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Biological and Molecular Analyses of Structurally Reduced Analogues of Endothelin-1

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SUMMARY

Structurally reduced analogues of endothelin-1 (ET-1) were synthesized through linking with an aliphatic spacer [aminocaproic acid (Aca)], segment 3–11 of ET-1 to carboxyl-terminal fragments of various lengths (16–21, 17–21, . . ., 21). The peptides were prepared in their linear or cyclic form, and a formyl group was or was not introduced on the Trp^{21} side chain. Pharmacological studies were carried out with the guinea pig lung parenchyma paradigm and the rat thoracic aorta bioassay. In the rat aorta, an ET_A receptor preparation, all of the analogues were inactive. However, in the lung parenchyma, we observed that among the linear formylated derivatives, [$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3–11)-Aca-(17–21)ET was a partial agonist. In this series, the presence of His^{16}, as in [$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3–11)-Aca-(16–21)ET, caused a decrease in contractile activity, suggesting that the imidazole group disfavors the proper interaction of the linear molecule with the ET_B receptors of the lung parenchyma. The loss of biological activity of the deformylated linear analogues strongly suggested that the formyl group played a stabilizing role in the structure of the linear molecules. Interestingly, molecular modeling studies indicated the adoption of different conformations by the formylated and the nonformylated analogues. In contrast, the stabilizing effect of the formyl group was not observed with the cyclic compounds. Furthermore, the presence of His^{16} favored the contractile activity of the cyclic peptides. Finally, the results demonstrated that the carboxyl-terminal residues 18–21 are required for the activity in the guinea pig lung parenchyma ET_B receptors.

ETs are mammalian regulatory peptides. This family includes ET-1 (1), ET-2 (2), ET-3 (3), and vasoactive intestinal contractor (4). They all contain 21 amino acid residues, free amino and carboxyl termini, and disulfide bonds between positions 1–15 and 3–11, respectively.

The wide distribution of ETs and their binding sites strongly suggests that these peptides possess functions in the regulation of a diversity of tissues. The most striking effects of ET-1 are observed in the cardiovascular system, where it is the most potent known vasoconstrictor (1). However, ETs present a large spectrum of nonvascular actions in a variety of tissues, including airways, kidneys, and central and peripheral nervous systems (5). The primary site of action of these peptides seems to be receptors on smooth muscles, and their interaction causes sustained vasoconstriction and bronchoconstriction. However, it has been shown that ETs can also participate in calcium transport, mitosis, neurotransmission, regulation of pituitary functions, and expression of several genes (5).

Molecular biology studies revealed the existence of two distinct genes that encode two receptors for ETs and are identified as ET_A (6) and ET_B (7). ET_A (selective for ET-1 and ET-2 and not for ET-3) is distributed predominantly in cardiovascular tissues, whereas ET_B (nonselective) is found in a larger variety of tissues, including kidney and brain. A third receptor, ET_C (selective for ET-3), was cloned in amphibians (8). Pharmacological results suggest that this putative ET_C receptor also exists in mammals (9, 10). Interestingly, recent reports have elicited pharmacological evidence of new ET_B receptor subtypes and incited the revision of their roles (10–17). It was suggested that these subtypes be called ET_{B1} and ET_{B2}. It is now generally accepted that ET_{B1} receptors, present in endothelial cells, cause vasodilation, whereas ET_A and ET_{B2} receptors, on smooth muscles, are involved in vasoconstriction (18).

A wide variety of structure-activity studies were carried out to precisely identify the pharmacophores of ETs (19–21). Structural modifications were also explored. For example, we showed that the formylation of the indole moiety

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The abbreviations that are used for the amino acids are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.* 138:9–37 (1984)]. L-Isomers of amino acids were used.

ABBREVIATIONS: ET, endothelin; Aca, ε-aminocaproic acid; BOC, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; For, formyl; Acm, acetamidomethyl.

of Trp^{21} did not affect the biological activity of ET-1 in the guinea pig trachea, a pharmacological preparation containing ET_B receptors (22). Furthermore, we observed that the formylated linear analogue of ET-1 still exhibited a potent effect in the guinea pig lung parenchyma but did not possess any vasodepressor activity in the rat blood pressure paradigm (11). These results suggested that different subtypes of ET_B receptors are present in the guinea pig trachea or lung parenchyma and the rat vascular system (12). Then, it seemed that some formylated ET-1 derivatives were able to discriminate between the ET_B-like effects measured with the rat blood pressure and the guinea pig pulmonary preparations. Therefore, to further explore the mode of action of ETs at the molecular level, we synthesized a series of hybrid molecules made by linking, with an aliphatic flexible spacer, the segment 3–11 of ET-1 to carboxyl-terminal fragments of various lengths (16–21, 17–21, . . ., 21). The analogues were investigated in their linear or cyclic form, and a formyl group was or was not introduced on the Trp^{21} side chain. Biological studies were conducted in the guinea pig lung parenchyma and the rat thoracic aorta bioassay. Finally, molecular modeling studies were used to analyze the structures of the peptides.

Materials and Methods

Reagents and solvents. BOC-protected aspartic acid, glutamic acid, histidine, isoleucine, leucine, methionine, and serine were purchased from Propeptide (Vert le Petit, France), and Aca, cysteine, and lysine were obtained from Bachem (Torrance, Ca). BOC-tryptophan (For) was obtained from Advanced ChemTech (Louisville, KY). BOP was purchased from Richelieu Biotechnologies (Montréal, Quebec, Canada). American Chemical Standards-grade dimethylformamide and methylene chloride were obtained from Anachemia Canada (Ville St.-Pierre, Quebec, Canada). Biograde TFA was purchased from Halocarbon (Hackensack, NJ). Diisopropylethylamine was obtained from Pfaltz and Bauer (Waterbury, CT) and was distilled from ninhydrin before use, and chloromethyl resin was purchased from Bio-Rad (Richmond, CA).

Peptide synthesis and cleavage. ET-1 and its analogues were synthesized with a homemade manual multireactor solid-phase peptide synthesizer according to a protocol that we described previously (23). A chloromethyl resin (24) was used as the solid support. The following side chain-protected BOC amino acids were used in the synthesis: cyclohexyl-aspartic acid, Acm-cysteine, cyclohexyl-glutamic acid, tosyl-histidine, 2-chlorobenzyloxycarbonyl-lysine, benzyl ether-serine, and For-tryptophan. BOC/amino acid couplings were performed in dimethylformamide with BOP reagent (25, 26) in the presence of diisopropylethylamine (27–29). Every coupling step was monitored with the use of the ninhydrin test (30).

Peptide-resins were cleaved from the solid support with liquid hydrofluoric acid (10 ml/g). The acid was condensed in a reactor already containing *m*-cresol (1 ml/g) and ethanethiol (1 ml/g) as scavengers. The reaction was carried out for 60 min at 0°, and then hydrofluoric acid was rapidly evaporated. The resin was washed with anhydrous diethylether, and the peptide was extracted with TFA. After evaporation of TFA, the remaining product was precipitated with ether. The collected material was completely dried and conserved at −20° until purification.

Purification and characterization of peptides. Crude peptides were purified through preparative reverse-phase HPLC with a Waters Prep LC 500A system equipped with a Waters 1000 PrepPak Module and a model 441 absorbance detector. A portion (600 mg) of the crude material was dissolved in degassed H_2O containing 0.06% TFA (1 liter) and then injected onto a Deltapak C_{18} (15 μm; 300 Å) column (30 × 5.7 cm). The material was eluted with a 3-hr linear gradient of (A) H_2O (0.06% TFA) and (B) CH_3CN (30%) in H_2O (0.06% TFA). The flow rate was maintained at 40 ml/min, and detection was at 229 nm. Individual fractions were analyzed through reverse-phase HPLC with a Waters Automated Gradient Controller coupled to a Lambda-Max model 481 LC spectrometer. The analyses were carried out on a Vydac (10 μm) reverse-phase column (30 × 0.39 cm) with (A) H_2O (0.06% TFA) and (B) CH_3CN as eluants with a linear gradient mode (20%-50% B in 15 min). The detection was at 230 nm, and the flow rate was fixed at 1.5 ml/min. The fractions corresponding to the purified peptide were pooled and lyophilized. To cyclize the peptides, they were dissolved in degassed aqueous acetic acid (80%), and a solution of iodine (50 Eq) in methanol was added dropwise. The reaction was continued until cyclization was evaluated as complete with the use of analytical HPLC (2–4 hr depending on the peptide). The reaction was stopped by the addition of ascorbic acid until complete discoloration. Most of the acetic acid was evaporated, and the remaining solution was diluted with H_2O (0.06% TFA) (1:100). The solution was injected onto a preparative HPLC column, and the product was purified as described above. After lyophilization, the peptides were finally deformylated with 0.1 N piperidine for 30 min at 0°. The reaction was quenched by freezing and lyophilization, and the deformylation reaction was verified with the use of analytical HPLC.

Each preparation was characterized through analytical HPLC on a Pharmacia SuperPac Pep-S (5 μm) column (25 × 0.4 cm) with a Waters 600E System Controller equipped with a Waters 484 Tunable Absorbance Detector set at 230 nm; the gradient was 25–45% (B) in 20 min with eluant (A) H_2O (0.06% TFA) and (B) CH_3CN. The flow rate was maintained at 1.5 ml/min. The peptides were also characterized through capillary electrophoresis (Applied Biosystems 270A; conditions: 20 mM sodium citrate buffer, pH 2.5; capillary, 35 cm × 50 μm; voltage, 7 kV; temperature, 30°; injection, 1 sec in vacuum mode; detection, at 200 nm).

Amino acid analysis were also performed on ET-1 and its analogues. Peptides were hydrolyzed with 6 N HCl containing 0.1% phenol over 24 hr at 110°. The samples were analyzed after drying and derivatization with phenylisothiocyanate, according to the method described by Waters Chromatography. The analyses were carried out with an HPLC system consisting of two Waters 510 pumps, a Waters 715 Ultra Wisp sample processor, a Waters TCM temperature controller coupled to a column heater module, and a PICO.TAG amino acid analysis column. During the analysis, the column was kept at 30°, and the elution of the PTC amino acid derivatives was achieved with a gradient of 1–100% (B) in 20 min. The solvents were (A) sodium acetate buffer, pH 6.4, containing 4% CH_3CN and 0.05% triethylamine and (B) 60% CH_3CN in H_2O. The detection was at 254 nm. The system was controlled, and the data were processed with the Waters Baseline 810 chromatography workstation software.

Biological activity study. The contraction induced by ET-1 and analogues was measured with the use of two different preparations: guinea pig lung parenchymal strips (ET_B receptors, Ref. 31) and rat aortic rings (ET_A receptors, Ref. 32). Dunkin-Hartley guinea pigs (either sex), weighing 250–300 g, were killed by cervical dislocation and rapidly bled. The lungs were quickly removed and placed in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer solution, and the parenchyma was delicately dissected. Strips were mounted in water-jacketed organ baths at 37° and irrigated with Krebs buffer containing 118 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.18 mmol/liter $MgSO_4 \cdot 7H_2O$, 1.18 mmol/liter KH_2PO_4, 25 mmol/liter $NaHCO_3$, 2.5 mmol/liter $CaCl_2$, 11.1 mmol/liter glucose, pH 7.0, at 37°. Peptides were dissolved in H_2O immediately before use. Mechanical responses were recorded isometrically on a Grass 7D polygraph with force-displacement transducers. Tissues were put under an initial tension of 2.0 g with a 60-min equilibration period. Thereafter, histamine (1×10^{-6} M) was injected

into each sample of parenchyma to verify its contractibility. The contractile effects of 1×10^{-11} to 3×10^{-8} M concentrations of ET-1 and analogues were recorded to obtain the corresponding concentration-response curves.

Male Sprague-Dawley rats were anesthetized, and the thoracic aorta was rapidly removed and cleaned of fat and connective tissue. The endothelium was removed by a gentle scrubbing. Rings (3–4 mm wide) were prepared and suspended under a tension of 2.0 g in water-jacketed organ baths (at 37°) containing oxygenated Krebs' buffer. Each preparation was allowed to equilibrate for 1 hr. Mechanical responses were recorded on the system described before. Concentration-response curves were measured for ET-1 and analogues from 1×10^{-10} to 3×10^{-7} M.

Molecular modeling. Molecular modeling analyses were carried out on selected peptides. Models were built in extended form with the use of the BIOPOLYMER module of the INSIGHT II program version 2.30 (Biosym Technologies, San Diego, CA) operating on a Silicon Graphics Indigo R4000 workstation. The structures were first submitted to 300 steps of steepest descent energy minimization using the consistent valence force field (DISCOVER module) and subsequently to 10,000 steps of conjugate gradient energy minimization with a maximum derivative criterion of 0.01 kcal/mol-Å. In all cases, the maximum derivative was reached in <10,000 steps.

Statistical analysis. The results are expressed as mean ± standard error of 4–16 experiments (three to six animals). Concentration-response curves were smoothed with the use of a nonlinear least-squares curve-fitting procedure available with the program SigmaPlot (Jandel).

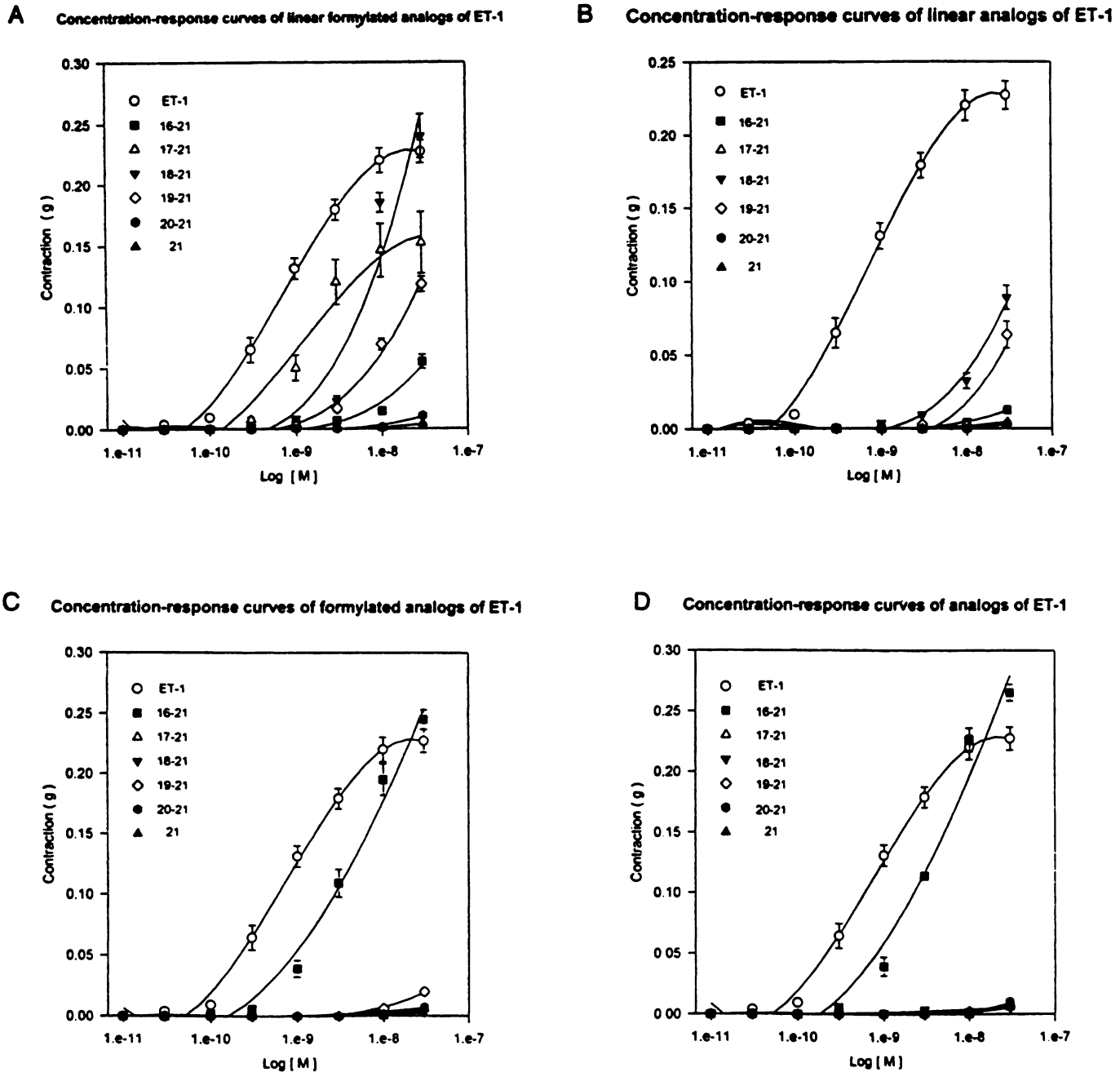

Fig. 1. Concentration-response curves of ET-1 and linear formylated analogues, [Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(*X*–21)ET (A); linear analogues, [Cys(Acm)^{3,11}]-(3–11)-Aca-(*X*–21)ET (B); formylated analogues, [Trp(For)^{21}]-(3–11)-Aca-(*X*–21)ET (C); and analogues, (3–11)-Aca-(*X*–21)ET (*X* = 16 to 21) (D) in the isolated guinea pig lung parenchyma. Results are the mean ± standard error of 8–16 experiments.

Results

Pharmacological studies. The evaluation of the biological activity in the guinea pig parenchyma bioassay showed that the linear formylated analogue containing the 17–21 terminal segment acted as a partial agonist with an EC_{50} of 1.4×10^{-9} M, whereas ET-1 exhibited an EC_{50} of 6.8×10^{-10} M (Fig. 1A). Further shortening of the carboxyl-terminal fragment to obtain analogues 18–21 and 19–21 gave peptides ~8- and ~45-fold less potent than the parent molecule, respectively (Table 1). Other deletions generated inactive analogues. Interestingly, the addition of His^{16} in the linear formylated analogues, as seen in [$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3–11)-Aca-(16–21)ET, caused a decrease in contractile activity ($EC_{50} \gg 10^{-7}$ M). Deformylation of the linear analogues had a detrimental effect on the biological activity in the parenchyma. In fact, only the derivatives with the 18–21 or the 19–21 carboxyl-terminal segment conserved a contractile activity, but they seemed to be >1000-fold less potent than ET-1 (Fig. 1B).

In the cyclic products, and in contrast to what was observed with the linear compounds, the presence of His^{16} favored the contracting activity because derivatives only 1 order of magnitude less potent than the parent molecule were obtained (Fig. 1, C and D). With or without a formyl group on the indole side chain of Trp^{21}, the residue 16 is necessary for the activity of the cyclic analogues, and any shortening of the carboxyl-terminal segment abolished the response in guinea pig lung parenchyma. Our results also showed that the disulfide bonds were not essential for the biological activity in the ET_B receptor pharmacological preparation that was used. Furthermore, the linear peptides seemed to need a formyl group on the Trp^{21} to induce a contraction similar to that of ET-1.

TABLE 1

Concentration of ET-1 analogues eliciting 50% of the maximal response to ET-1 in guinea pig lung parenchyma

Peptide	EC_{50}
	M
ET-1	6.8×10^{-10}
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(16-21)ET	$\gg 10^{-7}$
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(17-21)ET	1.4×10^{-9}[a]
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(18-21)ET	5.6×10^{-9}
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(19-21)ET	3.0×10^{-8}
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(20-21)ET	Inactive[b]
[$Cys(Acm)^{3,11}$,$Trp(For)^{21}$]-(3-11)-Aca-(21)ET	Inactive[b]
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(16-21)ET	Inactive[b]
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(17-21)ET	Inactive[b]
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(18-21)ET	$\gg 10^{-7}$
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(19-21)ET	$\gg 10^{-7}$
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(20-21)ET	Inactive[b]
[$Cys(Acm)^{3,11}$]-(3-11)-Aca-(21)ET	Inactive[b]
[$Trp(For)^{21}$]-(3-11)-Aca-(16-21)ET	3.6×10^{-9}
[$Trp(For)^{21}$]-(3-11)-Aca-(17-21)ET	Inactive[b]
[$Trp(For)^{21}$]-(3-11)-Aca-(18-21)ET	$\gg 10^{-7}$
[$Trp(For)^{21}$]-(3-11)-Aca-(19-21)ET	Inactive[b]
[$Trp(For)^{21}$]-(3-11)-Aca-(20-21)ET	Inactive[b]
[$Trp(For)^{21}$]-(3-11)-Aca-(21)ET	Inactive[b]
(3-11)-Aca-(16-21)ET	3.6×10^{-9}
(3-11)-Aca-(17-21)ET	Inactive[b]
(3-11)-Aca-(18-21)ET	Inactive[b]
(3-11)-Aca-(19-21)ET	Inactive[b]
(3-11)-Aca-(20-21)ET	Inactive[b]
(3-11)-Aca-(21)ET	Inactive[b]

[a] Partial agonist.
[b] Analogues inactive up to 3.0×10^{-8} M.

In rat aortic rings, the concentration-response curve of ET-1 is shown in Fig. 2. However, all of the analogues synthesized, whether linear or monocyclic, were inactive. The presence of a formyl group on the indole moiety of Trp^{21} had no effect.

Molecular modeling of ET-1 and selected analogues. As shown in Fig. 3, the carboxyl-terminal portion of ET-1 is folded toward the bicyclic part of the molecule. According to the molecular modeling results, the presence of a formyl group on Trp^{21}, as in [$Trp(For)^{21}$]ET-1, modified the orientation of the carboxyl-terminal segment, which then became elongated after the segment 12–15. The most evident modifications related to the amino acid side chains, between these two molecules, concerned Glu^{10}, Phe^{14}, His^{16}, and Asp^{18}. Furthermore, contrary to ET-1, the Asp^{8} of [$Trp(For)^{21}$]ET-1 is found far from the carboxyl-terminal segment of the molecule and, more precisely, from the side chain of Ile^{19}.

The general structure of [$Cys(Acm)^{3,11}$]-(3–11)-Aca-(17–21)ET is folded onto itself to form a square containing many residue side chains, in particular, Glu^{10}, Ile^{19}, and Trp^{21} (Fig. 4). Interestingly, the addition of a formyl group to this molecule gave a linear product in which the side chains were more accessible. Also, the orientations of the side chains in the segment 17–21 became very similar to those of ET-1. This important effect of the formyl group was not observed in the 18–21 linear compound. In fact, the conformation of the amino-terminal portions of both molecules, up to the aminocaproic acid residue, was identical. However, differences appeared at the Asp^{18} position. Indeed, the carboxyl-terminal tail of [$Cys(Acm)^{3,11}$]-(3–11)-Aca-(18–21)ET is linear, as is the entire molecule. The Trp^{21} side chain is distant from the others and oriented to be as far as possible from the peptide backbone. The addition of a formyl group to this analogue modified the orientation of the carboxyl-terminal end of the molecule. The

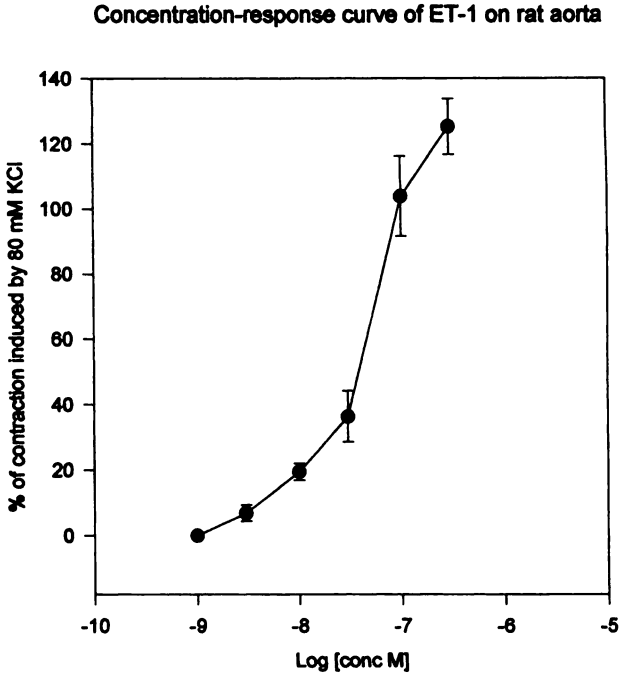

Fig. 2. Concentration-response curves of ET-1 in the isolated rat thoracic aorta. Results are the mean ± standard error of 11 experiments.

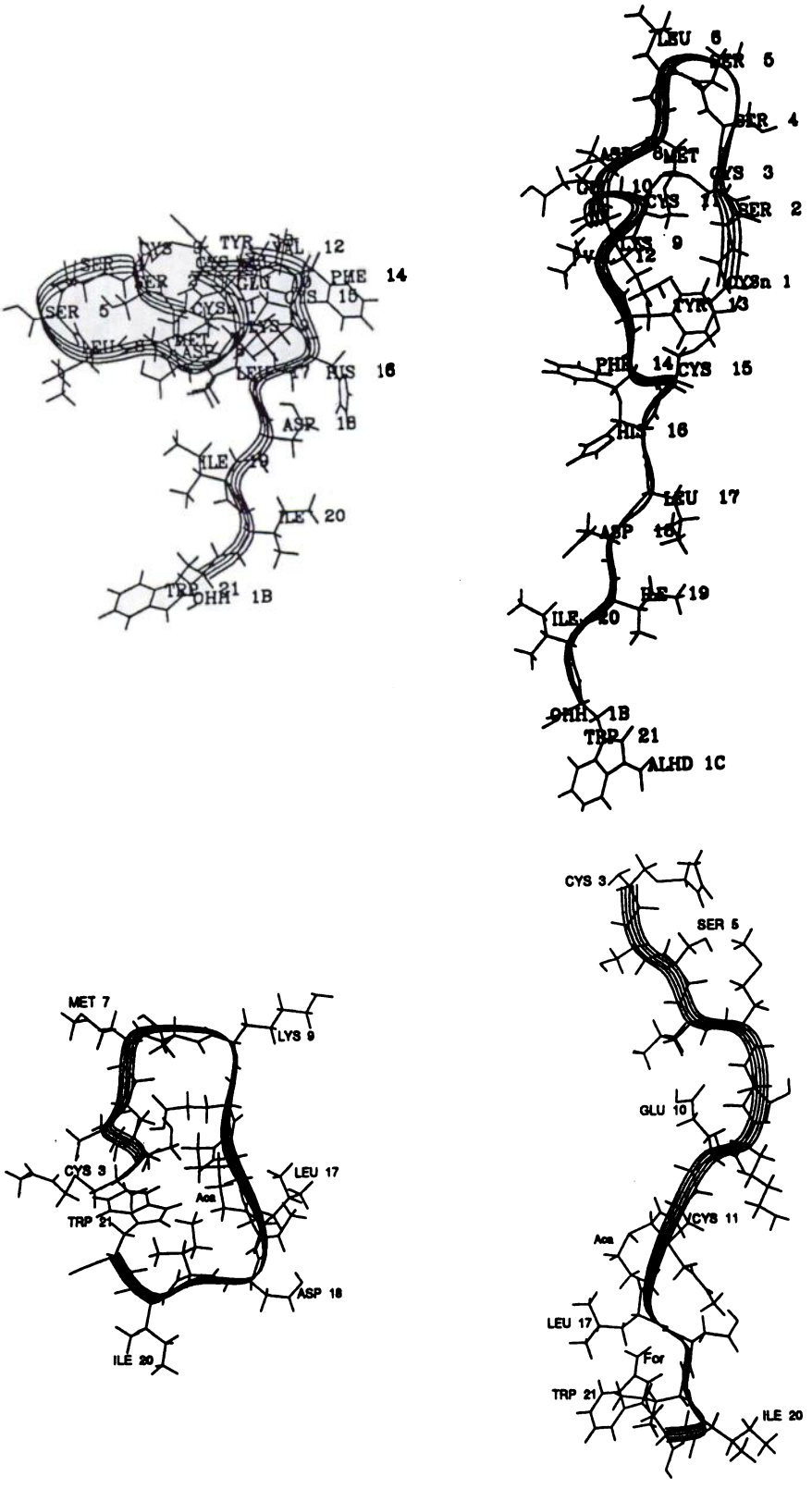

Fig. 3. Molecular modeling of ET-1 (A) and [Trp(For)^{21}]ET-1 (B) obtained after energy minimization with the program INSIGHT II (DISCOVERY module) (Biosym Technologies).

Fig. 4. Molecular modeling of [Cys(Acm)^{3,11}]-(3–11)-Aca-(17–21)ET (A) and [Cys(Acm)^{3,11}, Trp-(For)^{21}]-(3–11)-Aca-(17–21)ET (B) obtained after energy minimization with the program INSIGHT II (DISCOVERY module) (Biosym Technologies).

indole moiety of Trp^{21} is now folded toward the acetamidomethyl protecting group of Cys^{11} and the aminocaproyl spacer (Fig. 5).

Modeling studies on the cyclic analogues were performed with (3–11)-Aca-(16–21)ET and [Trp(For)^{21}]-(3–11)-Aca-(16–21)ET. The presence of a formyl group on the indole side chain did not have an effect on the three-dimensional conformation of the molecule. The cyclic core of these two derivatives did not become more compact, and their carboxyl-terminal segment and, more particularly, the indole moiety of Trp^{21} turned toward the side chains of Leu^{17} and Ile^{19} (Fig. 6).

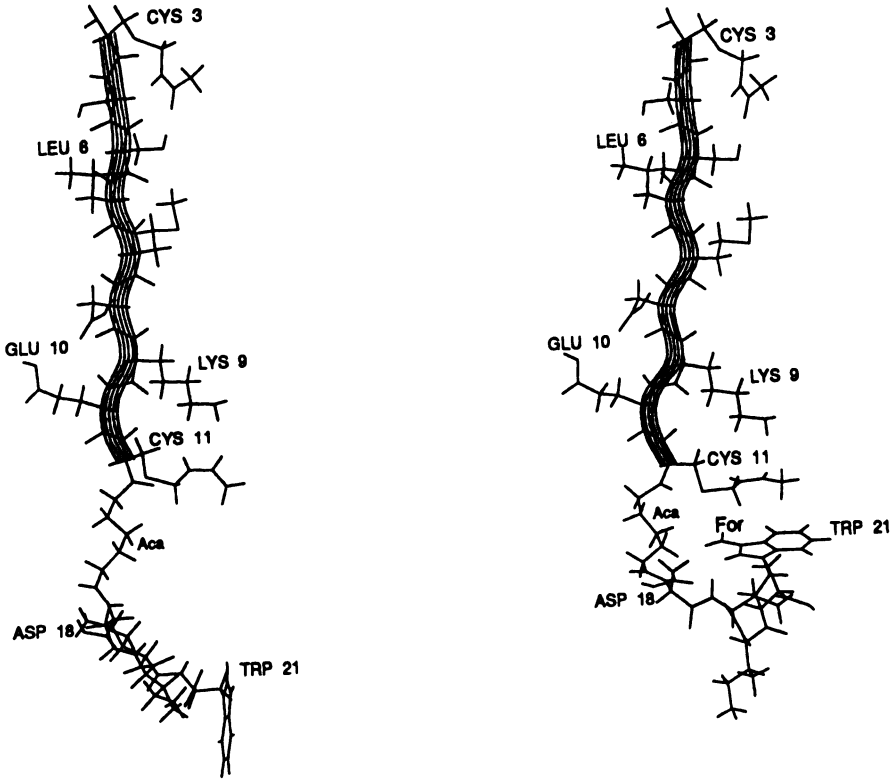

Fig. 5. Molecular modeling of [Cys(Acm)^{3,11}]-(3–11)-Aca-(18–21)ET (A) and [Cys(Acm)^{3,11}, Trp(For)^{21}]-(3–11)-Aca-(18–21)ET (B) obtained after energy minimization with the program INSIGHT II (DISCOVERY module) (Biosym Technologies).

Discussion

[Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(17–21)ET acted as a partial agonist in the guinea pig lung parenchyma bioassay. This linear molecule, as shown by modeling studies, is flexible and able to satisfy the binding requirements of the ET_B receptors found in this pharmacological preparation. When the formyl group was removed from this compound, the molecule became completely folded onto itself (Fig. 4). This showed the great flexibility of the carboxyl-terminal portion of the peptide and confirmed that a little chemical group such as CHO can modify the three-dimensional arrangement of the molecule. The similarity in the orientation of the carboxyl-terminal segment of [Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(17–21)ET and that of ET-1 can account for the biological activity of this compound. In fact, it is accepted that the activation of the ET_B receptors depends on the carboxyl-terminal stretch 16–21, a segment highly conserved in all of the ETs (5, 33). As described in a recent study of the crystal structure of human ET-1, Janes *et al.* (34) proposed that the entire carboxyl-terminal portion of the molecule is helical. Moreover, an important observation was that in the crystal structure, the side chain of Asp^8 is far from that of Leu^{17}. A similar spatial arrangement was observed with our formylated analogue, and this might be an essential feature for the agonistic activity. However, this hypothesis needs to be verified because the crystallographic results are not in agreement with those of NMR studies (35–37). Interestingly, the 17–21 linear formylated derivative acted as a partial agonist. As this analogue is a structurally reduced derivative of ET-1, this partial agonistic activity might result from a missing side chain or inadequate folding of the molecule due to the absence of any disulfide bond and to great flexibility resulting from the incorporation of the aminocaproic acid residue.

As viewed with molecular modeling (Fig. 4), the compact structure of [Cys(Acm)^{3,11}]-(3–11)-Aca-(17–21)ET and the inaccessibility of some side chains might account for the absence of activity, ≤30 nM. More particularly, amino acid side chains considered to be important for the activation of ET_B receptors, such as Glu^{10} and Trp^{21} (19, 38), are hidden inside the molecule (Fig. 4). Surprisingly, the addition of His^{16}, as seen in [Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(16–21)ET, caused a decrease in contractile activity, suggesting that the imidazole group disfavored, in the linear analogues, the proper interaction of the molecule with the receptor. These results suggest that hydrophobicity of the carboxyl-terminal tail of ET-1 might be an important parameter for the biological effect in guinea pig lung parenchyma. This additional hydrophobic character obtained with the withdrawal of His^{16} might favor the interaction of the peptide-ligand with the auxiliary hydrophobic binding pocket proposed by Cody *et al.* (39) for the ET receptors. Furthermore, these authors also reached the conclusion that the positive charge of His^{16} is not an essential feature for binding with the receptor.

The 18–21 linear formylated analogue was a complete agonist but was 10 times less potent than ET-1. Thus, the removal of Leu^{17} had an important effect. This residue and Phe^{14} are described as key amino acids for the activation of ET_B receptors (for a review, see Ref. 21). Our results showed that Leu^{17} and Phe^{14} are important but not essential because the analogue possessed, in the guinea pig lung parenchyma, an intrinsic activity similar to that of ET-1. In the 18–21 linear compounds, the formyl group also appeared to play a key role in the activity, probably by changing the orientation

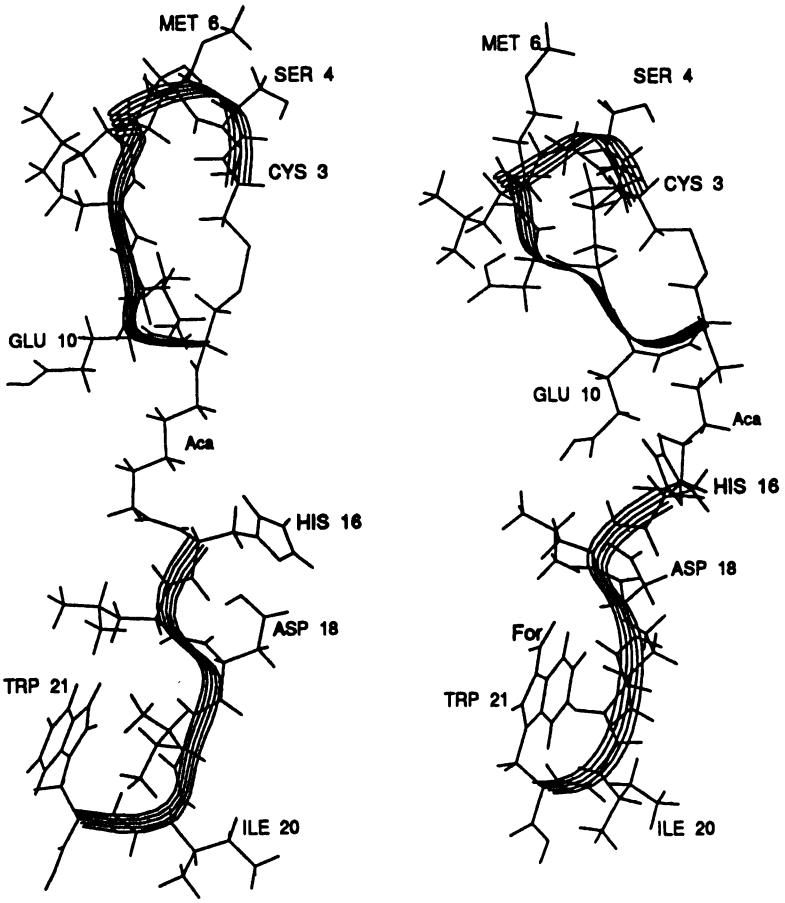

Fig. 6. Molecular modeling of (3–11)-Aca-(16–21)ET (A) and [Trp(For)^{21}]-(3–11)-Aca-(16–21)ET (B) obtained after energy minimization with the program INSIGHT II (DISCOVERY module) (Biosym Technologies).

of the indole moiety (Fig. 5). The resulting modification in the environment of Trp^{21} is probably in part responsible for the difference of activity observed between the 17–21 and 18–21 linear formylated compounds.

Further shortening of the carboxyl-terminal segment (segments 19–21, 20–21, 21) of the ET-1 analogues produced peptides ~100 times less potent (residues 19–21) than the parent molecule or inactive (residues 20–21, 21). As suggested by Saeki *et al.* (19), Asp^{18} is essential for good activity in a pharmacological preparation containing ET_B receptors. However, as seen with [Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(19–21)ET, the addition of a formyl group on the indole of Trp^{21} improved the biological activity. Therefore, according to our results, the orientation of the pharmacophores in the truncated analogue of ET-1 is more crucial than the presence of the negative charge of the Asp^{18} side chain.

The stabilizing effect of the formyl group was not present or apparent in the cyclic analogues, which suggested that the rigidity produced by the disulfide bond probably abolished it. Furthermore, in contrast to what was observed with the linear compounds, the presence of His^{16} favored the contracting activity. In fact, this residue, bearing a positive charge, seemed to be essential for the activity of the cyclic derivatives in the guinea pig lung parenchyma. The 16–21 cyclic analogues are as potent as [Cys(Acm)^{3,11},Trp(For)^{21}]-(3–11)-Aca-(17–21)ET but are full agonists. Interestingly, the side chains of Leu^{17} and Ile^{19} are in close proximity to that of Trp^{21} (Fig. 6). This important hydrophobic environment might play a determining role in the activity observed with these compounds. Consequently, the structure of the carboxyl-terminal segment of the 16–21 cyclic analogues is similar to that of ET-1, except for the orientation of the indole group of residue 21, which is folded toward the cyclic portion of the molecule, and for the distance between the side chains of Leu^{17} and the crucial cluster of charged residues (Asp^{8}-Lys^{9}-Glu^{10}). Indeed, according to Miasiro *et al.* (38), Lys^{9} is involved in tachyphylaxis, whereas Glu^{10} is important for the activation of the ET_B receptors in the guinea pig ileum.

The 24 derivatives of this series were unable to contract the rat thoracic aorta. Therefore, the ET_A receptors present in this pharmacological preparation possess structural requirements different than those of the ET_B receptors. The absence of the Cys^{1}—Cys^{15} bridge give a larger flexibility to the synthetic analogues in comparison to the native ET-1 molecule. The importance of this first disulfide bridge in the activation of the ET_A receptors has been verified (21, 40). Thus, the removal of the residues Cys^{1}, Ser^{2}, and Cys^{15} explains in part the lack of activity in the rat aorta.

With [Dpr^{1}-Asp^{15}]ET-1, in which the outer disulfide bridge was replaced by an amide bond, Spinella *et al.* (41) proposed, as a mechanism of ET-receptor interaction accounting for the long-lasting effect of this peptide, the formation of a disulfide bond between ET-1 and its receptor. This hypothesis, postulated after studies with guinea pig kidneys, a tissue containing ET_B receptors, suggested that Cys^{1}—Cys^{15} would react with a cysteine residue found on an extracellular loop of the receptor protein. Their model does not suggest that a disulfide bonded complex between ET and its receptor, *per se*, is responsible for receptor activation but rather that a disulfide exchange is a necessary intermediate step for ET to achieve

receptor activation. In our peptides, Cys^{1} and Cys^{15} are absent. Furthermore, we obtained active linear products in which Cys^{3} and Cys^{11} were protected with an acetamidomethyl group. Therefore, a mechanism involving a disulfide bridge formation with the receptor cannot be proposed to account for the activity in ET_B receptor preparations. Nevertheless, the mechanism proposed by Spinella *et al.* (41) might be appropriate for ET_A receptors for which the disulfide bonds of ET seemed to be important features for binding and activation.

Most of the differences among ET-1, ET-2, and ET-3 occur within residues 2–7. Then, as hypothesized by Coles *et al.* (42) after an ^{1}H NMR study of [α-aminobutyric acid^{1,15}]-ET-1, the variation of amino acid at position 2 (ET-1 and ET-2, Ser^{2}; ET-3, Thr^{2}), the spatial arrangement of residues 1–3, and the orientation of the amino-terminal charge of the three ETs were proposed to be responsible for the capacity of discrimination between ET_A and ET_B receptors. The results obtained with our structurally reduced analogues clearly demonstrated that residues 1 and 2 are not necessary for the activation of ET_B receptors but could be involved in the activation of ET_A receptors, a pharmacological preparation in which all of our analogues are inactive up to 3×10^{-7} M. Similarly, Cody *et al.* (39) investigated the role of the segment 3–11 of ET-1 in binding to ET_A and ET_B receptors (in rabbit renal artery vascular smooth muscle membranes and in rat cerebellar membranes, respectively). Thus, they tested the analogue (3–11)-(16–21)ET, which exhibited an IC_{50} of $>10^{-5}$ M for ET_B receptors. Their conclusion was that segments 1–3 and 11–15 are far more important in binding than is the portion 3–11. By comparison with our cyclic compounds containing these two segments of ET-1, we observed that the flexible aliphatic spacer (aminocaproic acid) introduced in the analogues played an important role in the activity in the guinea pig lung parenchyma (Table 1). This strongly suggests that the cyclic nucleus formed by residues 3–11 is certainly a structure recognizable by ET_B receptors. Furthermore, it seems that residues 12–15 would play a role for the ET_B receptors of the guinea pig lung parenchyma but not for the ET_A receptors of the rat aorta, a role that can be in part mimicked by the aliphatic spacer arm by fixing a proper distance between the amino- and carboxyl-terminal pharmacophores. The analogue (3–11)-Aca-(16–21)ET is only ~5 times less potent than ET-1 in the guinea pig lung parenchyma. According to the observation of Tam *et al.* (43) that the alanine substitution of Tyr^{13} or Phe^{14} caused a sharp decrease in binding affinity and following the hypothesis of Cody *et al.* (39) that the segment Val^{12}-Tyr^{13}-Phe^{14} interacts with an auxiliary hydrophobic binding pocket present in both receptor subtypes, the activity of the structurally reduced analogue on ET_B receptors probably could be improved by using a spacer containing a hydrophobic moiety.

A two-subdomain model was proposed to account for the selectivity for ET_A and ET_B receptors (33, 44). Following this hypothesis, each receptor would present one binding site for the amino-terminal segment of ETs and another for the carboxyl-terminal fragment. In agreement with previous studies (5) on the binding requirements of ET_B receptors, only the second site would be essential for their activation, whereas both sites would be necessary for binding to ET_A receptors. If only the carboxyl-terminal portion of the ET-1 analogues tested in this study binds to the receptor, this would explain the activity observed with ET_B receptor preparations and the absence of any response in a ET_A receptor bioassay. From our results in guinea pig lung parenchyma, residues 19–21 seemed to be crucial and Asp^{18} seemed to be important for linear compounds. In contrast, the entire carboxyl-terminal segment 16–21 would be required in cyclic analogues. Consequently, the three-dimensional arrangement of the side chains of these residues is decisive for the activation of ET_B receptors, and the disulfide bond plays an important role in the conformation of the 16–21 segment. Therefore, the activity observed in absence of the amino-terminal segment of the molecule supports the hypothesis of a multiple-subdomain model for ET receptors.

In conclusion, this study showed that the mechanism of ET-receptor interaction in guinea pig parenchyma is probably not related to a disulfide bridge formation. Furthermore, we observed that the incorporation of a formyl moiety on the indole of Trp^{21} played a structural role in the molecule. This effect would not be present or apparent in cyclic compounds. Also, in monocyclic analogues, residues 19–21 are essential for activation of ET_B receptors in guinea pig lung parenchyma, whereas Asp^{18} is important. The biological activity of these analogues missing residues 1–3 of ET-1 supports the two-subdomain model suggested for ET receptors and is in agreement with the postulate that the carboxyl-terminal segment of this peptide is sufficient for ET_B receptor activation but not for ET_A receptors in which all of the analogues were inactive. The structurally reduced derivatives of ET-1 synthesized for this study are selective for ET_B receptors. Finally, our results suggest that Leu^{17} and Ile^{19} form a hydrophobic environment around the side chain of Trp^{21}. This spatial arrangement might play a key role in the biological activity of ET-1.

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