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Structure–activity relationships of two *Rhodnius prolixus* calcitonin-like diuretic hormone analogs

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ABSTRACT

The calcitonin-like diuretic hormone (CT/DH) in *Rhodnius prolixus* influences various tissues associated with feeding-related physiological events. The receptors for this peptide have also been identified and shown to be expressed in these tissues. In the present study, we have investigated the effects of two *R. prolixus* CT/DH analogs (full-length form and N-terminal truncated form) on hindgut contractions and in a heterologous receptor expression system. The analogs contained the amino acid methyl-homoserine in place of methionine in order to prevent them from being oxidized and thus increase their stability. The full-length form of the analog retained all of its activity in our assays when compared to the endogenous peptide. Truncated analog displayed no activity in our assays.

1. Introduction

Rhodnius prolixus undergoes a period of rapid post-feeding diuresis (urine production) after ingesting large blood meals. It releases *Trypanosoma cruzi*, a parasite which causes Chagas disease, in its urine near the site of the bite after a blood meal. Infection by the parasite occurs when it enters the bloodstream of the host through the site of the bite. This post-feeding diuresis and enhanced haemolymph circulation involves a variety of tissues including the anterior midgut, Malpighian tubules (MTs), hindgut and dorsal vessel (see [8]). Various diuretic and anti-diuretic hormones (DHs and ADHs respectively) modulate these tissues in *R. prolixus*, including serotonin (5-hydroxytryptamine), corticotropin releasing-factor (CRF)-related diuretic hormone (CRF/DH), calcitonin-like diuretic hormone (CT/DH), kinin and CAPA (see [8]). Since these hormones control diuresis, they also influence the transmission of Chagas disease. The study of receptor–ligand interactions is important as it has great potential for screening many analogs. This will help in the development of pathway-specific peptidomimetics which could be used to control diuresis and prevent the spread of Chagas disease.

Rhodnius prolixus CT/DH (Rhopr-CT/DH) has been shown to stimulate low levels of MT secretion and to increase contractions of the anterior midgut, hindgut and dorsal vessel, *in vitro* (see [13]). Recently, we isolated and characterized two CT/DH receptors from *R. prolixus* (Rhopr-CT/DH-R1 and Rhopr-CT/DH-R2) [14]. Spatial expression analysis showed that both receptors are expressed in feeding-related tissues and hence provides further evidence for the involvement of this signaling system in post-feeding diuresis. In the present research, Rhopr-CT/DH analogs were synthesized and their activities compared with the native peptide using an *in vitro* biological assay and a cell-based functional receptor assay. We focused on the importance of peptide length and biostability, since peptide mimetics needs to be stable and small in size (lower production costs) for their use in pest-control to be economically feasible. The importance of the peptide length was assessed by using an N′-terminal truncated analog, which lacked the first 16 amino acid residues ([Hse(Me)]^{20}-Rhopr-CT/DH(17-31)). Moreover, the methionine residue was replaced by a methyl-homoserine residue to reduce its susceptibility to oxidation, since oxidized peptides show reduced biological activity, as has been shown for the vertebrate calcitonin (see [11]).

2. Materials and methods

2.1. Animals

Fifth-instar *R. prolixus* (8–10 weeks post-feeding as fourth instars) were obtained from a long standing colony at the University of Toronto Mississauga. Insects were reared in incubators

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in high relative humidity at 25 °C and routinely fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada).

2.2. Peptide synthesis

Rhopr-CT/DH (GLDLGLSRGFSGSQAAKHLMGLAAANYAGG-Pamide), which is identical in amino acid sequence to *Diploptera puntata* CT/DH (Dippu-CT/DH), was custom synthesized by GenScript (Piscataway, NJ, USA) at >95% purity. $[Hse(Me)]^{20}$-Rhopr-CT/DH and $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31) were synthesized as described earlier [4]. The purity of these peptide analogs was >99%, as estimated by HPLC. Stock solutions (1 mM) of all the peptides were made by dissolving them in water.

2.3. Hindgut contraction assay

Contraction assays were performed on isolated hindguts from unfed fifth-instar *R. prolixus* maintained under physiological saline [10,15]. Hindguts were incubated in 200 μL saline and secured by pinning a small piece of the ventral cuticle on a Sylgard (Dow Corning, Midland, Michigan, USA) coated dish. One end of a fine silk thread was tied between the anterior portion of the hindgut and a small portion of the posterior midgut while the other end of the thread was hooked to a miniature force transducer (AksjeselskapetMikro-elektronikk, Horten, Norway). Tissues were allowed to equilibrate in saline for a minimum of 10 min. The frequency of longitudinal contractions was monitored and recorded for 200 s using PicoLog recorder (Pico Technology, St. Neots, Cambridgeshire, UK) after the application of either saline or peptide. One-way ANOVA and Tukey post-test were used for the statistical analysis ($P < 0.05$).

2.4. Expression vector construction

The ORFs encoding *Rhopr-CT/DH-R1-B* and *Rhopr-CT/DH-R2-B* were amplified as described previously [14]. Kozak translation initiation sequence was also introduced at the 5′-end of these sequences; the resulting sequences were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). These were then subcloned into pIRES2-ZsGreen1 (Clontech, Mountain View, CA, USA) for mammalian cell expression.

2.5. Cell culture, transfections and bioluminescence assay

Chinese hamster ovary (CHO) cells stably expressing the human G-protein G16 (CHO/G16) used for the bioluminescence assay were cultured according to the conditions mentioned earlier [9]. X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN, USA) was used to transiently transfect CHO/G16 cells with the receptor expression vector (*Rhopr-CT/DH-R2-B*) and an expression vector containing cytoplasmic luminescent reporter aequorin at ratio of 2:1 (transfection reagent to expression vectors) according to the protocol supplied by the manufacturer. Since no difference in response was detected between CHO/G16 cells expressing Rhopr-CT/DH-R1-B either stably or transiently, previously established CHO/G16 cells stably expressing Rhopr-CT/DH-R1-B were used here [14]. These cells were then transiently transfected with aequorin as described above. The cells were incubated for 48 h post-transfection and used to perform the bioluminescence assay as mentioned earlier [14].

3. Results

3.1. Hindgut contraction assay

Previous work has shown that Rhopr-CT/DH increases the contraction frequency of *R. prolixus* hindgut in a dose-dependent manner [10]. Hence, we utilized this assay to compare the activity of the analogs against the native peptide. Rhopr-CT/DH and $[Hse(Me)]^{20}$-Rhopr-CT/DH at 10^{-6} M both increased the frequency of hindgut contractions, with no significant difference in the response to the two peptides (Fig. 1). There was no definite increase in the amplitude of contractions or the basal tonus. $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31) on the other hand had no effect on hindgut contractions compared to the saline controls (Fig. 1).

3.2. Bioluminescence assay

We expressed Rhopr-CT/DH-R1 and Rhopr-CT/DH-R2 in CHO/G16 cells and monitored ligand-receptor interactions using a calcium mobilization assay. Rhopr-CT/DH and $[Hse(Me)]^{20}$-Rhopr-CT/DH were both able to activate Rhopr-CT/DH-R1-B (EC_{50} = 260–340 nM) and Rhopr-CT/DH-R2-B (EC_{50} = 1.7–3.3 nm) at

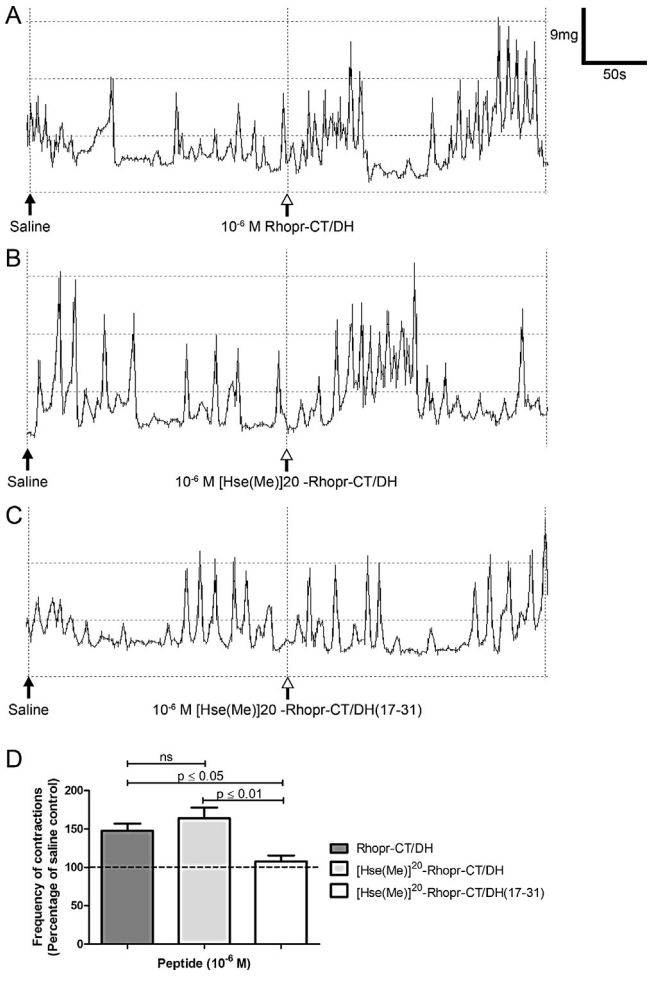

Fig. 1. Effect of Rhopr-CT/DH and its analogs on frequency of *R. prolixus* hindgut contractions. (A–D) Sample traces. Control contractions observed in saline prior to the application of 10^{-6} M Rhopr-CT/DH (A), $[Hse(Me)]^{20}$-Rhopr-CT/DH (B) or $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31) (C). Each preparation was thoroughly washed between different peptide applications. Saline was applied at the filled arrow and peptides were applied at the open arrow. (D) Bar graph showing the effects of the three peptides on the frequency of *R. prolixus* hindgut contractions. Bars represent mean ± s.e.m. of 6 preparations. Scale bars represent tension and time.

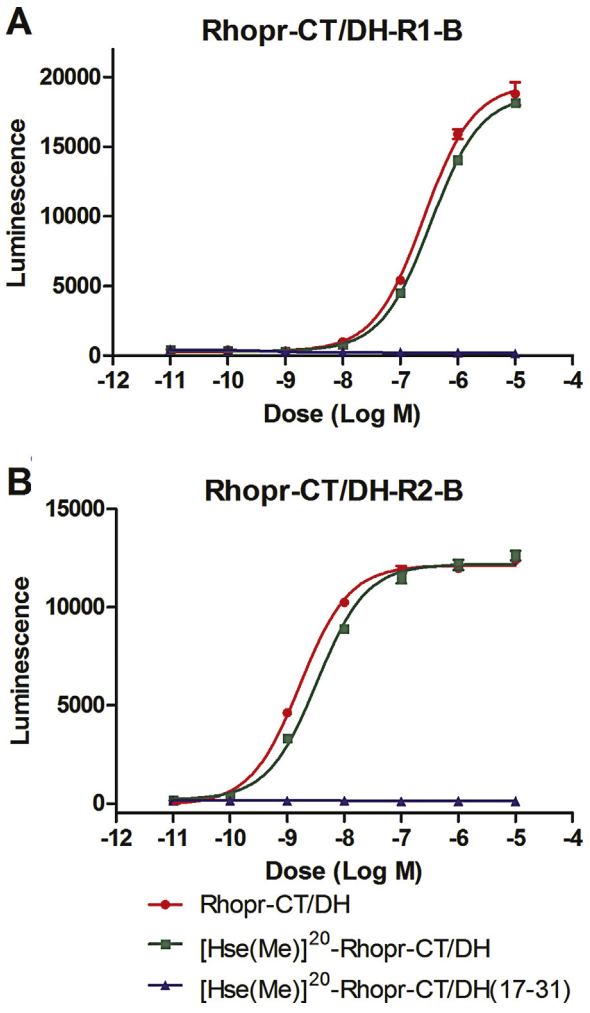

Fig. 2. Functional assay of *R. prolixus* CT/DH receptor isoforms (Rhopr-CT/DH-R1-B and Rhopr-CT/DH-R2-B) expressed in CHO/G16 cells. Dose-dependent effect on the bioluminescence response after addition of Rhopr-CT/DH, $[Hse(Me)]^{20}$-Rhopr-CT/DH or $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31) to CHO/G16 cells expressing Rhopr-CT/DH-R1-B (A) and Rhopr-CT/DH-R2-B (B). Vertical bars represent SEM ($n = 3$). Both receptors are activated by Rhopr-CT/DH and $[Hse(Me)]^{20}$-Rhopr-CT/DH but not by $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31).

equal sensitivities (Fig. 2). No response, however, was observed following the addition of $[Hse(Me)]^{20}$-Rhopr-CT/DH(17–31) to the cells that were transfected with either receptor (Fig. 2).

4. Discussion

In the present work, biostable analogs of Rhopr-CT/DH were synthesized and their activities compared with the native peptide. The methionine residue at position 20 was replaced by a methyl-homoserine residue to reduce its susceptibility to oxidation and thus improve peptide-stability. The full length form of the analog was as effective as the endogenous peptide when their activities were compared in both the cell-based receptor functional assay and the hindgut contraction assay; however, the N′-terminal truncated analog was inactive. Our results are in agreement with previous work where effects of similar modifications to Dippu-CT/DH were examined on secretion by *D. punctata* MTs [4]. Replacement of methionine with methyl-homoserine in Dippu-CT/DH did not alter the secretion rate [4], whereas the N′-terminal truncated analog had no effect on MT secretion. This is not surprising as there must be a selective pressure on maintaining the length of the peptide which has been conserved across protostomes and deuterostomes (see [13]); however, it is interesting that injection of the N-terminal truncated analog into locust nymphs resulted was anorexigenic [3], although it is not known which other receptors might be activated.

Development of novel insecticides is important due to various problems associated with current insect-control strategies. Neonicotinoids represent a class of neuro-active chemical insecticides that are currently the most widely used insecticide around the globe; however, several insect pests have already developed resistance to them (see [7]) and non-target beneficial species are also susceptible, including important pollinators [5,12]. Hence there is an urgent demand for novel environmentally friendly and highly selective insecticides. Peptide-based insecticides represent excellent candidates especially since some occur naturally as part of spider venoms [1]. Most peptides mediate their action *via* G protein-coupled receptors (GPCRs) to alter the physiology and behavior of insects. Targeting these receptors using biostable, species-specific peptide mimetics that alter the normal physiology of insects is a promising avenue to control insect populations [2,6]. In order for this approach of pest-control to be economically feasible, it is important that the peptide mimetics be small in size in order to reduce the production costs. Hence, future work should focus on CT/DH-R antagonists as opposed to agonists as the peptide length appears to be critical for activation of these receptors.

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