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Establishing A Crustacean Model of Stress Responses I.

Involvement of Serotonergic Neurons

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主持人:李奇英 國立彰化師範大學生物系

Tel.: 04-723-2105 ext. 3429; Fax: 04-724-7756;

E-mail: bicylee@cc.ncue.edu.tw

ABSTRACT

The present studies investigate the roles of serotonergic neuronal pathways in regulating release of CHH and of serotonin-regulated CHH release in stress responses. Eyestalk ganglia with intact X-organ-sinus gland complex were dissected from the crayfish Procambarus clarkii and incubated under various experimental conditions. Incubation media were then analyzed for the presence of released hyperglycemic factor using an in vivo bioassay. The results show that 5-HT enhanced release of hyperglycemic factor in a dose-dependent manner. Further, activity of the 5-HT-released hyperglycemic factor could be eliminated by adsorption of incubation media with anti-CHH serum, but not by pre-immune or anti-5-HT serum. On the other hand, thermal stress significantly increased blood glucose levels in intact animals, but not in eyestalk-ablated animals. Reserpine, a 5-HT-depleting drug, was effective in blocking stress-induced hyperglycemia. These results confirm the hypothesis that 5-HT enhances release of CHH that in turn elicits hyperglycemic responses and suggests that 5-HTergic neurons are involved in stress-induced hyperglycemia.

BACKGROUND

The X-organ/sinus gland complex located within the eyestalks of decapod crustaceans is an important endocrine system that produces and releases a host of regulatory neuropeptides (Cooke and Sullivan 1982; Keller 1992; Soyez 1997). Among these neuropeptides, crustacean hyperglycemic hormone (CHH) is involved in regulating blood glucose levels mainly through mobilization of glucose from the glycogen depots (see Santos and Keller 1993), although other regulatory functions have been proposed (e. g., Tensen et al. 1989; Charmantier-Daures et al. 1994; Yasuda et al. 1994; Liu et al. 1997). Sequence analysis of CHHs isolated from various decapods indicated that they are peptides of 72~73 amino acid residues with a considerable degree of homology (Soyez 1997). An intriguing feature of CHH is the existence of multiple molecular variants in a given species as has been reported in several species (Soyez et al. 1994, Yasuda et al. 1994; Aguilar et al. 1995; Soyez et al. 1997; Yang et al. 1997; Chung et al. 1998). Significance of this phenomenon of structural polymorphism remains unknown.

Basal and enhanced release of CHH in response to electrical and elevated [K^+] stimuli has been characterized (Keller et al. 1994; Richmond et al. 1995). Furthermore, several neuroactive substances are implicated in the regulation of CHH release. It has been reported that in vivo injection of dopamine, enkephalin, or serotonin (5-hydroxytryptamine, 5-HT) altered blood glucose levels, and suggested that the glycemic response was due to increased or decreased CHH release (Keller and Beyer 1968; Fingerman et al. 1981; Jaros 1990; Rothe et al. 1991; Kuo et al. 1995; Sarojini

et al. 1995; Lee et al. 2000a). Recently, it has been shown that CHH release in *P. clarkii* is indeed increased by dopamine injection (Juan, 2001).

It is known from several crustacean studies that stressors of various natures (extreme temperature, hypoxia, organic and inorganic pollutants, bacterial infection, etc.) induce hyperglycemia and/or CHH release (Fingerman et al. 1981; Reddy et al. 1994; Webster 1996; Lorenzon et al. 1997; Chang et al. 1998; the present study). However, the neural network conveying these environmental stress signals remain undefined. The objectives of the present study were to elucidate the roles of serotonergic neuronal pathways in regulating release of CHH and the roles of serotonin-regulated CHH release in stress responses.

MATERIALS AND METHODS

Stress experiments

Experimental animals (Procambarus clarkii) were acclimated to 24℃ for at least a week. In the experiment in which effect of the tested pharmacological agent were tested, reserpine was dissolved in crayfish saline (Van Harreveld, 1936) buffered with 20 mM HEPES and injected prior to stress manipulation into animals through the base of the chelae using an insulin syringe coupled to a 29 gauge needle. Animals were then transferred to either 24℃ (control) or 34℃ (stress). Hemolymph was withdrawn from animals at designated times after transfer. Four parts of the hemolymph were diluted immediately with one part of saline containing 0.01 M EDTA to inhibit clotting, and the mixture was centrifuged (2,000Xg, 20 min, 4 ℃) (Lee et al., 2000). The resultant supernatant was collected and immediately used for glucose analysis as described below.

In vitro Incubation

In order to study the effect of serotonin on CHH release, eyestalk ganglia with intact X-organ-sinus gland complex were dissected in ice-cold Van Harreveld saline buffered with 20 mM HEPES and incubated without or with various doses of serotonin (Lee et al., 2001). At designated times, incubation media were collected and analyzed for hyperglycemic activity using a bioassay as described previously (Lee et al., 2001). Specificity of the α-CHH serum used in immunoprecipitation has been described previously (Lee et al., 2001).

Glucose analysis

Blood glucose was quantified using the Trinder Glucose Assay Kit (# 315; Sigma) according to the manufacturer's protocol. Briefly, the hemolymph preparation (25 μl) and the Trinder reagent (200 μl) were added to the wells of a microplate, and mixed

using a microplate shaker. After standing at room temperature for 10 min, the optical density was read using an ELISA reader (Elx 808 IU, Bio-Tek Instruments Inc., Winooski, VT, USA) at 490 nm. A standard curve was constructed for each microplate using glucose standards (# 16-100, Sigma). Glucose concentration in samples was inferred from the standard curve and corrected for the dilution factor using KC4 software from Bio-Tek Instruments Inc.

RESULTS

Thermal stress (transfer from 24℃ to 34℃) significantly elevated glucose levels in intact animals, but not in eyestalk-ablated animals (Fig. 1). Pre-treating animals with reserpine, a serotonin-depleting drug, blocked hyperglycemic effect of thermal stress. Glucose levels increased more than 2 folds in stressed-animals receiving saline injection, but did not significantly alter in stressed-animals receiving saline injection (Fig. 2).

Blood glucose concentration in saline-injected eyestalk-ablated animals was 3.3 ± 0.2 mg/dL (n=8). In vitro incubated eyestalk ganglia released hyperglycemic factor into incubation media, the activity of which could be detected by a hyperglycemia bioassay, that is, injection of concentrated media derived from incubation of eyestalk ganglia significantly increased glucose concentration in eyestalk-ablated animals to 9.8 ± 2.1 mg/dL ($n=8$, $p < 0.01$). Serotonin (5-HT) enhanced release of hyperglycemic factor from eyestalk ganglia. Compared to media derived from incubation of eyestalk ganglia, injection of media obtained from incubation of 5-HT-treated ($1 \times 10^{-5} - 5 \times 10^{-4}$ M) eyestalk ganglia significantly increased glucose concentration 1.6 – 2.1 folds (Fig. 3). Identity of the hyperglycemic factor present in incubation of eyestalk ganglia was investigated using the immune serum. Adsorption of media derived from incubation of 5-HT-treated eyestalk ganglia by anti-CHH immune serum completely eliminated the hyperglycemic activity, whereas adsorption by either pre-immune serum or anti-5-HT serum did not have significant effect (Fig. 4).

DISCUSSION

Previous studies in the crayfish have demonstrated that 5-HT elicited significant hyperglycemic responses in intact, but not in eyestalk-ablated, animals suggesting that 5-HT-induced hyperglycemia is mediated by an enhanced release of eyestalk hyperglycemic factor, presumably CHH (Keller and Beyer 1968; Lee et al. 2000a). Direct supporting evidence for this hypothesis is provided by the present studies in which it was shown that in vitro incubated eyestalk ganglia, when challenged by 5-HT, released more hyperglycemic factor into incubation media as evidenced by a bioassay. Sáenz et al. (1997) reported that 5-HT induced firing and enhanced bursting activity

of a population of P. clarkii X-organ neurosecretory cells that are likely CHH-containing. The effective doses of 5-HT in enhancing release of hyperglycemic factor (see Fig. 1) are comparable to those in altering electrical activity of the neurosecretory cells (Sáenz et al. 1997). Keller et al. (1994) studied release of CHH from isolated X-organ-sinus gland of the crab Cardisoma carnifex. Although the assays employed are different (ELISA in Keller et al. vs. hyperglycemia assay in the present studies), the increase in hyperglycemic activity in response to 5-HT stimulation (see Fig. 3) falls within the range of increase in CHH release in response to various regimes of electrical stimulation (Keller et al. 1994).

It was demonstrated that the hyperglycemic activity present in media of 5-HT-treated tissue incubation could be completely absorbed by the anti-CHH serum, but not by the pre-immune serum, confirming that the 5-HT-released hyperglycemic factor is indeed CHH. The suggestion that 5-HT is responsible for the hyperglycemic activity contained in media of 5-HT-treated tissue incubation is rebutted by the observations that anti-5-HT serum was unable to absorb the hyperglycemic activity, and that injection of saline containing 5-HT did not significantly alter glucose concentration in eyestalk-ablated animals.

We have also demonstrated in the present studies that thermal stress significantly increased blood glucose levels (Fig. 1). The observation that reserpine, a 5-HT-depleting drug, effectively blocked stress-induced hyperglycemia (Fig. 2) suggests that 5-HTergic neurons are involved in stress-induced hyperglycemia.

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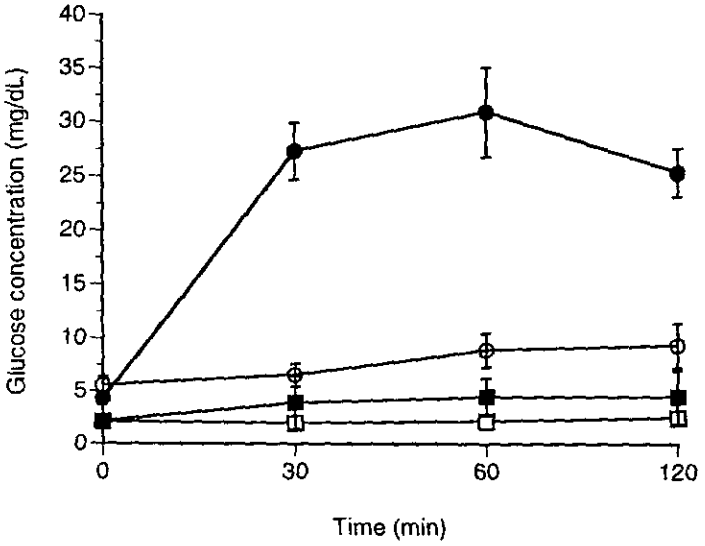

Fig. 1 Effect of thermal stress on the blood glucose levels. Intact (circle) and eyestalk-ablated (square) animals reared at 24°C were bled immediately before (0 min) and after (30, 60, 120 min) being transferred to 34°C (filled symbols) or retained at 24°C (open symbols).

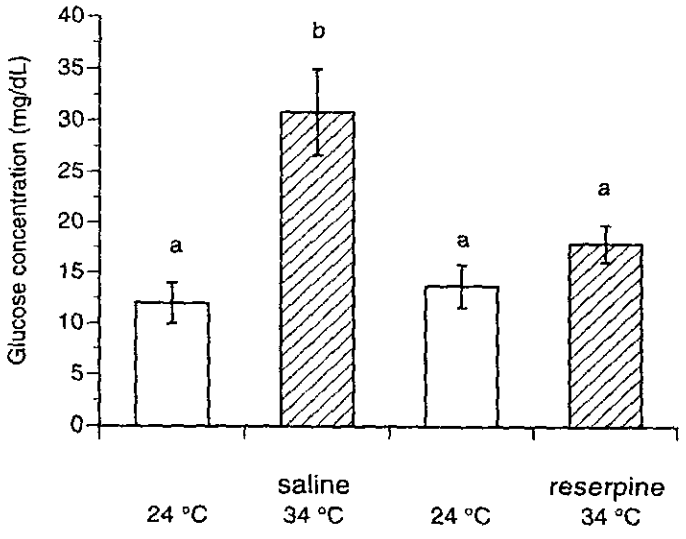

Fig. 2 Blcking of thermal stress-induced hyperglycemia in the crayfish, *Procambarus clarkii*, by reserpine. Animals acclimated to 24 °C for 1 week were bled before (open) receiving injections of saline or resperine (15 µg/g body weight), transferred to 34 °C tanks, and bled again 1 hour after transfer (hatched). Hemolymph withdrawn was used for glucose quantification. Values are mean ± SEM (n=8). Columns labeled with different letters are significantly different from each other at 1% levels.

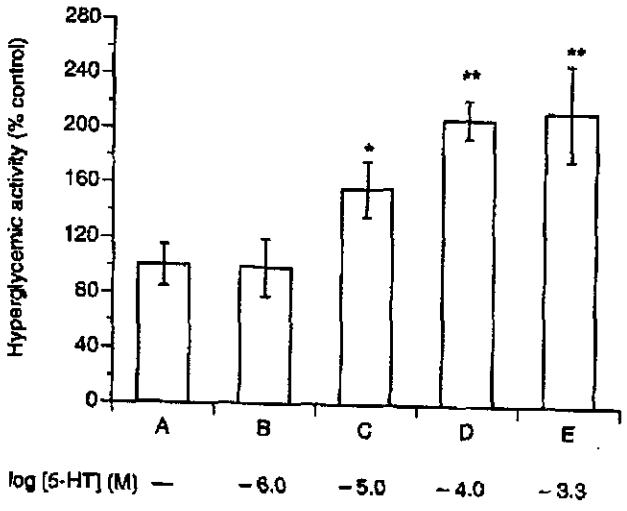

Figure 3 Dose-dependent effect of 5-HT on release of hyperglycemic factor. Eyestalk-ablated animals received an injection of concentrated media of two eyestalk ganglia incubated for 30 min in the absence (*A*) or presence of 5-HT (*B–E*) at the doses indicated. Hemolymph was withdrawn for glucose quantification 1 h after injection. Values are means ± SEM ($n = 8$) and are expressed as a percentage of control (*A*). A single asterisk indicates $P < 0.05$; two asterisks indicate $P < 0.01$ compared with control.

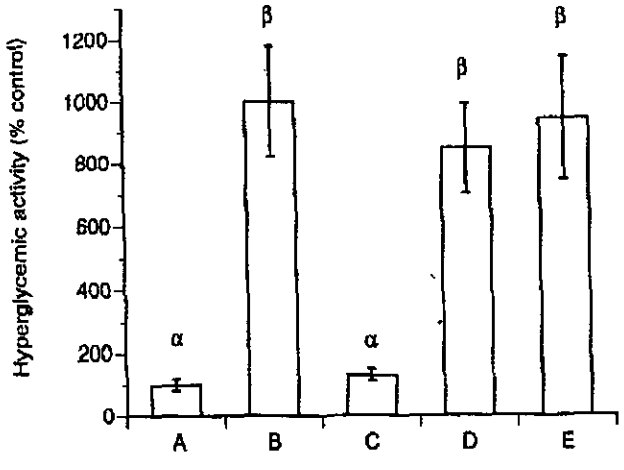

Figure 4. Immunoadsorption of hyperglycemic activity in 5-HT-treated tissue incubation. Eyestalk-ablated animals received an injection of saline (*A*) or concentrated/immunoadsorbed media (*B–E*) of two eyestalk ganglia incubated for 30 min with 10^{-4} M 5-HT. Sera used for immunoadsorption: *B*, none; *C*, anti-CHH; *D*, preimmune; *E*, anti-5-HT. Hemolymph was withdrawn for glucose quantification 1 h after injection. Values are means ± SEM ($n = 8$) and are expressed as a percentage of saline-injected control (*A*). Values labeled with different Greek letters are significantly different from each other ($P < 0.01$).