Proteolytic Processing of the *Aplysia* Egg-Laying Hormone and R3–14 Neuropeptide Precursors

Robert Newcomb and Richard H. Scheller

Department of Biological Sciences, Stanford University, Stanford, California 94305

A number of animal behaviors are influenced by the actions of neuropeptides that arise from the processing of complex protein precursors. In this report we investigate the proteolytic processing of neuropeptide precursors expressed in the *Aplysia californica* bag cells, which govern egg-laying, and neurons R3–14, which mediate aspects of cardiac output. Peptides were purified by fractionation on 2 high-pressure liquid chromatography systems followed by determination of amino acid compositions. Most of these compositions are indicative of processing products derived from the egg-laying hormone (ELH) and R3–14 precursors by cleavage at basic residues. We characterized 9 peptides that arise from the ELH precursor by cleavage of the signal sequence, as well as 7 out of 8 dibasic residues and at least 1 single Arg residue. The peptides range in size from 5 to about 60 amino acids. The R3–14 neuropeptide precursor is cleaved at 2 internal dibasic residues in addition to the signal sequence, resulting in 3 peptides. Shortened forms of several peptides probably result from amino- and carboxy-terminal peptidase action. It is likely that the complex mixtures of neuropeptides arising from these single protein precursors are co-secreted.

Neuropeptide precursors are often proteolytically cleaved into multiple peptide products possessing distinct biological actions. Examples include the pro-opiomelanocortin molecule, which is cleaved into fragments with activities ranging from the control of adrenal steroid secretion to a morphine-like activity (Mains et al. 1977), and the *Aplysia* bag cell egg-laying hormone (ELH) precursor, which contains peptides with distinct actions on isolated neurons (Scheller et al., 1983; Mayeri et al., 1985; Sigvardt et al., 1986). It is thought that the multiple active fragments of a neuropeptide precursor may act in a coordinated fashion to produce complex physiological and behavioral responses to particular stimuli.

One obstacle to the investigation of this hypothesis has been the complexity of the product mixture generated in the proteolytic cleavage of many neuropeptide precursors. The number of potential cleavage sites on a precursor protein molecule is often

large, and the isolation of all the potential fragments has, in the past, proven to be a difficult technical problem. As a result, studies of the actions of neuronal peptides have thus far focused on easily isolated major cleavage products, sidestepping the task of looking at the entire product mixture secreted by a peptidergic neuron. In view of the additional consideration that the product mixture contained in a peptidergic neuron appears to vary with secretory activity (Fleminger et al., 1984; Akil et al., 1985; Lorenz et al., 1985), our knowledge of the physiological roles of peptidergic neurons is limited by the lack of a complete characterization of the final proteolytic cleavage products.

To understand the information content of peptidergic neurotransmission, it will be necessary to define both the quantitative amounts and physiological activities of all peptides secreted under varying conditions of secretory demand. Defining the complete spectrum of physiological activities of each member of a complex mixture of peptides will probably remain a difficult task for some time to come. With technical advances in high-pressure liquid chromatography (HPLC) instrumentation, it is now possible to completely define the peptides that result from cleavage of some polypeptide precursors. Advances in automated sample handling and subsequent data analysis allow the resolution and characterization of increasingly complex mixtures of proteins and peptides, while advances in solvent delivery systems and fluorescence detectors allow the sensitive detection of released peptide mixtures by direct HPLC methods (Sigvardt et al., 1986).

As a model system to further investigate the biological roles of neurosecretion, the abdominal ganglion of the gastropod mollusc *Aplysia californica* offers several advantages. Peptides can be assayed directly for effects on identified neurons (Mayeri et al., 1985), thus making the difficult problem of comprehensive physiological characterization approachable. In addition, the genes encoding several neuropeptide precursors expressed in neurons of the abdominal ganglion have been cloned and characterized (Scheller et al., 1984). By determining the nucleotide sequence of genomic or cDNA clones, we have deduced the amino acid sequence of several peptide precursors, thus defining the potential peptide products.

The 271 amino acid precursor encoding ELH is expressed in the bag cell neurons localized in 2 homogeneous clusters on the rostral side of the abdominal ganglion (Mahon et al., 1985). These neurons send secretory processes primarily into the sheath surrounding the ganglion and the pleural abdominal connectives. At least 2 biologically active peptides, ELH and α -bag cell peptide (α -BCP), have been purified from bag cell extracts (Chiu et al., 1979; Mayeri et al., 1985; Sigvardt et al., 1986). Furthermore, bag cell discharge leads to a stereotyped egg-laying

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Correspondence should be addressed to Richard H. Scheller at the above address.

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behavior with multiple separate components (Arch and Smock, 1977). By characterizing the effects of peptides on identified neurons it may be possible to describe egg-laying behavior in terms of the summed actions of individual peptides on identified neurons. A prerequisite for these studies is a description of the peptide mixture cleaved from the ELH precursor.

We report here the results of an analysis of peptides obtained from bag cells and the abdominal ganglion using a 2-system reverse-phase HPLC approach. A consistent set of peptides was obtained and appears to account for all but one of the significant cleavage products of the ELH precursor. In addition, we identified several products that arise from a peptide precursor expressed in the R3–14 neurons of the abdominal ganglion. These neurons send processes into the sheath, as well as to the heart, and have roles in modulating cardiac output. The data define the spectrum of peptides potentially secreted by the bag cells and R3–14, thus providing information necessary for physiological studies and further investigations of protein packaging and trafficking.

Materials and Methods

Instrumentation. A Beckman instrument was used for preparative HPLC separations. This consists of 2 model 112 pumps, a model 210 sample injector (2 ml loop), and a model 165 absorbance detector. Amino acid analysis was performed with a Gilson system, including 2 model 302 pumps, a model 231-401 autosampling injector, a model 704 gradient control, and data analysis system. This system utilized Gilson Spectra-Glo and Perkin Elmer LS-1 fluorescence detectors. For all first system separations, a 3.0×40 mm guard column was used. This was removed for the second system separations.

Abdominal ganglion peptides. Abdominal ganglia were dissected from 30 animals (400–600 gm) and the bag cell clusters removed. The tissue was stored at -70°C until used. The ganglia were thawed and immediately boiled for 5 min in 5 ml of 0.1 N HCl, homogenized, and then centrifuged at low speed. Multiple 0.8 ml injections were made to load the extract for preparative HPLC (1 ml sample loop). Each injection contained 0.6 ml of extract and 0.2 ml of a saturated sodium acetate solution, which was added to neutralize the acid extract. After loading the entire extract volume, peptides were eluted with a linear gradient of 0–100% methanol (MeOH) in 0.1% trifluoroacetic acid (TFA) over 90 min at a flow rate of 1 ml/min; 1 ml fractions were collected. The separation column for this and the second system separations was a 4.3×250 mm column packed in the laboratory with Vydac 218 TP 54 octadecylsilica (ODS), 10 μm particle diameter. At the termination of the experiments reported here, its performance was measured at 1000 plates/250 mm.

The individual 1 ml fractions obtained above were dried to $\frac{1}{2}$ to $\frac{1}{3}$ original volume with 200 μl saturated sodium acetate added to minimize surface adsorption. These samples were rechromatographed on a second system using linear gradients of 0–100% MeOH over 90 min in 0.025 M sodium phosphate, pH 6.2. Initial peptide detection was by absorbance at 220 and 280 nm. The 1 ml fractions were further characterized by amino acid analysis.

Isolation of bag cell peptides. For this experiment, 50 freshly dissected bag cell clusters from 25 animals (200–400 gm) were homogenized in 4 ml of HCl:acetone:water (1:40:6). The extract was centrifuged at low speed and the resulting supernatant dried to about $\frac{1}{3}$ the original volume with nitrogen. Following neutralization with sodium acetate, the extract was injected onto a 4.3×250 mm ODS column (Vydac 218 TP 54, 5 μm particle diameter). Elution was at 1 ml/min with a MeOH gradient of 0–15% over 20 min, then 15–100% over 210 min in 0.025 M sodium phosphate, pH 6.2. Fractions of 1 ml were collected and aliquots subjected to amino acid analysis. Following evaluation of amounts and purity, fractions were pooled as shown in Figure 1 and rechromatographed. Second system separations were over the same 5 μm particle diameter ODS column but with the following gradient of MeOH in 0.1% TFA: 0–35% over 20 min, 35–45% over 10 min, 45–65% over 40 min, 65–75% over 10 min, and 75–100% over 10 min. Elution was monitored at 280 and 220 nm. At the end of these experiments, the performance of the separation column was measured at 7000 plates/

250 mm. This entire experiment was repeated with bag cell clusters from 25 400–600 gm animals. The identical set of peptides was obtained.

Amino acid analysis. Peptides were characterized by amino acid analysis essentially as described (Lindorth and Mopper, 1979; Newcomb, 1983). For initial screening of fractions, hydrolysis was performed in Teflon-stoppered autosampler vials (Alltech no. 95171). For compositional analysis, hydrolysis was done in sealed Pyrex tubes under air with 50 μl 6 N HCl (Sigma) containing 2–5% anisole (Sigma). Derivatization with *o*-phthalaldehyde 2-mercaptoethanol was automated with the 231-401 autoinjector. The reagents (about 10 mg/ml and 50 μl /ml, respectively) were premixed in 0.5 M potassium borate, pH 10. For each analysis, 40 μl of the reagent was added by the autosampler to each hydrolysate. Peptide hydrolysates were loaded onto the autosampler rack (Gilson rack code 9) in 50–70 μl water:MeOH (3:1). A total injection volume of 80 μl was used with an 80 μl sample loop. Typically, 50 samples (run over 25 hr) were loaded with amino acid standards constituting 1 of every 8 analyses. Other analysis conditions were identical to those previously described (Newcomb, 1983) but with amino acid amounts determined from peak area integration performed by the Gilson 704 data analysis system.

Results

We characterized peptides from bag cell clusters or abdominal ganglia that are cleaved from the ELH and R3–14 neuropeptide precursors utilizing a 2-system reverse-phase HPLC approach to isolate the majority of the predominant peptides. Two different extraction procedures (0.1 N HCl, 100°C , and acid:acetone:water) and 2 different starting tissues (abdominal ganglia and bag cells) resulted in a reproducible and consistent set of peptides.

To insure that the peptides we isolated correspond to *in vivo* cleavage products, studies were done to examine the stability of peptides in the extraction mixture. Abdominal ganglia and bag cells were extracted and homogenized in 0.1 and 0.2 N HCl at 100°C , cold 0.2 N HCl, and HCl:acetone:water (1:40:6). These mixtures were allowed to incubate at room temperature for 2–3 hr and then examined on HPLC. In the 0.1 and 0.2 N HCl, 100°C , and the HCl:acetone:water extracts, the major peptides were stable at all time periods investigated. This was not the case with the cold 0.2 N HCl extracts. Therefore, the peptides we characterized are likely to be the result of *in vivo* processing events. However, some caution must be exercised with the fragments present in minor molar amounts.

An example of the chromatographic pattern obtained from a bag cell extract is shown in Figure 1. Approximately 25 peptide peaks were easily resolved, mostly between 60 and 180 min, corresponding to MeOH concentrations ranging between approximately 30 and 80%. Chromatographic patterns obtained from abdominal ganglion extracts were similar but exhibited a 5-fold greater density of peaks. Most of these additional abdominal ganglion peaks were relatively minor compared to those displayed in the bag cell chromatogram.

Because of the complexity of the protein/peptide mixtures, only the predominant peptides were of sufficient purity to be identified on the basis of amino acid composition after a single HPLC separation. As a result, it was necessary to further purify many of the peptides over a second gradient system, as shown in Figure 2 and described in Materials and Methods. As expected, these chromatograms were considerably less complex, in general revealing only 1–4 peaks in the case of bag cells and 1–4 major peaks with many minor peaks in the case of the abdominal ganglion (Fig. 2).

Peptides were characterized by amino acid analysis using the *o*-phthalaldehyde, 2-mercaptoethanol precolumn derivatization procedure (Lindorth and Mopper, 1979). The advantages

Figure 1. First-dimension HPLC. Chromatogram of an acid acetone extract of bag cell clusters from 200–400 gm *Aplysia californica* ($n = 25$). The gradient is increasing concentrations of methanol in 0.025 M sodium phosphate pH 6.2. Fractions eluting after the ELH and before II (RLRFD) contain very little peptide material and were not subjected to second system analysis. R14 indicates various peptides from the R14 neuropeptide precursor. Following determination of amounts and purity of protein by amino acid analysis, 1 ml fractions were pooled for second system chromatography. The late eluting fraction of the acidic peptide (peak R) contains an impurity that does not separate on the second system we used. All other fractions, A–T, were rechromatographed on the second system of Figure 2.

of this procedure are that it is rapid and sensitive, and the derivatization procedure can be automated allowing the large number of analyses required for the approach used here. Results of amino acid analysis are given in Table 1.

Given the multiple determinations of each amino acid composition, the data are of sufficient accuracy to allow comparison to amino acid sequences of previously characterized neuropeptide precursors as defined from our analysis of recombinant DNA clones. Two unexpected peptides, which apparently do not derive from previously characterized precursors, were isolated in pure form. These peptides are likely to be of neuronal origin and the composition of the larger, lysine-rich product is presented in Table 1. This peptide was found to elute at 35 and 33 min (Fig. 2, C, D). The composition of the peptide found in the bag cells is similar to the major abdominal ganglion form of the peptide, which was found in multiple forms. The other peptide is quite small, having the determined composition of Glu:1 Ser:1 Ala:1. These 3 amino acids occur in characterized abdominal ganglion precursors, including the R3–14 precursor; however, release of this product is not consistent with cleavages at or near basic residues. Both peptides are present in approximately equivalent abundance to the bag cell and R3–14 peptides.

Most of the other compositions obtained are consistent with peptides derived from the ELH and R3–14 peptide precursors

(Table 1; Figs. 3, 4). This might be expected, given that both of these groups of neurons synthesize large amounts of product and send processes to the connective tissue sheath surrounding the ganglion. As a result, comparatively large amounts of peptide are stored in the ganglion rather than being transported to peripheral targets.

For the shorter peptides, the composition data were sufficient to specify exact sequences after comparison with the gene sequence. Minor errors in the analysis did not allow this for the larger peptides, but it was still possible to identify the likely cleavage sites with a high degree of confidence. We discuss cleavage products based on the best fit to the data. These fits predict that all of the longest forms of the peptides arise from cleavage at basic residues and the signal sequence. All peptides are in exact agreement with cleavage at these sites, with the minor exception that some of the determined values for aspartate seem to be off by about 10%. In Table 1 and Figure 4, we specify the limits of the peptides according to the standard errors of the analysis procedure (see legend Table 1). Peptides from the bag cells were, in general, completely pure, and gave rise to all but one of the ELH precursor compositions given in Table 1. R3–14 and the major ELH precursor peptides were present in such large quantities in the abdominal ganglion that their amino acid compositions could also be obtained.

The 271 amino acid sequence of the ELH precursor is shown

Figure 2. Second-dimension chromatograms. Fractions A–T from the first chromatographic system were rechromatographed in 0.1% trifluoroacetic acid with increasing methanol. All detectable A_{220} peaks were subjected to amino acid analysis. Peptides determined to be on the ELH precursor are identified by their position on the precursor; error limits on the analysis are given in Table 1. Peptides present on the ELH precursor that could only be approximately localized are denoted by parentheses. Peptides obviously not on the ELH precursor are denoted by an *asterisk*. Other peaks are not peptide or were present in insufficient amounts for identification.

in Figure 3A. The precursor contains 8 paired basic residues, and our data demonstrate that all but one of these sites are proteolytically processed to generate peptides in the bag cells. In addition to cleavage of the signal sequence, a single arginine residue clearly serves as a processing site in maturation of the product mixture (Figs. 3, 4). These 9 cleavages of the ELH

precursor predict the occurrence of 10 peptides. We would not expect to isolate the signal sequence peptide, since, by analogy to other systems, this cleavage is likely to occur on the nascent protein chain while traversing the membrane of the endoplasmic reticulum, followed by fast turnover of the signal peptide. Of the remaining 9 products, 8 were isolated (peptides K1, I, II,

Table 1. Amino acid compositions of purified peptides

Peptide	Position					M_w	N									
A1	ELK 110(−0, +3)–144(−0, +1)					3896	4									
	D	E	S	M	H	G	T	A	T	R	V	F	I	L	K	
Calc.	8.4	4.4	3.8	+	0	2.5	1.2	3.5	0.81	1.9	1.9	2.5	0	1.1	0	
SD	1.1	0.57	0.67			0.63	0.10	0.49	0.24	0.43	0.20	0.53		0.18		
Obs.	9	4	4	1	0	2	1	3	1	2	2	3	0	1	0	
C1	ELH 110(−0, +3)–148(−0, +0)					4408	14									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	8.4	5.0	3.8	+	0	2.3	1.2	3.1	0.88	2.9	1.9	2.8	0	2.0	0	
SD	1.5	0.50	0.42			0.36	0.30	0.22	0.13	0.53	0.15	0.39		0.18		
Obs.	10	5	4	1	0	2	1	3	1	3	2	3	0	2	0	
D1	ELH 188(−0, +0)–203(−1, +0)					1564	9									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	1.9	6.4	0	0	2.0	0.99	0	0	0	0	0	1.0	3.3	0	
SD		0.41	1.2			0.31	0.12						0.07	0.14		
Obs.	0	2	6	0	0	2	1	0	0	0	0	0	1	3	0	
E1	ELH 150(−0, +0)–158(−0, +0)					1123	5									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	0	1.2	–	0	0.56	0	1.2	0.91	2.1	0	0.88	0	2.2	0	
SD			0.23			0.21		0.28	0.18	0.18		0.19		0.25		
Obs.	0	0	1	0	0	0	0	1	1	2	0	1	0	2	0	
H1	ELH 103(−1, +4)–148(−0, +4)					5409	9									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	8.2	4.9	4.4	+	0	2.9	1.4	3.5	1.0	7.7	2.0	4.0	0	3.3	0	
SD	1.3	0.66	0.51			0.59	0.27	0.36	0.18	0.81	0.09	0.51		0.22		
Obs.	11	5	4	1	0	2	1	3	1	7	2	4	0	3	0	
H2	ELH 185(−0, +1)–203(−1, +0)					1863	7									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	1.9	6.6	0	0	2.4	0.98	0	0	0	0.96	0	0.98	4.8	0	
SD		0.51	1.0			0.78	0.16				0.10		0.09	0.75		
Obs.	0	2	7	0	0	2	1	0	0	0	1	0	1	4	0	
K1	ELH 33(−5, +0)–93(−6, +2)					6718	17									
	D	E	S	M	H	G	T	A	T	R	V	F	I	L	E	
Calc.	10.0	4.9	10.1	+	0.85	4.0	1.3	5.0	1.8	4.0	5.3	3.8	0	2.2	2.6	
SD	1.8	0.79	1.4		0.56	0.87	0.27	0.71	0.26	0.67	0.73	0.61		0.42	1.3	
Obs.	13	4	10	1	1	4	1	4	2	4	6	4	0	2	3	
Q1	ELH 245(−0, +0)–271(−0, +0)					2843	5									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	4.3	4.1	4.7	0	0	1.08	0.99	1.1	0	1.2	1.1	0	1.08	6.2	2.06	
SD	0.41	0.27	0.18			0.04	0.01	0.11		0.05	0	0	0.05	0.15	0.11	
Obs.	4	4	5	0	0	1	1	1	0	1	1	0	1	6	2	
I	ELH 96(−0, +0)–100(−0, +0)					727	3									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	0	0	0	0.59	0.31	0	0	0	2.0	0	1.0	0	0.95	0	
SD					0.03	0.16				0.04		0		0.04		
Obs.	0	0	0	0	1	0	0	0	0	2	0	1	0	1	0	
II	ELH 103(−1, +0)–107(−0, +1)					706	2									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0.72	0	0	0	0	0	0	0	0	2.0	0	0.93	0	1.0	0	
SD	0.54									0.56		0.10		0.25		
Obs.	1	0	0	0	0	0	0	0	0	2	0	1	0	1	0	

Table 1. Continued

Peptide	Position					M_w	N									
III	ELH 150(-, +0)-157(-1, +0)					1010	3									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	0	0.80	0	0	2.5	0	1.5	1.1	2.2	0	0.91	0	1.2	0	
SD			0.21			1.6		0.36	0.10	0.53		0.15		0.17		
Obs.	0	0	1	0	0	0	0	1	1	2	0	1	0	1	0	
R14 I	R14 24(+1, -0)-49(+0, -1)					2917	6									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	6.9	4.3	0.99	0	0	1.1	2.8	1.1	0	0	2.9	2.0	0	3.0	0.93	
SD	0.28	0.43	0.23			0.09	0.37	0.16			0.2	0.11		0.27	0.09	
Obs.	7	4	1	0	0	1	3	1	0	0	3	2	0	3	1	
R14 II	R14 24(+1, -0)-47(-0, +0)					2673	1									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	5.7	4.0	1.0	0	0	2.3	2.7	1.2	0	0	1.5	2.2	0	3.3	0	
Obs.	6	4	1	0	0	1	3	1	0	0	3	2	0	3	0	
R14 III	R14 52(+0, -0)-63(+0, -0)					1380	3									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	3.0	1.1	+	0	0	1.1	2.1	0	1.0	0	0.98	0	0.96	0	
SD		0.29	0.21				0	0.32		0.06		0.02		0.03		
Obs.	0	3	1	1	0	0	1	2	0	1	0	1	0	1	0	
R14 IV	R14 52(+0, -0)-62(+0, -0)					1267	3									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	3.1	0.88	+	0	0	0.91	2.2	0	1.1	0	1.1	0	0	0	
SD		0.26	0.11				0.11	0.17		0.07		0.11				
Obs.	0	3	1	1	0	0	1	2	0	1	0	1	0	0	0	
R14 V	R14 54(+0, -0)-63(+0, -0)					1180	3									
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	0	2.0	1.1	+	0	0	0.87	1.3	0	1.0	0	1.1	0	1.1	0	
SD		0.16	0.14				0.11	0.06		0.10		0		0.06		
Obs.	0	2	1	1	0	0	1	1	0	1	0	1	0	1	0	
Lysine-rich peptide	$N = 11$															
	D	E	S	M	H	G	T	A	Y	R	V	F	I	L	K	
Calc.	4.4	6.4	3.1	0	1.0	1.3	3.75	0	0	0	1.0	0.96	1.6	1.9	10.8	
SD	0.82	1.0	0.40		0.52	0.17	0.47				0.06	0.10	0.20	0.18	1.5	

The composition of a novel lysine-rich peptide is also given (lowest row). Conservative limits of the position of each peptide on the precursor are given as + or - (in parentheses) the most likely position based on the best fit of the data. The limits were obtained by moving 1 end away from the best fits of the peptides until any amino acid would be more than 1 SD $\pm$ 0.5 from the mean value of the determination. By this criterion peptides H1 and K1 could be moved a considerable distance along the precursor. However, the assigned sequence represents the best fit to the data and predicts that logical cleavage sites are used to generate the peptides. All values are from 24 hr hydrolysates except for ELH 33-93 Val, Phe and Leu, which are from 8-48 hr hydrolysates. (This peptide contains a very hydrophobic sequence and Val values are still slightly low.) Errors for R14 II amino acid residues 24-47 ($n = 1$) were assumed to be the same as for R14 24-49. Note that the *o*-phthalaldehyde technique does not determine Pro or Cys. The amino acid composition of the lysine-rich peptide was from the peak eluting at 35 and 33 min in Figure 2, C and D; it reflects a mixture of very closely related forms of this peptide. All R3-14 peptides were obtained from the abdominal ganglion. All ELH peptides were from the experiments depicted in Figure 2 except for ELH 150-157, which was obtained from the second preparation of bag cells (400-600 gm animals), and RLRFD, which was obtained from the abdominal ganglion. All of the ELH peptides in this table are identified according to their elution in Figure 2 or by Roman numerals as described in the legend to Figure 5. All of the ELH peptides except RLRFD and ELH 150-157 were also identified in the abdominal ganglion. A 0 in the compositions means a value less than 0.2; in general, these values were less than 0.05.

C1, E1, H2, V, and Q1), and the ninth (peptide IV, Fig. 4) is presumed to exist because of the occurrence of both flanking peptides. Peptide IV has a number of charged amino acids and most likely was missed on our chromatographic systems, although we cannot rule out the possibility that it is degraded *in vivo*. All of the peptides were found in both bag cell and abdominal ganglion extracts with the exception of RLRFD, RLRFDH, ELH 150-157, and ELH 110-145. Because of differences in chromatographic procedures and the ways that the fractions were pooled, these peptides were found in only 1 tissue.

A number of shortened forms of the peptides that are cleaved at basic residues were isolated. These peptides most likely arise from either amino or carboxy terminal peptidase activities. An exception is ELH 110-144, which could arise from either cleavage at a single arginine at position 146 or simply carboxy-terminal trimming. Because of the series of control experiments described earlier, it is likely that these peptides arise *in vivo* and greatly increase the complexity of the peptide product mixture. An additional peptide was found that may represent an intermediate in the processing pathway. Peptide H1 appears to be

A

ELH PRECURSOR

1 Met Lys Arg Pro Asn Asn Arg Pro Thr Asn Thr Met Ser Leu Ile Leu Cys Leu Thr Leu Ser Ser Ser Gln Ser Ala Ser Val [▼]
 33 His Gly Lys Asn Phe Ala Thr Asn Arg Ala Val Lys Ser Ser Prophe Val Val Leu Ser Pro Asp Asp Asn Val Val Ser Met Ser Gly Glu
 65 Asn Gly Tyr Arg Ser Ala Leu Arg Glu Ala Phe Asp Lys Ser Ser Arg Asp Tyr Asp Asn Gly Glu Asp Val Phe Ser Asn Glu Lys Arg Arg
 97 β -BCP Lys Arg Arg Phe Arg Phe Asp Arg Asp Gln Asp Glu Gly Asn Phe Arg Arg Phe Pro Thr Asn Ala Val Ser Met Ser Ala
 129 γ -BCP Lys Arg Arg Phe Arg Phe Asp Arg Asp Gln Asp Glu Ala Val Tyr Gln Arg Asp Leu Arg Ala Pro Arg Leu Arg Phe Tyr Ser Leu Arg Lys
 161 Asp Glu Asn Ser Pro Phe Asp Leu Ser Asn Glu Asp Gly Ala Val Tyr Gln Arg Asp Leu Arg Ala Pro Arg Leu Arg Phe Tyr Ser Leu Arg Lys
 Arg Ala Ala Gly Met Glu Gln Ser Glu Gly Gln Asn Pro Glu Thr Glu Ser His Ser Arg Arg Lys Arg Ser Val Leu Thr Pro Ser Leu Ser
 193 Ser Leu Gly Glu Ser Leu Glu Ser Gly Ile Ser Lys Arg Ile Ser Ile Asn Gln Asp Leu Lys Ala Ile Thr Asp Met Leu Leu Thr Glu Gln Ile
 225ELH.....
 Arg Glu Arg Gln Arg Tyr Leu Ala Asp Leu Arg Gln Arg Leu Leu Glu Lys Gly Lys Arg Ser Ser Gly Val Ser Leu Leu Thr Ser Asn Lys Asp
 257 Glu Glu Gln Arg Glu Leu Lys Ala Ile Ser Asn Leu Leu Asp ***
 Acidic

B

R3-14 PRECURSOR

1 Met Gln Val Leu His Leu Cys Leu Ala Val Ser Ile Ala Val Ala Leu Leu Ser Gln Ala Ala Trp Ser Glu Glu Val Phe Asp Asp Thr Asp Val [▼]
 33 Gly Asp Glu Leu Thr Asn Ala Leu Glu Ser Val Leu Thr Asp Phe Lys Asp Lys Arg Glu Ala Glu Glu Pro Ser Ala Phe Met Thr Arg Leu Arg
 65 Arg Gln Val Ala Gln Met His Val Trp Arg Ala Val Asn His Asp Arg Asn His Gly Thr Gly Ser Gly Arg His Gly Arg Phe Leu Ile Arg Asn
 97 Arg Tyr Arg Tyr Gly Gly His Leu Ser Asp Ala ***

Figure 3. Amino acid sequence of the ELH and R3-14 peptide precursors. *A*, The ELH precursor. Amino acids are numbered 1–271 above the sequence. The basic residues that are cleaved to generate the peptides found in this study are *underlined*. The *arrow* indicates the best fit for cleavage of the signal sequence. α -, β -, and γ -BCP peptides are labeled, as are ELH and the acidic peptide. *B*, The R3-14 peptide precursor. Again, the *arrow* indicates the position of cleavage of the signal sequence and the basic residue cleavage sites are *underlined*.

Figure 4. Processing of the ELH and R3-14 peptide precursors. *A*, The ELH precursor. Two related precursors of the ELH gene family are diagrammed. The peptide A precursor is expressed in the atrial gland and the ELH precursor in the bag cell neurons. Dashed lines between the precursors depict regions of insertions and deletions. Large arrows above the precursor denote the location of cleavage of the signal sequence; small arrows and lines are positions of internal proteolytic cleavages that occur at dibasic and single basic residues, respectively. Sequences related to peptide A (**), ELH (■), the acidic peptide (▨), and ArgLeuArgPhe (□) are indicated. Cysteine residues are indicated below the precursors. The arrows between the peptide A and ELH precursors indicate regions that are conserved between the *A. californica* and *A. parvula* species. The active peptides are indicated by brackets above the ELH precursor. The dashed bracket indicates a suspected active peptide. Each identified cleavage product is indicated as a bar below the ELH precursor. The smaller internal bars demarcate the possible errors in the determination. Each peptide is labeled with an identifying letter corresponding to the second-dimension chromatograph and also a number corresponding to the ELH precursor peak in that chromatograph. Peptides not found in the set of analyses illustrated in Figure 1 are labeled with Roman numerals I–V. Peptide V is the ELH, which was not further characterized. All other peptides were found in other analyses with the exception of peptide IV. All peptides and compositions, as well as errors of the analysis, are summarized in Table 1. *B*, The R3-14 peptide precursor. The – signs indicate an acidic and the + signs a basic region of the precursor. Cleavage products and other symbols are as in *A*. Peptide VI was previously characterized (Kaldany et al., 1986; Campanelli and Scheller, in press).

comprised of peptides II and C1 with the ArgArg dibasic at amino acids 108 and 109 uncleaved (Figs. 3, 4). All of the other dibasic residues are LysArg pairs, which suggests that different combinations of dibasics may be processed at different rates. Alternatively, the processing pathway may not be unique, and cleavage at 1 site may preclude cleavage at a second position.

The R3-14 precursor is only 108 amino acids, making its processing considerably easier to interpret. This study identified a series of peptide cleavage products that are consistent with those found by injecting labeled amino acids into neuron R14 followed by isolation and sequencing of the radiolabeled peptide products (Kaldany et al., 1986). Cleavage of the signal sequence occurs after the Ser at position 23, and 2 internal dibasic residues are further processed (Figs. 3B, 4B). Various shortened forms of the resulting cleavage products were observed as described above.

Discussion

We report experiments that define peptide cleavage products arising from the R3-14 and ELH precursor proteins. While we cannot be certain that this study defines complete sets of peptides, all major peaks that elute from our HPLC systems were analyzed. Each precursor gives rise to peptides via cleavage of a signal sequence and internal basic residues. A variety of studies of the processing of these precursors have been reported (Wilson, 1971; Arch, 1972; Loh and Gainer, 1975; Berry, 1976; Aswad,

1978); however, only with the knowledge of the precursor structure afforded by the analysis of recombinant DNA clones have precise studies become feasible.

Previous studies defined the primary sequence of ELH (Chiu et al., 1979), the acidic peptide (Scheller et al., 1983), and α -BCP (Rothman et al., 1983). From the precursor structure we predicted additional cleavages that would give rise to peptides we called the β -, γ -, and δ -BCPs. Cleavages that give rise to β - and γ -BCP were shown to occur. However, no cleavage at the ArgArg sequence at positions 117 and 118 was noted; thus, no δ -BCP was detected. This result is consistent with evolutionary studies demonstrating that α -, β -, and γ -BCP regions are conserved, while the δ -BCP region of the precursor is not found in another species, *Aplysia parvula* (Nambu and Scheller, 1986). These observations are also consistent with the studies utilizing the β -, γ -, and δ -BCPs that we synthesized based on the DNA sequence (Rothman et al., 1985a, b).

It is worth noting that we did not detect products that would arise via processing at the single Arg residue, which is cleaved in a related gene to generate the biologically active peptide A found in the atrial gland (Fig. 4). This may be due to the existence of different enzymes in the 2 tissues or different conformations of the precursor proteins near this cleavage site. While many single Arg residues are not cleaved, the amino terminal end of the α -BCP results from cleavage of such a residue. This points to the importance of surrounding sequences and protein structure in cleavage enzyme recognition.

Prior to isolation of cDNA and genomic clones encoding the R3–14 peptides, little information was available on the peptides produced in these cells (e.g., Aswad, 1978). This study and our previous work (Kaldany et al., 1986) precisely define the peptide products and illustrate the importance of combining biochemical and molecular genetic studies to define secretory products.

Pulse chase (Kaldany et al., 1986) and peptide isolation approaches yield a consistent picture of the processing of the R3–R14 neuropeptide precursor. Cleavage at paired basic residues and removal of these basic residues generates the predominant peptides, while further exopeptidase digestion yields minor products: the intact forms of the precursor amino terminal fragment and central portion were found in near-equal amounts (about 1 nmol/ganglion; see also Rothman et al., 1985a, b; Campanelli and Scheller, in press), with the shortened peptides being minor products.

The peptide products that arise from the ELH precursor were not isolated in stoichiometric amounts, as can be seen by a comparison of peak heights in Figure 1 and consideration of amino acid compositions. Amino acid analysis showed recovery of 3–1 nmol per bag cell cluster of ELH (V), the acidic peptide peptide (Q1), and peptides spanning residues 185–203 (D1 and H2). Peptides spanning residues 33–93 (K1 and various minor products in chromatograms I–N of Fig. 2) and 103–148 (H1, C1, and A1) were recovered in total amounts of 0.1–0.2 nmol per bag cell cluster. β -BCP was recovered in even lower amounts. The reason for the nonstoichiometric yields may simply be differential recovery of the peptide products after extraction and HPLC separation. Peptide K1 may also be substantially degraded, as a large number of distinct forms of the peptide were obtained.

Electron-microscopic immunohistochemical studies demonstrate the coexistence of γ -BCP and ELH immunoreactivity in bag cell dense-core granules (Kreiner et al., 1986). Furthermore, antibodies directed against 3 regions of the R3–14 precursor all react with the same granules. These studies in conjunction with the data presented here suggest that the many, or more likely all, products of the peptide precursors are likely to be co-released. In the case of the bag cells, more than 10 peptides may be secreted simultaneously. While we know at least 2 peptides are active, further physiological analysis must be done before the complete spectrum of activities can be elucidated.

We have recently generated evidence that the basic peptide arising from the carboxy-terminal end of the R3–14 precursor stimulates contractile activity in a variety of muscles in *Aplysia* (Campanelli and Scheller, in press). In this precursor, it is probably the case that the acidic amino terminal portion of the precursor forms ionic interactions with the very basic, active peptide, thus facilitating packaging into dense-core granules. The R3–14 neurons also may use glycine as a co-transmitter substance (Price and McAdoo, 1981), further expanding the repertory of substances secreted by these cells.

Interestingly, ELH has an amidated carboxy-terminal end, and the active peptide in the R3–14 neurons is blocked at the amino-terminal end, probably by the formation of a pyro-glutamic acid residue (Kaldany et al., 1986). Both peptides are known to be active, and these modifications may serve to protect biologically active products from the amino and carboxy peptidase actions we observed. Alternatively, the α -BCP is found in 2 forms differing by single amino acids from the carboxy-terminal end. The 1–9 form of α -BCP is about 30-fold less active

than the 1–8 form, suggesting, in this case, that the carboxypeptidase activity may be important in generating the active peptide (Rothman et al., 1983). Extension of an active sequence may then have a similar role to amidation in protecting an active-core peptide from degradation. We do not know if any of the other amino or carboxypeptidase trimmed forms of the peptides we characterized have functional significance.

The complex roles of neuropeptides in behavioral processes are only beginning to be understood in cellular and molecular terms. Definition of the processing products resulting from cleavage of the ELH and R3–14 peptide precursors defines the set of peptides involved in mediating egg-laying behavior and cardiac output, respectively, and allows further studies of the complex regulation of posttranslational processing events.

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