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A conserved heptapeptide sequence in the waterborne attractin pheromone stimulates mate attraction in *Aplysia*

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Abstract

Mate attraction in the marine mollusk *Aplysia* involves long-distance waterborne chemical signaling via the release of the peptide pheromone attractin during egg laying. *Aplysia californica* attractin attracts conspecifics, reduces the latency to mating, and stimulates hermaphroditic mating. Four additional members of the *Aplysia* attractin family have recently been characterized from *Aplysia brasiliana*, *Aplysia fasciata*, *Aplysia depilans*, and *Aplysia vaccaria*. The five sequences differ significantly, but share six cysteine residues and the strictly conserved sequence Ile^{30}-Glu-Glu-Cys-Lys-Thr-Ser^{36}. Attractin is attractive to geographically and evolutionarily distant species, suggesting that the conserved heptapeptide region may be important for mate attraction. Consistent with this prediction, a synthetic constrained cyclic peptide that contains the conserved heptapeptide sequence is significantly attractive in T-maze bioassays. The attractins are the first family of waterborne peptide pheromones characterized in invertebrates and are unique in that family members are not species-specific pheromonal attractants.

Keywords: *Aplysia*; Attractin; Albumen gland; Pheromone

1. Introduction

Pheromones are thought to play an important role in coordinating reproductive behavior in many aquatic species, but few waterborne peptide pheromones in invertebrates have been rigorously characterized by both chemical and behavioral methods to date: attractin from the marine opisthobranch gastropod mollusk *Aplysia californica* [4,5,13,14,19] and nereithione from the marine polychaete worm *Nereis* [17,22]. *A. californica* is a simultaneous hermaphrodite that does not normally fertilize its own eggs. Field studies have shown that *A. californica* are solitary animals most of the year, but move into breeding aggregations during the reproductive season, where animals mate and lay eggs. The aggregations usually occur where egg cordons are laid, often deposited one on top of another. Egg cordons are attractive in T-maze assays, and attraction is due to chemical, and not visual, cues [12].

Following ovulation, eggs travel to the fertilization chamber which is surrounded by the albumen gland [3,15]. The albumen gland packages eggs into a long string-like cordon that has a high surface-to-volume ratio. Egg cordons are considered to be a source of both waterborne and contact pheromones that attract animals to the area and induce them to mate and/or lay eggs [2,12], but only albumen gland attractin has been identified to date.

Attractin, a 58-residue peptide with three intramolecular disulfide bonds [14,19], was isolated from *A. californica*, a slow-moving Pacific Coast species, and bioassayed in *Aplysia brasiliana*, a fast-swimming species found in the Gulf of Mexico; this was required since *A. californica* tend to crawl out of T-mazes before being exposed to chemical test stimuli. Attractin is attractive to *A. brasiliana*, reduces the latency to mating and stimulates hermaphroditic mating [13,14]. The amount of attractin that was attractive to conspecifics and induced potential mating behaviors (1–10 pmol in 6 l artificial seawater) was in the range of concentrations normally observed with pheromones, demonstrating that attractin has pheromonal activity [13,14,19]. These findings are consistent with field observations in which multiple *Aplysia* species are often associated with a single breeding aggregation, for example, *A. californica* and *A. vaccaria* from the Pacific Coast ([9,16], and S. LePage, Marine Research and Educational Products, Carlsbad, CA, personal communication), and *Aplysia fasciata* and *Aplysia depilans*

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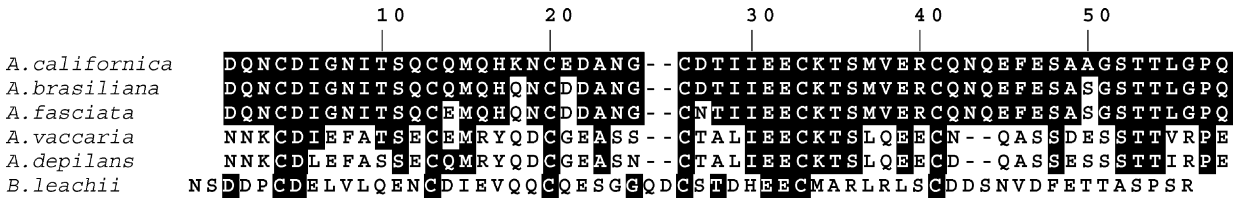

Fig. 1. Comparison of amino acid sequences of *Aplysia californica* (protein information resource accession no. A59061), *A. brasiliana* (accession no. B59060), *A. fasciata* (accession no. A59447), *A. vaccaria* (accession no. A59424), *A. depilans* (accession no. A59446), and *Bursatella leachii* attractin (accession no. 59453). Identities are shaded black.

from the Mediterranean Sea [1]. We hypothesized that each species secretes its own unique attractin-related attractant, but that the attractant may not be species-specific.

We recently sequenced the attractin-related peptides from four additional species of *Aplysia*: *A. brasiliana*, which are attracted to *A. californica* attractin; *A. fasciata*; *A. depilans*; and *Aplysia vaccaria* (Fig. 1; manuscript in preparation). A single, distinct attractin-related sequence was found in each species, and two groups were identified: [1] *A. californica*, *A. brasiliana*, and *A. fasciata* (91–95% sequence identity with *A. californica* attractin); and [2] *A. depilans* and *A. vaccaria* (39–43% identity). The five attractin sequences represent three subgenera and ∼15% of all *Aplysia* species (Table 1). The 3D nuclear magnetic resonance structure of *A. californica* attractin has recently been determined [5].

Aplysia californica attractin is attractive not only to other *Aplysia* species but also to the distantly related freshwater pulmonate gastropod *Lymnaea stagnalis* (A. ter Maat, unpublished observations). Although an attractin-related peptide has not yet been identified in *Lymnaea*, we recently characterized the attractin-related peptide from another member of the Aplysiid family, *Bursatella leachii* (subfamily: notarchinae; manuscript in preparation). The sequence differed significantly (Fig. 1), but shared all six cysteines and conserved Asp^{5}, Glu^{31}, and Glu^{32} residues (*Aplysia* residue numbers). Since the five *Aplysia* sequences share the strictly conserved sequence Ile^{30}-Glu-Glu-Cys-Lys-Thr-Ser^{36} and attractin is attractive to geographically and evolutionarily distant species, we hypothesized that the heptapeptide (IEECKTS) domain might be important for mate attraction. Consistent with this suggestion, a constrained cyclic peptide that contains the conserved heptapeptide sequence is significantly attractive in T-maze bioassays. In contrast to the species-specific waterborne peptide pheromonal attractants in amphibians [7,8,20,21], the attractins are, to our knowledge, the first peptide pheromone family in invertebrates and vertebrates that are not species specific.

Table 1
Subgenera and species that *Aplysia* attractin-related peptides have been characterized

Subgenus	Species
Pruvotaplysia	*parvula, punctata*
*Neoaplysia**	*californica**
*Varria**	*brasiliana**, *fasciata**, *cervina, cornigera, cronullae, dactylomela, denisoni, extraordinaria, gigantean, gracilis, inca, keraudreni, kurodai, maculata, morio, oculifera, pulmonica, rehderi, reticulata, robertsi, sagamiana, sowerbyi, syneyensis, willcoxi, winneba*
*Aplysia**	*depilans**, *vaccaria**, *cedrosensis, dura, juliana, nigra*
Phycophyla	*euchlora*

Subgenera and species that have been examined for attractin-related peptides are indicated by asterisks. Modified from Kandel [6].

2. Material and methods

2.1. Pheromonal attraction of Aplysia

Aplysia brasiliana was used as the experimental animal in T-maze experiments because it is more reproductively active than *A. californica* [14]. Previous T-maze assays [12–14] showed that *A. brasiliana* is attracted to a caged non-laying conspecific (stimulus animal) when attractin is placed in the artificial seawater (ASW; 6 l volume; 30–32 ppt) adjacent to a stimulus animal, and displays male mating behaviors. *A. brasiliana* were housed in individual cages in large aquaria containing ASW, and fed *Gracilaria* at 16:00–18:00 h; the light:dark cycle was 14 h:10 h. Egg-laying activity was monitored twice daily (08:00–09:00 h, 16:00–18:00 h) and egg cordons removed. All animals used in assays were sexually mature as defined by the ability to lay eggs spontaneously or following injection of atrial gland extracts [12] that contain egg-laying hormone-related peptides [10,11,18].

A schematic diagram of the T-maze has been published [13]; the ASW was stationary during experiments. A non-laying conspecific was placed in one of the perforated stimulus cages at each end of the two arms of the T-maze, and a potential attractant added to the adjacent fresh ASW; this is the stimulus animal. After 5 min, a non-laying animal (test animal) was placed in the base of the maze and observed for up to 20 min. The test animal exhibited one of three patterns of behavior. [1] It swam to the top of the maze while moving the head from side to side, and then moved into one arm. [2] It swam around in the maze, often visiting both cages before choosing a cage. [3] It remained at the base of the maze. The negative control assay was a

non-laying conspecific with nothing placed in the adjacent ASW. The potential attractant (1–1000 nmol synthetic peptide) was alternated between arms in consecutive assays. A response was considered to be: positive if the test animal traveled to the stimulus cage within 20 min and maintained contact for 5 min; negative if the test animal traveled to the cage in the opposite arm and maintained contact for 5 min; or no choice if the test animal did neither. Fifteen assays were performed for every dose tested. Statistical significance was assessed by chi-square analysis. In each case, test animals were choosing between a stimulus in one arm and no stimulus in another.

2.2. Peptide synthesis

Two peptides were synthesized by bioWORLD (Dublin, OH). The first peptide, D^{27}TIIEESKTSMVER^{40}, corresponded to residues 27–40 of *A. californica* attractin, with the exception that Cys^{33} was replaced by a serine residue in order to prevent the formation of dimers. The second, a constrained cyclic peptide, $\overline{C^{26}\text{DTIIEESKTSMVERC}^{41}}$, corresponded to residues 26–41 of *A. californica* attractin, with the exception that Cys^{33} was replaced by a serine residue; the two cysteine residues were joined by a disulfide bridge. The linear peptide was resuspended in 0.1% heptafluorobutyric acid (HFBA) prior to reverse phase (RP)-HPLC; the cyclic peptide was resuspended in 1.5 M guanidine, 0.125 M Tris–HCl (pH 8.2) prior to RP-HPLC. Each synthetic peptide was purified on a semi-preparative Vydac C18 RP-HPLC column (10 mm × 250 mm) using a linear gradient (0.1% HFBA for 5 min; 0–60% CH_3CN/0.1% HFBA in 200 min) at a flow rate of 1.75 ml/min, and fractions from multiple runs were pooled, aliquoted, and quantified by amino acid compositional analysis. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) verified the identity of each peptide and demonstrated that the measured masses of the HPLC-purified linear and cyclic peptides were 1637.9 (predicted: 1637.82) and 1841.8 (predicted: 1842.1), respectively. Amino acid compositional analysis indicated that the amino acid ratios of the HPLC-purified linear peptide were: Asp, 0.93 (1); Thr, 1.54 (2); Ser, 1.83 (2); Glu, 2.99 (3); Val, 1.33 (1); Ile, 1.24 (2); Lys, 0.92 (1); Arg, 0.97 (1); Gly, 0.56 (0); Ala, 0.15 (0); Leu, 0.16 (0). Amino acid compositional analysis indicated that the amino acid ratios of the HPLC-purified cyclic peptide were: Asp, 0.87 (1); Thr, 1.35 (2); Ser, 1.83 (2); Glu, 3.00 (3); Val, 1.72 (1); Cys, 0.39 (1); Met, 0.81 (1); Ile, 1.46 (2); Lys, 0.38 (1); Arg, 0.44 (1); Gly, 0.21 (0); Ala, 0.17 (0); His, 0.21 (0).

3. Results

In control assays, four animals (27%) entered the right arm and remained, two (13%) entered the left arm and remained, and nine (60%) did neither (Fig. 2A). Of the six animals making a choice, only three went to the stimulus cage, two of which were in the right arm and one of which was in the left arm of the maze. These control assays established chance levels of attraction at three animals.

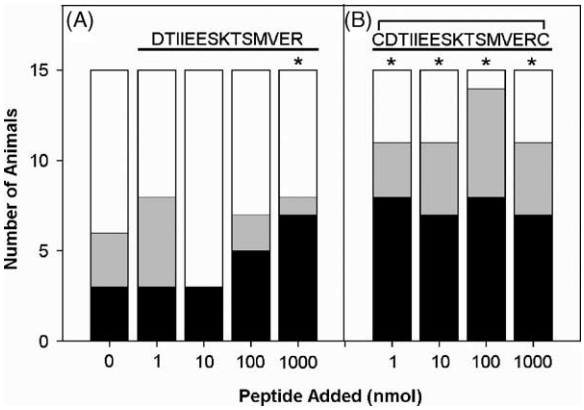

Fig. 2. Synthetic peptides that contain the conserved heptapeptide attractin sequence are attractive to *A. brasiliana*. (A) The number of *A. brasiliana* individuals attracted to a non-laying conspecific (0 nmol) was increased by placing either 100 or 1000 nmol of the linear synthetic peptide D^{27}TIIEESKTSMVER^{40} in the adjacent seawater; for both doses, fewer animals went to the opposite arm (negative) of the T-maze and fewer failed to make a choice (no choice) when peptide was added ($^*P < 0.05$). In each assay, animals chose between a stimulus in one arm and no stimulus in the other. Solid bars: positive responses. Shaded bars: negative responses. Open bars: no choice responses. (B) The number of *A. brasiliana* individuals attracted to a non-laying conspecific was significantly increased by placing either 1, 10, 100, or 1000 nmol of the synthetic cyclic peptide $\overline{C^{26}\text{DTIIEESKTSMVERC}^{41}}$ in the adjacent seawater; for all four doses tested, fewer animals failed to make a choice (no choice).

The response pattern changed when 100 or 1000 nmol of D^{27}TIIEESKTSMVER^{40} was placed in the ASW adjacent to the stimulus animal: 5 of 15 animals (33%; 100 nmol) and 7 of 15 animals (47%; 1000 nmol) were attracted to this synthetic peptide; in both cases, fewer animals went to the opposite arm and fewer failed to make a choice (Fig. 2A). The response pattern to 1000 nmol differed significantly from that to a non-laying conspecific alone ($\chi^2(2) = 7.11$; $0.025 < P < 0.05$).

Compared to the ASW control, the response pattern changed significantly when 1, 10, 100, or 1000 nmol of the constrained cyclic peptide $\overline{C^{26}\text{DTIIEESKTSMVERC}^{41}}$ was placed in the ASW adjacent to the stimulus animal: at the lowest dose tested (1 nmol), 8 of 15 animals (53%) were attracted to this cyclic peptide; fewer animals failed to make a choice (Fig. 2B). The response pattern to each of the four doses that were tested differed significantly from that to a non-laying conspecific alone (1 nmol: $\chi^2(2) = 11.11$; $0.001 < P < 0.005$; 10 nmol: $\chi^2(2) = 8.44$; $0.01 < P < 0.025$; 100 nmol: $\chi^2(2) = 18.44$; $P < 0.001$; 1000 nmol: $\chi^2(2) = 8.44$; $0.01 < P < 0.025$). Due to the limited seasonal availability (May to August) and limited lifespan

of *A. brasiliana*, assays testing lower doses of the cyclic peptide were not possible.

4. Discussion

In the five attractin sequences that have been characterized in *Aplysia* to date (Fig. 1), all share the strictly conserved sequence Ile[30]-Glu-Glu-Cys-Lys-Thr-Ser[36] (IEECKTS). When the Aplysiidae opisthobranch *B. leachii* attractin sequence is also considered (Fig. 1), all attractin-related peptides characterized to date have six conserved cysteines and conserved Asp-5, Glu-31, and Glu-32 residues (residue numbers). Our recent 3D nuclear magnetic resonance solution structure of A. californica attractin shows that all of these residues are solvent exposed [5].

Previous studies of *A. californica* attractin have demonstrated that: attractin is progressively degraded from the C-terminus but not from the N-terminus, and only the first 41–47 amino acids are required for attraction activity [14]; the cysteines are joined by disulfide bridges (Cys-4 to Cys-41, Cys-13 to Cys-33, Cys-20 to Cys-26; [19]); *N*-glycosylation at Asn-8 is not required for attraction [13,14,19]; the native structure of attractin is compact and relatively resistant to proteolysis [5,19]; circular dichroism and nuclear magnetic resonance spectra demonstrate that a substantial part of the 3D structure is helical [5,19]; and attractin affects behavior in all aquatic gastropod mollusks examined to date (manuscript in preparation).

We hypothesized that (I/L)IEECKTS constitutes a critical binding domain, and we assessed the importance of this domain by designing and synthesizing a linear (D[27]TIIEESKTSMVER[40]) and a cyclic peptide $\overline{\text{C}^{26}\text{DTIIEESKTSMVERC}^{41}}$. In the context of the tertiary structure, this domain is very likely to have a unique structure, and therefore a cyclic peptide better reflects the native structure by minimizing conformational entropy. In the (I/L)IEECKTS motif, we mutated the cysteine to serine to avoid disulfide-bonded dimers. Consistent with our hypothesis, the constrained cyclic peptide $\overline{\text{C}^{26}\text{DTIIEESKTSMVERC}^{41}}$ was significantly active in T-maze assays at all doses tested, while the linear peptide D[27]TIIEESKTSMVER[40] was only moderately active. The amount of synthetic peptide tested was higher than the amount of native and recombinant attractin that is attractive to conspecifics (1–10 pmol in 6 l ASW), therefore it is not possible to directly compare the potency of the cyclic peptide with full-length attractin. Nevertheless, the data demonstrate that the (I/L)IEECKTS domain is involved in pheromonal attraction and may account for the interspecific attraction activity of attractin. We hypothesize that the conserved solvent-exposed charged residues in the *Aplysia* attractin pheromone family of pheromones (Glu-31, Glu-32, and/or Lys-34) may be involved in receptor binding and pheromonal attraction, and may account for the interspecific attraction activity of attractin.

Attractins form a family of structurally homologous proteins, each of which is sequence-specific for a given *Aplysia* species. Nevertheless, we hypothesize that each attractin is recognized by, and attracts individuals from, other species due to structural similarities among different family members, specifically the (I/L)IEECKTS domain. The combined structural and behavioral observations suggest a potential mechanism to explain why attractin is not a species-specific pheromone. They open the door to studies of the *Aplysia* olfactory system, an important sensory modality that has not been studied in detail, and provide insights into studies to characterize the attractin receptor.

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