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Effects of elevated ecdysteroid on tissue expression of three guanylyl cyclases in the tropical land crab *Gecarcinus lateralis*: possible roles of neuropeptide signaling in the molting gland

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Summary

Two eyestalk (ES) neuropeptides, molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH), increase intracellular cGMP levels in target tissues. Both MIH and CHH inhibit ecdysteroid secretion by the molting gland or Y-organ (YO), but apparently through different guanylyl cyclase (GC)-dependent pathways. MIH signaling may be mediated by nitric oxide synthase (NOS) and NO-sensitive GC. CHH binds to a membrane receptor GC. As molting affects neuropeptide signaling, the effects of ecdysteroid on the expression of the land crab *Gecarcinus lateralis* β subunit of a NO-sensitive GC (Gl-GC-Iβ), a membrane receptor GC (Gl-GC-II) and a NO-insensitive soluble GC (Gl-GC-III) were determined. Gl-GC-Iβ isoforms differing in the absence or presence of an N-terminal 32-amino acid sequence and Gl-GC-III were expressed at higher mRNA levels in ES ganglia, gill, hepatopancreas, ovary and testis, and at lower levels in YO, heart and skeletal muscle. Three Gl-GC-II isoforms, which vary in the length of insertions (+18, +9 and +0 amino acids) within the N-terminal ligand-binding domain, differed in tissue distribution. Gl-GC-II(+18) was expressed highly in striated muscle (skeletal and cardiac muscles); Gl-GC-II(+9) was expressed in all tissues examined (ES ganglia, YO, gill, hepatopancreas, striated muscles and gonads); and Gl-GC-II(+0) was expressed in most tissues and was the dominant isoform in ES and thoracic ganglia. ES ablation, which increased hemolymph ecdysteroid, increased Gl-GC-II(+18) mRNA level in claw muscle. Using real-time RT-PCR, ES ablation increased Gl-GC-Iβ, Gl-GC-III and ecdysone receptor mRNA levels in the YOs ~ten-, ~four- and ~twofold, respectively, whereas Gl-GC-II mRNA level was unchanged. A single injection of 20-hydroxyecdysone into intact animals transiently lowered Gl-GC-Iβ in hepatopancreas, testis and skeletal muscle, and certain Gl-GC-II isoforms in some of the tissues. These data suggest that YO and other tissues can modulate responses to neuropeptides by altering GC expression.

Key words: guanylyl cyclase, molting, ecdysteroid, Crustacea, Arthropoda, Y-organ, gene expression, skeletal muscle, nervous system, digestive gland, molt-inhibiting hormone, crustacean hyperglycemic hormone, ecdysone receptor, mRNA.

Introduction

Guanylyl cyclases (GCs) catalyze the production of the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP) in response to various signals, such as nitric oxide (NO), peptide ligands and fluxes in intracellular Ca^{2+} levels (Lucas et al., 2000; Padayatti et al., 2004; Potter, 2005; Pyriochou and Papapetropoulos, 2005). In crustaceans, NO-sensitive GC (GC-I) and membrane receptor GC (GC-II) are implicated in the signaling pathways for two neuropeptide hormones, molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH), respectively. Both hormones are produced and released from the X-organ/sinus gland complex in the eyestalk (ES) ganglia (Skinner, 1985). MIH inhibits ecdysteroid synthesis and secretion in the molting glands or Y-organs (YOs), which are a pair of epithelioid glands located in the anterior cephalothorax (Lachaise et al., 1993; Skinner, 1985). MIH induces a large increase of cGMP in the YOs (Chung and Webster, 2003; Saïdi et al., 1994; Sedlmeier and Fenrich, 1993). A NO synthase (NOS) is phosphorylated in activated YOs, which suggests that a NO-sensitive GC mediates the MIH-induced increase in cGMP (Kim et al., 2004; Lee and Mykles, 2006). The current thinking is that CHH binds to a GC-II membrane receptor, which increases cGMP levels in various tissues (Chung and Webster, 2003; Goy, 1990; Goy et al., 1987; Pavloff and Goy, 1990; Scholz et al., 1996; Sedlmeier and Keller, 1981).

Crustacean tissues express several different GCs, including membrane receptor GCs and both NO-sensitive and NO-insensitive soluble GCs (Aonuma, 2002; Goy, 1990; Goy, 2005; Prabhakar et al., 1997; Scholz, 2001; Scholz et al., 1996; Scholz et al., 2002). cDNAs encoding the β subunit of a NO-sensitive GC (GC-Iβ), a membrane receptor GC (GC-II) and a NO-insensitive GC (Gl-GC-III) have been cloned (Lee et al., 2007; Liu et al., 2004; Zheng et al., 2006). In *G. lateralis*, isoforms of

Gl-GC-Iβ and Gl-GC-II appear to be generated by alternative splicing. Two Gl-GC-Iβ isoforms differ in the absence (Δ32N) or presence (Δ0N) of a 32-amino acid sequence at the N terminus (Lee et al., 2007). Three Gl-GC-II isoforms differ in length within the N-terminal ligand-binding domain (+18, +9 and +0 amino acid insertions) (Lee et al., 2007). As molting can affect YO sensitivity to MIH and CHH (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004; Nakatsuji et al., 2006), the effects of ES ablation and ecdysteroid injection on GC mRNA levels in YOs and other tissues were determined.

Materials and methods

Animals

Blackback land crabs *Gecarcinus lateralis* Fréminville 1835 were collected from Puerto Rico or the Dominican Republic. They were kept in covered plastic cages with aspen bedding moistened with tapwater at ~27°C and 50% humidity and were fed raisins, carrots and lettuce twice a week. A 12 h:12 h dark:light cycle was used. Molt stage was monitored by regeneration of an autotomized walking leg; initiation of regenerate growth [Regeneration index >10 (Skinner, 1985)] indicated entry into proecdysis. Only intermolt animals with basal regenerates [Regeneration index <8 (Skinner, 1985)] were used.

Two methods were used to increase hemolymph ecdysteroid levels *in vivo*: ES ablation (ESA) and injection of intact animals with 20-hydroxyecdysone (20E). ESA removes the primary source of MIH, thus stimulating YO ecdysteroidogenesis (Skinner, 1985); wounds were cauterized with a heated spatula. A 20E (Sigma-Aldrich, St Louis, MO, USA) stock solution (10 mg ml^{-1}) in 10% ethanol was injected into the hemolymph through the arthrodial membrane at the base of a walking leg at 0.41 μg 20E g^{-1} body mass. Control animals received the same volume of 10% ethanol. Hemolymph ecdysteroid was quantified by radioimmunoassay (Chang and O'Connor, 1979).

Tissue expression of land crab guanylyl cyclases

Tissues were dissected from 3–5 crabs and immediately placed in RNA*later* RNA Stabilization Reagent (Qiagen, Germantown, MD, USA) and stored at –20°C or flash frozen in liquid N_2 and stored at –80°C. Total RNA was isolated using either the RNeasy Mini column (Qiagen) or TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and treated with DNase I to remove any contaminating genomic DNA. RNA concentration was determined by UV absorbance at 260/280 nm and stored at –80°C. First strand cDNA was synthesized in a reaction (20 μl) containing ~1 μg total RNA, 50 mmol l^{-1} Tris-HCl (pH 8.3), 75 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} dNTPs, 40 units of RNaseOUT ribonuclease inhibitor, 200–500 ng oligo$(dT)_{12–18}$ primer, and 200 units SuperScript III RT (Invitrogen). The reaction mixture was incubated for 60 min at 50°C, inactivated at 70°C for 15 min, treated with *E. coli* RNase H (2 units), and stored at –20°C.

PCR was performed in a Perkin Elmer 9600 cycler (Waltham, MA, USA) using Takara Ex Taq HotStart polymerase (Takara, Inc., Madison, WI, USA). Sequence-specific primers to Gl-GCIβ, GC-II, Gl-III and Gl-EF2 were synthesized by Integrated DNA Technologies, Inc. (Table 1). Gl-EF2 (GenBank accession no. AY552550), which is constitutively expressed, served as an internal control (Kim et al., 2005a; Kim et al., 2005b). Reactions (30 μl) contained 1 μl first strand cDNA mixture, 3 μl 10× Takara EX Taq buffer, 2 μl 250 μmol l^{-1} dNTPs, 0.2 μl Takara EX Taq DNA polymerase (5 units μl^{-1}), and 18.8 μl PCR grade deionized water. The PCR conditions were an initial denaturation at 96°C for 5 min, then 35 cycles of denaturation at 96°C for 20 s, annealing at 62°C for 20 s, and extension at 72°C for 30 s, followed by 5 min at 72°C as a final extension. Identities of the PCR products were confirmed by sequencing a subset of samples from specific tissues. Samples (15 μl) were separated on 2% agarose gels with a 100-bp PCR Molecular Ruler DNA size ladder (Bio-Rad, Richmond, CA, USA) and stained with Ethidium Bromide. The gel image was reversed and analyzed by Quantity One software (Bio-Rad). Expression data were normalized to an internal positive control (Gl-EF2).

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify the effect of ESA on the expression of the three GCs, ecdysone receptor (EcR) and EF2 in the YO. RNA was purified from pooled YOs from 4–5 animals at each time point (0, 1, 3 and 7 days post-ES ablation) and reverse transcribed as described above. Real-time PCR was performed in a LightCycler 2.0 (Roche Applied Science, Nutley, NJ, USA) using LightCycler FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science) and sequence-specific primers to Gl-GCIβ, Gl-GC-II, Gl-GC-III, Gl-EcR and Gl-EF2 (Table 2). Reactions (20 μl) contained 2 μl first strand cDNA

Table 1. *Primers used for tissue expression of land crab GC-Iβ, GC-II, GC-III and EF2*

Name	Sequence	T_m (°C)	Description
cGC-Iβ-F4	5′-ACA CTG TCA ACA TCA CTT CGA GGA CG-3′	62.3	GC-Iβ FP, C-terminal
cGC-Iβ-R3	5′-GCG AGG CGA AAC ATT CAT TTC CTT CC-3′	62.4	GC-Iβ RP, C-terminal
cGC-Iβ-F100	5′-ATG TGT TGT GTT CCT CTG TGA GGA CGC-3′	62.4	GC-Iβ FP, 5′UTR
cGC-Iβ-R100	5′-CGA ACA GTT CCA GAA TGG CGT TGG C-3′	62.5	GC-Iβ RP, N-terminal
cGC-II-F83	5′-CCA CGA CAA TAA CGG AAA CAG ACC ACG G-3′	62.8	GC-II FP
cGC-II-R83	5′-CTT GTC CTT GAT AAG CGT GCC GTT GC-3′	62.2	GC-II RP
cGC-III-F42	5′-GTG TCG CCC TTC ACC CAG AAA ATG TTG G-3′	63.4	GC-III FP
cGC-III-R41	5′-CCT GCT GGA CGT TGG AAT GAT TGT GAG G-3′	62.9	GC-III RP
cEF2-F1	5′-TTC TAT GCC TTT GGC CGT GTC TTC TC-3′	62.6	EF2 FP
cEF2-R1	5′-TGA TGG TGC CCG TCT TAA CCA GAT AC-3′	62.1	EF2 RP

EF2, elongation factor 2 (GenBank accession no. AY552550); FP, forward primer; GC, guanylyl cyclase [for accession numbers, see Lee et al. (Lee et al., 2007)]; RP, reverse primer; T_m, melting temperature.

Table 2. *Primers used for quantification of land crab GC-Iβ, GC-II, GC-III, EcR and EF2 in the Y-organ by real-time RT-PCR*

Name	Sequence	T_m (°C)	Descriptions
cGCIB-F4	5′-ACA CTG TCA ACA TCA CTT CGA GGA CG-3′	62.3	GCIβ FP
cGCIB-R3	5′-GCG AGG CGA AAC ATT CAT TTC CTT CC-3′	62.4	GCIβ RP
cGCII-F50	5′-TAC GCG ATG AGG AGA GAA AGA CCA AGG A-3′	62.2	GCII FP
cGCII-R50	5′-GGA CGG TGG TGG ATA ACG AAC TTG TGT T-3′	62.2	GCII RP
cGCIII-F42	5′-GTG TCG CCC TTC ACC CAG AAA ATG TTG G-3′	63.4	GCIII FP
cGCIII-R41	5′-GTG AGA GAT CGT GGA GAA CGT GAA GAA CGG-3	63.0	GCIII RP
cEF2-F1	5′-TTC TAT GCC TTT GGC CGT GTC TTC TC-3′	62.6	EF2 FP
cEF2-R1	5′-TGA TGG TGC CCG TCT TAA CCA GAT AC-3′	62.1	EF2 RP
cEcR-F1	5′-AAG AAT GCC GTG TAC CAG TGT AAA TAT G-3′	62.1	EF2 RP
cEcR-R1	5′-GCT GCT CGA AGC ATC ATG ACC TC-3′	62.1	EF2 RP

EcR, ecdysone receptor (GenBank accession no. AY642975); EF2, elongation factor 2; FP, forward primer; GC, guanylyl cyclase; RP, reverse primer; T_m, melting temperature.

mixture, 4 μl 5× SYBR Green Master Mix, 1 μl of 10 μmol l^{-1} forward primer and 10 μmol l^{-1} reverse primer each, and 12 μl PCR-grade water. The amount of YO cDNA from intact animals was doubled in the PCR to compensate for the low levels of Gl-GCIβ and GC-III transcripts relative to the their levels in YOs from ES-ablated animals. The PCR conditions were an initial denaturation at 95°C for 10 min, 42 cycles of denaturation at 95°C for 5 s, annealing at 62°C for 5 s, and extension at 72°C for 20 s, followed by melting curve analysis between 65°C and 95°C. Transcript copy number was calculated with the Roche LightCycler software using standard curves of serial dilutions (10 fg to 10 ng) of each template (Kim et al., 2005a; Kim et al., 2005b). Transcript levels of the GCs and EcR were normalized to EF2 copy number. As the copy numbers differed by as much as 5 orders of magnitude between the different transcripts, expression was related to transcript levels in YOs from intact (0 day post-ES ablated) animals so that the data could be combined in a single graph.

Statistical analyses

Statview 5.0.1 software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. Regression analysis was used to determine the correlations between ecdysteroid concentration and GC mRNA level. One-way analysis of variance (ANOVA) *post-hoc* tests (Bonferroni–Dunn, Tukey–Kramer and Fisher's PLSD tests) were used to determine significant differences in GC mRNA levels in response to ES ablation and 20E injection.

Results

Most tissues expressed the three guanylyl cyclases, although the levels of expression varied among tissues in intact animals (Fig. 1). RT-PCR was used with primers directed to the C-terminal region of Gl-GC-Iβ (Table 1) that amplified the same product from both isoforms. Gl-GC-Iβ was expressed at higher levels in ES ganglia, gill, hepatopancreas, ovary and testis. Expression was lower in YO, heart, claw muscle and thoracic muscle. RT-PCR with primers (Table 1) that flanked the insertion in the ligand-binding domain quantified the expression of the three Gl-GC-II isoforms (+18, +9 and +0). The reactions were separated on a polyacrylamide gel to distinguish the three products. Gl-GC-II(+18) was expressed at highest levels in heart and skeletal muscle; Gl-GC-II(+9) was expressed in all tissues, with the highest levels in gill, hepatopancreas and gonad; and Gl-GC-II(+0) was expressed at low levels in most tissues, but was the predominant isoform in nervous tissues, including ES ganglia (EG; Fig. 1) and thoracic ganglion (data not shown). Gl-GC-III showed an expression pattern similar that of Gl-GC-Iβ (Fig. 1). Elongation factor 2 (Gl-EF2) was expressed at similar levels in all tissues (Fig. 1A).

Each GC was quantified by RT-PCR after 35 cycles and normalized to Gl-EF2, which was constitutively expressed (Fig. 1A) (see also Kim et al., 2005a; Kim et al., 2005b). Terminating the PCR at 35 cycles was within the exponential phase for generation of products. For example, the amounts of the PCR products of the three GC-II isoforms in claw and thoracic muscles in intact, 1 day post-ESA and 3 days post-ESA animals were quantified at 33, 35 and 37 cycles. As expected, the slopes (log products *vs* cycle number) were ~2 for GC-II(+18) and GC-II(+9); the means ± s.d. were 2.066±0.099 (N=30) and 1.914±0.122 (N=30), respectively. The slope was <2 for GC-II(+0) (1.688±0.071; N=30), indicating that the PCR conditions may underestimate the amount of this isoform at higher mRNA levels. Similar experiments on GC-Iβ and GC-III showed an approximately twofold response per cycle between 33 and 37 cycles (data not shown). These experiments validate this method for quantifying GCs. In addition, the analysis enabled quantification of the relative expression of the three Gl-GC-II isoforms.

The effects of ESA, which increased ecdysteroid levels in the

Table 3. *Effect of eyestalk ablation on hemolymph ecdysteroid levels in land crabs*

Treatment	Hemolymph [ecdysteroid] (pg μl^{-1})	N
Intact (control)	11.45±0.86	29
1 day post-ESA	44.06±3.68	20
3 days post-ESA	83.35±5.98	20
7 days post-ESA	132.49±11.20	20

ESA, eyestalk ablation.

Ecdysteroid was quantified with radioimmunoassay; values are means ± 1 s.e.m.

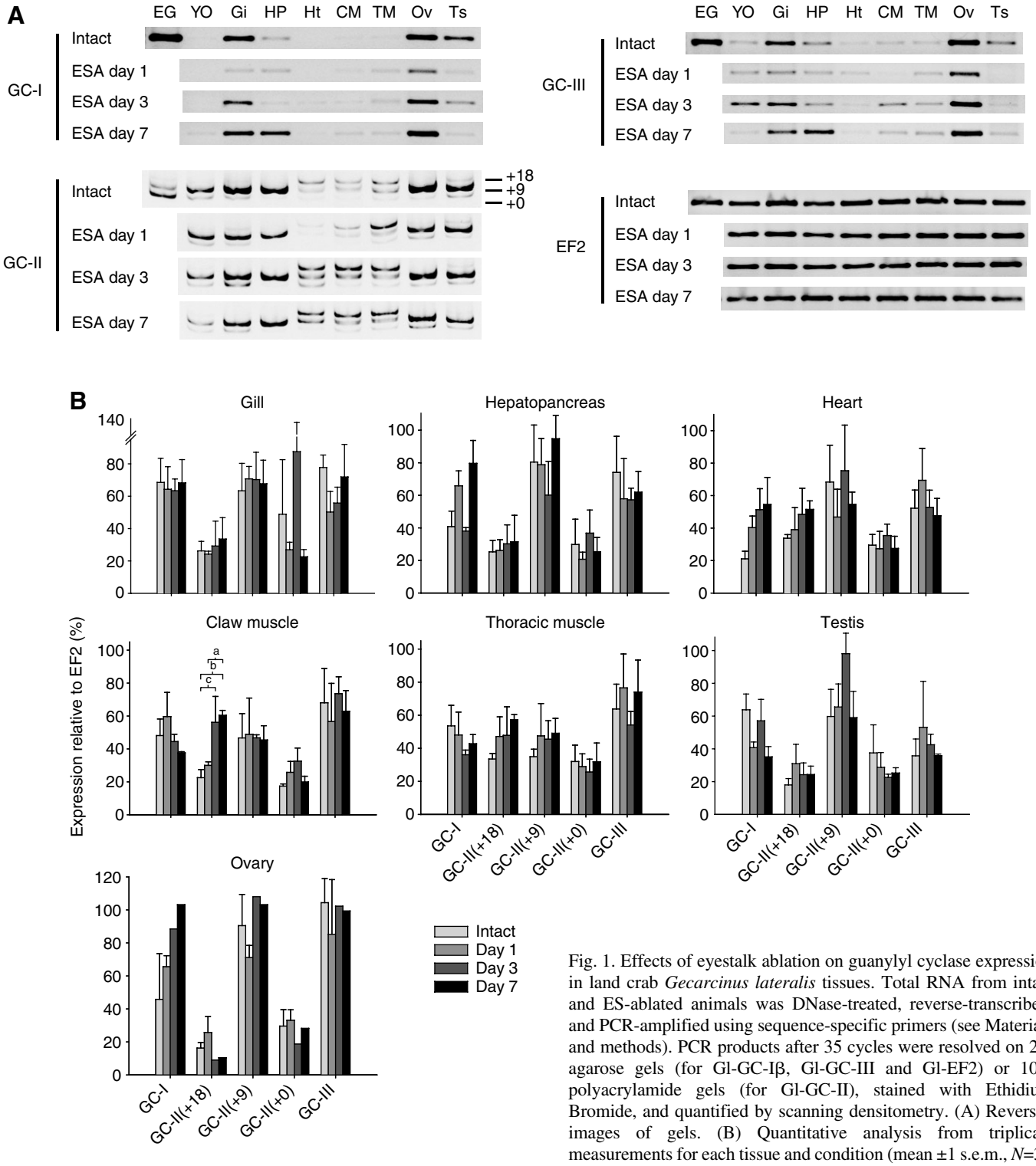

Fig. 1. Effects of eyestalk ablation on guanylyl cyclase expression in land crab *Gecarcinus lateralis* tissues. Total RNA from intact and ES-ablated animals was DNase-treated, reverse-transcribed, and PCR-amplified using sequence-specific primers (see Materials and methods). PCR products after 35 cycles were resolved on 2% agarose gels (for Gl-GC-Iβ, Gl-GC-III and Gl-EF2) or 10% polyacrylamide gels (for Gl-GC-II), stained with Ethidium Bromide, and quantified by scanning densitometry. (A) Reversed images of gels. (B) Quantitative analysis from triplicate measurements for each tissue and condition (mean ±1 s.e.m., N=3). Both Gl-GC-1β (Δ0N and Δ32N isoforms were not distinguished; primers were directed to a common sequence in the C terminus) and Gl-GC-III were expressed at high levels in ES ganglia (EG), gill (Gi), ovary (Ov) and testis (Ts) from intact animals. Tissues from intact animals differed in relative expression of the three Gl-GC-II isoforms. Gl-GC-II(+9) was expressed at varying levels in all tissues and was the dominant isoform in Y-organ (YO), gill, hepatopancreas (HP) and gonad (Ov and Ts). Gl-GC-II(+18) was expressed at highest levels in heart (Ht), claw muscle (CM) and thoracic muscle (TM). Gl-GC-II(+0) was expressed at low levels in most tissues except ES ganglia, in which it was the major isoform. ES ablation had no significant effect on Gl-GC-Iβ (GC1), Gl-GC-II (GC2) and Gl-GC-III (GC3) expression in most tissues (B). The only exception was the increased expression of the Gl-GC-II(+18) isoform in claw muscles from 3 days and 7 days post-ESA animals. Gl-EF2 was expressed at similar levels in all tissues. Significant differences between means were determined by one-way ANOVA *post-hoc* test (Fisher's PLSD); P values are indicated with brackets (a, P<0.034; b, P<0.013; c, P<0.023).

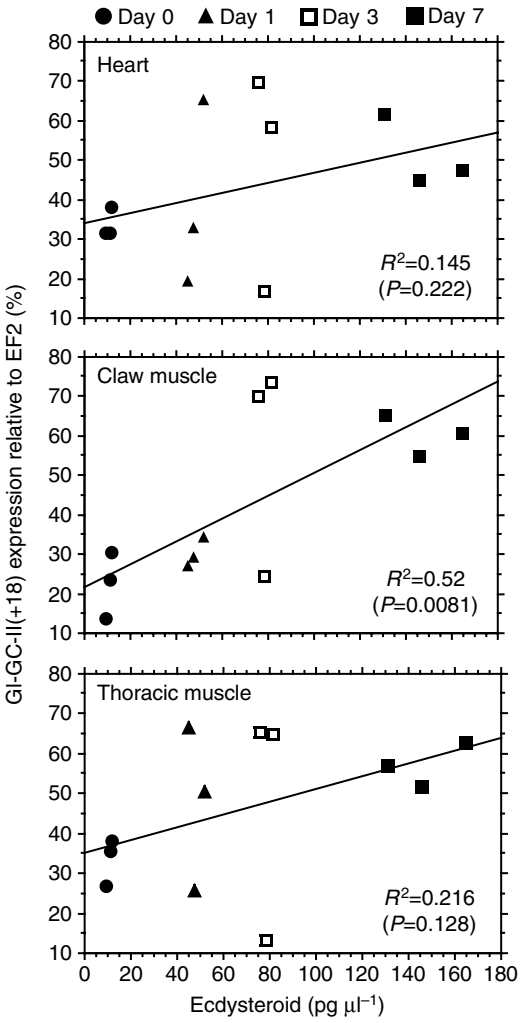

Fig. 2. Regression analysis of Gl-GC-II(+18) isoform expression in striated muscles as a function of ecdysteroid concentration in ES-ablated land crabs. Gl-GC-II(+18) was significantly correlated with hemolymph ecdysteroid concentration in claw muscle, but not in thoracic muscle or heart.

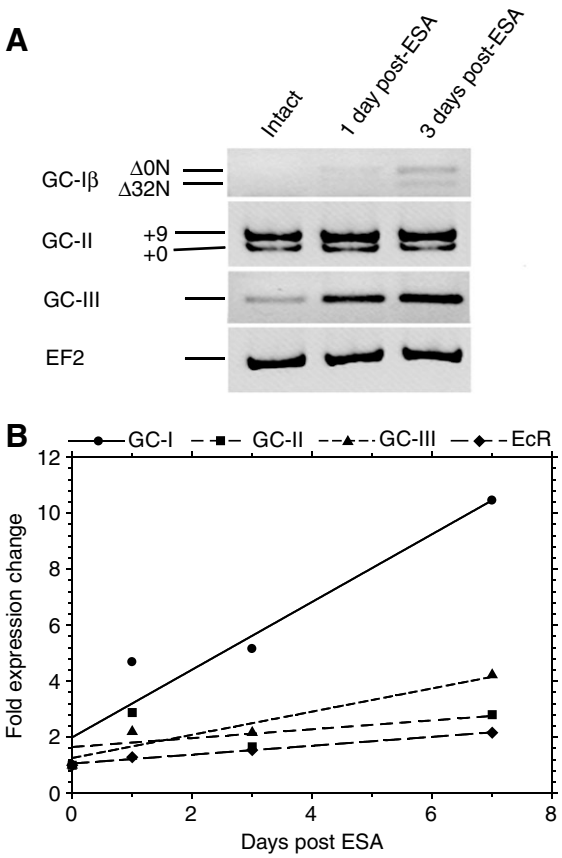

Fig. 3. Effects of eyestalk ablation (ESA) on guanylyl cyclase and ecdysone receptor expression in the Y-organ of land crabs. Total RNA from intact and ES-ablated animals was DNase-treated, reverse-transcribed and PCR-amplified using sequence-specific primers (see Materials and methods). (A) PCR products after 35 cycles were resolved on a 2% agarose gel (for Gl-GC-Iβ, Gl-GC-III and Gl-EF2) or a 10% polyacrylamide gel (for Gl-GC-II) and stained with Ethidium Bromide (reversed images). ESA increased mRNA levels of the two Gl-GC-Iβ isoforms (Δ0N and Δ32N) and Gl-GC-III, but had no effect on mRNA levels of Gl-GC-II(+9), Gl-GC-II(+0) and Gl-EF2. (B) Real-time RT-PCR quantification of Gl-GC-1β, Gl-GC-II, Gl-GC-III and EcR normalized to Gl-EF2. The Gl-EF2 transcript copy numbers were 3.58×10^8 at Day 0, 2.15×10^8 at Day 1, 1.81×10^8 at Day 3 and 1.56×10^8 at Day 7 (The amount of cDNA from intact animals was doubled in the PCR to compensate for low mRNA levels of Gl-GC-Iβ and Gl-GC-III; see Materials and methods). The data are presented as the fold difference with respect to transcript levels in YOs from intact animals (0 day post-ESA). The transcript copy numbers in intact YOs before normalization were 1.01×10^{10} for Gl-GC-Iβ, 1.30×10^8 for Gl-GC-II, 3.47×10^{10} for GC-III and 5.00×10^5 for Gl-EcR. Gl-GC-Iβ, Gl-GC-III and Gl-EcR mRNA levels increased ~tenfold, ~fourfold and ~twofold, respectively, by 7 days post-ESA, whereas Gl-GC-II level was not significantly correlated with days post-ESA.

hemolymph (Table 3), were determined on GC expression in various tissues. RT-PCR from a single set of animals suggested that GC expression was responsive to ESA (Fig. 1A). However, analysis of tissues from 3 sets of animals (N=3 for each tissue at each time period) showed no significant effect of ESA in most tissues (Fig. 1B). The only exceptions were the expression of the Gl-GC-II(+18) isoform in claw muscle and Gl-GC-Iβ Δ0N and Δ32N isoforms and GC-III in YO. Gl-GC-II(+18) expression increased about threefold in 3- and 7-day post-ESA animals (Fig. 1B). Moreover, Gl-GC-II(+18) mRNA level was significantly correlated with ecdysteroid concentration in claw muscle, but not in heart or thoracic muscle (Fig. 2). The expression of the Gl-GC-Iβ Δ0N and Δ32N isoforms and Gl-GC-III in YO increased in response to ESA, while the expression of the Gl-GC-II(+0) and Gl-GC-II(+9) isoforms and Gl-EF2 remained unchanged (Fig. 3A).

Real-time RT-PCR was used to quantify more accurately the effect of ESA on the mRNA levels of the three GCs and EcR in the YO. The method did not distinguish the different Gl-GC-Iβ and Gl-GC-II isoforms, as primers targeted to the variant sequences were unsuitable. Consequently, the data constitute the sum of all the transcripts encoding either Gl-GC-Iβ or Gl-GC-II. ESA had the largest effect on Gl-GC-Iβ, increasing the mRNA level 10.45-fold after 7 days post-ESA (Fig. 3B; R^2=0.925; P=0.0381). Gl-GC-III and Gl-EcR mRNA levels

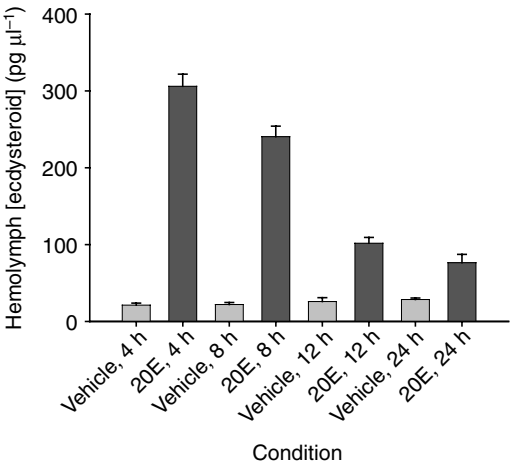

Fig. 4. Effect of 20-hydroxyecdysone (20E) injection on hemolymph ecdysteroid concentration. Intact land crabs were injected with 20E or vehicle (10% ethanol) and sampled at 4 h, 8 h, 12 h and 24 h after injection. Ecdysteroid was quantified with radioimmunoassay. 20E caused a large, transient increase in ecdysteroid concentration in the hemolymph (values are mean ± 1 s.e.m., N=10).

increased 4.23-fold (R^2=0.915; P=0.0435) and 2.16-fold (R^2=0.988; P=0.0060), respectively (Fig. 3B). In contrast, Gl-GC-II mRNA level was not significantly affected by ESA (Fig. 3B; R^2=0.288; P=0.4629). These results are consistent with those from the RT-PCR after 35 cycles (Fig. 3A).

ESA removes the primary source of MIH, CHH and other neuropeptides; the increase in hemolymph ecdysteroid is a secondary response to reduced MIH level. Therefore, an injection of a single dose of 20E was used to determine the direct effects of ecdysteroid in intact animals. Animals were injected with 20E or vehicle (10% ethanol) and tissues were collected at 4 h, 8 h and 12 h after injection. The 20E injection caused a transient elevation of ecdysteroid levels in the hemolymph, whereas the vehicle had no effect (Fig. 4). GC mRNA levels in hepatopancreas, testis, claw muscle and thoracic muscle were determined by RT-PCR analysis. Injection of 20E did affect GC expression in the four tissues (Fig. 5). In general, if there was an effect on a particular GC, it was usually an initial reduction in mRNA levels at high hemolymph 20E concentrations (4 h post-20E injection) followed by a return to pre-injection levels at 8 h and 12 h post-injection. The only exceptions were a significant increase in GC-Iβ mRNA in hepatopancreas 12 h post-injection and continued suppression of Gl-GC-II(+0) mRNA in claw muscle at 8 h and 12 h post-injection (Fig. 5). The level of Gl-GC-Iβ mRNA was lowered or remained low at 4 h post-20E injection in hepatopancreas, testis, claw muscle and thoracic muscle. There were significant decreases in Gl-GC-II(+9) mRNA at 4 h in the testis and in Gl-GC-II(+0) mRNA at 4 h in hepatopancreas and thoracic muscle. The response of Gl-GC-II(+18) to 20E injection was similar in claw and thoracic muscles, although the decrease at 4 h post-injection was only significant in claw muscle. There was no significant effect of 20E on Gl-GC-III expression in all four tissues (Fig. 5).

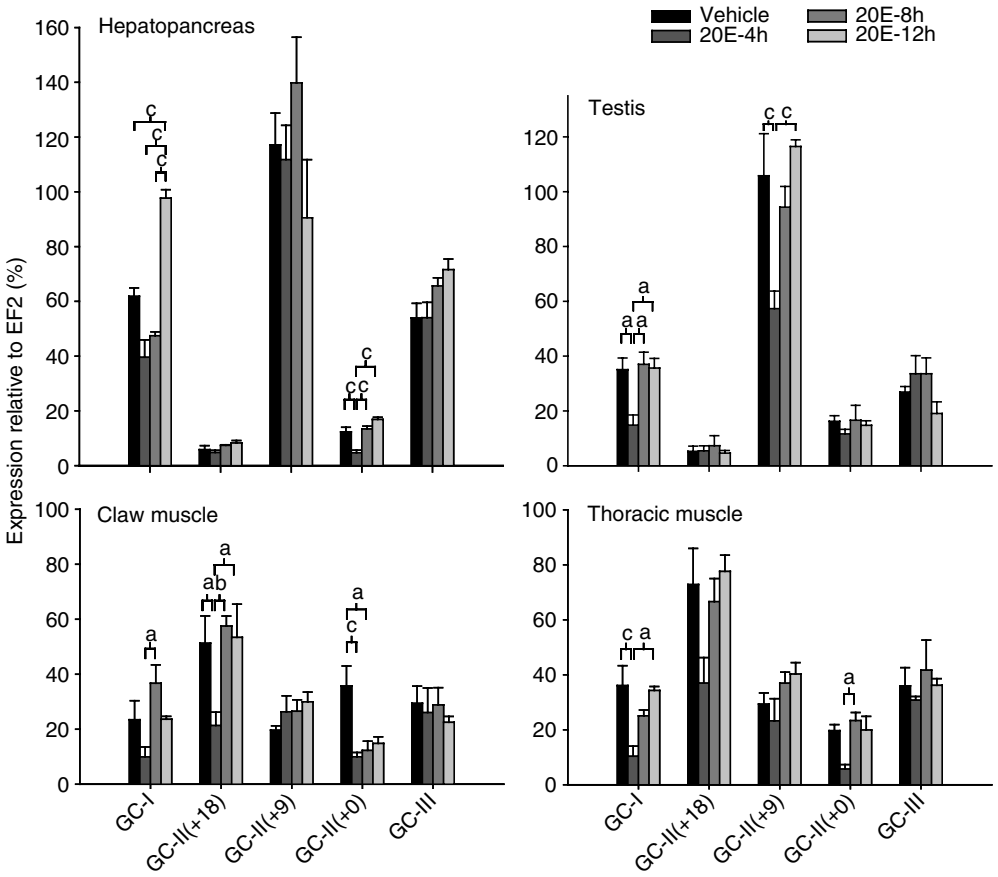

Fig. 5. Effects of 20-hydroxyecdysone (20E) injection on expression of guanylyl cyclases in hepatopancreas, testis, claw muscle and thoracic muscle. Intact land crabs were injected with 20E or vehicle (10% ethanol) and tissues collected at 4 h, 8 h and 12 h after injection. Total RNA was DNase-treated, reverse-transcribed and PCR-amplified using sequence-specific primers (see Materials and methods). PCR products after 35 cycles were resolved on 2% agarose gels (for Gl-GC-Iβ, Gl-GC-III and Gl-EF2) or 10% polyacrylamide gels (for Gl-GC-II), stained with Ethidium Bromide, and quantified by scanning densitometry. GC expression was normalized to Gl-EF2 (values are mean ± 1 s.e.m., N=3). Significant differences between means, obtained by one-way ANOVA *post-hoc* test (Bonferroni–Dunn), are indicated with brackets (a, P<0.05; b, P<0.01; c, P<0.002).

Discussion

Invertebrate GCs are grouped into four classes, designated GC-I, -II, -III and -IV (Lee et al., 2007; Morton and Simpson, 2002), and carry out diverse physiological functions in arthropods (Morton and Hudson, 2002). In crustaceans, GCs are essential components of signal transduction pathways that regulate nerve and muscle activity, molting, carbohydrate metabolism, and ion and water balance (Fanjul-Moles, 2006; Goy, 2005; Scholz, 2001; Spaziani et al., 1999). Our particular interest is the role of GCs in MIH and CHH signaling in the crustacean YO; both neuropeptides inhibit molting by suppressing YO ecdysteroidogenesis (Chung and Webster, 2003; Webster and Keller, 1986). Thus, entry into premolt is correlated with reduced MIH synthesis in the eyestalk and a corresponding increase in hemolymph ecdysteroid (Chen et al., 2007; Lee et al., 1998). Various environmental stressors inhibit molting, which may be mediated by elevated CHH in the hemolymph (Chang et al., 1998; Chung and Webster, 2003; Chung and Webster, 2005).

CHH increases cGMP levels in target tissues by way of a membrane receptor GC (Chung and Webster, 2003; Goy, 1990; Goy et al., 1987; Pavloff and Goy, 1990; Scholz et al., 1996; Sedlmeier and Keller, 1981). Given its pleiotropic effects, one would expect the CHH receptor to be expressed in all tissues. Crayfish, blue crab and land crab membrane receptor GCs are widely expressed (Liu et al., 2004; Zheng et al., 2006) (Fig. 1). Moreover, the Gl-GC-II(+9) is the dominant isoform in hepatopancreas and YO (Fig. 1), both of which have high-affinity CHH receptors (Chung and Webster, 2003; Kummer and Keller, 1993; Webster, 1993). The different Gl-GC-II isoforms may confer tissue-specific responses to CHH isoforms that differ in biological activities (Bulau et al., 2003; Bulau et al., 2005; Chung et al., 1999; Chung et al., 1998; Dircksen et al., 2001; Gu and Chan, 1998; Marco et al., 2003; Serrano et al., 2003; Soyez et al., 1994; Yang et al., 1997; Yasuda et al., 1994).

The identity of the MIH receptor remains controversial. YO membranes have distinct receptors for both CHH and MIH (Chung and Webster, 2003; Webster, 1993). As MIH and CHH are members of the same neuropeptide family, it seems reasonable to expect that they would be ligands for related membrane receptors. While there is data supporting the CHH receptor as a GC-II, the data do not support the MIH receptor as another GC-II. Only one GC-II has been isolated in crustacean tissues and its wide tissue distribution is not consistent with its role as a MIH receptor (Lee et al., 2007; Liu et al., 2004; Zheng et al., 2006). As MIH-binding capacity is highest in YO membranes (Asazuma et al., 2005), a receptor for MIH would be expected to be expressed preferentially in the YO. This is not the case for any GC-II isoform (Fig. 1). Moreover, cross-linking prawn ^{125}I-MIH with YO membrane labeled a ~70 kDa protein (Asazuma et al., 2005), which is about one-half the estimated nonglycosylated masses of the membrane GC cDNAs from crayfish, blue crab and land crab (Lee et al., 2007; Liu et al., 2004; Zheng et al., 2006). The actual mass of the GC-II is probably larger, due to extensive N-linked glycosylation in the extracellular domain, which is necessary for ligand binding and activation (Padayatti et al., 2004). Finally, the increase (~tenfold) of Gl-GC-Iβ but not Gl-GC-II in YOs of ES-ablated animals (Fig. 3B) supports the hypothesis that a NO-sensitive GC, and not a membrane receptor GC, mediates MIH signaling. Although these data suggest that the MIH receptor is not a membrane GC, the identity of the MIH receptor will not be resolved until the protein is isolated and characterized fully.

As MIH signaling in the YO involves both cAMP and cGMP as secondary messengers (Baghdassarian et al., 1996; Böcking and Sedlmeier, 1994; Chung and Webster, 2003; Mattson and Spaziani, 1985; Mattson and Spaziani, 1986; Saïdi et al., 1994; Sedlmeier and Fenrich, 1993; Spaziani et al., 1999; Von Gliscynski and Sedlmeier, 1993), we propose a pathway involving a G protein-coupled MIH receptor, adenylyl cyclase, NOS and GC-I (Kim et al., 2004; Lee and Mykles, 2006). YOs express a trimeric G protein (Han and Watson, 2005), NOS (Kim et al., 2004) and NO-sensitive GC (Figs 1, 3). Activators of adenylyl cyclase inhibit YO ecdysteroidogenesis (Mattson and Spaziani, 1985; Mattson and Spaziani, 1986; Saïdi et al., 1994; Sedlmeier and Fenrich, 1993). MIH stimulates cGMP-dependent and cAMP-dependent kinases (Baghdassarian et al., 1996; Böcking and Sedlmeier, 1994; Spaziani et al., 1999; Von Gliscynski and Sedlmeier, 1993). This pathway is similar to that inhibiting ecdysteroidogenesis in blowfly ovary, except that cAMP and cGMP are antagonists. Stimulation of ecdsteroid secretion by cAMP is blocked by cGMP (Maniere et al., 2003). A cGMP analog and phosphodiesterase inhibitors (IBMX and MDBAMQ) suppress ecdysteroid secretion by previteollogenic and vitellogenic ovaries *in vitro* (Maniere et al., 2003). Moreover, sodium nitroprusside, a NO donor, inhibits ecdysteroid secretion (Maniere et al., 2003). Reduced ecdysteroid secretion results from the activation of protein kinase G (PKG), as KT 5823, a specific PKG inhibitor, increases secretion by previteollogenic and vitellogenic ovaries (Maniere et al., 2003). Taken together, these data suggest an inhibitory pathway involving NOS and GC-I in blowfly ovary. Zheng et al. (Zheng et al., 2006) report unpublished data that sodium nitroprusside has no effect on ecdsteroid secretion on blue crab YOs *in vitro*. However, negative results are difficult to interpret without knowing if the NO donor actually raised intracellular cGMP levels. NO is highly unstable and may have been metabolized before reaching levels needed to activate the GC-I. Our data from land and green crab YOs show that NO donors inhibit ecdysteroidogenesis *in vitro* and will be included in a separate publication.

NO-sensitive GC has a more restricted tissue distribution than membrane receptor GCs. As NO is rapidly metabolized, it must be produced at or near the target tissue. Thus, it is not surprising that the tissue expression is similar between NOS and GC-Iβ. Both NOS and GC-I are highly expressed in nervous tissues of arthropods and mammals (Aonuma, 2002; Collmann et al., 2004; Mahadevan et al., 2004; Nighorn et al., 1998; Prabhakar et al., 1997; Scholz et al., 1996; Scholz et al., 2002). Lobster tissues with significant NO-sensitive GC activity are intestine, hepatopancreas, nerve cord, heart and gill (Prabhakar et al., 1997). In land crab, NOS and GC-Iβ are co-expressed in ES ganglia, thoracic ganglion, gill, ovary, YO and testis (Kim et al., 2004) (Fig. 1).

Little is known about the functions of the ‘atypical’ GCs. Only two GC-III cDNAs (Gl-GC-III and Ms-GC-I from land

crab and hawk moth, respectively) and one GC-IV cDNA (Ms-GC-β3 from hawk moth) have been characterized. Ms-GC-I, which is expressed in the nervous system, has high NO-insensitive GC activity (Nighorn et al., 2001; Simpson et al., 1999). Although it lacks a transmembrane domain, it is associated with membranes *in vivo* (Nighorn et al., 2001; Simpson et al., 1999). Ms-GC-β3 has a specialized function in the nervous system of holometabolous insects; it is activated by eclosion hormone, which triggers the sequence of behavioral movements during pupal ecdysis (Morton and Simpson, 2002). The tissue distribution of Gl-GC-III is similar to that of Gl-GC-Iβ, as its highest expression is in ES ganglia, gill, hepatopancreas, ovary, YO and testis (Fig. 1). Moreover, ES ablation upregulates Gl-GC-III in the YO (Fig. 3). This coordinated expression suggests that Gl-GC-III augments NO signaling in target tissues.

The activation of ecdysteroid-responsive genes regulates development, reproduction, growth and molting in arthropods (Chang, 1993; Kozlova and Thummel, 2000; Skinner, 1985; Truman and Riddiford, 1999). Ecdysteroid regulates crustacean gene expression through a heterodimeric nuclear hormone receptor consisting of ecdysone receptor (EcR) and retinoic-X receptor (RXR) (Durica et al., 2002; Wu et al., 2004). Gl-GC-II(+18) mRNA increased in claw muscle in response to ESA and was correlated with hemolymph ecdysteroid, but not in heart or thoracic muscle (Fig. 1B, Fig. 2). This differential response to ES ablation is consistent with a previous study showing that ESA stimulates expression of EcR and calpain T in claw muscle specifically (Kim et al., 2005a). The different sensitivities of claw and thoracic muscles may be due to differences in the expression of RXR isoforms, which dimerize with EcR to form ecdysteroid receptors that differ in ligand and DNA binding properties (Kim et al., 2005b; Wu et al., 2004).

YO activation coincides with an upregulation of the soluble GCs. ESA of intermolt animals induced large increases in Gl-GC-Iβ (~tenfold) and Gl-GC-III (~fourfold) mRNAs in YOs (Fig. 3B). There is also a ~twofold increase in EcR mRNA (Fig. 3B). These data suggest that the YO responds to the absence of neuropeptide(s) by increasing its sensitivity to hormones. The YOs of other decapods display altered responses to ecdysteroid and neuropeptides over the molt cycle (Chung and Webster, 2003; Dell et al., 1999; Nakatsuji and Sonobe, 2004). YOs from premolt animals are refractory to MIH, even though MIH receptor binding remains unchanged (Chung and Webster, 2003; Nakatsuji and Sonobe, 2004; Nakatsuji et al., 2006). This reduced sensitivity in premolt is correlated with an increased capacity to degrade cGMP, which keeps cGMP levels low when MIH is present. YOs from premolt crayfish have increased phosphodiesterase activity (Nakatsuji et al., 2006). This is correlated with reduced MIH- or CHH-induced cGMP levels in YOs from premolt green crabs (Chung and Webster, 2003). A general conclusion one can make from these data is that the YOs from intermolt animals are more responsive to MIH and CHH than YOs from premolt animals. Moreover, the significance of the land crab data (Fig. 3) is that the intermolt YO is capable of increasing cGMP-mediated signaling in the absence of ES neuropeptides.

As ESA removes the major site for the synthesis of MIH, CHH and other neuropeptides, the response of tissues may be due to reduced ES factors, increased ecdysteroid, or a combination of both. 20E was injected into intact animals to determine the direct effects of ecdysteroid on GC expression in hepatopancreas, testis, claw muscle and thoracic muscle. A common pattern for those GCs showing significant changes was a reduced or low mRNA level at 4 h post-injection followed by restored or increased levels at 8 h and 12 h post-injection. These data suggest that high ecdsyteroid levels, which were comparable to those at the end of the premolt period (Skinner, 1985), can repress expression of Gl-GC-Iβ in all four tissues and certain Gl-GC-II isoforms in certain tissues (Fig. 5).

GCs mediate neuropeptide control of various physiological processes in arthropods. The expression of the three GC classes and NOS (Kim et al., 2004) in crustacean tissues other than the nervous system suggests that cGMP has functions in addition to neuromodulation. The inhibition of YO ecdysteroidogenesis by both MIH and CHH is associated with large increases in intracellular cGMP (Chung and Webster, 2003; Saïdi et al., 1994). It appears that the two neuropeptides accomplish this using two different cGMP-mediated signaling pathways. CHH acts directly *via* binding to a membrane receptor GC (Goy, 1990; Scholz et al., 1996). MIH may stimulate GC-I by activating NOS (Kim et al., 2004; Lee and Mykles, 2006). The two methods (ESA and 20E injection) used to increase hemolymph ecdysteroid levels elicited different responses by tissues. ESA increased Gl-GC-II(+18) in claw muscle, suggesting it has a role in molt-induced atrophy (Mykles, 1997). ESA also increased Gl-GC-Iβ, Gl-GC-III and EcR expression in the YO, suggesting that the tissue is compensating for low ES neuropeptide levels in the hemolymph. Ecdysteroid (20E) at high doses generally had a transient inhibitory effect on the expression of Gl-GC-Iβ and some Gl-GC-II isoforms, most likely resulting from the rapid clearance of 20E from the hemolymph. These data indicate that there are interactions between ES neuropeptides and ecdysteroids that can affect the expression of GCs in crustacean tissues.

List of abbreviations

20E	20-hydroxyecdysone
cGMP	3′,5′-cyclic guanosine monophosphate
CHH	crustacean hyperglycemic hormone
EcR	ecdysone receptor
EG	ES ganglia
ES	eyestalk
ESA	ES ablation
GC	guanylyl cyclase
Gl-EF2	*Gecarcinus lateralis* elongation factor 2
MIH	molt-inhibiting hormone
NO	nitric oxide
NOS	NO synthase
PKG	protein kinase G
RT-PCR	reverse transcription polymerase chain reaction
YO	Y-organ
Δ32N / Δ0N	absence / presence of 32 amino acid sequences at N terminus of Gl-GC-Iβ

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