Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 180-186

IDENTIFICATION OF AN ARGININE VASOPRESSIN-LIKE DIURETIC HORMONE FROM LOCUSTA MIGRATORIA

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Received October 19, 1987

SUMMARY: We have identified two neuropeptides (F1 and F2) from suboesophageal and thoracic ganglia of *Locusta migratoria*, which we isolated earlier based on their immunological similarity to arginine vasopressin. The more abundant and hydrophilic factor, F1, has sequence Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2, but its biological role is unknown. The less abundant factor, F2, is an antiparallel dimer of F1, and functions as a diuretic hormone of this species. It appears to act through the intermediacy of cyclic AMP. The properties of the native neuropeptides were identical with those of samples synthesized from appropriately protected L-amino acids.

Insects depend for their survival upon mechanisms that critically control their internal water balance. Although insect diuretic hormones have long been implicated in this regulation, no diuretic hormone has hitherto been isolated from any insect species (1). Immunological studies (2) have established the existence in several insect species of material that cross-reacts with antibodies raised against AVP, the antidiuretic hormone of most mammals. Moreover, this AVP-like material has been shown to have diuretic properties in *Locusta migratoria* using two different assays (3); it is localized predominantly in the SOG and the TG (4,5). The AVP-like factor was characterized (6) as a peptide with M_r ≈2,500. Recently, we isolated (7) not one, but two AVP-like factors (F1 and F2) from SOG and TG, and now report their identification.

ABBREVIATIONS: AVP, arginine vasopressin; AVT, arginine vasotocin; AVP-like IDH, AVP-like insect diuretic hormone; BSA, bovine serum albumin; Acm, acetamidomethyl; Boc, *t*-butyloxycarbonyl; Bzl, benzyl; 4-MeBzl, 4-methylbenzyl; Npys, 3-nitro-2-pyridinesulfenyl; MT, Malpighian tubules; RCM, reduced and carboxymethylated; RIA, radioimmunoassay; RPLC, reversed-phase liquid chromatography; SOG, suboesophageal ganglion; TG, thoracic ganglion; Tos, tosyl; TFA, trifluoroacetic acid.

0006-291X/87 $1.50

MATERIALS AND METHODS

Peptide analysis. The isolation of F1 and F2 from 51,000 SOG and TG of *Locusta migratoria* has been reported (7) using sequential RPLC purifications. Fractions were assayed with an RIA for AVP. Diuretic activity was checked at key purification steps with an *in vivo* assay (3).

Amino acid analysis was performed on hydrolysates of duplicate aliquots of F1 and F2, with one set of samples pre-oxidized with performic acid (8). Purified F1 and F2 were treated with 125 μg of dithiothreitol (>1,000 x molar excess) in 6M guanidine·HCl, 0.5 M Tris·HCl, pH 8.5. After reduction, thiols were alkylated with ≃400 μg (200 μCi, a molar excess) of [2-^{3}H]ICH_2CO_2H. The RCM derivatives were pre-purified using a C_2 reversed-phase cartridge prior to RPLC separation on a 10 x 0.21 cm Aquapore C_4 column; fractions from RPLC were tested by liquid scintillation counting and RIA. For both RCM-F1 (~280 pmol) and RCM-F2 (~160 pmol) most radioactivity and immunoreactivity eluted in the same fraction, which also corresponded to a UV-absorbing peak. These fractions were concentrated and sequenced (performed by R. Blacher, Applied Biosystems) using an Applied Biosystems 470A gas-phase sequencer with on-line RPLC analysis of PTH amino acids (Applied Biosystems 120A). PTH-Cys(CH_2CO_2H) was determined by radioassay of an aliquot from each cycle.

Synthesis. Details of these syntheses will be published elsewhere. Peptide F1 was synthesized on *p*-methylbenzhydrylamine resin using N^{α}-*t*-butyloxycarbonyl (Boc) or N^{α}-*t*-amyloxycarbonyl amino acids coupled through activated ester intermediates. The resulting peptidyl resin, Boc-Cys(EtS)-Leu-Ile-Thr(Bzl)-Asn-Cys(4-MeBzl)-Pro-Arg(Tos)-Gly-NH-resin, was cleaved with HF containing anisole and cyclized by disulfide disproportionation to give F1. The corresponding acid was synthesized similarly, using hydroxymethylated polystyrene resin. Treatment of F1 with 9 molar equivalents of Et_3N and 3 molar equivalents of acetic acid (9) gave a mixture of the dimers D1 and D2, together with F1. The dimers were purified by reversed phase LC.

Samples of D1 and D2 were cleaved with thermolysin. Fragments were separated by RPLC and sequenced (Applied Biosystems 477A) to determine configuration of the dimers.

The parallel dimer was synthesized from an Acm protected monomer, Cys(Acm)-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2, prepared in a manner analogous to F1. Oxidation with $K_3Fe(CN)_6$ formed an acyclic dimer containing a Cys^6-$Cys^{6'}$ disulfide bond. The N-termini were protected by Boc- derivatization and the product was treated with I_2 to form an intramolecular disulfide bond at Cys^1-$Cys^{1'}$. Removal of the Boc groups gave a product that coeluted with D2.

The antiparallel dimer was prepared by reaction of the above Acm monomer with another monomer containing an Npys protecting group: Cys(Npys)-Leu-Ile-Thr-Asn-Cys(Acm)-Pro-Arg-Gly-NH_2. The resulting acyclic dimer, which contained a Cys^6-$Cys^{1'}$ disulfide bond, was treated with I_2 to remove the Acm groups and form a Cys^1-$Cys^{6'}$ disulfide bond. The major product coeluted with D1.

In vitro bioassay. The biological activities of synthetic and native peptides were assayed using a new *in vitro* test (Proux *et al.*, unpublished) based on the excretion of primary urine by isolated MT. A set of MT were freed of trachea, but left attached to a piece of midgut. One end of the midgut was ligated around a cannula; the other end was ligated off. The preparation was placed in a 1-ml bathing chamber containing oxygenated saline. The cannula reached a second chamber filled with paraffin oil *via* a hole in the chamber walls. Urine flowed into the oil, forming droplets. The flow rate was determined by measuring at intervals the size of urine droplets with a graduated ocular. Saline was changed hourly; assay solutions were added at the second hour. Responses are expressed as percentage change in urine output between the first and the second hour.

RESULTS AND DISCUSSION

Amino acid compositions of F1 and F2 proved to be identical and suggested that each contained nine residues (Table 1). Moreover, both RCM-peptides were

Table 1. Amino acid compositions of F1 and F2. 64 pmol of F1 and 24 pmol of F2 were hydrolyzed, based on comparison with 104 pmol of norleucine added. Cysteic acid and proline were quantified from a separate aliquot with only external standard comparison with other residues; proline was assigned a value of 1.00. The other amino acids were normalized on an apparent total of six residues. Amino acids not shown were not detected.

	# of Residues	
Amino acid	F1	F2
Asx	0.84 (1)	0.89 (1)
Thr	0.88 (1)	0.89 (1)
Gly	1.13 (1)	1.32 (1)
Ile	1.01 (1)	0.93 (1)
Leu	1.13 (1)	1.03 (1)
Arg	1.02 (1)	0.93 (1)
Cys	1.66 (2)	1.66 (2)
Pro	1.00 (1)	1.00 (1)
Total	9	9

found to have the identical primary sequence Cys(CH_2CO_2H)-Leu-Ile-Thr-Asn-Cys(CH_2CO_2H)-Arg-Pro-Gly. These results were surprising considering the substantially different retentions of the native factors on LC.

Nonapeptides with the sequence determined for F1/F2 were synthesized using manual solid-phase methodologies (10) as both the carboxyl-terminal amidated and free acid forms. Nonlabeled RCM-derivatives of these synthetic peptides were prepared and admixed with an aliquot of the [^{3}H]RCM derivative of F1. On reversed-phase LC analysis, the "native" [^{3}H]RCM-F1 coeluted with the synthetic RCM-amide (retention time 19.0 min), which eluted faster than the RCM-acid (retention time 22.1 min). Thus, the native peptide F1 must have an amidated carboxyl terminus. As we had observed that native RCM-F1 and RCM-F2 coelute, RCM-F2 must also have a carboxyl-terminal amide. The intact, synthetic nonapeptide Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 was also analyzed by LC; it had the same retention time as did immunoreactivity from native F1 (Fig. 1) rather than F2.

To resolve this paradox of identical primary structures for F1 and F2, we analyzed the factors using size-exclusion LC with RIA monitoring. The retentions of native F1 and F2 corresponded to M_r ≃700 and ≃1,470, respectively (Fig. 2). This immediately suggested that F2 might be a dimer of F1.

Synthetic F1 was treated using conditions known to convert oxytocin to a mixture including parallel and antiparallel dimers (9); separation on reversed-phase LC gave F1 (20.1 min) and two more retained peaks at 42.0 and 45.3 min. The latter were analyzed by size-exclusion LC; both had M_r ≃1,570. Comparative analyses by reversed-phase LC showed that the faster eluting dimer (D1) had retention (UV detection) virtually coincident with that of native F2 (separate analysis, fractions monitored by RIA).

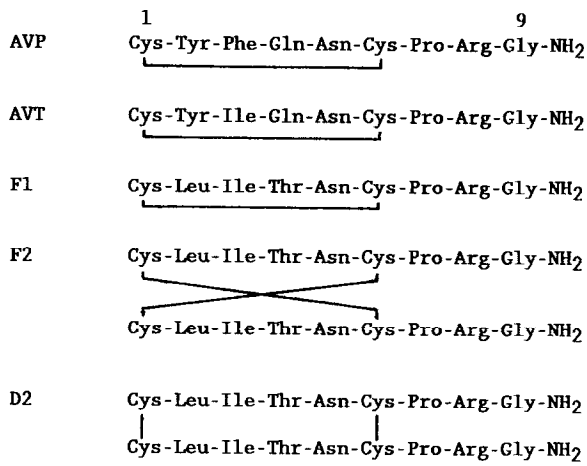

Fig. 1. Amino acid sequences of AVP, AVT, F1, F2, and the synthetic parallel dimer D2. The faster eluting, synthetic antiparallel dimer D1 is identical with F2.

To ascertain the relative configurations of the dimers, we synthesized each of them specifically, using protecting groups that allow directed

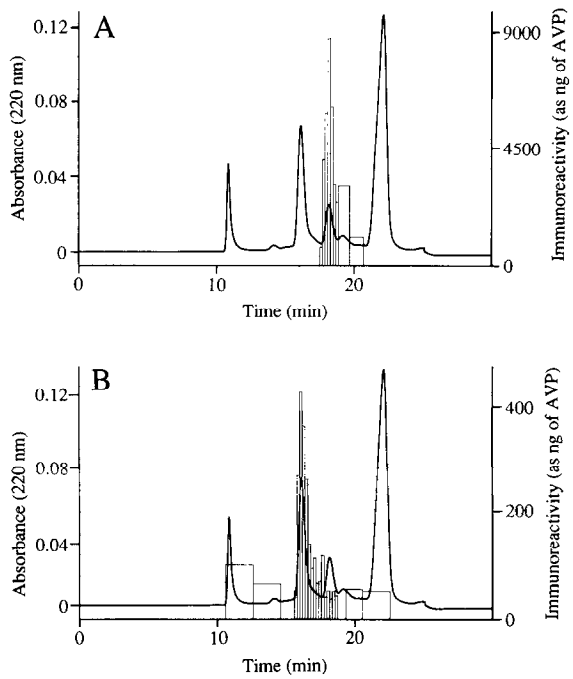

Fig. 2. Size-exclusion LC of native and synthetic F1 and F2. Immunoreactivity from native F1 (A) and F2 (B) was determined in 0.2 min fractions and plotted as a histogram, which is overlaid on UV absorbance signals from separate injections of synthetic F1 (18.2 min) and a mixture of D1 and D2 (16.1 min). The TSK 2000SW column was eluted at 0.5 ml/min with 40% CH_3CN/0.1% TFA. Exclusion and inclusion volumes are defined by BSA (10.9 min) and acetone (22.1 min). The column was calibrated with linear peptide standards; the apparent M_r values of F1 and F2 were ~75% of the true M_r based on sequence results.

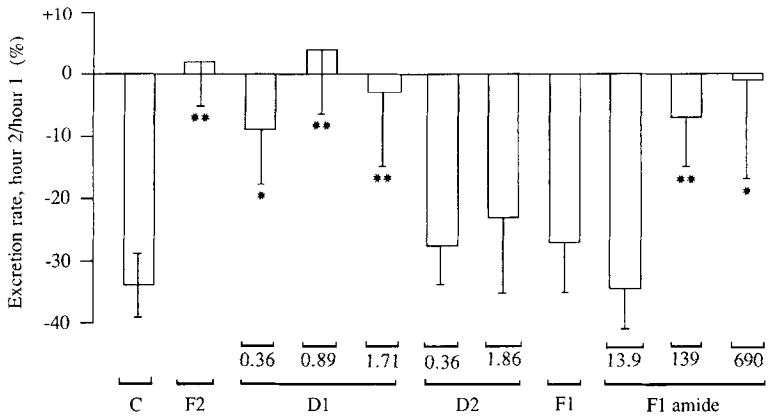

Fig. 3. Diuretic responses induced by native and synthetic F1 and F2. Responses are expressed as % change (mean ± s.e.m.) in urine output from measurements at hour 1 and hour 2. A diuretic effect is indicated by little change between the first and second hour; the lack of stimulation in the control group leads to decreased flow (34%) in the second hour. * and **: $p<0.05$ and $p<0.01$ respectively. C: control; F2: pure, native F2 (≈1.7 nM); D1: antiparallel dimer; D2: parallel dimer; F1: purified, native F1 (≈7.7 nM); F1 amide: synthetic F1. Numbers below data bars give concentration of synthetic factors (nM).

formation of disulfide bonds (11). Comparison of these dimers by LC with those synthesized nonspecifically showed that D1 corresponds to the antiparallel and D2 to the parallel isomer. Also, digestion of synthetic D1 and D2 with thermolysin followed by LC separation gave different peptide fragments. The major fragments were sequenced, showing that thermolysin cleaved each chain at the Leu-Ile bond. Structures of the fragment peptides were only consistent with D1 being the antiparallel, and D2 the parallel, dimer.

We examined the immunological relationship of the synthetic factors with an RIA (12) for AVP. Displacement curves of ^{125}I-AVP competed against F1 amide and acid, and dimers D1 and D2, were established and compared with AVP standard curves. Their cross-reactivities with anti-AVP antibody were defined as the concentration of peptide, relative to AVP, which caused 50% inhibition of binding of ^{125}I-AVP. These cross-reactivity ratios were 4.5 for dimer D1, 9.6 for D2, 10.4 for F1 (amide form), and 3×10^4 for the acid form of F1.

The biological activities of synthetic and native peptides were assayed *in vitro* using isolated Malpighian tubules; the results are shown in Fig. 3. The lack of stimulation in controls leads to gradually decreasing flow. In contrast, the flow remains roughly constant when the medium is complemented with physiological doses of D1 or native F2, and with high doses of synthetic F1. Physiological doses of D2 and native F1 have no effect.

To check whether the diuretic hormone acts on MT via cAMP, intact MT were incubated with D1 for various periods of time, then extracted and assayed for cAMP using an RIA (13). As shown in Table 2, addition of D1 elevates tissue cAMP concentration at all times tested.

Table 2. Effect of synthetic F2 (1.71 nM) on endogenous cAMP level in intact Malpighian tubules as a function of incubation time. mean ± s.e.m., n = 5 for each group, **: p <0.01. cAMP was quantified by RIA (13) while protein was quantified as described (22).

	cAMP (pmol/mg of protein)	
Incubation time	Control	Stimulated
1 min	7.29 ± 0.85	16.3 ± 5.8 **
5 min	7.37 ± 1.17	20.5 ± 9.7
10 min	6.91 ± 0.88	17.5 ± 11.3

Taken together, these data show the presence in SOG and TG of *Locusta* of an antiparallel dimeric peptide (F2), which we call the AVP-like insect diuretic hormone (AVP-like IDH). The sequence homology between the AVP-like IDH and the vertebrate neurohypophyseal peptides is clear: Cys-(2)-(3)-(4)-Asn-Cys-Pro-(8)-Gly-NH_2 (14). The AVP-like IDH and AVP differ only at positions 2, 3, and 4, whereas the homology to the ancestral molecule AVT (14) is even stronger, with differences restricted to positions 2 and 4.

Neurophysin-like immunoreactivity exists in the same cells of *L. migratoria* that contain AVP-like immunoreactivity (4). This association parallels that of neurophysin and AVP in the vertebrate neurohypophysis. Neurophysin is well known to form dimers whose properties are strongly affected by binding to AVP (15). It is conceivable that a neurophysin-like protein or its dimer may serve as the template for dimerization of F1 to give F2. Naturally occurring dimeric forms of the vertebrate neurohypophyseal peptides have not been reported, but could conceivably occur. In fact, the existence of a neuropeptide as a dimer has only two precedents. Transforming growth factor-β, a dimer, is encoded by a gene containing one copy of the 112-amino acid monomer (16). β-Human atrial natriuretic polypeptide is an antiparallel dimer of the 28-amino acid α-human atrial polypeptide (17). The latter dimer also coexists in tissue with its corresponding monomer, a situation analogous to F1 and F2.

Corpora cardiaca of *L. migratoria* reportedly contain a diuretic peptide, which has been partially sequenced after a two step LC purification procedure (18). Its sequence is unrelated to the AVP-like IDH.

Two unrelated insect neuropeptides have both biological and sequence homology with vertebrate counterparts (19,20), while another insect neuropeptide has only structural homology with a vertebrate neurohormone (21). The sequence homology of F1 and F2 with AVT (78%) is the highest yet observed for any insect neurohormone, and suggests that further research based on homology of vertebrate and invertebrate neurohormone families will be rewarding.

ACKNOWLEDGEMENTS

We thank H. Röller for discussions and comments on the manuscript, R. Schroeder (The Salk Institute) and B. Cesarin for amino acid analyses, A. Chen

for synthesizing a sample of F1, H. Kataoka for suggesting use of thermolysin, R. Troetschler and S. J. Kramer for discussions and determining stimulation of cAMP induced by F2 in MT of *Schistocerca nitens*, and the members of the Laboratoire de Neuroendocrinologie, Bordeaux, for supplying locust ganglia.

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