Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9817–9821, December 1986
Neurobiology

Isolation of <Glu-Gly-Arg-Phe-NH_2 (Antho-RFamide), a neuropeptide from sea anemones

(coelenterate/neurotransmitter/evolution/radioimmunoassay/HPLC)

C. J. P. GRIMMELIKHUIJZEN AND D. GRAFF

Zoological Institute, University of Heidelberg, Im Neuenheimer Feld 230, 6900 Heidelberg, Federal Republic of Germany

Communicated by John Kendrew, September 2, 1986

ABSTRACT **A radioimmunoassay has been developed for peptides containing the carboxyl-terminal sequence Arg-Phe-NH_2 (RFamide). Using this radioimmunoassay and applying cation-exchange chromatography and HPLC, we have isolated an RFamide peptide from acetic acid extracts of the sea anemone *Anthopleura elegantissima*. Three different methods established that the structure of the *Anthopleura* RFamide peptide (Antho-RFamide) is <Glu-Gly-Arg-Phe-NH_2. Comparison of synthetic and natural Antho-RFamide and their enzymatic breakdown products on six different HPLC columns confirmed the structure of the sea anemone peptide. Using synthetic Antho-RFamide as a standard in our radioimmunoassay, we measured high concentrations (3.2 nmol/g wet weight) of this peptide in extracts of *Anthopleura*. It is proposed that Antho-RFamide is a transmitter at neuromuscular synapses in sea anemones.**

Coelenterates have the simplest nervous systems of the animal kingdom, and it was probably within this group of animals that nervous systems first evolved. Ultrastructural and electrophysiological studies have shown that most synapses in the nervous systems of coelenterates are chemical (for reviews see refs. 1–3). Despite this evidence, however, the nature of neurotransmitter substances in coelenterates has remained unknown. Recently, using immunocytochemistry and radioimmunoassays, we have demonstrated substances in the nervous systems of coelenterates that are related to vertebrate and invertebrate neuropeptides (see ref. 4 for a review). In particular, peptides resembling the molluscan neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide; ref. 5) are ubiquitous in coelenterates (2–4, 6–10). Of antisera against different fragments of FMRFamide, those raised against RFamide were found to be the most potent in staining coelenterate nervous tissue, indicating that the coelenterate peptides have the sequence RFamide in common with FMRFamide but the amino terminus is different (2, 3, 8, 9). This paper describes the development of a radioimmunoassay for the coelenterate RFamide-like peptides and the purification and sequencing of such a peptide from the sea anemone *Anthopleura elegantissima*.

METHODS

Radioimmunoassay. Four rabbits were immunized with RFamide (Bachem, Bubendorf, Switzerland) that was coupled by carbodiimide to bovine thyroglobulin (Sigma). The antisera were treated overnight at 4°C with bovine thyroglobulin at 1 mg/ml, followed by centrifugation, to remove antibody populations specific for the carrier protein. One antiserum (code 145IV) was selected for the present study. ^{125}I-labeled Tyr-Phe-Met-Arg-Phe-NH_2 was used as a tracer in the radioimmunoassay. It was prepared by adding 5 μl (0.5 mCi; 18 MBq) of $Na^{125}I$ (Amersham) to 15 μl of 0.2 M sodium phosphate buffer, pH 7.0, containing 0.22 μg of Tyr-Phe-Met-Arg-Phe-NH_2 (Peninsula Laboratories, San Carlos, CA), followed by addition of 10 μl of phosphate buffer containing 1.4 μg of chloramine-T. After mixing, the reaction was stopped after 20 sec with 500 μl of 0.3% $Na_2S_2O_5$. The mixture was immediately loaded on a methanol/water-treated Sep-Pak C_{18} cartridge (Waters Associates), which was quickly rinsed with 40 ml of twice-distilled water, to remove unreacted ^{125}I. Labeled material was eluted with 2-ml portions of 40%, 60%, 80%, and 90% (vol/vol) methanol and three times with 2-ml portions of 100% methanol. Each fraction was collected in 6 ml of RIA buffer, consisting of 0.05 M sodium phosphate at pH 7.4 and 1% bovine serum albumin (Sigma). The major portion of the tracer was eluted at 90% or 100% methanol. The quality of an aliquot (3000 cpm) of the tracer fractions was tested by overnight incubation, at 4°C, in 1 ml of RIA buffer to which no antiserum, or antiserum 145IV (1:1000 final dilution) was added. Antiserum-bound tracer (*B*) and free tracer (*F*) were determined as described below. For the selected fraction, B/F of 20–25 was usually obtained in tubes with antiserum, and B/F of 0.1 in tubes without. For radioimmunoassays (cf. Fig. 1), a mixture of tracer (3000 cpm/ml of RIA buffer) and antiserum 145IV (1:10,000 in RIA buffer) was prepared. To tubes with 1-ml portions, 1–10 μl of standards or samples were added, and, after mixing, the contents were incubated for 48 hr at 4°C. Subsequently, 0.1 ml of bovine gamma globulin solution (Cohn fraction II, Sigma; 1 mg/ml in RIA buffer) and, after mixing, 1 ml of ice-cold 25% polyethylene glycol (M_r, 5000–7000; Roth, Karlsruhe) were added. After thorough mixing for 5 sec, the tubes were incubated on ice for 1 hr and then centrifuged at 5000 × *g* for 15 min. Free (supernatant) tracer was decanted and the radioactivities of both bound (precipitated) and free tracer were measured on a γ counter (Kontron, Eching, F.R.G.).

Sea Anemone Extract. *Anthopleura elegantissima*, harvested in May 1984, were purchased from Biomarine Laboratories (Venice, CA) and transported on dry ice to Heidelberg. Frozen tissue was minced and boiled for 15 min in twice-distilled water (3 ml/g wet weight). After cooling to 0°C, the mixture was brought to 0.5 M acetic acid (pH 3.2) and homogenized with an Ultra-Turrax (IKA, Staufen). After centrifugation (5000 × *g*, 15 min), the pellet was reextracted with 0.2 M acetic acid (3 ml/g wet weight). The combined supernatants were brought to pH 7.0 with ammonia and pumped through eight linearly connected methanol/water-treated Sep-Pak C_{18} cartridges. At regular intervals, the first cartridge was removed and a fresh cartridge was connected at the end of the row. After the extract had been pumped through, all cartridges were washed with twice-distilled water

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Abbreviations: FMRFamide, Phe-Met-Arg-Phe-NH_2; RFamide, Arg-Phe-NH_2; DABITC, 4-*N*,*N*-dimethylaminoazobenzene 4′-isothiocyanate.

and eluted with 60% methanol (10 ml per cartridge). Subsequently, the methanol was removed by rotary evaporation and the remaining aqueous solution was brought to the same ammonium acetate molarity as the starting buffers for cation-exchange chromatography.

Chromatography. For cation-exchange chromatography, CM-Sephadex C-25 (Pharmacia) was used. After swelling and equilibration with ammonium acetate buffer, the gel was packed into the column and washed for at least 12 hr (24 ml/hr) with buffer, before the gradient was started. In CM-Sephadex C-25 batches used in this study, *Anthopleura* RFamide peptide was always eluted at 0.6–0.8 M ammonium acetate (cf. Fig. 3). In batches that were purchased more recently, however, the same material was eluted at 1.4–1.5 M. The reason for this might be variations in capacity of the gel. Columns (Pharmacia) were connected to a Multirac/Uvicord II chromatography equipment of LKB. HPLC was carried out with either a Bio-Rad 1330 (Fig. 4), or Shimadzu LC-6A (Figs. 5 and 6) system. The HPLC columns Spherisorb ODS-2, Spherisorb C-8, and Partisil ODS-3 were purchased from Latek (Heidelberg), Nucleosil C-18, Nucleosil propyl-phenyl, and Nucleosil propylnitrile from Macherey & Nagel.

Peptide Chemistry. After overnight hydrolysis in 6 M HCl at 115°C, the amino acid compositions of peptides were determined by dansylation (volume: 20 μl) and separation of the dansylated amino acids on 2.5 × 2.5 cm F1700 polyamide sheets (Schleicher & Schuell; Dassel) (11, 12). Stoichiometry of the amino acids was kindly determined by H. Ponstingl (Deutsche Krebsforschungszentrum, Heidelberg) and R. Frank (European Molecular Biology Laboratory, Heidelberg), using derivatization with ninhydrin or *ortho*-phthalaldehyde. The amino acid sequence was determined by dansyl–Edman degradation (13) and by the 4-*N*,*N*-dimethylaminoazobenzene 4′-isothiocyanate (DABITC) method (14). To test for tryptophan, 5- to 10-nmol samples of sea anemone peptide or model peptides containing or lacking tryptophan were spotted on filter paper. After spraying with Ehrlich reagent (Sigma), the paper was heated until color appeared. Enzymatic removal of pyroglutamate residue (<Glu) was carried out by addition of 6 milliunits of pyroglutamate aminopeptidase (L-pyroglutamyl-peptide hydrolase; 5-oxoprolyl-peptide hydrolase; EC 3.4.19.3; obtained from Serva, Heidelberg) to 20 nmol of peptide, dissolved in 50 μl of freshly prepared 0.1 M $NaHCO_3$, followed by incubation at 37°C for 30 or 60 min. The enzymatic reaction was stopped by addition of 50 μl of 2 M acetic acid, which was followed by lyophilization and subsequent purification on HPLC (Fig. 6). <Glu-Gly-Arg-Phe-NH_2, Arg-Leu-NH_2, and Arg-Met-NH_2 were prepared as custom syntheses by Bachem. Phe-Met-Arg-Phe-NH_2 and [Arg^8]vasopressin were purchased from Bachem; Leu-Pro-Leu-Arg-Phe-NH_2, Phe-Met-Arg-Phe-, and Trp-Met-Asp-Phe-NH_2 were from Peninsula Laboratories; Leu-Thr-Arg-Pro-Arg-Tyr-NH_2 was from UCB-Bioproducts (Brussels, Belgium); and Phe-Met-Lys-Phe-NH_2 was from Cambridge Research Biochemicals (Harston, Cambs., U.K.).

RESULTS

Radioimmunoassay for RFamide Peptides. A radioimmunoassay has been developed for the dipeptide RFamide (Fig. 1). This assay recognizes free RFamide and amino-terminal elongated peptides containing the RFamide moiety, such as Phe-Met-Arg-Phe-NH_2 and Leu-Pro-Leu-Arg-Phe-NH_2 (Fig. 2). The elongated peptides, however, are recognized slightly better than RFamide itself (Fig. 2). Peptides terminating with -Arg-Tyr-NH_2 can also be measured, but with a much lower efficiency (1/100th of that for peptides containing RFamide). Only peptides containing arginine followed by an amidated

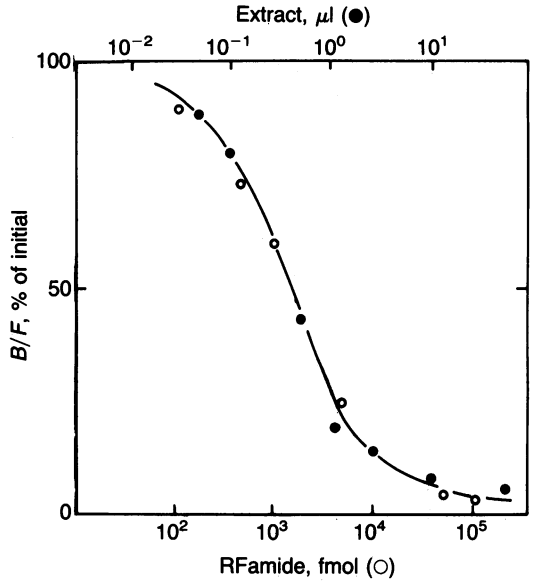

FIG. 1. RFamide radioimmunoassay of an acetic acid extract of the sea anemone *Anthopleura elegantissima* (0.4 g wet weight per ml of extract). The displacement curves of standard RFamide and extract are identical.

aromatic amino acid are recognized properly in the assay. Phe-Met-Lys-Phe-NH_2, a Phe-Met-Arg-Phe-NH_2 analogue in which the arginine is replaced by the other positively charged amino acid, lysine, reacts poorly (1/2000th as efficient as RFamide peptides). Other peptides, in which the Phe-NH_2 terminus is not preceded by a positively charged amino acid, such as the carboxyl terminus of gastrin/cholecystokinin (Trp-Met-Asp-Phe-NH_2), are not recognized in the assay (Fig. 2). Peptides containing arginine followed by an amidated nonaromatic amino acid, such as Arg-Leu-NH_2 or Arg-Met-NH_2, do not displace efficiently (1/500th as much as RFamide). When no aliphatic side chain is present in the amino acid following arginine, such as in vasopressin (whose carboxyl terminus is Pro-Arg-Gly-NH_2), the peptide is not recognized. Also, the carboxyl-terminal amidation is essential for recognition, as Phe-Met-Arg-Phe does not react (Fig. 2).

Purification of the Sea Anemone Peptide. With the RFamide radioimmunoassay, high amounts (10 nmol RFamide equiv-

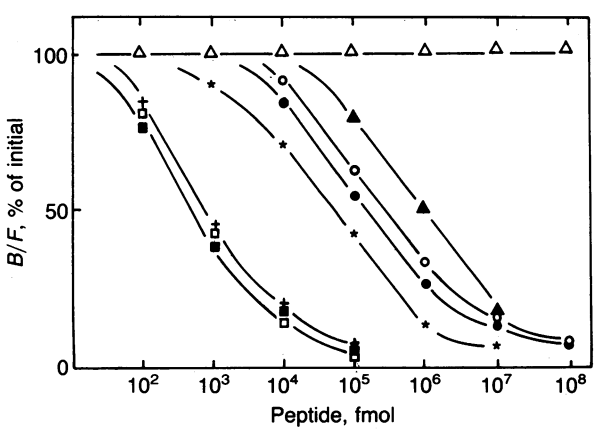

FIG. 2. Specificity of the RFamide radioimmunoassay. +, RFamide; □, Phe-Met-Arg-Phe-NH_2; ■, Leu-Pro-Leu-Arg-Phe-NH_2; *, bovine pancreatic polypeptide-(31–36)-hexapeptide (Leu-Thr-Arg-Pro-Arg-Tyr-NH_2); ●, Arg-Met-NH_2; ○, Arg-Leu-NH_2; ▲, Phe-Met-Lys-Phe-NH_2; and △, Phe-Met-Arg-Phe, cholecystokinin-(30–33)-tetrapeptide (Trp-Met-Asp-Phe-NH_2), or [Arg^8]vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2).

alents/g wet weight) of immunoreactive material can be measured in acetic acid extracts of the sea anemone *Anthopleura elegantissima* (Fig. 1). Using this assay as a monitoring system, we have purified the RFamide-like substance from an acetic acid extract of 400 g of sea anemones. Initial purification, desalting, and concentration of the immunoreactive material involved passage through Sep-Pak C_{18} cartridges. A successful subsequent purification method was cation-exchange chromatography on CM-Sephadex C-25, using a salt and a pH gradient. As could be expected from a positively charged peptide (see above discussion of the specificity of the radioimmunoassay), the immunoreactive material was strongly bound to the gel. This material could be eluted at 0.7–0.8 M ammonium acetate (pH 5.3), when most of the contaminating substances had been removed (Fig. 3). After further cation-exchange chromatography at pH 7.0 and pH 8.5 (not shown, but cf. refs. 2 and 3), the material was pure enough to be analyzed on HPLC. On a Spherisorb ODS-2 column (20-min gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid), the *Anthopleura* RFamide-like peptide appeared as one single peak at 8 min (Fig. 4). This material was pure, as was shown by subsequent HPLC under different conditions (cf. Fig. 5).

Structure of the Sea Anemone Peptide. Aliquots (1 nmol) of the HPLC-purified material (Fig. 4) were hydrolyzed overnight in 6 M HCl and subsequently dansylated. The dansylated amino acids were separated on small polyamide sheets, and identified as glutamate, glycine, arginine, and phenylalanine. A test for tryptophan in the peptide (Ehrlich reagent) was negative. An end-group determination, using dansyl chloride, showed that no reactive end group was present. As glutamate was one of the amino acids in the hydrolysate, the amino terminus could be pyroglutamate. Incubation of the peptide with a critical (low) amount of pyroglutamate aminopeptidase, followed by HPLC, yielded a new peak of immunoreactive material (cf. Fig. 6). This material contained

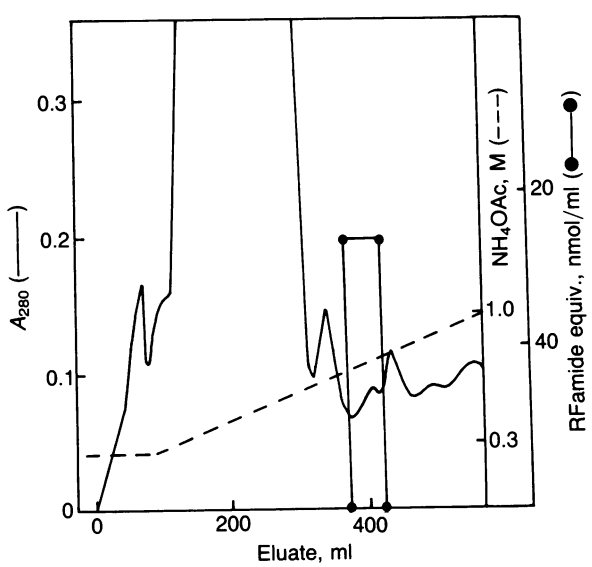

FIG. 3. Cation-exchange chromatography on CM-Sephadex C-25 of an acetic acid extract of *Anthopleura elegantissima* (400 g wet weight; after desalting of the extract on Sep-Pak and elution with 60% methanol). The column (2.6 × 39 cm; void volume, 80 ml) was equilibrated with 0.3 M ammonium acetate, pH 5.0. Subsequently 80 ml of the extract was applied, and a linear gradient of 0.3 M ammonium acetate, pH 5.0, to 1.0 M ammonium acetate, pH 7.0, was started (24 ml/hr, 20 hr). After this gradient, the column was washed with 250 ml of 2.0 M ammonium acetate, pH 7.0. All immunoreactive material was eluted at 0.7–0.8 M ammonium acetate (1900 nmol of RFamide equivalents). The concentration of RFamide equivalents given at the right ordinate is that of the combined immunoreactive fractions.

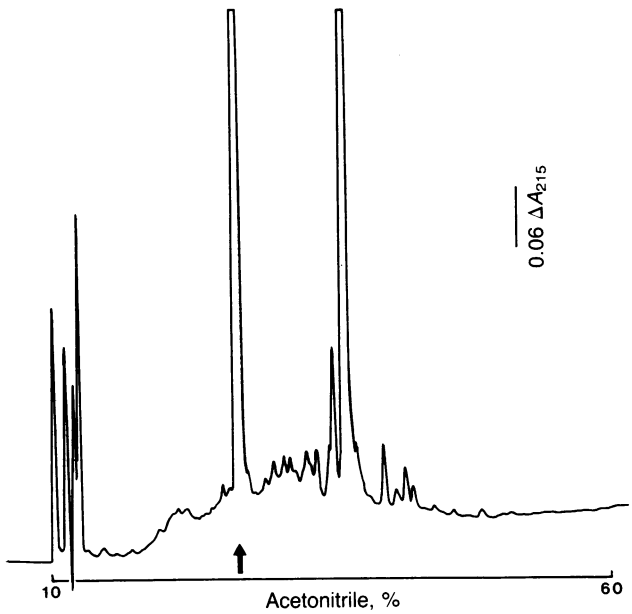

FIG. 4. HPLC of a portion (90 nmol RFamide equivalents) of immunoreactive material purified by cation-exchange chromatography (after desalting with Sep-Pak C_{18} cartridges). An analytical C_{18} column (Spherisorb ODS-2, 5-μm particle size, 80-Å pore size, dimensions 4 × 250 mm) was equilibrated with 10% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid. Subsequently a gradient was started from 10% to 60% acetonitrile in 0.1% trifluoroacetic acid (20 min, 1 ml/min). The RFamide peptide was eluted at 8 min (arrow). This peptide was found to be very pure, both by subsequent HPLC and by amino acid analyses.

Gly, Arg, and Phe, but not Glu. Thus only one Glu had to be present in the peptide, being in the form of an amino-terminal <Glu. At this stage we asked two colleagues (H. Ponstingl and R. Frank) to determine the stoichiometry of the amino acid residues, using their amino acid analyzers. Both scientists found a stoichiometry of 1:1:1:1. As there is only one Glu in the peptide, there must also be one Gly, Arg, and Phe. From our knowledge of the specificity of the radioimmunoassay, by which only Gly-Arg-Phe-NH_2 can be recognized, and no other amidated combination of the three amino acids (see above), the sequence of the peptide had to be <Glu-Gly-Arg-Phe-NH_2. This sequence was confirmed by dansyl–Edman degradation (13). After enzymatic removal of <Glu, we found Gly as an end group and, after degradation cycles, Arg and Phe-NH_2. The sequence of the sea anemone peptide was also confirmed by using the DABITC method (14). In contrast to the dansyl–Edman degradation, this method does not destroy tryptophan, which further established that no Trp is present in the peptide.

Comparison of Synthetic and Natural Peptide. <Glu-Gly-Arg-Phe-NH_2 has been synthesized commercially. The synthetic peptide was subsequently compared with the natural peptide on several types of reversed-phase HPLC columns. HPLC on columns containing Spherisorb ODS-2 (80 Å pore diameter), Partisil ODS-3 (60 Å) and Nucleosil C-18 (120 Å) which, in addition to their properties in reversed-phase chromatography, also discriminate between peptides of slightly different sizes, showed that synthetic and natural peptide were always eluted at the same positions (Fig. 5 *a–f*). The same results were obtained after HPLC with material containing different ligands such as Spherisorb C-8, Nucleosil propylphenyl, and Nucleosil propylnitrile (Fig. 5 *g–l*). Also, the products of enzymatic digestion of both peptides by pyroglutamate aminopeptidase appeared at exactly the same positions during HPLC (Fig. 6).

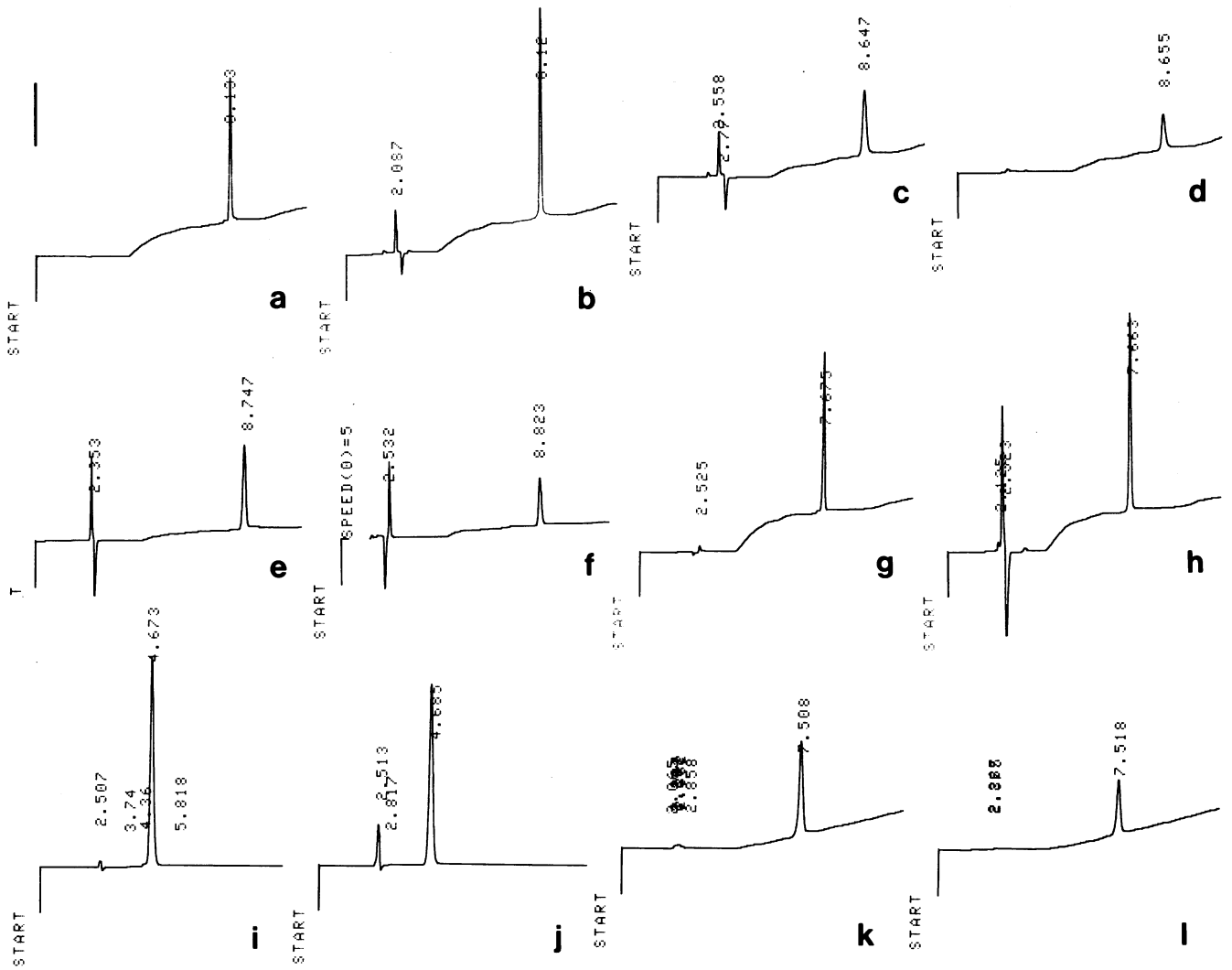

FIG. 5. Comparison of synthetic and natural *Anthopleura* RFamide peptide on different HPLC columns, using different acetonitrile gradients. Peptide (2–6 nmol) was injected in columns equilibrated with starting eluent (wavelength setting, 215 nm; bar represents ΔA of 0.5). The synthetic and natural peptide always appeared at the same retention time (variability of retention time of the HPLC system is ±1%; the peaks appearing at 2–3 min are injection artefacts). (*a*) Synthetic peptide appearing at 8.103 min. Column: Spherisorb ODS-2, particle size 5 μm, pore size 80 Å, dimensions 4 × 250 mm; gradient: 20 min, 1 ml/min, 10–60% acetonitrile in 0.1% trifluoroacetic acid. (*b*) Natural peptide appearing at 8.120 min (same conditions as *a*). (*c*) Synthetic peptide appearing at 8.647 min. Column: Partisil ODS-3, 5 μm, 60 Å, 4 × 250 mm; gradient: 10 min, 1 ml/min, 10–20% acetonitrile in 0.1% trifluoroacetic acid. (*d*) Natural peptide appearing at 8.655 min (same conditions as *c*). (*e*) Synthetic peptide appearing at 8.747 min. Column: Nucleosil C-18, 5 μm, 120 Å, 4 × 250 mm; gradient: 10 min, 1 ml/min, 10–20% acetonitrile in 0.1% trifluoroacetic acid. (*f*) Natural peptide appearing at 8.823 min (same conditions as *e*). (*g*) Synthetic peptide appearing at 7.675 min. Column: Spherisorb C-8, 5 μm, 80 Å, 4 × 250 mm; gradient: 20 min, 1 ml/min, 10–60% acetonitrile in 0.1% trifluoroacetic acid. (*h*) Natural peptide appearing at 7.663 min (same conditions as *g*). (*i*) Synthetic peptide appearing at 4.673 min. Column: Nucleosil propylnitrile, 7 μm, 300 Å, 4 × 250 mm; gradient: 10 min, 1 ml/min, 0–5% acetonitrile in 0.1% trifluoroacetic acid. (*j*) Natural peptide appearing at 4.685 min (same conditions as *i*). (*k*) Synthetic peptide appearing at 7.508 min. Column: Nucleosil propylphenyl, 7 μm, 120 Å, 4 × 250 mm; gradient: 10 min, 1 ml/min, 10–17% acetonitrile in 0.1% trifluoroacetic acid. (*l*) Natural peptide appearing at 7.518 min (same conditions as *k*).

Concentration of the Sea Anemone Peptide. Using synthetic <Glu-Gly-Arg-Phe-NH_2 as a standard in our radioimmunoassay, we measured 3.2 ± 0.5 nmol/g wet weight in three different extracts of *Anthopleura*.

DISCUSSION

In four different ways we have established the structure of the *Anthopleura* RFamide peptide. These are (*i*) a combination of the amino acid analyses of intact peptide and its enzymatic breakdown product with a knowledge of the specificity of the RFamide radioimmunoassay ("sequencing without sequencing"), (*ii*) sequencing with the dansyl–Edman degradation, (*iii*) sequencing with the DABITC method, and (*iv*) chemical synthesis and comparison of synthetic and natural peptides (and their enzymatic breakdown products) on six different HPLC columns. From this we can safely conclude that the structure of the *Anthopleura* RFamide peptide is <Glu-Gly-Arg-Phe-NH_2. From now on, we shall call this peptide "Antho-RFamide."

Immunocytochemical studies using RFamide antisera have shown that peptides containing a RFamide-like carboxyl terminus are abundant in sensory and motor neurons innervating ecto- and endodermal muscles in sea anemones (ref. 3; C.J.P.G., D.G., and I. D. McFarlane, unpublished data). Antho-RFamide, therefore, is also likely to be produced by such neurons. <Glu-Gly-Arg-Phe-NH_2 itself, however, is certainly not visualized with our immunocytochemical technique, as this peptide lacks free amino or carboxyl groups, and cannot be linked to the surrounding tissue by fixatives. During the incubation procedures necessary for immunocytochemical staining, the peptide will thus be washed away.

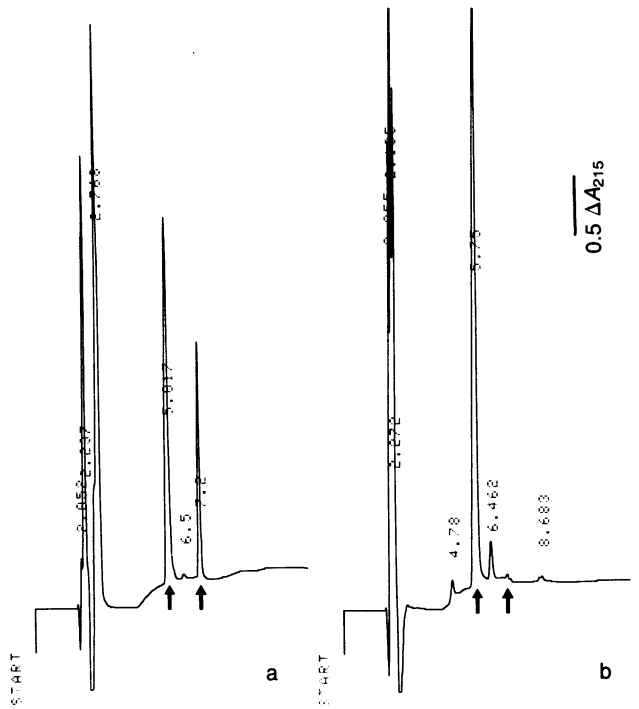

FIG. 6. HPLC after degradation of synthetic and natural *Anthopleura* RFamide peptide by pyroglutamate aminopeptidase. Column: Partisil ODS-3, 5 μm, 60 Å, 4 × 250 mm; gradient: 20 min, 1 ml/min, 10–60% acetonitrile in 0.1% trifluoroacetic acid. (*a*) Products of a 30-min degradation of 15 nmol of synthetic peptide. Only two peaks are immunoreactive (arrows). The peak at 7.200 min is the intact peptide, that at 5.817 min is a fragment that lacks Glu. (*b*) Products of a 60-min degradation of 15 nmol of natural peptide. All peptide has now been degraded to an immunoreactive product that lacks Glu and that appears at the same position as the degradation product of the synthetic peptide (5.750 min; retention-time variability is ±1%).

The material stained by the RFamide antisera in sea-anemone neurons is probably a fixable precursor of <Glu-Gly-Arg-Phe-NH_2, which, because of the amide requirement for antibody recognition, must be amino-terminal-elongated Gln-Gly-Arg-Phe-NH_2.

Antho-RFamide is present in high concentrations in *Anthopleura elegantissima* (3.2 nmol/g wet weight). This concentration is much higher than that of any neuropeptide in the mammalian brain. Neuropeptide Y, for example, which is the most abundant neuropeptide in the brain of mammals, occurs in concentrations of up to 0.5 nmol/g wet weight in specialized regions of the human brain (15) and of up to 1 nmol/g wet weight in certain regions of the brain of the rat (16). Averaged over the whole brains, those numbers must be at least 10-fold lower. When whole animals are compared, the concentration of Antho-RFamide in *Anthopleura* is certainly more than 1000-fold higher than that of neuropeptide Y in mammals. This indicates that a high percentage of neurons in the sea anemone produces high amounts of Antho-RFamide, and it illustrates that this peptide must have an extremely important role in the anthozoan nervous system.

The role of Antho-RFamide is unknown yet, but from its probable localization in motor neurons (see above), this peptide could be a transmitter at neuromuscular synapses. This is further suggested by the fact that Phe-Met-Arg-Phe-NH_2, which is structurally related to Antho-RFamide, has a transmitter-like action at neuromuscular synapses in molluscs (17). <Glu-Gly-Arg-Phe-NH_2 appears to be perfectly constructed for a peptide transmitter. It is small, allowing many copies to be accommodated on a common precursor protein (cf. ref. 18), and it is protected at both the amino and carboxyl termini (<Glu and -NH_2) against unspecific amino- and carboxypeptidases.

Antho-RFamide is a neuropeptide that shares the RFamide moiety with the molluscan neuropeptides Phe-Met-Arg-Phe-NH_2 (5), <Glu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (19), Ser-Asx-Pro-Phe-Leu-Arg-Phe-NH_2 (20), Gly-Asx-Pro-Phe-Leu-Arg-Phe-NH_2 (20), the avian brain peptide Leu-Pro-Leu-Arg-Phe-NH_2 (21), and three bovine brain peptides: γ_1 melanocyte-stimulating hormone [carboxyl terminus: Trp-Asp-Arg-Phe-NH_2 (22)] and two peptides terminating with Pro-Gln-Arg-Phe-NH_2 (23). Whether this implies evolutionary conservation of the RFamide sequence or simply reflects some adaptive process (convergence) cannot be decided.

We thank Drs. H. Ponstingl and R. Frank for their amino acid analyses, Dr. W. Stöcker for introducing the DABITC method to us, Mrs. S. L. Himanen for typing the manuscript, and the Deutsche Forschungsgemeinschaft for financial support (Gr 762/4). C.J.P.G. is the recipient of a Heisenberg Fellowship (Gr 762/1).

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