Review

Peptide and protein pheromones in amphibians

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Received 27 January 2001; received in revised form 29 March 2001; accepted 3 April 2001

Abstract

Purification, characterization and biological activity of urodele and anuran sex-pheromones were reviewed. Female-attracting pheromones obtained from the abdominal gland of Cynops pyrrhogaster and C. ensicauda males are peptides consisting of 10 amino acid residues being designated sodefrin and silefrin, respectively. Each pheromone attracted only conspecific females. Molecular cloning of cDNAs encoding sodefrin and silefrin revealed that both are generated from precursor proteins. Synthesis of these pheromones is regulated by prolactin (PRL) and androgen. Responsiveness of the female vomeronasal epithelium to sodefrin is enhanced by PRL and estrogen. The submandibular gland of the male terrestrial salamander, Plethodon jordani secretes a 22-kD proteinaceous pheromone that enhances female receptivity. It was revealed that every salamander synthesizes multiple isoforms of this pheromone, Plethodontid receptivity factor. The magnificent tree frog, Litoria splendida breed in an aquatic environment. The skin glands of the male secrete a female-attracting peptide pheromone, splendipherin, comprising 25 amino acid residues. The significance of the structure of the amphibian sex-pheromone as peptide and protein is discussed in terms of their species specificity.

Keywords: Sex-pheromone; Newt; Salamander; Frog; Courtship behavior; Mating behavior; Abdominal gland; Skin gland

1. Introduction

In urodeles, the most significant factor in sex recognition and courtship is chemical communication through the products (pheromones) of various glands that exhibit a marked development during the reproductive period. Effects of chemo-

This paper was submitted as part of the proceedings of the 20th Conference of European Comparative Endocrinologists, organized under the auspices of the European Society of Comparative Endocrinology, held in Faro, Portugal, 5–9 September 2000.

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abdominal gland of the sword-tailed newt, *C. ensicauda* (Yamamoto et al., 2000).

In anurans, on the other hand, acoustical communication has been thought to play an important role in sex recognition and mating (Holliday and Tejedo, 1995; Sullivan et al., 1995). However, the presence of anuran sex-pheromone was recently demonstrated for the first time in the magnificent tree frog, *Litoria splendida* (Wabnitz et al., 1999).

In this article, purification, characterization and biological activity of both urodele and anuran pheromones, molecular cloning of cDNA encoding *Cynops* pheromones and the involvement of endocrine factors in the secretion of and response to *Cynops* pheromones will be reviewed.

2. Female-attracting pheromones in two species of genus *Cynops*

2.1. Isolation and characterization of pheromones

Treatment of sexually undeveloped males or hypophysectomized and castrated males of *C. pyrrhogaster* with a combination of prolactin (PRL) and androgen elicits courtship behavior (Toyoda et al., 1993, 1996) and development of the abdominal gland (Kikuyama et al., 1975). The courting males attract their partners by sending the water around the cloaca toward the female’s snout by vibrating their tail vigorously. During courtship the males project numerous minute tubules that are connected to the abdominal gland from the cloaca. This suggests that the male newts emit a female-attracting pheromone from the cloaca and that the pheromone is secreted by or through the abdominal gland. In fact, it was demonstrated that the water in which sexually developed males had been kept attracts female newts, whereas the water in which abdominal gland-ablated male newts had been kept does not (Toyoda et al., 1994).

On the basis of this observation, an attempt was made to isolate and characterize the substance(s) possessing this female-attracting activity (Kikuyama et al., 1995). Female-attracting pheromone activity was monitored by a preference test (Toyoda et al., 1994). An aqueous extract of the abdominal glands of sexually developed red-bellied newts exhibited a considerable female-attracting activity. When the extract was placed in a sponge block in a container filled with 3000 ml of water, the minimum effective amount required to attract a sexually mature female was the equivalent of 0.1% of the abdominal gland content.

The active substance in the abdominal gland was revealed to be soluble in water but not in organic solvents. When an aqueous extract of the abdominal glands was subjected to gel-filtration column chromatography on Sephadex G100, the female-attracting activity emerged in a fraction with a relative molecular mass of less than 5000. When this fraction was incubated with pronase, its female-attracting activity was completely lost, indicating the active substance was a peptide. After two purification cycles of reverse-phase high-performance liquid chromatography (HPLC), the active peptide was isolated from the gel-filtration fraction. Yield of the final product was 0.6 μg/gland. The final product was revealed to be a decapeptide with the amino acid sequence SIP-SKDALLK. C-terminal analysis by carboxypeptidase-P digestion revealed that the C-terminal residue was a free Lys residue. The relative molecular mass spectrometry corresponded with that calculated from the amino acid sequence. The peptide showed no sequence homology with any known peptide and was designated sodefrin, which derived from the ancient Japanese word ‘sodefuri’, meaning ‘soliciting’ (Kikuyama et al., 1995).

Ten nanograms of native sodefrin absorbed by a sponge block was enough to attract the sexually developed female but not male newts in a container filled with 3000 ml of water (Toyoda et al., 1995; Toyoda et al., 1995). Synthetic sodefrin exhibited female-attracting activity similar to that of the native material, with a minimum effective concentration within the range of 0.1–1.0 pM.

Frozen sections of abdominal glands immunolabeled with a fluorescent antibody against sodefrin showed that the apical portion of the epithelial cells contained immunoreactive sodefrin. An immunoelectron microscopic study of the abdominal gland using sodefrin antiserum and goat anti-rabbit IgG labeled with gold particles as a second antibody showed the particles to be localized mainly within the secretory granules (Toyoda et al., 1995). This indicates that sodefrin is secreted by the epithelial cells of the abdominal gland.

If sodefrin acts as a courtship pheromone, it would be expected to exert a species-specific action and thus contribute to reproductive isolation. In order to verify this assumption, we chose a congeneric species of newt, *C. ensicauda*, to use as a model. *C. ensicauda* females were not attract-
ed to sodefrin, but they were attracted to a water extract of abdominal glands from males of their own species (Kikuyama et al., 1995). However, *C. pyrrhogaster* females responding to sodefrin were not sensitive to the water extract of the abdominal glands from *C. ensicauda* males. These results indicate that the female-attracting substances differ between these two species of the genus *Cynops*.

In order to ascertain whether the abdominal gland of *C. ensicauda* possesses substances that immunoreact with the sodefrin antiserum, an aqueous extract of the abdominal gland was subjected to a sodefrin radioimmunoassay (RIA). This extract showed no cross-reactivity in this RIA system, whereas a water extract from the abdominal gland of *C. pyrrhogaster* showed an inhibition curve parallel to the sodefrin standard (Yamamoto et al., 1996).

Subsequently, female-attracting pheromone in the abdominal gland of *C. ensicauda* was obtained by adopting purification steps similar to those used for obtaining sodefrin (Yamamoto et al., 2000). The yield of the final product was 0.55 µg/gland. This substance comprised 10 amino acid residues with the sequence of SILSKDAQLK, which was different from that of sodefrin by two amino acid residues with substitutions Leu for Pro and Gln for Leu at position 3 and 8, respectively. This peptide was designated silefrin, a combination of the first three N-terminal amino acids, SIL and efrin derived from sodefrin. Both native and synthetic silefrin exerted an equipotent activity in attracting conspecific females. As anticipated, silefrin was not effective in attracting *C. pyrrhogaster* females.

### 2.2. Molecular cloning of pheromone precursor proteins

To further our understanding of female-attracting peptide pheromones at a molecular level, sodefrin precursor cDNA was prepared and examined (Iwata et al., 1999). A sodefrin precursor cDNA isolated from a cDNA library constructed from *C. pyrrhogaster* abdominal gland mRNA was found to contain 1364 base pairs (bp) with an open reading frame of 567 bp and to encode a sodefrin precursor protein of 189 amino acids residues. The precursor protein included a predicted signal peptide and, in a region close to the C-terminus, the sodefrin molecule. Northern blot analysis of sodefrin mRNA in the abdominal gland revealed the size to be approximately 1.5 kb and sodefrin mRNA to be expressed exclusively in the abdominal gland. It was noted that the sodefrin sequence is not sandwiched by two pairs of dibasic amino acids. Accordingly, sodefrin seems to be generated in a way different from commonly observed processing of peptide hormone precursors. Synthetic sodefrin C-terminally extended with isoleucine, serine and alanine (C-terminal portion of sodefrin precursor consisting of 13 amino acid residues) does not attract the females, indicating that these three amino acid residues must be removed from the C-terminus of this molecule for acquisition of biological activity.

A cDNA clone encoding silefrin precursor protein was also isolated from a *C. ensicauda* abdominal gland cDNA library. Its nucleotide sequence showed 93% homology with *C. pyrrhogaster* cDNA. The deduced amino acid sequence showed 82% homology with the *C. pyrrhogaster* molecule. The differences in the structure and female-attracting properties of the pheromone in *C. pyrrhogaster* and *C. ensicauda* appear to be part of the mechanism which is responsible for reproductive isolation.

It is worth mentioning that a cDNA comparable to cDNAs encoding *Cynops* female-attracting pheromones was isolated from a *Triturus cristatus* abdominal gland cDNA library (Cardinali et al., 2000), although the bioactivity of the putative pheromone molecule has not been tested.

### 2.3. Hormonal effect on the pheromone secretion

The effects of PRL, androgen, and PRL plus androgen on the sodefrin content in hypophysectomized and castrated red-bellied newts was investigated using an RIA for sodefrin. A combination of PRL and androgen is known to elicit the structural development of various organs related to reproduction including the abdominal gland (Iwata et al., 2000a) as well as the reproductive behavior (Toyoda et al., 1993, 1996). Treatment of hypophysectomized and castrated male newts with androgen but not PRL significantly increased the sodefrin content of the abdominal glands. A combination of both hormones produced a synergistic effect resulting in a further increase in the sodefrin content (Yamamoto et al., 1996). This observation is consistent with the finding that the treatment of *C. pyrrhogaster* males with PRL plus androgen
enhances the release of female-attractant into the water (Toyoda et al., 1994). Further evidence that PRL and androgen stimulate sodefrin synthesis comes from Northern blot analysis using sodefrin precursor cDNA (Iwata et al., 1999) as a probe. Sodefrin mRNA levels were elevated to some extent by the treatment with either PRL or androgen and markedly by the administration of both PRL and androgen (Iwata et al., 2000a). Since androgen receptors reside in the nuclei of the epithelial cells of the abdominal gland (Matsumoto et al., 1996) and PRL receptor mRNA has been expressed in the gland (Kato et al., 1997), these two hormones are considered to be major factors for enhancement of the pheromone production.

Likewise, it was confirmed that silefrin precursor mRNA in the abdominal gland are increased by the treatment with a combination of PRL and androgen in the abdominal gland of C. ensicauda (Iwata et al., 2000b). Recently, we found that arginine vasotocin (AVT) caused a decrease in the content of sodefrin in the abdominal gland, suggesting that it induces the discharge of sodefrin. Administration of a V1 (vasopressor) receptor antagonist, but not that of a V2 (antidiuretic) receptor antagonist, suppressed the decrease in sodefrin content of abdominal glands in sexually developed intact males (Toyoda et al., 1997). Therefore, AVT is considered to induce the discharge of sodefrin through the V1 receptor. The existence of an actin-like protein in a structure around the ducts of the abdominal gland suggests that AVT acts on that contractile structure to induce the discharge of the sodefrin through the ducts (Kikuyama et al., 1999).

2.4. Olfactory response to a pheromone sodefrin

Sodefrin is considered to act through the olfactory organ of female newts since attraction to this substance was abolished by bilateral nostril plugging or nerve transection between the nasal cavity and the olfactory bulb (Toyoda et al., 1995).

The olfactory system of urodeles consists of two morpologically distinct epithelia, namely, the main olfactory epithelium and the vomeronasal epithelium (Eisthen, 1992). In C. pyrrhogaster, the main chamber of the nasal cavity is lined with the main olfactory epithelium consisting of sensory and non-sensory cells. The sensory epithelium consists of both ciliated and micravillar cells. Lateral to the main chamber of the nasal cavity there is a diverticulum, which is lined with vomeronasal epithelium. The sensory epithelium of this region contains only microvillar cells (Jones et al., 1994). The axons of olfactory receptor cells terminate in the main olfactory bulb at the rostral portion of the telencephalon, whereas the axons of the vomeronasal receptor cells project to the accessory olfactory bulb located dorsocaudally to the main olfactory bulb (Toyoda et al., 1999).

Electrophysiological studies revealed that sodefrin evoked a marked electro-olfactogram (EOG) response when applied to the central region of the vomeronasal epithelium of the female newt (Toyoda et al., 1999; Toyoda and Kikuyama, 2000). In sexually developed female newts, the threshold concentration of sodefrin required for the induction of the EOG response was 0.1 pM. This concentration is close to the minimum effective concentration (0.1–1.0 pM) required to attract female newts (Kikuyama et al., 1995). Interestingly, the vomeronasal epithelium of sexually undeveloped females scarcely responded to sodefrin. Treatment of sexual undeveloped females with PRL and gonadotropin restored responsiveness to the pheromone (Toyoda et al., 1999). Likewise, a combination of PRL and estrogen markedly enhanced EOG responses in the ovariectomized and hypophysectomized female newts. The EOG response to the pheromone was also moderately enhanced by treatment with either PRL or estrogen alone. However, a slight but significant elevation was observed in castrated and hypophysectomized males receiving PRL plus estrogen or estrogen alone. Thus, it was concluded that the main site of action of sodefrin resides in the vomeronasal epithelium and that sensitivity to sodefrin is under the control of PRL and estrogen. The existence of sexual dimorphism in olfactory responsiveness to the hormones and/or the pheromone was noted.

3. A pheromone enhancing female receptivity in a terrestrial salamander, Plethodon jardani

Male P. jardani develops a mental gland underneath the chin that produces courtship pheromone during reproductive period. During courtship performed on the land, the female walks forward with the male by straddling his tail and resting her chin on his tail base. The male turns back toward the female and brings his mental gland in contact with her nares. Then the male deposits the spermatophore and the female picks it up through her
It has been presumed that the male mental gland secretes substance(s) that enhance female’s receptivity and shorten the duration of the time spent for courtship.

Rollmann et al. (1999) isolated a courtship pheromone from the male’s mental gland extract using anion-exchange HPLC and gel-filtration chromatography. SDS-polyacrylamide gel electrophoresis and amino acid sequencing confirmed that four isoforms of 22 kD protein exist. They were termed plethodontid receptivity factor (PRF). These isoforms showed 90% homology in their N-terminal amino acid sequence. Molecular cloning of cDNAs encoding these isoforms revealed sequence homology to members of the interleukin-6 cytokine family.

4. A female-attracting pheromone in a tree frog, *Litoria splendida*

The skin glands of anurans have been known to secrete defence compounds including various types of bioactive peptides (Erspamer, 1993). In the magnificent tree frog, *Litoria splendida* sends out biologically active (antimicrobial) peptides from the parotoid and rostral glands. Warbnitz et al. (1999) monitored the peptide content of secretions from the parotoid and rostral gland of male and female frogs every month for 3 years by HPLC and found one component that exists only in male secretions. This peptide comprised 25 amino acid residues with the sequence of GLVSSIGKALGGL-LADVVKSKGQP A. Behavioral test revealed that it attracts female frogs kept in a tank containing water at the final concentration of 10 pM but not conspecific males or females of different species, *L.* caerulea. This peptide pheromone designated splendipherin increases in concentrations during the breeding season up to 1% of total peptide content of the glandular secretion, suggesting that its synthesis is under hormonal control.

Recently, Pearl et al. (2000) provided evidence that the breeding glands of male dwarf African clawed frogs (*Hymenochirus* sp.) release a female-attracting substance. Females subjected to a Y-maze test showed a positive chemotaxis to the water in which males had been kept and to the water containing the breeding gland homogenate but not to the water in which conspecific females or breeding gland-ablated males had been kept. Isolation and characterization of the active substance remains to be done.

5. Concluding remarks

Since the discovery of the amphibian sex-pheromone sodefrin, three other pheromones have been identified. Among them two are peptides which attract females in the water and the remaining one is a proteinaceous pheromone that acts on females on the land. However, it may be premature to conclude that in amphibians the peptide pheromone is for aquatic animals and the proteinaceous pheromone is for terrestrial ones.

Peptide and protein molecules are ideal as species-specific reproductive pheromones, since many variant forms can be generated by the modification of the nucleotide sequence of the pheromone gene.

Sodefrin was demonstrated electrophysiologically to act on the vomeronasal epithelium of the female. Recently, we have demonstrated that among the enzymatically dispersed vomeronasal epithelial cells, there are a few cells that respond to sodefrin to increase cytosolic calcium concentrations. The fact that the responsiveness to sodefrin is dependent on PRL and estrogen suggests that these hormones may have some influence upon the expression of sodefrin receptor molecules. The identification and characterization of pheromone receptors is definitely needed to shed further light on the mechanism of chemical communication during reproduction in amphibians.

Acknowledgments

Part of this work was supported by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and research grants from the Promotion and Mutual Aid Corporation for Private School of Japan, Asahi Glass Research Foundation and Uehara Memorial Research Foundation to S.K.

References


