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Heterogenous endothelin receptors mediate relaxation and contraction in the guinea-pig ileum

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Abstract

IRL1620, a specific endothelin ET_B receptor agonist, induced relaxation followed by contraction in the guinea-pig ileum, as did endothelin-1. Both components of the response were concentration-dependent in the range studied. Repeated administration of IRL1620 induced tachyphylaxis only of the contractile component, whereas endothelin-1 desensitized both components. BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]), a specific endothelin ET_A receptor antagonist, did not inhibit the relaxation induced by either agonist, although it did inhibit the contraction induced by endothelin-1, but not by IRL1620. PD145065 (Ac-(D-Bhg-Leu-Asp-Ile-Ile-Trp) (D-Bhg = 5*H*-dibenzyl[*a*,*d*]cycloheptene-10,11-dihydroglycine)), a combined endothelin ET_A/endothelin ET_B receptor antagonist, inhibited the contractile effects of both endothelin-1 and IRL1620 and also inhibited the relaxation induced by IRL1620. Apamin, a Ca^{2+}-activated K^+ channel blocker, inhibited only the endothelin-1-induced relaxation. Our studies suggest that two endothelin ET_B receptor subtypes mediate relaxation in the guinea-pig ileum: one is less sensitive to PD145065 but apamin-inhibitable, and the other is more sensitive to PD145065 but not apamin-inhibitable. Our results also suggest that both endothelin ET_A and endothelin ET_B receptor subtypes mediate contraction in the ileum.

Keywords: Endothelin; IRL1620; Endothelin receptor subtype; PD145065; BQ-123; Tachyphylaxis

1. Introduction

The endothelin/sarafotoxin family comprises the 21-residue peptide isoforms, endothelins 1, 2 and 3 (Yanagisawa et al., 1988; Inoue et al., 1989), sarafotoxins S6a, S6b, S6c and S6d (Kloog et al., 1988; Bdolah et al., 1989) and the vasoactive intestinal contractor polypeptide (Saida et al., 1989). These peptides elicit diverse biological effects on vascular (Marsden et al., 1989; D'Orleans-Juste et al., 1989) and nonvascular (Maggi et al., 1989; Änggård et al., 1990) tissues through at least two distinct receptor types, which have been cloned: the endothelin ET_A receptor, selective for endothelin-1 and endothelin-2, and the endothelin ET_B receptor, equally selective for all three endothelins (Arai et al., 1990; Sakurai et al., 1990). Recently, a receptor for endothelin-3 was also cloned from *Xenopus laevis* dermal melanophores (Karne et al., 1993). However, it is not yet certain whether it actually is the putative endothelin ET_C receptor, which is highly selective for endothelin-3 (Emori et al., 1990) or whether it represents a *Xenopus* variant of the endothelin ET_B receptor.

In blood vessels, both endothelin ET_A and ET_B receptors are present in vascular smooth muscle and may mediate vasoconstriction (Sumner et al., 1992), whereas the endothelin ET_B receptor is expressed in endothelial cells and mediates vasodilator effects through the release of nitric oxide. The endothelin ET_B receptor-dependent vasoconstriction varies between vessels and species, being more apparent in veins than in arterioles (Moreland et al., 1992). In addition to these receptors, there are others in various systems that do not fulfill the current criteria of endothelin ET_A or endothelin ET_B receptors. Thus, there may be subtypes of endothelin ET_B and endothelin ET_A receptors, non-ET_A/non-ET_B endothelin recep-

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tors, atypical endothelin ET_A receptors, etc. (Douglas et al., 1994).

Binding of endothelin to either endothelin ET_A or endothelin ET_B receptors on smooth muscle cells causes phosphatidylinositol (4,5)-biphosphate hydrolysis via G protein-coupled phospholipase C, generating IP_3 and diacylglycerol which ultimately induce or modulate contraction of the muscle. At least two G proteins, differentiated by pertussis toxin sensitivity, appear to mediate endothelin-induced phospholipase C activity. Recently, it was shown that the endothelin ET_B receptor subtype mediating vasoconstriction is pharmacologically distinct from the subtype that is located on the vascular endothelium and mediates vasorelaxation (Douglas et al., 1994). In the endothelium, the activation of endothelin ET_B receptors causes activation of phospholipase C and gating of a non-voltage-dependent Ca^{2+} channel, leading to an increase in $[Ca^{2+}]_i$ and protein kinase C activation. The resulting increase in $[Ca^{2+}]_i$ activates a Ca^{2+}/calmodulin-dependent isoform of nitric oxide synthase inducing release of nitric oxide (EDRF) which relaxes the underlying smooth muscle layer by activating guanylyl cyclase. Protein kinase C, in turn, activates a cascade of Ser/Thr kinases causing activation of mitogen-activated protein kinase (MAPK). MAPK phosphorylates a cytosolic type of phospholipase A_2, liberating arachidonic acid, which is metabolized to prostacyclin and thromboxane A_2.

Less is known about the transduction mechanisms of receptors that do not resemble the typical endothelin ET_A and endothelin ET_B type. Thus, the signal transduction mechanisms activated by endothelin receptors are complex and may evoke diverse signalling pathways by activating multiple G proteins, which in turn may activate a single or multiple effectors, depending on the cell/tissue (for a review see Simonson, 1993; Takuwa, 1993).

Endothelin-3 is known to be a relatively selective endothelin ET_B receptor agonist, whereas sarafotoxin S6c (Williams et al., 1991) and Suc-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21) (IRL1620; Watakabe et al., 1992) are examples of potent and specific agonists for the endothelin ET_B receptor. Recently, it was shown that IRL1620 induces endothelium-dependent relaxation of the rat aorta (Karaki et al., 1993) and rabbit mesenteric artery (Shetty et al., 1993), and that it also induces contraction of guinea-pig trachea (Takai et al., 1992) and porcine coronary artery (endothelium-independent) (Shetty et al., 1993). This agrees with the above-mentioned evidence for two functionally distinct endothelin ET_B receptors in vascular endothelium and smooth muscle.

We have previously shown that endothelin-1 and endothelin-3 induce a biphasic effect in the guinea-pig ileum by activating two different endothelin receptor subtypes: one specific for endothelin-1, possibly an endothelin ET_A receptor, and another, non-selective one, possibly an endothelin ET_B receptor subtype (Miasiro and Paiva, 1990,1992). To better characterize the endothelin ET_B receptor subtype and further investigate the functional heterogeneity of the endothelin receptors in visceral smooth muscle, we have studied the endothelin ET_B-selective agonist, IRL1620, and compared its action to that of endothelin-1 in the guinea-pig ileum. We have also studied the effects of PD145065, a potent combined endothelin ET_A/endothelin ET_B receptor antagonist (IC_{50}: 2.6 nM and 19 nM, respectively, Doherty et al., 1992) and BQ-123, a selective endothelin ET_A receptor antagonist (IC_{50}: 7.3 nM, Ihara et al., 1991), on the responses induced by IRL1620 and endothelin-1 in the guinea-pig ileum.

2. Materials and methods

Guinea-pigs of either sex (200–250 g) were stunned by a blow to the head and bled. A 20-cm portion of the terminal ileum was removed and washed with Tyrode solution at room temperature. Segments of the ileum (3.5–4.0 cm) were mounted in a 5-ml organ chamber containing Tyrode solution maintained at 37°C and bubbled with a constant stream of O_2. The isometric contractions were recorded through a Narco BioSystems force transducer, model F-60, and an ECB model 102-B recorder. The resting tension was adjusted to 1 g.

Unless otherwise stated, the agonists were left in contact with the preparation for 3 min and the time interval between administrations was sufficient to avoid tachyphylaxis. Responses to high concentrations were obtained in separate fresh preparations. At the beginning of each experiment, 60 mM KCl was always applied to elicit a maximal control response. The responses are expressed as percentages of this maximal KCl response.

In competition curves, the tissues were exposed either to PD145065 for 30 min or to BQ-123 for 20 min, prior to the application of the agonists. The concentrations of the antagonists used in this study were based on their foregoing IC_{50} values. In all experiments, parallel control curves were always obtained and in order to diminish variability, all data from the same batch of endothelin-1 were pooled.

For the study of tachyphylaxis, the guinea-pig ileum was submitted to four successive treatments of 3-min duration (at 10-min intervals) with a concentration of the peptide that produced a near-maximum contractile response. Quantitative/qualitative changes of the response observed during the execution of this protocol were indicative of tachyphylaxis.

2.1. Solutions and drugs

The Tyrode solution had the following composition (in mM): NaCl 137; KCl 2.7; $CaCl_2$ 1.36; $MgCl_2$ 0.49; $NaHCO_3$ 11.9; NaH_2PO_4 0.36 and glucose 5.1. The sodium-deficient solution was obtained by isosmotic replacement of the NaCl with D-glucose to give a solution containing 80 mM Na^+. The solutions in the organ bath were stirred with a stream of air and frequently replaced. The pH measured during the experiments was 8.0 ± 0.1.

Synthetic endothelin-1 was purchased from the Peptide Research Institute (Osaka, Japan; batch Nos. 420621, 431003). IRL1620 (Suc-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21), BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]) and PD145065 [Ac-(D-Bhg-Leu-Asp-Ile-Ile-Trp) (D-Bhg = 5*H*-dibenzyl[*a*,*d*]cycloheptene-10,11-dihydroglycine)] were kindly donated by Dr. T. Okada (Ciba-Geigy Japan), by Dr. M. Yano and Dr. M. Ihara (Banyu Pharmaceutical Co.), and by Dr. A.M. Doherty (Parke-Davis Pharmaceutical Division), respectively. Angiotensin II was synthesized by the solid phase method as previously described (Paiva et al., 1974). Apamin was from Sigma Chemical Co. (St. Louis, MO, USA). The inorganic salts were from Merck, Darmstadt, Germany.

All agonists and inhibitors were prepared in aqueous concentrated solution and were diluted with saline (NaCl, 0.9% w/v) before addition to the bath. The maximum volume used for the addition of the peptides to the bath was 200 μl.

2.2. Analysis of the results

The results are presented as the means ± S.E.M. Statistical analysis of the data was performed using Student's *t*-test. *P* values less than 0.05 were considered significant. The concentration-response curves were, in most cases, fitted using InPlot (GraphPad Software), a non-linear regression computer program.

3. Results

3.1. Isometric responses in normal and in low-sodium medium

IRL1620 induced a biphasic effect (a relaxation followed by contraction) in the guinea-pig ileum as did endothelin-1. The concentration-response curve for the contractile component induced by IRL1620 was steeper (slope = 2.55 ± 0.34) than that induced by endothelin-1 (slope = 0.63 ± 0.11, Fig. 1A) and reached only about 40% of the contraction induced by 60 mM KCl, even at the highest concentration tested (400 nM). This component of the response was transient and after washout of the agonist a slow relaxation was observed until a new resting level was reached. In contrast, endothelin-1 induced a maximum contraction similar in amplitude to the maximum KCl response (Fig. 1A), and the relaxation upon washout was slower than that observed in the case of IRL1620.

The relaxation induced by IRL1620 was also concentration-dependent (Fig. 1B) and differed from that induced by endothelin-1 in that it was long-lasting even at high concentrations of the peptide (see Fig. 4A), whereas endothelin-1, at high concentrations, induced a fast, brief relaxation (see Fig. 4B).

When the Na^+ gradient was reduced by exposing the tissues to a low-Na^+ medium, both components of the response induced by 200 nM IRL1620 were greatly reduced. Thus, the contractile component of the response was decreased from 35.8 ± 3.0% (n = 11) in normal medium to 5.0 ± 0.2% (n = 4) in low-Na^+

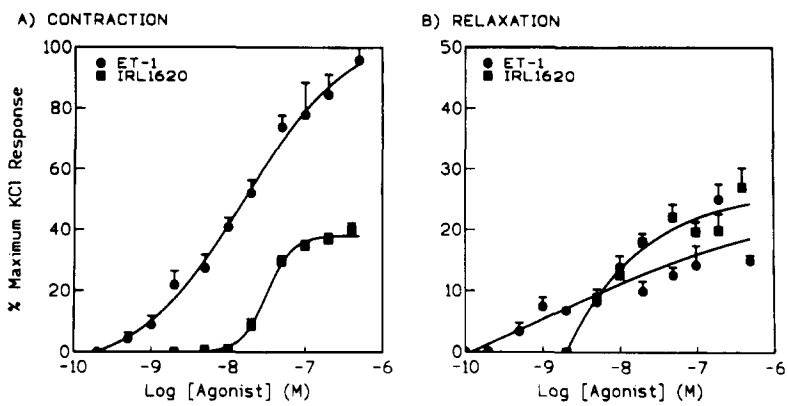

Fig. 1. Concentration-response curves for the contractile (A) and the relaxant (B) components of the guinea-pig ileum responses to IRL1620 and endothelin-1 (ET-1). The response amplitudes of both components are expressed as percentages of the phasic response induced by 60 mM KCl. Each point represents the mean ± S.E.M. of 10–20 experiments. The duration of the exposure was 3 min and the time interval between administrations was sufficient to avoid interference by desensitization. Responses to high concentrations (above 50 nM) were obtained in separate, fresh preparations.

medium ($P < 0.001$) while the relaxant component was decreased from 16.6 ± 3.1% ($n = 11$) to 5.5 ± 1.6% ($n = 4$) ($P < 0.05$).

3.2. Effects of BQ-123, PD145065 and apamin on the responses induced by IRL1620 and endothelin-1

Preincubation of the tissue for 20 min with 1.7 μM BQ-123 did not affect the biphasic response induced by IRL1620 (Fig. 2A and C). When the tissue was preincubated for 30 min with PD145065, in the concentration range 20–200 nM (Fig. 2B) the concentration-response curve for the contraction induced by IRL1620 was shifted to the right, with no change in the maximum response (Schild analysis, $pA_2 = 8.05$). With 2.1 μM PD145065 there was no contractile response to IRL1620 (Fig. 2B). As for the relaxant component of the response, treatment with 20–200 nM PD145065 did not affect significantly the concentration-response curve to IRL1620, whereas a higher PD145065 concentration (2.1 μM) was inhibitory and displaced the concentration-response curve to the right (Fig. 2D).

Endothelin-1 also induced a biphasic effect in the guinea-pig ileum. However, in contrast to IRL1620, both BQ-123 and PD145065 inhibited the contractile component of the response (Fig. 3A and B). In the presence of 34 nM BQ-123, there was a parallel shift of the concentration-response curve towards the right, with no decrease in the maximum response. In contrast, with 340 nM BQ-123 there was a decrease in the maximum contractile response with no additional parallel rightward shift (Fig. 3A). Using a different batch of endothelin-1, in the presence of PD145065 (100 nM and 2.1 μM), however, there was a shift to the right and also a decrease in the maximum contractile response that was about 80% of the maximum KCl response (Fig. 3B). BQ-123 did not inhibit the relaxation phase and PD145065 (2.1 μM) inhibited significantly the endothelin-1-induced relaxation only at the dose of 2×10^{-9} M. BQ-123 and PD145065 increased both the duration (not shown) and the amplitude of the relaxation induced by higher concentrations of endothelin-1 (Fig. 3C and D).

In order to investigate the mechanism underlying the endothelin-induced relaxation, apamin, a blocker of Ca^{2+}-activated K^+ channels, was used. On its own, apamin at 100 nM transiently increased the tonus and the spontaneous contractions of the guinea-pig ileum. Pretreatment with apamin for 20 min had markedly different effects on the responses to endothelin-1 and to IRL1620. In the case of endothelin-1, the relaxant component was completely blocked by 100 nM apamin (from 15.9 ± 1.2% ($n = 7$) to 0.0% ($n = 3$) of the maximum KCl response) while the amplitude of the contractile component was not significantly affected (from 67.1 ± 2.8% ($n = 7$), to 67.4 ± 7.4% ($n = 3$). In contrast, apamin did not inhibit the relaxation induced by IRL1620 but increased it from 20.6 ± 2.3% to 41.3 ± 2.1% of the maximum KCl response ($n = 5$, $P < 0.05$). Apamin also did not significantly decrease the contrac-

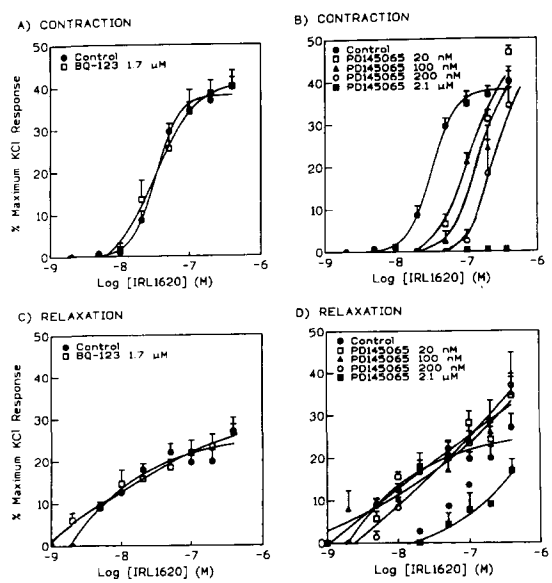

Fig. 2. Effect of preincubation for 20 min with BQ-123 (A, C) or for 30 min with PD145065 (B, D) on the concentration-response curves for the contractile (A, B) and relaxant (C, D) components of the responses induced by IRL1620 in the guinea-pig ileum. Each point represents the mean ± S.E.M. of 3–5 experiments (except for control curves: 10–20 experiments). * Significantly different from control value ($P < 0.05$).

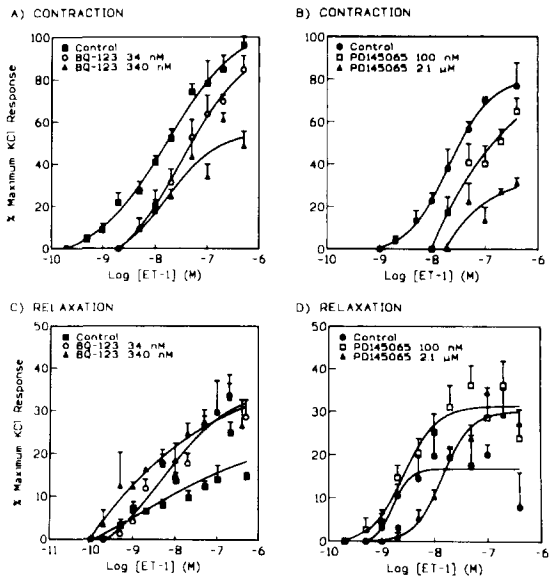

Fig. 3. Effect of preincubation for 20 min with BQ-123 (A, C) or for 30 min with PD145065 (B, D) on the concentration-response curves for the contractile (A, B) and relaxant (C, D) components of the responses induced by endothelin-1 (ET-1) in the guinea-pig ileum. Each point represents the mean ± S.E.M. of 3–5 experiments (except for control curves: 10–20 experiments). * Significantly different from control value ($P < 0.01$).

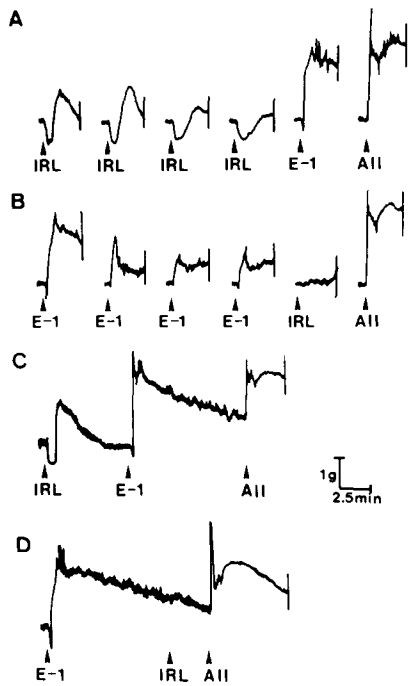

Fig. 4. (A) Effect of repeated treatments with 200 nM IRL1620 (IRL) on the guinea-pig ileum response to subsequent treatments with 100 nM endothelin-1 (E-1) and 100 nM angiotensin II (AII). (B) Effect of four treatments with 100 nM endothelin-1 on the response to subsequent treatments with 200 nM IRL1620 and 100 nM angiotensin II. The time of contact between the agonists and the tissue was 3 min, and the interval between treatments was 10 min. Isometric recordings of the isolated guinea-pig ileum responses to: (C) 400 nM IRL1620 (IRL) followed by the addition of 400 nM endothelin-1 (E-1) and 100 nM angiotensin II (AII); (D) 400 nM endothelin-1 followed by the addition of 400 nM IRL1620 and 100 nM angiotensin II.

tile component induced by IRL1620 (from 35.9 ± 4.5% to 24.9 ± 7.9%, $n = 5$).

3.3. Desensitization

Repeated administration of 200 nM IRL1620, at 10-min intervals, caused unusual tachyphylaxis of the tissue, compared to endothelin-3 and endothelin-1 (Fig. 4B), as evidenced by the decreasing amplitude of the contractile component without any marked alterations in the ability of the tissue to relax (Fig. 4A). This desensitization was specific since 100 nM angiotensin II given after this treatment elicited its characteristic tachyphylactic response upon repeated administration (not shown).

After desensitization of the tissue by repeated exposure to 200 nM IRL1620 at 10-min intervals, further application of 100 nM endothelin-1 induced its normal biphasic response (Fig. 4A), which was not significantly different from the control responses in fresh preparations. In contrast, tissues rendered tachyphylactic by four treatments with 100 nM endothelin-1 did not respond to the addition of 200 nM IRL1620 (Fig. 4B), although angiotensin II was still able to induce a normal response, indicating that the responsiveness of the tissue was intact.

Similar results were obtained in additivity experiments with IRL1620 and endothelin-1 in which one of these agonists was added to the bath after the guinea-pig ileum had reached a stable response to the other agonist. Fig. 4C illustrates an experiment in which, after the transient effect induced by 400 nM IRL1620, the further addition of 400 nM endothelin-1 still elicited a biphasic response. However, when this procedure was reversed, treatment with 400 nM endothelin-1 blocked the response to a subsequent addition of 400 nM IRL1620, while the addition of 100 nM angiotensin II still yielded a normal response (Fig. 4D).

4. Discussion

Similarly to endothelin-1 and endothelin-3 (Miasiro and Paiva, 1990, 1992; Lin and Lee, 1990), IRL1620, a specific endothelin ET_B receptor agonist, induced a biphasic effect (relaxation followed by contraction) in the guinea-pig ileum. The responses to IRL1620 were inhibited in low-Na^+ medium, indicating sensitivity to decreases in the Na^+ gradient across the smooth muscle cell membrane, as was also observed with endothelin-1 and endothelin-3 (Miasiro and Paiva, 1990,1992). Repeated administration of IRL1620 caused unusual tachyphylaxis, consisting mainly of a gradual decrease in the contractile component, leaving the relaxant component almost unaltered. In the case of endothelin-1 and endothelin-3, both components of the response are affected immediately in tachyphylaxis (Miasiro and Paiva, 1990, 1992).

The initial transient relaxant response to IRL1620 was more pronounced than that to endothelin-1. The relaxation was not inhibited by the endothelin ET_A specific receptor antagonist, BQ-123 (Fig. 2C), suggesting that it might be mediated by an endothelin non-ET_A receptor. The endothelin ET_A/endothelin ET_B receptor antagonist, PD145065, inhibited the relaxation at 2.1 μM but not at 20, 100 and 200 nM (Fig. 2D). On the other hand, the transient relaxation induced by endothelin-1 was not inhibited by BQ-123 at any of the concentrations tested, and was inhibited by PD145065 only at the lowest agonist dose, suggesting that this effect is not mediated by an endothelin ET_A receptor subtype. Based on the different behaviour towards the various antagonists, we speculate that different endothelin ET_B receptor subtypes are involved in the relaxant responses to the two agonists studied: an IRL1620-sensitive subtype which is more inhibited by PD145065, and an endothelin-1-sensitive subtype which is less inhibited by PD145065. Our results are consis-

tent with those of Hori et al. (1994), who studied the endothelin-induced relaxation of the carbachol-stimulated guinea-pig ileum. These authors found that the relaxant response to IRL1620 was not inhibited by BQ-123, but was partially inhibited by the endothelin ET_B specific receptor antagonist, IRL1038, indicating that the relaxant response (composed of two phases) was due to two endothelin ET_B receptor subtypes.

Interestingly, Warner et al. (1993b) suggested that the direct contractile effect of endothelin/sarafotoxin peptides in the guinea-pig ileum is due to activation of endothelin ET_A receptors. They also reported that the endothelin ET_B receptor involved in the inhibition of the twitches induced by transmural stimulation was not influenced by BQ-123 or by PD142893 (another endothelin ET_A/endothelin ET_B receptor antagonist), thus differing from the endothelin ET_B receptors on the endothelium which were antagonized by PD142893 (Warner et al., 1993a). In our studies, PD145065, at a concentration of 2.1 μM, did inhibit the relaxation induced by low doses of IRL1620.

The twitch depressor effects of endothelins in the guinea-pig ileum were not affected by indomethacin, 8-(*p*-sulphophenyl)theophylline or glibenclamide, thus suggesting that they do not involve the release of eicosanoids, purines or activation of ATP-sensitive K^+ channels (Wiklund et al., 1991; Guimarães and Rae, 1992). However, endothelin-1 can inhibit acetylcholine release from myenteric nerves stimulated by field stimulation and also enhance exogenous acetylcholine-induced responses (Wiklund et al., 1989, 1991). These effects suggest simultaneous inhibitory pre-junctional and stimulatory post-junctional mechanisms. However, it should be noted that activation of the acetylcholine receptor induces contraction of the guinea-pig ileum and that the biphasic effect induced by endothelins could be observed even in the presence of tetrodotoxin and atropine (Miasiro and Paiva, 1990; Lin and Lee, 1990), suggesting a direct effect on smooth muscle.

It is known that ileal muscle contains dense innervation, most of it on densely packed ganglia of the myenteric plexus, and many enteric neurotransmitters (Makhlouf, 1985). Lin and Lee (1990) demonstrated that the relaxant effect induced by endothelin-1 was not affected by antagonists of α-adrenoceptors (phentolamine, tolazoline), β-adrenoceptors (propranolol), purines (8-phenyl-theophylline) and opiates (naloxone), which are substances that may inhibit ileum tone. Furthermore, the initial relaxation was also not affected by pretreatment of the ileum with L-NAME (N^{ω}-nitro-L-arginine methyl ester), hemoglobin, sodium nitroprusside or methylene blue (Lin and Lee, 1992; Miasiro and Paiva, 1990), indicating that nitric oxide and/or guanosine 3′,5′-cyclic monophosphate (cGMP) mechanisms are not involved in this effect.

The lack of effect of anthracene-9-carboxylic acid on the relaxant response to endothelin-1 suggests that activation of Cl^- channels is not involved in this effect (Lin and Lee, 1992). However, Ca^{2+}-activated K^+ channels appear to contribute to the intestinal relaxation, in view of the inhibitory action of apamin on the endothelin-induced relaxation in the guinea-pig ileum (Lin and Lee, 1992).

In the present studies, we confirmed that apamin inhibits the relaxation induced by the non-specific agonist, endothelin-1, while the relaxation induced by IRL1620 was increased rather than inhibited by this compound. These results suggest that distinct mechanisms of signal transduction are involved in the relaxation triggered by activation of the PD145065-sensitive and -insensitive endothelin ET_B receptors.

Interestingly, apamin itself induced a transient contraction and increased the spontaneous contraction of the ileum, suggesting that apamin-sensitive Ca^{2+}-dependent K^+ channels may be operating in the resting tonus. Bolger et al. (1992), studying guinea-pig ileal longitudinal smooth muscle, found that low concentrations (10^{-10} M) of endothelin-1 reduced the rate of spontaneous contractions, but higher concentrations ($> 10^{-9}$ M) inhibited all spontaneous contraction. Suppression of spontaneous contraction by endothelin-1 was also observed in the guinea-pig taenia coli (Usune et al., 1991) and was related to the activation of apamin-sensitive Ca^{2+}-dependent K^+ channels. These results suggest that endothelin-1 may modulate electrical slow waves, affecting the electrical excitability and rhythmicity of contractions through the activation of Ca^{2+}-dependent K^+ channels. The presence of these channels in the guinea-pig ileum smooth muscle cells has not yet been demonstrated by electrophysiological and single-channel patch-clamp studies, although apamin-insensitive Ca^{2+}-dependent K^+ channels were demonstrated in the membrane of cells from the circular smooth muscle layer of that tissue, where they probably participate in the falling phase and after-hyperpolarization of the action potential (Nakao et al., 1986). Interestingly, Feres et al. (1994) have shown that the hyperpolarizing effect of bradykinin in the guinea-pig ileum was due to the opening of apamin-sensitive Ca^{2+}-activated K^+ channels and that the increase in the contractile response consequent to stretching was probably due to inactivation of these channels.

The contractile component of the response to IRL1620 differed from that elicited by endothelin-1 because it was less sustained and the relaxation after IRL1620 washout was faster. IRL1620 showed less efficacy and its concentration-response curve was steeper than that induced by endothelin-1, suggesting a different mechanism of action. The rank order of contractile potency in which endothelin-1 $\gg$ IRL1620 is compatible with an action at the endothelin ET_A receptor. This conclusion is strengthened by the observa-

tion that the endothelin-1-induced contraction was inhibited by BQ-123 and also by PD145065. Nevertheless, the finding that IRL1620, a selective endothelin ET_B receptor agonist, was inhibited by the endothelin ET_A/endothelin ET_B receptor antagonist, PD145065, and not by the endothelin ET_A-specific receptor antagonist, BQ-123 (Fig. 2A), confirmed that IRL1620 exerts its contractile effect through endothelin ET_B receptors. It is noteworthy that although there was a parallel shift to the right of the concentration-response curve of IRL1620 for the contraction in the presence of PD145065, Schild analysis yielded a shallow slope (-0.46 ± 0.07, $r = -0.987$) and consequently, the calculated pA_2 of 8.05 was greater than that found with rabbit pulmonary artery, where ET_B-like receptors are predominant ($pA_2 = 7.1$, Cody et al., 1993). This result is suggestive of possible ET_B receptor heterogeneity in the guinea-pig ileum.

Cross-tachyphylaxis and additivity experiments also demonstrated these two distinct sites in the guinea-pig ileum. Tissues rendered tachyphylactic to IRL1620 did respond to endothelin-1 (Fig. 4A), and in the presence of IRL1620, endothelin-1 could still induce its own response (as could the positive control angiotensin II), thus revealing its distinct characteristic receptor (Fig. 4C). However, since endothelin-1 is a non-specific agonist, it may act on the IRL1620 site, explaining the lack of response to the latter agonist after pre-treatment of the tissue with endothelin-1 in cross-tachyphylaxis and additivity experiments (Fig. 4B and D). Binding data demonstrating that there is a mixed population of endothelin ET_A and endothelin ET_B receptors in the guinea-pig ileum (Hori et al., 1994) corroborate the above conclusions based on pharmacological data.

In conclusion, our results with guinea-pig ileum indicate that the contractile component of the response induced by endothelins may be mediated by both endothelin ET_A and endothelin ET_B receptor subtypes, whereas the relaxation may be mediated by at least two endothelin ET_B receptor subtypes: (1) an endothelin ET_{B1} receptor that is more selective for IRL1620, is not apamin-inhibitable and is more sensitive to PD145065 but not to BQ-123, and (2) an endothelin ET_{B2} receptor that is more selective for endothelin-1, is apamin-inhibitable, is not sensitive to BQ-123 and is less sensitive to PD145065.

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