. 3. Representative experimental traces showing an increase in respiratory resistance (A-a), and a decrease in respiratory compliance (A-b) induced by a bolus i.v. injection of 1.3 μg/kg endothelin-1, and the effects of a bolus i.v. injection of 30 μg/kg IRL 3630A on these parameters (B-a,b) in the anaesthetized guinea pig. A: saline containing 0.1% DMSO and 0.1% cremophor-EL (vehicle) was injected 5 min prior to endothelin-1 injection. B: IRL 3630A was injected 5 min prior to endothelin-1 injection. *: the amplified gain was changed at this point. This resulted in a decrease in resistance to 2/5 that of the original and a 2.5 times increase in compliance.

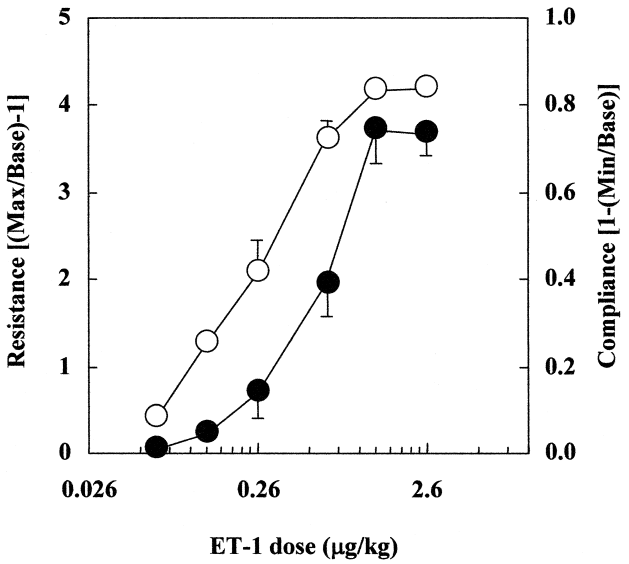

Fig. 4. Endothelin-1 dose-response curves for respiratory resistance (●) and compliance (○) in the anaesthetized guinea pig. Ordinates are represented as [(ratio of maximum value at transient change to basal level) − 1] for resistance and [1 − (ratio of minimum value at transient change to basal level)] for compliance. Results are given as the mean ± SEM of 6–8 experiments.

right and suppressed the maximal contraction to 74% that of the T_{max} value of the control (Fig. 2A). A combination of BQ-123 and IRL 2500 at a concentration of 10 μM each induced a drastic shift of the endothelin-1-response curve to the right (Fig. 2A). We then examined the effect of IRL 3630A on endothelin-1-induced tracheal contraction. This compound effectively inhibited contraction in a concentration-dependent manner (Fig. 2B). The pA_2 value was estimated as 7.1 from the Schild plot.

3.4. Effect of IRL 3630A (i.v.) on the respiratory actions of endothelin-1 in the anaesthetized guinea pig

Bolus i.v. injections of endothelin-1 into anaesthetized guinea pigs induced an increase in respiratory resistance and a decrease in respiratory compliance. Fig. 3A shows a typical time course of changes in resistance and compliance induced by endothelin-1 at a dose of 1.3 μg/kg. These changes in respiratory mechanics occurred in a biphasic manner: larger transient changes followed by smaller sustained changes significantly different from basal levels. The responses in both resistance and compliance to endothelin-1 were observed from a dose of 0.065 μg/kg of endothelin-1 and dose-dependently increased until a dose of 1.3 μg/kg (Fig. 4). The injection of a higher dose of endothelin-1, at 2.6 μg/kg did not elicit further changes in these parameters. The dose-response curves of the changes in resistance and compliance paralleled each other, giving half-maximal effective doses (ED_{50}) of 0.60 and 0.26 μg/kg, respectively.

Bolus i.v. administrations of IRL 3630A of up to 30 μg/kg did not affect the basal levels of resistance and compliance (Fig. 3B). IRL 3630A, at doses of up to 3 μg/kg, did not significantly inhibit the changes in both

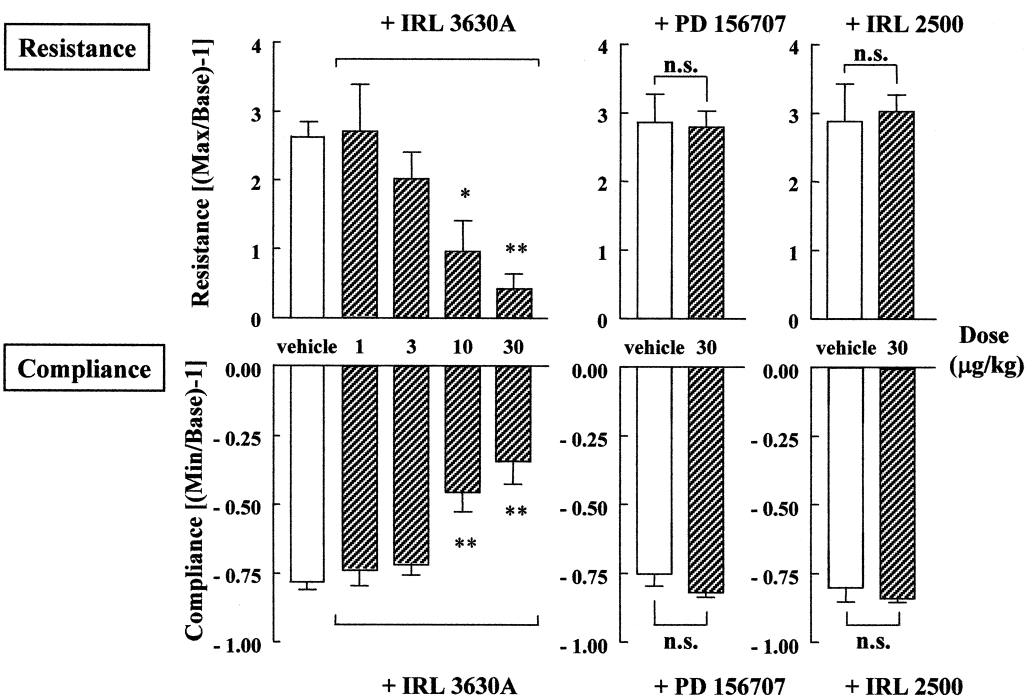

Fig. 5. Effects of IRL 3630A, PD 156707 and IRL 2500 on peak values of 1.3-μg/kg endothelin-1-induced changes in respiratory system resistance and compliance in the anaesthetized guinea pig. Values are expressed as the mean ± SEM of 4–12 animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ are significantly different from the vehicle control groups.

resistance and compliance induced by endothelin-1 at 1.3 μg/kg (Fig. 5). Higher doses of IRL 3630A at 10 μg/kg or above, inhibited the response of these respiratory parameters to 1.3 μg/kg endothelin-1 in a dose-dependent manner (Fig. 5). Representative experimental traces of the inhibitory effects of 30 μg/kg IRL 3630A are shown in Fig. 3B. On the other hand, PD 156707 and IRL 2500, at doses of up to 30 μg/kg had no effect on these respiratory parameter responses to 1.3-μg/kg endothelin-1 (Fig. 5).

4. Discussion

This paper reports the pharmacological properties of a newly developed endothelin receptor antagonist, IRL 3630A, characterized by in vitro binding and functional experiments, and secondly, reports the compound's effect on respiratory mechanics investigated by in vivo tests using anaesthetized animals.

In receptor binding experiments using recombinant human endothelin receptors, IRL 3630A showed almost equal affinities to endothelin ET_A and ET_B receptors, giving apparent K_i values of 1.5 and 1.2 nM, respectively (Table 1). The antagonistic potency of this compound for cellular function mediated by each of endothelin ET_A and ET_B receptors was evaluated by measuring inhibitory activities for endothein-1-induced $[Ca^{2+}]_i$ increases in EBTr and hGH cells which predominantly express endothelin ET_A and ET_B receptors, respectively. As shown in Fig. 1, IRL 3630A effectively, in concentration-dependent manners, inhibited both of the endothelin ET_A and ET_B receptor mediations at almost identical potency, reflecting highly balanced binding affinities to both receptor subtypes. Further confirmatory evidence for the high potency of IRL 3630A as a bifunctional antagonist was provided at the tissue level by examining its effect on the endothelin-1-induced contraction of epithelium-denuded trachea isolated from guinea pigs, in which the smooth muscle cells co-express both endothelin ET_A and ET_B receptors. As illustrated in Fig. 2, this compound potently antagonized the endothein-1-induced tracheal contraction ($pA_2 = 7.1$) which was only partially attenuated by the endothelin ET_A receptor antagonist BQ-123 (10 μM) and scarcely attenuated by the endothelin ET_B receptor antagonist IRL 2500 (10 μM). This effect of IRL 3630A was in accordance with the results of the combined use of BQ-123 and IRL 2500, which markedly antagonized the contraction. As seen in Fig. 2, a combination of BQ-123 and IRL 2500 at 10 μM each, and IRL 3630A alone at 10 μM, shifted the control curve to the right at almost the same efficacy. This is clearly attributed to the affinities of IRL 3630A for both endothelin ET_A and ET_B receptors, which are approximately equal to the affinity of BQ-123 for the endothelin ET_A receptor and the affinity of IRL 2500 for the endothelin ET_B receptor, respectively (Table 1).

Thus, only the dual blockade of endothelin ET_A and ET_B receptors produced effective antagonism in the guinea pig tracheal contraction induced by endothein-1. Similar phenomena have also been observed in human bronchi (Fukuroda et al., 1996) and rat trachea (Henry, 1993). However, there has been no sufficient explanation for such phenomenon. The endothein-1-induced contractile mechanisms in these tissues are likely to be considerably complicated. Our recent study (Inui et al., 1999) revealed that the endothein-1-induced contraction of the guinea pig trachea depends totally on Ca^{2+} influx, and that the Ca^{2+} influx pathways activated by endothelin ET_A and ET_B receptors are completely different, namely, endothelin ET_A and ET_B receptors open voltage-independent Ca^{2+} channels and L-type voltage-dependent Ca^{2+} channels, respectively. Furthermore, stimulation of endothelin ET_A receptors, but not of endothelin ET_B receptors, activates a phospholipase C/Ca^{2+}/protein kinase C signaling pathway which contributes to the contraction by increasing the Ca^{2+} sensitivity of the contractile apparatus. It, however, is still unclear as to whether these pathways are simultaneously involved in the endothein-1-induced contraction. We previously confirmed that BQ-123 at 10 μM did not affect the endothelin ET_B receptor-mediated contraction induced by endothein-3 (≤ 100 nM) in guinea pig isolated tracheal smooth muscle (Inui et al., 1994). Hence, the present results of minute inhibition by IRL 2500 and partial inhibition by BQ-123 in the endothein-1-induced contraction show that the endothelin ET_A receptor-mediated contraction is more potent than the endothelin ET_B receptor mediation in the guinea pig trachea. Furthermore, the results indicate that the endothein-1-induced contraction cannot be represented by a simple sum of the signals from the endothelin ET_A and ET_B receptors. The endothelin-1-induced tracheal contraction, rather, seems to be caused by signals from only endothelin ET_A receptors. The contribution of the endothelin ET_B-receptor–effector system is scarcely visible. A simple explanation of this is that the guinea pig tracheal smooth muscle cells may possess excess signal capacity built into the endothelin receptor system. Hence, even if one receptor subtype is blocked, enough signals may be produced from another remaining receptor population to induce maximal or nearly maximal contraction. The endothelin ET_A-receptor–effector system seems to predominate, and the endothelin ET_B-receptor system may be the reserved one. Consequently, the full inhibition of endothein-1-induced tracheal contraction requires the dual blockade of both endothelin ET_A and ET_B receptors. IRL 3630A resulted in effective antagonism for the guinea pig tracheal contraction induced by endothein-1.

The present study on the antagonistic effect of IRL 3630A was extended to in vivo experiments using anaesthetized guinea pigs. Respiratory system resistance and compliance responded to bolus i.v. administrations of endothein-1 at doses ranging from 0.065 to 1.3 μg/kg in a parallel fashion (Fig. 4). As shown in Fig. 5, bolus i.v.

administrations of IRL 3630A dose-dependently suppressed the increase in resistance and decrease in compliance induced by 1.3-μg/kg endothelin-1. In particular, IRL 3630A at a dose of 30 μg/kg caused an inhibition of 86% of the control in resistance and 56% of the control in compliance. On the other hand, neither the endothelin ET_A receptor antagonist PD 156707 nor the endothelin ET_B receptor antagonist IRL 2500 at 30 μg/kg each affected these respiratory parameters (Fig. 5). These results reflect the in vitro experimental results using isolated tracheal rings. Nagase et al. (1995) studied effects of the endothelin ET_A receptor antagonist BQ-123 and the endothelin ET_B receptor antagonist *N-cis*-2,6-dimethylpiperidinocarbonyl-(L)-γ-methyl-leucyl-(D)-1-methoxycarbonyltryptophanyl-(D)-norleucine (BQ-788) in anesthetized guinea pigs. Their results showed that the combined administration (bolus i.v. injection) of BQ-123 and BQ-788 at 2 mg/kg each inhibited the response of respiratory mechanics to endothelin-1 at 10^{-8} mol/kg (2.5 μg/kg, bolus i.v. administration) far more potently than a single administration of BQ-123 or BQ-788. Our present in vivo experimental results are basically in accordance with the results of Nagase et al (1995). The doses of endothelin-1 used in their experiment and our experiment were comparable, but the administration doses (2 mg/kg each) of antagonists used by them were much higher than that of the IRL 3630A used in the present experiments. IRL 3630A demonstrated sufficient efficacy at several tens of micrograms per kilogram. The recent in vitro study by Emanueli et al. (1998) revealed that, in the guinea pig tracheal tube preparation, epithelium- and nitric oxide-dependent relaxation to endothelin-1 is mediated by endothelin ET_A receptor activation. In the present in vivo test, however, the endothelin ET_A receptor antagonist PD 156707 at 30 μg/kg showed no effect on the endothelin-1-induced changes of the respiratory parameters (Fig. 5). On the other hand, in the in vitro experiment, the endothelin ET_A receptor antagonist BQ-123 partially attenuated the endothelin-1-induced contraction of the epithelium-denuded tracheal preparation (Fig. 2A). From these results, the lack of effect of PD 156707 in the in vivo test may be due to compensation by inhibition of relaxation and partial attenuation of the constriction in the airway. It is possible that the simultaneous inhibition by IRL 3630A of the endothelin ET_A and ET_B receptor mediations will predominate over the inhibition of relaxation. This is the first report showing a single compound effectively suppressing endothelin-1-induced changes in respiratory mechanics in vivo.

It has been postulated that endothelin-1 is involved in the pathophysiology of pulmonary disorders including asthma and pulmonary hypertension (Cernacek and Stewart, 1989; Springall et al., 1991; Hay et al., 1993; Hay and Goldie, 1995; Redington et al., 1995; Chalmers et al., 1997). The pulmonary artery, as well as the airway, possesses both endothelin ET_A and ET_B receptors. Combined treatment with endothelin ET_A and ET_B receptor antagonists has been reported to produce effective inhibition of endothelin-1-induced contraction of the pulmonary artery (Fukuroda et al., 1994; Sato et al., 1995; Higashi et al., 1997). Therefore, bifunctional antagonists for endothelin ET_A and ET_B receptors might exhibit therapeutic properties in certain pulmonary diseases. IRL 3630A is a compound with promising leads for such purposes.

Acknowledgements

We thank Ms. M. Hayakawa for excellent in vivo experimental assistance. Thanks are also due to Ms. T. Kimura and Ms. C. Sayo for the technical support.

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