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Hauser, Frank; Al-Ribaty, Tara; Stebegg, Marisa; Thygesen, Gedske; Grimmelikhuijzen, Cornelis J.P.

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Cloning and deorphanization of three inotocin (insect oxytocin/vasopressin-like) receptors and their ligand from the tick *Ixodes scapularis*

Frank Hauser, Tara Al-Ribaty, Marisa Stebegg, Gedske Thygesen, Cornelis J.P. Grimmelikhuijzen

Section for Cell and Neurobiology, Department of Biology, University of Copenhagen, Universitetsparken 15, DK-2100, Copenhagen, Denmark

**A B S T R A C T**

Many insects produce the cyclic neuropeptide inotocin (CLITNCPGamide), which is the insect orthologue of the mammalian neuropeptides oxytocin and vasopressin. These insects also have one inotocin G protein-coupled receptor (GPCR), which is the orthologue of the mammalian oxytocin and vasopressin receptors. The tick *Ixodes scapularis* belongs to the subfamily Chelicerae, an arthropod taxon different from insects, to which also spiders, scorpions, and mites belong. *I. scapularis* is an ectoparasite and a health risk for humans, because it transfers pathogenic microorganisms to its human host during a blood meal, thereby causing serious neurological diseases, among them Lyme disease and tick-borne encephalitis (TBE). By annotating the genomic sequence of *I. scapularis*, we previously found one presumed tick inotocin preprohormone gene and, in contrast to insects, three genes coding for presumed inotocin GPCRs. We now find that these GPCR genes cluster on one genomic contig, suggesting that they originated by recent gene duplications. Closely located on the same contig are also four adipokinetic hormone/corazonin-related peptide (ACP) GPCR genes, and one crustacean cardioactive peptide (CCAP) GPCR gene, suggesting evolutionary relationships. These evolutionary relationships are confirmed by phylogenetic tree analyses of their gene products. We also cloned the tick inotocin preprohormone, which has a structural organization closely resembling mammalian oxytocin and vasopressin preprohormones, including the presence of a conserved neurophysin sequence, having seven cystine bridges. This neurophysin sequence has two cysteine-knot domains, but in contrast to mammalian neurophysins, the tick neurophysin contains a canonical prohormone convertase cleavage signal and a peptide C-terminal amidation sequence (GKR), suggesting cleavage into two biologically active cysteine-knot peptides. This cleavage/amidation sequence occurs in neurophysins from most hard tick species, but not in other chelicerates. Mature tick inotocin is different from insect inotocin and has the sequence CFITNCPPGamide. Finally, we cloned and stably expressed the three tick inotocin receptors in Chinese Hamster Ovary cells and found that each of them was activated by nanomolar concentrations of tick inotocin (EC\textsubscript{50} for ITR1 = 1.6 × 10\textsuperscript{-8} M; EC\textsubscript{50} for ITR2 = 5.8 × 10\textsuperscript{-9} M; EC\textsubscript{50} for ITR3 = 9.3 × 10\textsuperscript{-9} M), thereby establishing that they are genuine tick inotocin receptors.

1. Introduction

Most multicellular animals belong to two evolutionary lineages, the Deuterostomia (such as mammals, and other chordates), and the Protostomia (such as insects, molluscs and other invertebrates). Nearly all multicellular animals use neuropeptides and neuropeptide G protein-coupled receptors (GPCRs) for the control of reproduction, development, feeding, homeostasis, growth, and many other physiological processes. The first neuropeptides to be isolated from mammals were oxytocin and vasopressin, which were sequenced by the groups of Du Vigneau and Acher in 1953 [1–3]. Other neuropeptides followed in the 1960ies, such as the hypothalamic releasing hormones [4–6] and in the 1970ies, when additional gut neuroendocrine peptides were discovered [7]. In the 1980ies, it became clear that orthologues of some of the neuropeptides that first were discovered in mammals, also existed in protostomes. This was a surprise, as it was often believed that protostomes and deuterostomes had their own taxon-specific neuropeptides. The first two deuterostome neuropeptide orthologues to be discovered in protostomes were insect sulfakinin, which was the orthologue of the mammalian neuroendocrine peptides gastrin and cholecystokinin [8], and conus snail vasopressin (called conopressin),...
occurred in some insect taxa and not in others [27]. For example, the with a sequenced genome, we found that inotocin and its receptor only also identified its receptor. By screening the genomes from other insects its GPCR [27,28]. These experiments confirmed the original claim [23]—mals, but diuretic. Antiparallel dimers with biological activity had never L. migratoria was not antidiuretic, as is known for vasopressin in mam-
趣味度の高い表現で記述されている。
Fig. 1. Alignment of the three tick inotocin receptors Isca-ITR1 (I. scapularis inotocin receptor1), Isca-ITR2, Isca-ITR3, the beetle inotocin receptor Tcas-ITR, and the wasp inotocin receptor Nvit-ITR. Amino acid residue positions common to at least three aligned receptors are highlighted in yellow. The position of a common intron shared by the five receptor genes is marked by a blue star. For GenBank accession numbers see Supplementary file 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
products were ligated into the vector pCR4-TOPO using the TOPO TA Cloning kit (Thermo Fisher Scientific), sequenced by Eurofins Genomics and analyzed with the CLC Main Workbench software package. The coding sequences of the receptors were inserted into the expression vector pIRE2-EGFP (Takara Bio Europe), using the EcoRI restriction sites and the Rapid DNA Dephos and Ligation kit (Merck).

Transfection of Chinese Hamster Ovary (CHO) cells was done as in Ref. [30]. The bioluminescence assay was performed on a Wallac Victor^2 multilabel counter (PerkinElmer Life Sciences) [31]. Tick inotocin was from Genemed Synthesis.

MUSCLE and MEGA 11 software were used for protein alignments and phylogenetic tree analyses. Neighbor-Joining trees were bootstrapped using 1000 replicates. Accession numbers are given in Supplementary file 2. SignalP 6.0 software [32] was used to predict the signal peptide. Deep TMHMM software [33] was used to predict transmembrane helices. Dose-response curves and EC_{50} values were calculated using Prism (GraphPad Software).

3. Results and discussion

3.1. Cloning of the three tick inotocin receptors

We searched the sequenced genome from I. scapularis [29] with a query corresponding to the beetle inotocin GPCR, which resulted in the identification of three presumed tick inotocin receptors (tick ITR1, ITR2, ITR3). We cloned these GPCRs, using standard molecular cloning techniques and PCR primers based on the genomic sequences of the three receptors (Fig. 1, Supplementary file 1). The protein sequences of both ITR1 and ITR2 were 471 amino acid residues long, while the length of ITR3 was 465 residues (Fig. 1). Each receptor had seven transmembrane helices and the canonical DRY sequence at the border of TM3 and the second intracellular loop (Fig. 1).

3.2. Phylogenetic tree analyses of arthropod oxytocin/vasopressin-like receptors

Fig. 2 (left side, with yellow background) shows a phylogenetic tree of various oxytocin/vasopressin-like receptors and the canonical DRY sequence at the border of TM3 and the second intracellular loop (Fig. 1).

![Phylogenetic tree of various oxytocin/vasopressin-like receptors](image-url)
3.3. Evolutionary relationships of the tick inotocin receptors with other neuropeptide receptors

The three tick inotocin receptor genes are lying on one genomic contig (NW_024609837), which has a size of 92 Mb (Fig. 3A). ITR2 and ITR3 are separated by only 0.7 Mb, which suggests that they originated by recent gene duplications. Also located on the same contig is one crustacean cardioactive peptide (CCAP) receptor gene and four adipokinetic hormone/corazonin-related peptide (ACP) receptor genes (ACPR2, ACPR3, ACPR4, and ACPR5) (Fig. 3A). A second contig contains a fifth ACP receptor gene (ACPR1) and a third contig contains a corazonin receptor gene (CRZR) (Fig. 3A).

The presence of three inotocin receptor genes, four ACP receptor genes, and one CCAP receptor gene on one contig (NW_024609837) suggests that these genes might be evolutionarily related. This conclusion is confirmed, when we look at other arthropods, for example at the malaria mosquito Anopheles gambiae, where one genomic contig (NT_078266) houses one adipokinetic hormone receptor (AKHR) gene, one CCAP receptor gene, one corazonin receptor gene, and one ACP receptor gene (Fig. 3B). It is interesting that A. gambiae has an AKH receptor gene that is lacking in I. scapularis and that I. scapularis has three inotocin receptor genes that are lacking in A. gambiae.

The evolutionary relationships between the inotocin, CCAP, AKH, corazonin, and ACP receptor genes are also confirmed, when we compare their gene products in a phylogenetic tree analysis. Fig. 3C shows such an analysis, where we used receptor protein sequences from both I. scapularis and A. gambiae. It is interesting that this tree shows two major branches: One branch, containing the inotocin and CCAP receptors, and one branch, containing the corazonin, AKH, and ACP receptors. Abbreviations: ACPR1, ACPR2, ACPR3, ACPR4, ACPR5, adipokinetic hormone/corazonin-related peptide receptor1, -2, -3, -4, -5; AKHR, adipokinetic hormone receptor; CCAPR, crustacean cardioactive peptide receptor; CRZR, corazonin receptor; Dmel-ASTAR1, Drosophila melanogaster allatostatin-A receptor 1; ITR1, ITR2, ITR3, inotocin receptor -1, -2, -3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Intron/exon organizations of the inotocin receptor genes

All three tick inotocin receptor genes share one common intron that is located at a position corresponding to an amino acid residue in the receptor proteins between transmembrane helix 6 (TM6) and TM7 (marked by a blue star in Fig. 1).

An alignment of the intron/exon organizations of the three tick inotocin receptors with those from the human oxytocin receptor and three vasopressin receptor genes shows a complete conservation of their gene structures (Supplementary file 3). Other chelicerates, myriapods, and insects have either identical or similar gene structures, but always one common intron with the human oxytocin and vasopressin receptor.
genes (Supplementary file 3). These results support the close evolutionary relationships between human and arthropod oxytocin/vasopressin receptor genes.

3.5. Cloning of the tick inotocin preprohormone

We have previously annotated the inotocin preprohormone gene from *I. scapularis* [29]. In the current paper, we have cloned and further analyzed this preprohormone. Fig. 4A and B show that the preprohormone gene consists of three exons (exon 1, 2, and 3). Exon 1 codes for the signal peptide and the immature inotocin sequence. Exon 2 codes for the neurophysin region of the preprohormone. Exon 3 codes for the C-terminal part of the protein.

The tick preprohormone has the same structural features as the mammalian oxytocin and vasopressin preprohormones: The immature inotocin sequence is located immediately after the signal sequence and is followed by a neurophysin part that contains fourteen cysteine residues, which form seven cystine bridges (Fig. 4C). After the signal peptide is removed by signal peptidase during RER membrane transport (left arrow), immature tick inotocin is liberated from its prohormone by cleavage at the canonical KR prohormone convertase (PC1/3) cleavage signal (middle arrow), followed by the conversion of the C-terminal G residue into a C-terminal amide group by peptidylglycine α-hydroxylating monooxygenase [37]. Mature tick inotocin has the sequence CFITNCPPGamide and differs from insect inotocin (CLITNCPRGamide) by two amino acid residues. Mature tick inotocin is cyclic after oxidation of its cysteine residues and formation of a cystine bridge. The neurophysin part of the tick inotocin preprohormone has two domains, one containing four cystine bridges and one containing three bridges (Fig. 4C). In between the two domains is a second canonical prohormone convertase cleavage site (PKR, highlighted in green and pink) [37]. Therefore, it seems likely that tick neurophysin is cleaved into two cystine-knot peptides of which one peptide...
and pest,

**I. scapularis**

selected cell clones that stably expressed one of the receptors. These cells coding for each of the three presumed tick inotocin receptors and

complementary file 5). The genome from the cattle tick,

_Haemaphysalis longicornis_, contains two genes for the inotocin preprohormone (Supplementary file 5).

The genome from the citrus mite,

normally, chelicerates have only one gene for an inotocin preprohormone. However, we found that the genome from the

locust, _Locusta migratoria_, contains five genes, of which five genes might possibly be pseudogenes (Supplementary file 5).

We transfected Chinese Hamster Ovary (CHO) cells with cDNA, coding for each of the three presumed tick inotocin receptors and selected cell clones that stably expressed one of the receptors. These cells also stably expressed the prionlike G protein _G_12 (which couples the receptors to the 

IP_3_/Ca_{2+}^{2-} second messenger pathway) and transiently expressed the cnidarian protein apoaequorin. Shortly before the assay, the cnidarian compound coelenterazine was added to the cells. Binding of the ligand to the GPCRs expressed in these pre-treated cells initiates a second messenger cascade, leading to an intracellular Ca_{2+}^{2-} pulse and a

Ca_{2+}^{2-}-mediated bioluminescence response, which can be quantified on a plate reader [27, 31].

Fig. 4E shows that tick inotocin induced a strong bioluminescence response in CHO cells stably expressing the tick ITR1 receptor (EC_{50} = 1.6 × 10^{-8} M). Also CHO cells stably expressing ITR2 (EC_{50} = 5.8 × 10^{-9} M) and CHO cells stably expressing ITR3 (EC_{50} = 9.2 × 10^{-9} M) showed strong bioluminescence responses after addition of nanomolar concentrations of tick inotocin (Fig. 4F and G). These results deorphanized the three receptors.

CRediT author statement

FH and CJPG: conceptualization, supervision, resources; FH, TAR, MS, and GT: investigation, formal analysis, validation; FH: visualization; CJPG: writing-Original Draft; CJPG and FH: writing-Review & Editing, project administration.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cornelis Grimmelikhuijzen reports financial support was provided by Carlsberg Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.09.009.

References


