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IMMUNOREACTIVE HELODERMIN-LIKE PEPTIDES IN RAT : A NEW CLASS OF MAMMALIAN NEUROPEPTIDES RELATED TO SECRETIN AND VIP

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Helodermin is a peptide from the venom of the lizard Heloderma Suspectum (Gila Monster) showing a high degree of sequence similarity with VIP, PHI and secretin in its N-terminal moiety. The present data support the presence of peptide(s) closely related to helodermin in the brain, gut and salivary glands of rat. In our radioimmunoassays, we routinely used one of the three specific antisera obtained from rabbits that were immunized against lizard helodermin coupled to bovine serum albumin with carbodiimide. Heat- and acid-stable immunoreactive helodermin-like material was more abundant in striatum, hippocampus and anterior pituitary than in cerebral cortex and hypothalamus. High levels of helodermin-like material were also present in salivary glands, duodenum and jejunum. When submitted to gel permeation chromatography on a TSK-G 2000 SW column, the apparent molecular radius of most of the immunoreactive material ranged from 6 to 12 KDa.

Helodermin is a basic pentatriacontapeptide amide (His-Ser-Asp-Ala-Ile-Phe-Thr-Gln-Gln-Tyr-Ser-Lys-Leu-Leu-Ala-Lys-Leu-Ala-Leu-Gln-Lys-Tyr-Leu-Ala-Ser-Ile-Leu-Gly-Ser-Arg-Thr-Ser-Pro-Pro-Pro-NH_2) recently isolated from the venom of the lizard Gila Monster (1,2). The N-terminal 1-27 sequence of helodermin shows a high degree of sequence similarity with peptides of the secretin/VIP/PHI/GRF/glucagon family from mammal and bird. Helodermin is also biologically active based on its ability to stimulate mammalian adenylate cyclase activities and its capacity to increase cyclic AMP levels in intact pancreatic acini (1,3). It interacts with either VIP-

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preferring or with secretin-preferring receptors depending on the tissue considered (4,5,6). In the present paper, we tested the hypothesis that lizard helodermin might be the prototype of a new class of regulatory peptides in mammalian tissues. For this purpose, we immunized rabbits against helodermin in order to develop a radioimmunoassay, and searched rat tissue extracts for the presence of immunoreactive helodermin-like material.

MATERIALS AND METHODS

Peptides

Natural helodermin (1-35)-NH_2 was prepared as described (1). The batch of helodermin used for rabbit immunization and for calibration of the radioimmunoassay was that previously utilized to establish the primary amino acid sequence of the peptide (2). Helodermin (1-35)-NH_2 and helodermin fragments (1-27)-NH_2, (7-35)-NH_2, (13-35)-NH_2, (17-35)-NH_2, (18-35)-NH_2 and (22-35)-NH_2 were synthesized by solid-phase methodology (7). Synthetic VIP and GRF(1-29)-NH_2 were generous gifts from Dr. D. Coy (Tulane University, MO, USA). Synthetic secretin, synthetic GRF(1-40)-OH, natural porcine glucagon, and natural chicken VIP were kindly given by, respectively, Dr. W. König (Hoechst Aktiengesellschaft, Frankfurt/Main, F.R.G.), Dr. J. Rivier (Peptide Biology Laboratory, The Salk Institute, San Diego, CA, USA), Dr. A. Moody (Novo Industri, Copenhague, Denmark) and Dr. S. I. Said (Okhlahoma City, OK, USA). PHI was purchased from Peninsula Laboratories Inc (San Carlos, CA, USA).

Induction of antibodies

1 mg helodermin was coupled to 9 mg bovine serum albumin in a total volume of 800 µl (made of 650 µl of 0.05 M sodium phosphate buffer (pH 7.45) and 150 µl of dimethylformamide), in the presence of 70 mg carbodiimide and 100,000 cpm ^{125}I-labelled helodermin as a tracer. The mixture was stirred gently at room temperature for 15 hrs and protected from light. It was then dialyzed for 24 h against 0.01 M phosphate buffer (pH 7.45) containing 0.15 M NaCl. The efficacy of coupling was 83 % as estimated by counting the radioactivity remaining in the dialysate. Three albino rabbits were each injected intradermally on the back at multiple sites with the equivalent of 80 µg helodermin as a conjugate emulsified with Freund's complete adjuvant and pertussis vaccine. Starting 3 weeks after the first injection, each rabbit was reinjected with a 80 µg helodermin equivalent of conjugate in the emulsion (without Freund's adjuvant) and injected again with the same dosis one month later. The rabbits were bled 2 weeks after each injection. The antisera obtained after the third injection were used for RIA (see below).

Radioimmunoassay (RIA) for helodermin

The assay was performed in a buffer containing 0.01 M sodium phosphate (pH 7.5), 0.9 % sodium chloride, 0.1 % sodium azide and 0.05 % Tween 20. The incubation started at 5° C with 0.2 ml of the sample dissolved in assay buffer and 0.3 ml of helodermin antiserum diluted in assay buffer enriched with 0.2 % normal rabbit serum. After 16-20 hrs incubation in the cold room, 0.05 ml of (^{125}I)iodohelodermin in assay buffer (10,000 cpm/tube) were added and the incubation was continued for a further 40 hrs period. Bound and free labelled peptides were separated by the second antiserum precipitation method using sheep antirabbit serum. The precipitation reaction was enhanced by adding 3.3 % polyethylene glycol (8). After a 5 min incubation at room temperature, the tubes were centrifuged at

1500 x g for 60 min at 4° C, the supernatant was discarded, and the pellet counted in a gamma counter. Helodermin was iodinated using the chloramine T method (6) and purified by cellulose absorption as previously described (9).

Tissue extraction

Male Wistar albino rats of 200-250 g were killed by decapitation and ensanguinated. The tissues were rapidly dissected out, weighed and frozen in liquid nitrogen. They were later quickly homogenized in distilled water (1 ml for 100 mg tissue), using a Polytron for 20 sec, then immediately boiled for 5 minutes in a hot water bath. The samples were then rapidly chilled over ice and an equal volume of 1.0 M acetic acid was added. The samples were rehomogenized and centrifuged at 30,000 x g at 4° C for 50 min. Each supernatent was lyophilized. For radioimmunoassay and for chromatography the material was redissolved in, respectively, the RIA buffer (0.01 M sodium phosphate buffer (pH 7.4), 0.15 M NaCl, 0.1 % sodium azide and 0.05 % Tween 20) or in a 30 % 1-propanol, 0.4 M ammonium acetate buffer (pH 7.0). If necessary, the material was further centrifuged at 30,000 x g for 30 min and any insoluble material was discarded.

Gel permeation chromatography

A 0.2 ml aliquot of the redissolved lyophilizate was applied to a 7.5 x 600 mm TSK-G 2000 SW high performance column (L.K.B., Bromma, Sweden) equilibrated in 30 % 1-propanol, 0.4 M ammonium acetate buffer (pH 7.0) and eluted at room temperature with the same buffer at 0.1 ml/min. The column was calibrated with bovine serum albumin (mol wt 67,000), myogobin (mol wt 17,000), cytochrome C (mol wt 12,500), insulin (mol wt 6,000), helodermin (mol wt 3,800), insulin chain A (mol wt 2,500), and bacitracin (mol wt 1,500). Five min fractions were collected, lyophilized, then redissolved with RIA assay buffer.

RESULTS

The three rabbits injected with BSA-coupled helodermin developed antibodies recognizing helodermin when tested after the third immunization (Fig. 1). No cross reactivity of the three antisera with either VIP (from chicken and pig), PHI, GRF(1-29)-NH_2, GRF(1-40)-OH or glucagon was detected. A population of immunoglobulins in antiserum 13-3 recognized efficiently the C-terminal helodermin fragments (7-35)-NH_2 and (13-35)-NH_2 but not fragments (17-35)-NH_2 and (22-35)-NH_2. Antiserum 14-3 cross reacted with fragment (7-35)-NH_2 but did not cross react significantly with fragment (13-35)-NH_2 and shorter C-terminal fragments. Antiserum 15-3 appeared to be highly specific for the N-terminal part of helodermin as it could not recognize significantly any of the C-terminal fragments. This 15-3 antiserum was chosen for further studies as : 1) it was the most sensitive antiserum for helodermin (with a limit of sensitivity of 2 pg and 50 % inhibition of tracer binding at 20 pg of natural helodermin); 2) its N-terminal specificity made it appropriate to search for helodermin-like

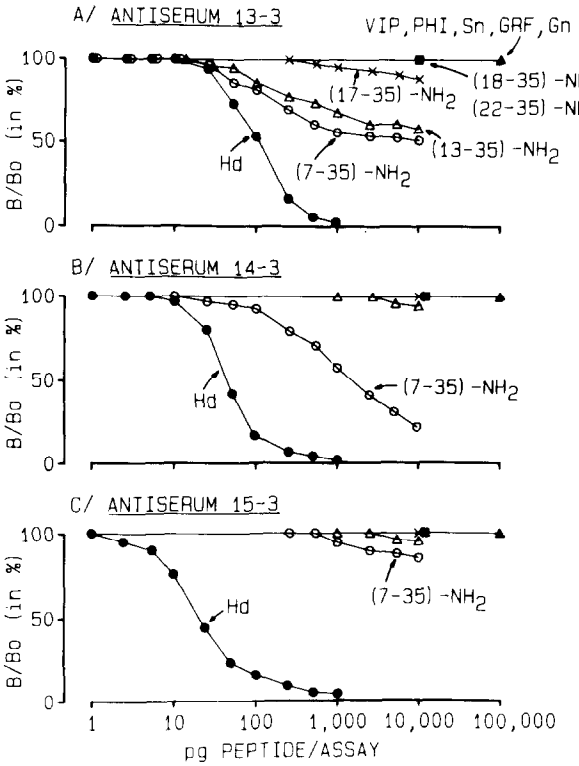


Fig. 1. Displacement of ^{125}I-helodermin bound to antiserum 13-3 (panel A), 14-3 (panel B) and 15-3 (panel C) by increasing concentrations of pure natural helodermin (●), synthetic fragments of helodermin (7-35)-NH_2 (o), (13-35)-NH_2 (△), (17-35)-NH_2 (x), (18-35)-NH_2 (■) and (22-35)-NH_2 (▪), as well as by VIP, PHI, secretin, GRF, and glucagon (▲). The results were expressed in % of tracer bound in absence of added peptide. The tracer bound (Bo) represented 32 %, 48 %, and 37 % of the radioactivity offered for antiserum 13-3, 14-3 and 15-3, respectively. Final antiserum dilutions were 1:20,000, 1:30,000 and 1:30,000 for, respectively, antiserum 13-3, 14-3 and 15-3.

material with potential biological activity; 3) it did not cross react with mammalian neuropeptides of the VIP family.

Figure 2 shows serial dilution curves of rat tissue extracts using antiserum 15-3 in the helodermin RIA. Extracts of salivary glands, central nervous system, gut and lung displaced dose-dependently the binding of (^{125}I)iodohelodermin to the antiserum. Displacement curves of the extracts were, however, not parallel with the standard curve, preventing an estimation of the absolute amount of helodermin-like material present in the extracts. The values reported in Table I must therefore be considered as indicative only, resting on the amount of extract inducing 50 % tracer

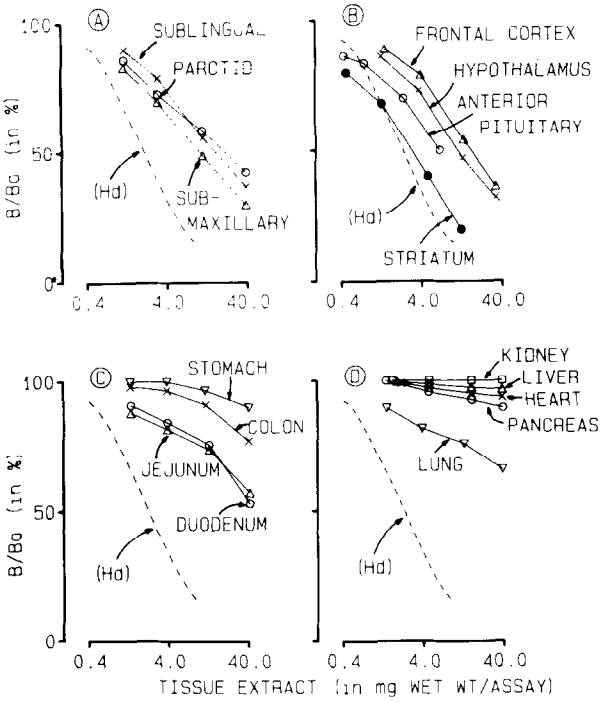


Fig. 2. Inhibition curves obtained with serial dilutions of extracts from salivary glands (panel A), central nervous system (panel B), gut (panel C), and other peripheral tissues (panel D) in the radioimmunoassay of helodermin using antiserum 15-3 (final dilution 1:30,000). The slope of the standard curve with natural helodermin (Hd) was represented by the dashed line.
Panel A : submaxillary (△), parotid (o), and sublingual (x) glands.
Panel B : striatum (●), anterior pituitary (o), hypothalamus (x), and frontal cortex (△).
Panel C : jejunum (△), duodenum (o), colon (+), and stomach (▽).
Panel D : lung (▽), pancreas (o), heart (x), liver (△), and kidney (□).

displacement (whenever possible) or on the highest displacement observed when a 50 % inhibition could not be attained. Extracts from kidney, liver and heart contained no detectable immunoreactive helodermin-like material. In addition, the presence of such a material in pancreas and stomach remained questionable.

Figures 3,4, and 5 show the elution profile, by TSK-G 2000 SW permeation chromatography, of the immunoreactive helodermin-like material extracted from salivary glands, central nervous system, and gut. The immunoreactive material from cerebral cortex, hypothalamus and duodenum eluted as a single, sharp peak of apparent mol wt 10-12 KDa. The corresponding material in parotid gland, striatum, jejunum, and colon

TABLE I. CONCENTRATION OF IMMUNOREACTIVE HELODERMIN-LIKE PEPTIDES IN RAT TISSUES

TISSUE	n	pg/g wet wt tissue
CEREBRAL CORTEX	4	1660
HIPPOCAMPUS	2	6500
STRIATUM	2	5250
MEDULLA	2	550
HYPOTHALAMUS	2	1600
ANTERIOR PITUITARY	3	2840
SUBMAXILLARY GLAND	4	1640
SUBLINGUAL GLAND	4	2300
PAROTID GLAND	4	2600
STOMACH	3	200
DUODENUM	3	1050
JEJUNUM	3	1100
COLON	3	550
LIVER	2	N.D.
PANCREAS	2	150
KIDNEY	2	N.D.
HEART	2	N.D.

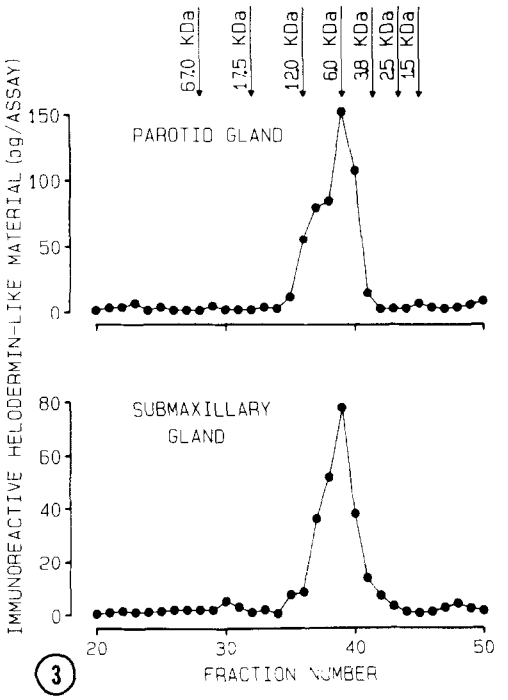
n : number of samples

N.D.: not detectable (less than 50 pg/g tissue).

extracts eluted as two distinct peaks, a 10 - 12 KDa component and another one of about 6 KDa. The rather large peak eluted with a submaxillary gland extract reflected probably also the presence of two similar components. In all tissues tested so far, the immunoreactive helodermin-like materials eluted before the 3.8 KDa helodermin standard.

DISCUSSION

The venom of Helodermae lizards is endowed with a biological activity similar to that of VIP and secretin (10). The purification of the corresponding active material was monitored by its ability to stimulate rat pancreatic membrane adenylate cyclase activity (1). The pure peptide obtained is a biological analog of VIP and secretin (3,4,5,6). Its amino acid composition and sequence indicates marked homology between helodermin



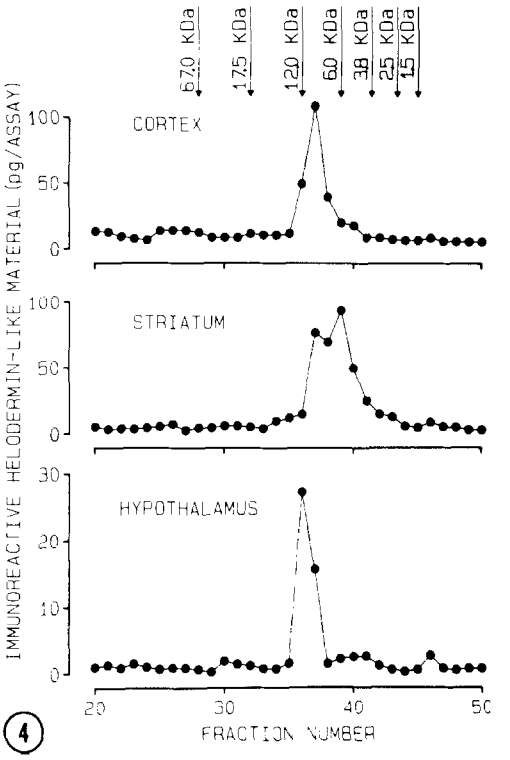


Fig. 3. Elution profiles of immunoreactive helodermin-like activity from extracts of parotid and submaxillary glands on TSK - G 2000 - SW column. Experimental details are given in Materials and Methods. The extracts corresponding to, respectively, 200 mg parotid gland and 120 mg submaxillary gland were used. In each chromatographic profile it was verified that the tracer remained stable during the radioimmunoassay (that means 90 % precipitable with 10 % TCA) in all fractions tested. The molecular weight markers are indicated by an arrow.

Fig. 4. Elution profiles of immunoreactive helodermin-like activity from cerebral cortex (200 mg), striatum (40 mg), and hypothalamus (45 mg) extracts on TSK-G 2000 SW column. Same experimental conditions as in Figure 3.

and all peptides of the VIP/secretin/PHI/ glucagon/GRF family, especially when the N-terminal part of the molecule is considered (2). This characteristic moiety is essential for the biological activity of all members of this family of peptides. By contrast, the C-terminal part of helodermin is remarkably distinct from that of the parent peptides, even when considering the molecular extensions of VIP, PHI, glucagon, and GRF precursors (11,12,13). The C-terminal sequence Arg-Thr-Ser-Pro-Pro-Pro-NH_2 of helodermin, in particular, has no equivalent in the other peptides. We suspected, therefore, that helodermin could be the prototype of a new class of peptides rather than a simple ontogenic variant of a known pattern.

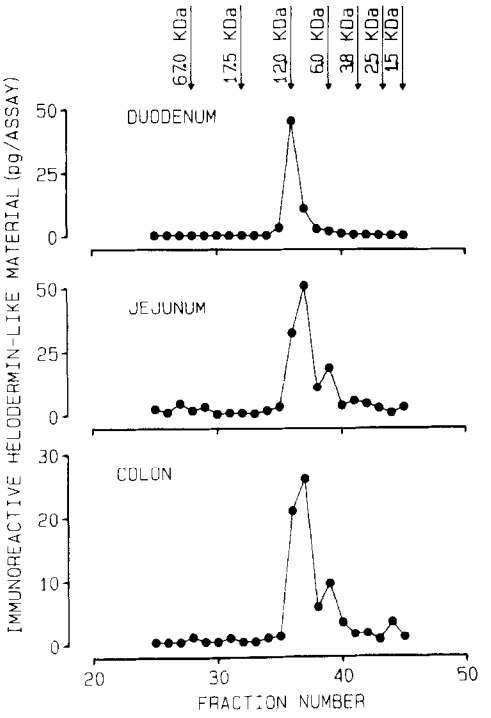


Fig. 5. Elution profiles of immunoreactive helodermin-like activity from duodenum (200 mg), jejunum (200 mg), and colon (200 mg) extracts on TSK-G 2000 SW column. Same experimental conditions as in Figures 3 and 4.

The present results support the existence of a new class of peptides in the brain, salivary glands and gut of a mammal, that belongs to the same family yet is distinct from VIP, secretin, PHI, glucagon and GRF. The highly sensitive helodermin radioimmunoassay we developed, although concerned with a N-terminal specific antiserum, was, indeed, unable to interfere with VIP, PHI, secretin, glucagon or GRF but clearly capable to identify cross reacting material in extracts from rat salivary glands, central nervous system, and gut. This immunoreactive material was heterogeneous when submitted to gel permeation chromatography, the apparent molecular weight ranging from 6 to 12 KDaltons. The tissue distribution of this material was original : in the central nervous system, higher levels were found in striatum and hippocampus than in cerebral cortex and hypothalamus; high levels were found in salivary glands; the concentration was higher in duodenal and jejunal extracts than in colonic extracts; finally, pancreatic extracts were almost devoid of immunoreactivity. This

distribution contrasts with that of VIP (and PHI) in the central nervous system and in peripheral tissues (14,15,16) and also with that of GRF which is preferentially located in hypothalamus and pancreas (17). The previously reported presence of large amounts of immunoreactive glucagon in salivary glands (18-19) was probably artifactual due to tracer degradation during the radioimmunoassay (20).

To conclude, the present study supports the presence, in rat, of a new brain - gut - salivary gland peptide belonging to the VIP, secretin, PHI, glucagon, and GRF family, and closely related to lizard helodermin.

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