Sodefrin: a novel sex pheromone in a newt

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The abdominal gland in the male red-bellied newt, Cynops pyrrhogaster, is the source of a female-attracting pheromone. An attempt was made to isolate and characterize the female-attracting pheromone in the abdominal glands of male newts. The active substance, named sodefrin (from the Japanese ‘sodefuri’ which means ‘soliciting’) has been isolated and shown to be a novel decapeptide with the sequence, Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys. Its minimum effective concentration in water is 0.1–1.0 pmol l⁻¹. Synthetic sodefrin shows a female-attracting activity similar to that of the native peptide, and acts through the olfactory organ of female newts. Electrophysiological studies reveal that sodefrin evokes a marked electro-olfactogram response in the vomeronasal epithelium in sexually mature females and in ovariectomized females treated with prolactin and oestrogen. The pheromonal activity of sodefrin appears to be species-specific since it does not attract females of a congeneric species, the sword-tailed newt C. ensicauda. However, C. ensicauda has a variant of sodefrin differing from that in C. pyrrhogaster by substitutions of Leu for Pro at position 3 and Gln for Leu at position 8. The C. ensicauda variant sodefrin does not attract C. pyrrhogaster females. Genes encoding the sodefrin precursor protein have been cloned in both C. pyrrhogaster and C. ensicauda. Immunostaining of the abdominal gland using the antiserum against sodefrin shows that sodefrin occurs in the epithelial cells, predominantly within the secretory granules. Sodefrin content, detected by immunoassay, in C. pyrrhogaster males decreases after castration and hypophysectomy and increases markedly in the castrated and hypophysectomized newts after treatment with androgen and prolactin. This combination of hormones also enhances sodefrin mRNA content in the abdominal gland as assessed by northern blot analysis using sodefrin cDNA.

In urodeles, chemical communication through the products of various glands is the most significant factor in sex recognition and courtship (Houck, 1986; Feldhoff et al., 1988; Thompson et al., 1988). However, very little work has been done to elucidate the biochemical nature of these substances.

We have identified a novel female-attracting peptide pheromone, sodefrin, in the abdominal glands of Cynops pyrrhogaster (red-bellied newt) males (Kikuyama et al., 1995). In this article, we review the isolation, molecular cloning, and localization of sodefrin, together with its hormonal control, secretion and mode of action. We demonstrate the presence of a variant type of sodefrin in a congeneric species, C. ensicauda (sword-tailed newt), which attracts female C. ensicauda but not C. pyrrhogaster.

Abdominal gland as a source of female-attracting pheromone

In male newts of the genus Cynops, the abdominal gland of the cloaca is the source of the chemical substances that attract females during the breeding season. During courtship, the male Cynops attracts his partner by directing the water around his cloaca toward the female’s snout by vibrating his tail. Then he moves in front of the female and she follows him with her snout in contact with his folded tail (Fig. 1). Numerous minute tubules connected with the abdominal gland project from the male cloacal orifice during courtship to facilitate the response of female-attracting pheromones.

Treatment of sexually inactive C. pyrrhogaster males with a combination of prolactin and androgen (or gonadotrophin) elicits courtship behaviour (Toyoda et al., 1993, 1996) and development of the abdominal gland (Kikuyama et al., 1975). The water in which these hormone-treated males have been kept attracts sexually mature female newts but the water in which saline-injected male newts have been kept does not. The female-attracting activity of the water in which males have been kept is reduced markedly when the abdominal glands of the males are surgically removed (Toyoda et al., 1994). Sexually inactive females do not respond to the male-conditioned water.

Isolation and characterization of female-attracting pheromone

An aqueous extract of the abdominal glands of sexually developed red-bellied newts exhibits a substantially greater female-attracting activity than extracts prepared from sexually undeveloped newts (Toyoda et al., 1994). When the extract is placed in a sponge block in a container filled with 3000 ml water, the minimum effective amount required to attract a sexually mature female is the equivalent of 0.1% of the abdominal gland. The active substance in the abdominal gland...
in a container filled with 3000 ml water (Kikuyama is enough to attract sexually mature female but not male newts (Kikuyama ancient Japanese word 'sodefuri', which means 'soliciting' any known peptide and was designated sodefrin from the Leu-Leu-Lys. This peptide shows no sequence homology with the amino acid sequence, Ser-Ile-Pro-Ser-Lys-Asp-Ala-

filtered column chromatography, the female-attracting activity emerges in a fraction with a relative molecular mass < 5000. When this fraction is subjected to pronase digestion, it loses its female-attracting activity, indicating that it is a peptide (Kikuyama et al., 1995, 1997).

After two purification cycles of reverse-phase high-performance liquid chromatography, the active peptide isolated from the gel filtration fraction yields a final product with the amino acid sequence, Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys. This peptide shows no sequence homology with any known peptide and was designated sodefrin from the ancient Japanese word 'sodefuri', which means 'soliciting' (Kikuyama et al., 1995).

Ten nanograms of native sodefrin absorbed by a sponge block is enough to attract sexually mature female but not male newts in a container filled with 3000 ml water (Kikuyama et al., 1995; Toyoda et al., 1995). Synthetic sodefrin exhibits female-attracting activity similar to that of the native material with a minimum effective concentration within the range 0.1–1.0 pmol l⁻¹.

**Olfactory response to sodefrin**

Sodefrin is detected by the female red-bellied newts through the olfactory system. If the nostrils of sexually active females are plugged or the nerves between the nasal cavity and the olfactory bulb are transected, the pheromonal activity of sodefrin is not observed (Toyoda et al., 1995, in press).

The olfactory system of urodeles consists of two morphologically 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 sensory and non-sensory epithelia. The sensory epithelium consists of both ciliated and microvillar cells. Lateral to the main chamber of the nasal cavity there is a diverticulum 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. Electrophysiological studies revealed that sodefrin evokes a marked electro-olfactogram (EOG) response when applied to the vomeronasal region (Toyoda et al., in press). In sexually developed female newts, the threshold concentration of sodefrin required for the induction of an EOG response is 10⁻¹³ mol l⁻¹. This concentration is close to the minimum effective concentration (10⁻¹³–10⁻¹² mol l⁻¹) required to attract female newts (Kikuyama et al., 1995).

The vomeronasal epithelium of sexually undeveloped females scarcely responds to sodefrin. Treatment of sexually undeveloped females with prolactin and gonadotrophin restores responsiveness to the pheromone (Toyoda et al., in press). Likewise, a combination of prolactin and oestrogen enhances EOG responses in the ovariectomized female newts.

**Molecular cloning of cDNA encoding sodefrin**

A sodefrin precursor isolated from a cDNA library constructed from *C. pyrrhogaster* abdominal gland mRNA was found to contain 1360 bps with an open reading frame of 567 bps, and to encode sodefrin precursor protein of 189 amino acids residues. The precursor protein includes 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 a size of about 1.5 kb and that sodefrin mRNA is expressed exclusively in the abdominal gland (Iwata et al., 1998). However, sodefrin seems to be generated in a way different from the commonly observed processing of peptide hormone precursors, since its sequence is not sandwiched by two pairs of diphasic amino acids.

A sodefrin precursor cDNA isolated from *C. ensicauda* has a nucleotide sequence showing 93% homology with *C. pyrrhogaster* cDNA. The deduced amino acid sequence shows 82% homology with the *C. pyrrhogaster* molecule. The sodefrin-like peptide from *C. ensicauda* has two substitutions (Leu for Pro at position 3 and Gln for Leu at position 8) compared with sodefrin. [Leu³, Gln⁸]sodefrin attracts *C. ensicauda* females but not *C. pyrrhogaster* females. Likewise, sodefrin attracts only *C. pyrrhogaster* females (Iwata et al., 1998). These differences in the structure and female-attracting properties of sodefrin in two species of newt appear to be part of the mechanism that is responsible for reproductive isolation.

**Localization of sodefrin**

Frozen sections of abdominal glands immunolabelled with an antiserum against sodefrin using a fluorescent marker show that the epithelial cells stain positively for sodefrin (Fig. 2). An immuno-electron microscopic study of the abdominal gland using sodefrin antiserum and goat anti-rabbit IgG labelled with gold particles as a second antibody showed that gold particles were localized mainly on the secretory granules (Fig. 3; Toyoda et al., 1995), indicating clearly that sodefrin is secreted by the epithelial cells of the abdominal gland.
Hormonal control of sodefrin secretion

A radioimmunoassay has been developed to measure sodefrin, and was used to investigate the effects of prolactin, androgen, and prolactin plus androgen on sodefrin content and concentration in hypophysectomized and castrated animals. Prolactin and androgen stimulate the development of the abdominal gland (Kikuyama et al., 1975). Androgen receptors occur in the nuclei of the epithelial cells of the abdominal glands in C. pyrrhogaster (Matsumoto et al., 1996) and prolactin receptors have been demonstrated in the abdominal gland of C. ensicauda (Kato et al., 1997). Treatment of hypophysectomized and castrated male newts with androgen but not prolactin significantly increases the sodefrin content of the abdominal glands. A combination of both hormones produces a synergistic effect resulting in a further increase in sodefrin content (Yamamoto et al., 1996). This observation is consistent with the finding that the treatment of male C. pyrrhogaster with prolactin plus androgen enhances the release of female attractant into the water (Toyoda et al., 1994). Further evidence that prolactin and androgen stimulate sodefrin synthesis comes from northern blot analyses using sodefrin precursor cDNA as a probe. Sodefrin mRNA concentrations are increased moderately by the treatment with either prolactin or androgen and markedly by the combined administration of prolactin and androgen (Kikuyama et al., 1997).

Conclusions

The existence of pheromones in the class Amphibia has long been known. Sodefrin identified in C. pyrrhogaster is the first amphibian pheromone to be identified, and is the first peptide pheromone identified in a vertebrate. Given the fact that, in most urodeles, reproduction takes place in an aquatic environment, a non-volatile but water-soluble peptide is an
appropriate sex pheromone. Analysis of cloned sodefrin cDNA reveals that it encodes a peptide molecule larger than the sodefrin molecule, indicating the existence of a pheromone precursor molecule. A congeneric species, C. etschata, has a sodefrin-like peptide with two amino acid substitutions compared with sodefrin. Both this peptide and sodefrin attract only conspecific females. This finding raises the possibility that amphibian species belonging to the same genus have species-specific female-attracting pheromones. Interspecies differences in the structure of sodefrin-like peptides may be significant in ensuring reproductive isolation. Peptide 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 (precursor) gene. The identification of the pheromones in the wide range of amphibian species is now needed to establish the relationship between reproductive isolation mechanisms and the molecular structure of reproductive pheromones. Sodefrin acts on the vomeronasal epithelium. Efforts should be directed to the identification and characterization of the sodefrin receptor to throw light on the mechanism of chemical communication during reproduction in urodèles.

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References

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