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Physiological effects of biostable kinin and CAPA analogs in the Chagas disease vector, *Rhodnius prolixus*

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ABSTRACT

In the Chagas disease vector *Rhodnius prolixus*, the kinin and CAPA family of neuropeptides are implicated in feeding and diuresis-related behaviours, with Rhopr-kinins stimulating contractions of the midgut, salivary glands, and hindgut, and RhoprCAPA-2 functioning as an anti-diuretic hormone. The current study examined the effects of kinin and CAPA neuropeptides and their analogs on feeding and diuresis, and on hindgut contractions and MT fluid secretion in *R. prolixus*. The biostable Aib-containing kinin analog 2139[Φ1]wp-2 was found to have antifeedant effects, and to be more potent than Rhopr-kinin 2 in stimulating hindgut contractions. The CAPA analog 2129-SP3[Φ3]wp-2 induced the intake of a larger blood meal, and increased the rate of post-prandial rapid diuresis. RhoprCAPA-2, but not its analog, potentiated hindgut contractions induced by Rhopr-kinin 2. Potentiation was observed with the CAPA analog on 5-HT-stimulated increases in frequency of hindgut contractions, whereas RhoprCAPA-2 inhibited this 5-HT-mediated stimulation. The CAPA analog induced hindgut contractions and prevented the inhibition induced by RhoprCAPA-2 on 5-HT-stimulated MT secretion. These results demonstrate novel interactions between Rhopr-kinin and RhoprCAPA-2 on the hindgut, possibly influencing post-feeding excretion. The kinin analog is a potent agonist of the kinin receptor, and the CAPA analog an antagonist of the CAPA receptor. The use of neuropeptide mimetics is a promising approach to vector control as they can disrupt behaviours, and the effects of these neuropeptide analogs highlight their value as lead compounds, given their ability to interfere with epidemiologically-relevant behaviours.

1. Introduction

The blood-gorging hemipteran *Rhodnius prolixus* is a domestic vector of Chagas disease in Central and South America, caused by the transmission of *Trypanosoma cruzi*, a flagellate parasite (Moncayo, 2003; Dujardin et al., 1998). *R. prolixus* consumes a blood meal that can be up to 10 times its body weight during each instar, following which the insect rids itself of the excess water and salt from the blood meal. The transmission of the parasite to its host occurs during this post-prandial diuresis, and so an understanding of the physiology of feeding and diuresis-related behaviours is essential in understanding disease transmission (Orchard, 2006; Orchard and Paluzzi, 2009). The systems controlling diuresis include the Malpighian tubules (MTs), midgut and hindgut, which are subject to control by various diuretic and anti-diuretic hormones and myotropic factors (Coast et al., 2002; Orchard, 2006; Orchard and Paluzzi, 2009).

Many essential processes within insects are regulated by neuropeptides which act upon target tissues through their release as neurohormones into the haemolymph or as neuromodulators (Schoofs et al., 2017). Insect kinins, first isolated from *Leucophaea maderae* head extracts, share the C-terminal pentapeptide sequence FX^1X^2WG-amide (where X^1 can be Ser, Phe, His, Asn, or Tyr and X^2 can be Ser, Pro or Ala) (Holman et al., 1986a, b; Holman et al., 1987a, b). Kinins have been implicated in various behaviours, such as hindgut contraction (Bhatt et al., 2014), MT fluid secretion (O'Donnell et al., 1998; Terhzaz et al., 1999; Rosay et al., 1997), ecdysis (Kim et al., 2006), and more recently locomotor activity and metabolic rate (Zandawala et al., 2018). The *R. prolixus* kinin (Rhopr-kinin) transcript encodes for eighteen predicted kinins and precursor associated peptides (Te Brugge et al., 2011a; Bhatt et al., 2014) and these kinins stimulate *R. prolixus* hindgut, anterior midgut, and salivary gland contractions (Orchard and Te Brugge, 2002; Te Brugge et al., 2009; Te Brugge et al., 2001; Bhatt et al., 2014). Interestingly, co-localization of Rhopr-kinin has been shown with corticotropin-releasing factor (CRF)-like diuretic hormone (Rhopr-CRF/DH) within the *R. prolixus* central nervous system (CNS) and endocrine cells of the midgut. A decrease of CRF-like and kinin-like

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immunoreactive staining in neurosecretory cells is seen up to 2.5 h after feeding, with levels being restored 1 day after feeding, suggesting a role of CRF and kinin in feeding-related behaviours (Te Brugge et al., 1999, 2001; Te Brugge and Orchard, 2002; Mollayeva et al., 2018). In the *R. prolixus* excretory system, the MTs play a dominant role in the regulation of urine volume and composition (Coast, 2009), primarily under control by the diuretic neurohormones serotonin [5-hydroxytryptamine (5-HT)], calcitonin-like diuretic hormone (DH_{31}), and Rhopr-CRF/DH (Maddrell et al., 1971; Te Brugge et al., 2005, 2011b), and the anti-diuretic hormone *R. prolixus* CAPA (Rhopr-CAPA) (Orchard and Paluzzi, 2009; Ianowski et al., 2009; Paluzzi et al., 2008). CAPA peptides are encoded by the *capability* genes, initially identified in *Drosophila melanogaster* (Kean et al., 2002). The first two CAPA peptides encoded by the gene containing the consensus carboxyl terminal sequence A/PFPRV-NH_2, with the third peptide containing the consensus carboxyl terminal sequence G/MWFGPRL-NH_2, typically referred to as a pyrokinin (PK)-related peptide (Paluzzi, 2012; Paluzzi et al., 2008). CAPA peptides were originally discovered in *Manduca sexta* for their cardioacceletatory effects and display diuretic, anti-diuretic, and myotropic effects in a variety of species (Huesmann et al., 1995; Predel and Wegener, 2006; Paluzzi et al., 2008). The RhoprCAPA transcript encodes three CAPA peptides, as found in other species: RhoprCAPA-1, RhoprCAPA-2, and RhoprCAPA-pk1. RhoprCAPA-2 has been found to inhibit 5-HT-stimulated secretion by the MTs and absorption from the anterior midgut (Ianowski et al., 2009; Orchard and Paluzzi, 2009; Paluzzi et al., 2008). The effects of the CAPA peptides are mediated by the RhoprCAPA G protein-coupled receptor (GPCR) (*capa-r*) (Paluzzi et al., 2010). Expression of the *capa-r* transcript has been confirmed in the hindgut of *R. prolixus*, suggesting that CAPA peptides may influence hindgut contractions (Paluzzi et al., 2010).

From an agrochemical and medical perspective, neuropeptides and neuropeptide analogs are compounds of interest for the disruption of critical functions as a means of pest control (Jiang et al., 2015). Neuropeptide analogs have been synthesized with a modified chemical structure in order to overcome limitations associated with delivery of the compound (eg. movement through the cuticle, endopeptidases, exopeptidases) (Nachman et al., 1997). In *Musca domestica*, addition of alpha-aminoisobutyric acid (Aib) to a kinin analog resulted in resistance to hydrolysis by angiotensin converting enzyme (ACE) and neprilysin (NEP) and the analog was found to be potent in inducing myotropic activity in *L. maderae* hindguts (Nachman et al., 1997). Recently, the kinin analog 2139 was shown to stimulate fluid secretion in *D. melanogaster*, and significantly reduced survival under desiccation stress (Alford et al., 2019a). Decreased survival was also exhibited in *Myzus persicae* and *Macrosiphum rosae* under cold stress exposure after kinin analog treatment (Alford et al., 2019b). In *R. prolixus*, an Aib-containing kinin analog was found to have antifeedant effects, as insects only consumed 60% of a blood meal that contained the analog (Lange et al., 2016).

The CAPA analog 2129-SP3[Φ3]wp-2 has been designed with the addition of hydrophobic moieties to the N-terminus to increase greater *in vivo* stability, and also possesses a steric hindrance adjacent to the alpha carbon in the C-terminal position, directing its binding to a CAPA receptor whilst interfering with any PK receptor binding (Zhang et al., 2011; Jurenka, 2015; Alford et al., 2019a). This CAPA analog also influences desiccation and starvation survival, as it significantly improved *D. suzukii* desiccation survival, while significantly increasing the desiccation and starvation mortality in *M. persicae* and *M. rosae* (Alford et al., 2019a, 2019b).

In this study, we examine the effects of the biostable Aib-containing kinin analog 2139[Φ1]wp-2 and an antagonist of the CAPA receptor, CAPA analog 2129-SP3[Φ3]wp-2 (Table 1) on feeding and diuresis-related behaviours, including changes in blood meal size, hindgut contractions, and excretion rate. Due to the presence of CAPA receptors on the hindgut (Paluzzi et al., 2010), we also further investigated the role of members of the RhoprCAPA family of peptides on hindgut contractions. Examining the effects of the kinin and CAPA analogs on the physiology of *R. prolixus* will assist in determining the potential value of these analogs in wide scale pest control strategies.

Table 1
Structure of Kinin and CAPA analogs.

Analog	Structure
Kinin: 2139[Φ1]wp-2	FF **[Aib]** WGa
CAPA: 2129-SP3[Φ3]wp-2	**2Abf-Suc-A**TPRIa

2. Materials and methods

2.1. Animals

5th instar male and female *R. prolixus* were obtained from an established colony at the University of Toronto Mississauga. Insects were reared at 25 °C and 50% humidity in incubators, and were fed defibrinated rabbit blood (Cedarlane Laboratories, Burlington, ON, Canada) once in each instar. Tissues were dissected from 5th instar *R. prolixus* 3–5 weeks post-feeding as 4th instars. All tissue dissections were performed in *R. prolixus* physiological saline, consisting of 150 mM NaCl, 8.6 mM KCl, 2.0 mM $CaCl_2$, 4.0 mM $NaHCO_3$, 8.5 $MgCl_2$, 0.02 mM HEPES and 34 mM glucose in pH 7.0.

2.2. Chemicals

Rhopr-kinin 2, RhoprCAPA-1, RhoprCAPA-2, and RhoprCAPA-pk1 were custom synthesized by Genscript (Piscataway, NJ, USA). The peptides were then reconstituted in double-distilled water into stock solutions at 10^{-3} M and stored at −20 °C. Stock solutions of the Aib-containing insect kinin analog (2139[Φ1]wp-2) with the amino acid sequence Phe-Phe-Aib-Trp-Gly-NH_2 (Nachman et al., 1997), and insect CAPA analog (2129-SP3[Φ3]wp-2) with the sequence 2Abf-Suc-ATPRIa synthesized as previously described (Nachman et al., 1997; Alford et al., 2019b) were prepared in 80% aqueous acetonitrile (ACN) containing 0.01% trifluoroacetic acid (TFA), and stored at 4 °C at a concentration of 10^{-3} M. Control experiments with appropriate dilutions of 80% ACN containing 0.01% TFA indicated that there was no effect on the physiological assays or on feeding following injection.

2.3. Hindgut contraction assay via force transducer

The *R. prolixus* hindgut was isolated under physiological saline, along with the cuticle at the posterior end and fixed onto a Sylgard-coated dish using minuten pins through the cuticle and bathed in 200 µl of physiological saline. One end of a fine silk thread was tied to the anterior end of the hindgut, with the other end tied to a Grass FT03 force transducer (Astro-Nova Inc., Rhode Island, USA). The amplitude of basal tonus changes were recorded using the PicoScope 2204 Oscilloscope (Pico Technology, Cambridgeshire, UK). Tissues were equilibrated in saline for 10 min. Peptides, including their analogs, were applied by addition of 100 µl of various concentrations of the peptides in saline concurrent with removal of 100 µl of the bath saline to ensure the bath volume remained constant. The preparations were washed with saline between test doses of peptide and the bath volume was maintained at 200 µl of saline. The recorded traces were analyzed for changes in basal tonus.

2.4. Hindgut contraction assay via impedance

The *R. prolixus* hindgut was isolated under physiological saline, along with the cuticle at the posterior end and fixed onto a Sylgard-coated dish using minuten pins through the cuticle and the anterior end and bathed in 200 µl of physiological saline. Electrodes were placed on

either side of the anterior region of the hindgut. Peristaltic contractions were monitored through a UFI impedance converter (Model 2991, Morro Bay, CA, USA). The frequency of the hindgut contractions was recorded using the PicoScope 2204 Oscilloscope (Pico Technology, Cambridgeshire, UK). Tissues were equilibrated with saline for 10 min. Peptides and analogs were applied onto the tissue by addition of 100 µl of various concentrations in saline concurrent with removal of 100 µl of the saline, ensuring that the volume of saline within the bath remained constant. To validate the recorded contractions, the tissue was observed visually to correlate which deflections represent contractions. The frequency of contractions was measured for 3 min, and the recorded traces were analyzed.

2.5. Feeding bioassay

Unfed 5th instar insects (3–5 weeks post feeding as 4th instars) were separated into 3 groups of 20 with similar average weights. Each group was injected through the membrane at the junction of the hind leg with the abdomen with one of the following: 1 µl Rhopr-kinin 2 (10^{-4} M), 1 µl kinin analog 2139[Φ1]wp-2 (10^{-4} M), 1 µl RhoprCAPA-2 (10^{-4} M), 1 µl CAPA analog 2129-SP3[Φ3]wp-2 (10^{-4} M), or 1 µl physiological saline. After a 2-h recovery period, each group was placed in a 10 cm diameter glass jar and fed on 20 mL of warm defibrinated rabbit blood for 20 min. Insects from each group were individually weighed immediately after feeding (time 0) and maintained in individual cubicles. Weights were later recorded at 1, 2, 3, and 4-h time points. Since 5th instar *R. prolixus* tend to take a blood meal 8–10 times their initial body weight, insects that fed less than 1 times their body weight were considered not fed and excluded from the data, as were those punctured during the weighing process.

2.6. Malpighian tubule secretion assay

Whole MTs from 5th instar insects were dissected under saline using glass probes and transferred to a Sylgard-coated Petri dish containing 20 µl drops of saline overlaid with water-saturated mineral oil. The proximal end of the tubule was pulled out of the saline and wrapped around a minuten pin. Excess tubule from the proximal end was cut prior to wrapping, and the tubules were nicked gently at the pin. The equilibrating saline was removed and replaced with 10^{-8} M 5-HT (Sigma, Oakville, ON, Canada), the CAPA analog 2129-SP3[Φ3]wp-2, a mixture of 10^{-8} M 5-HT and 10^{-7} M RhoprCAPA-2, or a mixture of 10^{-8} M 5-HT, 10^{-7} M RhoprCAPA-2 and different concentrations of the CAPA analog 2129-SP3[Φ3]wp-2. Tubules were allowed to secrete for 30 min. Droplets of secreted fluid from the nicked end of the tubule were then collected using an oil filled micropipette tip, and the diameter of the droplet was measured using an eyepiece micrometer on the bottom of the Sylgard-coated Petri dish. The droplet volume was then calculated using the equation $V = (\pi/6)d^3$ where d is the diameter of the droplet measured. At the end of the experiments, tubules were stimulated with 10^{-6} M 5-HT and the maximal rate of secretion was measured to check viability of the tissues.

3. Results

3.1. In vivo effects of kinin and CAPA analogs on feeding and diuresis

Injection of Rhopr-kinin 2 prior to feeding did not alter the size of blood meal consumed over a 20-min feed as compared to saline injected insects (Fig. 1A). On the other hand, injection of the kinin analog 2139[Φ1]wp-2 prior to feeding led to a significant decrease in the size of the blood meal consumed over a 20 min period (Fig. 1A). Rapid post-feeding diuresis occurs over the subsequent 3–4 h and this can be monitored by measuring the loss of weight due to excretion over time. Rhopr-kinin 2 and the kinin analog 2139[Φ1]wp-2 did not alter the rate of diuresis over 4 h (Fig. 1B).

The effects of RhoprCAPA-2 and the CAPA analog, 2129-SP3[Φ3]wp-2, were also examined on feeding and rate of diuresis in 5th instar insects. Injection of RhoprCAPA-2 had no effect on the size of the blood meal, whereas injection of the CAPA analog 2129-SP3[Φ3]wp-2, resulted in a larger blood meal being consumed (Fig. 1C). Injection of RhoprCAPA-2 did not influence the rate of diuresis over 4 h (Fig. 1D); however, the CAPA analog, 2129-SP3[Φ3]wp-2, which resulted in a larger blood meal taken appeared to have an increased rate of diuresis over the first hour after feeding (Fig. 1D).

3.2. In vitro effects of kinin and CAPA analogs on hindgut contractions

To further investigate the analogs, we turned to *in vitro* preparations of tissues associated with feeding, namely the hindgut and MTs. Both Rhopr-kinin 2 and the kinin analog, 2139[Φ1]wp-2, resulted in dose-dependent increases in basal tonus of the hindgut with an apparent threshold observed at 10^{-10} M for Rhopr-kinin 2 and 10^{-14} M for 2139[Φ1]wp-2, while significant changes occurring at 10^{-8} M and 10^{-9} M respectively (Fig. 2). In addition, 2139[Φ1]wp-2 induced stronger contractions than Rhopr-kinin 2. The EC_{50} value of 2139[Φ1]wp-2 is approximately 5.5×10^{-10} M, whereas the EC_{50} value for Rhopr-kinin 2 is approximately 5.5×10^{-9} M (Fig. 2C and D). The effects of 2139[Φ1]wp-2 were more difficult to reverse and required more washes in saline than Rhopr-kinin 2. Neither RhoprCAPA-1 (not shown), RhoprCAPA-2 (Fig. 3A) nor RhoprPK-1 (not shown) altered contractions of hindgut. Interestingly, the CAPA analog, 2129-SP3[Φ3]wp-2, stimulated dose-dependent increases in hindgut contractions (Fig. 3B and C). The threshold is at 10^{-10} M, EC_{50} of approximately 10^{-8} M, and maximum tension at 10^{-6} M (Fig. 3C). In order to examine for any interaction between Rhopr-kinin 2 and RhoprCAPA-2, the two peptides were applied simultaneously on the hindgut (Fig. 4). Application of Rhopr-kinin 2 along with RhoprCAPA-2 resulted in statistically significant increases in hindgut contractions relative to Rhopr-kinin 2 alone (Fig. 4A–C). Interestingly, this potentiation of hindgut contractions was not observed with the CAPA analog, 2129-SP3[Φ3]wp-2 (Fig. 4D).

To further examine any co-operative effects of RhoprCAPA-2 and the CAPA analog 2129-SP3[Φ3]wp-2, changes in 5-HT-stimulated increases in the frequency of hindgut contractions were measured. As the contractions induced by 5-HT are not easily monitored by the force transducer (Te Brugge and Orchard, 2002; Bhatt et al., 2014), an impedance monitor was used to assess changes in the frequency of hindgut contractions. Varying concentrations of RhoprCAPA-2 and 2129-SP3[Φ3]wp-2 were each applied simultaneously with 10^{-8} M 5-HT on the hindgut (Figs. 5 and 6). 2129-SP3[Φ3]wp-2 potentiated the effects of 10^{-8} M 5-HT, with a statistically significant increase in hindgut frequency observed at a concentration of 10^{-7} M 2129-SP3[Φ3]wp-2 (Fig. 5B and C). However, Rhopr-CAPA 2 was found to inhibit this 5-HT-mediated increase in frequency, with a statistically significant reduction in frequency at 10^{-6} M (Fig. 6B and C).

3.3. In vitro effects of CAPA analogs on malpighian tubule secretion

To determine the effects of the CAPA analog 2129-SP3[Φ3]wp-2 on diuresis *in vitro*, varying concentrations of the analog were tested on unstimulated tubules, and 5-HT-stimulated tubules. The analog had no effect on unstimulated tubules (not shown) and failed to have any potentiation effect on tubules stimulated with 10^{-8} M 5-HT (Fig. 7A). Varying concentrations of the CAPA analog 2129-SP3[Φ3]wp-2 were mixed with 10^{-8} M 5-HT and 10^{-7} M RhoprCAPA-2. As previously shown, RhoprCAPA-2 inhibits 5-HT stimulated secretion (Fig. 7B). The analog prevented the anti-diuretic effect of RhoprCAPA-2 with a statistically significant difference observed at 10^{-6} M but failed to return the tubules to its initial rate of 5-HT-stimulated secretion (Fig. 7B).

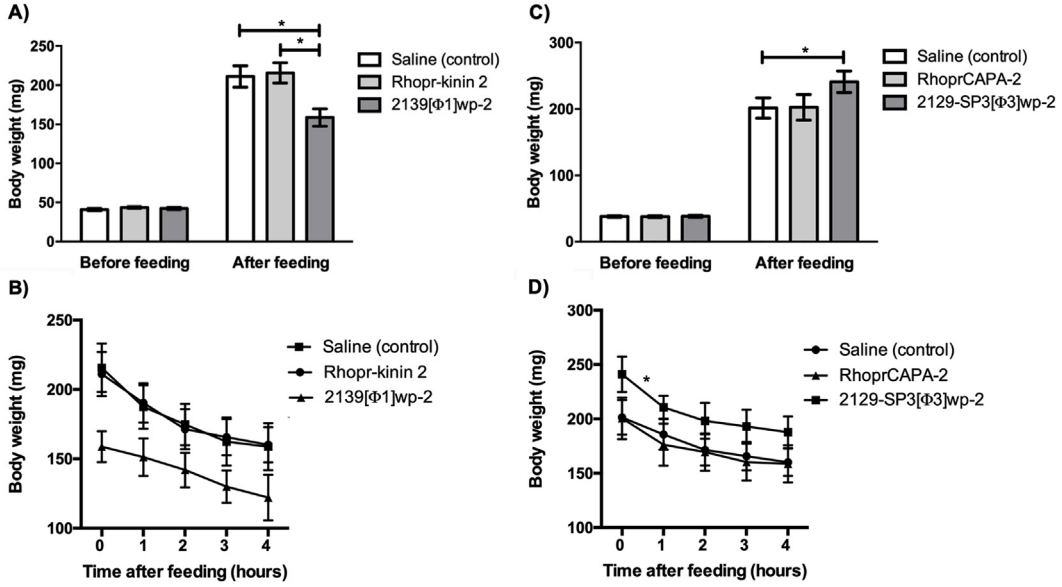

Fig. 1. A) The effects of injection of 1 μl saline, 1 μl Rhopr-kinin 2 (10^{-4} M), and 1 μl of the kinin analog, 2139[Φ1]wp-2 (10^{-4} M), on the size of blood meal taken and B) rate of diuresis over 4 h by 5th instar *R. prolixus*. C) The effects of injection of 1 μl saline, 1 μl RhoprCAPA-2 (10^{-4} M) and 1 μl CAPA analog, 2129-SP3[Φ3]wp-2 (10^{-4} M), on the size of blood meal taken and D) rate of diuresis by 5th instar *R. prolixus*. Weight of insects was measured after 20 min of blood-feeding (time 0) and at 1 h increments post-feeding for 4 h (One-way ANOVA followed by Tukey's post-hoc test, slopes tested for significance using an F-test, * = $p < 0.05$. Data are means ± SEM of n = 16–20).

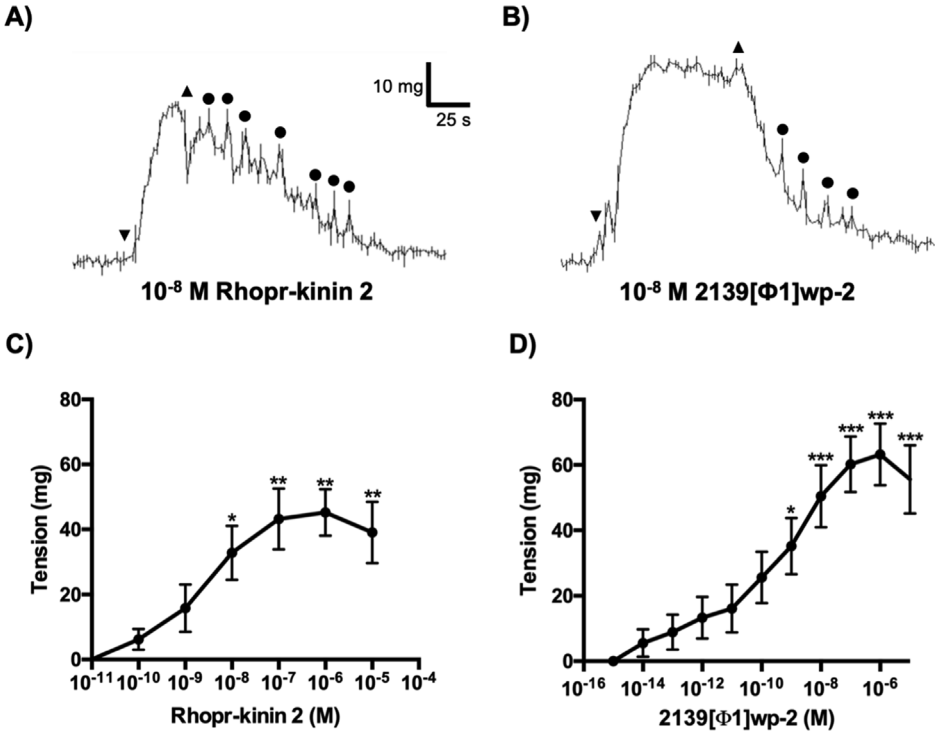

Fig. 2. Example traces of changes in basal tonus of hindgut contractions in response to A) 10^{-8} M Rhopr-kinin 2 and B) 10^{-8} M kinin analog, 2139[Φ1]wp-2. Downward arrowheads denote application of peptide, upward arrowheads denote the start of saline wash, and circles denote vertical deflections due to wash. Dose-response curves displaying changes in basal tonus of hindgut contractions in response to C) Rhopr-kinin 2 and D) kinin analog, 2139[Φ1]wp-2. (One-way ANOVA followed by Dunnett's multiple comparison test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Data are means ± SEM of n = 5).

4. Discussion

In *R. prolixus*, the precisely timed events that occur during post-prandial rapid diuresis are governed by the neuroendocrine system, therefore investigating the neuroactive chemicals that may be associated with these specific behaviours and physiology provides insight into how these processes occur. The endocrine system can be used as a target to disrupt epidemiologically-relevant behaviours in order to prevent disease transmission. Neuropeptides show great promise in the development of next-generation insecticides, due to their specificity in terms of function and binding to GPCRs (Audsley and Down, 2015).

As blood feeding is a requirement for the initiation of many developmental and reproductive processes and the transition to the next instar within *R. prolixus*, interference with these events would prove to be quite detrimental (Lange et al., 2016). Insects that were injected with the kinin analog 2139[Φ1]wp-2 prior to feeding had a significantly reduced blood meal compared to saline injected insects which was consistent with previous work where Aib-containing analogs were found to have antifeedant effects on 5th instar *R. prolixus* (Lange et al., 2016). In the hemipteran *Acyrthosiphon pisum*, while the presence of

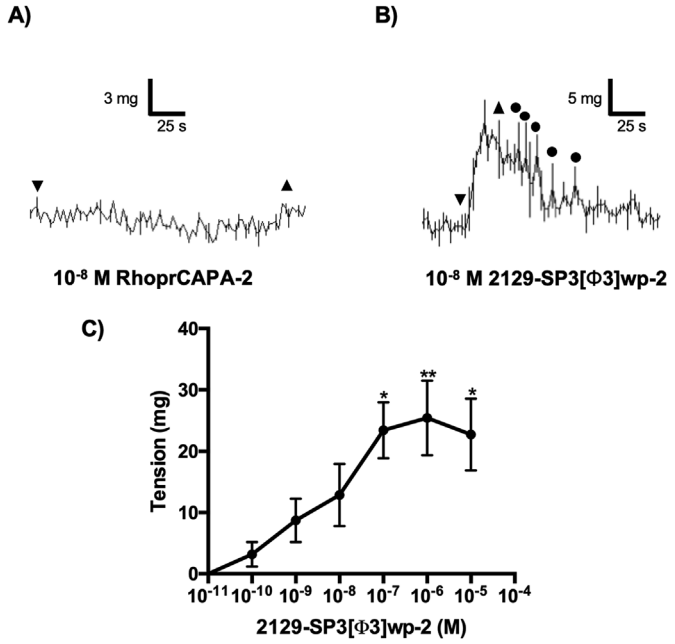

Fig. 3. Example traces of changes in basal tonus of hindgut contractions in response to A) 10^{-8} M of RhoprCAPA-2 and B) 10^{-8} M of CAPA analog, 2129-SP3[Φ3]wp-2. Downward arrowheads denote application of peptide, upward arrowheads denote the start of saline wash, and circles represent vertical deflections due to wash. C) Dose-response curve displaying changes in basal tonus of hindgut contractions in response to the CAPA analog, 2129-SP3[Φ3]wp-2 (One-way ANOVA followed by Dunnett's multiple comparisons test, * = $p < 0.05$, ** = $p < 0.01$. Data are means ± SEM of n = 5).

Aib-containing analogs within the aphid diets resulted in reduced feeding, and aphicidal activity, 2139[Φ1]wp-2 failed to reduce aphid fitness under desiccation and survival stress in *M. persicae* and *M. rosae* (Alford et al., 2019b; Smagghe et al., 2010). These differences may be due to the specificity of the kinin analogs, as species-specific effects of neuropeptide analogs have been previously observed in *D. melanogaster* (Alford et al., 2019a). Injection of the kinin analog did not influence the rate of post-prandial rapid diuresis which is consistent with the fact that kinins do not play a direct role in post-prandial rapid diuresis (Lange et al., 2016, Te Brugge and Orchard, 2002; Te Brugge et al., 2009).

In contrast to Rhopr-kinins, RhoprCAPA functions as an anti-diuretic hormone through the inhibition of MT fluid secretion and anterior midgut fluid transport (Paluzzi et al., 2008; Ianowski et al., 2009). Insects injected with the CAPA analog 2129-SP3[Φ3]wp-2 prior to feeding took a significantly increased blood meal compared to saline injected insects and had a significantly greater rate of post-prandial rapid diuresis within the first hour. As the CAPA analog 2129-SP3[Φ3]wp-2 is likely acting upon Rhopr-CAPA receptors as an antagonist (Jiang et al., 2015), this increased rate of rapid diuresis is likely due to the blocking of the CAPA receptor, thus preventing the anti-diuretic effects of RhoprCAPA-2. The changes in blood meal size upon injection of 2129-SP3[Φ3]wp-2 suggests that RhoprCAPA may also influence feeding. Once an insect has successfully fed, it has obtained enough nutrients required to moult into the next instar, and so does not require another blood meal (Buxton, 1930). Since RhoprCAPA-2 is released towards the end of diuresis, it may also serve as a signal to prevent additional feeding events. Within *R. prolixus*, multiple neurohormones such as sulfakinin (Rhopr-SK-1) and Rhopr-CRF/DH have also been identified as influencing feeding (Al-Alkawi et al., 2017; Mollayeva et al., 2018), and therefore may co-operatively function in regulating satiety and the motivation to feed. The injection of RhoprCAPA-2 did not have an impact on the size of blood meal, which suggests that RhoprCAPA-2 might not influence satiety, but is more a signal to prevent additional feeding events.

The hindgut of *R. prolixus* plays an essential role during post-feeding diuresis, as it is responsible for the excretion of accumulated urine (Maddrell, 1976). In addition, the *T. cruzi* parasite is present in the hindgut in its highly infectious stage, and so contraction of the hindgut results in the release of the parasite along with urine onto the host (Bern et al., 2011). Aib-containing analogs have previously been shown to be more effective in eliciting myotropic and diuretic effects than their endogenous counterparts in *L. maderae* and *Acheta domesticus*

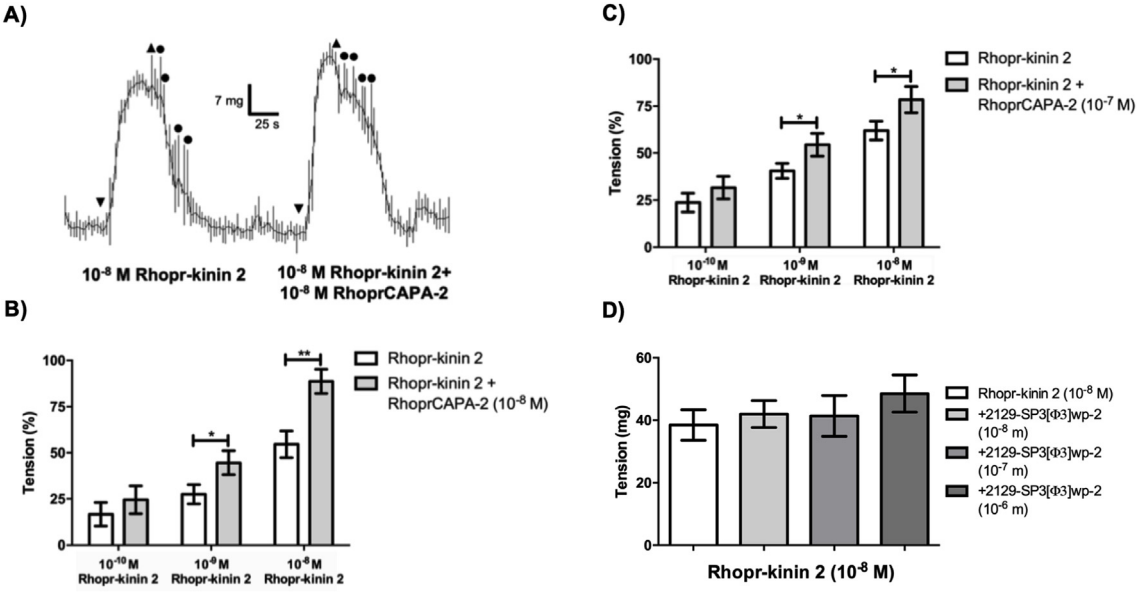

Fig. 4. A) Example traces of changes in basal tonus of hindgut contractions in response to 10^{-8} M Rhopr-kinin 2 and a mixture of 10^{-8} M Rhopr-kinin 2 + 10^{-7} M RhoprCAPA-2. Downward arrowheads denote application of peptide, upward arrowheads denote the start of saline wash, and circles denote vertical deflections due to wash. B) 10^{-8} M RhoprCAPA-2 and C) 10^{-7} M RhoprCAPA-2 potentiates the change in basal tonus elicited by varying concentrations of Rhopr-kinin 2. Change in tension is represented as a percent of maximum tension induced by 10^{-8} M Rhopr-kinin 2 on each preparation. D) The effects of varying concentrations of the CAPA analog, 2129-SP3[Φ3]wp-2 on the changes in basal tonus elicited by 10^{-8} M Rhopr-kinin 2, with no statistically significant differences found (Two-way ANOVA followed by Tukey's post-hoc test, * = $p < 0.05$, ** = $p < 0.01$. Data are means ± SEM of n = 5).

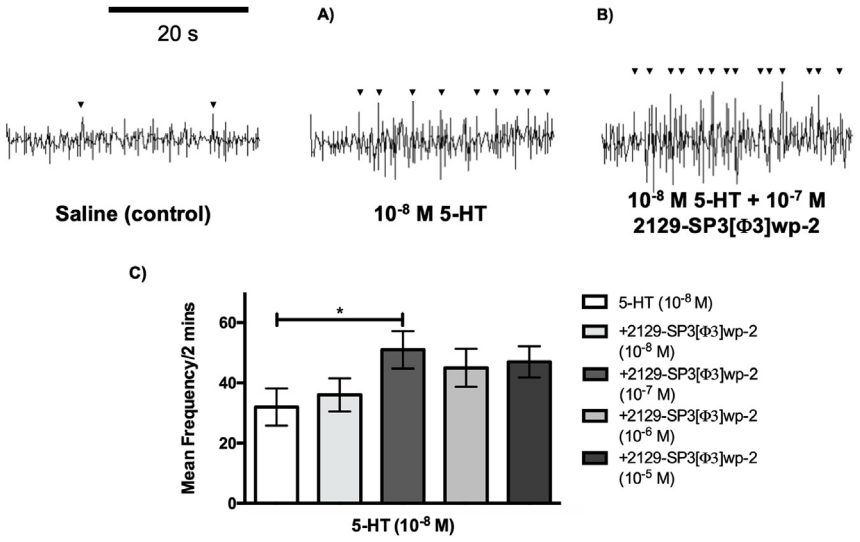

Fig. 5. Example traces of changes in the frequency of hindgut contractions in response to A) 10^{-8} M 5-HT and B) a mixture of 10^{-8} M 5-HT and 10^{-7} M of the CAPA analog 2129-SP3[Φ3]wp-2. Downwards triangles denote vertical deflections of hindgut contractions. C) The effects of varying concentrations of the CAPA analog 2129-SP3[Φ3]wp-2 on the frequency of hindgut contractions elicited by 10^{-8} M 5-HT over a 2-min period (One way-ANOVA followed by Dunnett's multiple comparison test, * = $p < 0.05$. Data are means ± SEM of n = 8).

(Nachman et al., 1997; Taneja-Bageshwar et al., 2009). Aib-containing analogs tested on *R. prolixus* hindgut were found to be more biologically active than Rhopr-kinins, and in some cases eliciting irreversible changes in basal tonus (Bhatt et al., 2014). Similar results were obtained with the kinin analog 2139[Φ1]wp-2, which caused dose-dependent increases in basal tonus of the hindgut and was active at concentrations as low as 10^{-14} M. As described for other Aib-containing analogs in *R. prolixus*, the effects of 2139[Φ1]wp-2 were more difficult to wash off. As these analogs were synthesized to prevent degradation by endogenous peptidases, the analog may have a prolonged effect on its target tissue by also influencing the binding to the receptor (Nachman et al., 2003). These potent changes in physiology induced by the kinin analogs highlight their promise in future studies for pesticide development.

Despite the presence of CAPA receptors (and indeed pyrokinin receptors) (Paluzzi, 2012; Paluzzi et al., 2008;Paluzzi and O'Donnell, 2012) on the hindgut, none of the three CAPA neuropeptides (Rhopr-CAPA-1, RhoprCAPA-2, or Rhopr-pk1) were found to have any direct effect on hindgut contractions. However, the CAPA analog 2129-SP3[Φ3]wp-2 induced hindgut contraction in a dose-dependent manner, but its effects were not as intense as Rhopr-kinin 2 or the kinin analog 2139[Φ1]wp-2. Interestingly though, RhoprCAPA-2 potentiated the effect of Rhopr-kinin 2. Since RhoprCAPA-2 is released as a signal to terminate diuresis, it may also assist the hindgut in excretion of the remaining urine. This potentiation effect was not observed with RhoprCAPA-1 or Rhopr-pk1, nor was it seen with the CAPA analog 2129-SP3[Φ3]wp-2.

The potentiation effect of RhoprCAPA-2 may be due to the interaction of separate second messenger pathways after GPCR activation. As the kinin receptor has not yet been characterized within *R. prolixus*, the associated second messenger pathway is currently unknown. Within other insects, the kinin receptor has been associated with an increase in intracellular Ca^{2+} via the inositol phosphate (IP_3) pathway (Radford et al., 2002; Terhzaz et al., 1999; Beyenbach, 2003; Pietrantonio et al., 2005). In *R. prolixus*, activation of the CAPA receptor in the MTs results in an increase in cGMP, likely activating a phosphodiesterase that degrades cAMP, thereby lowering cAMP levels. (Paluzzi et al., 2008; O'Donnell and Spring, 2000). Here, we propose that the CAPA receptor may be constitutively active within the hindgut, with cGMP stably keeping cAMP levels low. Upon activation of both the kinin and CAPA receptors, the increase in cGMP may participate in the IP_3 pathway to increase intracellular Ca^{2+} resulting in stronger contractions. In vertebrates, cGMP signaling is implicated in both stimulating or inhibiting contraction (Fischmeister et al., 2005; Fellner and Arendshorst, 2009).

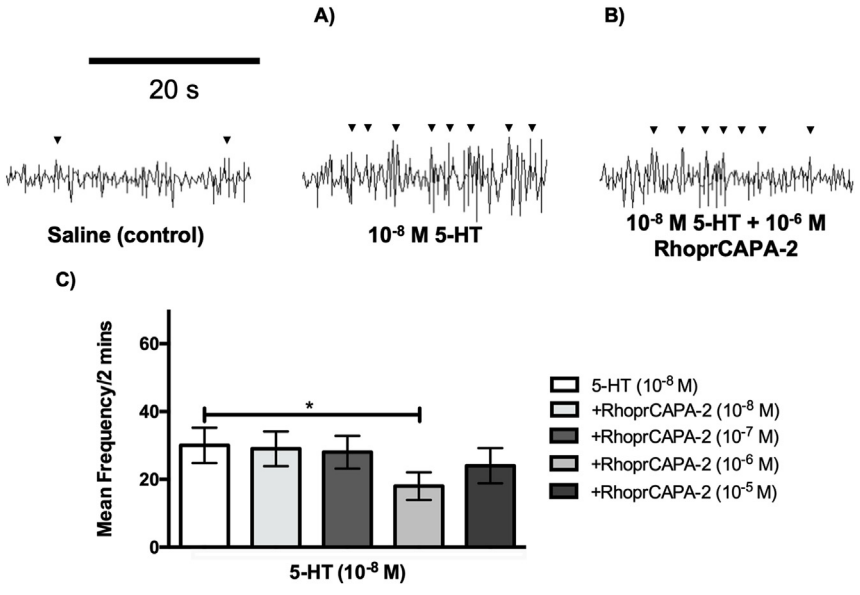

Fig. 6. Example traces of changes in the frequency of hindgut contractions in response to A) 10^{-8} M 5-HT and B) a mixture of 10^{-8} M 5-HT and 10^{-6} M RhoprCAPA-2. Downwards triangles denote contractions. C) The effects of varying concentrations of RhoprCAPA-2 on the frequency of hindgut contractions elicited by 10^{-8} M 5-HT over a 2-min period (One way-ANOVA followed by Dunnett's multiple comparison test, * = $p < 0.05$. Data are means ± SEM of n = 8).

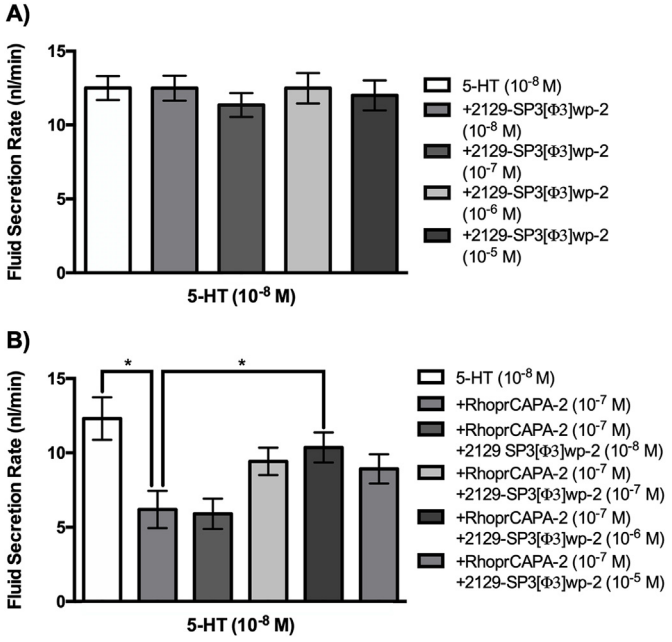

Fig. 7. A) The effects of varying concentrations of the CAPA analog 2129-SP3[Φ3]wp-2 on the fluid secretion rate of MTs stimulated by 10^{-8} M 5-HT. No statistically significant differences were found. B) The antagonist effects of various concentrations of the CAPA analog 2129-SP3[Φ3]wp-2 on RhoprCAPA-2 (10^{-7} M) resulting in blocking the inhibitory effect of Rhopr-CAPA-2 on MTs stimulated with 10^{-8} M 5-HT (One-way ANOVA followed by Dunnet's multiple comparison test, * = $p < 0.05$. Data are means ± SEM of n = 6).

Within vertebrate cardiac muscle, cGMP has shown to have excitatory effects through the stimulation of Ca^{2+} channels, resulting in an increase of intracellular Ca^{2+}. These Ca^{2+} channels are responsible for the excitation-contraction coupling within the muscle (Wang et al., 2000; Fischmeister et al., 2005; Kuo and Ehrlich, 2015). In contrast are the stimulatory effects of the CAPA analog 2129-SP3[Φ3]wp-2. This analog appears to be an antagonist of the CAPA receptor and so blocks the cGMP-mediated cAMP degradation via activation of a phosphodiesterase, in turn allowing cAMP to increase and stimulate hindgut contractions. In order to further investigate the possible mechanism by which this kinin/CAPA interaction occurs, the effects of RhoprCAPA-2 and 2129-SP3[Φ3]wp-2 were assessed with 5-HT. 5-HT has myostimulatory effects on the hindgut, via an increase in cAMP levels (Orchard, 2006). A potentiation effect was observed with co-application of 5-HT and 2129-SP3[Φ3]wp-2 on the frequency of hindgut contractions. This is likely due to the antagonist action on the CAPA receptors, preventing the RhoprCAPA-mediated cAMP decrease. Conversely, the effects of 5-HT were inhibited by RhoprCAPA-2, due to the increase in cGMP, activation of a phosphodiesterase, and degradation of cAMP.

The MTs are critical in allowing *R. prolixus* to return to a homeostatic state following a blood meal. As diuretic hormones such as 5-HT, Rhopr-DH_{31}, and Rhopr-CRF/DH function to stimulate secretion within the tubules, Rhopr-CAPA 2 is required as a signal to abolish this secretion to prevent excess ion and water loss (Paluzzi et al., 2008; Orchard, 2006). The CAPA analog 2129-SP3[Φ3]wp-2 was found to interfere with Rhopr-CAPA 2's ability to inhibit 5-HT-stimulated MT secretion, that is to say, it is an antagonist of the CAPA receptor. Within *D. suzukii*, 2129-SP3[Φ3]wp-2 had a protective effect as females injected with the analog had a significantly increased survival rate. Within *D. melanogaster*, CAPA functions as a diuretic hormone (Kean et al., 2002), with desiccation survival linked to the regulation of fluid secretion (Terhzaz et al., 2015). This supports the function of 2129-SP3[Φ3]wp-2 as a CAPA receptor antagonist, as it prevents CAPA-stimulated fluid secretion in *D. melanogaster*, thereby increasing survivability under desiccation stress (Alford et al., 2019a). Within *M. persicae* and *M. rosae*, however, injection of 2129-SP3[Φ3]wp-2 resulted in accelerated mortality under desiccation and starvation stress (Alford et al., 2019b). These effects of the CAPA analog may be due to the lack of MTs within aphids, therefore CAPA may not play a direct role in desiccation (Jing et al., 2015).

In summary, these results display the efficacy in which the biostable neuropeptide analogs are able to induce potent changes in physiology, thus showing potential use in the development of pest control strategies. The need for highly coordinated release of neuroactive chemicals within *R. prolixus* is a necessity in order for the insect to successfully gorge on blood. Given the bugs susceptibility to predators in its engorged state, rapid elimination of water and salt is required (Orchard, 2006). These analogs are able to successfully disrupt this coordination. The novel interaction between Rhopr-kinin 2 and RhoprCAPA-2 further highlights the importance of coordinated release of these neuropeptides throughout the life cycle of *R. prolixus*. Investigating the intracellular mechanisms by which the actions of the neuroactive chemicals function provides insight into the mode of action of epidemiologically-relevant behaviours, which can also aid in the development of next-generation pest control strategies.

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