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The presence and distribution of gonadotropin-releasing hormone-liked factor in the central nervous system of the black tiger shrimp, *Penaeus monodon*

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Abstract

The distribution and presence of gonadotropin-releasing hormone (GnRH) in the central nervous system (CNS) of *Penaeus monodon* were examined by immunocytochemistry, high performance liquid chromatography (HPLC), and radioimmunoassay (RIA). We demonstrated the existence of octopus (oct)GnRH-liked immunoreactivity (ir-octGnRH) and lamprey (l)GnRH-III-liked immunoreactivity (ir-lGnRH-III) in cell bodies of medium-sized neurons of the anterior part (protocerebrum) of the supraesophageal ganglion (brain). In addition, only the ir-octGnRH was detected in the nerve fibers located in the brain and segmental ganglia (subesophageal, thoracic, and abdominal ganglia). Moreover, some branches of these fibers also innervated the neurons in the middle (deutrocerebrum), posterior (tritocerebrum) brain and segmental ganglia. There was no ir-lGnRH-I and ir-salmon (s)GnRH detected in the shrimp CNS. The results from HPLC and RIA showed ir-GnRH in the CNS using anti-lGnRH-III, but not with anti-mammalian (m)GnRH. The data from immunocytochemistry, HPLC and RIA suggest that ir-GnRH in shrimp may be more similar to octGnRH and lGnRH-III than the other forms. These findings support the hypothesis that GnRH-liked factor(s) may be an ancient peptide that also exists in this decapod crustacean.

Keywords: GnRH; Immunocytochemistry; Nervous system; Crustacean; *Penaeus monodon*

1. Introduction

GnRH is a well-known decapeptide that is a major mediator in the brain–pituitary–gonadal axis in vertebrates (Fernald and White, 1999; Morgan and Millar, 2004; Millar, 2005). Fourteen isoforms of GnRH have been reported in vertebrates and have been classified into three groups: GnRH1, 2, and 3 (Fernald and White, 1999; Tsai, 2006). Silver et al. (2004) proposed that there is the fourth group of vertebrate GnRH based on phylogenetic analysis, function, neuronal distribution, and developmental origin. In tetrapods, GnRH1 is synthesized in neurons of forebrain, transported to median eminence, and finally released into hypothalamo-hypophyseal portal circulation to stimulate the release of gonadotropins, i.e., follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. GnRH2 is localized in the midbrain, and is speculated to serve as neurotransmitter/neuromodulator (Tsai, 2006). GnRH3 which is present in the telencephalon and the terminal nerves could be involved in sex-associated behaviors (Fernald and White, 1999). The lamprey GnRHs (lGnRH-I and -III) form the fourth group, GnRH4, which are both hypothalamic neurohormone and are derived from diencephalon/ventricular origin (Silver et al., 2004). In addition, GnRH is also detected in extra-pituitary tissues such as gonad, liver, kidney, placenta, breast, and prostate gland (González-Martínez et al., 2004).

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Recently, many studies have reported the presence of GnRH in several invertebrates, which implies that this peptide could be conserved structurally as well as functionally throughout animal phyla (Young et al., 1999; Anctil, 2000; Tsai et al., 2003; Gorbman and Sower, 2003). Ten GnRH isoforms have been isolated and their primary structures determined in invertebrates, i.e., nine in tunicates and one in octopus (Powell et al., 1996; Iwakoshi et al., 2002; Adams et al., 2003; Kavanaugh et al., 2005). GnRH-like immunoreactivities have also been reported in an ascidian (Tsutsui et al., 1998), mollusks (Goldberg et al., 1993; Di Cosmo and Di Cristo, 1998; Young et al., 1999; Zhang et al., 2000; Di Cristo et al., 2002; Iwakoshi et al., 2002; Tsai et al., 2003; Iwakoshi-Ukena et al., 2004), a cnidarian (Anctil, 2000), a platyhelminthes (Anctil and Tekaya, 2005), and a coral (Twan et al., 2006). Although the structures have not yet been identified, the existence of the ir-GnRH in a variety of invertebrates suggests the ancestral GnRH has predated the emergence of vertebrate, and that GnRH is an ancient peptide that exists throughout vertebrate and invertebrate phyla (Rastogi et al., 2002; Gorbman and Sower, 2003; Tsai, 2006). GnRH has been reported to be functionally conserved as a reproduction-regulating factor in a few invertebrate species (Fang et al., 1991; Young et al., 1999; Di Fiore et al., 2000; Zhang et al., 2000; Adams et al., 2003; Gorbman et al., 2003; Iwakoshi-Ukena et al., 2004; Twan et al., 2006). In this report, we demonstrated ir-GnRH present in the CNS of the black tiger shrimp, *Penaeus monodon*, which may suggest the involvement of GnRH in the shrimp reproduction.

2. Materials and methods

2.1. Animals

Sexually mature female shrimp, with average weight of 180–250 g were caught from the Gulf of Thailand, and maintained at the Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand. The shrimp were maintained in a cement tank filled with seawater, at a temperature of about 25–28 °C, salinity at 30 ppt, with continuous aeration. They were fed with minced squid and kept under a normal day/light cycle (12/12 h). Approximately 70% of the seawater was replaced once a day. The shrimp were acclimatized for at least 7 days before being sacrificed. For chromatographic and radioimmunological studies, 30 whole CNS (supraesophageal and segmental ganglia) were dissected, and immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Antibodies and GnRH peptides

For immunocytochemitry, four available antibodies against GnRHs were used. The first was antibody against octGnRH (anti-octGnRH, Lot 9779, generously provided by Dr. Pei-San Tsai). This antibody was selected since octopus is considered to be more related to shrimp as both are protostomes. The other three antibodies were generated from one basal vertebrate (lamprey) and one basal teleost (salmon) which included anti-sGnRH, Lot 1667 (a kind gift of Dr. Judy King), anti-lGnRH-I, Lot 1467, and anti-lGnRH-III, Lot 3952 (produced in the laboratory of Dr. Stacia A. Sower, Sower et al., 1993). The lamprey as a basal vertebrate may have retained ancentral characteristics found in invertebrate GnRHs. The sGnRH is considered as GnRH3, whereas the lGnRHs are classified as GnRH4. Antibody against mGnRH (GnRH1, Lot R1245) was also used for determining the existence of a mGnRH-liked peptide in the RIA. The RIA using anti-mGnRH showed no immunoreactivity (see Section 3), thus this antibody was excluded from further use in immunocytochemistry. For peptides used in preabsorption study, octGnRH peptide (a gift from Dr. Hiroyuki Minakata and Dr. Pei-San Tsai), lGnRH-I, and lGnRH-III peptides (from the laboratory of Dr. Stacia A. Sower, purchased from American Peptide at 95% purity) were used.

2.3. Immunocytochemistry

The eyestalk, supraesophageal ganglion, and segmental ganglia were removed from non-gravid female shrimps. They were fixed in Bouin's fixative overnight, dehydrated, embedded in paraffin blocks, and then sectioned at 7 μm thick. The sections were deparaffinized with xylene, rehydrated through a graded series of ethanol (100–70%). The sections were immersed in 1% H_2O_2 in 70% ethanol for 15 min to eliminate endogenous peroxidase, then covered with 0.1% glycine and 4% BSA in 0.1 M phosphate buffer saline (PBS), pH 7.4, for 15 min for blocking free-aldehyde and non-specific binding, respectively. The sections were then incubated with the primary antisera including anti-octGnRH (1:500), anti-sGnRH (1:500), anti-lGnRH-I (1:1000), and anti-lGnRH-III (1:4000), at 4 °C for overnight. Negative controls were performed by incubating the sections in PBS or in primary antisera which were preabsorbed with GnRH peptides (50 μg/0.1 ml antisera at working dilution). Thereafter, sections were rinsed in several baths of PBS containing 0.1% Tween-20, and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) diluted at 1:1500 for 1 h. The presence of ir-GnRH in the tissues was enhanced and visualized by DAB enhanced liquid substrate system (Sigma). The sections were then dehydrated, cleared, mounted, and observed under a light microscope. The experiments were repeated for at least three times for each tissue. In addition, some sections were processed by conventional method, and then stained with hematoxylin (H) and eosin (E) dyes for histological characterization of the tissues being studied.

In order to compare the staining intensity of ir-GnRH in experimental and controlled sections which were probed with preabsorbed anti-GnRHs, densitometric analysis of stained neurons was performed using ImageJ software. A box of 50×50 pixels was generated and placed over the positively-stained areas. On the same section, the areas which showed negative staining were also measured and considered as background staining. These background values were then used to subtract from the values measured from the positively-stained areas. At least 10 areas from both positive and negative immunoreactive areas were randomly measured in one section, and three sections from each experiment were analyzed.

2.4. GnRH peptide extraction and HPLC analysis

The shrimp CNS were extracted according to the methods described by Fahien and Sower (1990) at the Faculty of Science, Mahidol University, Thailand. Briefly, the CNS parts were weighed, and then homogenized at 4 °C with a polytron in 2.0 M ice-cold acetic acid. The homogenate was centrifuged at 10,000*g* for 45 min. The supernatant was subsequently dried in Speed Vac Concentrator, and then kept at −80 °C. Further processing of the extracts was performed at the Department of Biochemistry and Molecular Biology, University of New Hampshire, USA. The extract was resuspended with Milli-Q water, and purified by a Sep-Pak C18 cartridge column pretreated with 100% methanol. The peptide was eluted from the column with 70% acetronitrile. The elute was then dried on Speed Vac Concentrator followed by resuspension in Milli-Q water. Insoluble material was filtered using an ARCO LC 13 (0.45 μm). The filtrate was subsequently injected into a 20 μl the injection loop of a Perkin–Elmer HPLC system filled with a Percosphere 3CR C18 (0.46×8.3 cm) reverse-phase column. The isocratic mobile phase consisted of 7.4 g ammonium acetate and 3.04 g citric acid in 1000 ml of 19% acetronitrile with flow rate of 2 ml/min. The fractions were collected every 18 s.