

Methionine-Enkephalin Induces Hyperglycemia Through Eystalk Hormones in the Estuarine Crab *Scylla serrata*

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Abstract. The hypothesis is tested that methionine-enkephalin, a hormone produced in and released from eyestalk of crustaceans, produces hyperglycemia indirectly by stimulating the release of hyperglycemic hormone from the eyestalks. Injection of methionine-enkephalin leads to hyperglycemia and hyperglucosemia in the estuarine crab *Scylla serrata* in a dose-dependent manner. Decreases in total carbohydrate (TCHO) and glycogen levels of hepatopancreas and muscle with an increase in phosphorylase activity were also observed in intact crabs after methionine-enkephalin injection. Eyestalk ablation depressed hemolymph glucose (19%) and TCHO levels (22%), with an elevation of levels of TCHO and glycogen of hepatopancreas and muscle. Tissue phosphorylase activity decreased significantly during bilateral eyestalk ablation. Administration of methionine-enkephalin into eyestalkless crabs caused no significant alterations in these parameters when compared to eyestalk ablated crabs. These results support the hypothesis that methionine-enkephalin produces hyperglycemia in crustaceans by triggering release of hyperglycemic hormone from the eyestalks.

Introduction

In decapod crustaceans, hemolymph sugar level is regulated by hyperglycemic hormone. Abramowitz *et al.* (1944) were the first to demonstrate that injection of eyestalk extract induced hyperglycemia in *Callinectes*. Since then, hyperglycemia as a response to injection of eyestalk extract

has been observed in almost all groups of crustaceans (see review by Keller, 1992). This neurohormone is stored in and released from the sinus gland. The chemical nature, mode, and site of action of hyperglycemic hormone has been extensively studied in a number of crustaceans (see reviews by Keller *et al.*, 1985; Sedlmeier, 1985). The amino acid sequence of hyperglycemic hormones has been determined from a large number of crustaceans (see La Combe *et al.*, 1999, for review). The gene for hyperglycemic hormone was also cloned from crabs (Kegel *et al.*, 1989), lobster (Tensen *et al.*, 1991), prawn (Ohira *et al.*, 1997), isopod (Martin *et al.*, 1993), and crayfish (Kegel *et al.*, 1991; Huberman *et al.*, 1993; Yasuda *et al.*, 1994). Recently, we reported the expression of hyperglycemic hormone gene at different molt stages in *Homarus americanus*, the American lobster (Reddy *et al.*, 1997).

Since the discovery of opioid peptides in decapod crustaceans by Mancillas *et al.* (1981), several workers have attempted to determine the physiological function of these peptides, but the results are fragmentary. Sarojini *et al.* (1995, 1996, 1997) provided evidence that methionine-enkephalin slowed ovarian maturation in the fiddler crab *Uca pugilator* and the crayfish *Procambarus clarkii*, and suggested that methionine-enkephalin produces this effect indirectly by stimulating the release of gonad-inhibiting hormone from eyestalks. In *Uca pugilator*, methionine-enkephalin appears to stimulate release of the concentrating hormones for black and red pigment cells (Quackenbush and Fingerman, 1984) and the dark-adapting hormone for distal retinal pigment cells (Kulkarni and Fingerman, 1987). We reported a neurotransmitter role for methionine-enkephalin in regulating the hemolymph sugar level of the freshwater crab *Oziotelphusa senex senex*, and hypothesized that methionine-enkephalin produces hyperglycemia indi-

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rectly by stimulating release of hyperglycemic hormone (Reddy, 1999).

The objectives of the present study were threefold: (a) by extending our studies to the estuarine crab *Scylla serrata*, to test our hypothesis, generated by the study of *Oziotelphusa senex senex*, that methionine-enkephalin produces hyperglycemia in decapod crustaceans; (b) to determine the changes in levels of tissue carbohydrates and phosphorylase activity during methionine-enkephalin treatment; and (c) to provide evidence that supports the triggering of release of hyperglycemic hormone during methionine-enkephalin treatment.

Materials and Methods

Individuals of *Scylla serrata* (15 ± 2 cm in carapace width; 110 ± 5 g wet weight) were collected from the Chennai coast, India. They were kept in large aquaria with continuous aeration and acclimatized to laboratory conditions for one week under constant salinity (25 ± 1 ppt), pH (7.2 ± 0.1), and temperature ($23 \pm 2^\circ\text{C}$). During this period the crabs were fed fish flesh. Feeding was stopped 24 h before the beginning of the experiments, and no food was given during experimentation. Only intermolt (Stage C₄), intact, male crabs were used in the present study.

Methionine-enkephalin (Sigma Chemical Co.) was dissolved in physiological saline (Pantin, 1934). In these experiments, each of the 10 groups of crabs used consisted of 10 individuals. The first group served as normal and received no treatment. A second group served as control, with each crab in this group receiving an injection of $10\ \mu\text{l}$ of physiological saline (Pantin, 1934) through the base of the coxa of the 3rd pair of the walking legs. In groups 3–5 respectively, each crab received an injection of 10^{-7} , 10^{-8} , and 10^{-9} mole methionine-enkephalin in $10\ \mu\text{l}$ volume. Both eyestalks were ablated from all the crabs in groups 6–10. The eyestalks were extirpated by cutting them off at the base, without prior ligation but with cautery of the wound after operation. Twenty-four hours after eyestalk ablation, these groups were used for experimentation. Crabs in group 6 served simply as eyestalkless animals, and crabs in group 7 received $10\ \mu\text{l}$ crustacean Ringer solution and served as eyestalkless controls. In groups 8–10 respectively, each crab was injected with 10^{-7} , 10^{-8} , and 10^{-9} mole methionine-enkephalin in $10\ \mu\text{l}$ volume. Based on preliminary kinetic studies, the crabs were sacrificed for analysis 2 h after injection (Figs. 1, 2).

Hemolymph ($500\ \mu\text{l}$) was aspirated by syringe, through the arthrodial membrane of the coxa of the 4th pair of walking legs. The other tissues (hepatopancreas

and muscle from chela propodus) were then quickly dissected out, weighed, and analyzed by the procedures outlined below.

Hemolymph total carbohydrate level. Hemolymph total carbohydrate (TCHO) levels were estimated in trichloroacetic acid supernatant (10% TCA w/v) according to the method of Carroll *et al.* (1956).

Hemolymph glucose level. For measurement of glucose, $100\ \mu\text{l}$ of hemolymph was mixed with $300\ \mu\text{l}$ of 95% ethanol. After deproteinization (4°C , $14,000 \times g$, 10 min), the sample was combined with a mixture of glucose enzyme reagent (glucose-6-phosphate dehydrogenase and NADP) and color reagents (phenazine methosulfate and iodonitrotetrazolium chloride) (kit from Sigma). After 30 min, the intensity of the color was measured at 490 nm and quantified with standards.

Tissue TCHO and glycogen levels. TCHO levels in the tissues (hepatopancreas and muscle) were estimated in the 10% TCA supernatant (5% w/v), and glycogen was estimated in the ethanolic precipitate of TCA supernatant, according to the method of Carroll *et al.* (1956).

To $0.5\ \text{ml}$ of clear supernatant was added $5.0\ \text{ml}$ of anthrone reagent, and the combination was boiled for 10 min in a water bath. The samples were then immediately cooled. A standard sample containing a known quantity of glucose solution was always tested along with the experimental samples. Absorbance was measured at 620 nm against a reagent blank.

Tissue phosphorylase activity. Phosphorylase activity was assayed in hepatopancreas and muscle by colorimetric determination of inorganic phosphate released from glucose-1-phosphate by the method of Cori *et al.* (1955). First, $0.4\ \text{ml}$ of the enzyme was incubated with $2.0\ \text{mg}$ of glycogen for 20 min at 35°C , then the reaction was initiated by the addition of $0.2\ \text{ml}$ of $0.016\ M$ glucose-1-phosphate (G-1-P) to one tube (phosphorylase *a*) and a mixture of $0.2\ \text{ml}$ of G-1-P and $0.004\ M$ adenosine-5-monophosphate (phosphorylase *ab*) to another tube.

The reaction was incubated for 15 min for determining total phosphorylase and for 30 min for active phosphorylase. The reaction was terminated by the addition of $5.0\ \text{ml}$ of $5\ N$ sulfuric acid. Released inorganic phosphate was estimated by the method of Taussky and Shorr (1953).

Protein determination. Total protein levels in the enzyme source were estimated following the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Table 1

Effect of eyestalk ablation (ESX) (24-h post-ablation) and injection of methionine-enkephalin into intact and ablated crabs on hemolymph total sugar and glucose levels in *Scylla serrata*

	No treatment	Ringer injection	10^{-7} mol/crab	10^{-8} mol/crab	10^{-9} mol/crab	Dunnet's comparison test
Total Sugar (mg/100 ml)						
Intact (Group 1)	12.11 ± 1.01	12.73 ± 1.84^a (5.12)	28.8 ± 2.18^b (126.23)	19.64 ± 1.41^b (54.28)	16.52 ± 1.94^b (29.77)	$F_{(4,45)} = 137.160$
ESX (Group 2)	9.41 ± 1.13^b (-22.22)	$9.34 \pm 1.03^{b,c}$ (-0.74)	$9.41 \pm 1.13^{b,c}$ (0.74)	$9.43 \pm 1.01^{b,c}$ (0.96)	$9.21 \pm 1.08^{b,c}$ (-1.39)	$F_{(4,45)} = 0.099$
Two-way ANOVA: $F_{1,90}$ (Between groups) = 772.002, $P < 0.001$; $F_{4,90}$ (Among treatments) = 98.747, $P < 0.001$; $F_{4,90}$ (Interaction) = 94.552, $P < 0.001$.						
Glucose (mg/100 ml)						
Intact (Group 1)	6.41 ± 0.62	6.55 ± 0.76^a (2.16)	12.07 ± 1.34^b (84.27)	11.44 ± 1.28^b (74.65)	9.13 ± 0.78^b (39.38)	$F_{(4,45)} = 75.613$
ESX (Group 2)	5.19 ± 1.01^b (-19.03)	$5.52 \pm 0.81^{b,c}$ (6.35)	$5.19 \pm 1.01^{b,c}$ (-5.97)	$5.21 \pm 0.91^{b,c}$ (-5.61)	$5.44 \pm 0.77^{b,c}$ (-1.44)	$F_{(4,45)} = 0.387$
Two-way ANOVA: $F_{1,90}$ (Between groups) = 440.810, $P < 0.001$; $F_{4,90}$ (Among treatments) = 40.092, $P < 0.001$; $F_{4,90}$ (Interaction) = 44.753, $P < 0.001$.						

Values are mean $\pm$ SD of 10 individual crabs. Values in parentheses are percent change from control. For calculation of percent change for eyestalk-ablated (ESX) crabs and Ringer-injected intact crabs, intact crabs served as control; for met-injected crabs, Ringer-injected crabs served as control.

^a Not significant compared with intact crabs.

^b $P < 0.001$ compared to intact crabs.

^c Not significant compared to eyestalkless crabs.

Statistical analysis. Statistical analysis of the results was made using a two-way ANOVA test followed by Dunnet's multiple range test (preceded by one-way ANOVA), using SPSS version 10.0 (SPSS Inc., Chicago, IL).

Results

Effects of methionine-enkephalin on carbohydrate metabolism of intact crabs

Injection of methionine-enkephalin into intact crabs resulted in significant hyperglycemia and hyperglucosemia in a dose-dependent manner (Table 1), whereas injection of physiological saline had no effect on hemolymph carbohydrate levels. At doses between 10^{-9} mol/crab (36.41%) and 10^{-6} mol/crab (147.81%), the effect of methionine-enkephalin was statistically significant. For doses lower than 10^{-9} mol/crab, however, methionine-enkephalin did not elicit a hyperglycemic response (Fig. 1). A time course for methionine-enkephalin-induced hyperglycemia is shown in Figure 2 for a 10^{-7} mol/crab dose, which is a nearly saturating dose. The hemolymph glucose level increased significantly within 30 min of methionine-enkephalin injection, reached a peak at 2 h, then declined gradually.

Hepatopancreas glycogen and TCHO levels in crabs that received methionine-enkephalin were significantly lower than those of control crabs (Table 2). Decreases in muscle glycogen and TCHO levels were also significant after the injection of methionine-enkephalin (Table 3), suggesting

the possible mobilization of glucose molecules from hepatopancreas and muscle to hemolymph.

Phosphorylase (both total and active) activity levels were significantly increased in both hepatopancreas and muscle

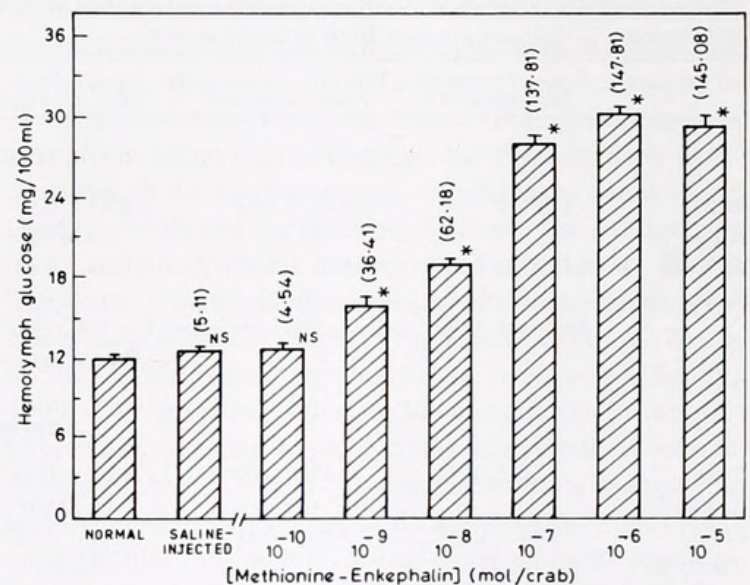


Figure 1. Dose-dependent effect of methionine-enkephalin on the hemolymph glucose levels in intact *Scylla serrata*. Two hours after injection of saline (10 μ l/animal) or methionine-enkephalin at the doses indicated, hemolymph was withdrawn from crabs for glucose determination. Each bar represents a mean $\pm$ SD ($n = 10$). Numbers in parentheses indicates the percent increase from the normal values. * Significant difference from normal crabs at $P < 0.001$. NS Not significant.

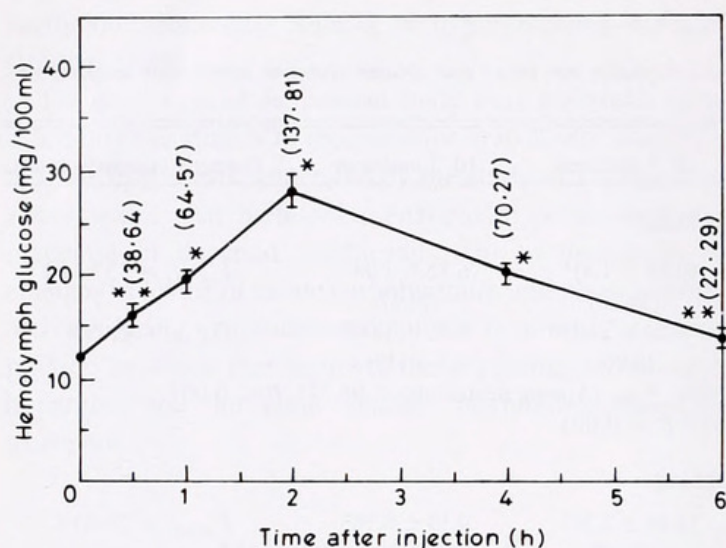


Figure 2. Time course of methionine-enkephalin-induced hyperglycemia. After injection of methionine-enkephalin (10^{-7} mol/crab), hemolymph was withdrawn from intact crabs at the time points indicated for glucose determination. Each point represents a mean $\pm$ SD ($n = 10$). Numbers in parentheses represent percent change from zero time controls. * Significant difference from zero time control at $P < 0.001$. ** Significant difference from zero time control at $P < 0.001$. ^{NS} Not significant from zero time control.

after the injection of methionine-enkephalin (Tables 4, 5). The ratio of active to total phosphorylase also increased in the tissues of crabs after the injection of methionine-en-

kephalin, indicating conversion of inactive to active phosphorylase.

Effects of bilateral eyestalk ablation and injection of methionine-enkephalin into ablated crabs on carbohydrate metabolism

Bilateral eyestalk removal caused a significant decrease in hemolymph carbohydrate level (Table 1). Enhancement of TCHO level of hepatopancreas and muscle was also significant in eyestalk-ablated crabs (Tables 2, 3). The increase was greater in muscle. Glycogen level in hepatopancreas increased significantly in eyestalkless crabs. A similar pattern was observed in muscle. Tissue phosphorylase activity levels decreased significantly in eyestalk-ablated crabs (Tables 4, 5).

Injection of methionine-enkephalin into eyestalkless crabs did not significantly change hemolymph carbohydrate levels compared to Ringer-injected eyestalkless crabs (Table 1). The levels of tissue TCHO and glycogen and activity levels of total and active phosphorylase were also not significantly altered in eyestalkless crabs after methionine-enkephalin injection (Tables 2–5).

Discussion

The effect of eyestalk hormones on tissue carbohydrate levels and phosphorylase activity has been extensively stud-

Table 2

Effect of eyestalk ablation (ESX) (24-h post-ablation) and injection of methionine-enkephalin into intact and ablated crabs on hepatopancreas total carbohydrate (TCHO) and glycogen levels in Scylla serrata

	No treatment	Ringer injection	10^{-7} mol/crab	10^{-8} mol/crab	10^{-9} mol/crab	Dunnet's comparison test
TCHO (mg/g)						
Intact (Group 1)	13.66 $\pm$ 1.54	13.84 $\pm$ 1.61 ^a	8.47 $\pm$ 0.97 ^b	9.01 $\pm$ 1.51 ^b	9.47 $\pm$ 1.49 ^b	$F_{(4,45)} = 38.033$ $P < 0.001$
ESX (Group 2)	17.87 $\pm$ 1.94 ^b	18.01 $\pm$ 1.97 ^{b,c}	17.44 $\pm$ 1.43 ^{b,c}	17.81 $\pm$ 1.62 ^{b,c}	17.93 $\pm$ 1.59 ^{b,c}	$F_{(4,45)} = 0.229$
	(30.96)	(0.67)	(-0.74)	(-0.96)	(-1.39)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 566.317, $P < 0.001$; $F_{4,90}$ (Among treatments) = 19.027, $P < 0.001$; $F_{4,90}$ (Interaction) = 14.896, $P < 0.001$.						
Glycogen (mg/g)						
Intact (Group 1)	1.22 $\pm$ 0.10	1.23 $\pm$ 0.09 ^a	0.58 $\pm$ 0.14 ^b	0.61 $\pm$ 0.13 ^b	0.64 $\pm$ 0.21 ^b	$F_{(4,45)} = 148.477$ $P < 0.001$
ESX (Group 2)	2.04 $\pm$ 0.29 ^b	2.06 $\pm$ 0.31 ^{b,c}	2.11 $\pm$ 1.18 ^{b,c}	2.09 $\pm$ 0.21 ^{b,c}	2.07 $\pm$ 0.28 ^{b,c}	$F_{(4,45)} = 0.230$
	(67.21)	(0.98)	(2.42)	(1.45)	(0.48)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 1658.593, $P < 0.001$; $F_{4,90}$ (Among treatments) = 24.964, $P < 0.001$; $F_{4,90}$ (Interaction) = 27.016, $P < 0.001$.						

Values are mean $\pm$ SD of 10 individual crabs. Values in parentheses are percent change from control. For calculation of percent change for ESX crabs and Ringer-injected intact crabs, intact crabs served as control; for met-injected crabs, Ringer-injected crabs served as control.

^a Not significant compared with intact crabs.

^b $P < 0.001$ compared to intact crabs.

^c Not significant compared to eyestalkless crabs.

Table 3

Effect of eyestalk ablation (ESX) (24 h post-ablation) and injection of methionine-enkephalin into intact and ablated crabs on muscle total carbohydrate (TCHO) and glycogen levels in *Scylla serrata*

	No treatment	Ringer injection	10 ⁻⁷ mol/crab	10 ⁻⁸ mol/crab	10 ⁻⁹ mol/crab	Dunnet's comparison test
TCHO (mg/g)						
Intact	4.39 ± 0.53	4.41 ± 0.49 ^a	2.94 ± 0.31 ^b	3.01 ± 0.37 ^b	3.12 ± 0.92 ^b	$F_{(4,45)} = 30.829$
(Group 1)		(0.46)	(-33.33)	(-31.74)	(-29.25)	$P < 0.001$
ESX	6.26 ± 0.71 ^b	6.31 ± 0.81 ^{b,c}	6.31 ± 0.76 ^{b,c}	6.25 ± 0.84 ^{b,c}	6.33 ± 0.92 ^{b,c}	$F_{(4,45)} = 0.045$
(Group 2)	(42.59)	(0.80)	(0)	(-0.95)	(0.31)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 579.612, $P < 0.001$; $F_{4,90}$ (Among treatments) = 8.707, $P < 0.001$; $F_{4,90}$ (Interaction) = 9.114, $P < 0.001$.						
Glycogen (mg/g)						
Intact	0.66 ± 0.06	0.64 ± 0.09 ^a	0.34 ± 0.09 ^b	0.37 ± 0.06 ^b	0.41 ± 0.08 ^b	$F_{(4,45)} = 45.114$
(Group 1)		(-3.03)	(-46.87)	(-42.18)	(-35.31)	$P < 0.001$
ESX	1.01 ± 0.09 ^b	1.02 ± 0.11 ^{b,c}	0.99 ± 0.14 ^{b,c}	1.07 ± 0.33 ^{b,c}	1.03 ± 0.21 ^{b,c}	$F_{(4,45)} = 0.188$
(Group 2)	(53.03)	(0.99)	(-2.94)	(4.90)	(0.98)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 422.031, $P < 0.001$; $F_{4,90}$ (Among treatments) = 6.391, $P < 0.001$; $F_{4,90}$ (Interaction) = 6.713, $P < 0.001$.						

Values are mean (mg glucose/g tissue) ± SD of 10 individual crabs. Values in parentheses are percent change from control. For calculation of percent change for ESX crabs and Ringer-injected intact crabs, intact crabs served as control; for met-injected crabs, Ringer-injected crabs served as control.

^a Not significant compared with intact crabs.

^b $P < 0.001$ compared to intact crabs.

^c Not significant compared to eyestalkless crabs.

ied (Keller, 1965; Ramamurthi *et al.*, 1968; Sagardia, 1969). Eyestalk removal inactivates the phosphorylase system and activates uridine-diphosphate-glucose glycogen transglucosylase (glycogen synthetase) (Keller, 1965; Ramamurthi *et al.*, 1968). Ramamurthi *et al.* (1968) also observed stimulation of uptake and incorporation of glucose ¹⁴C into the glycogen fraction of muscle tissue after eyestalk removal; this stimulation was accompanied by a decrease in hemolymph sugar level. Injection of eyestalk extract reversed these changes. The hyperglycemic hormone of eyestalks of the crab *Ozotellphusa senex senex* and the prawn *Penaeus monodon* enhances the activity of the phosphorylase system (Reddy *et al.*, 1982, 1984; Reddy, 1992).

An increase in phosphorylase activity and a decrease in glycogen and TCHO levels in hepatopancreas and muscle of *Scylla serrata*, followed by hyperglycemia after the injection of methionine-enkephalin, indicate glycogenolysis and mobilization of sugar molecules from tissues to hemolymph. This is in agreement with other findings (see review by Reddy and Ramamurthi, 1999). Though the hormone that elevates hemolymph sugar is conventionally called crustacean hyperglycemic hormone (CHH), Hohnke and Scheer (1970) suggested that the primary function of the CHH is not to elevate hemolymph sugar level, but to elevate intracellular glucose through the degradation of glycogen by activating the enzyme phosphorylase. The conversion of phosphorylase from its inactive to active form results in

glycogenolysis, and the resultant glucose molecules leak into the hemolymph, causing hyperglycemia. This view has been supported by Telford (1975).

Our results clearly demonstrate that methionine-enkephalin is involved in the regulation of carbohydrate metabolism in the crab *Scylla serrata*. In the present study, we show that methionine-enkephalin elicited a hyperglycemic response in *S. serrata* in a dose-dependent manner (Fig. 1). Methionine-enkephalin-induced hyperglycemia has been similarly demonstrated in the freshwater crab *Ozotellphusa senex senex* (Reddy, 1999) and the brackish-water prawns *Penaeus indicus* and *Metapenaeus monocerus* (Kishori *et al.*, 2001). The doses of methionine-enkephalin that induced hyperglycemia ranged from 10⁻⁹ to 10⁻⁶ mol/animal (Fig. 1), which is comparable to those reported for *O. senex senex* (Reddy, 1999). Our observation that methionine-enkephalin was ineffective in inducing hyperglycemia in eyestalk-ablated *S. serrata* (Table 1) is also consistent with those obtained in crabs (Reddy, 1999) and prawns (Kishori *et al.*, 2001) and suggests that the hyperglycemic effect of methionine-enkephalin results from an enhanced release of CHH (Keller, 1992; Soyey, 1997).

Injection of methionine-enkephalin into intact *S. serrata* also has two other effects. It activates the phosphorylase system, which causes degradation of glycogen. It also results in accumulation of sugar molecules in the tissues; these molecules are ultimately mobilized to hemolymph,

Table 4

Effect of eyestalk ablation (ESX) (24 h post-ablation) and injection of methionine-enkephalin into intact and ablated crabs on hepatopancreas phosphorylase activity levels in *Scylla serrata*

	No treatment	Ringer injection	10 ⁻⁷ mol/crab	10 ⁻⁸ mol/crab	10 ⁻⁹ mol/crab	Dunnet's comparison test
Phosphorylase <i>a</i>						
Intact (Group 1)	2.62 ± 0.29	2.67 ± 0.33 ^a (1.91)	3.87 ± 0.46 ^b (44.94)	3.63 ± 0.34 ^b (35.95)	3.60 ± 0.42 ^b (34.83)	$F_{(4,45)} = 28.430$ $P < 0.001$
ESX (Group 2)	1.72 ± 0.31 ^b (-34.35)	1.67 ± 0.29 ^{b,c} (-2.33)	1.69 ± 0.11 ^{b,c} (1.19)	1.81 ± 0.22 ^{b,c} (8.38)	1.84 ± 0.31 ^{b,c} (10.17)	$F_{(4,45)} = 1.473$
Two-way ANOVA: $F_{1,90}$ (Between groups) = 716.848, $P < 0.001$; $F_{4,90}$ (Among treatments) = 23.852, $P < 0.001$; $F_{4,90}$ (Interaction) = 18.208, $P < 0.001$.						
Phosphorylase <i>ab</i>						
Intact (Group 1)	4.52 ± 0.41	4.56 ± 0.44 ^a (0.89)	5.81 ± 0.67 ^b (27.41)	5.69 ± 0.52 ^b (24.78)	5.56 ± 0.73 ^b (21.92)	$F_{(4,45)} = 15.846$ $P < 0.001$
ESX (Group 2)	4.06 ± 0.44 ^b (-10.18)	4.08 ± 0.41 ^{b,c} (0.49)	4.10 ± 0.39 ^{b,c} (0.49)	4.12 ± 0.34 ^{b,c} (0.98)	4.09 ± 0.51 ^{b,c} (0.24)	$F_{(4,45)} = 0.044$
Two-way ANOVA: $F_{1,90}$ (Between groups) = 169.103, $P < 0.001$; $F_{4,90}$ (Among treatments) = 11.291, $P < 0.001$; $F_{4,90}$ (Interaction) = 9.985, $P < 0.001$.						

Values are mean (iP released/mg protein/h) ± SD of 10 individual crabs. Values in parentheses are percent change from control. For calculation of percent change for ESX crabs and Ringer-injected intact crabs, intact crabs served as control; for met-injected crabs, Ringer-injected crabs served as control.

^a Not significant compared with intact crabs.

^b $P < 0.001$ compared to intact crabs.

^c Not significant compared to eyestalkless crabs.

causing hyperglycemia. Methionine-enkephalin might have elevated the phosphorylase system in intact crabs in several different ways—for example, by triggering release of hyperglycemic hormone or by mimicking the action of this hormone. However, because methionine-enkephalin was not able to produce these changes in eyestalkless crabs, it seems most likely that methionine-enkephalin exerted its hyperglycemic effect by triggering release of hyperglycemic hormone from the sinus gland of eyestalks. This supports our earlier results that sinus glands in the eyestalks of crabs are the main release site for hyperglycemic hormone (Reddy and Ramamurthi, 1982).

The mechanisms whereby methionine-enkephalin causes release of neurohormones are still uncertain. In mammals, endogenous opioid peptides are involved in regulating the release of neurohypophysial peptides (Bicknell *et al.*, 1988; Yamada *et al.*, 1988; Sasaki *et al.*, 2000). In crustaceans, opioid-peptide-like (methionine-enkephalin-like, leucine-enkephalin-like and *b*-endorphin-like) hormones were isolated and characterized from X-organ sinus gland complexes of eyestalks (Fingerman *et al.*, 1983, 1985). However, there is little information about the effect of opioid peptides on release of neurohormones in crustaceans. Sarojini *et al.* (1995, 1996), using highly selective opioid antagonists, provided evidence that methionine-enkephalin exerts its effect by acting through delta-type opioid receptors in regulating ovarian maturation in *Procambarus*

clarkii. *In vivo* studies with tissues of *P. clarkii* showed that methionine-enkephalin exerted its effect by at least modulating the release of eyestalk peptide hormone (Sarojini *et al.*, 1997). Recently, we provided evidence for a neurotransmitter role for methionine-enkephalin in causing hyperglycemia in the crab *O. senex senex* (Reddy, 1999). Methionine-enkephalin also triggers the release of red-pigment-concentrating hormone, black-pigment-dispersing hormone (Quackenbush and Fingerman, 1984), and dark-pigment-adapting hormone (Kulkarni and Fingerman, 1987). Three facts make it seem likely that this hyperglycemic action of methionine-enkephalin in the present study on *S. serrata* is also indirect and involves stimulation of release of CHH. Methionine-enkephalin-like material is present in the neuroendocrine complex of the eyestalk of crustaceans (Fingerman *et al.*, 1983, 1985). Methionine-enkephalin mediation of release of neurohormones has been demonstrated (Reddy, 1999). In cases where methionine-enkephalin has been found to stimulate neurohormone release, it does not act in the absence of neuroendocrine organs. As further support for the conclusion, eyestalk extract from methionine-enkephalin injected prawns showed significantly less activity than the normal eyestalk extract in inducing hyperglycemia (Kishori *et al.*, 2001).

Although the mechanisms that trigger release of CHH are still unknown, it is noteworthy that 5-hydroxytryptamine (5-HT), or serotonin, triggers CHH release in the crayfish

Table 5

Effect of eyestalk ablation (ESX) (24 h post-ablation) and injection of methionine-enkephalin into intact and ablated crabs on muscle phosphorylase activity levels in *Scylla serrata*

	No treatment	Ringer injection	10 ⁻⁷ mol/crab	10 ⁻⁸ mol/crab	10 ⁻⁹ mol/crab	Dunnet's comparison test
Phosphorylase a						
Intact	1.92 ± 0.09	1.94 ± 0.14 ^a	3.26 ± 0.36 ^b	3.01 ± 0.12 ^b	3.02 ± 0.26 ^b	$F_{(4,45)} = 84.853$
(Group 1)		(1.04)	(68.04)	(55.15)	(55.67)	$P < 0.001$
ESX	0.99 ± 0.08 ^b	1.02 ± 0.11 ^{b,c}	1.01 ± 0.09 ^{b,c}	1.04 ± 0.13 ^{b,c}	1.06 ± 0.21 ^{b,c}	$F_{(4,45)} = 0.368$
(Group 2)	(-48.44)	(3.03)	(-0.74)	(1.96)	(3.92)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 1711.188, $P < 0.001$; $F_{4,90}$ (Among treatments) = 58.745, $P < 0.001$; $F_{4,90}$ (Interaction) = 52.927, $P < 0.001$.						
Phosphorylase ab						
Intact	2.49 ± 0.45	2.52 ± 0.49 ^a	3.49 ± 0.41 ^b	3.46 ± 0.44 ^b	3.44 ± 0.51 ^b	$F_{(4,45)} = 16.086$
(Group 1)		(1.21)	(38.49)	(37.30)	(36.50)	$P < 0.001$
ESX	2.22 ± 0.32 ^b	2.18 ± 0.31 ^{b,c}	2.22 ± 0.34 ^{b,c}	2.24 ± 0.42 ^{b,c}	2.25 ± 0.41 ^{b,c}	$F_{(4,45)} = 0.061$
(Group 2)	(-11.65)	(-0.91)	(1.83)	(2.75)	(3.21)	
Two-way ANOVA: $F_{1,90}$ (Between groups) = 136.048, $P < 0.001$; $F_{4,90}$ (Among treatments) = 10.259, $P < 0.001$; $F_{4,90}$ (Interaction) = 8.734, $P < 0.001$.						

Values are mean (iP released/mg protein/h) ± SD of 10 individual crabs. Values in parentheses are percent change from control. For calculation of percent change for ESX crabs and Ringer-injected intact crabs, intact crabs served as control; for met-injected crabs, Ringer-injected crabs served as control.

^a Not significant compared with intact crabs.

^b $P < 0.001$ compared to intact crabs.

^c Not significant compared to eyestalkless crabs.

Orconectes limosus (Keller and Bayer, 1968), *Astacus leptodactylus* (Strolenberg and Van Herp, 1977), and *Procambarus clarkii* (Lee *et al.*, 2000). Strolenberg and Van Herp (1977), working with *A. leptodactylus*, and Martin (1978), working with *Porcellio dilatatus*, found that the sinus glands of specimens injected with 5-HT show increased numbers of exocytotic profiles, suggestive of increased CHH release. Exocytosis in *A. leptodactylus* was maximal 2 h after 5-HT was injected, and the hemolymph glucose concentration peaked 4 h after the injection (Strolenberg and Van Herp, 1977). In *P. dilatatus*, hyperglycemia induced by 5-HT is mediated by 5-HT₁- and 5-HT₂-like receptors in triggering release of CHH (Lee *et al.*, 2000).

In summary, we have shown that methionine-enkephalin is a potent hyperglycemic regulator in the crab *Scylla serrata*. The most likely site of action of methionine-enkephalin is the eyestalks, where the X-organ-sinus glands may respond to methionine-enkephalin stimulation by releasing CHH. Based on these results, experiments are being conducted to determine whether methionine-enkephalin enhances the release of CHH in crustaceans.

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