Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part A 148 (2007) 55–63
CBP
www.elsevier.com/locate/cbpa

Vascular aspects of water uptake mechanisms in the toad skin: Perfusion, diffusion, confusion ☆

Niels J. Willumsen [a,*], Arne L. Viborg [a], Stanley D. Hillyard [b]

[a] Institute of Molecular Biology and Physiology, August Krogh Building, University of Copenhagen, Denmark
[b] School of Dental Medicine, University of Nevada, Las Vegas, NV, USA

Received 17 July 2006; received in revised form 29 December 2006; accepted 31 December 2006
Available online 12 January 2007

Abstract

Blood cell flow (BCF) in the water absorbing "seat patch" region of toad skin was measured with laser Doppler flow cytometry. BCF of dehydrated toads increased by a factor of 6–8 when water contact was made and declined gradually as toads rehydrated. Water absorption was initially stimulated and declined in parallel with BCF. Water absorption measured during the initial rehydration period did not correlate with BCF and hydrated toads injected with AVT increased water absorption without an increase in BCF indicating the lack of an obligate relation between blood flow and water absorption. Aquaporins 1–3 were characterized by RT-PCR analysis of seat patch skin. AQP 1 was localized in the endothelium of subepidermal capillaries and serves as a pathway for water absorption in series with the apical and basolateral membranes of the epithelium. Dehydrated toads rehydrated more rapidly from dilute NaCl solutions than from deionized water despite the reduced osmotic gradient. BCF of toads rehydrating on 50 mM NaCl was not different than on deionized water and blocking Na^+ transport with 100 μM amiloride did not reduce water absorption from 50 mM NaCl. Thus, neither circulation nor solute coupling explains the greater absorption from dilute salt solutions. Rehydration from 10 mM $CaCl_2$ was stimulated above that of DI water by a similar degree as with 50 mM NaCl suggesting the anion might control water permeability of the skin.

Keywords: Amphibian skin; Toad; Water uptake; Dehydration; Aquaporins; Seat patch; Blood flow

1. Introduction

Except for the breeding period, toads (Bufonidae) spend the greater fraction of the year terrestrially. At this time the amphibian antidiuretic hormone, arginine vasotocin (AVT), reduces glomerular filtration, stimulates reabsorption of stored bladder water and stimulates the water permeability of the skin to facilitate rapid rehydration (Bentley, 1966). Water absorption occurs primarily through a specialized, richly vascularized region of the pelvic skin termed the 'seat patch' and may amount to as much as 20–30% of the body mass per hour. Word and Hillman (2005) recently showed that water absorbed across the seat patch of *Bufo marinus* is taken up entirely into the circulation and lead us to re-examine the question: Is water absorption limited by blood flow to the skin (perfusion), osmotic flow across the skin (diffusion) or some combination of these mechanisms? Christensen (1975) observed the high rate of AVT-stimulated water absorption across isolated seat patch skin from *Bufo bufo* only when the vasculature was perfused with a Ringer's solution. In contrast, Baldwin (1974) found significant stimulation of water flow across isolated, non-perfused, seat patch skin from *B. punctatus* following treatment with the mammalian antidiuretic hormone arginine vasopressin (AVP). However, values for isolated seat patch skin were about half that of water absorption by dehydrated *B. punctatus*, in vivo, held with modeling clay to apply the seat patch to a calibrated pipette (McClanahan and Baldwin, 1969). The first section of this review will describe experiments designed to test the hypothesis that seat patch blood flow is a limiting factor for water absorption by conscious, unrestrained toads and will present new data that identify aquaporin 1 as a water conducting pathway in cutaneous capillaries.

☆ This paper was presented in the session "Water transport" at the society of experimental biology's annual meeting at the university of Kent, Canterbury, UK April 2nd–7th 2006.

* Corresponding author. Institute of Molecular Biology and Physiology, August Krogh Building, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen O, Denmark. Tel.: +45 3532 1635; fax: +45 3532 1567.
E-mail address: nwillumsen@aki.ku.dk (N.J. Willumsen).

1095-6433/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpa.2006.12.032

AVT also stimulates Na^+ transport across isolated amphibian skin. This has been measured as short circuit current (I_{sc}) in Ussing chamber preparations and shown to be the result of an increased number of epithelial Na^+ channels (ENaCs) in the apical membrane of the epithelium (Helman et al., 1983). AVT stimulation of I_{sc} across toad skin (*Bufo woodhouseii*) is much greater in the seat patch than in pectoral skin (Baker and Hillyard, 1992) and NaCl transport has been shown to be coupled to water absorption, *in vitro* (Nielsen et al., 2007). In live toads, *B. bufo* were observed to rehydrate 64% more rapidly from 20 mM NaCl than from deionized water (Ferreira and Jesus, 1972) and it was suggested water uptake was coupled to Na^+ transport. In the second section of this review we present our own studies that have shown enhancement of water absorption by dilute salt solutions (Hillyard and Larsen, 2001) and describe experiments aimed at elucidating the still unresolved mechanism for this phenomenon (confusion).

2. Methods

2.1. Animal sources and maintenance

Bufo bufo and *B. marinus* were provided by commercial suppliers. Toads were maintained in a 3 × 5 m room with water and dry surface available ad lib. *B. punctatus* were captured in the Spring Mountains, Clark County Nevada, under permit from the Nevada Department of Wildlife and maintained in 30 terraria with rocks, sand and water arranged to approximate their natural environment. All species were maintained and experiments conducted at ambient (20–23 C) temperature. All were maintained on a regular photoperiod, usually 12:12 light:dark, and fed on a regular basis to maintain or increase their body mass. Under these conditions toads were considered to maintain a hydrated state (Jørgensen, 1994) and normally stored dilute urine in their urinary bladders to offset dehydration. The weight of a hydrated toad with an empty bladder, the standard weight (Ruibal, 1962), was used as a reference for evaluating levels of dehydration.

2.2. Seat patch blood flow and water uptake rates

Viborg and Rosenkilde (2004) utilized laser Doppler flow cytometry to measure seat patch blood flow in *B. bufo*. An external probe measured the velocity and density of blood cells in the cutaneous circulation to a depth of approximately 1 mm. The output of the probe is expressed in units of mV and gives a relative measure of Blood Cell Flow (BCF) rather than an absolute value for volume flow. The probe was positioned in the floor of a Lucite chamber and toads were placed individually in the chamber with the seat patch over the probe. BCF was initially measured on hydrated toads before and after placing them in a water filled beaker to determine whether water contact, per se, stimulated BCF. Toads were then dehydrated between 10–20% of their standard weight. BCF was again measured before and after water exposure to determine if water exposure in addition to dehydration is necessary for stimulating BCF. After the initial measurement of BCF, toads were allowed to remain in water for 30 min periods at which time BCF and water uptake, evaluated as weight gain, were recorded. Viborg and Hillyard (2005) and Viborg et al. (2006) further developed this method with the laser-Doppler probe incorporated into a calibrated water reservoir so water absorption could be measured simultaneously with BCF. This allowed blood flow and water absorption to be compared during the initial period following water contact when both parameters are maximally stimulated. Because dehydration increases the plasma AVT concentration of *B. marinus* (Konno et al., 2005) one might expect a parallel increase in BCF to accompany the increase in water absorption (Parsons and Mobin, 1991). For this reason, Viborg and Rosenkilde (2004) also measured BCF and water absorption in hydrated *B. Bufo* injected with exogenous AVT (100 ng/100 g BW).

2.3. Water uptake routes in the toad skin

A polyclonal antibody was raised in rabbits against the terminal twenty two amino acid sequence of rat AQP 1; GQVEEYDLDADDINSRVEMKPK. The antibody was affinity purified and used in a 1:100 or 1:300 dilution. Paraffin sections (10 μM) were dewaxed and rehydrated. Endogenous peroxidase was blocked by 0.5% H_2O_2 in absolute methanol. To reveal antigens, the sections were boiled in 1 mM Tris, pH 9 supplemented with 0.5 mM EGTA. Non-specific binding was quenched by incubating the sections in 50 mM NH_4Cl and blocked in phosphate-buffered salt solution (PBS) supplemented with 1 % bovine serum albumen (BSA), 0.05% saponin and 0.2% gelatin. The sections were incubated overnight at 4 C with the antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. After washing, the sections were incubated with horseradish peroxidase conjugated goat anti rabbit IgG (P0448 1:200 Dako) diluted in PBS supplemented with BSA and Triton X-100. The peroxidase stain was visualized by 0.05% 3,3′ diaminobenzidine tetrahydrochloride dissolved in PBS with 0.1% H_2O_2. Mayer′s haematoxylin was used for counterstaining and the sections were dehydrated and mounted in hydrophobic Eukitt mounting medium (O. Kindler, Freiburg, Germany). For control experiments, comparisons were made between sections labeled with a 1:100 dilution of the primary antibody alone and preabsorbed with 30 μg of the peptide.

We have also begun to examine the expression of aquaporins the pelvic and pectoral skin of *B. bufo* that were taken directly from the maintenance terraria, dehydrated or kept in standing water to insure complete hydration. Reverese transcriptase (RT)-PCR was conducted on homogenates of epithelial cells using sets of forward and reverse primers designed from base sequences of *B. marinus* AQP 1-3 obtained from GenBank:

Aquaporin 1: Accession Number AF020620
AQP-t1Fw: GGCGGTGATAGCCGAGTTCT
AQP-t1Rw: CGGTCCGTTAAGTCGCTGGT
Aquaporin 2: Accession Number AF020621
AQP-t2Fw: TGAATTGGCAGTCGGCACTT
AQP-t2Rw: CCTGGGCTGTTCTTGTCTTTCC