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Investigation of the potential involvement of eicosanoid metabolites in anti-diuretic hormone signaling in *Rhodnius prolixus*

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ABSTRACT

The use of naturally occurring plant-derived compounds for controlling insect pests remains an attractive alternative to potentially dangerous synthetic chemical compounds. One prospective plant-based compound, isoforms of the so-called jack bean urease (JBU) from the jack bean, *Canavalia ensiformis*, as well a derived peptide, Jaburetox-2Ec, have insecticidal effects on an array of insect species. In the Chagas' disease vector, *Rhodnius prolixus*, some of the physiological effects attributed to these urease isoforms include inhibition of serotonin (5-HT)-stimulated fluid secretion by the Malpighian tubules (MTs). Here, we investigated whether the effects of these exogenous urease isoforms were targeting the neuroendocrine network involved in the anti-diuretic hormone (RhoprCAPA-2) signaling cascade. We show that pharmacological agents known to interfere with eicosanoid metabolite biosynthesis do not affect RhoprCAPA-2 inhibition of 5-HT-stimulated fluid secretion by MTs. In addition, we demonstrate that RhoprCAPA-2 inhibition of MTs is independent of extracellular or intracellular calcium. Using a heterologous system for analysis of receptor activation, we show that neither JBU nor Jaburetox-2Ec are agonists of the anti-diuretic hormone receptor, RhoprCAPAr1. Finally, activation of the receptor using sub-maximal doses of the natural ligand, RhoprCAPA-2, was not influenced by the presence of either JBU or Jaburetox-2Ec indicating that the urease isoforms do not compete with RhoprCAPA-2 for binding and activation of RhoprCAPAr1. Taken together, these results suggest that at least two distinct mechanisms leading to inhibition of fluid secretion by MTs exist in *R. prolixus* and, unlike the urease-related effects, the eicosanoid metabolite pathway is not involved in RhoprCAPA-2 mediated anti-diuresis.

1. Introduction

Rhodnius prolixus is a haematophagous insect that has been identified as a principal vector of Chagas' disease in Central and South America [56]. Transmission of the protozoan parasite *Trypanosoma cruzi*, that causes Chagas' disease in humans, occurs in close association with the rapid diuresis in *R. prolixus* that facilitates excretion of excess fluid and ions from the blood meal [32]. *T. cruzi* trypomastigotes are transmitted through fecal contamination of the bite wound produced by blood feeding [29]. The enormous blood meal imbibed by *R. prolixus* during each post-embryonic stage is required for nymphal development and also contributes to increased egg production in adult females [15]. The rapid diuresis that follows engorgement of vertebrate blood has been studied in detail and involves rapid rates of transport of ions that are present in excess in the blood meal, mainly sodium and chloride, as well as osmotically obliged water. The excess water and salts associated mainly with the non-nutritive plasma portion of the blood meal is removed by absorption across the lumen of the anterior midgut that is matched with secretion of primary urine by the MTs so that salt and water homeostasis is maintained within the haemolymph [25].

Two diuretic hormones have now been identified in *R. prolixus*: the first hormone, a biogenic amine, is serotonin (5-hydroxytryptamine; 5-HT); and, the second is a peptide, related to the corticotropin-releasing factor family of vertebrate peptides, RhoprCRF/DH [50]. Both of these diuretic hormones lead to rapid increases in fluid and ion transport across the anterior midgut epithelium [4,14,18,48,50] and are responsible for

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increasing secretion rates by MTs over a thousand-fold relative to unstimulated tubules [23,24,26,51,52]. Secretion rates of this magnitude would result in disruption of haemolymph homeostasis within a short period of time if diuresis were not stringently controlled, and *R. prolixus* must be capable of down regulating diuresis to counter this effect [25,39,40]. Interestingly, however, although members of other diuretic peptide families are known to exist in *R. prolixus* [49,50,53,59], only 5-HT and RhoprCRF/DH are diuretic hormones acting on the anterior midgut [48,50,54] and MTs [12]. In order to prevent excessive loss of water and salts, the rapid diuresis terminates around 3–4 h post-feeding. An anti-diuretic neurohormone, namely RhoprCAPA-2, has been shown to inhibit 5-HT-stimulated absorption by the anterior midgut as well as inhibit 5-HT-stimulated secretion by MTs [18,33,34,36]. RhoprCAPA-2 exerts its effects on the anterior midgut and MTs through activation of the anti-diuretic hormone receptor, RhoprCAPAr1 [35].

Interestingly, isoforms of the jack bean urease (JBU) from the jack bean plant, *Canavalia ensiformis*, as well as a peptide derived from JBU, Jaburetox-2Ec (JBX), have been shown to have inhibitory actions on 5HT-stimulated secretion by MTs [46]. More specifically, the JBU effects were shown to be dependent on eicosanoid metabolites and calcium ions, whereas the effects of JBX may involve cyclic GMP [46].

Given that the primary targets eliciting the JBU and JBX effects in *R. prolixus* are unknown, we sought to elucidate if these exogenous plant-derived factors were acting on cellular targets of the native anti-diuretic peptide, RhoprCAPA-2. First, we determined whether the eicosanoid metabolite pathway inhibitors could block RhoprCAPA-2 inhibition of 5-HT-stimulated tubule fluid secretion. We next studied the involvement of intracellular and extracellular calcium in RhoprCAPA-2 inhibition of tubule fluid secretion stimulated by 5-HT. Lastly, we investigated if the anti-diuretic hormone receptor, RhoprCAPAr1, could be activated by either JBU or JBX, and furthermore we tested if RhoprCAPA-2-induced receptor activation could be modulated in the presence of either JBU or JBX. Our findings suggest that the JBU and JBX mediated inhibition of tubule fluid secretion is independent of the native anti-diuretic peptide signaling mechanism leading to the cessation of diuresis.

2. Methods

2.1. Animals

R. prolixus Stål were obtained from a laboratory colony at the Department of Biology, University of Toronto Mississauga. Insects were maintained at 25 °C and high relative humidity and were routinely fed on defibrinated rabbit blood (Cedarlane, Burlington, ON) using an artificial feeding membrane. Unfed fifth-instar insects were used in all experiments, approximately 6–8 weeks post-feeding as fourth instars.

2.2. Malpighian tubule (MT) fluid secretion assays

Insects were dissected under physiological saline as described previously [36]. Malpighian tubules were excised whole and transferred on glass probes to 20 μl drops of saline submerged in water-saturated paraffin oil in Sylgard-coated Petri dishes. The entire upper/distal (secretory) segment of the tubule was placed in the saline droplet and the lower/proximal end of each tubule was wrapped around a minuten pin. The length of the lower (reabsorptive) tubule section for each tubule that was excised was not standardized, as fluid secretion rates were found to be unaffected by the length of the lower tubule [27,39]. Tubule secretion was monitored after rupturing the lower tubule segment between the saline droplet and the minuten pin with fine forceps. Transfer of secreted droplets following set intervals was accomplished using a micro-aspirator. The diameter of the secreted droplet was used to calculate volume (V) and determined by use of an ocular micrometer and calculated using the formula $V = (\Pi/6)d^3$, where d represents the diameter of the droplet measured by an ocular micrometer.

Stimulation of fluid secretion by MTs with 5-HT was carried out using either 100 nM or 1 μM. The lower dose was used to produce sub-maximal rates of fluid secretion [54] that could be challenged with the anti-diuretic peptide, RhoprCAPA-2, while the higher dose of 5-HT was used to achieve maximal secretion rates. To ensure functionality of the excised MTs and exclusion of damaged preparations, the first treatment involved sub-maximal concentrations of 5-HT over 30 min and were used to determine control secretion rates. Subsequently, the tubules were washed with saline and incubated for 30 min with sub-maximal 5-HT in combination with an experimental compound (endogenous anti-diuretic peptide alone or in presence of pharmacological agent). Following a saline wash, tubules were treated with a high dose of 5-HT for 30 min in order to determine maximal secretion rates which were used for normalization of data if required. In order to determine if extracellular calcium was required for anti-diuretic peptide signaling, fluid secretion assays were carried out in calcium-free saline containing the calcium chelator, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; 1 mM) and supplementing with $MgCl_2$ to maintain saline osmolarity. The involvement of intracellular calcium stores in anti-diuretic peptide signaling was tested using the intracellular calcium antagonist, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) [11]. Serotonin hydrochloride (5-hydroxytryptamine hydrochloride; 5-HT), TMB-8, dexamethasone and indomethacin were purchased from Sigma (Oakville, Ontario, Canada). RhoprCAPA-2 was obtained as described previously [36].

2.3. Jack bean urease and recombinant peptide, Jaburetox-2Ec

Jack bean urease (JBU) was purchased (Sigma, Oakville, ON) and prepared as a 1 mM stock solution as described previously [46]. The V5-His6 tagged recombinant peptide, Jaburetox-2Ec (JBX), was purified as described previously [30]. In brief, *Escherichia coli* BL21(DE3)-strain bacteria (NEB, Pickering, ON) were transformed with pET101-JBX recombinant vector and cells were grown overnight on LB plates containing ampicillin. Colonies containing recombinant vector were confirmed following standard plasmid minipreparation (BioBasic, Markam, ON) and DNA sequencing (Center for Applied Genomics at the Hospital for Sick Children, Toronto, ON). An aliquot of recombinant bacteria liquid culture grown overnight was taken and added to fresh media (250 mL) containing ampicillin. Cells were grown at 37 °C with shaking (~300 rpm) until an OD_{600} = 0.6–0.8 at which point isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final titer of 0.5 mM. Following a 3 h induction, cells were harvested, resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 7.0, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100 and 10 mM imidazole), sonicated and centrifuged (14,000 × *g* for 20 min). The supernatant was processed on a nickel affinity column (Ni-NTA-agarose; Qiagen, Mississauga, ON), which was previously equilibrated with buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole). After 30 min, the column was washed with 20 mL of the same buffer, containing 20 mM imidazole. The protein was eluted with the equilibration buffer containing 200 mM imidazole and quantified following the Bradford assay [5]. The samples were dialyzed against 20 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β-mercaptoethanol, pH 7.5, 0.02% sodium azide.