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BIOLOGY OF ENTEROSTATIN.II. DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR ENTEROSTATIN (Val-Pro-Asp-Pro-Arg), THE PROCOLIPASE ACTIVATION PEPTIDE

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Summary: Enterostatins belong to a family of pentapeptides (e.g., Val-Pro-Asp-Pro-Arg in pig, horse, dog, and rat; Ala-Pro-Gly-Pro-Arg in human and chicken; and Val-Pro-Gly-Pro-Arg in rat) derived from the amino-terminus of procolipase after the action of trypsin. Pharmacologic studies with Val-Pro-Asp-Pro-Arg have suggested a role for this peptide in appetite regulation and pancreatic insulin secretion. Studies into the distribution of enterostatins or the role of endogenous peptides have not been possible due to the lack of a suitable method for enterostatin assay. To this end, we raised a highly specific antibody and developed an enzyme-linked immunosorbent assay for Val-Pro-Asp-Pro-Arg . Using the newly developed assay we have shown the presence of Val-Pro-Asp-Pro-Arg-like immunoreactivity (2455±440 pmol/g) in the rat brain.

Enterostatin is a pentapeptide that is generated by the action of trypsin on procolipase in the intestinal lumen (1). The structure of enterostatin is highly conserved in evolution with an amino acid sequence of VPDPR in pig, horse, dog, and rat, APGPR in human and chicken, and APE-R in dogfish (2). The structure of rat enterostatin was determined earlier to be VPDPR by amino acid sequence of pure rat pancreatic procolipase (2); however, in a recent study based on the structure of colipase cDNA, the amino terminal of procolipase was found to be VPGPR (3).

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Abbreviations Used: APGPR, Ala-Pro-Gly-Pro-Arg; BS, bis(sulfosuccinimidyl) suberate; DSDPR, Asp-Ser-Asp-Pro-Arg; ELISA, enzyme-linked immunosorbent assay; HVPDPR, His-Val-Pro-Asp-Pro-Arg; KHL, Keyhole limpet hemocyanin; PBS, phosphate buffered saline; PTPS.NH_2, Pro-Thr-Pro-SerNH_2, VEPIPY, RSA, rabbit serum albumin; Val-Glu-Pro-Ile-Pro-Tyr; VGDE, Val-Gly-Asp-Glu; VPL, Val-Pro-Lys; VPL,VPL,VPDPR, Val-Pro-Asp-Pro-Arg; VPDPRC Val-Pro-Asp-Pro-Arg-Cys; VPDPRH, Val-Pro-Asp-Pro-Arg-His; VPDPRRY, Val-Pro-Asp-Pro-Arg-Arg-Tyr; VPDPRVPDPRC , Val-Pro-Asp-Pro-Arg-Val-Pro-Asp-Pro-Arg-Cys; VPGPR, Val-Pro-Gly-Pro-Arg; YVPDPR, Tyr-Val-Pro-Asp-Pro-Arg

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In 1988, Erlanson-Albertsson et al were the first to show that enterostatin was a biologically active peptide that decreased food intake in rats (4,5). Since that initial discovery, this pentapeptide not only has been shown to cause anorexia in rats (4,5) and sheep (6) but to be a selective inhibitor of fat intake in rodents (7-9). In addition, enterostatin has also been shown to inhibit secretion of insulin (10) and pancreatic enzymes (11) as well as to augment corticosterone output in rodents (8). Further progress into the distribution and action of enterostatin has been slow due to the lack of a sensitive assay for this peptide. To this end, we have developed a sensitive and highly specific assay for this peptide and have measured its distribution in rat serum.

MATERIALS AND METHODS

1) Peptides and reagents for ELISA assay :

The peptides HVPDPR, VPDPRH, YVPDPR, VPDPRRY, VPDPRC, APGPR, and VPDPRVPDPRC were synthesized by LSUMC Core Laboratories, New Orleans, or Alpha Diagnostics, San Antonio, TX, USA. The purity of the peptides was determined by one or more of the following methods: mass spectroscopy, high-pressure liquid chromatography, amino acid analysis, and amino acid sequence analysis. Porcine pancreatic colipase and the peptides VPDPR, VPL, VEPIPY, VGDE, PTPS.NH_2 and DSDPR were products of Sigma Chemical Co. The second antibody, substrate, and all other reagents for ELISA were purchased from Alpha Diagnostic International, Inc., San Antonio, TX, USA.

2) Production of polyclonal antibody:

VPDPRVPDPRC was coupled to Keyhole limpet hemocyanin (KHL) using MBH bifunctional agent. Two adult New Zealand rabbits received primary injection of peptide-KLH conjugate (0.3-0.4 mg / rabbit) emulsified in Freund's complete adjuvant. All injections were made at multiple sites by subcutaneous and intramuscular routes. A booster injection was given with peptide-KLH conjugate (0.3-0.4 mg / rabbit) emulsified in Freund's incomplete adjuvant at 2-week intervals. The first bleed was taken 1 week after the fifth injection, and the antibody titer was checked as described in the methods. Thereafter, animals were boosted every 2 weeks and bled the following week.

3) Enzyme-linked immunosorbent assay (ELISA):

i) Preparation of coating antigen: One ml of a bis(sulfosuccinimidyl) suberate (BS) solution (5 mg/ml) in 10mM phosphate-buffered saline (PBS) containing 0.1% NaN_3, pH 7.2 was added dropwise to 2 ml of rabbit serum albumin (RSA) solution (10 mg/ml) in PBS and stirred for 2 hrs at 22^{0}C. Excess BS was removed by gel filtration through a G-50 column. The column was eluted with PBS and 50 (2 ml) fractions were collected. Protein-containing fractions (fraction 7-8) were pooled together. The pooled protein fraction (4.0 ml) was incubated overnight at 4^{0}C with 1 mg VPDPRC, followed by 0.4 ml of 1 M glycine in PBS for 2 hrs at 22^{0}C. The RSA-BS-VPDPRC solution was dialyzed against 500 ml of 0.9% NaCl (saline) for 48 hrs with 4 changes of dialysis buffer. The protein content of RSA-BS-VPDPRC preparation was measured using BCA protein assay, diluted with saline containing 0.1% NaN_3 to a protein concentration of 0.5 mg/ml, and stored frozen at -20^{0}C in 0.1 ml aliquot.

ii) Procedure for competitive ELISA: RSA-BS-VPDPRC (0.5 mg/ml) was diluted to 1.0 µg/ml in coating buffer (50 mM sodium phosphate, 145 mM NaCl, pH 7.4 containing an antigen stabilizer). The wells of high-binding microtiter plates were coated with 0.1 ml of RSA-BS-VPDPRC (1.0 µg/ml) by overnight incubation at

4^{0}C. All further operations were performed at room temperature (22-23^{0}C). To wash the wells of the microtiter plate or to remove its contents, the plate was rapidly inverted and the contents forcefully dashed into a tray. Each well was washed 3 times with 0.3 ml wash buffer (50 mM sodium phosphate, 145 mM NaCl, 0.05% Tween, 0.1% NaN_3, pH 7.4 containing an antigen stabilizer), blocked for 3 hours with 0.2 ml of blocking buffer (10% bovine serum albumin, 50 mM sodium phosphate, 145 mM NaCl, pH 7.4 containing an antigen stabilizer), and the buffer removed. To each well, an unknown sample or increasing amounts of VPDPR or other peptides in a total volume of 50 µl, 50 µl of the VPDPR-antibody diluted 1:4000 in ELISA buffer (1.5% bovine serum albumin, goat/fetal bovine serum, 0.1% NaN_3 containing an antigen stabilizer), and 150 µl of ELISA buffer was added and incubated for 3 hours. At the end of incubation, plates were washed 3 times with wash buffer, and 0.1 ml of goat anti-rabbit IgG conjugated with horseradish peroxidase (diluted 1:2000 in ELISA buffer) was added and incubation continued for an additional 30 minutes. Plates were washed 5 times with wash buffer, the enzymatic reaction was initiated by the addition of 0.1 ml of TMB substrate solution (50 mM tetramethylbenzidine, 1% dimethylsulfoxide, 0.01% hydrogen peroxide containing an antigen stabilizer) and terminated 15 minutes later by the addition of 0.1 ml of stop solution (0.2 M sulfuric acid in water). The absorbance was measured at 450nm using an ELISA plate reader.

4) Preparation of brain extracts for ELISA:

Adult Sprague-Dawley rats (Hilltop Lab Animals, Inc) were killed by decapitation, and the brains were removed and homogenized in ice-cold 0.4 M perchloric acid (10 ml/g tissue) using a Virtis Polytron (for 3 X 15 sec at a setting of 60). The homogenate was centrifuged at 2,000 X g for 30 min at 4^{0}C, and the supernatant was adjusted to pH 7.2 with saturated K_2CO_3, incubated on ice for 60 min and then recentrifuged as above. The clear supernatant was lyophilized to dryness and then resuspended (1 ml/brain) in ELISA buffer for assay or in TBS (50 mM Tris.HCl, 150 mM NaCl, 3.1 mM NaN_3, pH 7.4) for chromatography. If necessary, samples were centrifuged to eliminate residual potassium perchlorate before assay or chromatography.

5) Chromatography:

One ml of brain extract was loaded on a Sephadex G-25 column (l = 60 cm, d = 1.2 cm, v = 67.8 ml) equilibrated with TBS, the column was eluted with TBS, and 60 fractions (1.2 ml / fraction) were collected. Each fraction was subjected to ELISA for VPDPR using 5 different dilutions of the sample (1:2, 1:4, 1:8, 1:16, and 1:32), and the peptide concentration was calculated using the linear portion of the competition curve. In our scheme of dilution, 1:2 and 1:4 dilutions are equnalent to 50% and 25% dilutions, respectively.

RESULTS

We experimented with different conditions (pH, temperature, duration of incubation, etc.) and composition of reagents to establish an optimal condition for ELISA assay, which is described under methods. The presence of antibody to VPDPR was detected using ELISA. A high antibody titer was achieved by week 9, and thereafter, the titer remained almost constant for at least additional 6 weeks. The antibody (R151B2) used in this study was bleed #2 from rabbit #151.

The data presented in Figure 1 show that addition of synthetic VPDPR to the assay well led to a dose-dependent decrement in the binding of VPDPR-antibody to RSA-BS-VPDPRC attached to the well and, therefore, to a decrease in A_{450nm}. Under the conditions described in this assay, the limit of detection was about 300 pg per well or 10.2 nM. The useful range of the standard curve, however, extended up to 20 ng/well or 0.68 μM. The intra-assay inter-assay coefficients of variation derived from three replicate samples containing 0.5, 1.0, 5.0, 10.0, or 20 ng VPDPR per well were 5.6 and 9.8%, respectively. The addition of rat brain extracts to the assay well reduced A_{450nm} in proportion to its VPDPR content in a manner parallel to the synthetic VPDPR (Figure 1). These data suggest an immunoidentity between brain VPDPR-like immunoreactivity and synthetic VPDPR.

To further characterize the properties of VPDPR-antibody (R151B2), we compared the inhibition of binding of VPDPR-antibody to RSA-BS-VPDPRC by VPDPR, colipase, and 12 VPDPR-analogs or peptides with some structural homology with VPDPR. The results of this comparison are summarized in Table 1. The antibody exhibited the highest affinity for VPDPRVPDPRC, the peptide used as an antigen to generate the antibody. The addition of amino acids on the amino-

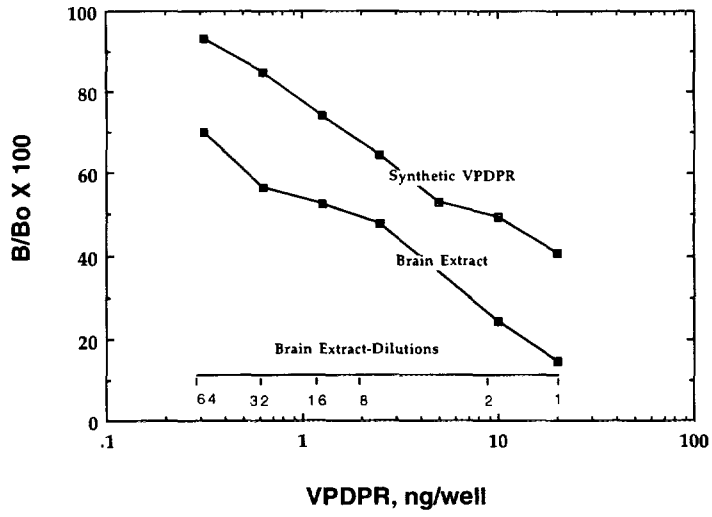

Figure 1. The analysis of immunoidentity between synthetic VPDPR and VPDPR-like immunoreactivity in the rat brain extracts. The addition of synthetc VPDPR to ELISA wells resulted into a dose-dependent decrement in the binding of VPDPR-antibody to RSA-BS-VPDPRC attached to the well and, therefore, a decrease in A_{450nm}. The inhibition curve generated with serial dilutrions of the brain extract was parallel to the authentic VPDPR. B_0 and B refer to A_{450nm} in the absence and presence of added VPDPR or brain extract, respectively.

Table 1: The specificity of rabbit anti-VPDPR antibody

Peptide	Cross-reactivity (%) #
Val-Pro-Asp-Pro-Arg	100.00
Val-Pro-Asp-Pro-Arg-His	100.00
Tyr-Val-Pro-Asp-Pro-Arg	21.45
His-Val-Pro-Asp-Pro-Arg	3.70
Val-Pro-Asp-Pro-Arg-Arg-Tyr	<0.125
Val-Pro-Asp-Pro-Arg-Arg-Cys	153.85
Val-Pro-Asp-Pro-Arg-Arg-Val-Pro-Asp-Pro-Arg-Arg-Cys	312.50
Ala-Pro-Gly-Pro-Arg	<0.125
Val-Pro-Leu	<0.125
Val-Glu-Pro-Ile-Pro-Tyr	<0.125
Val-Gly-Asp-Glu	<0.125
Pro-Thr-Pro-SerNH2	<0.125
Asp-Ser-Asp-Pro-Arg	<0.125
Colipase	<0.125

Cross-reactivity is calculated on the basis of peptide doses needed to inhibit enzymatic reaction by 25% under conditions outlined in the methods.

terminus, but not the carboxy-terminus (with the exception of VPDPRRY), of VPDPR resulted in a significant loss of cross-reactivity. It must be pointed out that VPDPR-antibody (R151B2) exhibits no cross-reactivity for APGPR, the human form of enterostatin (2).

To further characterize the nature of VPDPR-like immunoreactivity in the rat brain, one whole brain was extracted with perchloric acid and the extract was subjected to gel-filtration on a Sephadex-G25 column as described under method. The column was washed and used to fractionate synthetic VPDPR under conditions identical to those used for the brain extract. Each fraction was assayed for VPDPR using ELISA and the results (Figure 2) show an almost identical elution profile of synthetic VPDPR and brain VPDPR-like immunoreactivity.

The data presented in Table 2 show a distribution of VPDPR-like immunoreactivity in 5 male and 6 female whole rat brains. Enterostatin was found in all samples examined with a mean of 2455 pmol/gram wet tissue and the range of 634 to 5132 pmol/g wet tissue. While the male brains appeared to contain more

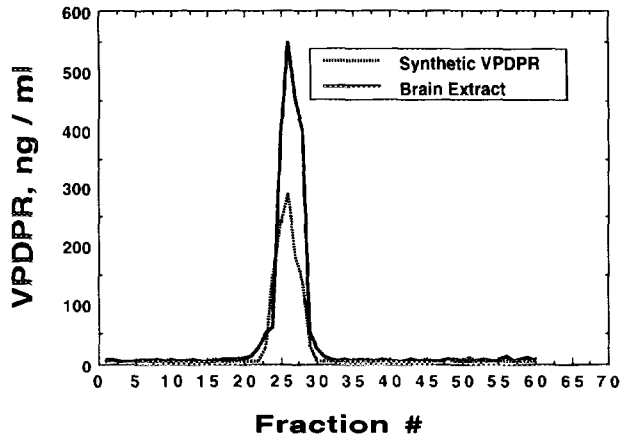

Figure 2. Chromatographic profile of synthetic VPDPR and brain extract VPDPR-like immunoreactivity on a Sephadex G-25 column.

VPDPR-like immunoreactivity than did the female brains, the differences were not statistically significant, due to large standard deviations and the small number of observations.

DISCUSSION

The results of the studies presented herein show for the first time i) the generation of a highly specific antibody to rat enterostatin (VPDPR) that does not recognize human enterostatin (APGPR), ii) the use of this antibody to develop

Table 2: Distribution of Enterostatin-like Immunoreactivity in Male and Female Rat Brains

Parameters	Enterostatin, pmol / g tissue		
	Male only	female only	male & female
Mean	3126.8	1895.2	2455.0
SD	1837.1	856.6	1459.7
SEM	821.6	349.7	440.1
Sample size	5	6	11
Minimum	634.2	787.7	634.2
Maximum	5132.4	3343.4	5132.4

ELISA for VPDPR, and iii) the presence of VPDPR-like immunoreactivity in the rat brain. The mean whole brain level of VPDPR was 2455 pmol/g tissue with SD, SEM, and a range of 1460, 440, and 634 to 5132, respectively (Table 2). The measurements (12, 13) of the levels of other enterostatin-like peptides using a rabbit anti-CYAPGPR antibody (12) have revealed significantly lower concentrations of APGPR in human urine (50-270 nM) and serum (5-40 nM), and VPGPR in the rat intestinal content (1.42±0.14 μM). However, the concentration of VPDPR in the rat brain reported here is very large compared with those of many other neuropeptides (14-19) including, met-enkephalin (340±22 pmols), neurotensin (90-144 pmols), leu-enkephalin (92 ±6 pmols), substance P (92.4 pmols), somatostatin (85.1 pmols), cyclo(His-Pro) (42.4 ±4.9 pmols), thyrotropin-releasing hormone (26.6 ±2.0 pmols), and bombesin (15.6 ±3.6 pmols)

VPDPR can arise from tryptic activation of procolipase (1); however, since there is no evidence of the presence of procolipase in the brain, the origin of brain VPDPR can only be speculated. It is conceivable that the brain may contain an endogenous procolipase-like peptide serving as an enterostatin precursor, or that enterostatin may arise from a source unrelated to procolipase. Since enterostatin exhibits a very low permeability across the gastrointestinal tract, the possibility that brain enterostatin may be derived from a peripheral peptide circulating in the blood seems unlikely (20).

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