Isolation, expression analysis, and functional characterization of the first antidiuretic hormone receptor in insects

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Diuresis following blood-gorging in *Rhodnius prolixus* is the major process leading to the transmission of Chagas' disease. We have cloned the cDNA of the first receptor known to be involved in an antidiuretic strategy in insects, a strategy that prevents diuresis. This receptor belongs to the insect CAPA receptor family known in other insects to be activated by peptides encoded within the *capability* gene. We characterize the expression profile in fifth-instars and find expression is localized to the alimentary canal. Highest transcript levels are found in Malpighian tubules and the anterior midgut, which are known targets of the antidiuretic hormone, RhoprCAPA-α2. Two transcripts were identified, *capa-r1* and *capa-r2*; however, the latter encodes an atypical G protein-coupled receptor lacking a region ranging between the first and second transmembrane domain. Our heterologous expression assay revealed the expressed *capa-r1* receptor is activated by RhoprCAPA-α2 (EC_{50} = 385nM) but not by RhoprCAPA-α1. Structural analogs of the inactive RhoprCAPA-α1 were capable of activating the expressed *capa-r1* receptor, confirming the importance of the C-terminal consensus sequence common to CAPA-related peptides. In addition, this receptor has some sensitivity to the pyrokinin-related peptide, RhoprCAPA-αPK1, but with an efficacy ≈40-fold less than RhoprCAPA-α2. Other peptides belonging to the PRXa-mide superfamily were inactive on the *capa-r1* receptor. Taken together, the neuroendocrinological relevance of this receptor in facilitating the antidiuretic strategy in *R. prolixus* may make this receptor a useful target for development of agonists or antagonists that could help influence the transmission of Chagas' disease that occurs during diuresis in this medically important insect-disease vector.

CAPA | Chagas' | G protein-coupled receptor | neurohormone | neuropeptide

A major physiological process that allows insects to adopt a variety of feeding strategies and environmental niches is their ability to regulate water and ion composition in their hemolymph. This process involves control over primary urine production via the insect kidney equivalent, the Malpighian (renal) tubules (MTs) and, in a number of insects, the reabsorption of essential salts and water from the hindgut before the final excretae is voided. Insect neurohormones, including peptides and biogenic amines, together with their cognate receptors, carry out an essential role in these and related physiological processes, and numerous neuroendocrine factors regulating fluid and ion balance in insects have been described (see ref. 1 for a review). One insect that has been used as a model for understanding the control of this diuresis is the hematophagous bug, *Rhodnius prolixus*. This insect imbibes enormous blood meals and must then rapidly eliminate large volumes of excess salts and water (2). The parasitic protozoan, *Trypanosoma cruzi*, is transmitted to humans in the urine, and so diuresis controls the transmission of Chagas' disease. Neurohormones control hemolymph salt and water homeostasis by acting on the anterior midgut and MTs (3, 4). The insect CAPA peptides, some of which are also referred to as periviscerokinins, usually contain the consensus carboxy terminal sequence FPRV-NH_2. These peptides are normally produced within the central nervous system and are known to be released into the hemolymph from peripheral neurohemal sites, where they modify activities of visceral tissues. In dipterans, these peptides activate nitric oxide synthase in principal cells of the main segment of the MTs, leading to increased fluid secretion (5, 6). Interestingly, these peptides are not stimulatory on MTs in all insects in which these peptides have been tested. For example, in the locust, *Schistocerca gregaria*, this family of peptides does not increase fluid secretion (6), nor does it increase levels of nitric oxide. In addition, CAPA-related peptides are inhibitory on MTs in the hematophagous insect, *R. prolixus* (7, 8). Recently, we established that *R. prolixus* contains two genes. Each gene codes for three peptides, two of them being CAPA-related peptides and the third being a pyrokinin-related peptide. The second encoded peptide in each prepropeptide, RhoprCAPA-α2(-β2), is identical in sequence in each paralog (9, 10) and has been shown to directly inhibit 5HT-stimulated secretion by the MTs (9, 10), as well as absorption of water and ions by the anterior midgut (4, 11). The availability of completed insect genomes has made the identification of receptors for these neuroendocrine factors more feasible. The first such receptor, belonging to the G protein-coupled receptor (GPCR) family, was annotated in the genome assembly of the fruit fly, *Drosophila melanogaster* (12), and was subsequently deorphaned by functional characterization by two separate research groups (13, 14). This gene, annotated as CG14575, was shown by functional ligand-receptor interaction assay to have an EC_{50} value of 150 to 230 nM and 69 to 110 nM (13, 14) for the *D. melanogaster* CAPA peptides, capa-1 and capa-2, respectively. Subsequent to these studies, a CAPA receptor was also identified in the malaria mosquito, *Anopheles gambiae* (15). In the present study, we report the isolation, transcript expression profile, and functional interaction analysis of the CAPA receptor in *R. prolixus*, referred to as *RhoprCAPA-R*. This receptor mRNA is localized to tissues of the alimentary canal (foregut, midgut, hindgut, and MTs), and exhibits highest transcript levels in the anterior midgut and MTs: tissues known to be regulated by the antidiuretic neurohormone RhoprCAPA-α2 (identical in sequence to RhoprCAPA-β2). In addition, the upper secretory segment of the Malpighian tubules contains the majority of the *RhoprCAPA-R* transcript expression, compared with the lower, nonsecretory reabsorptive segment.

Author contributions: J.-P.P. designed research; J.-P.P. performed research; Y.P., R.J.N., and I.O. contributed new reagents/analytic tools; J.-P.P. analyzed data; and J.-P.P. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The following have been deposited in the GenBank database: capa-r1 (GenBank accession: GU734127) and capa-r2 (GenBank accession: GU734128)

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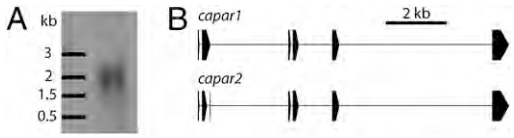

Fig. 1. *Rhodnius prolixus* CAPA receptor transcript size and predicted genomic organization. (*A*) Northern blot showing approximate size of the *RhoprCAPA-R* transcripts as determined by hybridization of alimentary canal mRNA. (*B*) Predicted gene structure showing exon/intron size and location based on amplification of cDNA and comparison with preliminary assembly of *R. prolixus* genome.

Functional ligand-receptor interaction assays demonstrate that the receptor has highest affinity for the antidiuretic neurohormone, RhoprCAPA-α2, encoded by both *R. prolixus* CAPA gene paralogs. To our knowledge, this study is unique in isolating and functionally characterizing an antidiuretic hormone receptor in any insect. The identification of such a receptor, and the knowledge acquired from the neuroendocrinological effects that it mediates, holds promise for influencing the transmission of Chagas' disease associated with blood-feeding by this medically important pest.

Results

***R. prolixus* CAPA Receptor.** The *R. prolixus capa-r* gene encodes two mRNA variants, *capa-r1* (GenBank accession: GU734127) and *capa-r2* (GenBank accession: GU734128), with a cloned transcript size of ≈1,437 bp and 1,344 bp, respectively (Fig. S1). Northern blot hybridization suggests the full-length transcripts may be slightly larger (Fig. 1*A*); however this is likely an extension of the 5′UTR because an in-frame stop signal (bases −54 to −52) (Fig. S1) is present upstream of the identified ATG translation start site. Both of these transcripts are encoded by six exons spanning ≈10.2 kb of the genome as predicted by the *R. prolixus* preliminary genome assembly (Fig. 1*B*). The data suggest the smaller transcript, *capa-r2*, is the product of splicing of an optional intron because of cryptic splicing signals located over the second exon sequence (Fig 1*B* and Fig. S1) that reduces the size of this exon from 282 bp to 189 bp and reduces the resulting protein by 31 amino acids. The *capa-r1* transcript encodes a predicted seven transmembrane-domain protein consistent with features present in GPCRs with an extracellular N-terminal sequence and intracellular C-terminal sequence (Fig S2*A*). The *capa-r2* transcript produces an atypical GPCR having only six predicted hydrophobic transmembrane domains and, in addition, both the N-terminal and C-terminal ends are predicted to be intracellular (Fig S2*B*).

Sequence and Phylogenetic Analysis. Sequence analysis of CAPA-R1 receptor reveals features characteristic of the Rhodopsin-like GPCR subfamily (or family-A GPCRs). These features include a slight variation of the NSxxNPxxY motif localized to the seventh α-helix membrane-spanning domain and a D/E-R-Y/F at the border between the third transmembrane and the second intracellular loop (16). Sequence comparison of the resulting translation of *capa-r1* and *capa-r2* transcripts with orthologous proteins predicted or identified in other insects revealed some interesting findings regarding the sequence similarity among the receptors (Fig. 2). Either character-based maximum parsimony or distance-based neighbor-joining analyses yield similar outcomes in tree topology (neighbor-joining results are shown). A monophyletic group con-

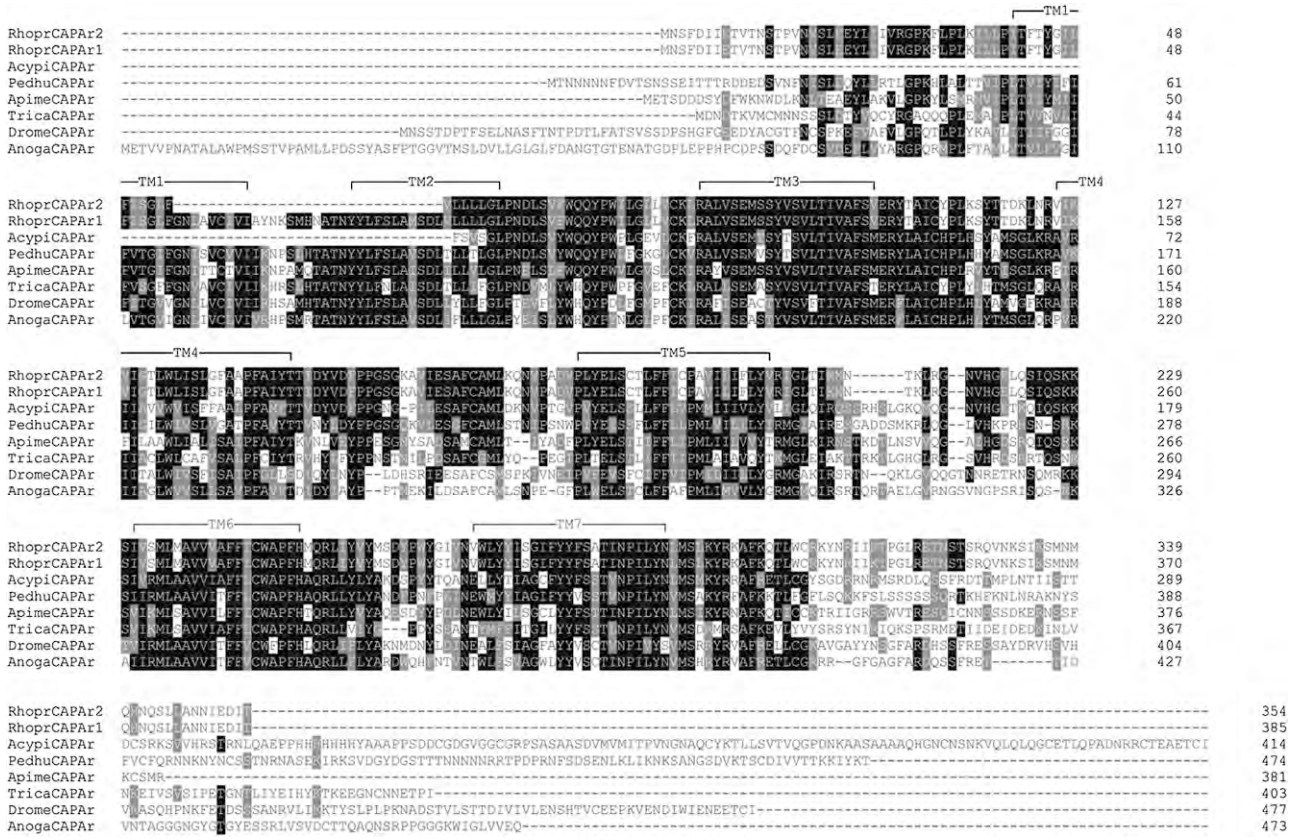

Fig. 2. Sequence analysis of CAPA receptors in insects. Protein alignment of the insect CAPA receptors identified or predicted based on genome sequence data. The predicted location of the seven transmembrane domains are noted above each row (TM1–TM7). Dark-gray shading denotes sequences identical in greater than 50% of that particular column and light-gray shading denotes similar residue to column-consensus residue.

tains the *R. prolixus* CAPA receptor sequences identified herein together with other insect CAPA receptors; the pyrokinin-1 receptors form a distinct clade (Fig. 3), with each having high bootstrapping support. The clade consisting of the two *R. prolixus* receptor sequences and, in addition, that includes the *D. melanogaster* and *A. gambiae* receptor sequences, also has very high bootstrapping support. Similarly, the monophyletic group consisting of two identified pyrokinin-1 receptors from the dipteran insects, *D. melanogaster* and *A. gambiae*, as well as an orphan receptor from *Aedes aegypti* (XP_001662936), which we classify as a pyrokinin receptor based on sequence analysis, also has high bootstrapping support. All other relationships among sequences were not highly supported (bootstrapping support ranging between 71–81%).

Expression Pattern of CAPA Receptor Transcript Variants. To predict potential physiological targets for the *R. prolixus* CAPA peptides, we investigated the spatial expression profile of the two transcript variants encoding the putative CAPA receptor in *R. prolixus*. Expression of *capa-r1* transcript is exclusively localized to the alimentary canal (Fig. 4*A*). More specifically, expression is predominantly in the MTs and the anterior midgut; however, expression also exists in the oesophagus posterior midgut, and hindgut, with the latter demonstrating lowest levels. Other tissues, such as the central nervous system, salivary glands, reproductive tissues, trachea, fat body, dorsal vessel, or abdominal nerves, do not exhibit any detectable levels of either transcript. In all tissues where *capa-r1* expression is identified, substantially lower expression of the second transcript variant, *capa-r2*, is evident, with values approximately ~350-fold less relative to *capa-r1* (Fig. 4 *A* and *B*). We also were interested in analyzing the expression of the two transcript variants encoding the putative RhoprCAPA receptor in distinct upper secretory and lower reabsorptive regions of the MTs. The *capa-r1* transcript expression is localized almost entirely to the upper lengths of the tubules, with greater than 60-fold higher levels, compared with the lower segment expression (Fig. 4*B*). Expression of the second transcript variant, *capa-r2*, demonstrates extremely low levels relative to *capa-r1* (Fig. 4*B*), consistent with the relative values identified in other tissues where the two transcripts are expressed (Fig. 4*A*).

Functional Ligand–Receptor Interaction Assay. Determination of the endogenous ligands for the two receptor variants was facilitated using a calcium mobilization assay in heterologously expressed *R. prolixus* CAPA receptor clones in CHO-K1 cells. First, we

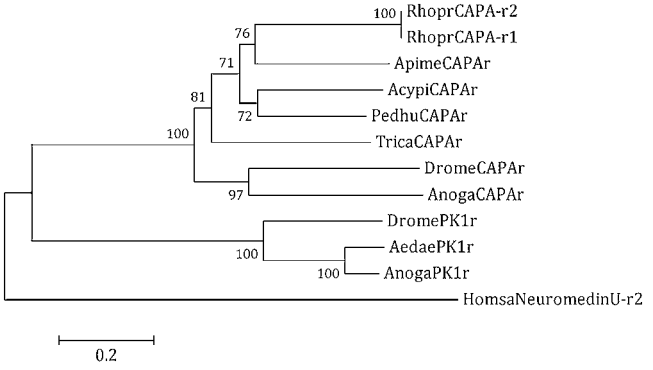

Fig. 3. Phylogenetic analysis of CAPA and pyrokinin-1 receptors in insects. The phylogenetic relationship of the insect CAPA and PK-1 receptors were deduced using the neighbor-joining method. Branch-length units are the number of amino acid substitutions per site. The numbers adjacent to the nodes represent bootstrap support for the clustering of associated sequences (bootstrap support below 70% is not shown). The closest human homolog to the insect CAPA receptors, the neuromedin U receptor-2, was included in the analysis and imposed as the out group.

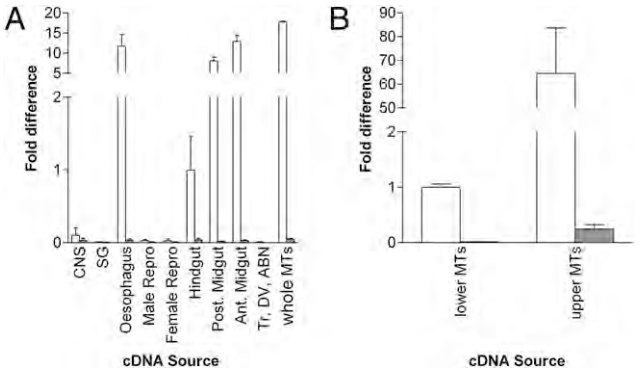

Fig. 4. CAPA receptor expression profile in fifth-instar tissues. The *capa-r1* transcript expression is denoted by open columns and *capa-r2* transcript expression is denoted by filled columns. (*A*) Expression was detected throughout the alimentary canal. Fold-difference in expression is relative to *capa-r1* expression in the hindgut. CNS, central nervous system; SG, salivary glands; Male Repro, male reproductive tissue; Female Repro, female reproductive tissue; Post. Midgut, Posterior midgut; Ant. Midgut, Anterior midgut; Tr, trachea; DV, dorsal vessel; ABN, abdominal nerves, diaphragm, and fat body; whole MTs, whole Malpighian tubules. (*B*) Upper and lower segments of MTs were dissected and separated before RNA isolation and cDNA synthesis. Fold-difference in expression is shown relative to the expression of the *capa-r1* transcript in lower tubule segments.

tested the endogenously expressed peptides encoded by the *RhoprCAPA-α* gene (Fig. 5*A*). The *capa-r1* clone has the greatest activity when tested with RhoprCAPA-α2 (EC_{50} = 385 nM). Activation of *capa-r1* is also evident with the pyrokinin-related peptide, RhoprCAPA-αPK1 (EC_{50} > 5 μM); however, at the highest dose tested, this accounted for only a 35% activation (≈40-fold lower efficacy) compared with the highest response observed with RhoprCAPA-α2. At all doses tested, the first peptide encoded in the *RhoprCAPA-α* gene, RhoprCAPA-α1, did not yield any detectable activation of the *capa-r1* receptor. The other receptor variant, *capa-r2*, was also tested against the peptides encoded by the *RhoprCAPA-α* gene; however, no response can be detected against any of the three native peptides (Fig. 5*B*). Insect CAPA peptides normally contain the consensus C-terminal sequence FPRV-NH_2, and because the noncanonical CAPA peptide, RhoprCAPA-α1, shows no activation of the *capa-r1* receptor, we tested structural analogs (Table 1) of this native peptide to identify which residues may be important for effective activation of this receptor. Although the native RhoprCAPA-α1 peptide is inactive on the *capa-r1* receptor, the modified analog, RhoprCAPA-α1ΔLRV-NH_2, with a C-terminal valine substituting the native alanine residue, shows some activation of the *capa-r1* receptor; however, at the highest dose tested (5 μM), this activity is ≈36% of the maximum response with RhoprCAPA-α2. A second modified analog, RhoprCAPA-α1ΔPRV-NH_2, having the native LRA-NH_2 substituted with the PRV-NH_2 C terminus, demonstrates complete recovery of activity on the *capa-r1* receptor (Fig. 5*C*). Similar experiments conducted on cell lines transfected with the *capa-r2* receptor variant demonstrate that these peptides/analogs are all inactive for calcium mobilization (Fig S3*A*). We next tested the transfected cell line with the structurally related peptides, human AVP (HomsaAVP) and *D. melanogaster* hugin γ (Dromehugγ). Neither of these peptides were active on the *capa-r1* receptor (Fig. 5*D*) or on the *capa-r2* receptor variant (Fig S3*B*). Finally, control cells that were transfected with empty vector showed no response when challenged with any of the peptides used in this study, indicating that the calcium mobilization results were indeed mediated by the transfected *capa-r1* receptor and not the result of activation of any endogenous receptors in the CHO cells.

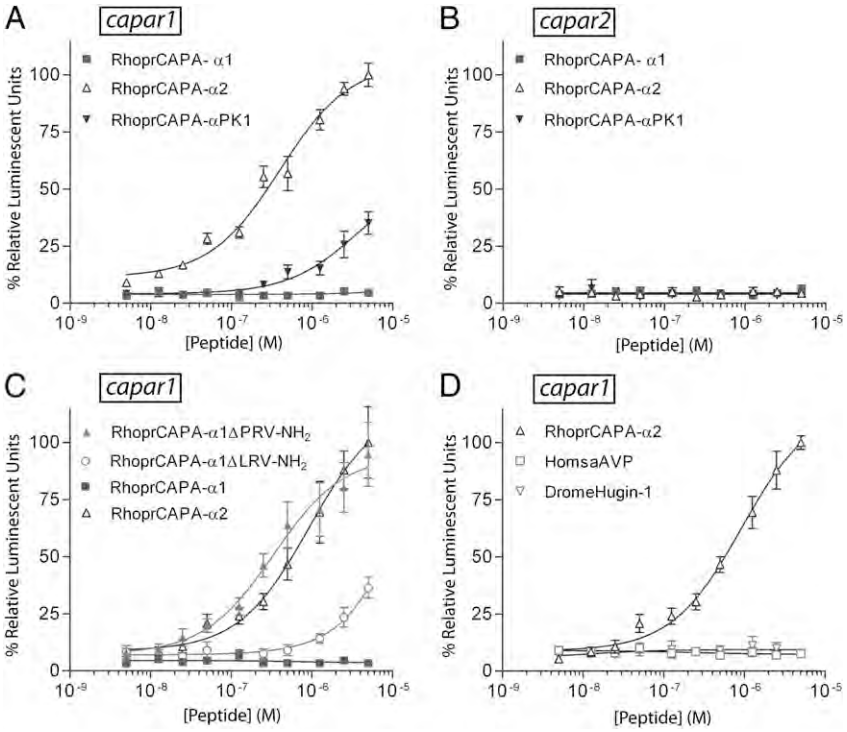

Fig. 5. Heterologous expression assay of the *R. prolixus* CAPA receptor in CHO-K1 cells. (*A*) Activity of the native *RhoprCAPA-α* gene products on the *capa-r1* receptor variant. Dose-response curve demonstrating activity of RhoprCAPA-α2 on the expressed *capa-r1* receptor. The pyrokinin-like peptide, RhoprCAPA-αPK1, also activates the expressed *capa-r1* receptor, but is ≈40-fold less potent. (*B*) Using the same ligands on the *capa-r2* receptor variant. None of the native peptides activate *capa-r2*. (*C*) Activity of structural analogs of the first encoded peptide of the *RhoprCAPA-α* gene, RhoprCAPA-α1. Activity of the RhoprCAPA-α1ΔPRV-NH_2 analog closely mimics the efficacy of RhoprCAPA-α2, whereas the RhoprCAPA-α1ΔLRV-NH_2 analog demonstrates an intermediate response. (*D*) Activity of structurally related nonnative peptides containing the PRXamide motif. Neither human AVP nor *D. melanogaster* hugγ (hugin-1) are active on the expressed *capa-r1* receptor at the doses tested. All peptides tested in *C* or *D* are inactive on the expressed *capa-R2* receptor (Fig S3). In each plot, vertical bars denote SEs and all values are plotted relative to the maximum response obtained by RhoprCAPA-α2 in cells expressing the *capa-r1* receptor. For *B*, maximum response values were taken from replicate plates done in tandem but challenged with cells expressing the *capa-R1* receptor.

Discussion

We have isolated and characterized the first antidiuretic hormone receptor in insects. This receptor, found in the blood-feeding Chagas' disease vector, *R. prolixus*, shows high amino acid sequence similarity to CAPA receptors identified in other insects, such as *D. melanogaster* (13, 14), *A. gambiae* (15) and *Tribolium castaneum* (17), as well as putative CAPA receptors predicted or annotated in the *Acyrthosiphon pisum*, *Pediculus humanus corporis*, and *Apis mellifera* genomes. The CAPA-related peptides in insects play key roles in regulating fluid secretion by MTs. In dipterans, these peptides are described as stimulating fluid secretion by the principal cells of MTs (6) by way of calcium signaling (18) and activation of nitric oxide synthase and soluble guanylate cyclase (6, 19). Unlike this stimulatory effect in dipteran MTs, the CAPA-related peptide in *R. prolixus*, RhoprCAPA-α2(-β2), acts as an inhibitor of MT fluid secretion (9), counteracting the stimulatory effect of the diuretic hormone 5-HT, and thus is referred to as an "antidiuretic hormone." In dipterans, it is unknown if these peptides regulate any other tissue in addition to the MTs. In *R. prolixus*, we have recently shown that the antidiuretic hormone, RhoprCAPA-α2(-β2), is also a potent inhibitor of anterior midgut absorption stimulated by 5-HT (4, 11).

In this study, we show expression data for a CAPA receptor homolog in *R. prolixus* that correlates with the physiological roles identified previously in the MTs (9) and anterior midgut (4, 11). Interestingly, we have identified that additional tissues that comprise the alimentary canal contain appreciable levels of CAPA receptor expression, namely in oesophagus posterior midgut, and hindgut, suggesting additional targets for the endogenous CAPA peptides. Future studies will investigate what physiological roles the native CAPA peptides may facilitate at these recently identified target tissues, but these tissues are not considered to be involved in the rapid postprandial diuresis in *R. prolixus*.

In previous studies where insect CAPA receptors have been identified, little data has been presented on their spatial expression characteristics (13–15). Expression has been shown associated with the thorax and abdomen in adult *D. melanogaster* (14) and more recently, tubule-specific expression was confirmed in *A. gambiae*, *Anopheles stephensi*, and *D. melanogaster* (6). In this latter study, attempts at amplifying an orthologous receptor from *A. aegypti* tubules were unsuccessful. However, in silico attempts at identifying a CAPA receptor ortholog in the *A. aegypti* genome revealed a receptor sequence sharing greatest similarity to the *D. melanogaster* and *A. gambiae* pyrokinin receptor 1 (Fig. 3); thus, it remains unclear if a CAPA receptor exists in this insect. Nevertheless, CAPA receptor expression associated with tissues other than the MTs remains elusive in insects and thus, this study is unique in comprehensively examining the tissue-expression profile for this receptor type. In the MTs, we investigated whether the CAPA receptor transcripts were differentially expressed in the

Table 1. Summary of peptides and analogs structurally related to the CAPA peptides in *R. prolixus* tested in the functional expression assays

Peptide/analog name(sequence)	EC_{50}(M)Rhopr-capaR1	EC_{50}(M)Rhopr-capaR2
RhoprCAPA-α1(SPISSVGLFPFLRA-NH_2)	Not active	Not active
RhoprCAPA-α2(EGGFISFPRV-NH_2)	3.85×10^{-7}	Not active
RhoprCAPA-αPK (NGGGGNGGGLWFGPRL-NH_2)	$>5 \times 10^{-6}$	Not active
RhoprCAPA-α1ΔLRV-NH_2 (SPISSVGLFPFLRV-NH_2)	$>5 \times 10^{-6}$	Not active
RhoprCAPA-α1 ΔPRV-NH_2 (SPISSVGLFPFPRV-NH_2)	3.35×10^{-7}	Not active
HomsaAVP(CYFQNCPRG-NH_2)	Not active	Not active
DromeHugin-1 (pQLQSNGEPAYRVRTPRL-NH_2)	Not active	Not active

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upper and lower tubule segments. We found the *capa-r1* transcript level is substantially greater in the upper secretory segment of the MTs, with relatively little expression in the lower reabsorptive segment. This finding correlates well with the profound antidiuretic effect on upper secretory segments stimulated with 5-HT. The reabsorption of water by the lower segment of the tubule has not been shown by any diuretic peptides or 5-HT (20), although 5-HT does lead to K^+ and Cl- reabsorption in the lower segment (21–23). Thus, the greater than 60-fold difference in expression of *capa-r1* between these functionally distinct MTs segments parallels the physiological effects previously documented for RhoprCAPA-α2 on MTs. The effects, if any, of these peptides on the lower segments will require further investigation. Although expression of the *capa-r2* transcript was detected in similar tissues as the *capa-r1* transcript, relative expression levels are substantially lower (approximately < 350-fold) and thus it is unclear what function, if any, the atypical GPCR product of this transcript may hold.

To support the phylogenetic analysis and expression profile data suggesting the identified receptor was a CAPA receptor homolog, we tested the receptor clones in a heterologous expression assay. The *capa-r1* expressed receptor was activated by low concentrations of RhoprCAPA-α2 with an EC_{50} value comparable to that determined for other heterologously expressed CAPA receptors (13, 14); however, this EC_{50} value does not match the observed in vitro potency observed for RhoprCAPA-α2, which lies in the low nanomolar range (4, 9), and this difference may be a result of poor coupling of the receptor to the calcium mobilization pathway leading to non-optimal activation of the aequorin reporter. In support of this prediction, our recent physiological results suggest that calcium is not involved in eliciting the natural RhoprCAPA-α2 inhibitory effect on the anterior midgut (4); thus, the endogenous receptor coupling in vivo differs from that in our heterologous expression system. The first encoded peptide, RhoprCAPA-α1, does not activate the receptor over the range of concentrations tested. This finding is in contrast to the result shown for CAPA receptors in other insects, where the receptors are activated more or less equally by the first two peptides produced by the CAPA prepropeptide (13–15). However, this result is not surprising considering that the sequence of the first peptide in each of the two *R. prolixus* CAPA precursors has lost the consensus CAPA peptide C-terminal FPRV-NH_2 sequence. Interestingly, the *capa-r1* receptor is also activated by the native pyrokinin-like peptide, RhoprCAPA-αPK1, although this peptide is about 40-fold less potent than RhoprCAPA-α2. In studies on the dipteran CAPA receptors, the pyrokinin-like peptides encoded by their respective CAPA precursors activate distinctive pyrokinin-1 receptors and do not activate the CAPA receptors, although the CAPA peptides have no activity on the pyrokinin-1 receptors (13–15, 24). Experiments testing structural analogs of the RhoprCAPA-α1 revealed that the lack of the consensus PRV-NH_2 motif is indeed responsible for the loss of activity. Structural analogs with the consensus motif partially or fully restored demonstrate activity on the expressed *capa-r1* receptor, although only the fully restored consensus analog, RhoprCAPA-α1ΔPRV-NH_2, had activity closely comparable to RhoprCAPA-α2. In support of these findings, alanine-replacement analogs of *Manduca sexta* CAP2b (also known as ManseCAPA-1) (25) tested for diuretic activity in the housefly, *Musca domestica*, demonstrated that the C-terminal residues are critical for biological function (26). Exogenous PRXamide peptides sharing limited structural similarity to the CAPA peptides, namely human AVP and *D. melanogaster* hugin-γ peptides, did not activate the *capa-r1* receptor. Similar results regarding the specificity of the insect CAPA receptor orthologs were shown previously with no response to mammalian AVP (13) or *D. melanogaster* hugin-γ (13, 14). Furthermore, the *A. gambiae* homolog of the hugin-γ peptide was similarly inactive on the mosquito CAPA-receptor (15). In contrast to the results obtained for the *R. prolixus capa-r1* receptor, all of the peptides tested in this study were inactive on the *capa-r2* expressed receptor. Although such a result would be expected considering the atypical predicted membrane topology of the CAPA-R2 receptor, the possibility of a functional role for this protein cannot be dismissed. A schematic overview of the *R. prolixus* CAPA neuropeptide/receptor signaling system, with emphasis of the known physiological roles for the antidiuretic hormone RhoprCAPA-α2, is presented (Fig S4) based on the receptor transcript spatial expression profile and the physiological effects previously identified (4, 7–9).

Receptors belonging to the GPCR super family are very often targets of pharmaceutical research, leading to treatment for malignancies and diseases, and it has been stated that the potential for future drug discovery is immense, considering many pharmaceuticals target only a handful of GPCRs (16). *R. prolixus* is a principal vector of Chagas' disease and both sexes must gorge on a blood meal during each nymphal stage for growth and development, and adult females require a blood meal to increase egg production. Thus, at the level of the individual insect, the opportunity to transmit disease can be 12 times as great compared with many mosquitoes, where only adult females will gorge on a blood meal. It has been shown that the CAPA peptide, RhoprCAPA-α2, plays a significant role in coordinating an antidiuretic strategy in *R. prolixus* (4, 9). Potentially, the development of biologically stable mimetic agonists or antagonists affecting the *R. prolixus* CAPA receptor could disrupt fluid and salt homeostasis and overall diuresis, and thereby impede the transmission of *T. cruzi* infection that occurs during the rapid postprandial diuresis and excretion in *R. prolixus*.

Materials and Methods

Animals. Fifth-instar *R. prolixus* Stål were reared at high relative humidity in incubators at 25 °C and routinely fed on rabbit blood. Insect tissues were dissected under physiological saline and stored as previously described (9).

Isolation of a Partial Coding Sequence of the *R. prolixus* CAPA Receptor Gene. CAPA receptor protein sequences identified or predicted in *D. melanogaster* (AAS65092) (13, 14), *A. mellifera* (NP_001091702) (27), *A. gambiae* (AAX84796) (15), and *T. castaneum* (XP_973937) (17) were aligned by ClustalW (28) and regions of high conservation were used for design of degenerate primers. A cDNA library from the upper segment of MTs was constructed following the Creator SMART cDNA Library Synthesis kit, as described previously (9), and was used as a template for PCR using the CAPA receptor degenerate primers (Table S1). Sequencing of positive amplicons was carried out at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, Ontario, Canada).

Rapid Amplification of cDNA Ends PCR of the *R. prolixus* CAPA Receptor Gene. Total RNA from MTs (1.5 μg) was used for cDNA synthesis using the 5′/3′ Rapid Amplification of cDNA Ends (RACE) Kit according to manufacturer instructions (Roche Applied Science). The partial sequence coding for the *R. prolixus* CAPA receptor gene obtained through PCR screening of the upper MT cDNA library was used to design gene-specific primers for application in RACE PCR (Table S2 and *SI Materials and Methods*).

Northern Blot Analysis. Fifth-instar alimentary canals (including foregut, midgut, hindgut, and MTs) were dissected and total RNA was isolated using the SV Total RNA Isolation System (Promega) and then further processed for isolation of mRNA using the PolyATtract mRNA Isolation System III (Promega). Isolated mRNA was quantified using a NanoDrop UV Spectrophotometer and 1 μg per lane was used for Northern blot analysis, as described previously (10). Digoxigenin-labeled RNA antisense probes were synthesized by in vitro transcription, as described previously (10), using cDNA template generated with the sense primer capaR_3raceFOR2 and antisense primer capaR_3endREV1 (Table S2).

Reverse-Transcriptase Quantitative PCR Tissue Expression Analysis. RNA was isolated as noted above from tissues of fifth-instar *R. prolixus* dissected from 20 to 25 insects fed 7 to 8 weeks previously as fourth-instars. Using 200 ng total RNA, first-strand cDNA was synthesized as previously described (10)

and reactions were diluted 5-fold using nuclease-free water and subsequently used as template for quantitative PCR (qPCR). Primers were designed over exon-exon splice boundaries and sense primers were designed specifically for the transcript variants of the *RhoprCAPA-R* gene (Table S2) to differentiate expression levels between these alternative transcripts. All qPCR reactions were carried out on a Mx4000 Quantitative PCR System (Stratagene) with cycling conditions and housekeeping control genes, rp49 and actin 5c, which have been validated as stable targets and used previously (10). Primer efficiencies were determined for each target and relative expression was determined following the delta-delta Ct method (29), and fold-differences were normalized to either of the housekeeping genes with similar relative expression values with each normalizer target (rp49-normalized data shown). Experiments were repeated for a total of three biological replicates with two technical replicates each that included a no-template control and no reverse-transcriptase control to ensure absence of contaminating template in reagents and possible genomic DNA contaminant amplification, respectively.

Preparation of Mammalian Expression Constructs. The full-length cDNA of the two alternative transcripts of the RhoprCAPA receptor (*capa-r1* and *capa-r2*) were amplified by primers designed at the 5′- and 3′-end (Table S2) and using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and cloned into the pGEM-T Easy vector (Promega). Sequencing of individual clones was used to differentiate between different splice variants. These clones were subsequently used as a template for PCR using primers that spanned the complete open-reading frame and that had the 5′ noncoding sequence modified by introducing a Kozak translation initiation sequence (Table S2) required for optimal translation by eukaryotic ribosomes (30–32). The *RhoprCAPA-R* ORFs were inserted into the pcDNA 3.1$^+$ (Invitrogen) for expression in mammalian cells.

Cell Line Expression and Functional Analysis of the RhoprCAPA Receptor. Transient expression of the *RhoprCAPA-R* gene was accomplished using a CHO-K1 cell line and subsequently prepared for the luminescence assay (see *SI Materials and Methods* for details). Each *RhoprCAPA-R* transcript variant expression construct was cotransfected with a construct carrying the cytoplasmic luminescent reporter aequorin (cyto-Aeq). Peptides encoded by the *RhoprCAPA-α* gene and derived analogs were synthesized by following methods described previously (9). The source of human arginine vasopressin (HomsaAVP) and *D. melanogaster* hugin γ (Dromehugγ) peptides were as described previously (13).

Sequence Analysis of the RhoprCAPA Receptor. The deduced amino acid sequence encoded by the small- and large-transcript variants of the *RhoprCAPA-R* gene were analyzed for predicted membrane topology using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Using ClustalX (28), the sequences were compared to other known or predicted CAPA receptor sequences and pyrokinin-1 receptors identified in other insects noted above or predicted (*SI Materials and Methods*) and also including a human homolog, the neuromedin U receptor 2 (33). Using MEGA 4.02, both maximum parsimony and neighbor-joining methods were used; however, both produced trees with highly similar topology. The reliability of the relationships between taxa was tested using the bootstrap test with 1,000 iterations (34).

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