On the Mode of Action of Methionine Enkephalin, FK 33–824 and Naloxone in Regulating the Hemolymph Glucose Level in the Fresh Water Field Crab *Oziotelphusa senex senex*

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The possible involvement of opioid system in the regulation of hemolymph glucose level in the fresh water crab *Oziotelphusa senex senex* Fabricius, was investigated. Opioid agonist and antagonist was also used in addition to methionine-enkephalin itself. Injection of the opioid, methionine-enkephalin and FK 33–824 significantly elevated hemolymph glucose level. In contrast, injection of naloxone in to crab resulted in decrease in hemolymph glucose level. Injection of naloxone prior to injection of methionine-enkephalin blocked the hyperglycemic action of methionine-enkephalin. Injection of methionine-enkephalin, FK 33824 and naloxone produced no significant effect on hemolymph glucose level in eyestalk-less crab. The alterations in the intact crab hemolymph glucose level hypothesised to be due to stimulation of release of hyperglycemic hormone during methionine-enkephalin and FK 33824 treatment and blocking of release of hyperglycemic hormone during naloxone treatment from the eyestalks of crab *Oziotelphusa senex senex*.

Introduction

In decapod crustaceans, the major neuroendocrine complex of the eyestalk, the medulla terminalis X-organ-sinus gland complex, is the source of a number of neuropeptides, which are of great importance for the regulation of a variety of physiological functions (see reviews Fingerman, 1997; Reddy and Ramamurthi, 1999). Among them is a neuropeptide with a potent hyperglycemic action called hyperglycemic hormone. This hormone has been isolated from the fresh water crab, *Oziotelphusa senex senex* (Reddy and Ramamurthi, 1982a). We have reported earlier the cyclic fluctuation of release of hyperglycemic hormone in the crab, *Oziotelphusa senex senex* (Reddy *et al.,* 1991a). The interspecific action of crustacean hyperglycemic hormone has also been demonstrated (Reddy, 1991b; 1992; Reddy and Ramamurthi, 1980; 1981; 1982b; Reddy *et al.*, 1982a). We have also observed that the exposure to pesticides like sumithion and hexachlorocyclohexane results in hyperglycemia in the crab *Oziotelphusa senex senex*, apparently triggering the release of hyperglycemic hormone from the eyestalks (Reddy *et al.*, 1982b).

Mancillas *et al.* (1981) first reported the presence of an opioid in crustaceans. Since then, presence of opioid peptides were demonstrated in several crustaceans (see review Nagabhushanam *et al.,* 1995), but the physiological significance of opioid peptides in crustaceans is not thoroughly established. Nagabhushanam *et al.* (1995) also summarised the possible functional roles of opioid peptides in crustaceans. Recently a neurotransmitter role for methionine-enkephalin in regulating hemolymph sugar level in the crab, *Oziotelphusa* was reported (Reddy, 1999). In view of the fact that opioid peptides act as neurotransmitters (Rothe *et al.*, 1991; Sarojini *et al.,* 1995), it is conceivable that these peptides could help in the secretion of hyperglycemic hormone from the neurosecretory cells that synthesize it. The study described below was designed to investigate further the action of opioid agonist (FK 33824) and antagonist (naloxone) on the regulation of hemolymph glucose level of crab, *Oziotelphusa senex senex*.

Materials and Methods

Adult, intact fresh water crabs *Oziotelphusa senex senex* (Fabricius), were obtained from paddy

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fields in and around Tirupati. Male crabs in inter molt stage C_4 (Reddy, 1990) and body weight 30–32 g, were maintained in glass aquaria containing aerated water at 27 °C with 12 hours of light daily. The water was changed daily and the crabs were fed on sheep meat *ad libitum* on alternate days.

Crabs were selected from the stock supply and divided into 11 groups of 9 each. The first group, which served as the normals did not receive any treatment. From this group, 100 µl of hemolymph was collected through the arthrodial membrane of the coxa of the 3rd pair of walking legs. Group 2 was the control, which received only 10 µl of physiological saline. The crabs in groups 3, 4 and 5 received 10^{-8} mol/crab of methionine-enkephalin, FK 33–824 and naloxone respectively in 10 µl volume. The crabs of group 6 received 10 µl of naloxone (10^{-8} mol/crab) followed by 10 µl of methionine-enkephalin (10^{-8} mol/crab) after 30 min. Methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) ($C_{27}H_{35}N_5O_7S$), Naloxone hydrochloride ($C_{19}H_{21}NO_4HCl$), FK 33–824 (D-ala2,MePhe4, Met(O)5-ol) ($C_{29}H_{41}N_5O_7S$) were obtained from Sigma.

Eyestalks were removed from the crabs in groups 7 to 11. Eyestalks were removed by cutting off the organs at the base, without prior ligation but with cautery of the wound after operation. After 24 h of bilateral eyestalk ablation, the crabs in group 7 was used for hemolymph collection without treatment. Group 8 to 10 received in 10 µl volume, 10^{-8} mol/crab of methionine-enkephalin, FK 33–824 and naloxone, respectively. As described for group 6, the crabs in group 11 received both naloxone (10^{-8} mol/crab) followed by methionine-enkephalin (10^{-8} mol/crab) after 30 min in 10 µl volume each. Hemolymph was collected from injected groups, 2 h after injection and used for the determination of hemolymph glucose level. For measurement of glucose, hemolymph (100 µl) was mixed with 300 µl of 95% ethanol in a 1.5 ml microtube. The proteins were precipitated by centrifuging at 14,000×*g* for 10 min at 4 °C. A mixture of glucose enzyme reagent (glucose-6-phosphate dehydrogenase and NADP) and colour reagents (phenazine methosulfate and iodonitrotetrazolium chloride) (kit from Sigma) was added (200 µl) to the protein-free sample. After 30 min, the intensity of the colour was measured at 490 nm and quantified with standards. One way ANOVA test was employed to analyze the data followed by Student Newman-Keul (SNK) test to determine the level of significance..

Results and Discussion

Hemolymph glucose level increased (62.7%) significantly ($p<0.001$) in the fresh water crab *Oziotelphusa senex senex* after methionine-enkephalin injection. Injection of FK 33824 also resulted in significant ($p<0.001$) increase (29%) in hemolymph glucose level in the intact crab *Oziotelphusa senex senex* (Table). Hemolymph glucose level in naloxone injected crabs decreased by 47%, which was statistically significant ($p<0.001$). Injection of methionine-enkephalin into naloxone-injected

Table. Hemolymph glucose level in normal and eyestalk ablated *Oziotelphusa senex senex* following different treatments.

Groups of crabs tested	Hemolymph glucose level (mg/100 ml)
Normal (intact) crabs	31.00 ± 3.46
Intact crabs injected with saline	32.63 ± 3.88[a] (0.53)
Intact crabs injected with Met-enk	50.43 ± 5.92[b] (62.68)
Intact crabs injected with FK 33–824	40.09 ± 3.01[b] (29.31)
Intact crabs injected with naloxone	16.33 ± 1.94[b] (−47.32)
Intact crabs injected with naloxone and Met-enk	13.89 ± 1.93[b] (−55.30)
Eyestalkless crabs (ESX)	15.41 ± 1.56[b] (−50.29)
ESX crabs injected with Met-enk	16.07 ± 2.12[a] (4.28)
ESX crabs injected with FK 33–824	16.86 ± 2.11[a] (9.29)
ESX crabs injected with naloxone	15.03 ± 1.56[a] (−2.47)
ESX crabs injected with naloxone and Met-enk	15.37 ± 1.37[a] (−0.71)
F ratio	440.10
p value	< 0.001

Values are Mean ± S. D. of 9 individual observations. Values in parenthesis are percent change over control. For calculation of % change and evaluation of 'p' for intact–injected and ESX crabs, normals served as control; for ESX– injected crab, ESX crabs served as control.

[a] Values are not significant from control crabs.

[b] Values are significant at $p<0.001$ from control crabs.

crabs, did not produce any increase in hemolymph glucose level.

Eyestalk ablation resulted in significant decrease (50%) in hemolymph glucose level in the crab *Oziotelphusa senex senex*. Injection of methionine-enkephalin, FK 33824, naloxone and naloxone followed by methionine-enkephalin into ablated crabs, resulted in no significant change in hemolymph glucose level from eyestalk ablated crabs (Table).

Bilateral eyestalk ablation significantly ($P<0.001$) decreased hemolymph glucose level. Ablation of eyestalks results in removal of X-organ-sinus gland complex, which is the secretory and storage site for hyperglycemic hormone, thereby eliminates hyperglycemic hormone from circulation, resulting in significant decrease in hemolymph glucose level. Injection of methionine-enkephalin significantly elevated the hemolymph glucose level in intact crabs, but this action of methionine-enkephalin appears to be an indirect one, because as is clear from data, methionine-enkephalin induced hyperglycemia only in intact crabs, presumably by triggering release of the hyperglycemic hormone. Methionine-enkephalin has been found to stimulate release of the neurohormones from the neurosecretory cells of eyestalks. Quackenbush and Fingerman (1984) demonstrated stimulation of release of red pigment concentrating and black pigment dispersing hormones from the neurosecretory cells of *Uca pugilator* after methionine-enkephalin injection. Naloxone blocked the action of methionine-enkephalin in intact crabs and isolated eyestalks (Quackenbush and Fingerman, 1984). Sarojini *et al.* (1995) provided evidence for methionine-enkephalin involvement in the release of gonad-inhibiting hormone from the eyestalks of the fiddler crab *Uca pugilator*. Recently, we have demonstrated a neurotransmitter role for methionine-enkephalin in the regulation of release of hyperglycemic hormone in the crab, *Oziotelphusa* (Reddy, 1999). From the present results, we can hypothesize that in *Oziotelphusa*, methionine-enkephalin is acting as a neurotransmitter that triggers hyperglycemic hormone release from the neuroendocrine cells but not as a hormone directly affecting the hemolymph glucose level. Evidence for the neurotransmitter role for methionine-enkephalin was also provided by Rothe *et al.* (1991); Luschen *et al.* (1991); Sarojini *et al.* (1995; 1996 and 1997).

FK 33–824, a stable methionine-enkephalin analogue, had a potent hyperglycemic effect in the intact crabs but not in eyestalkless crab. These results are consistent with previous data showing that methionine-enkephalin triggers release of hyperglycemic hormone from the sinus glands of eyestalks of *Oziotelphusa senex senex*, which would bring about elevated hemolymph sugar level (Reddy, 1999). In contrast, naloxone, a competitive opioid antagonist binding to opioid receptors, decreased hemolymph glucose level in intact crabs and suggests the antagonistic action of FK 33–824 in regulating hemolymph sugar level. Injection of naloxone into intact crabs, prior to injection of methionine-enkephalin blocked the hyperglycemic action of this opioid. In contrast, methionine-enkephalin does not affect the hemolymph glucose level in eyestalkless crabs, which is consistent with the hypothesis that enkephalin acts by stimulating hyperglycemic hormone release from the sinus glands of the eyestalks.

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Fingerman M. (1997), Crustacean physiology: A retrospective, prospective, and introspective analysis. Physiol. Zool. **70**, 257–269.

Luschen W., Buck F., Willig A. and Jaros P. P. (1991), Isolation, sequence analysis and physiological properties of enkephalins in the nervous system of the shore crab, *Carcinus maenas* L. Proc. Natl. Acad. Sci, USA. **88**, 8671–8675.

Mancillas J. R., Mc Ginty J. F., Selveerston A. I., Karten H. and Bloom F. E. (1981), Immunocytological localization of enkephalin and substance P in retina and eyestalk neurons of Lobster. Nature. **293**, 576–578.

Nagabhushanam R., Sarojini R., Reddy P. S. and Fingerman M. (1995), Opioid peptides in invertebrates: Localization, distribution and possible functional roles. Curr. Sci. **69**, 659–671.

Quackenbush L. S. and Fingerman M. (1984), Regulation of neurohormone release in the fiddler crab, *Uca pugilator*: Effects of gamma–aminobutyric acid, octopamine, met–enkephalin and beta–endorphin. Comp. Biochem. Physiol. **79C**, 77–84.

Reddy P. S. (1990), Neuroendocrine control of molting in the fresh water field crab *Oziotelphusa senex senex* Fabricius. Trop. Freshwater Biol. **2**, 213–222.

Reddy P. S. (1991a), Suppression of diurnal rhythm of oxidative metabolism by eyestalk removal in *Oziotelphusa senex senex* Fabricius. Arch. Internat. Physiol. Biochim. Biophys. **99**, 393–395.

Reddy P. S. (1991b), Crustacean hyperglycemic hormone induced alterations in millepede (*Spirostreptus asthenes*) carbohydrate metabolism. Arch. Internat. Physiol. Biochim. Biophys. **99**, 405–407.

Reddy P. S. (1992), Changes in carbohydrate metabolism of *Pila globosa* in response to crustacean hyperglycemic hormone. Arch. Internat. Physiol. Biochim. Biophys. **100**, 281–283.

Reddy P. S. (1999), A neurotransmitter role for methionine–enkephalin in causing hyperglycemia in the fresh water crab *Oziotelphusa senex senex*. Curr. Sci. **76**, 1126–1128.

Reddy P. S. and Ramamurthi R. (1980), Effect of crustacean eyestalk extract on the carbohydrate levels in the south Indian scorpion *Heterometrus fulvipes* (Koch). Experientia **36**, 318–319.

Reddy P. S. and Ramamurthi R. (1981), Interspecific effects of crustacean hyperglycemic hormone. Indian J. Exp. Biol. **19**, 472–474.

Reddy P. S. and Ramamurthi R. (1982a), Neuroendocrine control of carbohydrate metabolism in the rice field crab (*Oziotelphusa senex senex*). J. Rep. Biol. Comp. Endocrinol. **2**, 49–57.

Reddy P. S. and Ramamurthi R. (1982b), The effect *in vivo* of crustacean eyestalk extract on the aspects of carbohydrate metabolism in the slug, *Laevicaulis alte*. Arch. Internat. Physiol. Biochem. **90**, 9–14.

Reddy P. S. and Ramamurthi R. (1999), Recent trends in crustacean endocrine research. PINSA. **65B**, 15–32.

Reddy P. S., Bhagyalakshmi A., Ramamurthi R. and Chandrasekharam V. (1982a), Hyperglycemic activity of crab and scorpion hormones in grass hopper (*Poecilocerus pictus*). Experientia **38**, 811–812.

Reddy P. S., Ramesh Babu S. B. and Ramamurthi R. (1982b), Hyperglycemia in the fresh water field crab (*Oziotelphusa senex senex*) produced by a pesticide BHC. Z. Naturforsch. **37C**, 545–546.

Reddy P. S., Bhagyalakshmi A., Chandrasekharam V. and Ramamurthi R. (1984), Differential responsiveness of phosphorylase system and fat body carbohydrates of *Poecilocerus pictus* (Insecta) to hyperglycemic factors of crab (Crustacea) and scorpion (Arachnida). Gen. Comp. Endocrinol. **54**, 43–45.

Rothe H., Luschen W., Asken A., Willing A. and Jaros P. (1991), Purified crustacean enkephalin inhibits release of hypergycemic hormone in the crab *Carcinus maenas* L. Comp. Biochem. Physiol. **99C**, 57–62.

Sarojini R., Nagabhushanam R. and Fingerman M. (1995), Evidence for opioid involvement in the regulation of ovarian maturation of the fiddler crab, *Uca pugilator*. Comp. Biochem. Physiol. **111A**, 279–282.

Sarojini R., Nagabhushanam R. and Fingerman M. (1996), *In vitro* assessment of opioid agonists and antagonists on ovarian maturation in the red swamp crayfish, *Procambarus clarkii*. Comp. Biochem. Physiol. **115C**, 149–153.

Sarojini R., Nagabhushanam R. and Fingerman M. (1997), An *in vitro* study of the inhibitory action of Methionine–enkephalin on ovarian maturation in the red swamp crayfish , *Procambarus clarkii*. Comp. Biochem. Physiol. **117C**, 207–210.