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GLYCINE-EXTENDED PROCESSING INTERMEDIATE OF proVIP: A NEW FORM OF VIP IN THE RAT

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SUMMARY: The biosynthesis of many peptides including vasoactive intestinal polypeptide (VIP) requires enzymatic α-carboxyamidation via a glycine-extended intermediate form. In an effort to identify and quantify glycine-extended VIP in rat tissue extracts a radioimmunoassay specific for this peptide was developed. The concentrations of glycine-extended VIP ranged from 1.3 pmol/g in the brain to 83.9 pmol/g in the small intestine. The identity of the peptide was substantiated by cation-exchange HPLC. The ratio of glycine-extended VIP to amidated VIP varied considerably being highest (63%) in the small intestine. The natural occurrence of glycine-extended VIP in connection with our recent demonstration of its biological activity suggest a physiological role for this biosynthetic intermediate VIP form.

A large number of peptide hormones and transmitters mature through elaborate posttranslational modifications of which α-carboxyamidation is considered decisive for the biological activity (1-3). Precursors to the amidated peptides all contain a specific amino acid sequence, called the C-terminal amidation signal, in which a glycine-residue, followed by a pair of basic amino acids, lies adjacent to the residue which is α-amidated in the final peptide. Biosynthetic processing of the precursor thus includes cleavage at the C-terminus of the dibasic sequence by endopeptidase with a trypsin-like activity followed by removal of the two basic amino acid residues by carboxypeptidase(s) (5) and cleavage of the amide donor, glycine, by a specific amidation enzyme, PAM (2,3,6). The immediate preceding precursor of an amidated peptide is consequently the glycine-extended form.

ABBREVIATIONS: VIP, vasoactive intestinal polypeptide; PHI, peptide histidine isoleucine amide 1-27; PHM, peptide histidine methionine amide 1-27; PHV, peptide histidine valine 1-42; PACAP, pituitary adenylate cyclase activating polypeptide 1-38; GHRF, growth hormone releasing factor; MES, 2-(N-morpholino)-ethanesulfonic acid; PAM, peptidyl glycine α-amidating monooxygenase.

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Vasoactive intestinal polypeptide (VIP) is a 28 amino acid neuropeptide which has a C-terminal asparagine amide(Asn-NH_2). Analysis of the amino acid sequence of the VIP precursor, prepro-VIP, deduced from the cDNA coding for human (7) or rat VIP (8) revealed that prepro-VIP contains the α-amidation signal (Asn-Gly-Lys-Arg).

In the present study a newly developed radioimmunoassay specific for glycine-extended VIP has been used to demonstrate that this intermediate biosynthetic precursor of VIP occurs in high concentration in rat tissue, especially in the intestine.

MATERIALS AND METHODS

Peptides

VIP which has C-terminal extension of glycine (VIP-Gly) and VIP which instead of the C-terminal aspargine amide has a carboxy-group at the C-terminus (VIP-free acid) were obtained by custom synthesis by Bachem, Torrance, California, USA. A dodecapeptide, corresponding to sequence 20-28 of VIP extended with glycine-lysine-arginine (VIP 20-28 Gly-Lys-Arg) and a tetradecapeptide corresponding to sequence 20-28 of VIP extended with glycine-lysine-arginine-serine-serine (VIP 20-28 Gly-Lys-Arg-Ser-Ser) were obtained by custom synthesis from Cambridge Research Biomedical, Cambridge, UK. Synthetic VIP, PHI, PHM, PHV, PACAP, helodermin, GHRF, secretin and glucagon were all purchased from Peninsula Laboratories Europe, St. Helens, UK.

Tissue extraction

Eight rats were killed by anaesthetic overdose (Halothane). Samples of the brain cortex and the whole wall of the stomach, small and large intestine were rapidly removed, immediately frozen on dry ice and weighed. Following storage at -20 °C for less than a month VIP and VIP-Gly were extracted at 4 °C from each tissue specimen by homogenization in acidified ethanol (70% ethanol containing 0,74% HCl). Solids were removed by centrifugation and the supernatants decanted and dried under vacuum. Samples were reconstituted in assay buffer and analysed in duplicate in at least two different dilutions.

Radioimmunoassay

The concentrations of amidated VIP in tissue extracts and column eluates were measured immunochemically according to published method (9,10) using VIP-antiserum 5601-9. This antiserum does not crossreact with VIP-Gly or VIP-free acid.

Antibodies against glycine-extended VIP were raised in rabbits, immunized with VIP-Gly coupled at the amino terminal end to bovine serum albumin by means of glutaraldehyde. Twenty microliters of glutaraldehyde 25% was added dropwise to 1 ml phosphate buffer 0.25 mol/l, pH 7.4 containing 0.7 μmol bovine serum albumin and 2.1 μmol VIP-Gly. Under these conditions approximately 70% of the peptide was coupled. The coupled peptide was emulsified with a double volume of Freund's adjuvant before immunization of eight random breed white Danish rabbits. Each rabbit received a dose of the antigen mixture equal to 30 nmol VIP-Gly for initial immunization , and 15 nmol for boosters subcutaneously at multiple sites. The animals were boosted every eighth week. VIP-Gly was radiolabeled with ^{125}I using the chloramine T method and the label was purified by chromatography on a Sephadex G-50 superfine column as previously described for VIP (9). For measurement of VIP-Gly in tissue extract 500 μl of reconstituted sample or standard was incubated with 200 μl of antiserum 814-9 [diluted 1:4000 in assay buffer (0.04 mol/l phosphate buffer containing 58 μmol human serum albumin, pH 7.4)] for 48 h at 4 °C. After addition of 3.0 fmol radiolabeled VIP-Gly

(100 μl) and incubation for another 48 h, bound and free peptide were separated at 4 °C by absorption to plasma-coated activated charcoal. Synthetic VIP-Gly was used as standards.

Chromatography

Dried tissue extracts reconstituted in equilibrium buffer and filtered through Millex AA 0.8 μm filters (Millipore) were run on a cation-exchange column (HR5/5 Mono S, Pharmacia) equilibrated with 20 mmol/l KCl in 50 mmol/l MES and eluted with a linear gradient of 1.0 mol/l KCl in 50 mmol/l MES for 78 min at a flow rate of 0.7 ml/min. The column was in separate runs calibrated with synthetic VIP-Gly and VIP. The concentration of VIP-Gly and amidated VIP in eluted fractions of 1.4 ml were determined by radioimmunoassay.

RESULTS AND DISCUSSION

Competitive binding curves (Fig. 1) disclosed that the selected antiserum (code No. 814-9) was specific for VIP-Gly and failed to cross-react with amidated VIP and VIP-free acid. Extensions of the peptide beyond the glycine moiety did not seem to be detected since VIP 20-28 Gly-Lys-Arg and VIP 20-28 Gly-Lys-Arg-Ser-Ser were unable to displace [^{125}I]-VIP-Gly from the antibody. Furthermore, the structurally related peptides PHI, PHM, PHV, PACAP, helodermin, GHRF, secretin and glucagon did not cross-react in the radioimmunoassay (Fig. 1). The ID_{50} of the VIP-Gly radioimmunoassay was 60 pmol/l with intra-assay and interassay coefficients of variations of 6% and 9%, respectively. Mean recovery of standard VIP-Gly added to tissue before extraction (n=6) was 72% and serial dilutions of extracted tissue specimens containing high levels of VIP-Gly displaced antibody binding of radiolabel in parallel with the standard curve (Fig. 1).

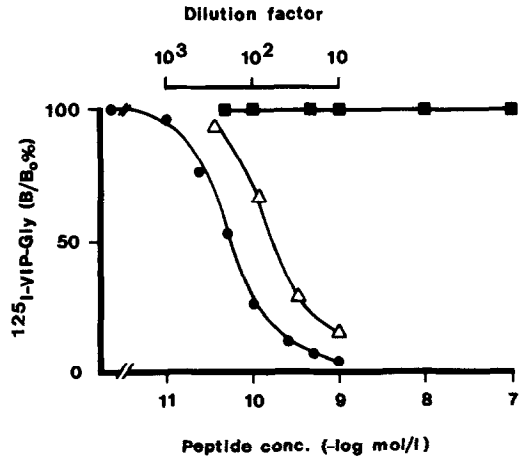

Fig. 1. Inhibition of the binding of ^{125}I-VIP-Gly to antiserum No. 814-9 by increasing concentrations of glycine-extended VIP (●—●). No cross-reactivity was observed with VIP 20-28 Gly-Lys-Arg, VIP 20-28 Gly-Lys-Arg-Ser-Ser, VIP-free acid, PHI, PHM, PHV, PACAP, helodermin, GHRF, secretin and glucagon (■—■). Serial dilution of extract from rat small intestine (Δ—Δ) was parallel to the standard curves.

Table 1
Tissue concentration of glycine-extended VIP (VIP-Gly) and amidated VIP (VIP-NH_2) in rat brain and gut

	VIP-Gly pmol/g	VIP-NH_2 pmol/g	VIP-Gly/VIP-NH_2 (%)
Brain	1.3 ± 0.1	43.2 ± 3.5	3.2 ± 0.3
Stomach	4.0 ± 0.4	120.8 ± 10.4	3.5 ± 0.3
Small intestine	83.9 ± 5.2	137.5 ± 8.1	62.8 ± 5.3
Large intestine	45.9 ± 3.9	204.3 ± 24.3	23.5 ± 1.4

Figures are means ± SEM for 8 specimens.

The concentrations of VIP-Gly and amidated VIP in acidified ethanol extracts of the brain and regions of the gastrointestinal tract of rat are shown in Table 1. Glycine-extended VIP was present in all tissues ranging from 1.3 pmol/g in the brain to 83.9 pmol/g in the small intestine. The ratio of glycine-extended to amidated VIP varied considerably between the regions, VIP-Gly constituting a major fraction in the small intestine. The identities of the two peptides in tissue extracts were substantiated by cation-exchange HPLC which demonstrated that the immunoreactive peaks eluted at the positions of synthetic VIP-Gly and VIP, respectively (Fig. 2).

The existence of this new VIP variant, VIP-Gly, which is most abundant in the rat intestine has not been reported previously. Even though a VIP antibody detects or cross-reacts with VIP-Gly, this peptide may be overlooked because it coelutes with VIP in current chromatographic systems. VIP-Gly serves as substrate for the amidation reaction mediated

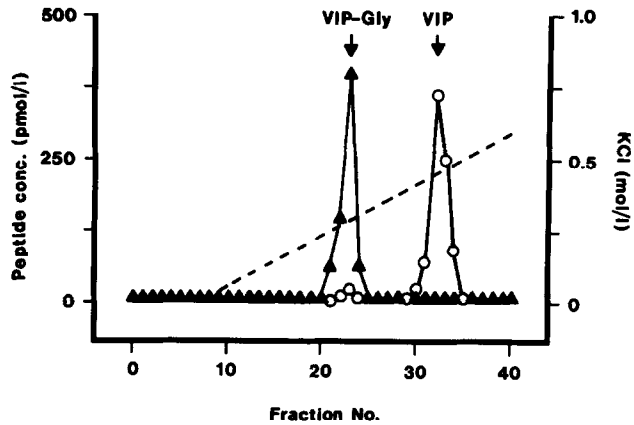

Fig. 2. Cation-exchange HPLC of small intestinal extracts on a Mono S column eluted with a linear gradient of KCl (------). Fractions were quantified for glycine-extended VIP (▲—▲) and VIP (o—o) by radioimmunoassay. The elution positions of synthetic VIP-Gly and VIP are indicated.

by PAM which depends on copper, ascorbic acid and molecular oxygen. It is likely that the relative abundance of VIP-Gly in the small intestine reflected low enzyme activity compared to that in the brain and stomach. Alternatively, VIP-Gly may constitute a poor substrate for rat intestinal PAM since the enzyme is present in several forms in various rat tissues (11). Since we have recently demonstrated that VIP-Gly has full biological activity (12), its natural occurrance might suggest that the peptide is of physiological importance.

ACKNOWLEDGMENT

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