

Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in *Aplysia*

(atrial gland/egg-laying hormone/amino acid sequence/molluskan peptides)

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ABSTRACT Two neuroactive peptides, A and B, have been isolated from the atrial gland in the reproductive tract of *Aplysia*. Each of the two peptides is able to induce egg-laying behavior in recipient animals. *In vitro* recordings from the abdominal ganglion show that both peptides also trigger long-lasting discharges in the bag cell neurons at concentrations around 0.1 μ M. The peptides were purified by a combination of ammonium sulfate precipitation, agarose gel filtration, and cation exchange chromatography. Each peptide has 34 amino acid residues. Microsequencing together with carboxypeptidase Y degradation and analysis of tryptic peptides revealed the following sequence for peptide A: H-Ala-Val-Lys-Leu-Ser-Ser-Asp-Gly-Asn-Tyr-Pro-Phe-Asp-Leu-Ser-Lys-Glu-Asp-Gly-Ala-Gln-Pro-Tyr-Phe-Met-Thr-Pro-Arg-Leu-Arg-Phe-Tyr-Pro-Ile. Peptide B differs from A in only four positions. The first nine residues of B are: Ala-Val-Lys-Ser-Ser-Ser-Tyr-Glu-Lys-, whereas residues 10-34 of B are identical to those of A. The calculated M_r of A is 3924 and that of B is 4032. The pI of peptide A as determined by isoelectric focusing in polyacrylamide gels is 7.9-8.1 and that of peptide B is 9.0-9.2. It is estimated that each atrial gland contains at least 150 μ g of peptide A and 50 μ g of B. Neither peptide resembles the egg-laying hormone isolated from bag cell neurons. It is postulated that the atrial gland peptides are released during copulation, and then by interacting with neuronal receptors in the head ganglia and pleuroabdominal connectives they cause the bag cells to afterdischarge, thereby releasing egg-laying hormone.

The neuropeptidergic bag cells in the abdominal ganglion of *Aplysia californica* are able to initiate egg laying and its associated behavior by secreting egg-laying hormone (ELH) during a long-lasting afterdischarge (1-3). This afterdischarge may be triggered by electric stimulation of a presumed afferent pathway from the head ganglia (4, 5). Neither the location in the head ganglia nor the transmitter of these input neurons to the bag cells are presently known.

When a homogenate of bag cells containing ELH is injected into the hemocoel of a sexually mature animal, a pattern of egg-laying behavior is triggered that results in egg laying within an hour after injection (6, 7). ELH has been purified and found to be a polypeptide of 36 amino acids with a M_r of 4385 and a pI of 9.0-9.2 (8). Copulation may be a natural stimulus for egg laying and it has recently been shown that the atrial gland in the reproductive tract of *Aplysia* contains a factor that is able to induce egg-laying behavior, in a similar way to ELH, when injected into recipient *Aplysia* (9, 10). Moreover, it was shown that the atrial gland factor resembles ELH in apparent molecular weight and pI, although in contrast to bag cell homogenates some bioactive material was at pH 7.4 on isoelectric focusing gels in addition to the pI 9.3 material (9, 10).

This paper describes the purification and primary structure of two atrial gland peptides, each of which is able to induce egg

laying in recipient *Aplysia*. We report, moreover, that both peptides also induce the characteristic afterdischarge of the bag cell neurons, suggesting that these neuroactive peptides induce egg laying indirectly by stimulating the release of ELH from the bag cell neurons.

MATERIALS AND METHODS

Egg-Laying Bioassay. *A. californica* was collected locally (Palos Verdes). Animals >250 g were isolated in individual baskets in seawater at $20 \pm 0.5^\circ\text{C}$ (11, 12). Fractions were assayed by injecting them through the foot into the hemocoel (6). With active fractions eggs appear at 30 ± 10 min after injection.

Purification of Atrial Gland Peptides A and B. Homogenization and Ammonium Sulfate Precipitation. Atrial glands (40; 9.6 g) were dissected from animals, suspended in 150 ml of 0.01 M Tris-HCl (pH 8.5), and homogenized in a VirTis 23 homogenizer at 23,000 rpm for 1 min. The homogenate was centrifuged for 15 min at $27,000 \times g$, and the supernatant (140 ml, 609 mg of protein) was brought to 45% saturation by the addition of solid ammonium sulfate. The mixture was stirred for 5 hr at 4°C , and the precipitate was collected by centrifugation at $27,000 \times g$ for 15 min.

Bio-Gel A-0.5 m Chromatography. The ammonium sulfate precipitate was dissolved in 0.01 M sodium phosphate, pH 6.5/6 M urea (4.2 ml, 477 mg of protein) and applied to a 1.5×88 cm Bio-Gel A-0.5 m column (Bio-Rad) equilibrated with the same buffer. Elution was with the same phosphate/urea buffer.

SP-Sephadex C-25 Chromatography. All SP-Sephadex C-25 (Pharmacia) chromatography runs were performed on a 2×7 cm (20-ml disposable syringe) column. The buffers used for runs at different pH were: 0.01 M sodium phosphate, pH 6.5 and 0.01 M sodium citrate, pH 5.0 and 3.5, all containing 6 M urea. The proteins were eluted with a linear gradient of sodium chloride as follows: at pH 6.5 and 5.0, from 0 to 0.2 M NaCl; at pH 3.5 (peptide B, Fig. 1C), from 0.1 to 0.4 M NaCl; and for pH 3.5 (peptide A, Fig. 1D), from 0.1 to 0.3 M NaCl. The total gradient volume in all runs was 200 ml. Urea was added because it was found that it enhanced the resolution of the peptides. Protein concentration was determined by measuring the absorption at 230 and 260 nm (13).

Amino Acid Analysis. Samples were hydrolyzed under decreased pressure at 115°C for 24 hr in 6 M HCl/0.5% phenol. After removal of HCl under decreased pressure, the hydrolysate was analyzed on a Beckman 120 B amino acid analyzer.

Isoelectric Focusing and Electrophoresis in Polyacrylamide Gels. Isoelectric focusing was performed in polyacrylamide gels (14) with Ampholine 3-10 (LKB Western Instruments). pH was determined directly on the gel with an MI-410 pH probe (Microelectrodes, NY). Protein bands could be

Abbreviations: ELH, egg-laying hormone; HPLC, high-performance liquid chromatography.

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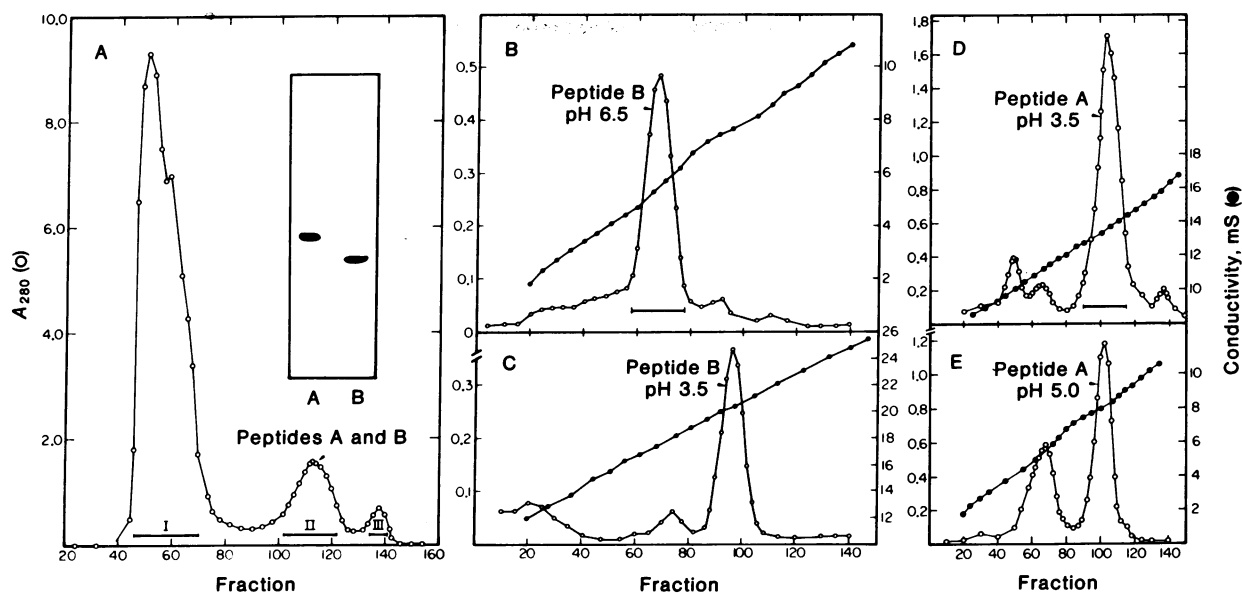


FIG. 1. Purification of peptides A and B. (A) Gel filtration of the ammonium sulfate precipitate on a 1.5×88 cm Bio-Gel A-0.5 m column. Fraction volume was 1 ml. Both peaks I and II had egg-laying activities. (Inset) Acid/urea/polyacrylamide gel electrophoresis of peptides A and B. (B) Chromatography of peak II (A) on a column of SP-Sephadex C-25, pH 6.5. Fraction volume was 1 ml. (C) Rechromatography of fractions 58–78 (B) on SP-Sephadex C-25 at pH 3.5. Peptide B emerged at fractions 88–105. (D) Material from peak II of A that was excluded on the SP-Sephadex column at pH 6.5, adjusted to pH 3.5, and applied to a SP-Sephadex column. Peptide A emerged at fractions 90–110. (E) Rechromatography of fractions 90–110 (D) on SP-Sephadex at pH 5.0. All SP-Sephadex columns were developed with a linear gradient of NaCl.

visualized as white precipitates by immersing the gels in a fixing solution of 10% trichloroacetic acid and 3% sulphosalicylic acid. Fixed gels were stained with a solution of 0.07% Coomassie blue G250 in 4% perchloric acid. Acid/urea (6.25 M)/polyacrylamide gel electrophoresis was performed according to Hurley (15).

Sequence Determination. Automated Edman degradation on peptides A and B was performed by using a sequenator program similar to that described (8). The only modification was one 10-min cleavage step to avoid proline overlap.

Succinylation, Trypsin Digestion, and Peptide Separation. Peptide A or B (1 mg each) was dissolved in 2 ml of 6 M guanidine-HCl/0.2 M NaHCO_3 - Na_2CO_3 buffer at pH 9.5. Succinic anhydride (75 mg) was added in small increments while the stirred solution was kept at pH 9.5 with the addition of 1 M KOH. The solution was stirred for 1 hr at room temperature,

Table 1. Amino acid composition of peptides A and B and of ELH

Amino acid	Peptide A, residues/mol		Peptide B, residues/mol		ELH (8), residues/mol
	a*	b†	c*	d†	e†
Asx	3.6	4	2.2	2	4
Thr	1.1	1	1.1	1	2
Ser	2.7	3	3.9	4	1
Glx	2.3	2	3.4	3	7
Pro	3.7	4	3.8	4	0
Gly	2.0	2	1.4	1	0
Ala	1.9	2	2.1	2	2
Val	0.9	1	1.1	1	0
Met	1.0	1	1.0	1	1
Ile	1.0	0	1.0	0	4
Leu	3.0	3	2.1	2	7
Tyr	3.3	3	3.6	4	1
Phe	3.1	3	3.1	3	0
Lys	2.1	2	2.7	3	2
Arg	2.3	2	2.0	2	5

* Determined by amino acid analysis after hydrolysis in 6 M HCl.

† Determined by microsequencing.

dialyzed against distilled H_2O , and lyophilized. The succinylated protein was dissolved in 1 ml of 0.1 M ammonium bicarbonate buffer (pH 8.0), and 30 μl of a trypsin solution at 1 mg/ml (Sigma, diphenyl carbamyl chloride-treated) was added. The mixture was left at 37°C for 16 hr and then 20 μl of the trypsin solution was added and the digestion was continued for another 4 hr. The reaction was stopped by lowering the pH to 3 with acetic acid and the mixture was lyophilized. The digest was dissolved in 150 μl of 49 mM KH_2PO_4 , pH 2.85, and applied to a Du Pont Zorbax C8 column attached to an Altex high-performance liquid chromatography (HPLC) apparatus. The column was developed with a gradient of the phosphate buffer to acetonitrile and emerging fractions were analyzed on the amino acid analyzer after acid hydrolysis.

Carboxypeptidase Y Sequence Determination. Carboxypeptidase Y (Sigma) was dissolved in H_2O at 1 mg/ml, and 25 μl of this solution was added to either peptide A or B (50 nmol) in 175 μl of 0.1 M pyridine/acetate buffer at pH 6.5. The digestions were carried out at room temperature for various lengths of time and stopped by lowering the pH to 3 with acetic acid. The digest was lyophilized and the released amino acids were determined directly on the amino acid analyzer.

Electrophysiological Recordings. All electrophysiological experiments were carried out at 14°C . Bag cell afterdischarges were recorded with extracellular suction electrodes placed over the bag cell clusters within the abdominal ganglion or over bag cell neurites as described in ref. 5. To achieve the final peptide concentrations, we added 100–500 μl of a more concentrated solution directly to the extracellular medium.

RESULTS

Bio-Gel A-0.5 m Chromatography. The elution profile of the ammonium sulfate precipitate that was applied to the column (477 mg) shows three main fractions: I, II, and III (Fig. 1A). Fractions I and II had egg-laying activities. Only fraction II, which contained peptides A and B, was studied.

SP-Sephadex C-25 Chromatography (pH 6.5 and 3.5): Peptide B. Fraction II (fractions 102–122, Fig. 1A, 20 ml, 67.9 mg of protein) was applied to the column at pH 6.5. The col-

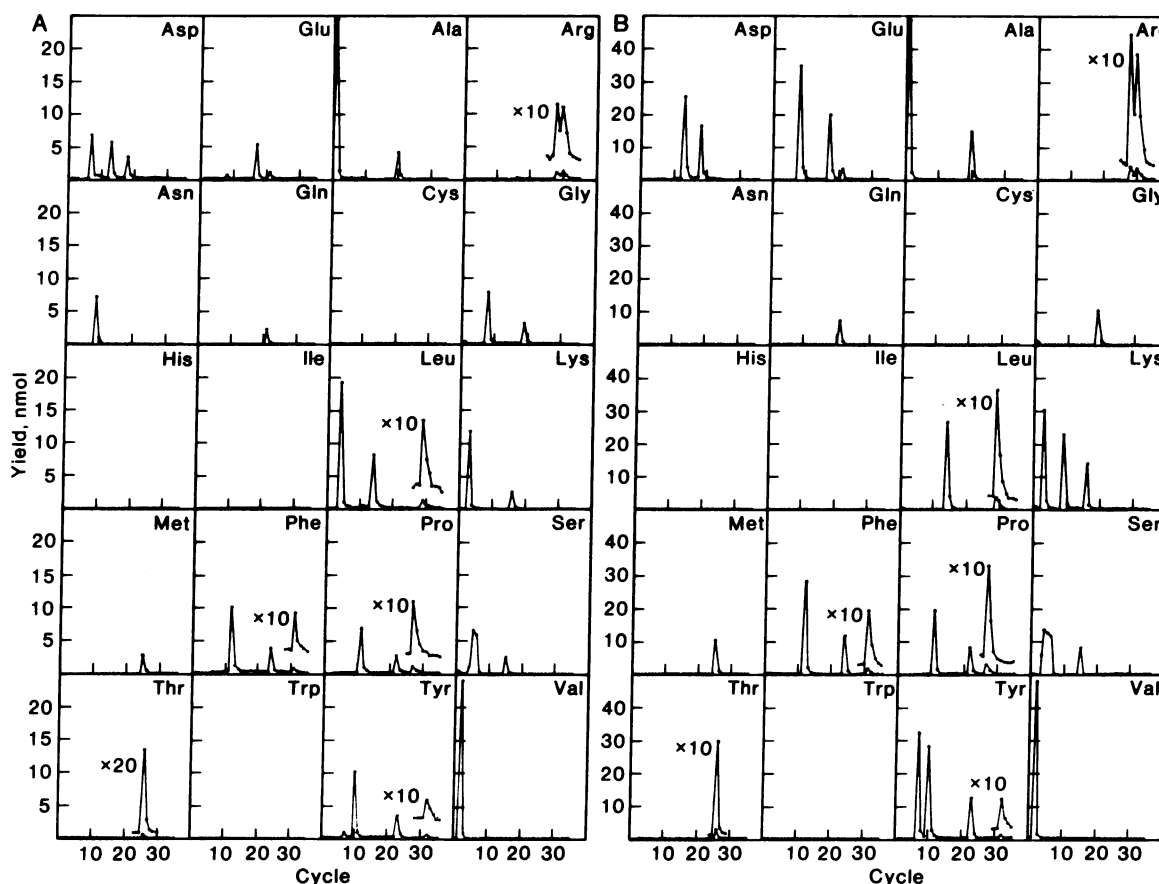


FIG. 2. Yields of phenylthiohydantoin-labeled amino acids from NH_2 -terminal sequenator analyses of peptides A (30 nmol) and B (60 nmol). Aliquots of each cycle were analyzed by HPLC; peak heights were converted to nanomoles for each derivative by using values for standard mixtures of phenylthiohydantoin-labeled amino acids, and the yields were normalized to a 100% injection.

umn was washed with the starting buffer until all unadsorbed protein was removed as determined at A_{280} . The excluded material (45 ml, 43.5 mg) showed egg-laying activity and was rechromatographed twice, resulting in peptide A, as described later. Elution of the material that was bound to the column with a linear gradient of NaCl resulted in one main peak that showed egg-laying activity (fractions 58–78, Fig. 1B, 21 ml, 2.9 mg). This fraction was rechromatographed on an SP-Sephadex column at pH 3.5. Peptide B emerged as a symmetrical peak (fractions 88–105, Fig. 1C, 1.8 mg).

SP-Sephadex C-25 Chromatography (pH 3.5 and 5.0): Peptide A. The excluded material from the initial cation exchange chromatography step at pH 6.5 (preceding paragraph) was adjusted to pH 3.5 and applied to an SP-Sephadex column at pH 3.5. Elution with a linear gradient of NaCl resulted in one

main peak (fractions 90–115, Fig. 1D, 25 ml, 12 mg protein) that showed egg-laying activity. This fraction was rechromatographed on an SP-Sephadex column at pH 5.0. Peptide A emerged as a symmetrical peak (fractions 90–110, Fig. 1E, 5.9 mg). Fractions 50–80 had no egg-laying activity.

Molecular Weight, Isoelectric Point, and Purity. When peptides A and B were applied to a 1.5×86 cm Bio-Gel A-0.5 m column equilibrated with 0.05 M sodium phosphate buffer, pH 7.5/6 M guanidine-HCl, each emerged as a single peak with an apparent M_r of 5500. The pI of peptide A as determined by isoelectric focusing in polyacrylamide gels was 7.9–8.1 and that of B was 9.0–9.2. Electrophoresis of A and B in acid/urea/polyacrylamide gels revealed a single band for each (Fig. 1A *Inset*). Peptide A had an R_F of 0.50 and peptide B had an R_F of 0.58. The sequenator analysis, however, revealed a small contamination (<5%) of peptide A by peptide B (Fig. 2A), although peptide B itself appeared pure by all criteria (Fig. 2B).

Composition and Sequence of Peptides A and B. The amino acid composition of peptides A and B is given in Table 1. The difference in composition between A and B explains the difference in pI, because peptide B with the extra lysine is more basic. Both A and B lack cysteine, histidine, and tryptophan. Comparison of the amino acid composition of A and B after acid hydrolysis with the data from an NH_2 -terminal sequenator analysis (Fig. 2) reveals that isoleucine, as well as one residue of proline, is missing in the sequence determination. We tried therefore to solve this discrepancy by determining the sequence near the COOH terminus with carboxypeptidase Y and also by trying to isolate the terminal peptide after tryptic digestion.

Table 2. Latencies and durations of bag cell afterdischarges induced by atrial gland peptides

	Delay, min	Duration, min
Crude atrial extract	7.3 ± 4.7 (7)	30.2 ± 13.8 (7)
Peptide A	14.1 ± 7.7 (6)	34.5 ± 14.9 (5)
Peptide B	17.9 ± 8.0 (4)	36.8 ± 10.7 (4)
Electrical stimulation	—	33.1 ± 20.6 (48)

Crude aqueous extract was added to the external medium of abdominal ganglia to a concentration corresponding to approximately 60 mg (wet weight) of atrial gland per ml. Peptides A and B were added at final concentrations of $6.8 \mu\text{g/ml}$. Electrical stimulation of the pleural end of the pleuroabdominal connectives was via a suction electrode (20 V, 2.5-msec pulses, six pulses per sec for 15 sec). The latency to onset of bag cell discharge and the duration of discharge are given (\pm SD) with the number of experiments in parentheses.

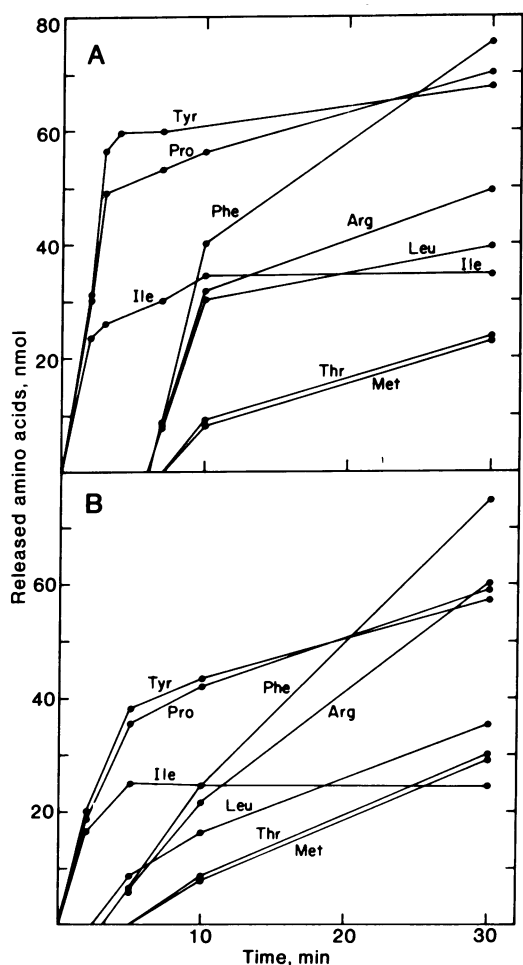


FIG. 3. Release of amino acids from the COOH terminus of peptide A (A) and B (B) by carboxypeptidase Y.

Carboxypeptidase Y Sequence Determination. The result of the release of amino acids from the COOH terminus of peptides A and B by carboxypeptidase Y is shown in Fig. 3. As can be seen, there is a fast release of tyrosine, proline, and isoleucine, although it is difficult to determine their exact release sequence. It is obvious, however, that the missing proline and isoleucine do indeed exist at the COOH terminus. The next group that is released is composed of phenylalanine, arginine,

and leucine. All this is in agreement with the sequence -Leu-Arg-Phe-Tyr-(Ile,Pro). Digestion of peptides A and B with carboxypeptidase A did not release any amino acids after incubation for 2 hr at 37°C, even after mild acid hydrolysis of the peptides in 0.03 M HCl (16).

Characterization of Tryptic Peptides. The profiles of the tryptic peptides of succinylated peptides A and B as separated by HPLC is shown in Fig. 4. Amino acid analysis of the various fractions revealed that one of the peptides in each run had the composition (Pro[1.0],Ile[1.0],Tyr[.90],Phe[1.1]) (Fig. 4). Microsequencing of this peptide from the tryptic digestion of peptide A as well as peptide B revealed the sequence: H-Phe-Tyr-Pro. Isoleucine did not show up in this sequence determination either, probably because it was washed out of the cup. Because we know, however, that the composition of this peptide is (Pro,Ile,Tyr,Phe), we can deduce that the sequence of this tryptic COOH-terminal peptide is Phe-Tyr-Pro-Ile. The sequence of peptides A and B is then as follows:

	5	10
Peptide A	H-Ala-Val-Lys-Leu-Ser-Ser-Asp-Gly-Asn-Tyr	
B	H-Ala-Val-Lys-Ser-Ser-Ser-Tyr-Glu-Lys-Tyr	
	15	20
A	Pro-Phe-Asp-Leu-Ser-Lys-Glu-Asp-Gly-Ala	
B	Pro-Phe-Asp-Leu-Ser-Lys-Glu-Asp-Gly-Ala	
	25	30
A	Gln-Pro-Tyr-Phe-Met-Thr-Pro-Arg-Leu-Arg	
B	Gln-Pro-Tyr-Phe-Met-Thr-Pro-Arg-Leu-Arg	
	34	
A	Phe-Tyr-Pro-Ile	
B	Phe-Tyr-Pro-Ile	

As can be seen, peptide A differs from peptide B in only four out of the first nine residues. All the substitutions, namely Leu → Ser, Asp → Tyr, Gly → Glu, and Asn → Lys, involve single base substitutions.

Effect of Atrial Gland Peptides on Bag Cells. We have found that the addition of crude aqueous extracts of the atrial gland or of the purified peptides A and B to the extracellular medium bathing the abdominal ganglion initiates a long-lasting discharge in the bag cells (Fig. 5). In addition, this effect could be obtained with asomatic bag cell neurites within isolated pleuroabdominal nerve preparations (5) and also when active material was applied to the attached head ganglia in an isolated compartment (17). Control extracts from other regions of the

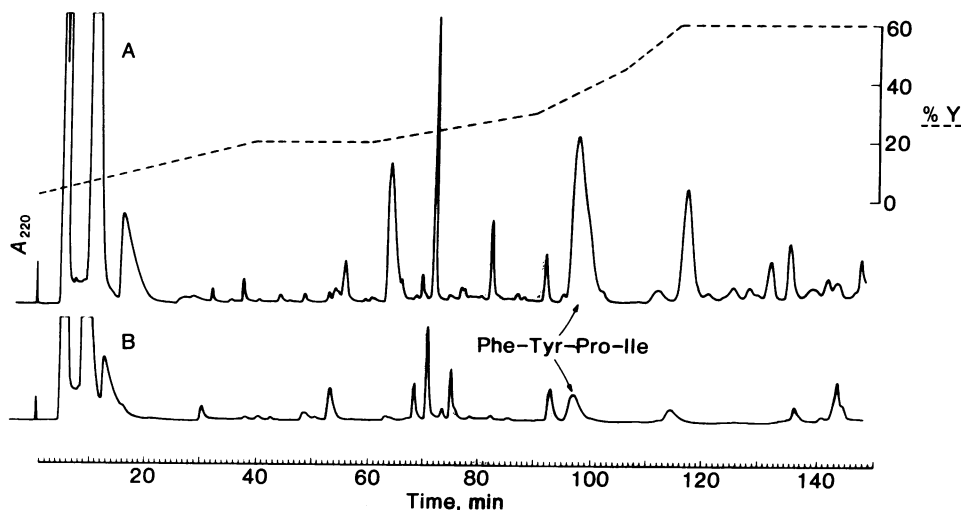


FIG. 4. Separation of tryptic digestion products of succinylated peptides A and B by HPLC. The column was developed with a gradient of 49 mM KH_2PO_4 /5.4 mM H_3PO_4 at pH 2.85 (X) to CH_3CN (Y) as indicated and monitored at 220 nm.

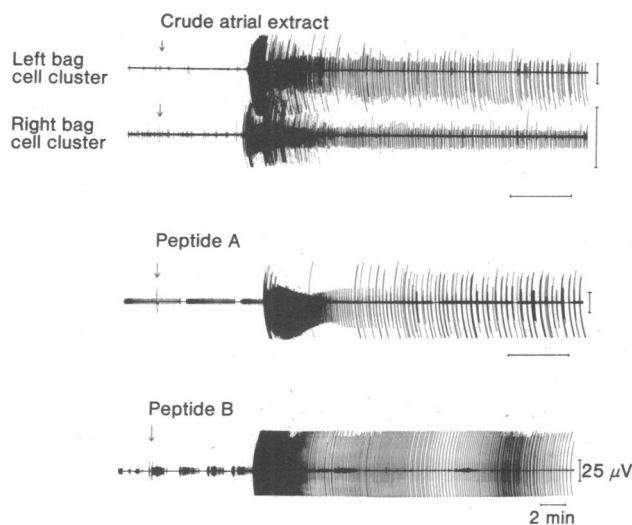


FIG. 5. The onset of discharge in the bag cells in abdominal ganglion preparations in response to (i) a crude aqueous extract of atrial gland (onset of discharge is shown in both left and right bag cell clusters, (ii) extracellular addition of purified peptide A (14 $\mu\text{g}/\text{ml}$), and (iii) that of peptide B (0.28 $\mu\text{g}/\text{ml}$). Only the response of the left bag cell clusters is shown for peptides A and B.

reproductive tract did not induce electrical activity in the bag cells. Concentrations as low as 1.39 $\mu\text{g}/\text{ml}$ (375 nM) for peptide A and 0.28 $\mu\text{g}/\text{ml}$ (75 nM) for peptide B proved capable of triggering bag cell discharges, although formal thresholds were not determined. No significant differences exist in the mean durations of discharges triggered by crude atrial extracts, by peptides A and B, or by electrical stimulation (Table 2).

DISCUSSION

This paper describes the purification and some properties of two neuroactive atrial gland peptides of *Aplysia* that are able to induce egg-laying behavior in recipient animals and afterdischarge in bag cells. Arch and his coworkers (9, 10) were the first to discover the existence of an egg-laying factor in atrial gland homogenates and to determine that the active substance could be located at two regions (pH 7.4 and 9.3) on isoelectric focusing gels. Peptides A and B have been purified by a combination of ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. They were judged pure on the basis of gel electrophoresis in the presence of urea/gel isoelectric focusing and amino acid composition, although microsequencing revealed a slight contamination of peptide A by peptide B. Peptide A was found to be a single-chain peptide with a calculated M_r of 3924 and a pI of 7.9–8.1 and peptide B was shown to be a single-chain peptide with a calculated M_r of 4032 and a pI of 9.0–9.2. The M_r and pI of peptide B closely resemble that of ELH (calculated M_r of 4385, pI 9.0–9.2) isolated from the bag cells of *Aplysia* (8), but their amino acid compositions (Table 1) and sequences (8) show no similarity. There is, however, a close resemblance between peptides A and B. In view of this similarity, it is likely that the peptides have similar biological activities, although we cannot be entirely certain that peptide A is as potent as peptide B because of the small amount of contamination by peptide B.

The failure of carboxypeptidase A to release any amino acid from the COOH terminus of peptides A and B in contrast to carboxypeptidase Y that has amidase activity (18) could indicate the possibility of an amidated isoleucine at the COOH terminus.

Degradation of peptides A and B with carboxypeptidase A after mild acid hydrolysis did not release any residues in contrast to what has been reported for substance P (16). It is known, however, that carboxypeptidase A fails to release proline or even a residue next to it (19). The pI of peptide A, calculated by the method of Edsall and Wyman (20) and assuming an unblocked COOH terminus, is in the range of 7.5–8.5 and agrees with the measured pI of 7.9–8.1. The calculated pI of peptide B, assuming an unblocked COOH terminus, is about 9.7, which also agrees with the measured pI of 9–9.2. The similarity between peptides A and B suggests that gene duplication and point mutation took place, as is suggested for many of the pituitary hormones (21) like oxytocin–vasopressin, growth hormone–prolactin, and others. It is not known at this stage, however, whether both peptides are actually released from the atrial gland or under what conditions. Another problem that remains unsolved at the moment is the presence and function of the high molecular weight material in the atrial gland that shows egg-laying activity (Fig. 1). It is conceivable that this material represents prohormones of peptides A and B. We tentatively suggest that copulation between *Aplysia* induces release of these atrial peptides and that these peptides act on neuronal receptors located in the head ganglia and the pleuroabdominal connectives to trigger bag cell afterdischarge (17). It remains to be determined whether these receptors occur on bag cell neurites, their input cells, or both.

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