SYNTHESIS OF FRAGMENTS OF THE VASOACTIVE INTESTINAL PEPTIDE (VIP) AND ANALYSIS OF THEIR RESIDUAL BIOLOGICAL ACTIVITIES

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The vasoactive intestinal (poly)peptide (VIP) is a linear peptide containing 28 amino acid residues, whose primary structure indicates a low metabolic stability. The following VIP fragments, as potential metabolites, and their analogues were prepared by synthesis on a solid: [His(Dnp)^{1}]VIP(1-10), VIP(11-14), [D-Arg^{12}]VIP(11-14), [Lys(Pac)^{15,21},Arg^{20}]VIP(15-22), and VIP(23-28). After purification, the peptides were characterized by amino acid analysis, mass spectrometry, RP HPLC, and capillary zone electrophoresis. In some tests, detailed examination of the biological activity of the substances in vivo and in vitro gave evidence of a low, residual activity of some fragments, viz. a depressoric activity in vivo for [His(Dnp)^{1}]VIP(1-10) and a stimulating activity for the release of α-amylase in vitro and in vivo for [Lys(Pac)^{15,21},Arg^{20}]VIP(15-22) and VIP(23-28).

The vasoactive intestinal (poly)peptide (VIP)**, which is a linear peptide containing 28 amino acid residues, was first isolated from pig duodenum[1]. Later it was disclosed that this substance is a neuropeptide which is present in both the central and peripheral parts of the nerve system[2,3]. As a neurotransmitter and neuromodulator, VIP plays an important part in the control of many motoric, secretive and metabolic processes. VIP exerts a relaxation effect on the smooth muscles of veins[4,5], respiratory organs[6], the gastrointestinal tract[7], and reproductive organs. The substance stimulates exocrine secretion in many tissues[8] and the growth and proliferation of nerve cells[9], and affect the immune functions[10]. The spectrum of its biological effects also suggests that VIP – its excess or

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** Abbreviations used: VIP, vasoactive intestinal (poly)peptide; Bzl, benzyl; CZE, capillary zone electrophoresis; Pac, phenylacetyl; TFA, trifluoroacetic acid; 2-BrZ, 2-bromobenzyloxycarbonyl. Symbols for amino acids and other abbreviations follow IUPAC recommendations (Biochem. J. *219*, 345 (1984)).

deficit – is an important factor in the pathogenesis of a number of diseases (cystic fibrosis, bronchial asthma, the pancreatic cholera syndrome, Hirschprung's and Crohn's syndromes) and hence, there is a high therapeutic potential in its molecule[11]. The main indications for VIP and its analogues are: asthma, coronary or peripheral ischemia, impotence, and gastrointestinal and some kinds of neurological disorder.

The primary structure of VIP is as follows:

His[1]-Ser-Asp-Ala-Val[5]-Phe-Thr-Asp-Asn-Tyr[10]-Thr-Arg-Leu-Arg-Lys[15]-Glu-Met--Ala-Val-Lys[20]-Lys-Tyr-Leu-Asn-Ser[25]-Ile-Leu-Asn-NH_2, which shows that the substance contains a number of amino acids whose peptidic bonds can be targets for hydrolytic enzymes (trypsin-like enzymes, convertase, chymotrypsin). With regard to the facts that peptide fragments can also participate in bonding interactions with receptors in physiological conditions and thus stimulate their own or antagonistic effects and that knowledge of such properties can serve as a basis for proposals for metabolically stabilized as well as shorter peptides with their own or inhibiting effects, we prepared some VIP fragments – presumed metabolic products – with a view to testing their residual biological activities.

EXPERIMENTAL

Amino acid derivatives and a VIP reference standard were purchased from Bachem (Bubendorf, Switzerland). Lysine(N^{ε}Pac) was kindly provided by Dr J. Pospisek of this Institute. Male rats (Wistar strain, 200–250 g) were obtained from the Research Institute for Pharmacy and Biochemistry (VUFB a.s., Czech Republic), Konarovice Division. Spofa Test Alpha-amylase was purchased from Slovakofarma (Hlohovec, Slovak Republic). Capillary zone electrophoresis (CZE) in 0.5 M acetic acid was performed on a homemade instrument[12]. Analytical RP HPLC was conducted on a Nucleosil C-18 column (5 μm, 4 × 250 mm) using an acetonitrile–water gradient in 0.1% TFA; absorbance was measured at 218 and 276 nm. Mass spectra were measured by the FAB method on a ZAB-EQ instrument (VG Analytical, Manchester, U.K.).

Peptide Synthesis

The following peptides were prepared by synthesis on a solid[13]:
His(Dnp)-Sr-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-OH, [His(Dnp)[1]]VIP(1-10) *I*
Thr-Arg-Leu-Arg-OH, VIP(11-14) *II*
Thr-D-Arg-Leu-Arg-OH, [D-Arg[12]]VIP(11-14) *III*
Lys(Pac)-Glu-Met-Ala-Val-Arg-Lys(Pac)-Tyr-OH, [Lys(Pac)[15,22],Arg[20]]VIP(15-22) *IV*
Leu-Asn-Ser-Ile-Leu-Asn-NH_2, VIP(23-28) *V*.

The peptides *I* through *IV* were prepared on a chloromethylated polystyrene carrier (Novabiochem, Laufelfingen, Switzerland) crosslinked with 1% divinylbenzene (0.96 mmol Cl/g). Carboxy-terminal tyrosine or arginine was attached by using the cesium bicarbonate method[14]. The bonded amino acid contents as determined by the picric test were as follows: 0.44 mmol/g Tyr and 0.28 mmol/g Arg. The synthesis was performed by using 0.68 g of the esterified carrier for peptides *I* and *IV* (0.3 mmol) and 1.07 g of the carrier for peptides *II* and *III* (0.3 mmol). Synthesis of peptide *V* was conducted with 4-methylbenzhydrylamine resin (Novabiochem, Laufelfingen, Switzerland), 0.37 g, at a substitution of 0.8 mmol NH_2/g. The Boc/Bzl strategy was applied; the amino acid side chains were pro-

tected as follows: His(Dnp), Ser(Bzl), Asp(Bzl), Thr(Bzl), Tyr(2-BrZ), Arg(Tos), Lys(Pac). The peptides were split off from the resin with liquid hydrogen fluoride containing anisole. The crude peptides were purified by RP HPLC on a Separon SGX-RPS column (10 μm, 250 × 16 mm, Tessek, Czech Republic) in a methanol–water gradient in 0.1% TFA. The structure of the peptides was verified by amino acid analysis[15] and by mass spectrometry applying the FAB method. Peptide *I*: Ala 1.2 (1), Asx 3.1 (3), His(Dnp) 1.0 (1), Phe 1.0 (1), Ser 1.1 (1), Thr 0.9 (1), Tyr 1.2 (1), Val 1.0 (1); mass spectrum (*m*/*z*): 1334 (M + 1). Peptide *II*: Arg 2.1 (2), Leu 1.2 (1), Thr 1.0 (1); mass spectrum (*m*/*z*): 546 (M + 1). Peptide *III*: Arg 2.0 (2), Leu 0.9 (1), Thr 1.2 (1); mass spectrum (*m*/*z*): 547 (M + 1). Peptide *IV*: Ala 1.0 (1), Arg 1.2 (1), Glx 1.0 (1), Lys 2.1 (2), Met 1.1 (1), Tyr 1.0 (1), Val 1.2 (1); mass spectrum (*m*/*z*): 1495 (M + 1). Peptide *V*: Asx 2.1 (2), Ile 1.0 (1), Leu 2.0 (2), Ser 1.1 (1); mass spectrum (*m*/*z*): 673 (M + 1).

Biological Activity Testing

Stimulation of α-amylase secretion by pancreatic acini cells in vitro. The stimulating activity for the secretion of α-amylase by acini cells of rat pancreas was determined by the method of Jensen and coworkers[16]. α-Amylase activity was determined by the method of Ceska and Birath[17].

Stimulation of pancreatic α-amylase secretion in vivo. The stimulating activity for the secretion of α-amylase was tested by the method of perfusion of the duodenum in rats in vivo. Male rats were placed in metabolic cages 20 h before the experiment; there they had free access to water only. After the application of urethane anesthesia (1.25 g/kg b.w., s.c.), a cannula was introduced into the vena femoralis. Following laparotomy, a segment of the duodenum with the bile duct was uncovered gently. The outlet cannula was introduced first (about 1 cm behind the bile duct end), the inlet cannula followed (1 cm before the bile duct end, nearer to the stomach). The places operated on were covered with a sterile wet application and the rat was laid on a thermopad whose temperature was held at 36 ± 1 °C. Perfusion of the duodenum was performed with physiological solution at a flow rate of 0.4 ml/min (a Gilson Minipuls 2 pump). The fractions were collected in 5 min intervals. The substances tested, dissolved to the resulting concentration in the physiological solution, were applied into the vena femoralis in a volume of 0.1 ml and washed with 0.2 ml of physiological solution. The concentration of the released α-amylase in perfusate was determined by the method of Ceska and Birath[17].

Relaxation of the stomach muscle in vitro. The relaxation activity of VIP and its fragments was studied on a tissue contracted with 5-hydroxytryptamine (70 nmol/l). 20 h before the experiment, male rats were placed in metabolic cages where they had free access to water only. Their stomachs were extracted after a short ether anesthesia and decapitation. A longitudinal muscle strip was prepared conventionally[18] and suspended in a test cell 7 ml in volume containing the Krebs–Henseleit medium thermostatted at 37 °C and saturated with a gas mixture of 95% O_2 + 5% CO_2 (pneumoxide). The muscle strip 2 × 15 mm was stretched to one gramme. Its contractions and subsequent relaxations were recorded, via a transducer and a contraction sensor[19], on a TZ 4620 recorder.

Blood pressure decrease in vivo. The depressoric activity of VIP and its fragments was studied by the direct method by blood pressure measurement in the arteria carotis. Male rats were subjected to urethane anesthesia (1.25 g/kg s.c.), and a cannula was introduced into the vena femoralis (for the application of substances) while a blood pressure measuring probe was introduced into the arteria carotis. Tracheotomy was performed in order to ensure regular breathing. Pressure changes were recorded with a TESLA LDP 102 pressure transducer.

Calculation of relative biological activities. The relative biological activities of the substances tested were determined based on the shifts of the parallel linearized segments of the dependences measured against the VIP reference standard.

RESULTS AND DISCUSSION

Peptide Synthesis

The peptides *I*–*V* were prepared by synthesis on a solid. The amino acid sequences of the pure peptides correspond to the sequence of the potential VIP metabolites; the modifications mentioned were chosen with regard to the planned future use in enzymatically catalyzed semisynthesis[20]. The peptides were characterized by their relative mobilities in RP HPLC and CZE (Fig. 1). CZE proved to be particularly well suited to the separation of the substances and impurities in them, if any. The fragments *I*, *II*, *IV*, and *V* were found to be nearly homogeneous (95–99%); only the fragment *III* was obtained in a lower purity (65%).

Biological Activities

The depressoric activity (Fig. 2) was tested for the following peptides (the ranges of doses applied are given in parentheses): VIP (0.02–2.0 nmol/rat), fragment *I* (20–2 000 nmol/rat), and fragments *II*, *IV*, and *V* (20–200 nmol/rat). Residual depressoric activity roughly amounting to 0.02% of the VIP activity was only observed for fragment *I*,

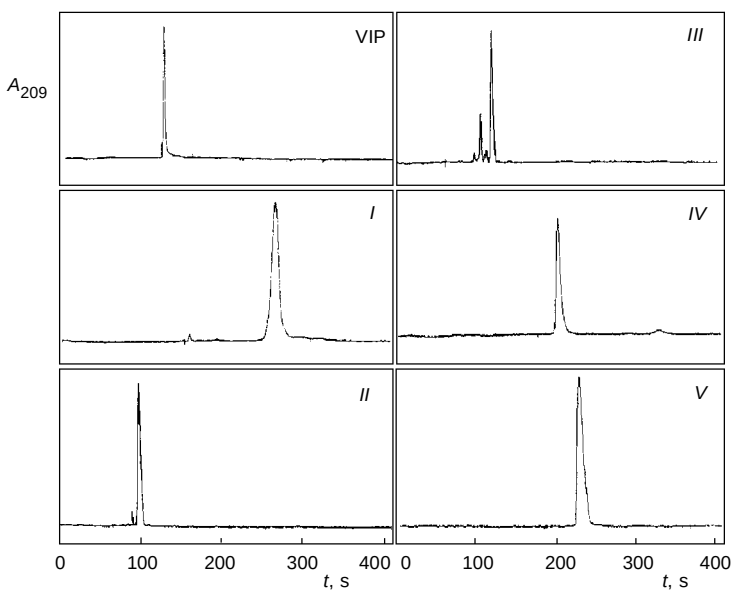

FIG. 1
Capillary zone electrophoresis of VIP and the following fragments: [His(DNP)$_1$]VIP(1-10) (*I*); VIP(11-14) (*II*); [D-Arg^{12}]VIP(11-14) (*III*); [Lys(Pac)$^{15;21}$;Arg^{20}]VIP(15-22) (*IV*); VIP(23-28) (*V*)

whereas the remaining fragments were inactive over the entire region examined. None of the fragments exhibited any inhibiting effect against VIP.

In the two tests of stimulating activity for the release of pancreatic α-amylase (Figs 3, 4), an appreciable activity was observed for fragment *V* (about 2% with respect to VIP) and a very weak activity for fragment *IV* (<0.01% in vivo and 0.1% in vitro). No stimulation was found for the remaining fragments.

The relaxation activity on the longitudinal stomach muscle was tested for the following peptides (the ranges of the resulting concentrations are given in parentheses): VIP (0.01–3.0 nmol/l), fragments *I*, *II*, *IV*, and *V* (0.020–57 μmol/l). While VIP at a concentration as low as 2 nmol/l completely relaxed the tissue stimulated with 5-hydroxytrypt-

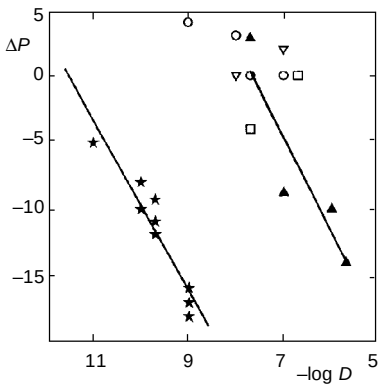

FIG. 2

Effect on blood pressure, Δ*P* (kPa) vs logarithm of the dose (*D*; mol/rat). * VIP; ▲ [His(DNP)$_1$]VIP(1-10) (*I*); ∇ VIP(11-14) (*II*); ❒ [Lys(Pac)$^{15;21}$;Arg^{20}]VIP(15-22) (*IV*); ❍ VIP(23-28) (*V*)

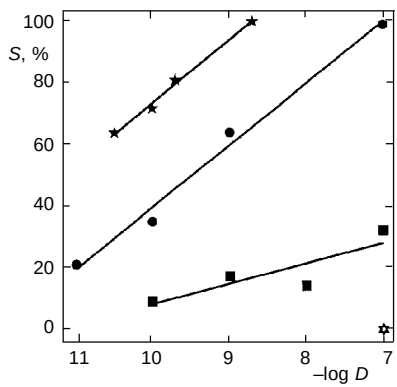

FIG. 3

Relative stimulation (*S*, %) of pancreatic α-amylase release vs logarithm of the dose (*D*; mol/rat). * VIP; Δ [His(DNP)$_1$]VIP(1-10) (*I*); ∇ VIP(11-14) (*II*); ■ [Lys(Pac)$^{15;21}$;Arg^{20}]VIP(15-22) (*IV*); ● VIP(23-28) (*V*)

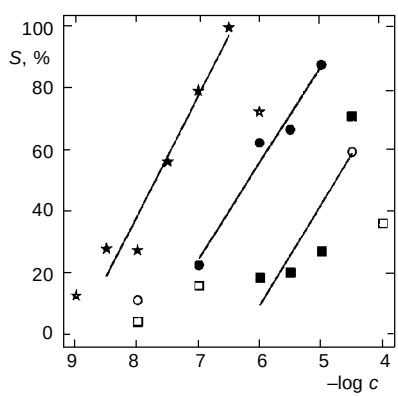

FIG. 4

Relative stimulation (*S*, %) of α-amylase release from pancreatic acini cells vs logarithm of the concentration (*c*, mol/l). * VIP; ■ [Lys(Pac)$^{15;21}$;Arg^{20}]VIP(15-22) (*IV*); ● VIP(23-28) (*V*); open symbols are used for points beyond the linear parts of bell-shaped curves

amine, all the VIP fragments were inactive over the entire concentration region; no inhibiting effect against VIP was observed either (as against the VIP(10-28) fragment which exerts a strong antagonistic effect on the relaxation activity of VIP(10-28)[21]).

Dissociation of the Individual Activities of VIP

Although all activities observed for the fragments were very low (Table I), the results indicate that a certain dissociation of the individual physiological activities can be achieved by dividing the VIP molecule into fragments. Both the agonistic and antagonistis activities for the relaxation of the stomach muscle seem to require the presence of a longer sequence.

TABLE I
Relative biological activities of VIP fragments

Test	Fragment				
	I	*II*	*IV*	*V*	VIP
Stimulation of pancreas in vivo	0	0	<0.01	2	100
Stimulation of acini cells in vitro	0	0	0.1	2	100
Decrease of blood pressure in vivo	0.02	0	0	0	100

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REFERENCES

1. Said S. I., Mutt V.: Science *169*, 1217 (1970).
2. Said S. I., Rosenberg R. N.: Science *192*, 907 (1976).
3. Fuxe K., Hokfelt T., Said S. I., Mutt V.: Neurosci. Lett. *5*, 241 (1977).
4. Brum J. M.: J. Am. Coll. Cardiol. *7*, 406 (1986).
5. Hand J. M., Lavaruso R. B., Will J. A.: Eur. J. Pharmacol. *98*, 279 (1984).
6. Grider J. R., Cable M. B., Said S. I., Makhlouf G. M.: Am. J. Physiol. *248*, G73 (1985).
7. Laurberg P.: Horm. Metab. Res. *18*, 230 (1986).
8. Fahrenkrug J., Holst-Pedersen J., Yamashita Y., Ottesen B., Hokfelt T., Lundberg J. M.: Regul. Pept. *18*, 51 (1987).
9. Magistreti P. J.: Peptides *7* (Suppl. 1), 169 (1986).

10. Rola-Plezczynski M., Bolduc D., St-Pierre S.: J. Immunol. *135*, 350 (1984).
11. Said S. I. in: *Advances in Peptide Hormone Research. Vasoactive Intestinal Peptide and Related Peptides* (S. I. Said and V. Mutt, Eds), Vol. 527. Ann. N.Y. Acad. Sci. *527*, 220 (1988). Raven Press, New York 1982.
12. Prusik Z., Kasicka V., Mudra P., Stepanek J., Smekal O., Hlavacek J.: Electrophoresis *11*, 932 (1990).
13. Barany G., Kreib-Cordonier N., Mullen D. G.: Int. J. Peptide Protein Res. *30*, 705 (1987).
14. Gisin B. F.: Helv. Chim. Acta *56*, 1476 (1973).
15. Moore S., Stein W. H.: Methods Enzymol. *6*, 819 (1963).
16. Jensen R. T., Lemp G. F., Garder J. D.: J. Biol. Chem. *257*, 5554 (1982).
17. Ceska M., Birath K.: Clin. Chim. Acta *26*, 437 (1969).
18. Vane J.: Br. J. Pharmacol. Chemother. *12*, 344 (1957).
19. Zenisek K., Barth T.: Cesk. Fysiol. *23*, 67 (1974).
20. Svoboda I., Zoulikova I., Velek J., Barth T., Hrbas P.: Unpublished results.
21. Chijiiwa Y., Murthy K. S., Grider J. R., Makhlouf G. M.: Regul. Pept. *47*, 223 (1993).