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Characterization of a tachykinin signalling system in the bivalve mollusc *Crassostrea gigas*

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ABSTRACT

Although tachykinin-like neuropeptides have been identified in molluscs more than two decades ago, knowledge on their function and signalling has so far remained largely elusive. We developed a cell-based assay to address the functionality of the tachykinin G-protein coupled receptor (Cragi-TKR) in the oyster *Crassostrea gigas*. The oyster tachykinin neuropeptides that are derived from the tachykinin precursor gene Cragi-TK activate the Cragi-TKR in nanomolar concentrations. Receptor activation is sensitive to Ala-substitution of critical Cragi-TK amino acid residues. The Cragi-TKR gene is expressed in a variety of tissues, albeit at higher levels in the visceral ganglia (VG) of the nervous system. Fluctuations of Cragi-TKR expression is in line with a role for TK signalling in *C. gigas* reproduction. The expression level of the Cragi-TK gene in the VG depends on the nutritional status of the oyster, suggesting a role for TK signalling in the complex regulation of feeding in *C. gigas*.

1. Introduction

Tachykinins (TKs) represent a large family of evolutionarily conserved brain/gut peptides in bilaterian animals. In mammals, the TK peptide family derives from alternate processing of three TAC genes (Steinhoff et al., 2014) (for review). TAC1 encodes substance P (SP), neurokinin A (NKA) as well as neuropeptide K (NPK) and neuropeptide γ (Npγ) (Carter et al., 1990). TAC3 (designated as TAC2 in rodents) only encodes neurokinin B (NKB) (Kotani et al., 1986). A third gene, TAC4 encodes endokinins A, B, C and D (EKA-D) as well as hemokinin-1 (HK-1) (Page et al., 2003). These genes are conserved from mammals to teleosts (Zhou et al., 2012) and a gene encoding two TK peptides was also characterized in the urochordate *Ciona intestinalis* (Satake et al., 2004). Outside the chordate phylum, TKs have also been characterized in insects, crustaceans, molluscs and annelids (Severini et al., 2002; Satake et al., 2003) (for review).

Chordate TK sequences display the conserved C-terminal pentapeptide signature FXGLM-amide, whereas protostome TKs share the C-terminal consensus sequence FX_1GX_2R-amide. Interestingly, some vertebrate-type TKs, derived from a distinct gene, have been identified in the salivary glands of cephalopod molluscs (Anastasi and Erspamer, 1963; Kanda et al., 2003) and insects (Champagne and Ribeiro, 1994) serving respectively as neurotoxins (Ruder et al., 2013) and as vasodilatory agents that act on vertebrate prey TK receptors (TKR) but not on endogenous receptors (Kanda et al., 2007).

TKs are widely distributed in the nervous systems of all bilaterian animal species. They have been shown to display regulatory roles in an extraordinarily diverse range of physiological processes. In addition to their modulatory role in the central control of respiration and cardiovascular activity, TKs, mainly via SP, also mediate pain, anxiety and motor coordination in the CNS of mammals (Vanden Broeck et al., 1999; Khawaja and Rogers, 1996). In arthropods, TKs are involved in odour perception and locomotion as shown in *Drosophila* (Winther et al., 2006) and in visual processing as suggested in crustaceans (Glantz et al., 2000). In bilateria, TKs have been shown to participate in the control of the activity of a wide array of peripheral organs and tissues. *In vitro* studies on organ preparations of protostome species suggest that TK signalling plays a role in the regulation of gut activity and visceral and skeletal muscle contractions (Palamiuc et al., 2017; Schoofs et al., 1990; Schoofs et al., 1990; Ikeda et al., 1993). Deficient TK functioning contributes to multiple disease processes in humans (Steinhoff et al., 2014).

In contrast to Ecdysozoa (Van Loy et al., 2010), which comprises arthropods and nematodes as major phyla, TK signalling has so far been largely unexplored in Lophotrochozoa, the protostome sister group of the Ecdysozoa. Only two studies, respectively in *Octopus* (Kanda et al., 2007) and in the worm *U. unitinctus* (Kawada et al., 2002), reported on the identification of a TKR in Lophotrochozoa. In bivalve molluscs, TK

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peptides have been molecularly characterized more than two decades ago in the mussel *Anodonta cygnea* (Fujisawa et al., 1993) and more recently in the oyster *Crassostrea gigas* (Stewart et al., 2014). The recent development of an extended transcriptomic database of *C. gigas* (Riviere et al., 2015) offers the opportunity to characterize neuropeptide receptors and thus establish their physiological role(s). The present study reports on the characterization of a TKR in the oyster *C. gigas* and shows that it is functionally activated by oyster TKs. In addition, we investigated the structure-activity relationship of ligand-receptor pairs by assessing the potency of a series of synthetic TK analogues. In order to further explore TK signalling in *C. gigas*, we determined the expression patterns of the genes encoding the TK precursor and the TKR at successive reproduction stages as well as in distinct nutritional conditions.

2. Material and methods

2.1. Peptide synthesis

All peptides were custom synthesized by GeneCust (Luxemburg). The sequences of *C. gigas* peptides were obtained from an in-house peptide database yielded by mass spectrometry analyses of tissue extracts and data mining (Stewart et al., 2014).

2.2. In silico analyses

Multiple sequence alignment was performed with TKR from various species (supplementary Table 1) using Clustal W (Thompson et al., 1994). To determine the relationship between Cragi-TKR and TKRs from other species (supplementary Table 2), a phylogenetic tree was generated by the maximum likelihood method using the phylogeny pipeline (www.phylogeny.fr) (Dereeper et al., 2008) connecting the following programs: MUSCLE for multiple alignment (full processing mode), Gblocks for alignment curation (minimum length of a block after gap cleaning: 10, no gap positions allowed in the final alignment, all segments with contiguous non-conserved positions higher than 8 rejected, minimum number of sequences for a flank position: 85%), PhyML for phylogeny (the default substitution model was chosen assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data Model). The reliability of internal branches was evaluated using an approximate likelihood-ratio test (aLRT). TreeDyn was used for tree drawing.

2.3. Reverse endocrinology

2.3.1. Molecular cloning of the Cragi-TKR and transfection of mammalian cells

In silico screening of the oyster transcriptomic database “GigaTon” (Riviere et al., 2015) resulted in the identification of a full length cDNA encoding *Cragi-TK*R (CHOYP_LOC100744404.1.1). The CDS of the *Cragi-TK*R gene was amplified by PCR (Pfu DNA polymerase, Promega) using gene-specific sense primer (5′-CACCATGGAGGGGAACAATTCAA CAAAAG-3′) harbouring a Kozak consensus sequence and antisense primer (5′-TCATAAATATTCAGCACTAGTTCTCCGCCC-3′). Ten nanogram of plasmid DNA (Pal 17.3 vector, Evrogen) from a *C. gigas* “all developmental stages and adult central nervous system” directional and normalized cDNA library (Fleury et al., 2009) was used as template. The resulting PCR product was directionally cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) and the correct insertion confirmed by sequencing. Human embryonic kidney (HEK293T) cells were transiently transfected with the *Cragi-TK*R/pcDNA3.1 construct using Fugene HD (Promega) according to the manufacturer’s instructions. As a first step, co-transfection was done with an expression construct for the human $G\alpha_{16}$ subunit, a promiscuous G protein that can direct intracellular signalling of GPCRs to the release of calcium via the phospholipase C_β pathway, regardless of the endogenous G protein coupling of the receptor (Mertens et al., 2004). To assess receptor activity independent of $G\alpha_{16}$, calcium responses were measured in cells expressing only *Cragi-TK*R. Cells for negative control experiments were transfected with empty pcDNA3.1 and $G\alpha_{16}$/pcDNA3.1 constructs.

2.3.2. Calcium fluorescence assay:

Activation of *Cragi-TK*R by oyster TK synthetic peptides was monitored using a fluorescence-based calcium mobilization assay. Briefly, transfected HEK293T cells were loaded with Fluo-4 Direct plus probenecid (qsp 2.5 mM final in the cell) (Invitrogen/Molecular Probes) for 1 h (45 min at 37 °C and 15 min at room temperature). Excitation of the fluorophore was done at 488 nm. The calcium response was measured for 2 min at 525 nm using the FLEXstation 3 (Molecular Devices) at 37 °C. Data were analysed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} M. Concentration-response measurements of activating ligands were conducted in triplicate and for at least three independent experiments. Half maximal effective concentrations (EC_{50} values) were calculated from concentration-response curves that were constructed using nonlinear regression analysis with a sigmoidal dose-response equation using Prism 5.0 (GraphPad software, USA).

2.3.3. cAMP luminescence assay

Cragi-TKR transfected HEK 293 T cells were incubated with Glosensor cAMP reagent (qsp 4% final in the medium) (Promega) for 2 h at room temperature prior to the injection of the candidate ligands. cAMP luminescence response was measured for 30 min after injection using a FLEX station 3 (Molecular Devices) at room temperature. Data were analysed using SoftMax Pro (Molecular Devices). Candidate peptide ligands were first tested at a final concentration of 10^{-5} M.

2.4. Animals and tissue sampling

Two-year old adult oysters *C. gigas*, purchased from a local farm (Normandie, France), were used for peptide characterization and transcription analyses. Stages of reproduction (Stage 0: resting undifferentiated stage, Stage 1: gonial multiplication stage, Stage 2: maturation stage, Stage 3: sexual maturity) were determined by histological analysis of gonad sections as described previously (Rodet et al., 2005). To study the influence of trophic conditions, one-year-old adult oysters were reared in water tanks either in absence of food or in presence of *Isochrysis galbana* (clone T-Iso) maintained at a concentration of 6 million of cells/mL during 4 weeks. Adult tissues (mantle, gill, labial palps, digestive gland, gonad, hemolymph, adductor muscle) were sampled, the visceral ganglia (VG) were carefully dissected out, thus limiting any contamination from the adjacent adductor muscles. All the samples were either placed in TriReagent (Sigma) or stored at −80 °C until use. For expression studies, adult tissues or VG and gonads during gametogenesis from 6 animals were mixed to generate 5 pools of each tissue. Individual VG from 19 and 17 animals were used to study gene expression in fed and starved animals respectively.

2.5. Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR analysis was performed using the iCycler iQ© apparatus (Bio-Rad). Total RNA was isolated from adult tissues using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Recovered RNA was further purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment during 20 min at 37 °C with 1 U of DNase I (Sigma) to prevent genomic DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotidic primers (Promega), 0.5 mM dNTPs and 200 U MMuLV Reverse Transcriptase (Promega) at 37 °C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70 °C for 10 min. The GoTaq® qPCR Master Mix (Promega) was used for real time monitoring of