Expression of a crustacean hyperglycemic hormone isoform in the shore crab, *Pachygrapsus marmoratus*, during adaptation to low salinity

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In Crustacea, neurosecretory cells that produce crustacean hyperglycemic hormone (CHH) have been located in different endocrine and neuroendocrine tissues like the X-organ/sinus gland complex (XO/SG) in the eyestalk, the pericardial organ (PO) around the heart and the gut. Aspects of the isolation, structural characterization, and functions of the neuropeptide CHH have been extensively studied in various species of Crustacea [1, 11]. Initially known as a hyperglycemic factor, that led to the appellation of the peptide, CHH is also involved in inhibition of ecdysteroid synthesis (i.e. in the molting process) and of methyl farnesoate synthesis, in stimulation of oocyte development, in lipid metabolism, and in secretion of digestive enzymes. Interestingly, studies have emphasized that CHH also plays a direct or indirect role in crustacean osmoregulation [7].

In crabs, different CHH sequence isoforms resulting from a splicing process have been described in neurohemal organs like the sinus gland and the pericardial organ [2, 9]. One of these isoforms, the so-called PO-type or unspliced form of CHH, exhibits the addition of about one hundred base pairs in the cDNA sequence compared to the classical SG-type CHH. This longer isoform could contribute to the control of hydromineral regulation [10]. In the crab *Carcinus maenas*, the PO-type CHH indeed appears to regulate neither hemolymph glycemia nor ecdysteroid synthesis, that are known functions of CHH [2], but could play a role in osmoregulating mechanisms [10]. These observations prompted us to examine whether PO-type CHH mRNA expression might change in relation to environmental variation of salinity. This study was conducted on the hyper-hyporegulating crab *Pachygrapsus marmoratus* during short-term acclimation from 2 to 48h to low salinity, using real-time quantitative PCR. This work intends to contribute to the knowledge on the neuroendocrine control of osmoregulation in crustaceans, and more especially on the involvement of CHH isoforms.

XO/SG complex and PO/heart were isolated from crabs adapted to seawater (SW, 36ppt) and to diluted seawater (DSW, 10ppt) and they were immediately preserved in RNA*later* (Ambion). These dissections were conducted on two sets of organs collected in 2003 and in 2004. Total RNA was purified from both type of organs isolated from 4 animals per sample with the RNAgents Total RNA Isolation System (Promega). The purified RNA was then analyzed for integrity and quantified using the Agilent Technologies 2100 Bioanalyzer. A normalized quantity of poly-A mRNA was reverse transcribed using oligo-dT primer and the SuperScript II reverse transcriptase (Invitrogen). The resulting cDNAs were then amplified by polymerase chain reactions performed with specific primers designed according to the sequence of the unspliced PO-type CHH [9]. PCR products were purified and prepared for direct sequencing on an ABI Prism 3100 automated sequencer (MDIBL). In real-time quantitative PCR (RT-QPCR), PO-type CHH cDNAs, analyzed in 1-µl triplicate aliquots, were amplified in the presence of Stratagene Brillant SYBR Green Master Mix using the Startagene MX4000 Multiplex Quantitative PCR System. To quantify relative mRNA expression, one of the test samples expected to yield high mRNA levels was used as the basis for comparison. An internal control (i.e. a "housekeeping" gene), arginine kinase, was amplified in the same templates using identical conditions of RT-QPCR.

Figure 1 represents the kinetics of mRNA expression of the CHH unspliced form (PO-type) in the PO/heart and the XO/SG complex from crabs adapted for a short-term to 10ppt. In XO/SG complex, results indicated that the expression of CHH unspliced form increased rapidly 2h after tranfer to low salinity. It has more than doubled after 4h and it was very significantly enhanced 6h after transfer in both 2003 and 2004 XO/SG samples ($p<0.0001$, ANOVA and Fisher's multiple-range LSD *post hoc* test). The expression returned to the initial level in SW (t=0) 2 days after transfer. We can notice that the PO-type CHH is really less expressed in PO/heart samples compared to the XO/SG complex samples. However, the PO-type CHH expression in PO/heart increased very significantly 6h after transfer from seawater to dilute medium in 2003 samples (from 0.04 ± 0.03 to 1.08 ± 0.17, $p<0.0001$) as well as in 2004 samples (from 0.19 ± 0.03 to 1.15 ± 0.23, $p<0.0001$), but PO/heart data in 2004 appears less impressive because of the scale of the graph due to the dramatic increase in XO/SG data. Moreover, the graphics indicate similar profile of results between the two groups of samples from 2003 and 2004 demonstrating reproducible experiments.

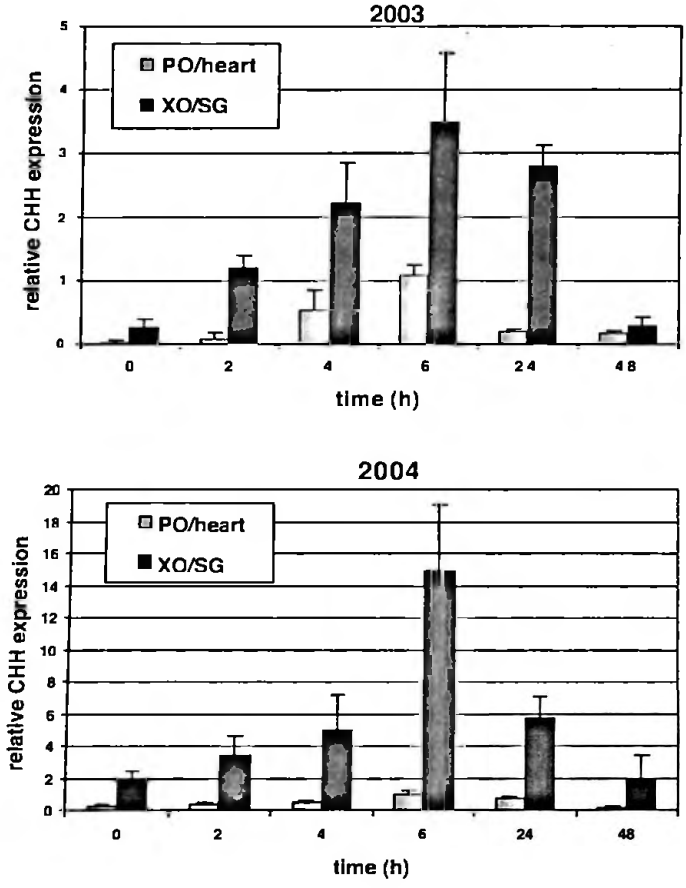

Fig. 1. Relative abundance of PO-type CHH mRNA from XO/SG complex and PO/heart samples from 2003 and 2004 at various intervals following transfer of the crab *Pachygrapsus marmoratus* from seawater (t=0) to diluted seawater of 10ppt (t=2 to 48h). Organs from 4 animals were pooled for each time point and relative mRNA expression was measured by real-time quantitative PCR in triplicate. cDNA transcribed from RNA of PO/heart at 6h exposure to 10ppt salinity served as the reference standard. Results are expressed as mean ± S.D. and statistically analyzed with analysis of variance and Fisher's multiple-range least significant difference *post hoc* test.

The mRNA expression of arginine kinase remained very stable and the variations were not significantly different from 2h to 48h after transfer to low salinity. The relative mRNA level varied between 0.75 ± 0.07 at 2h to 0.83 ± 0.02 at 6h and to 0.62 ± 0.01 at 48h in PO/heart samples and was at 0.60 ± 0.03 in SW, while it stayed constant from 0.11 ± 0.01 at 2h to 0.13 ± 0.01 at 48h in XO/SG samples and was at 0.10 ± 0.01 in SW.

In crabs, it has initially been reported that PO extracts can stimulate Na^+/K^+-ATPase activity and Na^+ uptake in isolated gills [3, 5]. Purified CHH from the sinus gland, containing the spliced and unspliced forms of CHH, was also shown to stimulate Na^+ influx in perfused gills of the crab *P. marmoratus* [8]. We have mentioned above that PO-type CHH seems not involved in functions such as glucose regulation or ecdysteroid synthesis, but the present results indicate a relation between PO-type CHH and salinity adaptation as the expression of this isoform of CHH strongly increases following tranfer to low salinity. In Astacidae Crustacea, including lobster and crayfish, a CHH polymorphism has been reported resulting from the post-translational isomerization of the phenylalanine residue on the third position of the N-terminus end from a L- to a D-configuration [6]. It has recently been demonstrated that the D-CHH isomer is more particularly effective on osmoregulatory parameters in this group of Crustacea [4]. These observations combined with the present result, suggest a differential specificity of CHH according to the isoforms and the groups of Crustacea.

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