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Localization of pyrokinin-like immunoreactivity in the brain of the crayfish *Astacus leptodactylus* (Crustacea)

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ABSTRACT. Pyrokinins are members of the pyrokinin/PBAN family of neuropeptides, which have been found in many insect species, and recently also in the crustacean *Litopenaeus vannamei*. Members of this family regulate reproductive processes in insects, including pheromone biosynthesis. Pyrokinin-like immunoreactivity has been studied in insects, but not in crustaceans. In this study, a polyclonal antibody against pyrokinin 1 of *Litopenaeus vannamei* was used to demonstrate the presence of pyrokinin-like material in the brain of the crayfish *Astacus leptodactylus*. Immunoreactivity was found in cells of the tritocerebrum, indicating that pyrokinin-like peptides are present in crayfish.

KEY WORDS: Pyrokinin, immunocytochemistry, neuropeptide, Crustacea.

INTRODUCTION

Pyrokinins are members of the pyrokinin/PBAN pheromone biosynthesis activating neuropeptide family of neuropeptides, which have been found to be responsible for a variety of functions in invertebrates such as myotropic activity of the hindgut in cockroach (NACHMAN et al., 1986), locust (SCHOOFS et al., 1991) and crayfish (TORFS et al., 2001), and stimulation of sex pheromone biosynthesis (GAZIT et al., 1990). Members of this family are characterized by the C-terminal amino acid sequence FXPRLamide. Pyrokinins (PK) are characterized by this typical C-terminus and by the fact that they were isolated through their myotropic activity. To date, 17 members of the pyrokinin subfamily have been characterized (Table 1). They were first isolated from the cockroach *Leucophaea maderae* (HOLMAN et al., 1986). Subsequently, pyrokinins were identified in the locusts *Locusta migratoria* (SCHOOFS et al., 1990a; 1990b; 1991, 1992b; 1993) and *Schistocerca gregaria* (VEELAERT et al., 1997), and the American cockroach *Periplaneta americana* (PREDEL et al., 1997, PREDEL et al., 1999; PREDEL & ECKERT, 2000). Most recently, our group has isolated the first members of this family from non-insects. Pev-PK 1 and Pev-PK 2 were isolated from the crustacean *Litopenaeus vannamei* through their ability to induce *L. maderae* hindgut contraction (TORFS et al., 2001). Both pyrokinins appear to be active at physiological concentrations on both insect (*L. maderae*) and crustacean (*Astacus leptodactylus* (Eschscholz, 1823)) hindgut.

The distribution of FXPRLamide-like immunoreactivity in the central nervous system of insect species has been investigated thoroughly (SCHOOFS et al., 1992a; TIPS et al., 1993; BRÄUNIG et al., 1996; PREDEL & ECKERT, 2000). Immunostaining in the brain was revealed in all investigated insect species. In crayfish, the presence of pyrokinin-like factors has not yet been reported. This paper reports the study of the distribution of pyrokinin-like immunoreactivity in the brain of the crayfish *Astacus leptodactylus*.

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TABLE 1

Sequence comparison of pyrokinins. Amino acids that are conserved throughout the pyrokinin subfamily are in boldface. pQ indicates a pyroglutamic acid residue.

Classis	Species	Peptide name	Sequence	Reference
Insecta	*L. maderae*	Lem-PK	pQTS**FT**P**RL**-**NH$_2$**	HOLMAN et al., 1986
	L. migratoria	Lom-PK I	pQDSGDEWPQQP**F**V**PRL**-**NH$_2$**	SCHOOFS et al., 1991
		Lom-PK II	pQSVPT**FT**P**RL**-**NH$_2$**	SCHOOFS et al., 1993
		Lom-MT I	GAVPAAQW**FS**P**RL**-**NH$_2$**	SCHOOFS et al., 1990a
		Lom-MT II	EGD**FT**P**RL**-**NH$_2$**	SCHOOFS et al., 1990b
		Lom-MT III	RQQP**F**V**PRL**-**NH$_2$**	SCHOOFS et al., 1992b
		Lom-MT IV	RLHQNGMP**FS**P**RL**-**NH$_2$**	SCHOOFS et al., 1992b
	S. gregaria	Scg-MT I	GAAPAAQ**FS**P**RL**-**NH$_2$**	VEELAERT et al., 1997
		Scg-MT II	TSSLFPH**PRL**-**NH$_2$**	VEELAERT et al., 1997
	P. americana	Pea-PK-1	HTAG**FI**P**RL**-**NH$_2$**	PREDEL et al., 1997
		Pea-PK-2	SPP**FA**P**RL**-**NH$_2$**	PREDEL et al., 1997
		Pea-PK-3	LVP**FR**P**RL**-**NH$_2$**	PREDEL et al., 1999
		Pea-PK-4	DHLPHVYS**PRL**-**NH$_2$**	PREDEL et al., 1999
		Pea-PK-5	GGGGSGETSGMW**FG**P**RL**-**NH$_2$**	PREDEL et al., 1999
		Pea-PK-6	SESEVPGMW**FG**P**RL**-**NH$_2$**	PREDEL & ECKERT, 2000
Crustacea	*L. vannamei*	Pev-PK 1	DFA**FS**P**RL**-**NH$_2$**	TORFS et al., 2001
		Pev-PK 2	ADFA**FN**P**RL**-**NH$_2$**	TORFS et al., 2001

MATERIAL AND METHODS

Animals

Mature specimens of the crayfish *Astacus leptodactylus* were purchased from a local seafood dealer (Colette, Belgium). They were maintained in freshwater tanks where the water was recirculating constantly through sand filtration units. The crayfish were kept at room temperature. Animals were anaesthetized by packing in ice for 30 min.

Production of antiserum

Synthetic Pev-PK 1 was coupled through the free carboxyl group of the N-terminal aspartate residue to bovine thyroglobulin using EDC (1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride). In this way, antisera against thyroglobulin bound DFAFSPRL-NH_2 would be mainly directed against the C-terminal portion of the peptide molecule. After overnight incubation, the water-soluble isourea, which is released as a by-product of the conjugation reaction, and the excess reagent were separated from the hapten-carrier complex by dialysis. The complex was dissolved in distilled water and emulsified with an equal amount of Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. Second, third and fourth boosts were given, using Freund's incomplete adjuvant, respectively two, four and six weeks after initial immunisation. The antiserum was characterized using immuno-dot-blot according to SALZET et al., 1997.

Immunocytochemistry

The brain was dissected in Bouin-Hollande's (10%) sublimate fixative. After 18 to 24 hr of fixation, the brains were rinsed in distilled water (12hr), dehydrated in an ethanol series (70, 95 and 100% for two times two hrs), cleared in Histosol plus and embedded in Paraplast. Alternating sections of 4 µm were made with an LKB Historange microtome using glass knives. The sections were processed using the peroxidase-antiperoxidase method (VANDESANDE & DIERICKX, 1976) with 3,3' diamino-benzidine as the peroxidase substrate. Method specificity was controlled by application of the preimmune rabbit antiserum taken from the same animal that produced the primary antiserum. Serum specificity was determined by absorption of the antiserum with the antigenic determinant it was directed against, i.e. Pev-PK 1.

RESULTS AND DISCUSSION

Characterization by immuno-dot-blot revealed that the produced antiserum, used in a dilution of 1/1000, recognized synthetic Pev-PK 1 (to which it was raised) and synthetic Pev-PK 2. Seven to eight tritocerebral cell bodies were labelled with anti-Pev-PK 1 (Fig. 1, top). No staining was observed when the anti-Pev-PK 1 serum previously inactivated with Pev-PK 1 was used, nor with control serum of the preimmunized rabbit (Fig. 1, bottom). Comparable results were obtained in the brain of several insect species. In *L. migratoria*, *P. americana* and *L. maderae* cell bodies were visualized in the tritocerebrum (TIPS et al., 1993). Our results confirm that

pyrokinin-related peptides have a wider distribution in Arthropoda than has been thought.

Of the various known functions of FXPRLamide-containing peptides, only the myotropic one has been studied in crustaceans up to now (Torfs et al., 2001). Further research has to be undertaken to assess the physiological significance of these crustacean pyrokinins. In crustaceans, urine-born pheromones play an important role in sexual behaviour. Although previous studies suggest that sex pheromones produced by females are important in courtship (Atema & Engstrom, 1971), no female sex pheromones have been chemically identified to date. In contrast with insects, little is known about the endocrinological processes underlying this phenomenon in crustaceans. The fact that pyrokinin-like immunoreactivity is found in the brain of *A. leptodactylus* could give great impetus towards sex pheromone research in crayfish and even astaciculture. The latter is of particular interest lately in many European countries, being promoted for its promising commercial prospects (Pérez et al., 1997).

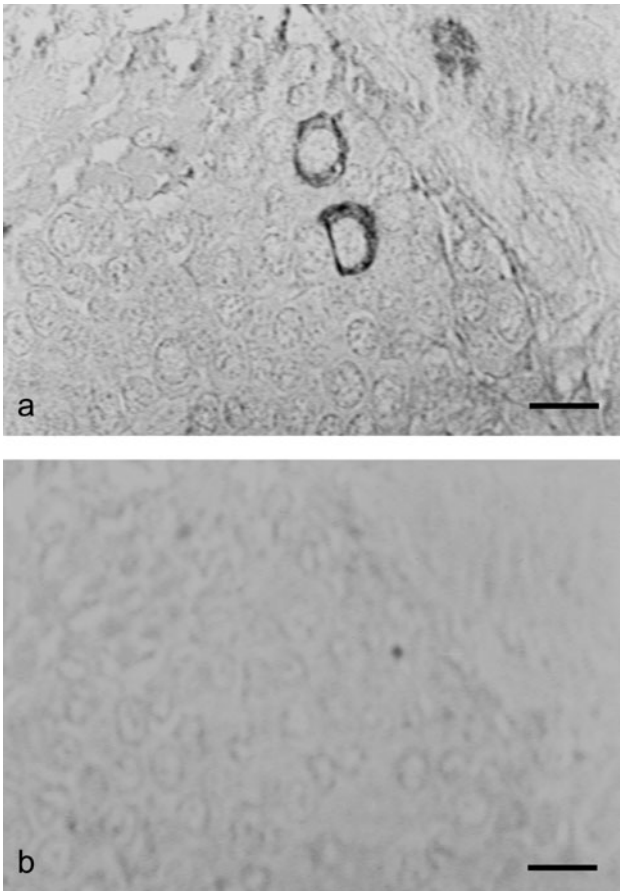

Fig. 1. – Pyrokinin-like immunoreactivity in the brain of *Astacus leptodactylus*. Top: Two immunostained cells in the tritocerebrum. Bottom: Control, alternate section showing no immunoreactivity with the serum of the preimmunized rabbit. Scale bar is 30 μm.

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