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Pharmacological characterization of endothelin-induced rat pulmonary arterial dilatation

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1 The aim of study was to characterize endothelin (ET)-induced vasodilatation in isolated extrapulmonary rat arteries (EPA) and in intrapulmonary arteries (IPA) preconstricted with 1 μM phenylephrine.

2 The ET-3 (1 nM – 100 nM)- and ET-1 (10 nM – 100 nM)-induced transient vasodilatations in EPA were more potent than those in IPA. The vasodilatation induced by ET-3 (100 nM) was larger than that induced by ET-1 (100 nM).

3 Both the ET_B antagonist, BQ788 (3 μM) and or endothelium denudation, but not the ET_A antagonist, BQ123 (3 μM), abolished the vasodilatation induced by ET-1 or ET-3 (100 nM each) in EPA and in IPA. The ATP-sensitive K + channel blocker, glibenclamide (20 μM) and the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 1 mM) suppressed the ET-induced vasodilatation in EPA and in IPA.

4 We conclude that the vasodilatation induced by endothelins is markedly reduced in rat isolated IPA, and suggest that the endothelial ET_B-mediated vasodilatation varies depending on rat pulmonary arterial regions. Furthermore, ET_B-mediated vasodilatation involves activation of ATP-sensitive K^+ channels and of nitric oxide synthase in rat isolated EPA and IPA.

Keywords: Endothelin-1; endothelin-3; vasodilatation; BQ788; BQ123; nitric oxide synthase; ATP-sensitive K^+ channel; ET_B receptor; intrapulmonary artery; extrapulmonary artery

Introduction

Endothelin (ET), now denoted as ET-1, was initially identified from culture medium conditioned by porcine aortic endothelial cells as the most potent endogenous vasoconstrictor described to date (Yanagisawa *et al*., 1988). It is one of a family of three isopeptides (ET-1, ET-2, and ET-3) collectively termed ETs. The functions of ETs are mediated by at least two distinct subtypes of receptors, namely ET_A and ET_B (Arai *et al*., 1990; Sakurai *et al*., 1990). Recently, because the ET-1 plasma concentrations are well below those required to produce pharmacological effects in healthy individuals, it is thought that ETs may function as local paracrine or autocrine hormones (Frelin & Guedin, 1994; Yanagisawa, 1994). However, the precise roles of ETs and distribution of ET receptor subtypes have not yet been fully determined.

It is now appreciated that intravenous injection of ET-1 in several species causes a transient vasodilatation followed by a sustained vasoconstriction (Yanagisawa *et al*., 1988; Thiemermann, 1991; Vane & Botting, 1992). Furthermore, numerous studies (De Nucci *et al*., 1988; Warner *et al*., 1989a; Eddahibi *et al*., 1993; Sudjarwo *et al*., 1993) indicate that ETs stimulate ET_B receptors in the endothelium and release endothelium-derived vasodilator substances, although the mechanism of the ET-induced vasodilatation is less clear. There is evidence both for (De Nucci *et al*., 1988; Warner *et al*., 1989a, b) and against (Hasunuma *et al*., 1990; Eddahibi *et al*., 1993) mediation by nitric oxide (NO). Some studies (Hasunuma *et al*., 1990; Eddahibi *et al*., 1993) indicate that an ATP-sensitive K^+ channel (K_{ATP} channel) is involved in ET-induced vasodilatation, whilst most studies indicate that cyclo-oxgenase metabolites do not mediate the vasodilatation (De Nucci *et al*., 1988; Warner *et al*., 1989b; Hasunuma *et al*., 1990).

It has been suggested that the response to ET is a combination of smooth muscle contraction and dilatation which are mediated via endothelial cell ET_B receptors and basally released endothelium-derived relaxing factor (EDRF, NO). As regards smooth muscle, ET_A and ET_B receptors contribute to the ET-1-induced contraction in some vascular tissues (Seo *et al*., 1994; Yanagisawa, 1994; Higashi *et al*., 1997). Recent findings suggest that the contributions of ET_A and ET_B receptors to the contractile responses vary greatly depending on the vascular regions studied. For instance, in human coronary arteries, the contractile response of segment 8 to ET-1 was probably mediated by ET_A. However, in segment 5 and 6, it is likely that more than one receptor subtype is involved in the contractile response to ET-1 (Godfraind, 1993). MacLean *et al*. (1994) suggested that in extrapulmonary artery of rats, the contractile responses to ET-1 are mediated by ET_A, whereas in rat pulmonary resistance arteries, ET_B receptors primarily contribute to these responses. Moreover, our previous data (Higashi *et al*., 1997) showed that the ET-1-induced constrictions, in rat intrapulmonary artery (IPA) are mediated by both ET_A and ET_B receptors located between extrapulmonary arteries (EPA) and resistance arteries. However, the ET-1-induced constriction is mediated only by the ET_A receptor in rat EPA. On the other hand, the relationship between ET-induced vasodilatation and vascular regions is still unclear. Some data suggest that endothelial cell release of vasodilator substances (basally released and ET_B mediated), which counteracts ET-induced constriction, varies depending on vascular regions. For example, the NO synthase inhibitor N^ω nitro-L-arginine methyl ester (L-NAME) potentiated the contraction to ET-1 in rat extrapulmonary arteries. However, it did not affect the contraction induced by the ET_B-selective agonist sarafotoxin S6c (SXS6c) in rat resistance pulmonary arteries (MacLean *et al*., 1994). Additionally, our previous data (Higashi *et al*., 1997) showed that endothelium-removal significantly augmented the contractile response to ET-3 in EPA but not in IPA, thus suggesting that the endothelial release of vasodilator substances (basally released and ET_B-mediated) are more effective in modulating the constriction of EPA than of IPA.

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Therefore, we postulate that the endothelial ET_B-mediated vasodilatation varies depending on the pulmonary arterial region. In the present study, we assessed the vasodilator effects of ET-1 and ET-3 in rat EPA and IPA. We further examined the effect of ET antagonists, a nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA) and a K_{ATP} channel blocker, glibenclamide, on ET-induced vasodilatation in rat IPA and EPA.

Methods

All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee, Fukui Medical School.

Animals

Male Sprague-Dawley rats weighing 300–350 g were used in this study. The animals were housed in a temperature-($25\pm2^\circ$C) and humidity (50%) controlled room with a 12 h light/dark cycle. They were given standard rat chow (CLEA Japan, Inc., Osaka, Japan) and tap water *ad libitum*.

Isolated pulmonary artery preparation

Following anaesthesia with pentobarbitone (50 mg kg^{-1}, i.p.) the heart and lungs were removed quickly *en bloc* and placed in aerated (21% O_2, 5% CO_2 and 74% N_2) Earle's balanced salt solution (EBSS) which has the following composition (mM): $CaCl_2$ 1.80, $MgSO_4$ 0.83, KCl 5.36, NaCl 116, Na_2HPO_4 0.40, glucose 5.50, $NaHCO_3$ 19.0 and sodium phenol red 0.03 (included as a pH indicator), pH 7.4, at 37°C. The right and left branches of EPA (2–3 mm o.d.) as well as distal IPA (1–2 mm o.d.) were isolated by use of small iris scissors under a stereoscopic microscope as previously described (Higashi *et al*., 1997). After the adventitia tissues had been carefully removed, the arteries were cut into rings of ~4 mm width. In some experiments, the endothelium was removed from the artery by gently rubbing the intimal surface. The rings were placed on steel wires attached to a force-displacement transducer (Grass FT 03) and suspended in a 10 ml water-jacketed organ bath containing the aerated EBSS at 37°C. IPA and EPA rings were progressively stretched and exposed to KCl (80 mM) and phenylephrine (1 μM) at each magnitude of stretch until the optimal point of the length-tension relationship had been reached; the optimal basal tension did not differ in IPA and EPA (each 500 mg). Resting passive tension (500 mg) was maintained throughout the experiments and was taken as baseline tension; all subsequent constrictor and dilatation-responses were recorded. After equilibration periods of 60 to 90 min, each preparation was first stimulated with 80 mM KCl. After readjustment to the resting tension, the rings were constricted with 1 μM phenylephrine, and the response to 1 μM acetylcholine (ACh) was assessed. Endothelial removal was confirmed by the lack of dilatator response to 1 μM ACh. Rings were washed again with EBSS to reach a resting tension and then 10 μM meclofenamate was added to the bath to inhibit prostanoid production. The constrictor responses to 80 mM KCl and 1 μM phenylephrine, and the dilator responses to 1 μM ACh are shown in Table 1. The dilator effect of ACh was expressed as % relaxation, where a return of vascular tone to the baseline was considered to be 100% relaxation. The % dilatation to ACh of EPA and of IPA did not differ (Table 1). The rings were pretreated either with vehicle, BQ123 (3 μM) (Ihara *et al*., 1992), an ET_A antagonist, BQ788 (3 μM) (Ishikawa *et al*., 1994), an ET_B antagonist, N^G-monomethyl-L-arginine (L-NMMA) (300 μM or 1 mM), N^G-methyl-D-arginine (D-NMMA) (1 mM) or glibenclamide (20 μM) and then 30 min later, ET-1 or ET-3 were added to baths of rings preconstricted with 1 μM phenylephrine, and changes in tension were recorded. Neither BQ123 (3 μM), BQ788 (3 μM), L-NMMA (300 μM or 1 mM), D-NMMA (1 mM) nor glibenclamide (20 μM) affected the resting tension.

Table 1 Constrictor effects of KCl (80 mM), phenylephrine (1 μM) and dilator effects of acetylcholine in rat isolated EPA and IPA

Treatment	*KCl (mg)*	*Phenylephrine (mg)*	*% dilatation to ACh*
Intact EPA ($n=110$)	1670 ± 40	1270 ± 30	81 ± 1
Intact IPA ($n=110$)	1720 ± 50	1260 ± 40	82 ± 1
Denuded EPA ($n=10$)	1550 ± 180	1510 ± 130	1 ± 1
Denuded IPA ($n=10$)	1400 ± 200	1400 ± 100	2 ± 1

Values are means ± s.e.mean of n rings.

ETs were examined by a single application. ET-induced vasodilatation was expressed as a % relaxation of the response to the preconstrictor (1 μM phenylephrine), where a return of vascular tone to the baseline was considered to be 100% relaxation.

Drugs

BQ123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)) and BQ788 (N-cis-2,6-dimethylpiperidinocarbonyl-L-r-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) was kindly supplied by Dr M. Yano (Banyu Pharmaceutical Institute, Japan) and glibenclamide by Hoechst Co. (Japan). L-NMMA was purchased from Calbiochem Co. (U.S.A.). ET-1 and ET-3 were obtained from Peptide Institute INC. (Japan). D-NMMA, EBSS, KCl, acetylcholine and meclofenamate were purchased from Sigma Chemical (St. Louis, U.S.A.). BQ123, D-NMMA and L-NMMA were dissolved in 0.9% NaCl. BQ788 and glibenclamide were dissolved in dimethyl sulphoxide (DMSO; final concentrations of DMSO were less than 0.1%). ET-1 and ET-3 were dissolved in 0.1% aqueous acetic acid.

Statistical analysis

The data are expressed as mean ± s.e.mean. Differences between groups were tested by the Bonferroni *post hoc* test when one-way ANOVA determined that some of the effects in our model were significantly different (Wallenstein *et al*., 1980). A P value <0.05 was considered to be significant.

Results

ET-3-induced vasodilatation in rat EPA and IPA

ET-3 concentration-dependently produced transient vasodilatation, which was followed by a sustained constriction by 1 nM in EPA and by 10 nM in IPA (preconstricted with 1 μM phenylephrine), respectively. A representative trace showing the effect of addition of 100 nM ET-3 to the organ bath is shown in Figure 1. The maximum values of ET-3 (100 nM)-induced vasodilatations were 50 ± 4% in EPA and 21 ± 1% in IPA, respectively. ET-3 at 1 nM, 10 nM and 100 nM produced more effective vasodilatations in EPA than in IPA (Figure 2a).

ET-1-induced vasodilatation in rat EPA and IPA

ET-1 produced transient vasodilatation, which was followed by sustained constriction, from 1 nM in EPA and from 10 nM in IPA which were preconstricted with 1 μM phenylephrine. The maximum values of ET-1 (100 nM)-induced vasodilator responses were 19 ± 2% and 9 ± 3% in EPA and IPA, respectively (Figure 2b). ET-1 at 10 nM and 100 nM produced more effective vasodilatations in EPA than in IPA and were of similar potency (Figure 2b).

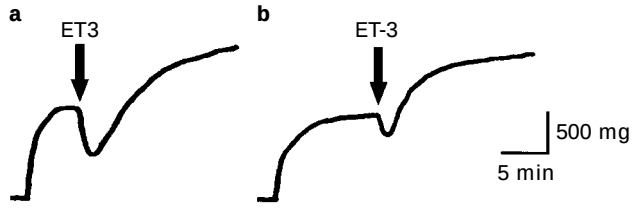

Figure 1 ET-3-induced transient vasodilatations (a) in rat extrapulmonary artery and (b) intrapulmonary artery preconstricted with 1 μM phenylphrine. Addition of 100 nM ET-3 to the organ bath caused transient vasodilatations, and then consistently constricted these pulmonary arteries.

Effect of ET antagonists, L-NMMA, glibenclamide and endothelial cell denudation on ET-3-induced vasodilatation

Figure 3A shows that addition of BQ788 (3 μM) completely inhibited the ET-3-induced vasodilatation of EPA and IPA. BQ123 (3 μM) did not affect the maximum values of ET-3 (100 nM)-induced vasodilatation of EPA and IPA. L-NMMA (300 μM or 1 mM) and glibenclamide (20 μM), but not D-NMMA (1 mM), suppressed the vasodilatations of EPA and IPA but were less effective than BQ788. The combination of L-NMMA (300 μM) and glibenclamide (20 μM) did not show any further suppression than that observed with either L-NMMA (300 μM) or glibenclamide (20 μM) alone. Removal of the endothelium completely inhibited the vasodilator responses in EPA and IPA.

Effect of ET antagonists, L-NMMA, glibenclamide and endothelium-removal on ET-1-induced vasodilatation

Figure 3B shows that addition of BQ788 (3 μM), but not BQ123 (3 μM), completely inhibited ET-1 (100 nM)-induced vasodilatation of EPA and IPA. Denudation of the endothelium also completely inhibited the vasodilatation of EPA and IPA. L-NMMA (300 μM or 1 mM) alone, glibenclamide (20 μM) alone and the combination of L-NMMA (300 μM) and glibenclamide (20 μM) significantly suppressed the ET-1-induced vasodilatation of EPA and IPA to a similar extent. On the other hand, D-NMMA (1 mM) did not affect the maximum values of ET-1 (100 nM)-induced vasodilatation of EPA and IPA.

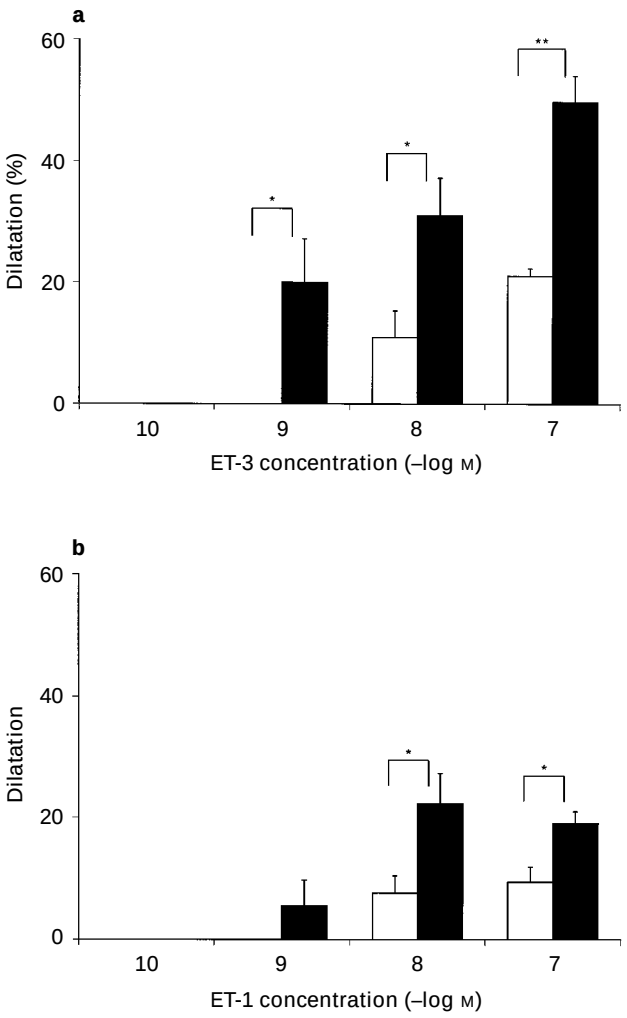

Figure 2 Relaxant effects of (a) ET-3 and (b) ET-1 in rat intrapulmonary (open columns) and extrapulmonary (closed columns) arteries preconstricted with 1 μM phenylephrine. The number of preparations in all groups was (a) 4 or (b) 6. Values are mean $\pm$ s.e.mean. $^*P<0.05$, $^{**}P<0.01$.

Discussion

The main findings of the present study are that the transient ET-3- or ET-1-induced vasodilatation in EPA is more potent than that in IPA and that this is mediated via ET_B receptors present on endothelial cells. This suggests that ET_B-mediated vasodilatations vary depending on the pulmonary arterial regions. However, the precise reason for this remains unclear. One possible explanation is that the population of functional ET_B receptors in the endothelium is greater in EPA than in IPA. In rat pulmonary artery smooth muscle, it appears that functional ET_A receptors greatly contribute to the ET-1-induced vasoconstriction in EPA, whilst functional ET_B receptors may gradually increase distally. However, in the resistance vessels, ET_B primarily contributes to the constriction (MacLean *et al*., 1994; Higashi *et al*., 1997). If this explanation is correct then, the functional ET_B receptors in endothelium of the rat pulmonary artery might gradually decrease in number on approaching the distal segment.

Alternatively, ET-induced constriction greatly affects the ET-induced vasodilatation in IPA in preference to EPA. Generally, in endothelium-intact vascular rings, the response to ET should be a balance between the following two factors: (i) vasoconstriction mediated by ET_A and/or ET_B, and (ii) vasodilatation mediated by endothelial ET_B with basal release of EDRF (NO). Our previous results (Higashi *et al*., 1997) demonstrated that the ET-1-induced constriction is mediated by only ET_A-receptors in rat EPA, but it is mediated by both ET_A- and ET_B-receptors in rat IPA. Hence, it appears that additional ET_B-mediated constriction in IPA causes a reduction of ET-induced vasodilatation. However, in endothelium-denuded rings, the sensitivity and potency of ET-1- or ET-3-induced constriction is not different between EPA and IPA (Higashi *et al*., 1997). Frelin & Guedin (1994) hypothesized, regarding the response of the ET-1-induced biphasic action (transient vasodilatation followed by a sustained constriction), that ET-1 first stimulates endothelial ET_B-receptors and occupies all endothelial ET_B-receptors and then diffuses into the media to act on receptors on smooth muscle cells. If the ET_B-mediated constriction counteracts the ET-induced vasodilatation faster than the ET_A-mediated constriction, the ET-induced vasodilatation in IPA (which possess ET_A and ET_B receptors in smooth muscle) may be reduced more than the vasodilatation in EPA (which possess only ET_A receptors in smooth muscle). This explanation cannot be completely excluded.

The consensus appears to be that the ET_A receptor has a greater selectivity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor shows almost equal affinities for all three isopeptides (Arai *et al*., 1990; Sakurai *et al*., 1990). However, in our present study, 100 nM ET-3-induced vasodilatation via activation of ET_B receptors was stronger than 100 nM ET-1-

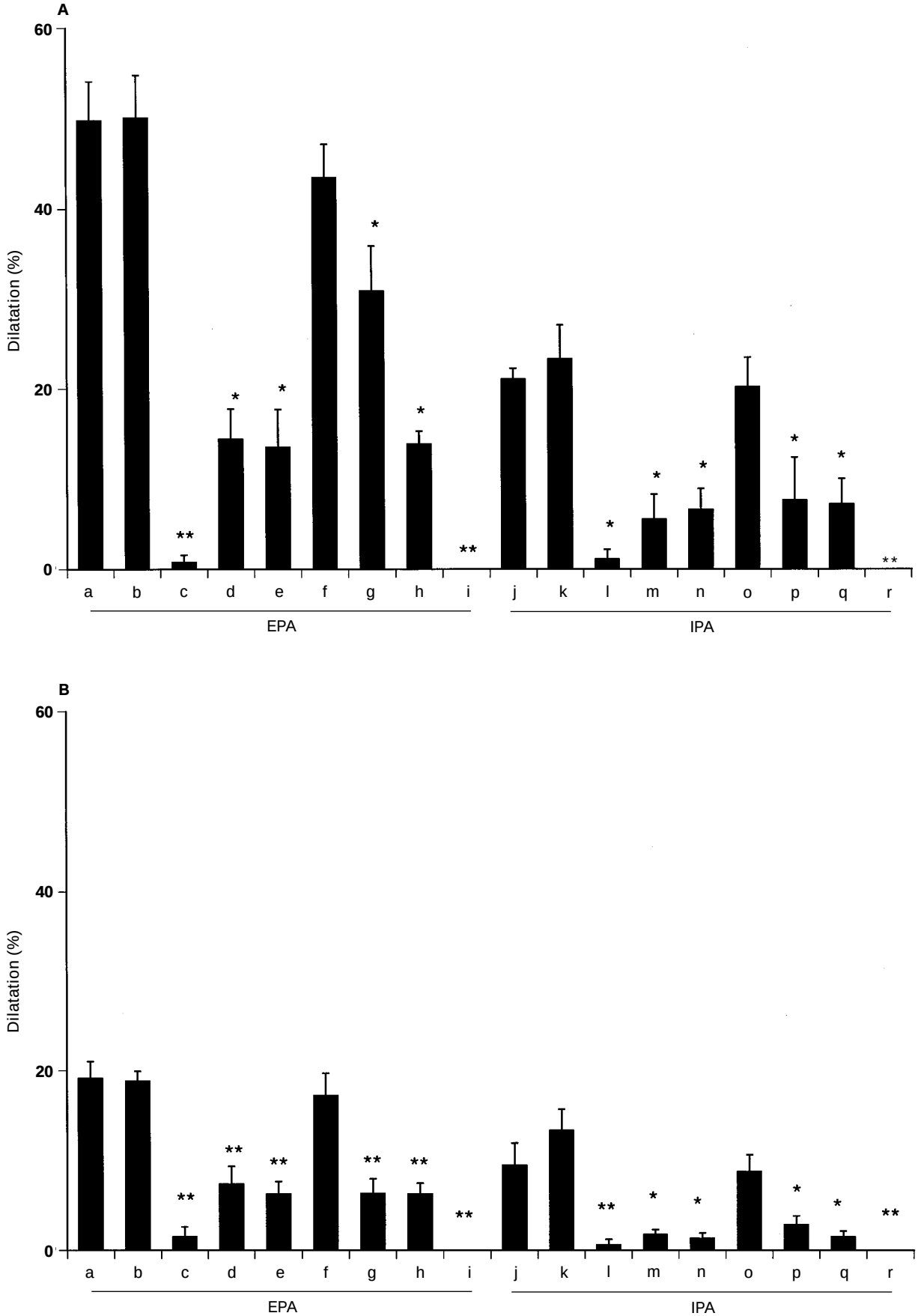

Figure 3 Relaxant effects of (A) 100 nM ET-3 and (B) 100 nM ET-1 in rat extrapulmonary (EPA) and intrapulmonary (IPA) arteries preconstricted with 1 μM phenylphrine. Control (a, j) responses and those in the presence of 3 μM BQ123 (b, k); 3 μM BQ788 (c, l); L-NMMA 300 μM (d, m) or 1 mM (e, n); 1 mM D-NMMA (f, o); 20 μM glibenclamide (g, p); 300 μM L-NMMA + 20 μM glibenclamide (h, q); or endothelium denuded (i, r). The number of preparations in all groups was (A) 4 or (B) 6. Values are mean $\pm$ s.e.mean. Control vs treated groups; $^{*}P<0.05$, $^{**}P<0.01$.

induced response (Figure 2). Our results are consistent with those of Eddahibi *et al*. (1993) who indicated that the vasodilator response to ET-3 was more potent than that to ET-1 in isolated perfused lungs. One possible explanation is that non ET_A and non ET_B-receptor subtypes with a higher affinity for ET-3 exist in the endothelium. In cultured endothelial cells from bovine carotid artery, the presence of specific ET-3 receptor subtype was shown (Emori *et al*., 1990). This could explain why ET-3 exerts a more potent vasodilator activity than ET-1. However, although new receptors have been postulated they have not been demonstrated. Assuming that there are no additional ET receptors, ET-1-induced constriction may counteract the vasodilatation faster and stronger than the ET-3 response. We previously indicated that contractile potency of ET-1 in rat pulmonary arteries is about 10 fold greater when compared to that of ET-3 (Higashi *et al*., 1997). If ET-1 diffuses into the media and binds to receptors on smooth muscle cells faster than ET-3, ET-1-induced vasodilatation may be much more reduced.

In the present study, L-NMMA, a nitric oxide synthase inhibitor, and glibenclamide, a K_{ATP} channel antagonist, partially but significantly suppressed ET_B-mediated vasodilatation in EPA as much as in IPA (Figure 3). These results and other evidence indicate that ET_B activation is involved in the activation of nitric oxide synthase (De Nucci *et al*., 1988; Warner *et al*., 1989a, b) and in the activation of a K_{ATP} channel (Hasunuma *et al*., 1990; Eddahibi *et al*., 1993). The incomplete suppression of the response by L-NMMA and by glibenclamide when compared with that of BQ788 or endothelium removal might be due to additional factors; ET_B-induced vasodilator mechanism(s) unrelated to NOS activation, K_{ATP} channel activation or prostacyclin (PGI_2); since a cyclo-oxygenase inhibitor was added in all our experiments. Eddahibi *et al*. (1993) found that ET-1 induced vasodilatation in the rat isolated lung is reduced by a (large conductance calcium-activated) K^+ channel blocker, tetraethylammonium ion (100 μM) and glibenclamide (100 μM), but not by a selective calcium-activated K^+ channel blocker, charybdotoxin (10 nM) or Na^+-K^+ ATPase inhibitor, ouabain (100 μM). They suggested that the mechanism of ET-1-induced vasodilatation of the rat isolated lungs involved activation of K_{ATP} channels but did not rule out a role of other channels. When L-NMMA and glibenclamide were simultaneously added no further additional inhibitory effects on the vasodilatation were observed. Murphy & Brayden (1995) showed that NO hyperpolarizes vascular smooth muscle in rabbit mesenteric arteries by activating K_{ATP} channels, then they suggested that hyperpolarization due to NO relaxed vascular smooth muscle via activation of K_{ATP} channels. Our results indicate that L-NMMA and glibenclamide suppress ET-induced vasodilatation by inhibiting the same pathway.

We conclude that the vasodilatation evoked by ETs is substantial in isolated extrapulmonary artery but of smaller magnitude in isolated intrapulmonary arteries. The vasodilatation due to activation of endothelial ET_B receptors involves the activation of NOS and ATP-sensitive K^+ channels.

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