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Effects of endothelin ET_B receptor agonists and antagonists on the biphasic response in the ileum [1]

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Abstract

In the guinea-pig ileum, both sarafotoxin S6c and IRL1620 (Suc-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21) induced a concentration-dependent biphasic effect (relaxation and contraction), but distinct tachyphylaxis of the tissue. Cross-tachyphylaxis and additivity experiments evidenced distinct receptors for these agonists. BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]), an endothelin ET_A receptor antagonist, did not affect the response induced by either agonist. PD145065 [Ac-(D-Bhg-Leu-Asp-Ile-Ile-Trp) (D-Bhg = 5*H*-dibenzyl[*a*,*d*]cycloheptene-10,11-dihydroglycine)], an endothelin ET_A/ET_B receptor antagonist, inhibited the contractions induced by IRL1620 and sarafotoxin S6c in competitive and noncompetitive manner, respectively. RES-701-1 [cyclic(Gly^1-Asp^9)(Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)], an endothelin ET_{B1} receptor antagonist, inhibited both components of the response induced by IRL1620, whereas it inhibited mainly the relaxation induced by low sarafotoxin S6c doses. Apamin and suramin had different effects towards the agonists. Our results suggest that two endothelin ET_B receptors with distinct signal transduction mechanism mediate the biphasic response: (1) the endothelin ET_{B1} receptor: sensitive to RES-701-1 and PD145065 and (2) the endothelin ET_{B2} receptor: less sensitive to RES-701-1 and PD145065.

Keywords: Sarafotoxin S6c; IRL1620; Ileum, guinea-pig; Endothelin receptor subtype; Tachyphylaxis

1. Introduction

Sarafotoxins are strong cardiotoxic isotoxins comprising five isopeptides (S6a, S6b, S6c, S6d and bibrotoxin) isolated and sequenced from the venom of the Israeli burrowing asp *Atractaspis engaddensis* and *Atractaspis bibroni* (Kloog et al., 1988; Takasaki et al., 1988; Bdolah et al., 1989; Becker et al., 1993). Bibrotoxin is suggested to be an isoform of the *A. engaddensis* sarafotoxin 6b, with Ala instead of Lys in position 4 (Becker et al., 1993). These sarafotoxins are structurally related to the endothelins, a family of vasoactive peptides consisting of endothelin-1, endothelin-2 (or its mouse variant, the vasoactive intestinal constrictor polypeptide) and endothelin-3 (Yanagisawa et al., 1988; Saida et al., 1989; Yanagisawa and Masaki, 1989). All three endothelin isoforms are expressed in the gut systems where they may have some physiological role (for a review, see Rae et al., 1995). Endothelin-3 is abundant in intestinal tissue (Matsumoto et al., 1989) and vasoactive intestinal constrictor polypeptide, whose gene is expressed only in the intestine, is a potent constrictor of the ileum (Ishida et al., 1989).

Endothelin/sarafotoxin peptides elicit diverse biological effects in several 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, which does not discriminate among the three natural endothelin isoforms (Arai et al., 1990; Sakurai et al., 1990), but is selectively activated by sarafotoxin S6c (Williams et al., 1991) and Suc-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21) (IRL1620; Watakabe et

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al., 1992). Although Southern blot analysis of human DNA reveals the existence of only two endothelin receptor genes (Masaki et al., 1994), probably corresponding to the endothelin ET_A and endothelin ET_B genes, two distinct human endothelin ET_B receptors were generated by alternative splicing from a single gene (Shyamala et al., 1994). Likewise, as a result of differential splicing, the cloning of the endothelin ET_B receptor cDNA from rat brain, revealed that the primary sequence of endothelin ET_B receptors in rat lung and brain differed by several amino acids near the N-terminal region (Cheng et al., 1993). In addition to the well-documented involvement of the membrane-spanning domains of the receptor in ligand binding, it is known that the N-terminal region of the endothelin receptor also plays an important role in forming the ligand binding sites. Thus, considerable differences in ligand–receptor interaction and function may occur between cells/tissues with these distinct endothelin receptor types and subtypes, as well as in the mechanism of signal transduction that might be complex (for reviews, see Simonson, 1993; Ohlstein et al., 1996).

There are several pharmacological data (Warner et al., 1993; Gellai et al., 1996; Ohlstein et al., 1996) indicating the existence of anatomically separate and distinct endothelin ET_B receptor subtypes; an endothelin ET_{B1} receptor on the endothelium that produces the release of nitric oxide to mediate vasodilation and an endothelin ET_{B2} receptor on the vascular smooth muscle that directly mediates vasoconstriction.

Functional studies based on the agonistic and antagonistic selectivity of the endothelin ET_B ligands, also support the existence of distinct endothelin ET_B receptor subtypes within certain tissues. In this regard, Sudjarwo et al. (1994) proposed that the rabbit saphenous vein displays two distinct venoconstrictor endothelin ET_B receptors, designated endothelin ET_{B1} and endothelin ET_{B2} subtypes (in addition to endothelin ET_{A1} and endothelin ET_{A2} receptor subtypes). Although both receptor subtypes are equally sensitive to blockade by BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-norleucine) and activation by sarafotoxin S6c, the endothelin ET_{B1} receptor subtype is selectively blocked by the endothelin ET_{B1} antagonists RES-701-1 and IRL1038 ([cyclic(Gly^1-Asp^9)(Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)] and {[Cys^{11}–Cys^{15}] ET-1 (11–21)}, respectively) and displays greater affinity for the agonist IRL1620 than the endothelin ET_{B2} receptor subtype (Karaki and Matsuda, 1996). This view has been substantiated by similar results in other tissues, such as the guinea-pig trachea (Yoneyama et al., 1995), as well as by the demonstration that endothelin ET_B receptors triggering nitric oxide release from endothelial cells (ET_{B1}-like) are more susceptible to blockade by the mixed endothelin ET_A/ET_B receptor antagonist PD142893 (Ac-(3,3-D-diphenylalanyl)-Leu-Asp-Ile-Ile-Trp trifluoroacetate), than those mediating contraction of the rat stomach or rabbit pulmonary artery (ET_{B2}-like; Warner et al., 1993).

Our previous pharmacological studies (Miasiro and Paiva, 1992; Miasiro et al., 1995b) and binding data (Hori et al., 1994) have demonstrated that there is a heterogenous population of endothelin ET_A and endothelin ET_B receptor types in the isolated guinea-pig ileum mediating its biphasic effect (relaxation and contraction). In order to better evaluate the subtypes of endothelin ET_B receptors and their mediated processes in the guinea-pig ileum, in the present study we further compared the actions of sarafotoxin S6c and IRL1620, selective endothelin ET_B agonists, and evaluated their susceptibility to blockade by BQ-123 (specific endothelin ET_A receptor antagonist, $IC_{50} = 7.3$ nM, Ihara et al., 1991), PD145065 (a potent combined endothelin ET_A/ET_B receptor antagonist, $IC_{50} = 2.6$ nM and $IC_{50} = 19$ nM, respectively, Cody et al., 1993) and the specific natural microbial endothelin ET_{B1} receptor antagonist, RES-701-1, isolated from *Streptomyces* sp. ($IC_{50} = 10$ nM, Tanaka et al., 1994).

2. Materials and methods

Guinea-pigs of either sex (250–350 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. Ileal segments (3.5–4.0 cm) were mounted in a 5-ml organ chamber containing Tyrode solution maintained at 37°C and bubbled with 95% CO_2–5% O_2, pH 7.4. The isometric contractions were recorded under 1 g resting tension, by means of a Narco BioSystems force transducer, model F-60, and an ECB model 102-B recorder.

In a first set of experiments, concentration–response curves to the biphasic effects of sarafotoxin S6c and IRL1620 were obtained by addition of single doses in stepwise increasing concentrations. 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. The responses are expressed as a percentage of the maximal KCl response (60 mM) always given at the beginning of each experiment.

In competition curves for the study of the antagonists, parallel control curves were always carried out. The tissues were exposed either to PD145065 for 30 min or to BQ-123 for 20 min or to RES-701-1 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.

For the study of tachyphylaxis, the guinea-pig ileum was submitted, at 10-min intervals, to three/four consecutive 3-min exposures to near-maximally effective concentration of the agonist. Tachyphylaxis and/or cross-

tachyphylaxis was assessed by the quantitative/qualitative changes in responsiveness of the tissue to each agonist. The additivity experiments were performed with the concentration of the agonist corresponding to its maximal response.

In order to better investigate the mechanism underlying the relaxations induced by the endothelin analogues, we used apamin (a blocker of Ca^{2+}-activated K^+ channels) and suramin (an antagonist of P_{2x} and P_{2y} purinergic receptors) in the final set of experiments.

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 Na^+-deficient solution was obtained by isosmotic replacement of the NaCl with D-glucose to give a solution containing 80 mM Na^+. These solutions were bubbled with a mixture of 95% O_2 and 5% CO_2 at 37°C and pH 7.4.

Synthetic endothelin-1 was purchased from the Peptide Research Institute (Osaka, Japan; batch # 441027) and sarafotoxin S6c was purchased from Peninsula Laboratories (CA, USA; batch # 031491 and # 430506). IRL1620 (Suc-[Glu^9,$Ala^{11,15}$]endothelin-1-(8–21), BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]), 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 (Banyu Pharmaceutical) and by Dr. A.M. Doherty (Parke-Davis Pharmaceutical Division), respectively. Natural RES-701-1 [cyclic(Gly^1–Asp^9)(Gly - Asn - Trp - His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)] isolated from *Streptomyces* sp. (Tanaka et al., 1994) was a generous gift from Dr. Y. Matsuda (Kyowa Hakko). Angiotensin II was synthesized by the solid phase method as previously described (Paiva et al., 1974). Apamin and suramin were from Sigma (St Louis, MO, USA) and Research Biochemicals International (Natick, MA, USA), respectively. The inorganic salts were from Merck, Darmstadt, Germany.

Endothelins and RES-701-1 were reconstituted in HAc 0.1% and dimethylsulfoxide, respectively; the concentrated solutions were further diluted properly 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 nonlinear regression computer program.

3. Results

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

All three endothelin receptor agonists, sarafotoxin S6c, IRL1620 and endothelin-1 induced dose-dependent biphasic responses (i.e., relaxation followed by contraction) in the guinea-pig ileum (Fig. 1A,B). At low concentrations, the selective endothelin ET_B receptor agonists sarafotoxin S6c and IRL1620 induced only relaxations of rather slow onset. Exposure to higher concentrations of either agonist induced faster relaxation and its duration was abbreviated by the onset of the contraction (Fig. 3A). These relaxations were more long lasting than that induced by high endothelin-1 concentrations, which were fast and brief (see Fig. 5, controls).

The concentration–response curve for the contractile component of the sarafotoxin S6c response was shallower than that of IRL1620 (slope: 0.29 ± 0.40 vs. 2.64 ± 0.40, respectively), and both peptides were less potent than endothelin-1 (Fig. 1A). This component of the response was transient for both agonists and tension returned to a new resting level, 5–10 min after peptide washout. In contrast, the amplitude of the maximum contractile response to endothelin-1 was similar to that caused by 60 mM KCl (Fig. 1A, slope: 0.63 ± 0.11), and the relaxation

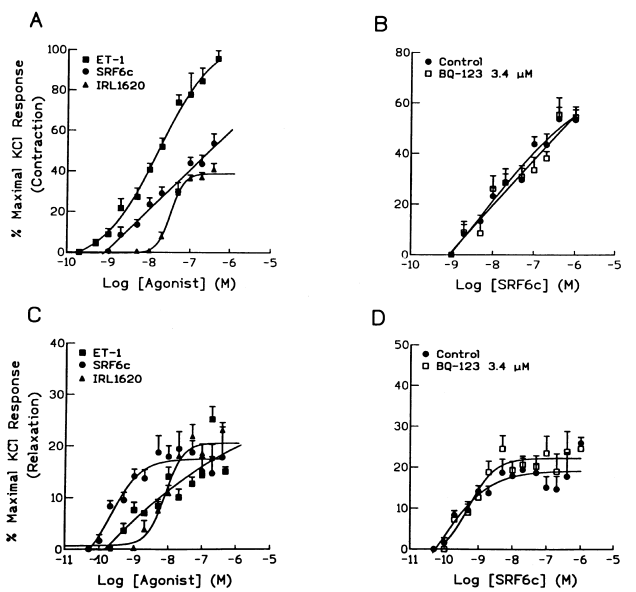

Fig. 1. Concentration–response curve obtained in the isolated guinea-pig ileum for the contractile (A) and relaxant (C) components of the responses to endothelin-1 (■), sarafotoxin S6c (●) and IRL1620 (▲). Effect of preincubation for 30 min with BQ-123 (□) on the contractile (B) and relaxant (D) responses induced by sarafotoxin S6c (control: ●). Each point represents the mean ± S.E.M. of four to seven experiments. Amplitudes of both components are expressed as percentages of the phasic response induced by 60 mM KCl. The duration of the treatments was 3 min and the time interval between administrations was sufficient to avoid interference by desensitization.

after washout was slower (about 15–20 min) than that observed in the case of sarafotoxin S6c and IRL1620 (data not shown).

When the Na^+ gradient was reduced by exposing the tissues to low-Na^+ medium (80 mM), both components of the responses induced by sarafotoxin S6c and IRL1620 (data previously shown; Miasiro et al., 1995b) were greatly reduced. Thus, the contractile component of the response induced by sarafotoxin S6c was decreased from $28.1 \pm 2.0\%$ ($n = 4$) in normal medium to $1.2 \pm 0.8\%$ ($n = 5$) in low-Na^+ medium ($P < 0.001$) while the relaxant component was decreased from $17.1 \pm 1.9\%$ ($n = 4$) to $5.8 \pm 1.1\%$ ($n = 5$) ($P < 0.001$).

3.2. Effects of BQ-123, PD145065 and RES-701-1 on the responses induced by sarafotoxin S6c and IRL1620

Preincubation of the tissue for 20 min with 1.7 μM or 3.4 μM BQ-123 did not affect the biphasic response induced by sarafotoxin S6c (Fig. 1B,D), similarly to what was previously described for IRL1620 (Miasiro et al., 1995b).

When the tissue was preincubated for 30 min with 2.1 μM PD145065, the concentration–response curve for the contraction induced by sarafotoxin S6c was shifted to the right with a decrease of the maximum response (Fig. 2A). Interestingly however, for concentrations of sarafotoxin S6c higher than 200 nM, the response in the presence of PD145065 was complex (see Fig. 5D): there was a fast sharp contraction, followed by a relaxation and another contraction. As for the relaxant component of the response, PD145065 only inhibited it at low concentrations of the agonist (slow relaxation—Fig. 2D).

Preincubation of the tissue with 2.0 μM RES-701-1 (selective endothelin ET_{B1} receptor antagonist) for 20 min did not significantly inhibit the contractile component of the response induced by sarafotoxin S6c (Fig. 2B). At high concentration of sarafotoxin S6c, the response was triphasic in the presence of RES-701-1 (see Fig. 4C) as was observed in the presence of PD145065. Concerning the relaxant component, RES-701-1 was more inhibitory on the responses to low doses of sarafotoxin S6c (Fig. 2E). However, in a few experiments, RES-701-1 was able to inhibit the fast phase of both relaxation and contraction induced by high doses of sarafotoxin S6c.

In the case of IRL1620, RES-701-1 inhibited significantly both components of the response. At 2.0 μM, RES-701-1 caused a pronounced shift to the right of the concentration–response curve for the contractile component (Fig. 2C) and concerning the relaxant component, it inhibited more significantly the responses induced by low doses of IRL1620 (Fig. 2F).

3.3. Tachyphylaxis / desensitization

Repeated administrations of 100 nM sarafotoxin S6c, at 10 min intervals, induced strong tachyphylaxis of both components of the response (Fig. 3B). In contrast, as previously shown (Miasiro et al., 1995b), repeated administrations of 100 nM IRL1620, at 10-min intervals, caused

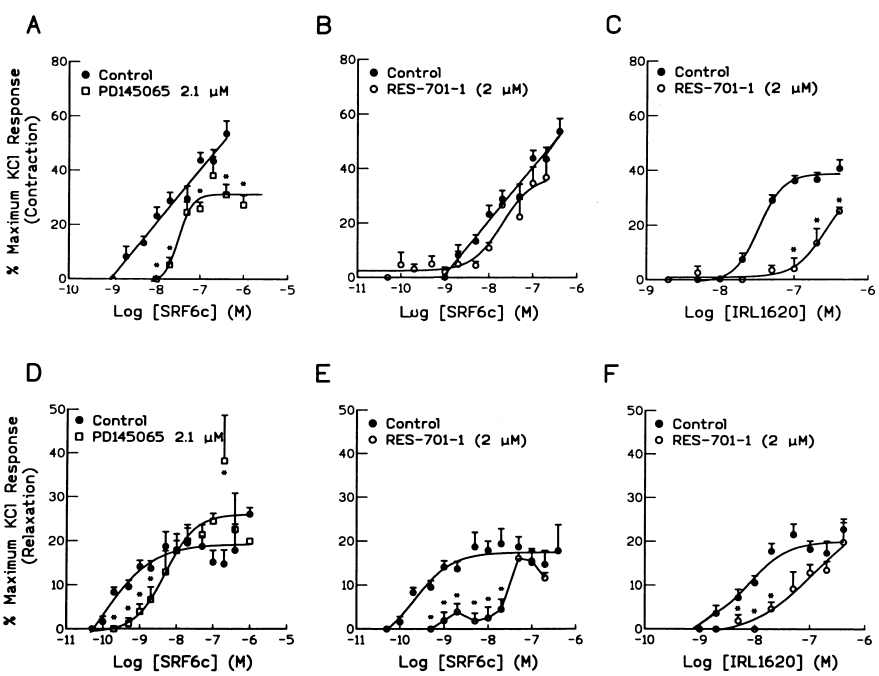

Fig. 2. Effect of preincubation with PD145065 (□) or RES-701-1 (○) on the contractile (upper panel) and relaxant (lower panel) components of the responses induced by sarafotoxin S6c (A, B, D, E—control: ●) and IRL1620 (C, F—control: ●) in the guinea-pig ileum. Each point represents the mean ± S.E.M. of three to seven experiments. Amplitudes of both components are expressed as percentages of the phasic response induced by 60 mM KCl. The duration of the treatment was 3 min and the time interval between administrations was sufficient to avoid interference by desensitization.

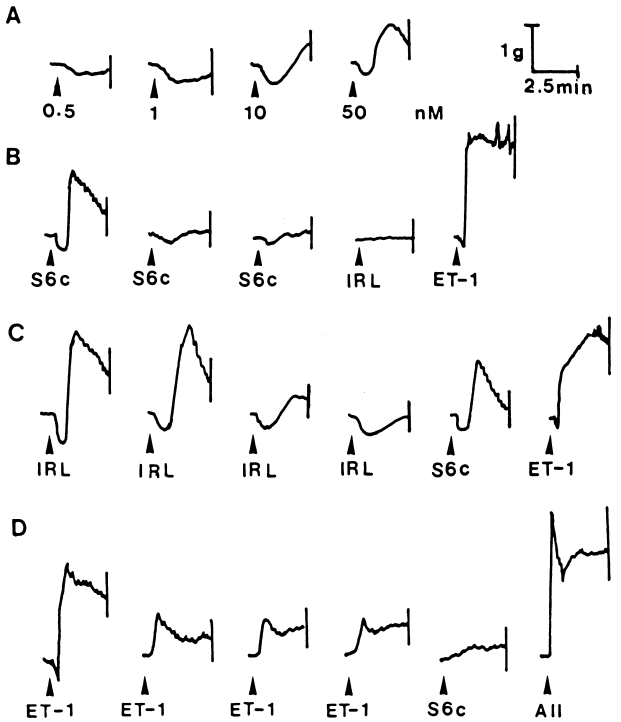

Fig. 3. (A) Isometric responses induced by increasing doses of sarafotoxin S6c on the guinea-pig ileum. (B) Effect of three successive treatments with 100 nM sarafotoxin S6c (S6c) on the responses to subsequent treatments with 100 nM IRL1620 (IRL) and 50 nM endothelin-1 (ET-1); (C) Effect of four successive treatments with 100 nM IRL1620 on the responses to subsequent treatments with 100 nM sarafotoxin S6c and 50 nM endothelin-1. (D) Effect of four successive treatments with 50 nM endothelin-1 on the response to subsequent treatments with 100 nM sarafotoxin S6c and 100 nM angiotensin II (AII). Duration of each treatment was 3 min, and the time interval between treatments was 10 min.

decreasing amplitude of the contractile component without significant alteration of the relaxing component (Fig. 3C). In both cases, desensitization was homologous, since 100 nM angiotensin II given after these treatments could elicit its characteristic tachyphylatic response upon repeated administrations (not shown).

In cross-tachyphylaxis studies, after desensitization of the tissue by repeated exposures to 100 nM sarafotoxin S6c, further application of 100 nM IRL1620 elicited no response, whereas subsequent addition of 100 nM endothelin-1 elicited a normal biphasic response (Fig. 3B). However, biological preparations rendered tachyphylatic to the contractile action of IRL1620 still responded in a typically biphasic manner to sarafotoxin S6c and to endothelin-1 given subsequently (Fig. 3C). On the other hand, four consecutive exposures of the preparation to 100 nM endothelin-1 clearly led to strong tachyphylaxis of both components of the response, and completely blocked the responses to subsequent addition of sarafotoxin S6c (Fig. 3D) or 100 nM IRL1620 (Miasiro et al., 1995b). However, a normal response to further addition of angiotensin II was observed, indicating that the responsiveness of the tissue was intact (Fig. 3D).

Fig. 4 shows somewhat similar results obtained in additivity experiments, in which agonists were added cumulatively at 3–4 min intervals. In the presence of 400 nM IRL1620, addition of 400 nM sarafotoxin S6c elicited a biphasic response, while further application of 100 nM endothelin-1 induced also a normal biphasic response (Fig. 4A). However, in similar additivity experiments, the presence of 100 nM endothelin-1 blocked the response to the subsequent additions of 400 nM sarafotoxin S6c or 400 nM IRL1620, while addition of 100 nM angiotensin II still yielded a normal response (data not shown). Fig. 4B illustrates an experiment in which, addition of 400 nM IRL1620 after the transient effect induced by 400 nM sarafotoxin S6c, elicited no response, but subsequent addition of 200 nM endothelin-1 elicited its characteristic biphasic effect. Similar protocol done in the presence of 3.4 μM BQ-123 resulted only in a biphasic response to sarafotoxin S6c (data not shown). After simultaneous

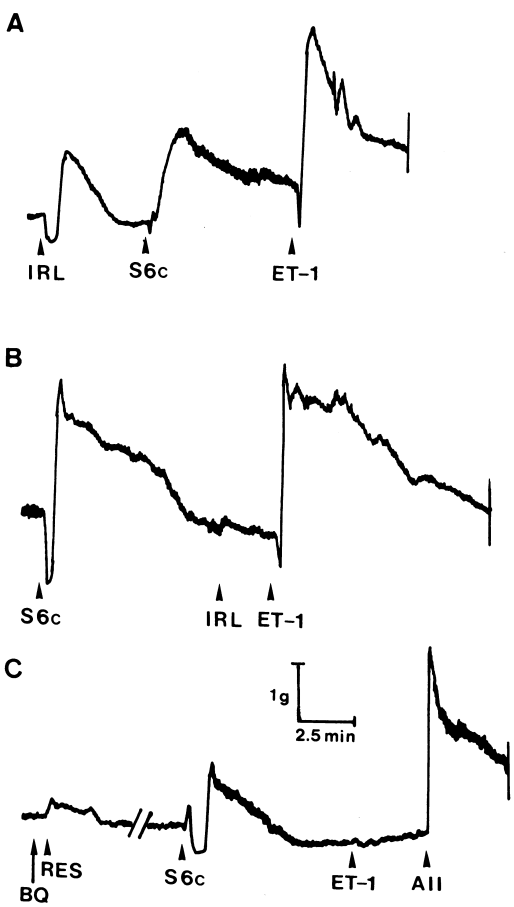

Fig. 4. Isometric responses of the isolated guinea-pig ileum to: (A) 400 nM IRL1620 (IRL) followed by additions of 400 nM sarafotoxin S6c (S6c) and 100 nM endothelin-1 (ET-1); (B) 400 nM sarafotoxin S6c followed by additions of 400 nM IRL1620 and 100 nM endothelin-1; (C) preincubation with 3.4 μM BQ-123 (BQ) and 2 μM RES-701-1 (RES) for 20 min, followed by additions of 400 nM sarafotoxin S6c, 100 nM endothelin-1 and 100 nM angiotensin II (AII). Upward arrows indicate addition of the agonists to the organ bath.

preincubation for 30 min with 3.4 μM BQ-123 and 2.0 μM RES-701-1, addition of 400 nM sarafotoxin S6c elicited a triphasic response of the tissue. In this condition, still in the presence of sarafotoxin S6c, further addition of 100 nM endothelin-1 caused no response, whereas addition of 100 nM angiotensin II elicited its characteristic contractile effect (Fig. 4C).

3.4. Effect of apamin and suramin on the biphasic response

On its own, 100 nM apamin 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 relaxant responses to endothelin-1, sarafotoxin S6c and IRL1620. Whereas the relaxant response to endothelin-1 was completely blocked (Fig. 5A), relaxations induced by IRL1620 and sarafotoxin S6c were not only resistant to inhibition but rather increased in its presence (Fig. 5B,C). Furthermore, the response to sarafotoxin S6c in the presence of apamin was typically triphasic (Fig. 5C), as previously observed in the presence of PD145065 (Fig. 5D) and RES-701-1 (Fig. 4C).

Prior incubation with suramin (450 μM, 40 min beforehand), an antagonist of P_{2x} and P_{2y} purinergic receptors, abolished the relaxation induced by endothelin-1 (Fig. 5A), as well as both components of the response induced by IRL1620 (Fig. 5B). However, rather than inhibiting the relaxant response to sarafotoxin S6c, suramin affected mainly the rapid phases of both relaxant and contractile responses, which became slower (Fig. 5C). Neither apamin (data not shown) nor suramin (Fig. 5D) altered the control contraction induced by angiotensin II (100 nM).

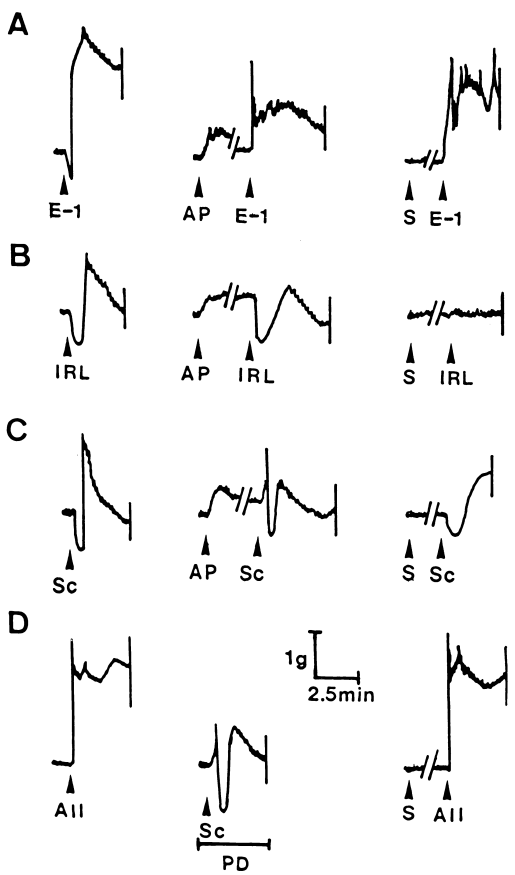

Fig. 5. Effect of preincubation for 20 min with 100 nM apamin (AP) and 450 μM suramin (S), respectively, on the biphasic responses induced by (A) endothelin-1 (E-1), (B) IRL1620 (IRL), (C) sarafotoxin S6c (Sc) and (D) angiotensin II (AII). (D) Effect of preincubation with 2.1 μM PD145065 (PD) on the biphasic response induced by 400 nM sarafotoxin S6c. The control responses of the agonists are shown on the left side of the traces.

4. Discussion

The present experiments demonstrate that in the guinea-pig ileum there is a heterogeneous population of endothelin ET_B receptor subtypes mediating the biphasic response (relaxation followed by contraction) induced by sarafotoxin S6c and IRL1620. This conclusion is evidenced by: (1) different effects of endothelin receptor antagonists; (2) distinct patterns of tachyphylaxis and cross-tachyphylaxis to IRL1620, sarafotoxin S6c and endothelin-1; and (3) different susceptibilities of the agonists-induced responses to modifications by apamin and suramin.

The ileal response to IRL1620 and sarafotoxin S6c, both endothelin ET_B agonists, similarly to other endothelin analogues (Lin and Lee, 1990, 1992; Miasiro and Paiva, 1990, 1992; Miasiro et al., 1993a,b, 1995a,b), were typically biphasic, concentration-dependent and were unaffected by the selective endothelin ET_A receptor antagonist BQ-123. However, their action profiles were affected in distinct ways by the mixed (i.e., nonselective) endothelin ET_A/ET_B receptor antagonist PD145065 (Cody et al., 1993) or the selective endothelin ET_{B1} receptor antagonist RES-701-1 (Karaki and Matsuda, 1996). Whereas the curves to the contractile component of the response to IRL1620 were equally shifted to the right, in a parallel (apparently competitive) fashion, by either PD145065 (Miasiro et al., 1995b) or RES-701-1 (present study), these antagonists exerted complex influences on the responses evoked by sarafotoxin S6c. Thus, PD145065 antagonized the contractile component induced by sarafotoxin S6c in an apparently noncompetitive fashion and RES-701-1, on the other hand, failed to affect the agonist-induced contractile curve. Furthermore, like PD145065, RES-701-1 unmasked a triphasic response of the ileum to high concentrations of sarafotoxin S6c, but not to IRL1620. It is interesting to observe that the same concentration of PD145065 (2.1 μM) completely inhibited the contraction induced by IRL1620 (Miasiro et al., 1995b).

These results are similar to those observed in rabbit pulmonary artery and saphenous vein (Karaki and Matsuda, 1996), in which RES-701-1 inhibits vasoconstriction

induced by IRL1620, but not by sarafotoxin S6c, suggesting the existence of two endothelin ET_B receptor subtypes. Indeed, the existence of two subtypes of the endothelin ET_B receptor is also supported by several other studies using different endothelin receptor agonists and antagonists (Clozel et al., 1992; Warner et al., 1993; Bax and Saxena, 1994; Douglas et al., 1995; Nishiyama et al., 1995).

Further confirmation of these two different endothelin ET_B receptor populations are the cross-tachyphylaxis and additivity experiments, which showed that the desensitization/tachyphylaxis of the receptor depends on which peptide is used for its induction. Cross-tachyphylaxis was observed between sarafotoxin S6c and IRL1620, but not with endothelin-1. However, tissues rendered tachyphylatic to IRL1620 did respond to sarafotoxin S6c and to endothelin-1 given thereafter, indicating additional endothelin sites for these two agonists. Neither agonist caused cross-tachyphylaxis to endothelin-1.

IRL1620 is a linear truncated endothelin analogue, with a modification in position 9 (Glu instead of Lys) and regarding to tachyphylaxis, it behaved differently from other endothelin analogues studied so far. Previous results obtained with Ala^9-endothelin-1 in the guinea-pig ileum have shown that position 9 is important but not essential for tachyphylaxis (Miasiro et al., 1995a). So, our present results strengthen the importance of position 9 on the phenomenon of tachyphylaxis and also corroborate our previous speculation that the endothelin's tachyphylatic domain resides at the N-terminal region of the molecule (Miasiro et al., 1993b). Interestingly, this same portion of the molecule has also been speculated to be critical for retaining the irreversible binding characteristic of the natural endothelins (Nambi et al., 1994) as compared to IRL1620 which showed reversible binding (Watakabe et al., 1992).

The different profiles of tachyphylaxis and cross-tachyphylaxis observed for both selective endothelin ET_B receptor agonists could be explained tentatively by a greater reversibility upon washout of IRL1620 from the endothelin ET_B receptor, as compared to sarafotoxin S6c, due to its structural difference. However, this possibility seems to be remote in view of the results of our series of additivity experiments, in which the preparations were exposed consecutively and cumulatively (i.e., without washouts in between additions) to IRL1620 and sarafotoxin S6c, or vice versa. Again, prior exposure to sarafotoxin S6c abolished responses to IRL1620, whereas the former still elicited significant (albeit smaller) relaxation and contraction when added after the latter. In both experiments, further addition of endothelin-1 could elicit its own biphasic response. These results demonstrate that the population of endothelin ET_B receptors in the guinea-pig ileum available for interaction with IRL1620 represents only a fraction of that which can be activated by sarafotoxin S6c, and further strengthen the view of two endothelin ET_B receptor subtypes in this tissue, in addition to endothelin-1-sensitive ET_A receptors.

All these results suggest that, besides the endothelin ET_A receptor, there are two distinct populations of endothelin ET_B receptors mediating the contractile effect in the guinea-pig ileum: one population of receptors more specific for IRL1620, sensitive to RES-701-1 and PD145065, and another one, specific for sarafotoxin S6c with less sensitivity to RES-701-1 and PD145065.

Concerning the relaxant component of the responses induced by sarafotoxin S6c and IRL1620, our results show that they are due also to activation of two endothelin ET_B receptors with distinct signal transduction mechanism as they were not inhibited by the endothelin ET_A specific receptor antagonist BQ-123, had different behaviour towards the endothelin antagonists and were distinctly affected by apamin and suramin.

In this way, 2.1 μM PD145065 (Miasiro et al., 1995b) and RES-701-1 (present studies) shifted to the right the concentration response curve for the IRL1620-induced relaxation, whereas they inhibited mainly the slow relaxation induced by low sarafotoxin S6c doses.

At low concentrations, both agonists induced only slow relaxations, however, at high concentrations of the agonists, which cause faster relaxation and also a contraction, RES-701-1 could inhibit some sarafotoxin S6c-induced relaxation, giving rise to a triphasic response. Furthermore, in some experiments, RES-701-1 could inhibit the fast phases of the relaxation and contraction induced by high doses of sarafotoxin S6c.

Depending on the agonist concentration range studied, it is possible that the concentration-related divergence of cellular responses to the agonists/antagonists may be due to different receptors that could result in the activation of distinct signalling systems; this is evidenced by our present results performed in order to investigate the relaxation mechanism.

Apamin, a small conductance Ca^{+2}-dependent K^+ channel blocker, inhibited only the endothelin-1-induced relaxation, but not the S6c- and IRL1620-induced relaxations, suggesting that two or more conductances may mediate these relaxant responses. In the endothelin-1-induced relaxation, there is a possible activation of Ca^{2+}-activated K^+ channels, as previously seen by Lin and Lee (1992) and Miasiro et al. (1995a). However, in the case of sarafotoxin S6c, its triphasic response observed not only in the presence of apamin, but also in the presence of PD145065 and RES-701-1, is indicative that in its complex relaxation there is at least one apamin-sensitive component, whose inhibition may lead to a fast contraction.

Interestingly, we have already observed previously (Miasiro et al., 1995a) that apamin also inhibited the relaxation induced by low doses of ATP and transformed it into a phasic contraction in the guinea-pig ileum, suggesting possible involvement of purines in this relaxation process. Like apamin, suramin, a P_{2x} and P_{2y} receptor

antagonist, inhibited the relaxation induced by endothelin-1 and the rapid phase of the relaxation induced by sarafotoxin S6c. However, it inhibited the relaxation as well as the contractile effect induced by IRL1620. It is interesting to observe that in the guinea-pig ileum longitudinal muscle, Shan et al. (1996) observed that all the relaxation induced by endothelin-1, sarafotoxin S6c and IRL1620 were abolished by 0.1 μM apamin, but were not affected by 300 μM suramin.

There are P_{2x}- (ligand-gated) and P_{2y}-(G-protein-coupled) purinoceptor families in several systems (Fredholm, 1994) and indeed, purinoceptors have been detected in intestinal smooth muscle, in intestinal ganglia and in endothelial and smooth muscle cells of vessels supplying the gastrointestinal tract (Hoyle and Burnstock, 1991; Burnstock et al., 1994). Endothelin-1 and endothelin-3 are both localized and expressed in the myenteric and submucous plexuses and nerve fibre bundles of the intestine, maybe having a paracrine role in neurotransmission or in modulation of neuronal function (Eaker et al., 1995). Our present results with suramin may well suggest the involvement of purinoceptors in the physiological and/or pathophysiological modulatory actions of the endothelins in the gastrointestinal systems.

Altogether, our results suggest that besides the endothelin ET_A receptor, there are two distinct populations of endothelin ET_B receptors mediating the biphasic effect in the guinea-pig ileum through distinct mechanisms of signal transduction: (1) the endothelin ET_{B1} subtype: sensitive to RES-701-1 and PD145065, specific for IRL1620 and non-specific to sarafotoxin S6c; and (2) the endothelin ET_{B2} subtype: less sensitive to RES-701-1 and PD145065, specific for sarafotoxin S6c.

The existence of distinct endothelin ET_B receptor subtypes is evidenced by the available pharmacological data, but better understanding of their mechanisms of action awaits further studies and new results from molecular cloning.

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