Endothelin ETA and ETB Receptors Mediate Endothelin-1-Induced Apamin-Sensitive Relaxation in the Guinea Pig Ileum

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ABSTRACT—Endothelin (ET) receptors involved in ET-1-induced responses of the longitudinal muscle of the isolated guinea pig ileum were studied. ET-1 caused concentration-dependent contractions, while ET-3 and selective ETB-receptor agonists, IRL1620 and sarafotoxin 6c (S6c), showed little or no effect. The ET-1-induced contractions were antagonized by BQ-123, an ETA-receptor antagonist, or PD142893, an ETA/ETB-receptor antagonist, indicating that the contraction is mediated by the ETA receptor. In preparations precontracted with carbachol, ET-1 elicited relaxations at lower concentrations and contractions at higher concentrations. ET-3, IRL1620 and S6c caused relaxations. These relaxations were little affected by BQ-123, but greatly antagonized by PD142893. The ET-1-induced relaxations were slightly affected by BQ-788, an ETB-receptor antagonist, but were markedly inhibited by the combination of BQ-788 and BQ-123. In ETB receptor-desensitized preparations, ET-1-induced relaxations were antagonized by BQ-123, whereas ET-3, S6c and IRL1620 showed no response. All these relaxations were abolished by apamin. These results indicate that ETA and ETB receptors mediate relaxation of the ileal smooth muscle through activation of apamin-sensitive K+ channels.

Keywords: Endothelin, Endothelin receptor, Apamin, Relaxation, Ileal smooth muscle (guinea pig)

Endothelin (ET)-1 is a potent vasoconstrictor peptide isolated from the culture media of porcine aortic endothelial cells (1). ET-1, ET-2 and ET-3 are a family of peptides predicted from three separate genes (2). These three isopeptides have been expressed in many tissues and are known to have a wide spectrum of biological activities in various tissues, including gut smooth muscles. The endothelins exert their effects through at least two distinct ET receptors, ETA and ETB (3). It is known that the ETA receptor is more selective to ET-1 and ET-2 than ET-3, whereas the ETB receptor is equisensitive to all the isopeptides. In vascular tissues, it has been shown that ET-1-induced vasoconstriction is mainly mediated by ETA receptors, although ETB receptors have also been reported to be involved in the vasoconstrictive effects in some blood vessels (3–10).

Abundance of ET-1 and ET-3 in intestinal tissues (11) implies that ET peptides may have physiological roles in gut systems. It has been reported that ET-1 induces biphasic responses, a transient relaxation, followed by a sustained contraction, in the guinea pig ileum (12–16). ET-1 has been shown to be more potent than ET-3 in inducing contraction of the smooth muscle, whereas ET-1 and ET-3 showed similar potency in inducing relaxation, suggesting involvement of ETB receptors in these relaxation responses (14, 15).

Recent development of selective and nonselective ET-receptor antagonists has made it possible to characterize the subtypes of ET receptors more precisely. A study using selective ETB-receptor antagonists indicated that in the guinea pig ileum stimulated by carbachol, the ET-induced relaxations are due to stimulation of ETB-receptor subtypes, including a novel ETB-receptor subtype (ETB2), which is resistant to some of selective ETB-receptor antagonists (17). Since heterogeneity of ET receptors on the same smooth muscle cells may complicate the pharmacological characterization of the receptor subtypes, the present experiments were designed to re-characterize the

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ET-receptor subtypes responsible for ET-induced contractions and relaxations of the longitudinal muscle of the isolated guinea pig ileum by using BQ-123 (cyclo(D-Asp-L-Pro-D-Val-L-Leu-d-Trp)-, an ETA-receptor antagonist); PD142893 (Ac-D-Dip-Leu-Asp-Ile-Ile-Trp, an ETA/ETB-receptor antagonist); and BQ-788 (N-cis-2,6-di-methyl-piperadino-carbonyl-L-γMeLeu-d-Trp(COOMe)-d-Nle-ONa, an ETB-receptor antagonist) (8, 18). The experiments were also performed after ETB-receptor desensitization by treatment with a selective ETB-receptor agonist, sarafotoxin 6c (S6c). To understand the physiological contribution of these receptors, receptor binding characteristics in this smooth muscle and the mechanism of the ET-induced relaxations were also analyzed.

MATERIALS AND METHODS

Measurement of mechanical responses

Male Hartley guinea pigs (Funabashi Farm, Funabashi) weighing 300–400 g were killed by a blow to the head and exanguination. The ileum was removed and rinsed with Krebs solution of the following composition: 118.4 mM NaCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 1.9 mM CaCl2, 4.7 mM KCl, 25.0 mM NaHCO3 and 10.1 mM D-glucose. The longitudinal muscle layer was isolated and the muscle strips (1.5–2.0 cm) were suspended in a 5-ml organ chamber containing the Krebs solution maintained at 37°C and gassed with a mixture of 95% O2 and 5% CO2. The contraction or relaxation of the muscle was measured isometrically with a transducer (TB-611T; Nihon Kohden, Tokyo) under a resting load of 0.3 g and recorded on a polygraph system (RMP-6004, Nihon Kohden). Before starting experiments, the preparations were equilibrated for 60 min. Agonists were added cumulatively to the organ chamber. The contractile responses induced by agonists were expressed in terms of percent of the response to 1 μM acetylcholine. The relaxant responses were examined during the sustained contraction induced by 50 nM carbachol and expressed as percentages of the tension induced by carbachol. Each dose of agonists was applied immediately after the response due to the preceding dose reached its full relaxation level. The ET-induced relaxation was a transient response in the guinea pig ileum, and the cumulative method might give values different from those obtained by a single dose application. However, the present experiments were performed by the cumulative method, because similar results were obtained qualitatively from these two methods in the preliminary experiments in our laboratory on the effects of endothelin receptor agonists and antagonists in the guinea pig ileum. In order to desensitize the ETB receptor, the muscle preparations were first precontracted with carbachol, and after the tension reached the steady level, the preparations were treated with 30 nM S6c for 30 min. This concentration of S6c elicited a transient relaxation, but the tension returned to the original level within 10 min. After pretreatment with 30 nM S6c for 30 min, a second addition of the same amount of S6c caused no response at all. Therefore, when the relaxant effects of the agonists were examined after pretreatment with 30 nM S6c for 30 min, the agonists were applied in the continuous presence of this concentration of S6c. The antagonists were applied 15 min before the addition of the agonists.

[125I]ET-1 binding assay

The ileal longitudinal smooth muscles (6.5 g wet weight) from 4 animals were homogenized with a Polytron-type homogenizer (Physcotron; Nition, Tokyo) in nine volumes of ice-cold 0.25 M sucrose solution containing 20 mM Tris-HCl (pH 7.4), 0.2 mM phenylmethyl-sulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin, 0.1 mM EDTA and 0.5 mM EGTA. The homogenates were centrifuged at 10,000 x g for 10 min and then the supernatants were centrifuged at 20,000 x g for 20 min at 4°C. The resulting pellets were washed three times with the sucrose solution described above. For displacement curves, the membranes were stored in aliquots at −80°C until use. The membranes (5 μg of protein) were incubated at 20°C for 2 hr with 15 pM [125I]ET-1 in the presence or absence of various concentrations of unlabeled ligands in a total volume of 0.56 ml of 20 mM HEPES-buffered salt solution (pH 7.4) containing 140 mM NaCl, 4 mM KCl, 1 mM K2HPO4, 1 mM MgCl2, 1 mM CaCl2, 10 mM D-glucose and 0.1% bovine serum albumin. The reaction was stopped by addition of 0.84 ml of the ice-cold HEPES-buffered salt solution, and then the unbound [125I]ET-1 was separated by centrifuging at 20,000 x g for 20 min at 4°C. The radioactivity in the pellet and the supernatant were measured by a gamma counter. Nonspecific binding was defined as the membrane-associated radioactivity in the presence of 0.1 μM unlabeled ET-1. Nonspecific binding was subtracted from the total binding, and the difference was defined as specific binding. ET-1 was iodinated with Na[125I] (15 Ci/mg; Amersham, Tokyo) by the chloramine T method, and the monoiiodinated [125I]ET-1 was purified by reverse phase high performance liquid chromatography. For Scatchard analysis, binding of [125I]ET-1 to total ETA and ETB receptors was determined with various concentrations of [125I]ET-1, while binding of [125I]ET-1 to ETB receptors was measured in the presence of 0.1 μM BQ-123 to mask the binding sites of the ETA receptor. Dissociation constants (Kd) and maximum binding capacities (Bmax) were calculated with a curve-fitting computer program.
Drugs and chemicals

ET-1, ET-3, S6c and apamin were purchased from Peptide Institute Inc. (Osaka). These peptides were dissolved in phosphate-buffered saline (pH 7.2), containing 0.05% bovine serum albumin. IRL1620 (Suc-[Glu9, Ala11–15]ET-1(8–21)) was obtained from International Research Laboratories, Ciba-Geigy Japan (Takarazuka), and dissolved in 0.01 N NaOH. BQ-123 was purchased from Peninsula Laboratories Inc. (Belmont, CA, USA), and dissolved in absolute ethanol. BQ-788 was obtained from Banyu Pharmaceutical Co. (Tokyo). PD142893 was synthesized at Research Laboratories, Nippon Chemiphar Co., Ltd. (Misato) and dissolved in absolute methanol.

RESULTS

Contraction of ileal smooth muscles

ET-1 (≥3 nM) caused concentration-dependent contractions of the ileal smooth muscles, while ET-3 and selective ETB-receptor agonists, IRL1620 and S6c, showed no effect when applied cumulatively (Fig. 1A). The contractile responses induced by ET-1 were markedly antagonized by 3 nM BQ-123, an ETA-receptor antagonist or 10 nM PD142893, an ETA/ETB-receptor antagonist (Fig. 1B). The contractile responses were not affected by 1 pM tetrodotoxin or 5 μM atropine (data not shown).

Relaxation of ileal smooth muscles

In preparations precontracted with carbachol, cumulative applications of ET-1 caused biphasic responses, relaxations at lower concentrations (0.1–10 nM) and contractions at higher concentrations (Fig. 2A). ET-3 (0.1–10 nM), S6c (0.1–10 nM) and IRL1620 (0.1–100 nM) elicited concentration-dependent relaxations similar to ET-1, but they showed little or no contraction (Figs. 2 and 3). The relaxations induced by these agonists were slightly affected by 3 μM BQ-123. In contrast, all the concentration-response curves for relaxation shifted markedly to the right by 10 μM PD142893, by 2 orders of magnitude (Fig. 3).

After desensitization of ETB receptors by pretreatment with S6c, ET-1 could still induce relaxations, followed by contractions (Fig. 4A), although 30 nM or higher concentrations of S6c showed no response at all, as described above. The EC50 values of ET-1 for the relaxation responses with or without S6c pretreatment were almost the same, 0.25 nM and 0.17 nM, respectively (Fig. 5A). These ET-1-induced relaxations in the preparations pretreated with S6c were greatly antagonized by 3 μM BQ-123 (Figs. 4B and 5A). The relaxation responses in the preparations untreated with S6c were slightly affected by 2 μM BQ-788, an ETB-receptor antagonist, but were markedly suppressed by the combination of BQ-788 and BQ-123 (Fig. 5B).

Mechanism of relaxation

The ET-1-, S6c- and IRL1620-induced relaxations were completely abolished by 0.1 μM of apamin, a small conductance Ca2+-activated K+ channel blocker (Fig. 6: A, B and C). Similarly, the relaxations induced by ET-1 after S6c pretreatment were also abolished by apamin (Fig. 6D). In preparations precontracted with 30 or 40 mM KCl
in place of carbachol, the ET-1-induced relaxations were markedly attenuated or abolished. The relaxations induced by these ET-related peptides were not affected by 0.2 μM of charybdotoxin, a large conductance Ca²⁺-activated K⁺ channel blocker; 10 μM of glibenclamide, an ATP-sensitive K⁺ channel blocker; 10 μM of indomethacin, a cyclooxygenase inhibitor; 100 μM of NΩ-nitroarginine, a nitric oxide synthase inhibitor; 300 μM of suramin, a purinoceptor antagonist; 15 μM of naloxon, an opiate receptor antagonist; and 1 μM of tetrodotoxin (data not shown).

**Binding experiments**

Saturable and specific binding for [¹²⁵I]ET-1 was detected in membranes of the longitudinal smooth muscle of the guinea pig ileum. In competitive binding assays, unlabeled ET-1 showed a monophasic inhibition curve with an IC₅₀ of 61 pM, and similarly unlabeled S6c showed an almost monophasic inhibition curve with an IC₅₀ of 145 pM (Fig. 7), suggesting that most of the [¹²⁵I]ET-1 binding sites were ET₁ receptors.

By Scatchard analysis, binding of [¹²⁵I]ET-1 to total ET₁ and ET₂ receptors showed a Kᵰ of 33.4 pM and Bₚᵰ of 1281 fmol per mg protein, while binding of [¹²⁵I]ET-1 to ET₂ receptors, which was measured in the presence of 0.1 pM BQ-123 to mask the binding sites of ET₁ receptors, showed a Kᵰ of 31.7 pM and Bₚᵰ of 1168 fmol per mg protein, suggesting 91.2% ET₂ receptors and 8.8% ET₁ receptors.

Unlabeled BQ-123 and IRL1620 showed biphasic curves (Fig. 7). About 10% of the [¹²⁵I]ET-1 binding sites was displaced by lower concentrations of BQ-123 (<0.1 μM), and the remaining 90% was displaced by higher concentrations of BQ-123 (>0.1 μM). Correspondingly,
about 90% of the $[^{125}]$ET-1 binding was displaced by lower concentrations of IRL1620 (<30 nM), while the remaining 10% was displaced by higher concentrations of IRL1620 (>30 nM). The Scatchard data and the results from the displacement curves indicate that the $[^{125}]$ET-1 binding sites in this tissue were composed of about 90% ET$_B$ receptors and about 10% ET$_A$ receptors.

DISCUSSION

In the present study, ET-1 caused concentration-dependent contractions of the longitudinal smooth muscle of the guinea pig ileum, while ET-3- and ET$_B$-receptor agonists, IRL1620 and S6c, showed no or very weak contractile effect. The ET-1-induced contractions were markedly antagonized by BQ-123, an ET$_A$-receptor antagonist, or PD142893, an ET$_A$/ET$_B$-receptor antagonist, indicating that the contractions are mediated by ET$_A$ receptors. Since tetrodotoxin and atropine did not affect the ET-1-induced contractions, this contractile effect of ET-1 may be a direct action of ET-1 on the smooth muscle and not due to stimulation of the myenteric plexus or release of acetylcholine from cholinergic neurons.

In preparations precontracted with carbachol, ET-1, ET-3, S6c and IRL1620 caused relaxations at low concentrations with almost the same potency. These relaxations were little affected by BQ-123, but greatly antagonized by PD142893. These results suggest that the ET$_B$ receptor is involved in the relaxations. Recently it has been suggested that ET-induced relaxations of the guinea pig ileal smooth muscle are composed of the initial transient relaxation and the following sustained relaxations, and that these relaxations are mediated by two types of ET$_B$ receptors, because selective ET$_B$-receptor antagonists, IRL1038 and RES-701-1, could inhibit only the transient phase (17, 19). In our experiments, however, the transient and the sustained phases of relaxations were not clearly distinguished, when a single-dose of these ET peptides was applied, and PD142893 could antagonize effectively all the relaxation responses (data not shown).
Further study will be necessary to clarify the involvement of heterogeneous ET$_B$-receptor subtypes in the relaxation responses in the guinea pig ileum.

In the preparations pretreated with S6c, subsequent applications of S6c failed to elicit any responses, indicating that ET$_B$ receptors on the ileal smooth muscle are...
strongly tachyphylactic as previously reported in vascular smooth muscles (9). After ET₂-receptor desensitization with S6c, only ET-1 could induce relaxation, with an EC₅₀ value almost the same as that obtained in the absence of S6c (Figs. 4A and 5A), and the ET-1-induced relaxation was clearly antagonized by BQ-123 (Figs. 4B and 5A), indicating involvement of ET₂ receptors in this case. The relaxation response to ET-1 in the absence of S6c was partly affected by BQ-788, an ET₂ receptor antagonist, but the combination of BQ-123 and BQ-788 was much more effective in inhibiting the relaxation. This synergistic inhibition of the ET-1-induced relaxation by BQ-123 and BQ-788 is in good agreement with the report on the ET-1-induced contraction of the rabbit pulmonary artery (8). These results indicate that the ET-1-induced relaxation of the guinea pig ileum is mediated by both
ET<sub>A</sub> and ET<sub>B</sub> receptors.

Since tetrodotoxin did not affect this relaxation, the relaxant effect of ET-1 appears to be a direct action on the longitudinal smooth muscle. Inhibitory modulation of smooth muscle activity through release of enkephalins, prostanoids, ATP or nitric oxide may not be involved in this relaxation response, because their antagonists and synthase inhibitors were all ineffective.

In the present experiments, the relaxations induced by ET-1 and related peptides were markedly attenuated or abolished in the presence of 30-40 mM KCl, suggesting that the relaxations are caused by activation of K<sup>+</sup> channels. Charybdotoxin or glibenclamide, a known inhibitor of a large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel or an ATP-sensitive K<sup>+</sup> channel, respectively (20, 21), did not affect these relaxations. Therefore the relaxations may not be due to activation of these two types of K<sup>+</sup> channels. Apamin, a blocker of a small conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (20, 21), abolished all the relaxations, in both S6c-treated and untreated preparations in our study. These results strongly suggest that activation of the apamin-sensitive, probably Ca<sup>2+</sup>-activated K<sup>+</sup> channel is essentially involved in the ET-induced relaxations in the guinea pig ileal smooth muscles. It is in agreement with the previous report that endothelins cause activation of the Ca<sup>2+</sup>-activated K<sup>+</sup> channel in the guinea pig ileum (15). Furthermore, the present results indicate that this effect is mediated by ET<sub>A</sub> as well as ET<sub>B</sub> receptors, although it has so far been reported that only the ET<sub>B</sub> receptor is responsible for activation of this K<sup>+</sup> channel (15). The presence of apamin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup> channels in the guinea pig ileum smooth muscle cells has not yet been demonstrated by electrophysiological and single-channel patch-clamp studies, but there have been several pharmacological studies that described involvement of the apamin-sensitive K<sup>+</sup> channels in the contractile effects of apamin and in agonist-induced relaxations of the intestinal smooth muscles (15, 22, 23). Although intracellular Ca<sup>2+</sup> is considered to be a primary regulator of this channel, the activation mechanism of this apamin-sensitive Ca<sup>2+</sup>-activated K<sup>+</sup> channel via ET receptors remains to be determined in more detail.

In the guinea pig ileum, about 80-90% of the ET binding sites is the ET<sub>B</sub> receptor, as shown in this report and others (17, 24). Although ET<sub>B</sub> receptors in some vascular smooth muscles are known to mediate potent vasoconstrictive effects of ETs (4, 8, 10), ET<sub>B</sub> receptors in the ileal longitudinal smooth muscles actually mediated only relaxation, in contrast to the contraction and relaxation responses through ET<sub>A</sub> receptors. These observations raise an interesting question of why the ET<sub>B</sub> receptor is inefficient in mediating contraction in this tissue. Further studies are required to reveal this unique signal transduction mechanism.

In conclusion, our present results demonstrate that ET-1-induced contraction is mediated by ET<sub>A</sub> receptors and that ET<sub>A</sub> and ET<sub>B</sub> receptors mediated ET peptides-induced relaxations in the smooth muscle of the guinea pig ileum.

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**Fig. 7.** Displacement curves of [125I]endothelin-1 (ET-1) binding to the membranes of the guinea pig ileum by unlabeled ET-1 (○), sarafotoxin 6c (□), IRL 1620 (○) and BQ-123 (■). Bars represent ±S.E.M. (n=6).
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