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Inhibition of Angiotensin Converting Enzyme and Potentiation of Bradykinin by Retro-Inverso Analogues of Short Peptides and Sequences Related to Angiotensin I and Bradykinin

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ABSTRACT. There is pharmacological evidence indicating that, in addition to the inhibition of angiotensin converting enzyme (ACE; EC 3.4.15.1), the potentiation of bradykinin (BK) responses may also involve the BK receptor or some binding site in the structures involved in the contractile response to this peptide. Dipeptides such as Val-Trp and some of its analogues as well as tripeptide homologues, including total and partial retro-inverso peptides, were synthesized and assayed for their ability to inhibit purified guinea pig plasma ACE and to potentiate the action of BK on the isolated ileum of the same species. The peptides containing the P_2-P_1, P_1-P'_1, and P'_1-P'_2 inverted amide bonds inhibited ACE, were resistant to hydrolysis, and, depending on the amino acid composition, some of them potentiated the contractile response to BK while others did not. Des-[Arg^1]-BK, which has an intrinsic activity at concentrations higher than 10^{-5} M, and the very dissimilar angiotensin I (AI) analogue [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide, which has no detectable contractile activity, were able to inhibit ACE and potentiate BK. In contrast to these peptides, BPP_{5a} and BPP_{9a} from *Bothrops jararaca* venom, and Potentiators B and C from *Agkistrodon halys blomhoffii* venom were more effective as BK potentiators than as ACE inhibitors. In conclusion, we have synthesized and assayed compounds that preferentially inhibit ACE, e.g. retro-inverso tripeptides, or potentiate the response of smooth muscle to BK, e.g. snake venom peptides. BIOCHEM PHARMACOL 51;8:1051–1060, 1996.

KEY WORDS. retro-inverso peptide; bradykinin potentiation; angiotensin converting enzyme inhibitors; peptide synthesis

Inhibitors of ACE† (EC 3.4.15.1) reduce blood pressure in several experimental models of hypertension in rats [1, 2], as well as in normotensive animals [3, 4]. Captopril and other ACE inhibitors have been used successfully in the treatment of human hypertension and congestive heart failure, and most of these effects have been ascribed primarily to the inhibition of ACE [5]. However, the mechanism of action of ACE inhibitors is claimed not to be restricted solely to the inhibition of ACE hydrolytic activities based on the following observations: (a) the lowering of blood pressure and the inhibition of plasma or lung ACE do not always parallel each other [6, 7], (b) the long-lasting effect of ACE inhibitors seems more likely to be due to the potentiation of BK than to the inhibition of AI conversion [8, 9], (c) a prolonged residual ("sensitizing") activity of snake venom BPP and some of their analogues has been described, even after the peptides were washed out [10], and (d) inhibition of the kininase activity of isolated duodenum preparations by BPP_{9a} and enalaprilat does not result in potentiation of BK-induced contraction or relaxation [11]. Therefore, in addition to a reduction in the degradation of BK by ACE, other sites of action for ACE inhibitors in the potentiation of kinin activities have been considered including an involvement at the level of the BK receptors as well as the inhibition of other proteolytic enzymes with kininase activity [12–17].

Verdini and colleagues [18, 19] synthesized partially modified retro-inverso analogues of BPP_{5a} [Gln-Lys-gPhe-(*S*)mAla-Pro] and BPP_{9a} [Gln-Trp-Pro-Arg-gLys-(*S*,*R*)mPhe-Ala-Pro]. The former BPP analogue was modified at the scissile bond for ACE, P_1-P'_1 (nomenclature proposed by Schechter and Berger [20]), and inhibited the enzyme without potentiating the effects of BK on rat blood pressure [18]. The

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† *Abbreviations:* ACE, angiotensin converting enzyme; AcOH, acetic acid; AI and AII, angiotensin I and II, respectively; An, anilyl; BK, bradykinin; Boc, *tert*-butyloxycarbonyl; BPP, bradykinin potentiating peptides; Bz, benzyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, dicyclohexylurea; FPLC, fast protein liquid chromatography; HOBt, hydroxybenzotriazole; OtBu, *tert*-butyl ester; OBu, butyl ester; OEt, ethyl ester; OMe, methyl ester; Suc, succinyl; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; gXaa, *gem*-diaminoalkyl analog of the indicated amino acid; and mXaa, malonyl residue corresponding to the indicated amino acid.

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latter analogue, modified at the P_2-P_1 position, inhibited ACE and potentiated BK contractile activity on isolated guinea pig ileum [19]. These observations with partial retro-inverso penta- and nonapeptide were obtained in different pharmacological preparations; more unequivocal information would be expected with shorter total retro-inverso peptides [21, 22], assayed in a pharmacological preparation and in an isolated ACE of the same animal.

Val-Trp is the shortest peptide described [23] with reasonable *in vivo* ACE inhibitory activity ($K_i < 10^{-6}$ M) and does not inhibit the AI contractile activity on the isolated guinea pig ileum, which results from the conversion of AI to AII. To gain further information on the BK-potentiating effects of ACE inhibitors using simple chemical structures, we synthesized Val-Trp analogues and their tripeptide homologues, and studied their efficacy as inhibitors of purified guinea pig plasma ACE and as potentiators of the contractile activity induced by BK on the isolated ileum of the same species. We also investigated the effects of retro-inverso and partial retro-inverso modifications of Val-Trp and its analogues on these same activities.

As a further step in examining the inhibition of ACE by the BK-potentiating peptides and their interaction with the BK receptor, we determined the inhibition constants (K_i) for plasma ACE and the BK-potentiating activity on isolated guinea pig ileum of two quite dissimilar peptides, [des-Arg^1]-BK and [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide. The former peptide possessed intrinsic contractile activity only at concentrations above 10^{-5} M; however, in spite of this weak agonistic activity, it should be potentially recognized by BK receptors. On the other hand, the hexapeptide [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide did not show detectable smooth muscle contractile activity and is unlikely to be recognized by BK receptors because of its structural and conformational similarities to AI [24].

The BK-potentiating peptides from *Bothrops jararaca* and *Agkistrodon halys blomhoffii* venoms are well-known potentiators of BK-induced contractions in the guinea pig ileum [25, 26]. To compare this pharmacological activity with their ability to inhibit ACE, we also determined the K_i values of BPP_{5a}, BPP_{9a} and Potentiators B and C on guinea pig plasma ACE.

MATERIALS AND METHODS

Chemicals

Sephadex G-200, Superose 12 and Mono Q column (HR 5/5) were purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. Protein molecular weight standards, *ortho*-phthaldialdehyde, Hip-Gly-Gly, and all amino acids were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade or the equivalent.

Peptides

BK, des-[Arg^1]-BK and [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide were synthesized by standard solid phase peptide synthesis [27], purified by preparative HPLC, and characterized by analytical HPLC and amino acid analysis. The BK-potentiating peptides (BPP_{5a}, BPP_{9a}, and Potentiators B and C) were gifts of Dr. Mineko Tominaga, Escola Paulista de Medicina, São Paulo, Brazil. The ACE substrate Hip-His-Leu and all the other di- and tripeptides were synthesized by classic solution methods using the general procedures previously described [28–30].

Retro-inverso Modified Peptides

These peptides were synthesized using coupling procedures involving DCC/HOBt, azide, and mixed anhydride. Boc or Z protecting amino groups were employed and were removed subsequenty by TFA or catalytic hydrogenation under palladium adsorbed on charcoal. Unless otherwise mentioned, the products were isolated by dissolving the reaction mixture in ethyl acetate and washing the organic phase with 5% $NaHCO_3$, cold 10% citric acid and NaCl saturated water, dried over Na_2SO_4, followed by evaporation and crystallization. The analytical data are shown in Table 1 and were obtained as follows: TLC was carried out on 0.25 mm silica-gel plates, and R_f (A), R_f (B) and R_f (C) values refer, respectively, to the solvent systems nBuOH:AcOH:water (4:1:1), nBuOH:AcOEt:AcOH:water (1:1:1:1) and nBuOH:pyridine:AcOH:water (30:20:6:24). Melting points were determined in a Kofler apparatus with a Reichter micro hot system and are uncorrected. Amino acid analyses were performed in a 6300 Beckman high performance amino acid analyzer after hydrolysis with 6 N HCl, *in vacuo*, at 110° for 72 hr. Peptides containing Trp were hydrolyzed with 3 N *p*-toluenesulfonic acid, in the presence of 1% indole for the determination of the amino acid contents. The description below gives only specific details for each one of the retro-inverted peptides obtained.

·OH-DVAL-MTRP-OH (PEPTIDE 4). mTrp-OEt (2 mmol), prepared as described by Greenstein and Winitz [31], was coupled to DVal-OMe · HCl (2.8 mmol) by the mixed anhydride procedure. The resulting diester (MeO-DVal-mTrp-OEt), which crystallized from ethyl ether (m.p. = 136–138°), was dissolved in ethanol and saponified with a 10% excess of 1 M KOH. Water was then added to the reaction mixture, the pH was adjusted to 2.5, and the peptide was extracted into ethyl acetate. The organic phase was washed with water and dried, and the compound was crystallized by solvent evaporation. Anal. calc. for $C_{17}H_{19}N_2O_4$: C, 64.00; H, 6.00; N, 8.80. Found: C, 66.50; H, 6.10; N, 8.97.

BZ-GGLY-DVAL-MTRP-OH (PEPTIDE 7). The first step was the synthesis of Bz-gGly-NH_2 · HCl, as previously described by Bergmann and Zervas [32] followed by coupling to Z-DVal by the DCC/HOBt procedure in dimethylformamide. After 24 hr, DCU was filtered off, the solvent was evaporated, and the oily material was dissolved in ethyl acetate/water and processed. The resulting Bz-gGly-DVal-Z crystallized from ethyl acetate/petroleum ether (m.p. = 129–133°). The next step was the synthesis of Bz-gGly-DVal-mTrp-OMe by coupling Bz-gGly-DVal-NH_2 (1.3

TABLE 1. Analytical data for the synthesized peptides

No.	Peptide	m.p. (°)	TLC			Amino acid analysis
			R_f (A)	R_f (B)	R_f (C)	
1	NH_2-Val-Trp-OH	230 (dec.)*	0.60	0.71	0.58	Trp:Val, 1.04:0.96
2	Suc-Val-Trp-OH	222 (dec.)	0.45	0.55	0.70	
3	Z-Val-Trp-OH	135–137	0.90	0.80	0.85	
4	OH-DVal-mTrp-OH	78–82	0.85	0.95	0.55	Val (80%)
5	Bz-Phe-Val-Trp-OH	124–128	0.90		0.75	Phe:Trp:Val, 0.98:0.99:1.02
6	Bz-Gly-Val-Trp-OH	186–190	0.80	0.90	0.70	Trp:Val:Gly, 1.01:0.96:103
7	Bz-gGly-DVal-mTrp-OH	180–185	0.90	0.92	0.72	Val (88%)
8	Bz-Phe-Gly-Trp-OH	128–133	0.81	0.94	0.64	Trp:Gly:Phe, 0.98:1.02:0.98
9	An-mPhe-Gly-Trp-OH	86–91	0.81		0.71	Trp:Gly, 1.02:0.98
10	Bz-gPhe-mGly-Trp-OH	133–138	0.90	0.90	0.65	Trp (76%)
11	Bz-Phe-gGly-mTrp-OH	158–161	0.82		0.70	
12	Bz-Gly-Arg-Trp-OH	189–192	0.35	0.45	0.65	Trp:Arg:Gly, 1.00:0.96:104
13	Bz-gGly-Arg-mTrp-OH	190–193	0.20	0.45	0.40	Arg (85%)
14	Bz-gGly-DArg-mTrp-OH	189–192	0.20	0.45	0.40	Arg (87%)
15	Bz-gGly-mGly-Gly-OH	195–198	0.50	0.55	0.50	
16	Bz-Gly-gGly-mGly-OH	158–160	0.48	0.60	0.45	Gly (89%)

* (dec.) = decomposition.

mmol) (obtained by hydrogenation of Bz-gGly-DVal-Z) to mTrp-OMe (2.0 mmol) by the DCC/HOBt procedure after which the mixture was processed and crystallized as described above (m.p. = 163–166°). The final compound was obtained by saponification of Bz-gGly-DVal-mTrp-OMe, which crystallized from ethanol/water (see Table 1 for analytical data). Anal. calc. for $C_{25}H_{28}N_4O_5$: C, 67.60; H, 6.30; N, 12.60. Found: C, 66.50; H, 6.30; N, 12.35.

An-mPhe-Gly-Trp-OH (Peptide 9). mPhe-OEt (27 mmol), obtained as previously described [33], was dissolved in DCM and coupled to aniline by the DCC/HOBt method. After 24 hr, DCU was removed by filtration, the reaction mixture was processed, and An-mPhe-OMe was crystallized from ethyl ether/petroleum ether (m.p. = 85–91°). The resulting product, An-mPhe-OMe (18.5 mmol), was saponified and coupled to Gly-OEt (20.4 mmol) by the DCC/HOBt procedure. After 24 hr, DCU was filtered off, the reaction mixture was processed, and An-mPhe-Gly-OEt was crystallized from petroleum ether (m.p. = 143–145°). This compound (1 mmol) was saponified and coupled to Trp-OMe by the DCC/HOBt procedure. The desired product (An-mPhe-Gly-Trp-OMe) was isolated and crystallized from ethyl ether (m.p. = 80–85°). The saponification of this compound led to the final product (peptide 9), which crystallized from ethyl ether/petroleum ether (see analytical data in Table 1).

Bz-gPhe-mGly-Trp-OH (Peptide 10). Bz-gPheNH_2 · HCl (1 mmol), obtained as previously described [32], and mGly-OEt (1.2 mmol), obtained by hemisaponification of diethylmalonate, were coupled by the DCC/HOBt procedure, processed, and Bz-gPhe-mGly-OEt was crystallized from petroleum ether (m.p. = 182–186°). This compound (0.4 mmol) was then saponified and coupled to Trp-OMe · HCl (0.47 mmol) by the DCC/HOBt procedure, processed, and Bz-gPhe-mGly-Trp-OMe was crystallized from petroleum ether (m.p. = 184–187°). Saponification of this product yielded Bz-gPhe-mGly-Trp-OH, which crystallized from ethanol/acidified water (see analytical data in Table 1). Anal. calc. for $C_{29}H_{28}N_4O_5 \cdot 2H_2O$: C, 62.26; H, 6.28; N, 8.81. Found: C, 61.40; H, 6.14; N, 8.97.

Bz-Phe-gGly-mTrp-OH (Peptide 11). Bz-Phe-Gly-$NHNH_2$ (2.4 mmol), synthesized as described by Greenstein and Winitz [31] (m.p. = 194–197°), was dissolved in 50% acetic acid containing 0.5 M HCl. This solution was cooled to 0°, and $NaNO_2$ (2.5 mmol), previously dissolved in water, was added dropwise. The reaction mixture was left under vigorous stirring for 3 min, and then an ethyl acetate/water mixture was added. The organic phase was processed, dried, dissolved in 20 mL of benzene containing benzylic alcohol, and the mixture left under reflux for 30 min. Bz-Phe-Gly-Z precipitated and was recrystallized from acetic acid (m.p. = 215–216°). This compound was catalytically hydrogenated to remove the Z protecting group and coupled to mTrp-OMe by the DCC/HOBt procedure. The reaction mixture was processed and Bz-Phe-gGly-mTrp-OMe was crystallized from petroleum ether (m.p. = 136–139°). This compound was saponified, and the final product, Bz-Phe-gGly-mTrp-OH, was crystallized from petroleum ether (see analytical data in Table 1). Anal. calc. for $C_{29}H_{28}N_4O_5$: C, 67.96; H, 5.40; N, 10.90. Found: C, 67.30; H, 5.26; N, 10.18.

Bz-gGly-Arg-mTrp-OH and Bz-gGly-dArg-mTrp-OH (Peptides 13 and 14). The procedure for the synthesis of the intermediate dipeptides Bz-gGly-(Tos)Arg-Boc (m.p. = 95–100°) and Bz-gGly-(Tos)DArg-Boc (m.p. = 104–106°) was essentially the same as that described above for peptide 7. These two compounds were treated with 2.5 N HCl in dioxane and then coupled to mTrp-OEt by DCC/HOBt. The reaction mixture was concentrated, processed as above, and crystallized from ethyl ether. The resulting Bz-gGly-(Tos)Arg-mTrp-OEt (m.p. = 85–90°) and Bz-gGly-(Tos)DArg-mTrp-OEt (m.p. = 110–112°) were saponified

leading to Bz-gGly-(Tos)Arg-mTrp-OH (m.p. = 128–130°) and Bz-gGly-(Tos)DArg-mTrp-OH (m.p. = 120–127°). The final compounds were obtained removing the tosyl protective group from Arg by treatment with hydrogen fluoride.

Bz-gGly-mGly-Gly-OH (Peptide 15). Bz-gGly-Z (4.6 mmol) obtained from Bz-gGly-$NHNH_2$, as described for peptide 11, was catalytically hydrogenated in the presence of concentrated HCl. The resulting product, Bz-gGly-$NH_2 \cdot HCl$ (2.2 mmol), was coupled to mGly OtBu (2.2 mmol), which was prepared as described by Greenstein and Winitz [31], using the DCC/HOBt procedure. The reaction mixture was processed, and Bz-gGly-mGly-OtBut was crystallized from ether (m.p. = 147–155°). One millimole of this product was treated with TFA and coupled to Gly-OEt (1.2 mmol) by the DCC/HOBt procedure. The final product was obtained by saponification of Bz-gGly-mGly-Gly-OEt and crystallized from ethyl acetate/ethyl ether (see analytical data in Table 1). Anal. calc. for $C_{17}H_{23}N_3O_7$: C, 53.5; H, 6.03; N, 11.02. Found: C, 56.68; H, 6.93; N, 13.48.

Bz-Gly-gGly-mGly-OH (Peptide 16). Bz-Gly-gGly-Z (2.3 mmol) obtained from Bz-Gly-GlyNHNH$_2$, as described for peptide 11, was hydrogenated catalytically and coupled to mGly-OtBu (1.7 mmol) by the DCC/HOBT procedure. The reaction mixture was processed and Bz-Gly-gGly-mGly-OBu was crystallized from ethyl acetate/ethyl ether (m.p. = 146–148°). The treatment of this product with TFA led to the final peptide, Bz-Gly-gGly-mGly-OH, which crystallized from ethyl ether (see analytical data in Table 1). Anal. calc. for $C_{15}H_{18}O_7N_3 \cdot AcOH$: C, 50.99; H, 5.09; N, 11.90. Found: C, 51.78; H, 4.92; N, 13.86.

Angiotensin Converting Enzyme Preparation

All procedures were carried out at 4°, except for the FPLCs, which were performed at room temperature. Heparin-treated guinea pig plasma was fractionated with $(NH_4)_2SO_4$ and the precipitate in the range 1.4 to 2.8 M containing most of the ACE activity, was suspended in 10 mM sodium phosphate buffer, pH 7.0, and was dialyzed for 24 hr against a 1 mM concentration of the same buffer. The protein solution was submitted to gel filtration through a 110 × 4 cm Sephadex G-200 column and eluted with 10 mM Tris–HCl buffer (pH 7.4) containing 0.3 M NaCl, at a flow rate of 25 mL/hr. The fractions presenting hydrolytic activity toward Hip-His-Leu were pooled and concentrated in a Centricell 20 Centrifugal Ultrafilter (Polyscience, Inc., Warrington, PA, U.S.A.). Using an FPLC system, the concentrate was applied to a Superose 12 prep grade HR 16/50 column, previously equilibrated with 10 mM Tris–HCl buffer (pH 7.4) containing 0.3 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 1 mL/min, and the fraction with peptidase activity was pooled and dialyzed overnight against 10 mM Tris–HCl buffer (pH 7.4). This solution was applied to a Mono Q HR 5/5 column previously equilibrated with 10 mM Tris–HCl buffer (pH 7.4), and a linear gradient of 0–450 mM NaCl in the same buffer was used to develop the column. The fractions containing ACE activity were collected and stored at −20°. The protein elution profiles were monitored at 280 nm, and the protein contents were determined by the Coomassie Blue method using bovine serum albumin as standard [34]. The SDS–PAGE of non-reduced samples was carried out as described by Laemmli [35].

Enzyme Assays

The enzymatic activity was followed during the purification by the method of Cushman and Cheung [36] adapted to a fluorimetric procedure by Friedland and Silverstein [37]. The reactions were carried out at 37° in 0.1 M potassium phosphate buffer, pH 8.3, containing 0.3 M NaCl and a 5 mM concentration of the substrate Hip-His-Leu. The His-Leu released was quantified fluorimetrically by the formation of a fluorescent adduct with *ortho*-phthaldialdehyde.

The kinetic parameters were calculated by the Lineweaver–Burk plots of initial hydrolysis velocities obtained for at least five substrate concentrations. For the the hydrolysis of AI, the incubations were carried out at 37° in 0.1 M sodium phosphate buffer, pH 8.0, containing 0.2 M NaCl. The His-Leu released was measured as described by Friedland and Silverstein [37]. The kinetic data for Hip-Gly-Gly hydrolysis were determined in 0.1 M potassium phosphate buffer, pH 7.4, in the presence of 0.3 M NaCl, at 37° as described by Yang *et al.* [38]. The hydrolysis of Hip-His-Leu was measured by the method of Cushman and Cheung [36] adapted for fluorimetric determination [37], as described above. The inhibition constants (K_i) were determined under the same conditions after a 5-min preincubation using Hip-His-Leu as the substrate. The kinetic data were analyzed using the *Grafit* computer program [39].

BK-Potentiating Activity in the Isolated Guinea Pig Ileum

The assays of BK-potentiating contractile activity in isolated guinea pig ileum were performed as previously described [40]. Before the potentiating assays, the preparations were adjusted to provide a reproducible log concentration–response to BK, and the peptides were tested for their intrinsic activity.

RESULTS

ACE was purified to apparent homogeneity from the 1.4 to 2.8 M ammonium sulfate precipitate of guinea pig plasma, followed by dialysis, gel-filtration on Sephadex G-200 and Superose 12, and ion exchange chromatography in a Mono Q column (Table 2). SDS–PAGE yielded a single broad protein band with an M_r of about 170 kDa following silver staining (Fig. 1). AI, Hip-His-Leu, and Hip-Gly-Gly were hydrolyzed by the purified protein in conditions described above, with K_m values of 6.3×10^{-5} M, 5.8×10^{-3} M, and 6.3×10^{-3} M, respectively. These hydrolyses were suppressed by dialysis or by EDTA, and could be recovered by the addition of Cl^- or Zn^{2+}, respectively.

TABLE 2. Purification of ACE from guinea pig plasma

Procedure	Protein (mg)	Activity (units)*	Specific activity (units/mg protein)	Purification (factor)
Plasma	3392	132	0.039	1.0
Ammonium sulfate†	1568	72	0.046	1.2
Sephadex G-200	26.8	12.9	0.480	12.3
Superose 12	1.48	4.3	2.9	74.4
Mono Q	0.064	2.3	36	923

* One unit was defined as the amount of enzyme that hydrolyzes 1 μM Hip-His-Leu/min at 37°.
† Ammonium sulfate concentration = 1.4 to 2.8 M.

The peptides containing one inverted peptide bond were obtained by coupling the malonic and the geminal diaminoalkyl analogues of the amino acids (peptides 10 and 11), while the insertion of a D-amino acid between them resulted in a total retro-inverso peptide (peptides 7 and 14). The effect of the L-enantiomer in this insertion was also examined by employing L-Arg (peptide 13). The anilide derivative of the Phe malonic analogue, when introduced into the dipeptide Gly-Trp, resulted only in the reversal of the first amide bond (peptide 9). All the peptides containing mPhe and mTrp were in the racemic (*R,S*) form, and no effort was made to separate them. The same rationale was employed to synthesize Bz-gGly-mGly-Gly and Bz-Gly-gGly-mGly where the second and third amide bonds were, respectively, inverted. The maximal solubility in an aqueous solution of peptides 5–11 was lower than 3×10^{-3} M, whereas the solubility of peptides 4 and 12–14 exceeded 10^{-2} M.

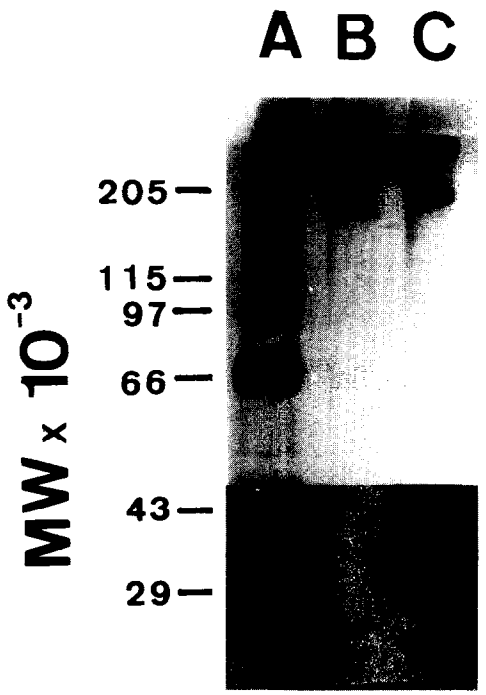

FIG. 1. SDS–PAGE: (7.5%). The proteins were visualized by silver staining. Lane A, molecular weight markers: myosin (205 kDa), β-galactosidase (115 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), egg albumin (43 kDa), and carbonic anhydrase (29 kDa). Lane B, affinity chromatography purified rabbit lung ACE (used as a standard; supplied by Dr A. R. Martins of the Universidade de São Paulo). Lane C, purified guinea pig plasma ACE.

Guinea pig plasma ACE was inhibited competitively by all the peptides obtained, except the succinyl and carbobenzoxy derivatives of Val-Trp, which were inactive. The inhibition constants (K_i) are presented in Table 3, and Fig. 2 exemplifies some of the inhibition curves obtained.

Val-Trp and all its synthetic analogues and homologues with natural peptide bonds and the ACE inhibitory activity had potentiating activity on the contractile response of the isolated guinea pig ileum to BK. The concentration dependence of the effect of the peptides with BK-potentiating activity in the guinea pig ileum was very similar to that of the peptides occurring in the venoms of *B. jararaca* and *A. halys blomhoffii* [40]. Figure 3A shows, as an example, this concentration-effect relationship for Val-Trp. The maximum potentiation observed with each of the peptides studied was around 5-fold, and all were specific for BK, since the responses to acetylcholine, AII and histamine were not potentiated. At the concentration range of 2×10^{-5} to 6×10^{-5} M, the concentration–effect curves showed a plateau at about a 2-fold potentiation, as illustrated in Fig. 3A. The minimum concentration that produced this 2-fold potentiation was obtained from the intercept of the plateau line with the least-squares straight line drawn through the points of the lowest BK-potentiating peptide concentrations (Fig. 3B). Using this method, the potentiation activity of all the peptides was determined (Table 3). The BK-potentiating action of retro-inverso peptides was observed to depend on the position of the inverted peptide bond and on the amino acid composition of the peptides. Namely, An-mPhe-Gly-Trp-OH (peptide 9) potentiated BK, while Bz-gPhe-mGly-Trp-OH and Bz-Phe-gGly-mTrp-OH (peptides 10 and 11) abrogated this activity, and Bz-gGly-DArg-mTrp-OH (peptide 14), in contrast to Bz-gGly-DVal-mTrp-OH (peptide 7), potentiated BK contractile activity at a concentration even lower than its K_i value for ACE inhibition (Table 3). In addition, the BK potentiation seemed not to depend on the chirality of the P'_1 residue because Bz-gGly-Arg-mTrp-OH (peptide 13) potentiated BK at a level equivalent to that of peptide 14.

Des-Arg^1-BK and [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide markedly inhibited ACE and also potentiated the BK

TABLE 3. Inhibition of guinea pig plasma ACE and BK-potentiating activity on guinea pig ileum by Val-Trp analogues, homologues, and some of their corresponding partial retro-inverso forms

No.	Peptides	K_i* (M)	Concentration for 2-fold potentiation (M)
1	NH_2-Val-Trp-OH	$(4.6 \pm 0.5) \times 10^{-6}$	2.4×10^{-5}
2	Suc-Val-Trp-OH	NI†	NP‡
3	Z-Val-Trp-OH	NI	NP
4	OH-DVal←mTrp-OH	$(5 \pm 1) \times 10^{-3}$	NP
5	Bz-Phe-Val-Trp-OH	$(1 \pm 0.1) \times 10^{-7}$	2.8×10^{-5}
6	Bz-Gly-Val-Trp-OH	$(1.8 \pm 0.3) \times 10^{-7}$	3.6×10^{-5}
7	Bz-gGly←DVal←mTrp-OH	$(1.7 \pm 0.2) \times 10^{-4}$	NP
8	Bz-Phe-Gly-Trp-OH	$(1.0 \pm 0.4) \times 10^{-6}$	3.2×10^{-5}
9	An←mPhe-Gly-Trp-OH	$(1.2 \pm 0.3) \times 10^{-6}$	1.1×10^{-4}
10	Bz-gPhe←mGly-Trp-OH	$(1.8 \pm 0.1) \times 10^{-4}$	NP
11	Bz-Phe-gGly←mTrp-OH	$(1.1 \pm 0.4) \times 10^{-3}$	NP
12	Bz-Gly-Arg-Trp-OH	$(1.8 \pm 0.2) \times 10^{-5}$	4.1×10^{-5}
13	Bz-gGly←Arg←mTrp-OH	$(3 \pm 1) \times 10^{-3}$	8.9×10^{-4}
14	Bz-gGly←DArg←mTrp-OH	$(1.4 \pm 0.6) \times 10^{-3}$	5.6×10^{-4}

* Values are means ± SD, N = 4.
† NI = No inhibition was detected up to an inhibitor concentration of 10^{-2} M.
‡ NP = No potentiation was detected up to a peptide concentration of 10^{-2} M for peptide 4 and 10^{-3} M for all others.

contractile activity in the isolated guinea pig ileum. The K_i values for ACE inhibition and the BK potentiation parameters obtained as in Fig. 2 are shown in Table 4. The K_i values for the inhibition of guinea pig plasma ACE by BK-potentiating peptides from the venoms of *B. jararaca* and *A. halys blomhoffi* are presented in Table 5, which also includes the potentiation parameters obtained by Tominaga *et al.* [40], under the same conditions as those used in the present work.

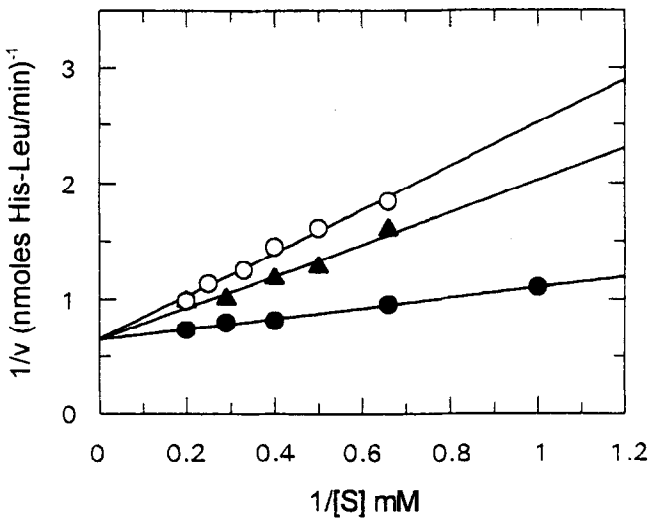

FIG. 2. Effect of Bz-Phe-Val-Trp and Bz-gPhe-mGly-Trp on guinea pig plasma ACE activity, measured using Hip-His-Leu as substrate. Key: Lineweaver–Burk representation in the absence (●) and in the presence of 1.5×10^{-7} M Bz-Phe-Val-Trp (▲) and 1.8×10^{-4} M Bz-gPhe-mGly-Trp (○).

DISCUSSION

The ACE plasma concentration is, generally, low in most mammals, except for guinea pig plasma, which contains quite a high amount of the enzyme [38, 41]. The potentiation of the guinea pig isolated ileum response to BK by a large number of peptides has been studied. In this work, we examined the effects of synthetic peptides in an isolated organ and on the inhibition of ACE in the same species following the purification of guinea pig plasma ACE to apparent homogeneity. The K_m values obtained for the hydrolysis of Hip-His-Leu (5.8×10^{-3} M) and AI (6.3×10^{-5} M) are very similar to those described previously for ACE of the same source [42] and for human plasma [43].

The retro-inverso peptides were obtained from the malonic analogues of tryptophan, phenylalanine, and glycine, and from the *gem*-diaminoalkyl analogues of phenylalanine and glycine. The latter compounds were generated by the Curtius rearrangement. The side-reactions previously described under similar conditions [44] were minimized by employing a slight excess (less than 10%) of benzyl alcohol and benzoylating Gly and Phe before the Curtius rearrangement.

The K_i value for the inhibition of guinea pig plasma ACE by Val-Trp is comparable to that obtained with rabbit lung ACE [23]. The unfavorable effect of a γ-carboxyl group at the P_1 position in Suc-Val-Trp (peptide 2) is in accord with the observed resistance of Hip-Glu-His-Leu hydrolysis by bovine lung ACE [45]. The introduction of a benzyloxycarbonyl (Z) group (peptide 3) in the N-terminal amino group resulted in the loss of inhibitory activity against ACE. This indicates that the urethane moiety impairs the

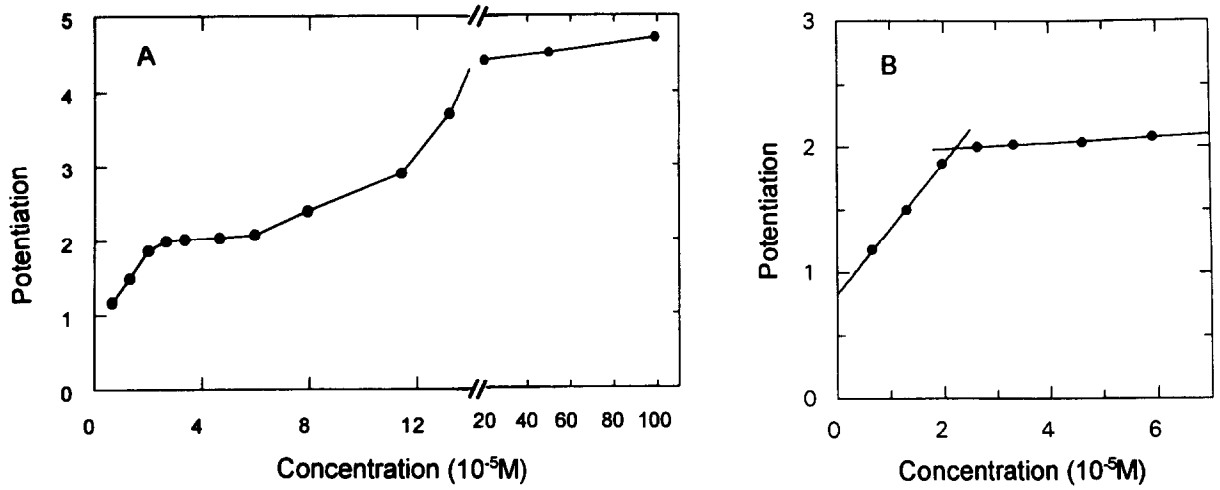

FIG. 3. (A) Potentiation of the response of the guinea pig isolated ileum to BK in the presence of different concentrations of Val-Trp. (B) Effect of low concentrations of Val-Trp on the guinea pig isolated ileum response to BK. The intersection of the lines in panel B represents the lowest Val-Trp concentration capable of causing a 2-fold potentiation of the effects of BK.

binding of Z-Val-Trp to ACE since the aromatic benzyl group at the P_1 position was expected to have favorable binding effects [46], as was also clearly demonstrated by the reduction of one order of magnitude in K_i values for Bz-Phe-Val-Trp and Bz-Gly-Val-Trp (peptides 5 and 6) in comparison to Val-Trp (peptide 1).

DVal-mTrp (peptide 4) can bind to ACE in two ways: (a) DVal-mTrp, where the free carboxyl of mTrp is located at the C-terminal position and the peptide binds to ACE with the amide bond inverted (NH ← C=O), and (b) mTrp-Val, where the free DVal carboxyl group is at the C-terminal position and the peptide binds to the enzyme with the amide group oriented in the natural direction (NH → C=O). The K_i value obtained for DVal-mTrp is three orders of magnitude higher than that for Val-Trp. This reduction in affinity may result from the inversion of the peptide bond and/or the presence of the free carboxyl at the N-terminal side of the peptide as discussed above. The introduction of Bz-gGly-NH at the DVal carboxyl group of DVal-mTrp leads to the total retro-inverso peptide Bz-gGly-DVal-mTrp (peptide 7), which presents a K_i value 10-fold lower than that of DVal-mTrp, but is three orders of magnitude higher than its analogue with the peptide bond in the natural direction.

A series of Bz-Phe-Gly-Trp (peptide 8) analogues were synthesized with the systematic inversion of each one of the three peptide bonds. Interestingly, the inversion of the first peptide bond (peptide 9) resulted in an analogue resistant to hydrolysis by ACE, without changing the affinity for the enzyme when compared with the parent peptide (peptide 8). The inversion of the other two peptide bonds resulted in an increase in the K_i values of one to two orders of magnitude (peptides 10 and 11). A similar effect was observed with Bz-Gly-Arg-Trp-OH (peptide 12) and its total retro-inverso analogue (peptide 14). The direction of the amide linkage between the residues P_1-P'_1 and P'_1-P'_2 has a significant role in the binding of the high affinity inhibitors to ACE, possibly because of the orientation of the inhibitors' residue side chains provided by the correct enzyme-inhibitor hydrogen bonds of their main chain amides. In addition, the orientation of peptide bonds and the P'_1 side chain of low affinity inhibitors seems to have a minor contribution to the ACE-inhibitor binding process since the K_i values obtained for peptides 13 and 14 were very similar and those of Bz-gGly-mGly-Gly [$3.1 \pm 0.5) \times 10^{-3}$ M] and Bz-Gly-gGly-mGly [$(5 \pm 1) \times 10^{-3}$ M] were not significantly different from the K_m [$(6.0 \pm 0.8) \times 10^{-3}$ M] for the hydrolysis of the substrate Hip-Gly-Gly.

The concentrations for the 2-fold potentiation of BK by Val-Trp (Fig. 3), Bz-Phe-Val-Trp, Bz-Gly-Val-Trp, and Bz-Phe-Gly-Trp, as calculated from the concentration–effect

TABLE 4. Inhibition of guinea pig plasma ACE and BK-potentiating activity on guinea pig ileum by BK and AI analogues

No.	Peptides	Name	K_i (M)	Concentration for 2-fold potentiation (M)
17	Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Des-[Arg^1]-BK	$(3.4 \pm 0.5) \times 10^{-7}$	7.6×10^{-7}
18	Cys-His Pro-Phe-His-Cys-NH_2	[Cys^5-Cys^{10}]-AI-(5-10)-amide	$(6 \pm 2) \times 10^{-5}$	5.0×10^{-5}

* Values are means ± SD, N = 4.

TABLE 5. Inhibition of guinea pig plasma ACE and BK-potentiating activity on guinea pig ileum by peptides from venoms of *B. jararaca* and *A. halys blomhoffii*

No.	Peptides	Name	K_i* (M)	Concentration for 2-fold potentiation† (M)
19	pGlu-Lys-Trp-Ala-Pro	BPP_{5a}	$(1.2 \pm 0.3) \times 10^{-6}$	5.8×10^{-8}
20	pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	BPP_{9a}	$(3.1 \pm 0.9) \times 10^{-8}$	5.8×10^{-9}
21	pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro	Potentiator B	$(8 \pm 2) \times 10^{-6}$	2.1×10^{-8}
22	pGlu-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	Potentiator C	$(7 \pm 1) \times 10^{-6}$	2.9×10^{-7}

* Values are means ± SD, N = 4.
† Data from Tominaga et al. [40].

curves, were very similar. The retro-inverso modification of the peptide bonds at P_1-P'_1 and P'_1-P'_2 in peptides 7, 10, and 11 resulted in ACE inhibitors lacking BK potentiation activity, at least up to a concentration of 10^{-3} M, which was the limit of their solubility in aqueous solutions. Peptide 4, which is more soluble in water due to its two carboxyl groups, also did not potentiate BK activity on guinea pig ileum up to 10^{-2} M. On the other hand, An-mPhe-Gly-Trp-OH (peptide 9), modified at the P_2-P_1, as well as Bz-gGly-Arg-mTrp-OH and Bz-gGly-DArg-mTrp-OH (peptides 13 and 14), which have P_1-P'_1 and P'_1-P'_2 peptide bonds inverted, potentiated BK at the same order of magnitude. It is noteworthy that the two latter peptides double the BK contractile activity at a concentration one order of magnitude lower than their K_i values for ACE inhibition, in contrast to the former peptide whose concentration that doubles the BK activity is two orders of magnitude higher than its K_i value (Table 3). The retro-inversion modification of peptides with ACE inhibition and BK-potentiating activities seems to have a different effect on each of these two activities. However, any further speculation regarding the dissociation of ACE inhibition from BK-potentiating activity of retro-inverso modified peptides is precluded by their low activity (10^{-4}–10^{-3} M), as well as by the complexity of the mechanism of BK-contracting activity on smooth muscles.

The two plateaus in the concentration–effect curves of BK potentiation were observed with all the peptides having BK-potentiating activity and were similar in shape to those previously described for larger BK-potentiating peptides from snake venoms [40]. The exceptions were peptides 9, 13, and 14 whose BK potentiation was not assayed in the high concentration range and des-[Arg^1]-BK for which the second plateau could not be determined since this peptide possesses intrinsic BK-like activity at concentrations higher than 10^{-5} M. The concentration of this peptide necessary to cause a 2-fold potentiation of the effects of BK was 7.6×10^{-7} M, which is approximately two orders of magnitude lower than the concentration for detectable intrinsic activity. If BK potentiation in the guinea pig ileum involves the BK receptor, these data suggest that des-[Arg^1]-BK interacts at two distinct sites of the receptor, one for agonistic activity and the other for BK potentiation. On the other hand, [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide, which was not expected to interact at the binding site of the BK receptor to cause muscle contraction, was able to potentiate the BK-induced responses at a concentration of 10^{-5} M, although it was less specific than des-[Arg^1]-BK.

The peptides from *B. jararaca* and *A. halys blomhoffii* venoms seem to be more effective as BK potentiators than as ACE inhibitors since the K_i values for ACE inhibition were one to two orders of magnitude higher than the concentration that caused a 2-fold BK potentiation. These data contrast with those obtained for des-[Arg^1]-BK or [Cys^5-Cys^{10}]-angiotensin-I-(5-10)-amide, for which the K_i and the concentrations for 2-fold BK potentiation were of the same order of magnitude. They also differed from those of Val-Trp and its analogues, which are more effective as ACE inhibitors than as BK potentiators. Therefore, these data suggest the snake venom peptides as candidate lead-compounds to design chemical structures that preferentially inhibit ACE or potentiate BK activities.

The results presented in this article, together with those reported in the literature, also suggest that the potentiation of BK contractile activity by very different peptide structures has a common general mechanism that possibly results from ACE inhibition and from a direct interaction of these peptides with ileum smooth muscle BK receptors or are in some other way involved in the cascade leading to muscle contraction. As the uses of ACE inhibitors are expanding continuously, particularly in the treatment of heart failure, it seems desirable to separate ACE inhibition by these compounds from their possible cellular effects.

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