Biostable insect kinin analogs reduce blood meal and disrupt ecdysis in the blood-gorging Chagas' disease vector, *Rhodnius prolixus*.

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Abstract

Rhodnius prolixus is a blood-gorging hemipteran that takes blood meals that are approximately 10 times its body weight. This blood meal is crucial for growth and development and is needed to ensure a successful molt into the next instar. Kinins are a multifunctional family of neuropeptides which have been shown to play a role in the control of feeding in a variety of insects. In this study, two biostable Aib-containing kinin analogs were tested to see if they interfere with blood-feeding and subsequent development into the next instar. One of the analogs, 1729 (Ac-R[Aib]FF[Aib]WGa), had no effect on the size of the blood meal or on the subsequent molting of the insect into the next instar. This analog also did not interfere with either short-term or long-term diuresis. The second analog, 1728 ([Aib]FF[Aib]WGa), appeared to be an antifeedant. Insects feeding on blood containing this analog (15 μ M) only consumed 60% of the blood meal taken by insects fed on blood without analog. Insects feeding on blood containing 1728 had a slower rate of rapid diuresis (diuresis in the first 3-5 h after feeding) leading to less urine being excreted by 5 days post feeding. The consequence of these effects was that insects fed on 1728 did not molt. This data indicates that the biostable Aib-containing analog 1728 disrupts normal growth and development in the blood-feeding insect, *R. prolixus*.

Keywords: diuresis, blood-feeding, molting, neuropeptide, kinin, peptide

1. Introduction

Neuropeptides are important regulators of vital functions in insects and therefore they have become lead compounds for the bio-rational design of insect control agents [1, 13]. One such family of peptides are the insect kinins which were first isolated from the cockroach, *Leucophaea maderae*, and shown to have myotropic activity on the hindgut [6-9]. The insect kinins are now known to be involved in a wide variety of feeding-related functions such as altering olfactory and gustatory responses in *Drosophila melanogaster* [11], stimulating secretion of primary urine by Malpighian tubules in a variety of insects [3, 10], stimulating hindgut contractions [2] and regulating digestive enzyme release [4, 5]. More recently, kinins have also been implicated in the coordination of ecdysis-related behaviours [12].

The *Rhodnius prolixus* kinin gene encodes 18 predicted kinins and precursor-associated or spacer peptides [2, 29]. All insect kinins possess a conserved C-terminal pentapeptide motif of $\text{FX}_1\text{X}_2\text{WGamide}$ where X_1 can be Ser, Phe, His, Asn, or Tyr and X_2 can be Ser, Pro or Ala [30]. Within the peptides coded on the Rhopr-kinin gene are 5 atypical WAamide kinins that have previously only been described in the shrimp, *Penaeus vannamei* [19]. In this blood-feeding insect, *R. prolixus*, kinins have been shown to be co-localized with the corticotrophin-releasing factor-like diuretic hormone (CRF/DH) in posterior-lateral neurosecretory cell bodies of the mesothoracic ganglionic mass (MTGM) and in neurohaemal sites on abdominal nerves. A decrease in intensity of immunoreactive staining for both the CRF/DH-like and kinin-like material within these cells and their neurohaemal sites suggests that these peptides are released within 2.5 hours after the start of blood-feeding [27]. In contrast to the effects of RhoprCRF/DH the Rhopr-kinins in *R. prolixus* do not appear to stimulate secretion by the Malpighian tubules or to increase anterior midgut absorption [26, 28], although they have been shown to depolarize midgut transepithelial voltage and resistance and to increase midgut and hindgut contractions. These effects suggest an involvement of Rhopr-kinins in digestion and excretion in *R. prolixus*.

Previous structure-activity studies on the kinins have used a peptidomimetic approach; replacing amino acids in the peptide with natural/unnatural structures to overcome the limitations for pest control development brought about by their chemical structure (e.g. their susceptibility to peptidases in the haemolymph) [15, 16, 18, 31-33]. Using this approach alpha-aminoisobutyric acid (Aib)-containing kinin analogs were designed and tested on a variety of physiological assays. These synthetic Aib-containing analogs were devised to prevent quick degradation by endogenous peptidases leading to a prolonged effect within the insect whilst retaining biological activity [13, 14]. Some Aib-containing kinin analogues have been shown to possess greater myotropic activity on the hindgut in *R. prolixus*, *L. maderae* and *Acheta domesticus* than the normal kinins [2]. These biostable Aib-containing kinins have also been particularly effective in feeding studies in the pea aphid, *Acyrtosiphon pisum*, where feeding on Aib-containing kinins resulted in antifeedant activity, with marked reduction in honeydew formation and very high mortality [22].

In this study we used two biostable Aib-containing kinins, 1729 (Ac-R[Aib]FF[Aib]WGa; also named K-Aib-3) and 1728 ([Aib]FF[Aib]WGa; also named K-Aib-1) (Fig. 1), to examine if kinin analogs alter feeding and short-term diuresis in the blood-feeding bug, *R. prolixus*. The kissing bug, *R. prolixus*, is a medically- important hemipteran that serves as a vector of Chagas' disease in humans since it transmits a protozoan parasite, *Trypanosoma cruzi*, via its urine following post-feeding diuresis. *R. prolixus* is an ideal model organism to study feeding-related changes since growth and development, ecdysis, and reproduction, are tightly regulated and timed by blood-gorging. *R. prolixus* remains in a state of arrested development until it takes a large blood-meal, which can be up to 10 times its body weight, and the nutrients obtained during the blood-meal are important for future growth and development of the insect into its next instar, and in adults for the ability to produce viable eggs. Therefore, using this model insect, we examined whether feeding on biostable kinin analogs led to a change in their ability to feed and therefore longer term effects on growth and development.

2. Methods

2.1 Animals

R. prolixus Stål were reared in a well-established colony at the University of Toronto Mississauga at 25°C and 50% humidity. The insects were fed on defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON) once during each larval stage using a latex-based artificial feeding membrane system.

2.2 Kinin Analogues

The two biostable Aib-containing insect kinin analogs, 1728 ([Aib]FF [Aib]WGa) and 1729 (Ac-R[Aib]FF[Aib]WGa), were synthesized, purified and quantified as previously described [25]. To solubilize the analogs, stock solutions were prepared in 40 µl of 80% aqueous acetonitrile (ACN) containing 0.01% trifluoroacetic acid (TFA).

2.3 Feeding Bioassay

Unfed fifth instars (4-6 weeks post-feeding as fourth instars) of similar weights were chosen and separated into groups of 16. Each group (n=16) was fed on 14 ml of warm defibrinated rabbit blood containing the kinin analogs or vehicle (80% ACN containing 0.01% TFA). The same volume of vehicle (40 µl) was added to the blood for all groups including the control groups. A trial feeding experiment was done with and without the vehicle and no difference in feeding behavior was seen, i.e. post-fed weight, feeding time, etc., indicating that it did not interfere with blood-feeding in *R. prolixus*.

Each group of insects (n=16) was placed in a 10 cm diameter glass jar and allowed to feed for 16 min. The time of feeding was determined by control feeding experiments in which normally all insects had finished feeding by this time, and had begun to move around the jar and to start diuresis. Individual insects from each group were weighed immediately (time 0) and maintained in individual containers kept in an incubator at 25°C and 50% humidity over the course of the experiment. Weights were recorded at

0.5, 1, 1.5, 2, 3, 4, 24 (1 day) and 120 (5 days) h after feeding. Time “0” was taken as the post-feeding weight. Insects were monitored daily over the next three weeks for deaths and molting into adults.

Normally fifth instar *R. prolixus* will take a blood meal approximately 10 times their body weight. Insects that fed less than 0.5 times their body weight were deemed not to have fed and excluded from the data. Also any insect that was injured during weighing was excluded, i.e. lost an appendage or were pierced with forceps and therefore lost blood content.

2.4 Statistical analyses

Graph Pad Prism (www.graphpad.com) was used to construct all graphical representations in this study and conduct statistical analyses. All data was analyzed by a One Way ANOVA followed by a Tukey’s Post-hoc test. Chi-Square statistics were performed on the molting data.

3. Results

Fifth instar *R. prolixus* were allowed to gorge for 16 min on rabbit blood during which time their body weight increased approximately 10 fold relative to their unfed body weight (Fig. 2 - 4). Rapid post-feeding diuresis then occurs for 3-5 hours during which time the insect loses approximately 24 % of its weight. This is followed by a much slower and smaller decrease in weight over 5 days (120 h). These insects then went through the process of growth and development leading to successful ecdysis into the adult stage.

We tested the two biostable Aib-containing analogs for their influence on feeding-related behaviors in *R. prolixus*. One of these, 1729 (Ac-R[Aib]FF[Aib]WGa), at two different concentrations tested (200 and 400 nmol per 14 ml of blood, approximately 14 μ M and 28 μ M, respectively), had little or no effects on either the weight of blood meal taken, the rate of diuresis or the ability of the insects to undergo ecdysis (Fig. 2, 3 and Table 1). Interestingly, addition of this analog to the blood meal resulted in a slight, though not significant, increase in initial post-fed weight at both concentrations tested. There was also no significant difference in body weight after 5 h (short-term diuresis) or 120 h (long-term diuresis) indicating again that this analog appears to not affect feeding or diuresis.

The second analog, 1728 ([Aib]FF [Aib]WGa), however, had a dramatic effect on feeding, diuresis and ecdysis (Fig. 4, 5 and Table 2). As shown in Fig. 4A and 5A, insects feeding on blood containing 200 nmol (approx. 27 μ M in the blood meal) of the kinin analog, 1728, only consumed 60% of the blood meal in comparison to controls. Interestingly, the higher dose of 400 nmol (approx. 54 μ M in the blood meal) 1728 was less effective with insects consuming approximately 85% of the blood meal as compared to control insects. In addition, the rapid diuresis over the first 3-5 hours progressed at a slower rate in the presence of 200 nmol of 1728 (loss of 19 mg body weight over 5 h for analog-fed insects versus 47 mg body weight over 5 h for control insects, Fig. 4) with less urine excreted from the insects by 120 h (Fig. 4A and 5C). At the end of 5 days the weight of insects having fed on analog 1728 was significantly lower

164 than controls (Fig. 5C). In addition to consuming significantly less blood meal (Fig. 5A), the majority of
165 insects that had fed on 1728 did not undergo ecdysis (Table 2).

4. Discussion

Gorging on blood results in short-term and long-term physiological and endocrinological changes in *R. prolixus* [20, 21]. On average, a 5th instar *R. prolixus* can consume blood meals that are 10 times its unfed body weight. Thus, in the short-term, the insect must eliminate excess water and salts during a rapid post-feeding diuresis which occurs during the 3 h following feeding. In the long-term, blood-gorging is the stimulus for endocrinological changes associated with growth and development, and blood-gorging of an appropriate weight of blood is required for each instar to develop and successfully go through ecdysis into the next instar. Thus, interfering with blood-gorging can have damaging consequences to the insect.

In light of data from another hemipteran, the aphid *A. pisum*, which illustrated that the presence of insect kinin analogs in their diet resulted in antifeedant and aphicidal activity [22], we tested for effects of similar Aib-containing kinin analogs on blood-gorging of 5th instar *R. prolixus*. One of the kinin analogs tested, 1728, disrupted blood-gorging in *R. prolixus*, with eventual negative influence on ecdysis to the adult. With this analog, the blood meal weight was only 60 % of the weight of control insects with diuresis rate similar, and only 18 % of the insects undergoing ecdysis relative to 80 % in control insects. Interestingly, this kinin analog was the same one that was shown to have both antifeedant and aphicidal activity in the pea aphid *A. pisum* [22]. The other Aib-containing kinin analog, 1729, was not active in this assay. Clearly, the presence of kinin analogs in the blood meal influences the amount of blood-gorging and consequently reduces the percentage of insects undergoing ecdysis and therefore continuing appropriate development into adults.

The mechanism of action of kinins/analogues is not well defined, but one could anticipate that it involves the kinin/analogues interaction with the *R. prolixus* kinin receptor and disrupting the timing of its normal physiological action. In *R. prolixus* kinins are potent stimulators of anterior midgut and hindgut contraction and of heart beat rate [2] and a recent study showed that some kinin analogs were more potent than native Rhopr-kinins on an *in vitro* hindgut assay [2]. In that study analogs 1728 and 1729 produced

significantly larger contractions of hindgut compared to Rhopr-kinin-2, and the effects were also very difficult to reverse upon washing with physiological saline. In addition, analogs 1728 and 1729 were effective at very low doses (10^{-16} M and 10^{-12} M respectively) versus Rhopr-kinin-2 which had a threshold between 10^{-10} M and 10^{-9} M (Bhatt et al., 2014). The increased potency of the analog 1728 compared to 1729 might explain its greater effect on feeding and diuresis. Interestingly, although Rhopr-kinins appear to be released along with RhoprCRF/DH in the first 3 hours after feeding, they do not appear to play any major role in post-feeding diuresis, which is in contrast to kinins which act as diuretic hormones in other insects [21].

Feeding kinin analogs to *R. prolixus* did not appear to influence post-feeding diuresis taking into account the different blood meal size, confirming other results regarding kinins involvement in diuresis [27, 28]. These slight differences in the rate of diuresis between the groups can be interpreted as resulting from the size of the weight of the blood meal. Disrupting normal contraction physiology of the digestive system could very well disrupt normal gorging behaviour with its associated feedback loops. In other studies, kinins have been shown to bind with high affinity to their receptors in ticks and mosquitoes and some of the Aib-containing kinin analogs were several fold more potent than a number of native kinins on these expressed receptors [23-25] matching the physiological effects shown on *R. prolixus* hindgut [2]. Notably, 1728 (K-Aib-1) is over 8-fold more potent than 1729 (K-Aib-3) on the mosquito kinin receptor [23-25]. The more potent effects of 1728 over 1729 in an in vitro *R. prolixus* hindgut contraction study [2], and in these in vivo feeding studies suggest that a similar difference in potency may exist with the interaction of these two Aib insect kinin analogs on the *R. prolixus* kinin receptor. As described in the aphid, it is also possible that taste receptors may be influenced by the presence of kinins/analogues, resulting in an influence on feeding behavior and less blood being gorged [22]. The higher activity observed for 1728 at the lower dose may reflect the onset of receptor de-sensitization, which has also been observed for insect kinins on an isolated cockroach hindgut contraction assay [6-9].

The data from *R. prolixus* matches in a global way the effects of these agents on aphids. In *A. pisum*, feeding on kinin analogs over a 3 day period resulted in antifeedant activity and high mortality [22]. The aphicidal activity (LC_{50}) of approximately 63 μ M of one of the analogs matched the potency of some commercially available aphicides [22]. *R. prolixus* also takes smaller blood meals when kinin analogs are present in the blood and so similar antifeedant activity is demonstrated in this blood-gorging hemipteran, at the lower dose of 27 μ M. This work extends that of previous studies whereby active biostable insect kinins represent potential leads in the development of novel, environmentally friendly pest control agents [13, 17].

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Table 1. Number of insects undergoing ecdysis after feeding on a blood meal containing 200 nmols or 400 nmols of the biostable Aib-containing kinin analog, 1729.

Treatment	Molted insects (#)	Non-molted insects (#)
Control	12	3
200 nmol	11	3
400 nmol	15	0

The Chi-square statistic is 3.606. The p-value is 0.164802. The result is not significant at $p < .05$.

Table 2. Number of insects undergoing ecdysis after feeding on a blood meal containing 200 nmols or 400 nmols of the biostable Aib-containing kinin analog, 1728.

Treatment	Molted insects (#)	Non-molted insects (#)
Control	12	3
200 nmol	3	13
400 nmol	6	10

The Chi-square statistic is 12.2568. The p-value is 0.00218. The result is significant at $p < .05$.

Figure Legends

Figure 1. Structures of the two biostable Aib-containing kinin analogues; (A) 1728 ([Aib]FF[Aib]WGa) and (B) 1729 (Ac-R[Aib]FF[Aib]WGa. Red color identifies the unnatural, sterically-hindered (bulky) α -aminoisobuteryl (Aib) residues, which confer biostability to the two insect kinin analogs.

Figure 2. Fifth instar female *Rhodnius prolixus* were fed to completion on rabbit blood or rabbit blood containing 200 or 400 nmol of the Aib-containing kinin analog, 1729 (Ac-R[Aib]FF[Aib]WGa). At the completion of feeding (16 min of feeding) insects were weighed to ascertain the weight of the blood meal and this was graphed at time 0h (Post-fed weight; mg). Insects were then weighed at 0.5, 1, 2, 3, 4, 5 and 120 h to determine weight loss through diuresis. Data is expressed as post-fed weight in mg (A) or as a percentage of weight compared to the post-fed weight at 0 h (B). Initial weight of insects was 30.4 ± 1.4 mg (controls); 30.4 ± 1.2 mg (Analog 1729, 200 nmol) and 32.1 ± 1.1 mg (Analog 1729, 400 nmol). Symbols are mean $\pm$ S.E.M. for 13-16 individuals.

Figure 3. Analog 1729 has no effect on size of blood meal and short-term or long term diuresis. (A) Post-fed weight (mg) taken at time 0 h after feeding insects on rabbit blood (control) or rabbit blood containing 200 or 400 mole of the Aib-containing kinin analog,. Insects were fed to completion (16 min). The weight of insects fed on blood containing 200 or 400 nmol of 1728 ([Aib]FF [Aib]WGa) after short –term diuresis at 5 h (B) or after long-term diuresis at 120 h (5 days) post-feeding (C). Bars are mean $\pm$ SEM of 13-16 insects. There is no statistical difference between any of the values (One way ANOVA, $p>0.05$)

Figure 4. Fifth instar female *Rhodnius prolixus* were fed to completion on rabbit blood or rabbit blood containing 200 or 400 nmol of the Aib-containing kinin analog, 1728 ([Aib]FF [Aib]WGa). At the completion of feeding (16 min of feeding) insects were weighed to ascertain the weight of the blood meal and this was graphed at time 0h (Post-fed weight; mg). Insects were then weighed at 0.5, 1, 2, 3, 4, 5 and 120 h to determine weight loss through diuresis. Data is expressed as post-fed weight in mg (A) or as a

percentage of weight compared to the post-fed weight at 0 h (B). Initial weight of insects was 30.4 ± 1.4 mg (controls); 30.4 ± 1.3 mg (Analog 1728, 200 nmol) and 32.5 ± 1.2 mg (Analog 1728, 400 nmol). Symbols are mean $\pm$ S.E.M. for 14-16 individuals.

Figure 5. Analog 1728 decreased the size of blood meal. (A) Post-fed weight (mg) taken at time 0 h after feeding insects on rabbit blood (control) or rabbit blood containing 200 or 400 mole of the Aib-containing kinin analog,. Insects were fed to completion (16 min). The weight of insects fed on blood containing 200 or 400 nmol of 1728 ([Aib]FF [Aib]WGa) after short-term diuresis (B) and after long-term diuresis (C). Bars are mean $\pm$ SEM of 14-16 insects. Asterisks indicate statistical difference of $p < 0.01$ (One way ANOVA followed by Tukey's post-hoc test)

A

B

A

B

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