Presence of helodermin-like peptides of the VIP-secretin family in mammalian salivary glands and saliva

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Helodermin is a biologically active peptide isolated from the venom of the Gila monster lizard *(Heloderma suspectum)* whose structure is related to that of vasoactive intestinal peptide and secretin. Using a specific radioimmunoassay based on antisera prepared by immunizing rabbits with natural helodermin, we demonstrated the presence of helodermin-like material in mammalian salivary glands, including parotid, submaxillary and sublingual glands from rat and dog, and parotid and submaxillary glands from man. All helodermin-like materials had an apparent molecular mass of 4–12 kDa. Dog saliva, collected after pilocarpine stimulation, revealed similar immunoreactivity with a major component around 6 kDa.

Helodermin *Gila monster venom* *Secretin/VIP peptide family* *Mammalian salivary gland* *Saliva*

1. INTRODUCTION

The presence, in the venom of Gila monster lizard (*Heloderma suspectum*), of a biologically active material closely related to peptides of the vasoactive intestinal peptide (VIP)/secretin/PHI family was discovered by Raufman et al. [1] and further substantiated by Amiranoff et al. [2] and our group [3,4]. The purification to homogeneity of this material was recently achieved [5,6] and the amino acid sequence determined independently by two groups of investigators [6,7]. The acronym helospectin was given by Parker et al. [6] to peptides with 37 and 38 amino acid residues and the acronym helodermin was proposed by our group to a 35 amino acid peptide [7]. The sequences of helodermin, helospectins, VIP, PHI, secretin, GRF and glucagon are compared in table 1. It is not yet clear whether helodermin and helospectin represent, indeed, distinct molecular species. We have now identified the presence of helodermin-like material in the salivary glands of mammals, using a specific radioimmunoassay.

2. MATERIALS AND METHODS

Salivary glands obtained from rat, dog and man were immediately frozen in liquid nitrogen. They were later homogenized in distilled water with an Ultraturrax and immediately immersed in a boiling water bath for 5 min. After cooling, the homogenate was mixed with an equal volume of 1.0 M acetic acid. After centrifugation, the supernatant was lyophilized then dissolved in either the radioimmunoassay buffer or in the buffer used for the chromatographic procedure (see below).

Antisera were prepared by immunizing three rabbits with natural pure helodermin [5] coupled to bovine serum albumin by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. One rabbit pro-

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duced, after the third immunization, antiserum (15-3) that was sufficiently active to be used for radioimmunoassay. [^{125}I]Iodohelodermin was prepared as described [8].

Radioimmunoassay was routinely performed in plastic tubes with a buffer system made of 0.01 M sodium phosphate (pH 7.5) completed with 0.9% sodium chloride, 0.1% sodium azide and 0.05% Tween 20. The incubation started with 0.2 ml of the sample dissolved in assay buffer and 0.3 ml of antiserum to helodermin (diluted 15000-fold with the assay buffer) and enriched with 0.2% normal rabbit serum. After 16–20 h at 4°C, 0.05 ml [^{125}I]iodohelodermin in assay buffer (± 10000 cpm/tube) were added and the incubation was continued at 4°C for a further 40 h period. Bound and free labelled peptides were separated by a second antibody precipitation method with sheep anti-rabbit γ-globulins, the precipitation being enhanced by adding 3.3% polyethylene glycol [9].

The main characteristics of the assay are illustrated in fig.1. Antiserum 15-3 reacted with natural helodermin, synthetic (1-37)-NH_2 helodermin, and the (1-27)-NH_2 helodermin fragment. Five synthetic C-terminal fragments of helodermin [(7-35)-NH_2, (13-35)-NH_2, (17-35)-NH_2, (18-35)-NH_2 and (22-35)-NH_2], tested at concentrations 100-fold higher than that allowing the recognition of the complete helodermin sequence, were not recognized by the antibodies, indicating that antiserum 15-3 was specific for the N-terminal part of helodermin. No cross reaction was observed with parent neurohormones and a few unrelated peptides, including chicken and porcine VIP, porcine secretin, porcine glucagon, porcine PHI, human GRF(1-40)-OH (kindly given by Dr J. Rivier), human GRF(1-29)-NH_2 (kindly given by Dr D. Coy), apamin, CCK-8, prolactin, and pancreatic polypeptide all tested at a dosis of 100 ng/assay.

3. RESULTS AND DISCUSSION

The acidic extracts obtained from parotid, sublingual, and submaxillary glands from rat and dog, as well as those prepared from human parotid and submaxillary glands (surgically removed for lithiasis) inhibited dose-dependently the binding of [^{125}I]iodohelodermin to antiserum 15-3. A typical example is given in fig.1 (panel B) with an extract of dog submaxillary gland. The competition curve

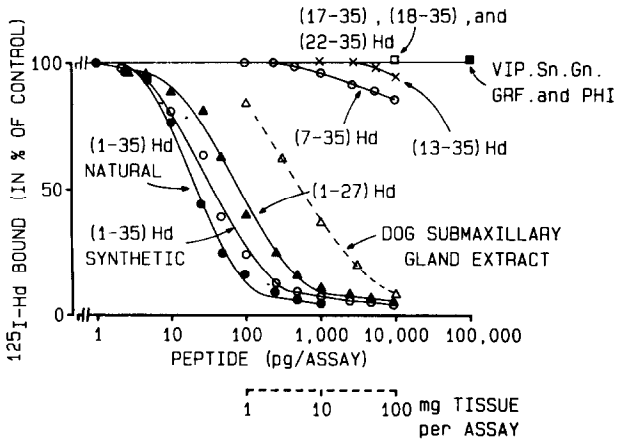

Fig.1. Main characteristics of the antihelodermin antiserum (15-3) utilized at a final 1:30000 dilution. The radioactivity bound (B_0) represented 37.8% of the radioactivity offered. The helodermin concentration required for 50% reduction of tracer binding (IC_{50}) was 23.0 ± 1.2 pg/assay (mean ± SE of 22 determinations) and the limit of detectability was 2 pg/assay. Specificity was tested comparing natural helodermin (●), fragment (7-35)-NH_2 (○), fragment (13-35)-NH_2 (×), and fragments (17-35)-NH_2, (18-35)-NH_2, and (22-35)-NH_2 (□) of helodermin to a dog submaxillary gland extract (△). VIP, secretin, glucagon, GRF(1-40)-OH, GRF(1-29)-NH_2 and PHI (■) were also tested. Average values of 2 experiments.

was not parallel to that of the reference peptide, preventing a precise estimation of the immunoreactive helodermin-like material. Similar results were obtained with all tissue samples tested. Considering the amount of extract provoking 50% inhibition of tracer binding, we tentatively estimated the concentration of helodermin-like material in rat and dog salivary glands to range from 2 to 8 ng/g wet wt tissue. In 4 pathological specimens of human salivary glands these values ranged from 0.8 to 8 ng/g wet wt tissue.

Acidic extracts of submaxillary glands were submitted to gel permeation chromatography on a TSK-G 2000 SW column of a model used to purify helodermin [5]. The column was eluted at 0.1 ml/min with 30% 1-propanol in 0.4 M ammonium acetate (pH 7.0) and 5 min fractions were collected. The fractions were evaporated and the dry residue, dissolved in assay buffer, was radioimmunoassayed with antiserum 15-3. Typical chromatograms of samples from submaxillary

glands of rat, dog and man are illustrated in fig.2. In the rat submaxillary extract, the rather asymmetrical peak suggested the coexistence of a major component of about 6 kDa with a larger molecular mass component. With dog submaxillary extract, the major peak eluted in the 12 kDa position but

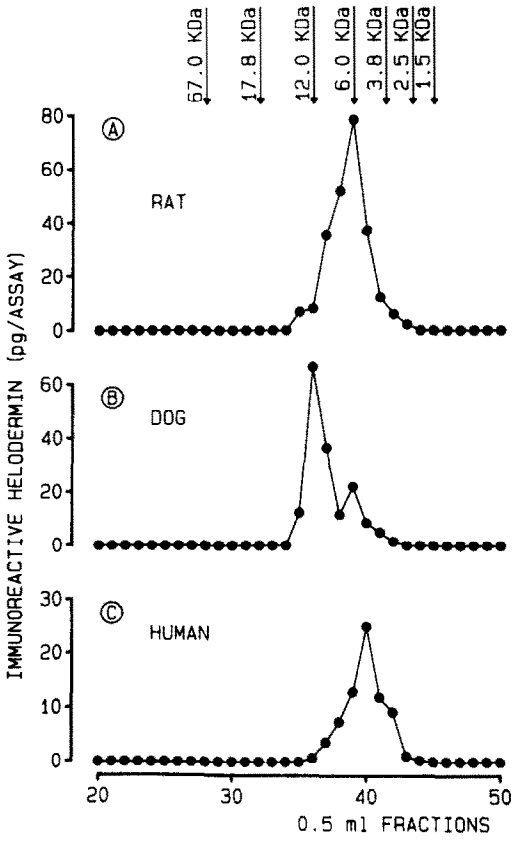

Fig.2. Gel permeation chromatography on TSK-G 2000 column of boiled acidic extracts corresponding to 250, 150 and 140 mg wet wt of submaxillary glands from, respectively, rat (panel A), dog (panel B), and human (panel C). The human specimen corresponded to a macroscopically intact area from a calculous gland. Elution was performed at a rate of 0.1 ml/min. 5 min fractions were collected and lyophilized; the residue was dissolved in 1.0 ml of assay buffer, 0.2 ml aliquots being used for radioimmunoassay with antiserum 15-3. Column calibration, as indicated by the arrows, was performed under similar experimental conditions with bovine serum albumin (67.0 kDa), myoglobin (17.8 kDa), cytochrome *c* (12.4 kDa), insulin (6.0 kDa), helodermin (3.8 kDa), insulin chain A (2.5 kDa) and bacitracin (1.5 kDa).

significant immunoreactivity was also present in the 6 kDa position. With human submaxillary extract, the main immunoreactive peak eluted with an apparent molecular mass between 4 and 6 kDa. Thus, in all salivary glands tested, the helodermin-like material had an apparent molecular mass of 4–12 kDa i.e. a molecular mass slightly higher than that of the reference helodermin from the venom of Gila monster lizard.

Besides, a sample of dog saliva, consisting mostly of sublingual and submaxillary secretions collected after stimulation with an i.m. injection of the muscarinic agonist pilocarpine, revealed after chromatography, a composite immunoreactive peak with a major component around 6.0 kDa (fig.3).

The preceding data did not reflect tracer degradation: [^{125}I]iodohelodermin remained stable (i.e. 90% precipitable by 5% trichloroacetic acid) in parallel incubations of tracer and tissue or saliva extracts, conducted under the radioimmunoassay conditions.

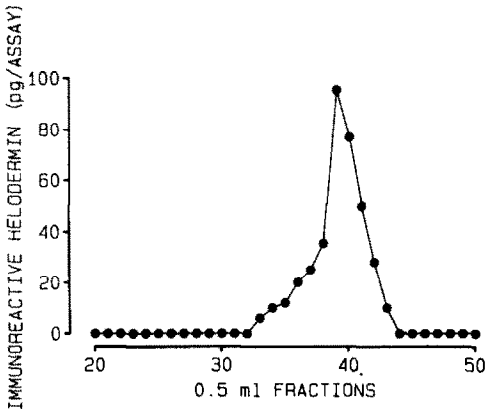

Fig.3. Gel permeation chromatography on TSK-G 2000 column of a boiled acidic extract corresponding to 0.5 ml of dog saliva collected at the ostium of the common duct of sublingual and submaxillary glands, after i.m. injection of 10 mg pilocarpine. The dog was anesthetized with nembutal. Saliva was collected over ice then boiled for 5 min. After cooling, an equal volume of 1 M acetic acid was added and the sample was centrifuged. The supernatant was lyophilized. The residue corresponding to 0.5 ml saliva was dissolved in the elution chromatographic buffer (see fig.2) and chromatographed. 5 min fractions were lyophilized, then dissolved in 1.0 ml assay buffer, 0.2 ml aliquots being used for radioimmunoassay with antiserum 15-3.

Table 1

Comparison of the amino acid sequence of helodermin with other members of VIP family

	1	5	10	15	20	25	30	35	40	45
Helodermin	H-S-D-A-I-F-T-Q-Q-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-A-S-I-L-G-S-R-T-S-P-P-P-OH									
Helospectin(s)	H-S-D-A-T-F-T-A-E-Y-S-K-L-L-A-K-L-A-L-Q-K-Y-L-E-S-I-L-G-S-S-T-S-P-R-P-P-S-(S)									
VIP (porcine)	H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N-OH									
PHI (porcine)	H-A-D-G-V-F-T-S-D-F-S-R-L-L-G-Q-L-S-A-K-K-Y-L-E-S-L-I									
Secretin (porc)	H-S-D-G-T-F-T-S-E-L-S-R-L-R-D-S-A-R-L-Q-R-L-L-Q-G-L-V-OH									
GRF(1-44) (hum)	Y-A-D-A-I-F-T-N-S-Y-R-K-V-L-G-Q-L-S-A-R-K-L-L-Q-D-I-M-S-R-Q-Q-G-E-S-N-Q-E-R-G-A-R-A-R-L									
Glucagon (porc)	H-S-Q-G-T-F-T-S-D-Y-S-K-Y-L-D-S-R-R-A-Q-F-V-Q-W-L-M-N-T									

We have thus established, by radioimmunoassay, that: (i) a group of peptides, immunologically related to helodermin (a biologically active peptide of the VIP/secretin/PHI/glucagon/GRF family present in the venom of Helodermae lizards), is present in mammalian salivary glands; (ii) these peptides are secreted in the oral cavity of mammals as well as a venomous lizard.

The sequence homology (table 1) between an exocrine secretory product such as lizard helodermin (and probably also its mammalian counterpart) on the one hand, and circulating hormones and/or neurotransmittors on the other supports the view of a unitary origin for exocrine and endocrine functions [10]. Examples of humoral messenger molecules appearing in exocrine secretions are relatively rare: epidermal growth factor produced by rodent salivary glands is preferentially secreted in saliva [11–13] and contributes slightly to circulating levels [14]; VIP, somatostatin and gastrin are found in gastric, pancreatic and intestinal secretions [15] but this represents probably an 'overflow' of neurohormonal secretion rather than direct exocrine secretion.

Due to the high degree of homology between helodermin and the other members of the VIP family, the new peptide(s) could, conceivably, represent mere ontogenic variant(s) of a well known model. The structure of each peptide of this family is, however, so conservative throughout evolution that helodermin might also be the prototype of a new class of regulatory peptides. Here we support the latter hypothesis by showing that helodermin-like material is present in large amounts in the salivary secretion and that the salivary glands are the main source of this material in both dog and man. These helodermin-like peptides represent original molecules, closely related yet different from other peptides of the VIP family considering that: (i) The antiserum utilized recognized preferentially the N-terminal portion of helodermin that displays the highest sequence homology with VIP, secretin, PHI, glucagon and GRF (more than 50% homology when considering the 1–15 sequence only; table 1). This N-terminal portion is critical for the biological activity of these peptides [16–19]; (ii) VIP, secretin, PHI, glucagon and GRF did not cross react with antihelodermin antiserum at a concentration of 100 ng/assay; (iii) VIP and PHI in mammalian salivary glands are strictly confined to nerve endings and ganglia [20,21]; (iv) it was previously reported that immunoreactive glucagon abounded in salivary glands [22–24] but this proved to be an artifact due to rapid tracer degradation by proteases during the radioimmunoassay [25]. Besides, glucagon cannot be demonstrated in salivary glands by immunohistochemcal techniques [26]; (v) the presence of secretin and GRF [27] has never been documented in salivary glands.

To attribute a precise role to the helodermin class of peptides will require: (i) the identification of helodermin in neurohormonal cells in the Gila monster lizard itself and (ii) the purification and chemical characterization of the helodermin-like material presently identified in mammals, as well as the appreciation of its distribution and biological activity.

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