Identification, functional characterization and expression patterns of a water-specific aquaporin in the brown dog tick, *Rhipicephalus sanguineus*

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**A B S T R A C T**

Much is known about the physiology of tick salivation, but nothing is known about the movement of water through the cell membranes of salivary glands, a phenomenon usually associated with water channels or aquaporins (AQPs). An AQP, *RsAQP1*, was identified in a salivary gland cDNA library of *Rhipicephalus sanguineus*. In the first functional characterization of an acarine AQP, *Xenopus* oocytes expressing *RsAQP1* became water permeable, whereas *RsAQP1* did not transport glycerol or urea. *RsAQP1* was inhibited by Hg2⁺ but not by triethylammonium. Treatment with a protein kinase A activator (cAMP) had no effect on *RsAQP1* transport, whereas treatment with a protein kinase C activator (phorbol 12,13-dibutyrate) reduced water flux by 60%. *RsAQP1* transcript was present in unfed larvae, nymphs and adult *R. sanguineus*, but absent in embryos. Partially fed female *R. sanguineus* expressed *RsAQP1* in gut, Malpighian tubules and was particularly abundant in salivary gland tissue, but absent in ovary and synganglion tissues. Because of the importance of water management in tick biology for both the off-host and on-host phases of the life cycle, our findings on tick AQP1 represent a major advancement in our understanding of tick osmoregulation that could potentially be exploited in tick control.

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1. Introduction

The salivary glands are of vital importance in tick biology: they act as the organs of osmoregulation both in the off-host and on-host phases of the tick life cycle. Salivary glands aid tick survival by enabling absorption of water vapour from air during the non-feeding phase and, thus, prevent desiccation. Conversely, the salivary glands allow the ticks to pass feeding phase and, thus, prevent desiccation. Important, it is this copious salivation into the host that is the route by which pathogens are transmitted as well as pro-feeding bioactive factors such as anticoagulant, anti-inflammatory and immunosuppressive salivary proteins (Valenzuela, 2004). Consequently, there has been much investigation into the physiology of tick salivary glands leading them to be, arguably, the best characterised salivary glands of any invertebrate. Much is known about the dopaminergic regulation of salivation, the role of cyclic AMP and protein kinase in salivation, the involvement of Na⁺/K⁺-ATPase and vacuolar H⁺-ATPase ion pumps and, more recently, regulation of exocytosis of salivary proteins (Kaufman, 1989; Sauer et al., 1995, 2000; Bowman and Sauer, 2004; Bowman et al., 2008). However, nothing is known about the fluid (i.e. water) transport in tick salivary glands.

Lipid bilayer membranes are essentially impermeable to water. But living cell membranes are rendered permeable to water by the presence of membrane-spanning water channels, termed aquaporins (AQPs) (for reviews see Borgnia et al., 1999; Zardoya, 2005; Krane and Goldstein, 2007; Campbell et al., 2008). AQPs act as specific pores allowing water molecules to passively traverse the membrane, the direction of the flow of water being solely dependent upon the osmotic concentration gradient across the membrane set up by various ion and solute active transporters. AQPs can be broadly divided into water-specific AQPs and those that additionally transport a limited range of non-polar solutes (e.g. glycerol and urea) and are termed aquaglyceroporins (or glycerol facilitation-like proteins, GLPs). Short-term regulation of cell membrane water permeability has been shown to be effected through phosphorylation of the AQP causing gating of the AQP pore or translocation from the cytosol to the plasma membrane or vice versa. Long-term regulation of cell membrane water permeability is effected through up- and down-regulation of gene expression. Not surprisingly, tissues that exhibit high water fluxes across them contain high levels of AQPs.

AQPs have been reported from organisms throughout the Plantae, Protista and Animalia kingdoms. There have been very few reports of AQPs in invertebrates and even fewer have been functionally characterised (for review see Campbell et al., 2008). With
the tremendous water movement through the salivary glands of ticks, this tissue would seem an ideal tissue for studying invertebrate AQPs. The presence of an AQP was postulated to explain the working model of tick salivary gland physiology put forward by Sauer et al. (2000). Later, evidence was presented for a role for a mercury-sensitive AQP in Ixodes ricinus salivary gland function in vitro (Bowman and Sauer, 2004). Recently, an AQP was cloned from the American dog tick, Dermacentor variabilis that was expressed in gut and ovaries, but was absent in the salivary glands (Holmes et al., 2008). Here, we report on the identification of an AQP from the salivary gland of the brown dog tick, Rhipicephalus sanguineus, and present the first functional characterization of an acarine AQP.

2. Materials and methods

2.1. Cloning of an aquaporin from R. sanguineus

A portion of a putative AQP was identified in a Rhipicephalus appendiculatus salivary gland EST database given the tentative consensus code TC77, accessed October 2004 on the TIGR Gene Indices website. This R. appendiculatus AQP was 873 bp and appeared to be lacking about 300 bp of the 5’-terminus. A variety of internal forward and reverse primers were designed based on TC77, and applied using a conventional PCR approach to amplify a TC77 homologue from an R. sanguineus salivary gland cDNA library (Lees, 2006). The 5’ and 3’ ends of the respective genes were identified using the gene specific primers (RsAQP1-1F GCCGCC/TTTCTG GGGATGG, RsAQP1-1R CCATCCCCCAAGA/GCGCCCG) in conjunction with M13 universal and reverse primers (M13-uni TGTA A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A A
10 min. The digested material was treated with proteinase K (Sigma–Aldrich) in the following reaction: 20 μl (2 μg) linearised plasmid, 1.3 μl (20 mg/ml) proteinase K, 0.5% SDS and 100 μl nuclease-free water. The reaction was mixed gently and incubated at 50 °C for 30 min prior to phenol:chloroform extraction. This material was then used for the in vitro generation of capped RNA (cRNA) using the mMessage mMachine kit (Ambion Ltd, Warrington, UK), as per the manufacturer’s protocol. The resultant cRNA was phenol:chloroform extracted, precipitated with isopropanol and dissolved in 30 μl DEPC water and quantified with an ND-1000 microspectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) prior to storage at −80 °C.

Microinjection was performed using a Harvard microinjection station. cRNA was diluted to 1 μg/μl and 25 nl of RsAQP1 cRNA solution was injected into the vegetal pole of each oocyte. 25 nl DEPC-treated water was similarly injected into some oocytes to act as a negative control. Oocytes were then transferred to fresh modified L-15 media and incubated at 15 °C for 48 h prior to osmotic challenge and visualisation.

2.5. Determination of transport properties of RsAQP1

Prior to osmotic challenge, oocytes were removed from modified L-15 media and equilibrated for 15 min in Barth’s modified saline (200 mOsm). Osmolalities were determined for all solutions with a Vapro 5520 vapour pressure osmometer (Wescor, Utah, USA). After equilibration, the oocytes were individually transferred to wells in a 12-well tissue culture plate containing hypotonic Barth’s media without added NaCl at 70 mOsm or containing Barth’s base with the appropriate solute (glycerol, urea, xylitol, sorbitol) added to a final osmolarity of 200 mOsm. Still images were captured every 5 s for 1 min using an inverted video microscope Axiovert 200M (Zeiss, Source), with high contrast black and white imaging. Each image was processed using SimplePCI2 software (Hamamatsu Corp, Brighton, UK) to measure the diameter and volume of oocytes over the time lapse. The osmotic permeability coefficient (P₀) is the rate of volume change over time, s. is the oocyte surface area, Vₘ is the molar ratio of water (18 cm³/mol) and (Osmₘ – Osmₜₜₜ) is the osmotic gradient across the membrane. Experiments were performed at least two times using different batches of Xenopus oocytes and cRNA preparations.

In some experiments, oocytes were pre-incubated for 15 min in modified Barth’s media (200 mOsm) containing various test compounds (100 μM HgCl₂, 50 μM 8-bromo-cAMP, 10 μM phorbol 12,13-dibutyrate (PdBu), or 10 and 100 μM tetraethylammonium (TEA)). After the pre-incubation period, the P₀ of the oocytes when exposed to hypotonic Barth’s media (70 mOsm) was determined, as above, except for experiments with TEA in which TEA was also present in the hypotonic Barth’s media.

2.6. Tissue and life stage distribution of RsAQP1 mRNA

Adult R. sanguineus (Charles River, Carrentripla, Ireland) were fed on guinea pigs and removed on day 8 post-infestation. Salivary glands, Malpighian tubules, synganglia (“brains”) and oviductal tissues were dissected from three partially fed individual female ticks and stored at −80 °C. Total RNA was prepared from these tissue samples using a Mini RNA Isolation Kit (Zymo Research, Orange, California, USA). Total RNA from egg, larval, and nymphal material was prepared by Trizol extraction of massed material. This material was then reverse transcribed using MMTV-RT (Promega). 1 or 2 μl total RNA was incubated at 70 °C with 1 μl of (T₂₀) in a total volume of 10 μl for 5 min. The material was then snap-chilled on ice for 5 min prior to the addition of 5 μl 5 × RT buffer, 1 μl dNTPs (25 μM each), 0.5 μl MMTV transcriptase and DEPC water to 25 μl. The reaction was then run for 75 min at 42 °C prior to arrest by heating to 70 °C for 5 min.

The presence of the RsAQP1 transcript was detected by PCR using a conventional PCR protocol and primers encompassing a 250 bp internal region of the ORF (RsAQP1-1F CCTCTCCCTTTGCTACACTG and RsAQP1-1R GTCCTTGGCAGATACCGGTG). A 50 μl final volume reaction was prepared constituted of 1 μl (200–300 ng) cDNA template, 5 μl 10 × reaction buffer, 1.5 μl 50 mM MgCl₂, 1 μl dNTPs, 1 μl primer mix (10 μM each), 0.5 μl (1.25 μl) Taq (Bioline, London, UK). The results and RT-PCR procedure were validated using R. sanguineus actin primers (RsAF, AGAGCAAGGTGATCCTC and RsAR AGCTCGTTGTAGAAGGTGTGG) generating a fragment of approximately 110 bp.

The PCR conditions were as follows: 1 cycle of 60 s at 94 °C, followed by 34 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 74 °C. Egg material consistently failed to produce a signal for RsAQP1 utilising this approach. This material was subjected to nested–PCR – the first round used primers encompassing the whole of the ORF used for the initial cloning of the gene (RsAQP1F₁ and RsAQP1R₂) for 20 cycles under standard conditions. The second, nested step utilised primers RsAQP1-1F₂ and RsAQP1-2R for 30 rounds.

2.7. Data presentation and statistical analysis

All data are presented as means ± SEM. Oocyte permeability experiments were performed at least two times using different batches of Xenopus oocytes and cRNA preparations with similar results. In experiments with multiple treatments, ANOVA was performed to test for treatment effects and, if appropriate, pairwise comparisons were performed using Tukey’s test (Minitab version 15). In experiments with two treatments, significant differences were determined by Student’s t-test.

3. Results

3.1. Cloning, topology and homology of RsAQP1

Using primers based on a putative AQP EST (TC77) in the R. appendiculatus database in conjunction with library vector primers, we cloned an AQP from an R. sanguineus salivary gland cDNA library. We have named this AQP as RsAQP1 (EMBL Accession no. FM210537). The full-length RsAQP1 cDNA was 1194 bp with an ORF of 872 bp and two untranslated regions of 120 bp and 202 bp at the 5’ and 3’ termini, respectively. A polyadenylation signal, AAATAA, was located 15 bp upstream of the poly(A) tail. The RsAQP1 ORF encodes a protein containing 292 amino acids with a predicted molecular weight of 30,929 kDa and isoelectric point of 6.4.

Topology and hydrophobicity predictions indicate RsAQP1 has 6 transmembrane-spanning regions and cytosolic N and C termini (Fig. 1) as is standard for AQP family members. RsAQP1 possesses two characteristic NPA motifs in transmembrane loops B and E at positions 73–75 and 205–207, respectively. A cysteine (Cys200) five characteristic NPA motifs in transmembrane loops B and E at positions 73–75 and 205–207, respectively. A cysteine (Cys200) five predicted to be mercury-sensitive. There are a total of 12 predicted phosphorylation sites in RsAQP1, however, many of these sites are likely to be mercury-sensitive. There are a total of 12 predicted phosphorylation sites in RsAQP1, however, many of these sites are likely to be phosphorylated by protein kinase C.

RsAQP1 shared high homology to several translated contigs within the R. appendiculatus (TC2934, 97% identity) and...
A. variegatum (TC579, 77% identity) EST databases (http://compbio.dfci.harvard.edu/tgi/tgipage.html) and the I. scapularis (VBpre_P011405, 77% identity) genome project (http://iscapularis.vectorbase.org/index.php). An AQP from D. variabilis (ABI53034, Holmes et al., 2008) was only 49% homologous to RsAQP1, however the I. scapularis genome contained a contig (32055.m000012) with 59% identity to the D. variabilis AQP. Phylogenetic analysis of these tick AQPs indicated that they branch into two clades (Fig. 2) which we have termed tick AQP1 and AQP2 groups. It is notable that the I. scapularis genome database contained both a tick AQP1 and tick AQP2 homologue.

In homology analysis excluding ticks, RsAQP1 was most similar to AQPs from the purple sea urchin (XP_786125, 50% identity), the gilted seabream (AAR13054, 44% identity), rainbow smelt (ABG24574, 45%) and within Mammalia human and pig AQP-10 (identities 45 and 44%, respectively). Of particular note, all these non-tick AQPs are classed as aquaglyceroporins based on amino acid sequence characteristics, though only the AQPs from the gilted seabream and humans have actually been demonstrated to transport urea and glycerol in addition to water. This would suggest that RsAQP1 is also an aquaglyceroporin and should be permeable to glycerol and urea.

3.2. Functional characterization of RsAQP1

RsAQP1 was successfully functionally expressed in Xenopus oocytes. Forty-eight hours post-injection of RsAQP1 cRNA, oocytes were permeable to water and swelled in a linear fashion over a short period when placed in hypoosmotic media (Fig. 3) and subsequently burst over a longer period. Though RsAQP1 readily transported water, oocytes expressing RsAQP1 did not appreciably transport any other tested non-polar solute above that level observed in water-injected control oocytes (Table 1). This absence of other solute transport was determined in several independent experiments with different RsAQP1 cRNA preparations and oocyte batches. As a positive control, oocytes expressing rat AQP9 transported glycerol and urea (data not shown).

Pre-incubation of oocytes in 100 μM HgCl2 (Fig. 4) reduced the Pf through RsAQP1 by 74% (P < 0.05) to a level not different from that of control oocytes (P > 0.05). However, RsAQP permeability was completely unaffected (P > 0.05) by pre-incubation in either 10 μM or 100 μM TEA (data not shown).

To determine if the phosphorylation status of RsAQP1 affected its permeability, oocytes expressing RsAQP1 were pre-incubated with the PKA agonist 8-Br-cAMP or the PKC agonist PdBu. Though PKA stimulation had no significant effect (P > 0.05) on RsAQP1 permeability, PKC stimulation reduced the Pf by 60% (P < 0.05) (Fig. 5).

3.1. Membrane topology of RsAQP1

Using predictive residue software the amino acid sequence for RsAQP1 was plotted using TeXtopo as it would appear in the plasma membrane of cells prior to complete folding of pore hemispheres into the hourglass shape. Loops are labelled A–E and transmembrane regions I–VI. Shaded red circles represent the two NPA motifs, the shaded black square indicates the putative Hg2⁺-sensitive cysteine and shaded green diamonds indicate predicted intracellular phosphorylation sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3 RsAQP1 mRNA expression pattern in different tissues and life stages

Semi-quantitative RT-PCR analysis of several tissues from partially fed adult female R. sanguineus demonstrated that RsAQP1 is expressed in the gut, Malpighian tubules and particularly abundant in the salivary glands (Fig. 6A). No RsAQP1 mRNA was detected in either the synganglion or the oviduct/ovary tissue.

Total RNA extracted from eggs/embryos and unfed larvae, nymphs and adult R. sanguineus was assessed for RsAQP1 mRNA expression. All life stages contained RsAQP1 mRNA except for the R. sanguineus eggs/embryos in which no RsAQP1 transcript could be detected either by a single round PCR or a nested-PCR approach (Fig. 6B).

4. Discussion

An AQP, named RsAQP1, was cloned from a salivary gland cDNA library of R. sanguineus. RsAQP1 was only weakly homologous to the recently reported AQP from D. variabilis (Holmes et al., 2008). Several other tick species’ salivary gland EST databases and the I. scapularis genome database were searched for homologues of R. sanguineus AQP1s and shared much sequence identity (Fig. 7), whereas tick AQPs indicated that they branched into two clear groups (Fig. 2), this would indicate that the split between tick AQP1 and tick AQP2 clades (Fig. 2), this would indicate that the split between tick AQP1 and tick AQP2 clades occurred before the Prostriata and Metastriata divergence in acarine evolution.

RsAQP1 was successfully expressed in Xenopus oocytes and rendered such oocytes permeable to water as demonstrated by their swelling and ultimately bursting when placed in hypoosmotic buffer. AQPs from five insect species have been functionally expressed and characterised, namely Rhodnius prolixus (Echevarria et al., 2001); Aedes aegypti (Duchesne et al., 2003); Cicadella viridis (LeCaherec et al., 1996); Drosophila melanogaster (Kaufmann et al., 2005).

Table 1

<table>
<thead>
<tr>
<th>Solute</th>
<th>Permeability coefficient (µm s⁻¹)</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>RsAQP1</td>
</tr>
<tr>
<td>Water</td>
<td>9.32 ± 8.29 (n = 9)</td>
<td>93.49 ± 10.05 (n = 14)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>−1.39 ± 1.31 (n = 7)</td>
<td>−2.90 ± 3.64 (n = 10)</td>
</tr>
<tr>
<td>Urea</td>
<td>0.58 ± 0.48 (n = 11)</td>
<td>3.11 ± 1.43 (n = 9)</td>
</tr>
<tr>
<td>Xylitol</td>
<td>3.23 ± 2.04 (n = 7)</td>
<td>1.62 ± 3.87 (n = 6)</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>0.43 ± 1.45 (n = 7)</td>
<td>7.85 ± 3.37 (n = 7)</td>
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Fig. 3. RsAQP1 is permeable to water. Xenopus oocytes injected with either 25 ng (25 nl) RsAQP1 cRNA or 25 nl water (control) were transferred to hypoosmotic buffer 48 h post-injection and the volume increase recorded by time-lapse videomicroscopy and image analysis. Data are means ± SEM (control n = 9, RsAQP1 n = 14) and are representative of 5 independent experiments with similar results.

Fig. 4. RsAQP1 is Hg²⁺ sensitive. Xenopus oocytes injected with either 25 ng (25 nl) RsAQP1 cRNA or 25 nl water (control) were transferred 48 h post-injection to hypoosmotic buffer with or without a 15 min pre-incubation in 100 µM HgCl₂ and the water permeability coefficient (Pₜ) determined by time-lapse videomicroscopy and image analysis. Data are means ± SEM (control n = 8, RsAQP1 n = 14 and RsAQP1 + Hg²⁺ n = 8) and are representative of 3 independent experiments with similar results. Columns not bearing the same letter are significantly different from each other (Tukey’s, P < 0.05).

Fig. 5. RsAQP1 is sensitive to phosphorylation status. Xenopus oocytes injected with either 25 ng (25 nl) RsAQP1 cRNA or 25 nl water (control) were transferred 48 h post-injection to hypoosmotic buffer with or without a pre-incubation in 10 µM PdBu for 15 min or in 50 µM 8-Br-cAMP for 30 min. Data are means ± SEM (control n = 15, RsAQP1 n = 12, RsAQP1 + PdBu n = 12 and RsAQP1 + cAMP n = 10) and are representative of 2 independent experiments with similar results. Columns not bearing the same letter are significantly different from each other (Tukey’s, P < 0.05).
and Polypedilum vanderplanki (Kikawada et al., 2008), but RsAQP1 is the first acarine AQP to be functionally characterised. RsAQP1 was inhibited by Hg2+ an almost universal property of invertebrate AQPs (Campbell et al., 2008), though Caenorhabditis elegans possesses three Hg2+-insensitive AQPs (Huang et al., 2007). Hg2+ inhibition is effected through a cysteine just a few residues upstream of the second NPA motif (Preston et al., 1993). In the case of RsAQP1, Hg2+ sensitivity is likely through the Cys200 just 5 residues upstream of the second NPA motif (Figs. 1 and 7). The tick AQP1s from R. appendiculatus and I. scapularis also contain a cysteine 5 residues upstream of the second NPA motif (Fig. 7) and similar Hg2+-sensitivity can be expected in these AQPs as observed for RsAQP1. We have previously shown that 10 μM HgCl2 completely inhibited dopamine-stimulated secretion by I. ricinus salivary glands in vitro without affecting the glands’ adenylate cyclase activity (Bowman and Sauer, 2004). Our observations on the whole salivary glands of I. ricinus (Bowman and Sauer, 2004) could be explained by the presence of the Hg2+-sensitive RsAQP1 homologue.

Tetraethylammonium (TEA) has been shown to be a potent, reversible inhibitor of mammalian AQP1 when expressed in oocytes (Brooks et al., 2000) or naturally in red blood cells (Yool et al., 2002). Further, mammalian AQP2 and AQP4 were shown to be sensitive to TEA and, by site-directed mutagenesis studies, the inhibition by TEA was shown to involve a tyrosine 6 residues upstream of the second NPA motif present in AQP1, AQP2 and AQP4 (Detmers et al., 2006). RsAQP1 possesses a tyrosine 7 residues upstream of the NPA motif (Fig. 7), but exhibited no inhibition by TEA. Mammalian AQP5 also has a tyrosine 7 residues upstream of the second NPA motif and it is also insensitive to TEA treatment (Detmers et al., 2006). Unfortunately, TEA cannot be used as an inhibitory reagent to further investigate the role of RsAQP1 either in vitro or in vivo.

Amongst non-tick species, RsAQP1 showed highest homology to AQPs in the purple sea urchin, the gilted seabream, rainbow smelt and human AQP-10. Interestingly, all of these AQPs are classed as aquaglyceroporins (GLPs) i.e. aquaporins that also transport non-polar solutes such as glycerol and urea. Despite the BLAST analysis indicating that RsAQP1 was most similar to the aforementioned GLPs, Xenopus oocytes expressing RsAQP1 did not transport any of the non-polar solutes tested, including glycerol. Water-specific AQPs and GLPs share a general common layout and the BLAST algorithm comparing primary sequence information for approximate sequence matching over the full-length determined that

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<th>AQP</th>
<th>GLP</th>
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<td>RsAQP1</td>
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Fig. 6. Relative tissue and life stage expression of RsAQP1. (A) Tissues were dissected from individual partially fed adult female R. sanguineus and the presence of RsAQP1 determined by RT-PCR using RsAQP1-specific primers (250 bp product). Actin (110 bp product) was used as a control. SG – salivary gland; MT – Malpighian tubules; Ovi – oviduct/ovary. Data are presented from one tick and are representative of three individual ticks with similar results. (B) RsAQP1 transcript was determined by RT-PCR in eggs or unfed larvae, nymph and adult ticks using RsAQP1-specific primers (250 bp product) and gel loading normalized to actin (110 bp product).

Fig. 7. Amino acid alignment of three full-length tick AQP1s from R. sanguineus (RsAQP1), R. appendiculatus (RaAQP1) and A. variegtatum (AvAQP1). The two NPA motifs are boxed. The four distinctive residues within the ar/R constriction (white text, black background) are shown together with the corresponding residues in water-specific AQPs (AQPs) and aquaglyceroporins (GLPs). The putative PKC phosphorylation sites (Thr82 and Ser274) are shaded in grey. Within the three tick AQP1s, “*” indicates residue identity; “.” indicates conserved residue and “.” indicates semi-conserved residue.
RsAQP1 is most similar to certain GLPs. Critical differences between water-specific AQPs and GLPs have been determined in the two constriction regions of the pore (Wu and Beitz, 2007), however these subtle differences would not be picked up by the BLAST algorithm. Indeed, according to the important residues put forward these subtle differences would not be picked up by the BLAST adenylate cyclase and increased levels of cAMP (Sauer et al., 2000; Bowman and Sauer, 2004) and involves, at least in constriction regions of the pore (Wu and Beitz, 2007). RsAQP1 possesses GLP-like residues at two of the four key residues, but not all of the four residues (Fig. 7). Overall, RsAQP1 does not have all the ar/R constriction residues for either water-specific AQPs or GLPs, but with a tendency to be more like a GLP. However, empirical determination of the transport properties of RsAQP1 clearly showed that it does not transport glycerol or urea. Of note, none of the arthropod AQPs characterised to date have been shown to be capable of transporting anything other than water (Campbell et al., 2008). However, we have recently identified a GLP in R. sanguineus that readily transports glycerol and urea (Ball et al., unpublished observations).

The water permeability of cells can dynamically change through the phosphorylation status of AQPs usually via stimulation of the tissue by diuretic or antidiuretic factors. For example, vasopression increases the water permeability of collecting ducts of mammalian kidneys through the phosphorylation of human AQP2 by protein kinase A (PKA) via an increase in intracellular levels of cAMP (Nejsum et al., 2005), whereas phosphorylation of human AQP1 via protein kinase C positively regulates water permeability (Zhang et al., 2007). Water flow through RsAQP1 was not affected by treatment with the PKA stimulant cAMP, but was reduced 60% by 15 min treatment with the PKC activator PdBu. Though RsAQP1 is predicted to have a total of 12 putative phosphorylation sites, only 4 of these residues (Ser4, Thr82, Thr272 and Ser274) are cytoplasmic which could be considered likely regulatory sites. Aligning the tick AQPs from R. sanguineus, R. appendiculatus and I. scapularis reveals two of these phosphorylation sites (Thr82 and Ser274, Fig. 7) are conserved and both are predicted to be PKC targets. Though we observed reduced water flux through RsAQP1 in oocytes, confirmation of the phosphorylation requires radiotracer studies or mutation of the putative phosphorylation sites. Reduced water permeability through human AQP4 via PKC phosphorylation has been reported in both oocytes (Han et al., 1998) and cultured mammalian kidney cells (Zelenina et al., 2002) treated with PdBu and in the kidney cells treated with dopamine (Zelenina et al., 2002).

It is well established that dopamine induces salivation by tick salivary glands in vitro and this is brought about by activation of adenylate cyclase and increased levels of cAMP (Sauer et al., 2000; Bowman and Sauer, 2004) and involves, at least in Amblyomma americanum, the phosphorylation of >12 unknown proteins by PKA (McSwain et al., 1985, 1987). It would appear that a similar scenario is at play in R. appendiculatus salivation, a close sibling tick species to R. sanguineus, in which salivary gland secretion and cAMP levels were increased by dopamine (Kaufman et al., 2001). Therefore, it had been postulated that the AQP in tick salivary glands would be phosphorylated by PKA and increase fluid secretion (Sauer et al., 2000) – this is clearly not the case, at least with RsAQP1. We now hypothesize that RsAQP1 in the salivary gland is not affected by dopamine-driven PKA phosphorylation and that the water permeability of the salivary gland cell remains constant, but that the rate of water flux is determined by ion pumps that are activated by dopamine and intracellular cAMP. The biological significance of the inhibitory effect of PKC phosphorylation on RsAQP1 is presently unknown.

In partially fed female ticks, RsAQP1 was not expressed in the oviduct or synganglion (“brain”), but transcript was expressed in the gut, the Malpighian tubules and was especially abundant in the salivary glands. In contrast, the AQP reported in D. variabilis, which we have classed in the tick AQP2 group, was absent in the salivary glands, but was present in high abundance in the ovary at 100× the level observed in the gut (Holmes et al., 2008). It would appear that RsAQP1 is present in tissues known to be involved in high rates of water flux i.e. gut and salivary gland. Water is absorbed from the blood meal across the gut wall and then expelled into the host via the salivary glands such that during the feeding cycle 80% of the total blood meal volume is excreted (Sauer and Hair, 1972; Kaufman and Phillips, 1973) and our results indicate that RsAQP1 is involved in this mass water movement. It is of interest that RsAQP1 is also present in the Malpighian tubules. Very little is known about tick Malpighian tubules but they are not thought to be involved in osmoregulation but, rather, they are reported to be involved in nitrogenous waste metabolism and transport and linked to the rectal sac. Following our results that the Malpighian tubules express RsAQP1, our interest was piqued and we observed that adult female I. ricinus fed in vitro (Kröber and Guerin, 2007) produce copious volumes of faeces that have a relatively high water content (Burdin and Bowman, unpublished observations). These findings would suggest high water flux and a role of RsAQP1 in the Malpighian tubules.

RsAQP1 mRNA transcript was present in unfed larvae, nymphs and adult R. sanguineus. Though not determined, it is expected that RsAQP1 is likely to be present in the salivary glands in these unfed stages and may play a role in osmoregulation where salivary glands are known to be involved in maintaining water balance through the absorption of water vapour from air (Needham and Teel, 1991). For example, it has been shown that larval, nymphal and adult R. sanguineus can readily absorb water vapour from air to redress red water balance when dehydrated (Yoder et al., 2006). Interestingly, embryonic R. sanguineus (~ 3 weeks post-oviposition) contained no trace of RsAQP1 transcript indicating that it is not required for water transport at this stage within the cleidoic egg.

In conclusion, a water-specific, Hg²⁺-sensitive aquaporin, RsAQP1, has been identified that has homologues in both prostriate (I. scapularis) and metastriate (R. appendiculatus and A. variegatum). Dopamine-stimulated secretion by tick salivary glands appears to be independent of any phosphorylation gating of RsAQP1. More likely, RsAQP1 is constitutively permeable to water and the increased water flux through RsAQP1 is due to dopamine activation of ion pumps to set up an appropriate osmotic gradient. RsAQP1 is present in tissues involved in mass water movement associated with concentrating the blood meal. Within this profile, the presence of RsAQP1 in the Malpighian tubules suggests a review of our understanding of the role of these little studied organs during the feeding phase of ticks.

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References


