ELSEVIER

European Journal of Pharmacology 278 (1995) 103–109

ejp

Role of positions 9 and 10 in the endothelin molecule for biological activity and discrimination of receptor subtypes

Nobuco Miasiro [a,*], Roberto De Castiglione [b,1], Antonio C.M. Paiva [a]

[a] *Department of Biophysics, Escola Paulista de Medicina, C.P. 20.388, 04034 São Paulo, SP, Brazil*
[b] *Pharmacia Farmitalia Carlo Erba, Via Giovanni XXIII 23, Nerviano (MI), Italy*

Received 25 October 1994; revised 13 February 1995; accepted 14 February 1995

Abstract

The importance of residues 9 and 10 in endothelin-1 was assessed by studying the responses of the guinea-pig ileum to [Ala^9]endothelin-1 and [Ala^{10}]endothelin-1. Both analogues induced relaxation followed by contraction. [Ala^9]Endothelin-1 showed similar ED_{50} values and maximum response to those of endothelin-1, whereas [Ala^{10}]endothelin-1 showed a larger ED_{50} value and was a partial agonist. Endothelin-1 and [Ala^{10}]endothelin-1 induced similar degrees of tachyphylaxis, whereas [Ala^9]endothelin-1 induced very little tachyphylaxis, indicating that Lys^9 is important for inducing tachyphylaxis. Apamin inhibited the relaxation induced by endothelin-1 and [Ala^9]endothelin-1 but not that induced by [Ala^{10}]endothelin-1. BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu), a specific endothelin ET_A receptor antagonist, inhibited [Ala^9]endothelin-1-, but not [Ala^{10}]endothelin-1-induced contraction. Cross-tachyphylaxis and additivity studies indicated that [Ala^9]endothelin-1, like endothelin-1, acts at the endothelin ET_A receptor, whereas [Ala^{10}]endothelin-1 behaved as an endothelin ET_B receptor agonist, like sarafotoxin S6c. Thus, the residue at position 10 plays a significant role in receptor activation and is a candidate for further exploration of receptor antagonism.

Keywords: Endothelin; Alanine analog; Receptor subtype; Ileum, guinea-pig; Sarafotoxin S6c; Tachyphylaxis

1. Introduction

The endothelin/sarafotoxin family of peptides comprises endothelin-l, endothelin-2 and endothelin-3 (Yanagisawa et al., 1988; Inoue et al., 1989) as well as the sarafotoxins SRF6a, SRF6b, SRF6c and SRF6d (Kloog et al., 1988; Bdolah et al., 1989) and the vasoactive intestinal contractor polypeptide (Saida et al., 1989), a mouse variant of endothelin-2, expressed only in the intestine. These isopeptides produce pleiotropic actions through multiple endothelin receptor subtypes that are widely distributed in many tissues (Yanagisawa and Masaki, 1989). A receptor subtype (ET_A), selective for endothelin-1, as well as a non-selective subtype (ET_B), have been cloned from the cDNA library, and shown to belong to the superfamily of heptahelical G-protein-coupled receptors (Arai et al., 1990; Sakurai et al., 1990). There is some evidence for a third subtype, the ET_C receptor (selective for endothelin-3), located primarily in brain and in endothelial cells, although its cDNA clone has not been isolated (Samson et al., 1990). All endothelin/sarafotoxin peptides consist of 21 amino acid residues with two intra-chain disulfide bridges and a common hydrophobic tail (residues 16–21). The most important difference between them resides at the N-terminal region, within the sequence of the Cys^3-Cys^{11} inner loop. The sequence Asp^8-Lys^9-Glu^{10} is conserved, except for the substitution of Glu^9 for Lys^9 in sarafotoxin S6c, a highly selective endothelin ET_B receptor agonist (Williams et al., 1991). Sarafotoxin S6c is the most acidic peptide of the family, and its low relative potency suggests that Lys^9 is very important for the vasoconstrictor activities of the sarafotoxin/endothelin peptides (Kitazumi et al., 1990).

* Corresponding author. Departamento de Biofísica, Escola Paulista de Medicina, Rua Botucatu, 862, 04023-062 São Paulo, SP, Brazil. Tel. 55-11-572-4583, fax 55-11-571-5780, e-mail ACM-PAIVA@BIOFIS.EPM.BR

[1] Presently retired.

SSDI 0014-2999(95)00109-3

We have previously found that endothelin-1 and endothelin-3 induce a biphasic effect (relaxation followed by contraction) on the isolated guinea-pig ileum, an effect which is characterized by a homologous desensitization and which is markedly dependent on the Na^+ gradient across the smooth muscle cell membrane. Cross-tachyphylaxis and additivity experiments indicated that there are at least two distinct receptor subtypes in the guinea-pig ileum: one selective for endothelin-1, possibly an endothelin ET_A receptor, and another one, non-selective, possibly an endothelin ET_B receptor, where endothelin-3 preferentially acts and induces desensitization (Miasiro and Paiva, 1990, 1992). In the present paper we have investigated the importance of positions 9 and 10 for the action of endothelin-1, by substituting alanine for the original amino acid in the endothelin-1 molecule, and comparing the effects of the new analogues with those previously described for endothelin-1 and endothelin-3 (Miasiro and Paiva, 1990, 1992).

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 which contained Tyrode solution maintained at 37° C and was bubbled with a constant stream of O_2. Recordings of isotonic contractions were made under 1-g load, on smoked drums, using frontal levers with 6-fold amplification. The isometric contractions were recorded through a Narco BioSystems force transducer, model F-60, and an ECB model 102-B recorder. Resting tension was adjusted to 1 g.

Unless otherwise noted, the agonists were left in contact with the preparation for 3 min and the time interval between administrations was sufficient to avoid interference by desensitization. Responses to high concentrations were obtained in separate fresh preparations. At the beginning of each experiment, 60 mM KCl was always applied to elicit the maximum control response, and the responses are expressed as percentages of this maximal KCl response. In competition experiments, the tissues were exposed to BQ-123 for 20 min prior to the application of the agonists.

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 measured pH during the experiments was 8.0 ± 0.1.

Synthetic endothelin-1 was from the Peptide Research Institute (Osaka, Japan; batch No. 420621); sarafotoxin S6c was from Peninsula Lab. (California, USA, batch No. 031491) and BQ-123 (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu) was kindly donated by Drs. M. Yano/M. Ihara (Banyu Pharmaceutical Co.). Angiotensin II (Paiva et al., 1974), [Ala^9]endothelin-1 and [Ala^{10}]endothelin-1 (De Castiglione et al., 1992) were synthesized by the solid phase method. Apamin was from Sigma Chemical Co. (St. Louis, MO, USA), and inorganic salts were from Merck (Darmstadt, Germany).

2.2. Analysis of the results

The results are presented as means ± S.E.M. Statistical analysis of the data was done by means of Student's *t*-test. *P* values less than 0.05 were considered

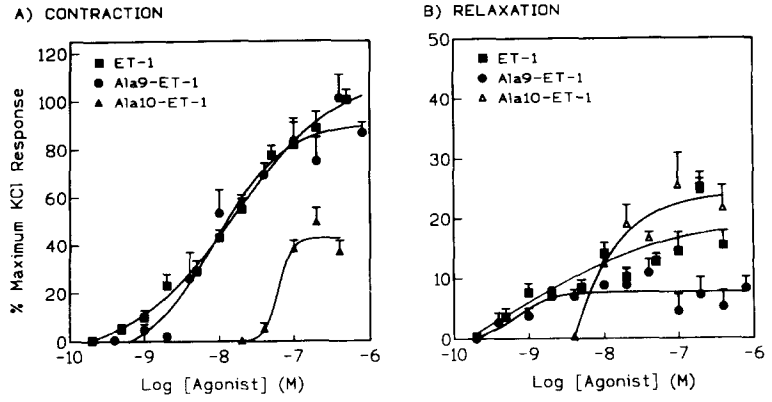

Fig. 1. Concentration dependence of the contractile (A) and of the relaxant (B) components of the responses of the guinea-pig ileum to endothelin-1, [Ala^9]endothelin-1 and [Ala^{10}]endothelin-1. Each point represents the mean ± S.E.M. of 4–7 experiments. Response amplitudes of both components are expressed 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.

significant. The concentration-response curves were in most cases fitted using InPlot (GraphPad Software), a non-linear regression computer program.

3. Results

3.1. Effect of [Ala^9]endothelin-1 on the guinea-pig ileum

In the guinea-pig ileum, [Ala^9]endothelin-1 elicited a biphasic effect (relaxation followed by contraction) that was concentration-dependent (Fig. 1). The contractile component of the response attained about 100% of the maximal response induced by KCl and had the same ED_{50} as endothelin-1 (Fig. 1A). The relaxing component was pronounced and long lasting at low concentrations, but at high concentrations it was overwhelmed by the contraction, resulting in a brief relaxation of rapid onset, as was observed with endothelin-1 (see Figs. 3A and 5A, Miasiro and Paiva, 1990).

When the Na^+ gradient was reduced by exposing the tissues to low-Na^+ medium, we observed that both components of the response induced by 400 nM [Ala^9]endothelin-1 were greatly reduced. The contractile component of the response was significantly reduced from 96.6 ± 9.5% ($n = 4$) in normal medium to 33.3 ± 6.7% ($n = 4$) in low-Na^+ medium ($P < 0.01$) and the relaxant component was reduced from 5.2 ± 2.7% ($n = 4$) to 1.6 ± 0.9% ($n = 4$).

Upon repeated administration, at regular intervals, [Ala^9]endothelin-1 induced less tachyphylaxis than endothelin-1. Thus, the isotonic response to the fourth consecutive dose in relation to the first dose was about 55% for [Ala^9]endothelin-1 (Fig. 2A), and about 25% for endothelin-1 (Fig. 2B). When the responses were recorded isometrically, tachyphylaxis was manifested by qualitative changes in the response to the following doses, which consisted of a decrease of the relaxant component, resulting in a faster onset of the contractile component of the response (Fig. 3A).

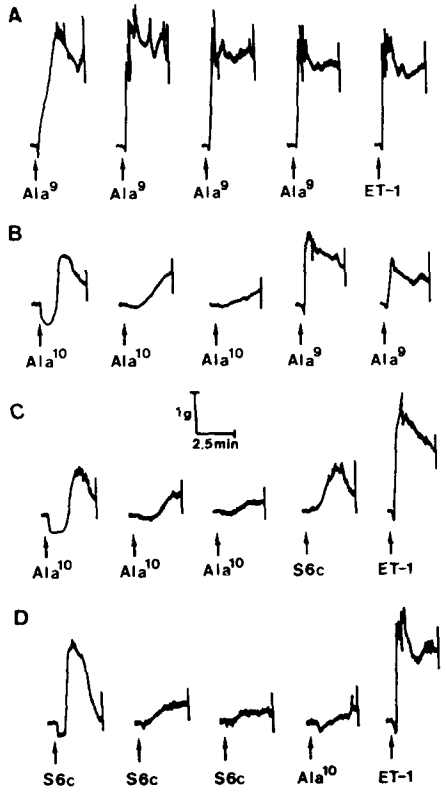

Fig. 3. Isometric recording of the responses induced by: (A) four successive administrations of 200 nM [Ala^9]endothelin-1 (Ala^9) followed by further addition of 100 nM endothelin-1 (ET-1); (B) three successive administrations of 200 nM [Ala^{10}]endothelin-1 (Ala^{10}) followed by two successive treatments with 100 nM [Ala^9]endothelin-1; (C) three successive administrations of 200 nM [Ala^{10}]endothelin-1 followed by subsequent additions of 100 nM sarafotoxin S6c (S6c) and 100 nM endothelin-1; (D) three successive administrations of 100 nM sarafotoxin S6c, followed by further consecutive additions of 200 nM [Ala^{10}]endothelin-1 and 100 nM endothelin-1. Time of contact of the agonists with the tissue was 3 min, and the time interval between treatments was 10 min. Upward arrows indicate addition of the agonist to the organ bath.

In tissues rendered tachyphylactic to [Ala^9]endothelin-1 (Fig. 2A) or to endothelin-1 (Fig. 2B), application of endothelin-1 or [Ala^9]endothelin-1, respectively,

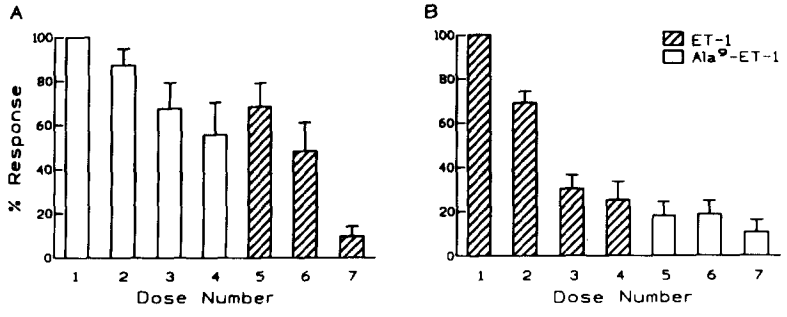

Fig. 2. Isotonic recordings of the responses of the isolated guinea-pig ileum to: (A) four successive treatments with 200 nM [Ala^9]endothelin-1 followed by three successive treatments with 100 nM endothelin-1; (B) four successive treatments with 100 nM endothelin-1 followed by three successive treatments with 200 nM [Ala^9]endothelin-1. In both cases, the first response to the agonist was taken as 100%. Contact time of the agonist with the tissue was 2 min, and the time interval between treatments was 5 min.

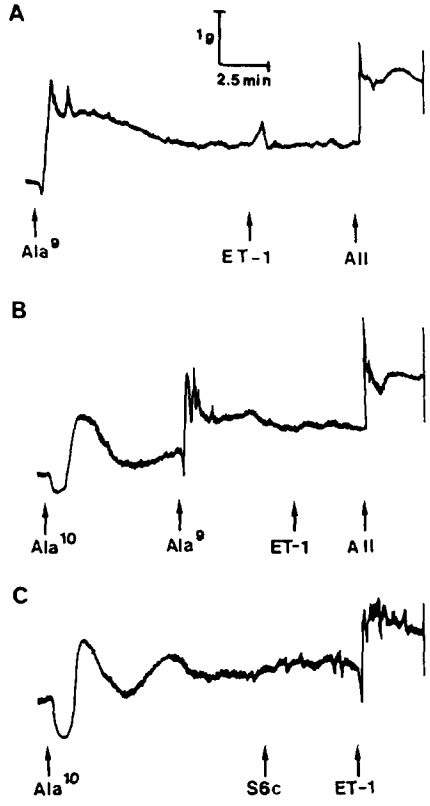

Fig. 4. Isometric recordings of the responses of the isolated guinea-pig ileum to: (A) 400 nM [Ala^9]endothelin-1 (Ala^9) followed by addition of 400 nM endothelin-1 (ET-1) and 100 nM angiotensin II (AII); (B) 400 nM [Ala^{10}]endothelin-1 (Ala^{10}) followed by addition of 400 nM [Ala^9]endothelin-1, 100 nM endothelin-1 and 100 nM angiotensin II; (C) 400 nM [Ala^{10}]endothelin-1 followed by addition of 200 nM sarafotoxin S6c (S6c) and 100 nM endothelin-1. The response to 60 mM KCl was always recorded 15 min before the addition of the peptides, as a control (not shown). Upward arrow indicates addition of the agonists to the organ bath.

induced a smaller response, indicating cross-tachyphylaxis. As endothelin-1 is more tachyphylactic than [Ala^9]endothelin-1, strong cross-tachyphylaxis was observed in the case of endothelin-1 (Fig. 2B). However, in the case of [Ala^9]endothelin-1 in isometric recordings (Fig. 3A), we observed that further addition of endothelin-1 induced almost the same response pattern as that of [Ala^9]endothelin-1 at that tachyphylactic level.

In additivity experiments, we observed that during the responses to [Ala^9]endothelin-1 (Fig. 4A) or to endothelin-1 (not shown) addition of endothelin-1 or [Ala^9]endothelin-1, respectively, could not induce any effect, whereas further addition of angiotensin II could elicit its normal response (Fig. 4A).

3.2. Effect of [Ala^{10}]endothelin-1 on the guinea-pig ileum

[Ala^{10}]Endothelin-1 also induced a concentration-dependent biphasic response (Fig. 1). The relaxant component of this response was more pronounced than that to [Ala^9]endothelin-1 and endothelin-1 (Fig. 1B), and was present even at high concentrations of the peptide (Fig. 3B), as seen also with sarafotoxin S6c (Fig. 3D). [Ala^{10}]Endothelin-1 was a partial agonist, the contractile response attaining only about 40% of the maximum KCl response (Fig. 1A).

When the Na^+ gradient was reduced by exposing the tissues to low-Na^+ medium, the contractile component of the response induced by 200 nM [Ala^{10}]endothelin-1 was completely inhibited, from $39.7 \pm 4.8\%$ ($n = 8$) in normal medium to $0.0 \pm 0.0\%$ ($n = 4$) in low-Na^+ medium ($P < 0.001$), and the relaxant component was reduced from $23.3 \pm 1.7\%$ ($n = 8$) to $6.3 \pm 2.2\%$ ($n = 4$) ($P < 0.001$).

Repeated administration of [Ala^{10}]endothelin-1 induced strong tachyphylaxis, as it did with sarafotoxin S6c (Fig. 3B and D), both components of the response being affected. Cross-tachyphylaxis studies between these two compounds showed that tissues rendered tachyphylactic to [Ala^{10}]endothelin-1 or to sarafotoxin S6c did not respond normally to sarafotoxin S6c (Fig. 3C) or to [Ala^{10}]endothelin-1 (Fig. 3D), respectively, given subsequently. However, endothelin-1 could still induce its biphasic effect in tissues rendered tachyphylactic to [Ala^{10}]endothelin-1 (Fig. 3C) or to sarafotoxin S6c (Fig. 3D). Similarly, a normal response to [Ala^9]endothelin-1 was observed in tissues rendered tachyphylactic to [Ala^{10}]endothelin-1 (Fig. 3B).

In additivity experiments, we observed that, in the presence of [Ala^{10}]endothelin-1, [Ala^9]endothelin-1 induced its biphasic effect, whereas further addition of endothelin-1 could not induce any response, but angiotensin II given subsequently could induce its normal characteristic response (Fig. 4B). However, when sarafotoxin S6c was added after the biphasic effect induced by [Ala^{10}]endothelin-1, it did not elicit its response, whereas endothelin-1 given thereafter could induce its characteristic biphasic effect (Fig. 4C).

3.3. Effect of apamin and BQ-123 on responses induced by [Ala^9]endothelin-1 and [Ala^{10}]endothelin-1

Apamin, a blocker of Ca^{2+}-activated K^+ channels, was used to investigate the mechanism underlying the endothelin-induced relaxation. On its own, apamin at 100 nM transiently increased the tonus and spontaneous contractions of the guinea-pig ileum. Pretreatment with 100 nM apamin for 20 min had markedly different effects on the responses to endothelin-1 and the alanine analogues (Fig. 5). In the case of [Ala^9]endothelin-1, the relaxant component was completely blocked by apamin, whereas the amplitude of the contractile component of the response was not significantly affected, but appeared faster (Fig. 5B). Similar behaviour was observed with endothelin-1 (not shown).

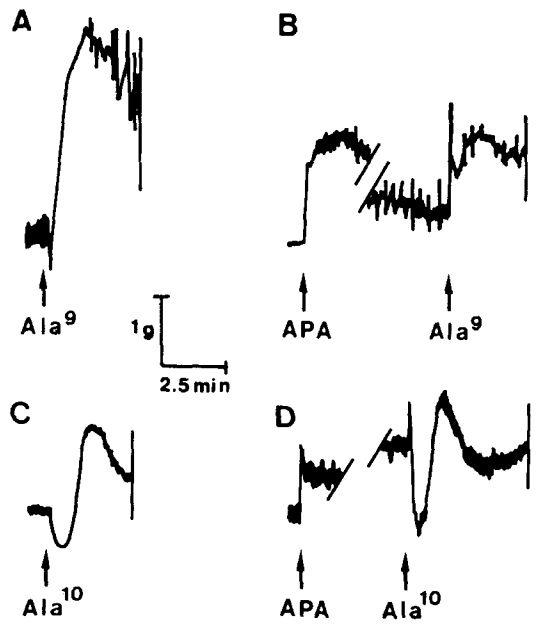

Fig. 5. Effect of 100 nM apamin (APA), preincubated for 20 min, on the response induced by: (B) 50 nM [Ala^9]endothelin-1 (Ala^9); (D) 200 nM [Ala^{10}]endothelin-1 (Ala^{10}). (A) and (C) show the respective control responses. Upward arrows indicate addition of the agents, and the broken lines indicate interruption of the recorder.

In contrast, the relaxation induced by [Ala^{10}]endothelin-1 was potentiated by apamin. Indeed, its response in the presence of apamin was complex, compared to the control (Fig. 5C): a sharp phasic contraction preceded the relaxation that was followed by a decreased contraction (Fig. 5D). A similar complex response was observed in the case of sarafotoxin S6c, a preferential endothelin ET_B receptor agonist (not shown).

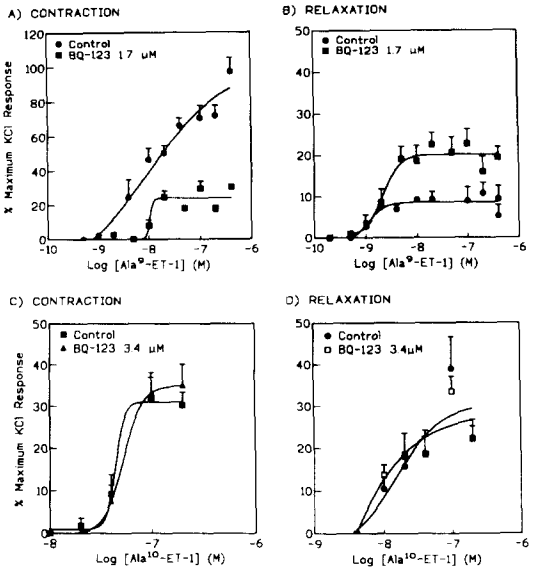

Fig. 6. Effect of pretreatment with BQ-123 for 20 min on the contractile component of the responses induced by [Ala^9]endothelin-1 (A) and [Ala^{10}]endothelin-1 (C), and on the relaxant component of the responses induced by [Ala^9]endothelin-1 (B) and [Ala^{10}]endothelin-1 (D). Each point represents the mean ± S.E.M. of 4–9 experiments.

Preincubation with 1.7 μM BQ-123 for 20 min shifted the concentration-response curve for the contractile component induced by [Ala^9]endothelin-1 to the right and also decreased the maximum response (Fig. 6A). The relaxing component was not inhibited but was rather increased in the presence of BQ-123 (Fig. 6B). In contrast, in the case of [Ala^{10}]endothelin-1, preincubation with 3.4 μM BQ-123 for 20 min did not inhibit either the contractile or the relaxant response (Fig. 6C and D).

4. Discussion

Our studies with the guinea-pig ileum demonstrate that, similarly to endothelin-1 (Miasiro and Paiva, 1990), [Ala^9]endothelin-1 induces a biphasic effect (relaxation followed by a contraction) which is dependent on the sodium gradient across the cell membrane. The relaxant component of the response was inhibited by apamin, indicating that Ca^{2+}-dependent K^+ channels are involved in the relaxation. BQ-123, a specific endothelin ET_A receptor antagonist, inhibited the contractile component similarly to what was seen in the case of endothelin-1 (Miasiro et al., 1993a), suggesting that it might be mediated by an endothelin ET_A receptor. [Ala^9]Endothelin-1 also resembled endothelin-1 in that repeated administration induced tachyphylaxis, which was characterized by a decrease in the amplitude of the isotonically recorded contractions, or by a decrease in the relaxant component and an increase in the rate of development of the contractile component recorded isometrically. Repeated treatment with either [Ala^9]endothelin-1 or endothelin-1 rendered the tissues equally tachyphylactic to both peptides (cross-tachyphylaxis), and additivity experiments showed that, during the response to one of them, the response to the other was impaired, indicating that [Ala^9]endothelin-1 and endothelin-1 interact with the same endothelin ET_A receptor subtype. Thus, our present results demonstrate that substitution of Lys^9 by Ala^9 in the endothelin-1 molecule did not affect the biological activity and functional properties of the peptide. Indeed, Hunt et al. (1991) have already suggested that Lys^9 is a tolerant site, since endothelin-1's activity on rat pulmonary artery is unaffected by leucine replacement at this position (Nakajima et al., 1989). Our results also agree with the finding that a D-Lys^9 substitution resulted in a more potent agonist than endothelin-1 (Galantino et al., 1992). Interestingly, however, [Ala^9]endothelin-1 induced less tachyphylaxis than endothelin-1, indicating that position 9 in the endothelin-1

molecule, although not important for biological activity, is important (though not essential) for inducing that phenomenon, thus supporting our previous speculation (Miasiro et al., 1993b) that the N-terminal portion is the tachyphylactic domain of the endothelin-1 molecule.

Concerning [Ala^{10}]endothelin-1, it also induced a biphasic effect, showed sodium dependency and tachyphylaxis, but its relaxing effect was not inhibited by apamin, as was also observed in the case of sarafotoxin S6c. [Ala^{10}]Endothelin-1 did not cross-react with [Ala^{9}]endothelin-1 in cross-tachyphylaxis experiments (Fig. 3B) and could induce its biphasic effect in the presence of [Ala^{10}]endothelin-1 in additivity experiments (Fig. 4B), suggesting that [Ala^{10}]endothelin-1 acts on a site distinct from the endothelin ET_A receptor. This view is strengthened by the fact that BQ-123, a specific endothelin ET_A receptor antagonist (Ihara et al., 1991), at 3.4 μM did not affect significantly the concentration-response curve for the contractile component induced by [Ala^{10}]endothelin-1. Furthermore, we observed cross-reactivity between sarafotoxin S6c and [Ala^{10}]endothelin-1 (Fig. 3C and D), but not to endothelin-1 (Fig. 3D) and, in additivity experiments, no response was observed to sarafotoxin S6c added during the response induced by [Ala^{10}]endothelin-1, whereas endothelin-1 given subsequently could elicit its own biphasic effect, due to the endothelin ET_A receptor (Fig. 4C). These results confirm that [Ala^{10}]endothelin-1 is an endothelin ET_B receptor agonist as it acts on the same receptor as sarafotoxin S6c, a known endothelin ET_B receptor agonist (Williams et al., 1991). These results, therefore, indicate that there is also an endothelin ET_B receptor subtype mediating contraction in the guinea-pig ileum.

Our results show that substitution of Glu^{10} by Ala^{10} resulted in large changes in the functional activity of the endothelin-1 molecule, transforming a non-specific agonist into a specific endothelin ET_B receptor agonist. [Glu^{9}]Sarafotoxin S6b, a chimeric peptide, is also an endothelin ET_B receptor agonist (Takayanagi et al., 1991), but this peptide contains the same Asp^{8}-Glu^{9}-Glu^{10} sequence found in sarafotoxin S6c, suggesting that not only position 9 is critical for functional activity. Indeed, Mahé et al. (1993) have shown that [Lys^{9}]sarafotoxin S6c retains its endothelin ET_B receptor agonistic activity. It is interesting to observe that the Ala^{10} substitution also led to a loss of potency and efficacy of the peptide. However, it should be noted that [Ala^{9}]endothelin-1's concentration-response curve overlapped that for endothelin-1 and that [Ala^{10}]endothelin-1's concentration-response curve almost also overlapped that for IRL1620 (unpublished results), strengthening the conclusion that [Ala^{10}]endothelin-1 is really an endothelin ET_B receptor agonist.

Concerning the relaxant component of the responses, apamin inhibited the relaxations induced by [Ala^{9}]endothelin-1 and by endothelin-1, but not those induced by [Ala^{10}]endothelin-1 and sarafotoxin S6c. Interestingly, in the presence of apamin, the responses to [Ala^{10}]endothelin-1 and to sarafotoxin S6c consisted of a rapid phasic contraction followed by increased relaxation and a small contraction (Fig. 5B). The reason for this complex response is unclear and needs to be further investigated.

It has been reported that, in the guinea-pig ileum, the relaxant response to endothelin-1 is due to activation of Ca^{2+}-activated K^+ channels, as it is inhibited by apamin (Lin and Lee, 1992), and inhibition of cholinergic neuronal activity (Wiklund et al., 1989). Our present studies confirm the results for endothelin-1 and extend them to [Ala^{9}]endothelin-1, which shares the same receptor subtype. However, [Ala^{10}]endothelin-1, like sarafotoxin S6c, behaves as an endothelin ET_B receptor agonist, and our results with apamin suggest that they both induce relaxation by triggering a signal transduction mechanism distinct from that of endothelin-1. ATP-induced hyperpolarization in the guinea-pig taenia caeci is via apamin-sensitive Ca^{2+}-activated K^+ channels (Maas and Den Hertog, 1979), and, interestingly, we have observed that exogenously applied ATP indeed induced relaxation of the guinea-pig ileum, and that its effect became a phasic contraction in the presence of apamin (unpublished observation).

In the presence of BQ-123 the relaxation induced by [Ala^{10}]endothelin-1 was not affected, whereas the relaxation induced by [Ala^{9}]endothelin-1 was increased rather than inhibited, maybe due to the decrease in the contractile component, as was observed in the case of endothelin-1 (Miasiro et al., 1993a). The lack of inhibition of the relaxation induced by the two analogues by BQ-123 suggests that this component may be due to an endothelin non-ET_A receptor, possibly an endothelin ET_B receptor.

Altogether, our studies demonstrate that: (a) *concerning receptor subtypes*, both endothelin ET_A and ET_B receptor subtypes may mediate contraction in the guinea-pig ileum, whereas there are at least two endothelin ET_B receptors mediating relaxation, one inhibited by apamin and another one not inhibited by apamin; (b) *concerning structural requirements for activity*, position 9 is important for tachyphylaxis and for binding whereas position 10 plays a significant role in receptor activation and is, therefore, a good candidate for further exploration of possible receptor antagonists.

Acknowledgements

This work was supported by grants from FINEP (No. 43.87.1044.00), CNPq (No. 303855/85-1 RN) and FAPESP (No. 93/4800-3), and done with the technical

assistance of Nelson A. Mora. We are grateful to Dr. M. Ihara and Dr. M. Yano (Banyu Pharmaceutical Co., Ltd.) for the gift of BQ-123.

References

Arai, A., S. Hori, I. Aramori, H. Ohkubo and S. Nakanishi, 1990, Cloning and expression of a cDNA encoding an endothelin receptor, Nature 348, 730.

Bdolah, A., Z. Wollberg, G. Fleminger and E. Kochva, 1989, SRFX-d, a new native peptide of the endothelin/sarafotoxin family, FEBS Lett. 256, 1.

De Castiglione, R., J.P. Tam, W. Liu, J.-W. Zhang, M. Galantino, F. Bertolero and F. Vaghi, 1992, Alanine scan of endothelin, in: Peptides, eds. J.A. Smith and J.E. Rivier (ESCOM Sci. Publ., Leiden) p. 402.

Galantino, M., R. De Castiglione, J.P. Tam, W. Liu, J.-W. Zhang, C. Cristiani and F. Vaghi, 1992, D-Amino acid scan of endothelin, in: Peptides, eds. J.A. Smith and J.E. Rivier (ESCOM Sci. Publ., Leiden) p. 404.

Hunt, J.T., V.G. Lee, P.D. Stein, A. Hedberg, E.C.-K. Liu, D. McMullen and S. Moreland, 1991, Structure-activity relationships of monocyclic endothelin analogs, Bioorg. Med. Chem. Lett. 1, 33.

Ihara, M., T. Fukuroda, T. Saeki, M. Nishikibe, K. Kojiri, H. Suda and M. Yano, 1991, An endothelin receptor (ET_A) antagonist isolated from *Streptomyces misakiensis*, Biochem. Biophys. Res. Commun. 178, 132.

Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto and T. Masaki, 1989, The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes, Proc. Natl. Acad. Sci. USA 86, 2863.

Kitazumi, K., T. Shiba, K. Nishiki, Y. Furukawa, C. Takasaki and K. Tasaka, 1990, Structure-activity relationship in vasoconstrictor effects of sarafotoxins and endothelin-1, FEBS Lett. 260, 269.

Kloog, Y., I. Ambar, M. Sokolovsky, E. Kochva, Z. Wollberg and A. Bdolah, 1988, Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain, Science 242, 268.

Lin, W.-W. and C.Y. Lee, 1992, Intestinal relaxation by endothelin isopeptides: involvement of Ca^{2+}-activated K^+ channels, Eur. J. Pharmacol. 219, 355.

Maas, A.J. and A. Den Hertog, 1979, The effect of apamin on the smooth muscle cells on the guinea-pig taenia coli, Eur. J. Pharmacol. 58, 151.

Mahé, E., S. Jouquey, M. Fortin, M. Harnois, B. Schoot, G. Hamon and D. Le-Nguyen, 1993, Synthesis and biological activities of some endothelin analogs, in: Peptides 1992, eds. C.H. Schneider and A.N. Eberle (ESCOM Sci. Publ., Leiden) p. 683.

Miasiro, N. and A.C.M. Paiva, 1990, Effects of endothelin-1 on the isolated guinea-pig ileum: role of Na^+ ions, Naunyn-Schmied. Arch. Pharmacol. 342, 706.

Miasiro, N. and A.C.M. Paiva, 1992, Effects of endothelin-3 on the isolated guinea-pig ileum: role of Na^+ ions and endothelin receptor subtypes, Eur. J. Pharmacol. 214, 133.

Miasiro, N., A.C.M. Paiva, M. Ihara and M. Yano, 1993a, Two receptor subtypes are involved in the contractile component of the guinea-pig ileum responses to endothelins, J. Cardiovasc. Pharmacol. 22 (Suppl. 8), S211.

Miasiro, N., C.R. Nakaie and A.C.M. Paiva, 1993b, Endothelin (16–21): biphasic effect and no desensitization on the guinea-pig isolated ileum, Br. J. Pharmacol. 109, 68.

Nakajima, K., S. Kubo, S. Kumagaye, H. Nishio, M. Tsunemi, T. Inui, H. Kuroda, N. Chino, T.X. Watanabe, T. Kimura and S. Sakakibara, 1989, Structure-activity relationship of endothelin: importance of charged groups, Biochem. Biophys. Res. Commun. 163, 424.

Paiva, T.B., G. Goissis, L. Juliano, M.E. Miyamoto and A.C.M. Paiva, 1974, Angiotensin-like and antagonistic activities of N-terminal modified [8-leucine] angiotensin II peptides, J. Med. Chem. 17, 238.

Saida, K., Y. Mitsui and N. Ishida, 1989, A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family, J. Biol. Chem. 264, 14613.

Sakurai, T., M. Yanagisawa, Y. Takuwa, K. Miyazaki, S. Kimura, K. Goto and T. Masaki, 1990, Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor, Nature 348, 732.

Samson, W.K., K.D. Skala, B.D. Alexander and F.L.S. Huang, 1990, Pituitary site of action of endothelin-selective inhibition of prolactin release in vitro, Biochem. Biophys. Res. Commun. 169, 737.

Takayanagi, R., K. Kitazumi, C. Takasaki, K. Ohnaka, S. Aimoto, K. Tasaka, M. Ohashi and H. Nawata, 1991, Presence of non-selective type of endothelin receptor on vascular endothelium and its linkage to vasodilation, FEBS Lett. 282, 103.

Wiklund, N.P., C.U. Wiklund, A. Ohlen and L.E. Gustafsson, 1989, Cholinergic modulation by endothelin in guinea-pig ileum, Neurosci. Lett. 101, 342.

Williams, Jr., D.L., K.L. Jones, D.J. Pettibone, E.V. Lis and B.V. Clineschmidt, 1991, Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes, Biochem. Biophys. Res. Commun. 175, 556.

Yanagisawa, M. and T. Masaki, 1989, Molecular biology and biochemistry of the endothelins, Trends Pharmacol. Sci. 10, 374.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature 332, 411.