European Journal of Pharmacology, 174 (1989) 23–31
Elsevier

EJP 51082

The activity of peptides of the endothelin family in various mammalian smooth muscle preparations

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Received 31 July 1989, revised MS received 13 September 1989, accepted 3 October 1989

The activity of natural endothelins such as ET-1, ET-2, ET-3 and of sarafotoxin S6b (SRFTX) as compared to that of the C-terminal hexapeptide ET-(16-21) was investigated in several smooth muscle preparations in the presence of indomethacin, captopril, bestatin and thiorphan. In some tissues (rat thoracic aorta, guinea-pig ileum, human urinary bladder, renal pelvis or renal artery), ET-(16-21) had little if any agonist activity. In other preparations (guinea-pig bronchus, rat vas deferens, rabbit pulmonary artery) ET-(16-21) was a full agonist. The amidated form of ET-(16-21) was either inactive or less potent than ET-(16-21). These findings provide evidence that at least two receptors exist for peptides of the ET family; these receptors were termed ET_A and ET_B. ET-(16-21) is a full agonist at ET_B receptors while being inactive or weakly active at ET_A receptors. The free acid of tryptophan in position 21 plays a key role in the activity of these peptides at ET_B receptors.

Endothelin; Bronchi (guinea-pig); Aorta (rat); Endothelin receptors; Endothelin-(16-21)

1. Introduction

The endothelins constitute a family of 21-amino acid peptides recently discovered on the human genome (Yanagisawa et al., 1988a,b; Inoue et al., 1989), and have been designated ET-1, ET-2 and ET-3 (ET = endothelin). ET-1 is identical to porcine endothelin, which was originally isolated as a product of cultured aortic endothelial cells (Yanagisawa et al., 1988b), while ET-3 has the same sequence as the peptide discovered on the rat genome (Yanagisawa et al., 1988a).

Endothelins are potent vasoactive agents in various isolated blood vessels as well as in intact animals, inducing either vasoconstriction or vasodilatation under different experimental conditions (Yanagisawa et al., 1988a,b; Lippton et al., 1988; Tomobe et al., 1988; Han et al., 1989; D'Orleans-Juste et al., 1989a,b; Walder et al., 1989; Wright and Fozard, 1988; Warner et al., 1989; De Nucci et al., 1988; Rodman et al., 1989). Endothelins are also potent contractile agents on certain non-vascular smooth muscles (Uchida et al., 1988; Maggi et al., 1989b,c; Hiley et al., 1989; Kozuka et al., 1989; Borges et al., 1989). Specific endothelin receptors have been demonstrated in various peripheral preparations (Power et al., 1989; Neuser et al., 1989; Hoyer et al., 1989; Davenport et al., 1989; Gu et al., 1989a).

Evidence obtained by different laboratories indicates that the actions of endothelins in mammalian tissues could be mediated by multiple receptors (Inoue et al., 1989; Warner et al., 1989).

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We found that the C-terminal hexapeptide, ET-(16-21), a sequence common to all endothelins, was inactive in the rat isolated aorta while it was a full agonist, although less potent than ET-1, in the guinea-pig isolated bronchus (Maggi et al., 1989a). Based on these results, the existence of two distinct receptors for endothelins, provisionally termed ET_A (for aorta) and ET_B (for bronchus) has been proposed.

We report here the results of a study in which the activity of ET-1, ET-2 and ET-3 was compared with that of their common C-terminal fragment, ET-(16-21), in various isolated organs from rats and guinea-pigs as well as on some isolated human preparations. The activity of these peptides was tested in the presence of indomethacin and a mixture of peptidase inhibitors in order to exclude indirect effects induced by the production of prostanoids (De Nucci et al., 1988) and to minimize the possible local metabolism by tissue enzymes (Kimura et al., 1988; Maggi et al., submitted). Sarafotoxin S6b (SRFTX), a 21-residue peptide isolated from the venom of the snake *Atractaspis engaddensis*, was also included in the study because of its close structural homology with the mammalian endothelins and its ability to interact with endothelin receptors (Kloog et al., 1988; Ambar et al., 1989; Gu et al., 1989b; Kloog and Sokolovsky, 1989).

2. Materials and methods

2.1. Animal tissues

Male albino guinea-pigs (220-300 g), male albino Wistar rats (300-350 g) and male albino rabbits (2.0-2.4 kg) were stunned and bled. The following organs were excised and placed in oxygenated Krebs solution at 37 °C containing 5 μM indomethacin: rat vas deferens pars prostatica (RVD), thoracic aorta (RTA) and jugular vein (RJV); guinea-pig ileum (GPI) and bronchus (GPB); rabbit pulmonary artery (RPA). The Krebs solution contained 5 μM indomethacin, 1 μM thiorphan, 1 μM captopril and 1 μM bestatin (Maggi et al., 1989a; Maggi et al., submitted). The vascular endothelium was removed from the RTA and RPA by gentle mechanical rubbing of the internal surface. The effectiveness of this maneuver was verified by the absence of a relaxant response to carbachol (10 μM) after the tone had been increased with phenylephrine (0.3 μM). The bronchial epithelium was removed from the GPB by gentle mechanical rubbing of the internal surface, as described previously (Maggi et al., 1989a,c). Zig-zag strips of the RTA were prepared. Rings of the GPB were connected to stainless steel hooks. The other preparations were arranged so as to record mechanical activity along their longitudinal axis (RVD segments 1.5 cm long; RJV segments 1.5 cm long; GPI segments 2 cm long) or circular axis (RPA strips 1 cm long). Activity was recorded isotonically from all preparations, which were maintained under a resting tension of 5 (RJV, GPB) or 10 mN (GPI, RVD, RPA, RTA). The RVD and GPI were electrically field-stimulated (5 Hz, 60 V, 0.25 ms pulse width, trains of 5 s every 60 s) by means of two platinum electrodes placed at the top and the bottom of the organ bath and connected to a GRASS S11 stimulator. The field stimulation-evoked contractions in the GPI and RVD were suppressed by 1 μM tetrodotoxin, which indicates their neural origin ($n = 4$).

2.2. Human tissues

Specimens of the human urinary bladder (HUB), renal pelvis (HRP) and renal artery (HRA) were obtained from patients undergoing total cystectomy for carcinoma of the bladder base ($n = 12$) or total nephrectomy for carcinoma of the kidney ($n = 8$). The preanesthetic medication and the induction and maintenance of anesthesia were as described previously (Maggi et al., 1989b). All experiments were carried out with preparations which had been stored overnight in chilled, oxygenated Krebs solution. Mucosa-free muscle strips from the dome of the urinary bladder were prepared as described previously (Maggi et al., 1989b). Longitudinal muscle strips of the renal pelvis (1 cm long, 2 mm wide) and rings of the renal artery were excised from the samples. Several preparations were made from each sample and were tested in parallel. The preparations were placed in 5 ml organ baths containing oxygenated

(96% O_2 4% CO_2) Krebs solution. For the urinary bladder and renal pelvis, tension was recorded by isometric force-transducers under a resting tension of 10 mN. The renal artery rings were suspended between two stainless steel hooks, placed in 5 ml organ baths and connected under a resting tension of 10 mN to an isotonic transducer.

2.3. Experimental protocol

All experiments started after a 90 min equilibration period. For the GPB, RJV, RPA, RTA and human tissues the contractile response to the endothelins is expressed as a % of the maximal contractile response produced by KCl (80 mM added to the bath). For the GPI and RVD the response to the peptides is expressed as a % variation of the response to electrical field stimulation. Only one peptide was tested in each preparation. Cumulative concentration-response curves were made for each peptide, the next concentration being added when the effect of the preceding one had reached a steady state.

2.4. Statistical analysis

Statistical analysis of the data was done with Student's t-test or analysis of variance, followed by Dunnett's test, when applicable. Each value in the text and figures is the mean ± S.E.M. of five to seven experiments. Regression analysis was done with the least-squares method. EC_{50} values and 95% confidence limits (c.l.) were calculated accordingly.

2.5. Drugs

The drugs used were: endothelin 1, endothelin 2 (Peninsula, St. Helens, England), endothelin 3 and sarafotoxin S6b (Novabiochem, Laufelfingen, Switzerland), indomethacin (Sigma, St. Louis, MO, USA), acetylcholine chloride (Merck, Darmstadt, FRG), phenylephrine HCl (Serva, Heidelberg, FRG), tetrodotoxin (Sankyo, Tokyo, Japan). ET-(16-21) and ET-(16-21) amide were synthesized by Dr. P. Rovero who used conventional solid phase methods.

3. Results

3.1. Animal tissues

In the RVD the endothelins produced a concentration-dependent (fig. 1) potentiation of the nerve-mediated contractions (5 Hz, 0.25 ms, 60 V trains of 5 s every 60 s). A small increase in tone was also evident in several preparations at high concentrations (cf. Borges et al., 1989). The maximal potentiation induced by ET-1, ET-2, ET-3, SRFTX or ET-(16-21) was similar (200-250% increase over resting values). Minor differences in the potency of the natural endothelins were observed in this tissue, ET-2 (the most potent) being 5-6 times more potent than ET-3. ET-(16-21) was a full agonist. The EC_{50} values and 95% c.l. of the various peptides are shown in table 1. The amidated form of ET-(16-21) was distinctly less effective than the parent peptide and produced a small effect only at 30-100 μM (fig. 1).

In the RPA the endothelins produced a concentration-dependent contraction (fig. 1). The maximal responses to the natural endothelins, SRFTX and ET-(16-21) did not differ from each other and ranged between 80-100% of the response to KCl. ET-2 was the most potent peptide tested (table 1). ET-(16-21) was a full agonist while its amidated form was completely inactive in concentrations up to 0.1 mM (fig. 1 and table 1).

In the GPB the endothelins produced a concentration-dependent contraction (fig. 1), as described previously (Maggi et al., 1989a,c; submitted). The maximal response induced by the natural endothelins, SRFTX and ET-(16-21) did not differ from each other and ranged between 75-90% of the maximal response to KCl. ET-3 was the most potent peptide tested (table 1). ET-(16-21) was a full agonist and its amidated form had a slight effect only at 10-100 μM (Maggi et al., 1989a).

In the RJV full concentration-response curves were obtained for ET-2 (the most potent peptide tested), ET-1 and SRFTX (fig. 2). The maximal response to these peptides was similar and ranged between 180-200% of the response to KCl (80 mM). ET-3 was distinctly less potent than the other peptides but a complete curve was not ob-

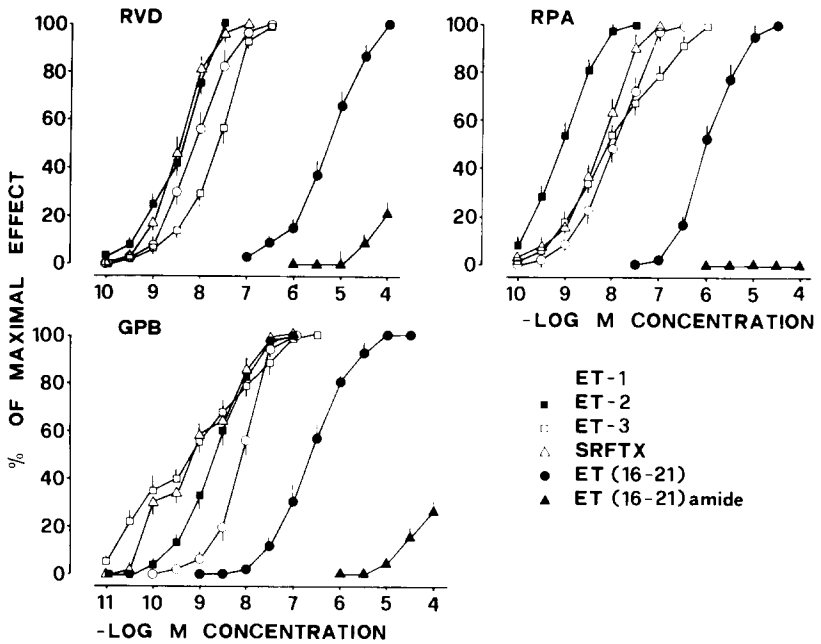

Fig. 1. Concentration-dependent response to the endothelins, sarafotoxin S6b (SRFTX), ET-(16-21) and ET-(16-21) amide of the rat isolated vas deferens (RVD), rabbit pulmonary artery (RPA) and guinea-pig bronchus (GPB). Each value is the mean ± S.E. of 5-7 experiments.

TABLE 1

EC_{50} values and 95% confidence limits of endothelins (ET), SRFTX, ET-(16-21) and its amidated form in various preparations. RTA = rat thoracic aorta; GPI = guinea-pig ileum; HRA = human renal artery; HRP = human renal pelvis; HUB = human urinary bladder; GPB = guinea-pig bronchus; RVD = rat vas deferens; RPA = rabbit pulmonary artery; NE = not evaluated.

Preparation	ET-1 (nM)	ET-2 (nM)	ET-3 (nM)	SRFTX (nM)	ET-(16-21) (μM)	ET-(16-21) amide (mM)
RTA	1.7 (1.4-2.0)	0.3 (0.1-0.5)	72 (32-194)	6.2 (4-12)	> 100	Inactive [b]
GPI	0.31 (0.2-0.4)	0.09 (0.03-0.15)	0.27 (0.1-0.3)	0.27 (0.1-0.4)	> 100	Inactive [b]
HRA	28 (14-83)	NE	≫ 0.3 μM	23 (7-115)	Inactive	Inactive [b]
HRP	48 (21-213)	NE	59 [a] (30-120)	43 (36-62)	> 100	Inactive [b]
HUB	17 (14-23)	5 (2-8)	≈ 1 μM	101 (35-670)	> 100	Inactive [b]
GPB	7 (4-12)	0.6 (0.5-0.7)	0.5 (0.4-0.6)	0.7 (0.6-0.8)	0.23 (0.11-0.62)	> 0.1
RVD	6 (4-12)	3.3 (2.5-5.4)	18 (15-55)	3.5 (2.7-5.3)	5.2 (4.6-6.0)	> 0.1
RPA	10 (9-12)	0.6 (0.2-0.8)	8 (7-9)	7 (4-23)	1.1 (0.9-1.3)	Inactive [b]

[a] Maximal response to ET-3 significantly different from that to ET-1 or SRFTX; [b] inactive at 0.1 mM.

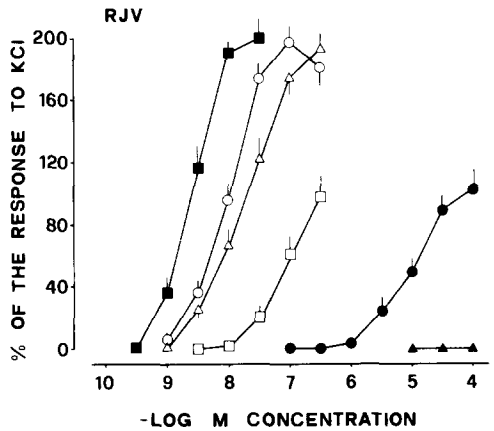

Fig. 2. Concentration-dependent response to the endothelins, sarafotoxin S6b, ET-(16-21) and ET-(16-21) amide of the rat jugular vein (RJV). Symbols used for the various peptides are the same as in fig. 1. Each value is the means ± S.E. of 5-7 experiments.

tained because of the limited supply of the peptide. ET-(16-21) was active but its maximal effect (at 0.1 mM) did not exceed 50% of the response evoked by ET-1, ET-2 or SRFTX (fig. 2). ET-(16-21) amide was inactive in concentrations up to 0.1 mM.

In the RTA the endothelins and SRFTX produced a concentration-dependent contraction (fig. 3). The maximal response to these peptides was similar and ranged between 85-100% of the response to KCl. The order of potency was ET-2 > ET-1 > SRFTX and ET-3 (fig. 3, table 1). As described previously (Maggi et al., 1989a), ET-(16-21) was weakly active at 30-100 μM and its amidated form was ineffective in concentrations up to 0.1 mM (fig. 3, table 1).

In the GPI the endothelins produced two effects (cf. Hiley et al., 1989) that is: (a) inhibition of nerve-mediated contractions and (b) a relaxation or contraction of the unstimulated ileum. This latter effect was characterized by a marked and long-lasting tachyphylaxis. Relaxation of the unstimulated ileum was observed at low concentrations of ET-1 which also inhibited the nerve-mediated contractions. Cumulative concentration-response curves for the inhibitory effect on nerve-mediated contractions were obtained, the

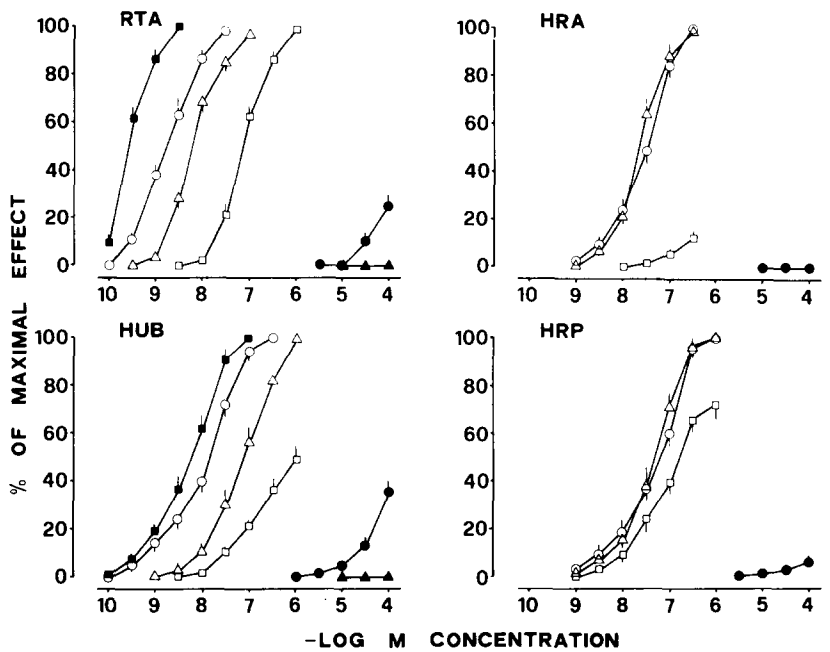

Fig. 3. Concentration-dependent response to the endothelins, sarafotoxin S6b, ET-(16-21) and ET-(16-21) amide of the rat thoracic aorta (RTA), human renal artery (HRA), human renal pelvis (HRP) and human urinary bladder (HUB). Symbols used for the various peptides are the same as in fig. 1. Each value is the mean ± S.E. of 5-7 experiments.

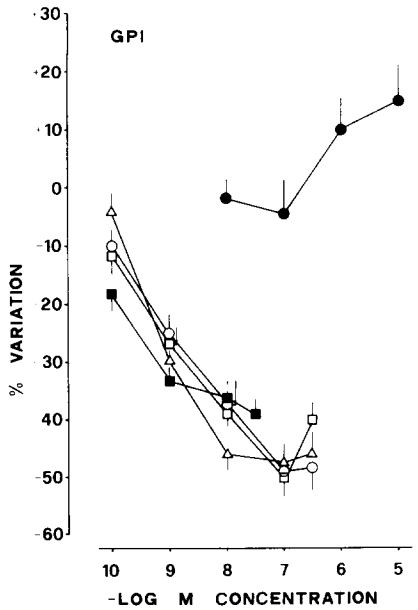

Fig. 4. Concentration-dependent response to the endothelins, sarafotoxin S6b, ET-(16-21) and ET-(16-21) amide of the electrically field-stimulated guinea-pig ileum (GPI). Symbols used for the various peptides are the same as in fig. 1. Each value is the mean ± S.E. of 5-7 experiments.

next concentration being added on a log 10 scale. Only this effect of the endothelins was systematically investigated in the present study. The endothelins produced a concentration-dependent (fig. 4) inhibition of the nerve-mediated contractions (5 Hz, 60 V, 0.25 ms, 5 s every 60 s) of the GPI, with a similar maximal effect (40-50% reduction). ET-2 was more potent than ET-1, ET-3 or SRFTX (table 1). ET-(16-21) had no significant inhibitory effect on the nerve-mediated contractions at concentrations up to 10-100 nM and enhanced the contraction amplitude at 1-10 μM. ET-(16-21) had a contractile effect at these concentrations, which prevented the study of its effect at higher concentrations.

3.2. Human tissues

ET-1 and SRFTX contracted muscle strips from the HUB, HRP and HRA in a concentration-dependent manner (fig. 3). In all these preparations ET-3 was less effective and/or potent than ET-1 or SRFTX. Indeed, the EC_{50} values for ET-3 in the HRA and HUB could not be calculated but were estimated to be $\gg 0.3\ \mu$M and near to 1 μM, respectively (table 1). In the HRP the maximal response to ET-3 did not exceed 70% of that produced by ET-1 or SRFTX. When taking this value as the maximal effect of ET-3, the corresponding EC_{50} value was close to that of the other two peptides. ET-(16-21) was either inactive (HRA) or weakly active (HRP, HUB) (fig. 3, table 1) whereas its amidated form was inactive in all preparations. ET-2 was tested on a few specimens of HRA and HRP (n = 2 for each tissue). In both cases ET-2 was more active than the other peptides tested but EC_{50} values were not be calculated because of the small number of experiments. ET-2 was the most potent peptide tested in the HUB (fig. 3).

3.3. Characteristics of the response in various tissues

In various tissues (GPB, RVD, RTA, RPA, HUB, HRA, HRP, RJV) the contractile effect of the endothelins and SRFTX developed slowly; the response to ET-2 developed the slowest, followed by that to ET-1, SRFTX and ET-3. The slow time course of action was particularly evident at low concentrations of the peptides. In contrast, the effects of ET-(16-21) developed rapidly in all the preparations in which the fragment was active (GPB, RVD, RJV, RPA). The maximum inhibitory effect of the peptides on the nerve-mediated contractions of the GPI was reached in 2-3 min. The potentiating effect on the electrically stimulated contractions of the RVD developed slowly at low endothelin concentrations. Typical tracings in fig. 5 show the time course of the potentiating effect produced by a maximally effective concentration of ET-1, ET-2 and ET-(16-21) on the RVD. Note that the action of ET-(16-21) reached a maximum in 1-2 min, while longer times were required for the natural peptides. The effect of ET-2, ET-1, SRFTX and ET-3 was quite difficult to remove by washing, particularly in the blood vessels, RVD and GPB. In contrast, the action of ET-(16-21) faded rapidly upon washing out. On the basis of EC_{50} values (table 1), the GPI appeared to be the most sensitive preparation while the human tissues were the least sensitive. Overall,

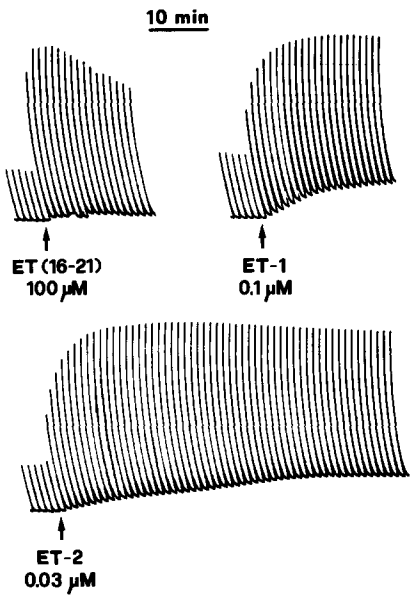

Fig. 5. Typical tracings illustrating the different time course for the potentiating effect produced by ET-(16-21), ET-1 or ET-2 on the electrically evoked (field stimulation, 5 Hz, 60 V, 0.25 ms pulse width, trains of 5 s every 60 s) contractions of the rat isolated vas deferens. The maximal potentiating effect was reached 2, 6 and 10 min after the addition of ET-(16-21), ET-1 and ET-2, respectively. Isotonic recording.

ET-2 was the most active peptide tested, the only exceptions being when it was tested in the GPB and RVD where ET-3 and SRFTX were equipotent with ET-2.

4. Discussion

The present findings indicate that peptides of the endothelin family and SRFTX possess potent contractile activity on various vascular and non-vascular smooth muscles. In addition, we confirm that these peptides powerfully inhibit the nerve-mediated contractions of the GPI (Hiley et al., 1989). This effect, similar to that found in the guinea-pig femoral artery (Wiklund et al., 1988), might involve a pre-junctional inhibition of evoked transmitter release. In contrast, a facilitatory effect on nerve-mediated contractions was observed in the RVD (cf. Hiley et al., 1989). These findings raise the point of a possible neuromodulatory action of the endothelins.

As observed by others, the action of the endothelins and SRFTX was, in most instances, very slow to develop although differences between the time taken to reach maximum responses were observed for the different peptides, ET-2 being the slowest and ET-3 the fastest. Kimura et al. (1988) reported that the removal of tryptophan (Trp) at position 21 reduced the contractile activity of ET-1 on the porcine coronary artery by about three orders of magnitude. Further, the response to ET-1(1-20) developed and decayed much more rapidly than the response to the natural peptide. This suggests that the hydrophobic C-terminal tail of the endothelins and particularly the C-terminal Trp might be responsible for the extremely slow association and dissociation kinetics of endothelins with their receptor(s). This view is now supported by our results with ET-(16-21) because, in all preparations in which this fragment showed activity, its agonism was characterized by fast rates of association and dissociation.

The C-terminal hexapeptide, ET-(16-21), was a full agonist in the GPB whereas it was inactive, either as agonist or antagonist, in the RTA (Maggi et al., 1989a). Based on these results, we proposed that two distinct endothelin receptors existed, which we provisionally termed ET_A and ET_B. This observation was extended in the present study to a variety of other smooth muscles to assess whether the fragment could discriminate between receptors present in different tissues. We now found that at least three preparations (GPB, RVD and RPA) appear to be endowed with ET_B receptors, that is, recognition sites at which the C-terminal hexapeptide possesses sufficient structural requirements to exert full agonistic activity. The RTA, HUB, HRP and HRA could be classified as being endowed with ET_A receptors, at which ET-(16-21) is either inactive or poorly active.

We found that, in those preparations that responded to ET-(16-21), amidation of the terminal COOH caused a dramatic loss of activity. The ET-(16-21) amide was > 440 times less active than the parent hexapeptide in the GPB and was completely inactive, in concentrations up to 0.1 mM, in the RPA. Accordingly, the Trp^{21} COOH function plays a crucial role in the stimulation of ET_B receptors (Maggi et al., 1989a). The existence

of multiple receptors for endothelins is further suggested when our findings are compared with those of Nakajima et al. (1989). These authors reported that ET-(16-21) was completely inactive in contracting the rat isolated pulmonary artery (putatively containing ET_A receptors) and that amidation of the terminal COOH of ET-1 yielded a peptide which was only 15 times less potent than ET-1 (Nakajima et al., 1989). Conceivably, the C-terminal COOH is more important for the activation of ET_B than ET_A receptors. However, removal of the Trp in position 21 resulted in a loss of activity in the rat isolated pulmonary artery (Nakajima et al., 1989). Taken together, these findings indicate that the C-terminal 'tail' of the endothelin molecule plays a role in activating all types of endothelin receptors, and that it is sufficient to exert full agonist activity only in selected preparations.

The response of the RJV and GPI to the endothelins did not allow us to make a simple classification of the receptors contained in these tissues for different reasons. In the RJV, the response to the fragment was apparently smaller than that to ET-1, ET-2 or SRFTX, that is, the fragment behaved as a partial agonist. In the GPI, the fragment was inactive in inhibiting the evoked contractions (an action which could be mediated by ET_A receptors) but produced strong contractions at μM concentrations. Further studies are needed to assess the nature of the responses to the endothelins in these tissues, which may contain more than one endothelin receptor.

The C-terminal hexapeptide tail has the same sequence in all endothelins. When analyzing comparative data in table 1, no particular associations are evident between the activity of ET-1, ET-2 or ET-3 on one hand and that of ET-(16-21) on the other. Clearly, data from a larger number of preparations are needed to establish whether the activity of the fragment is paralleled by a particular pattern of activity of the natural endothelins.

Acknowledgements

We wish to thank Prof. D. Turini and Dr. G. Barbanti, Department of Urology, University of Ferrara, Italy, for their cooperation in providing specimens of human tissues. Part of this work was supported by IMI, Rome, Grant No. 46287.

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