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Isolation of the neuropeptide pGlu-Gly-Arg-Phe-amide from the pennatulid *Renilla köllikeri*

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By using a radioimmunoassay for the sequence Arg-Phe-amide, the peptide pGlu-Gly-Arg-Phe-amide was isolated from the pennatulid *Renilla köllikeri* and sequenced. This peptide is a neuropeptide and is produced in high amounts in *Renilla* tissue (10 nmol/g wet wt).

Peptide isolation; Neuropeptide; Neurotransmitter; Evolution

1. INTRODUCTION

Coelenterates have the simplest nervous systems of the animal kingdom, and it was probably within this group of animals that nervous systems first evolved. From ultrastructural and electrophysiological investigations, it has become evident that neurotransmission in coelenterates is mainly chemical (reviews [1–3]). However, the nature of transmitter substances in coelenterates has long remained unknown. Recently, using immunocytochemistry with antisera to the sequence Arg-Phe-amide (RFamide), RFamide-like peptides could be demonstrated in the nervous systems of a variety of coelenterates [2–6]. Using a radioimmunoassay for RFamide, one such peptide, pGlu-Gly-Arg-Phe-amide, could be isolated and sequenced from the sea anemone *Anthopleura elegantissima* [7]. Here, we have isolated an RFamide-like peptide from the pennatulid *Renilla köllikeri*. The pennatulids (order Pennatulacea) and sea anemones (order Actinaria) are phylogenetically widely separated coelenterates, both belonging to the class of Anthozoa.

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The structure of the anthozoan neuropeptide pGlu-Gly-Arg-Phe-amide has been published in the proceedings of the following meetings: 'Invertebrate Peptides and Amines' (Bordeaux, April 1986), '14. Neurobiologentagung' (Göttingen, May 1986), 'Nervous Systems in Invertebrates' (Lennoxville, July 1986) and the '13th Conference of European Comparative Endocrinologists' (Belgrade, September 1986)

2. MATERIALS AND METHODS

R. köllikeri, harvested in May 1985, were purchased from Biomarine Laboratories (Venice, USA) and shipped on dry ice to Heidelberg. The same acetic acid extraction was carried out as described earlier for sea anemones [7], with the only modification being that far more acetic acid was needed to bring the homogenate to pH 3.2 (due to the presence of calcareous spicules in *Renilla*). Also the desalting procedure using Sep-pak, the cation-exchange chromatography using CM-Sephadex C-25, and details of the radioimmunoassay were described before [7]. The radioimmunoassay for RFamide has a high affinity for all peptides terminating with Arg-Phe-amide. Other peptides are only recognized (with a much lower efficiency), if they contain a carboxy-terminal amidation, as well as an uncharged aromatic or aliphatic amino acid in the carboxy-terminal position, preceded by Arg or Lys [2,7].

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HPLC was carried out with a Shimadzu LC-6A system; the HPLC columns were described earlier [7]. The amino acid composition of the peptide was determined by hydrolysis (6 N HCl, overnight at 115°C), subsequent dansylation and separation of the dansylated products on 2.5 × 2.5 cm polyamide sheets [7–9]. The stoichiometry of the amino acids was determined with an LKB amino acid analyzer using post-column derivatization with ninhydrin. Sequencing was carried out with the 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate (DABITC) method [10]. Enzymatic removal of pyroglutamate was performed by adding 6 mU pyroglutamate aminopeptidase (EC 3.4.19.3; from Serva, Heidelberg) to 10–20 nmol peptide dissolved in 50 μl freshly prepared 0.1 M $NaHCO_3$. After incubation for 1 h at 37°C, the reaction was stopped by addition of 50 μl of 2 M acetic acid, followed by lyophilization and purification by HPLC (fig.5). pGlu-Gly-Arg-Phe-amide was prepared as a customer synthesis by Bachem (Bubendorf).

3. RESULTS

Using a radioimmunoassay for RFamide, high amounts of immunoreactive material could be measured in acetic acid extracts of *R. köllikeri*. This material was retained on Sep-pak and could be eluted with 60% methanol [7]. By this procedure, the immunoreactive material was desalted, considerably purified and concentrated. After removal of the methanol, the material was further purified by cation-exchange chromatography using a salt gradient (fig.1). The *Renilla* RFamide-like peptide strongly bound to the resin, and was eluted at high ammonium acetate concentrations, when most of the contaminating substances had been removed (fig.1). After desalting with Sep-pak [7], the immunoreactive material was further purified by cation-exchange chromatography, using different pH values (figs 2,3). After these chromatographies, the material was pure enough to be analyzed by HPLC. Using a C-18 column and an acetonitrile gradient, the immunoreactive material was eluted as a single peak (fig.4). Rechromatography of this peak using different HPLC columns showed that this material was pure.

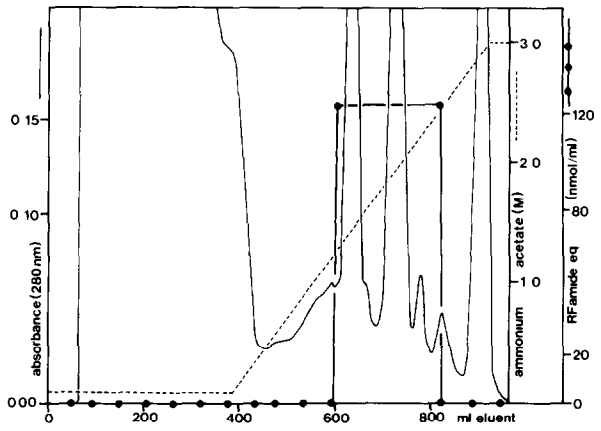

Fig.1. Cation-exchange chromatography of an acetic acid extract of 1.5 kg *R. köllikeri* (after desalting using Sep-pak [7]). The column (2.5 × 40 cm; void volume 75 ml) contained CM-Sephadex C-25 equilibrated with 0.1 M ammonium acetate, pH 7. After application of 100 ml sample, the column was rinsed with 220 ml buffer. Subsequently, a linear gradient of 0.1–3.0 M ammonium acetate (pH 7) was started. Several immunoreactive components were eluted between 1.2 and 2.4 M salt. These components were pooled (28 μmol RFamide equivalents).

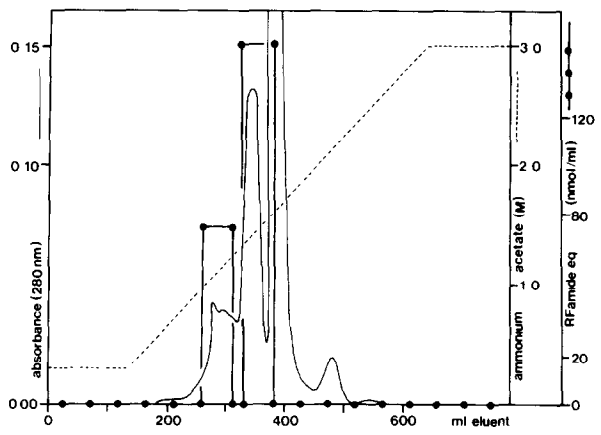

Fig.2. Cation-exchange chromatography of the pooled fractions of fig.1 (after desalting). A similar column to that in fig.1 was equilibrated with 0.3 M ammonium acetate, pH 8. After application of 60 ml sample, a linear gradient of 0.3–3.0 M ammonium acetate (pH 8) was started. A minor immunoreactive component was eluted at 1.0–1.2 M ammonium acetate (pooled, 4 μmol) and a major component at 1.3–1.6 M (8 μmol).

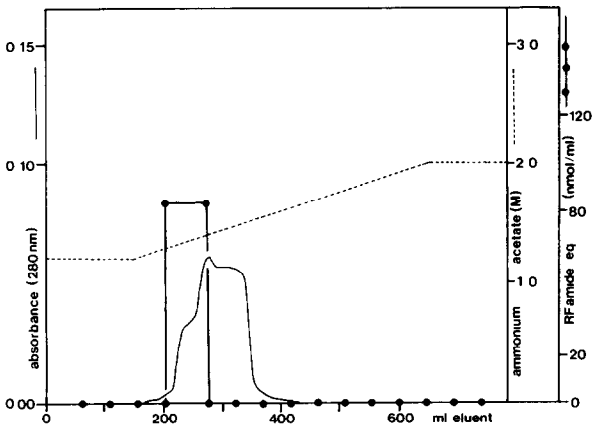

Fig.3. Cation-exchange chromatography of the major immunoreactive component of fig.2 (after desalting). A similar column to that in fig.1 was equilibrated with 1.2 M ammonium acetate, pH 5. After application of 77 ml sample, a linear gradient of 1.2 M (pH 5)–2.0 M (pH 8) ammonium acetate was started. Immunoreactivity was eluted at 1.3–1.4 M ammonium acetate (7 μmol).

Hydrolysis of the HPLC-purified material, followed by dansylation and chromatography of the dansylated amino acids, showed the following amino acids: Glu, Gly, Arg and Phe. End group determination using dansyl chloride did not reveal a reactive end group. As Glu was present in the hydrolysate, pyroglutamate (pGlu) could be the amino-terminus. Enzymatic digestion of the peptide with a low amount of pyroglutamate aminopeptidase, followed by HPLC, revealed a new peak of immunoreactive material (fig.5). This material contained Gly, Arg and Phe, but no longer any Glu. pGlu, therefore, is the amino-terminus of the peptide and no other glutamate or glutamine is present. Using an amino acid analyzer, the stoichiometry of the amino acids in the intact peptide was determined as 1:1:1:1. As the peptide only contains one Glu, the other amino acids must also be present as single copies. From our knowledge of the specificity of the radioimmunoassay (see section 2 and [2,7]), by which only Gly-Arg-Phe-amide can be recognized and no other amidated combination of the three carboxy-terminal amino acids, the structure of the intact peptide has to be: pGlu-Gly-Arg-Phe-amide. This sequence was confirmed using the DABITC method. After enzymatic removal of pGlu, Gly was found as an end group and, after degradation

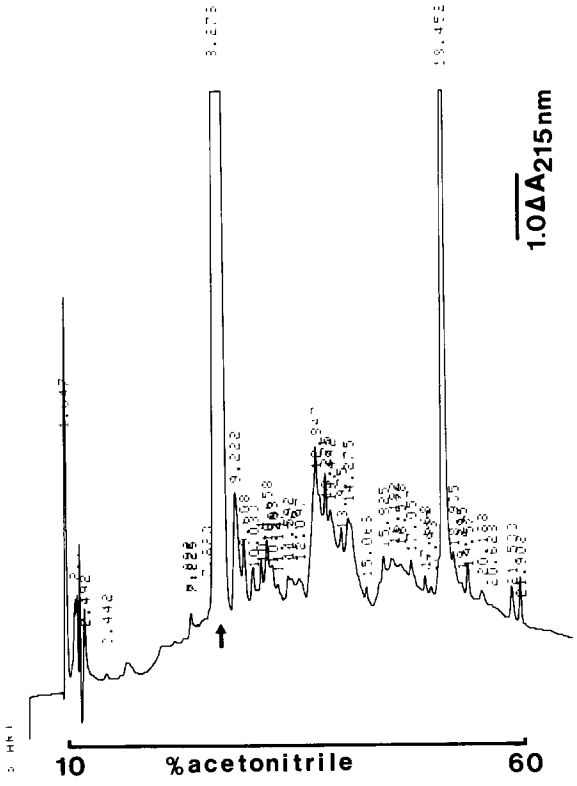

Fig.4. HPLC of a portion (500 nmol) of the immunoreactive material purified in fig.3 (after desalting). The column (Spherisorb ODS-2, 5 μm particle size, 80 Å pore size; dimensions: 4 × 250 mm) was equilibrated with 10% acetonitrile in 0.1% trifluoroacetic acid (TFA). After injection of the sample, a linear gradient of 10–60% acetonitrile in 0.1% TFA was started (20 min; 1 ml/min). The immunoreactive material was eluted as a single peak at 8.3 min (arrow).

cycles, Arg and Phe-amide.

The chromatographic behaviour of natural, purified *Renilla* peptide was compared with that of synthetic pGlu-Gly-Arg-Phe-amide, using HPLC and six different reversed-phase columns containing material with different ligands (C-18, C-8, propyl-phenyl, propyl-nitrile) and different pore diameters (60–300 Å). The natural and synthetic peptide were always eluted at exactly the same retention time (because of space problems this could not be shown, but cf. [7]). This further confirmed the structure of the *Renilla* peptide.

Using pGlu-Gly-Arg-Phe-amide as a standard in our radioimmunoassay, 15 ± 3 nmol/g wet wt of

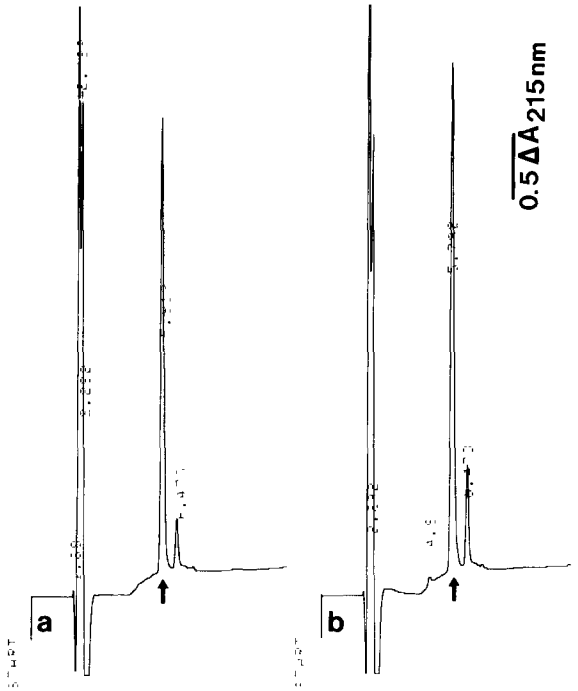

Fig.5. HPLC after degradation of natural and synthetic peptide by pyroglutamate aminopeptidase (column: Partisil ODS-3, 5 μm, 60 Å, 4 × 250 mm; gradient: 20 min, 1 ml/min, 10–60% acetonitrile in 0.1% TFA). Intact, undigested peptide is eluted at 7.2 min [7]. (a) Degradation product of 15 nmol natural *Renilla* peptide. Only one peak is immunoreactive (5.813 min; arrow). This material is a peptide fragment which lacks glutamate (pGlu). (b) Degradation product of 20 nmol synthetic pGlu-Gly-Arg-Phe-amide. This material (arrow) is eluted at the same position as the degradation product of the natural peptide (5.792 min; retention-time variability is ± 1%).

immunoreactive material was measured in three different acetic acid extracts of *Renilla*. Corrected for the contribution of the 'minor component' (fig.2), the concentration of pGlu-Gly-Arg-Phe-amide in *Renilla* is 10 ± 2 nmol/g.

4. DISCUSSION

pGlu-Gly-Arg-Phe-amide was isolated earlier from the sea anemone *A. elegantissima* [7]. Here, we have isolated and sequenced the same peptide from the pennatulid *R. köllikeri*. As two, phylogenetically widely separated anthozoans produce the same peptide, it is likely that this peptide occurs generally in anthozoans.

Using antisera to the sequence Arg-Phe-amide, we have stained numerous neurons in *Renilla*, many of which were associated with muscles. Non-neuronal tissue was not stained by the antisera (not shown, but an extensive anatomical study is in preparation; cf. also [5,6]). The peptide pGlu-Gly-Arg-Phe-amide, therefore, is likely to be produced by neurons, and thus be a neuropeptide.

pGlu-Gly-Arg-Phe-amide is present in high concentrations in *Renilla* tissue (10 nmol/g wet wt). This concentration is 3-times higher than that of pGlu-Gly-Arg-Phe-amide in sea anemones and 100-times higher than that of any neuropeptide in the mammalian brain [7]. This clearly illustrates that pGlu-Gly-Arg-Phe-amide must perform an important role in pennatulids. This role is unknown, but from its probable location in motor neurons, one function of the peptide could be that of a neurotransmitter at neuromuscular junctions.

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