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Role of cutaneous surface fluid in frog osmoregulation

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ABSTRACT

The study investigated whether evaporative water loss (EWL) in frogs stems from water diffusing through the skin or fluid secreted by mucous glands. Osmolality of cutaneous surface fluid (CSF) of *Rana esculenta* (*Pelophylax* kl. *esculentus*) subjected to isoproterenol or 30 °C–34 °C was 191 ± 9.3 and 181 ± 7.5 mosm/kg, respectively, as compared to lymph osmolality of, 249 ± 10 mosm/kg. Cation concentrations of CSF were likewise independent of pre-treatment with averages of, $[Na^+]$ = 65.5 ± 5.1 and $[K^+]$ = 14.9 ± 1.6 mmol/L, and lymph concentrations of 116 mmol Na^+/L and 5.1 mmol K^+/L. The relatively high $[K^+]$ confirms that CSF is produced by submucosal glands. Since the chemical energy of water of CSF was always higher than that of body fluids, diffusion of water would be from CSF to the interstitial fluid and not in the opposite direction. It is concluded that volume and composition of CSF are regulated by subepidermal exocrine gland secretion balanced by EWL into the atmosphere and ion reuptake by the epidermal epithelium. Previously discovered regulatory mechanisms of epithelial ion absorption, hitherto not ascribed a body function, fit well with a role in regulating turnover of CSF. As a regulated external physiological compartment, CSF would be of importance for the immune defenses that amphibians employ in protecting their skin.

1. Introduction

Frogs are adapted to live in aquatic as well as terrestrial environments. Water-acclimated frogs exhibit high rates of glomerular filtration ensuring the cutaneous uptake of water being balanced by voiding of highly diluted urine (Schmidt-Nielsen and Forster, 1954; Mayer, 1969). On land, urine production decreases significantly because of a decreased glomerular filtration rate and an increased fractional water reabsorption in early distal renal tubules. The urine produced is stored in the bladder and recycled into the body fluids for maintaining water balance during evaporation of water into the atmosphere (Bentley, 1966; Tran et al., 1992; Jørgensen, 1997). Early studies on water metabolism of amphibians led to the conclusion that the evaporative water loss (EWL) is due to an unavoidable diffusion of water across a highly water permeable skin (Overton, 1904; Adolph, 1932; Rey, 1937). Since then numerous studies have measured EWL in species under different laboratory conditions such as environmental temperature, air humidity and water balance of the animal (Hillman et al., 2009). Lillywhite reviewed the literature on the 'resistance of the diffusive water loss' (R_{EWL}) in tetrapod integument including that of amphibians. He concluded that in some cases R_{EWL} is correlated with the habitat of the species. However, the overall analysis did not indicate a consistent rule about this. For example semiaquatic frogs and terrestrial toads exhibit similar low R_{EWL}-values of 0.05–1.6 s/cm and 0–5 s/cm, respectively (Lillywhite, 2006). Notably, some R_{EWL} values of both species are very low and comparable to R_{EWL} of a free water surface, which is zero.

The skin of several amphibians is kept moist by a cutaneous surface fluid (CSF) secreted by subepidermal mucosal glands. CSF prevents desiccation of the epidermal cells, is of importance for skin respiration, and makes the animal slippery helping it escape from predators (Lillywhite, 1971; Duellman and Trueb, 1994; Lillywhite et al., 1998). Mucous secretion also helps in shedding of the stratum corneum that takes place at regular intervals of days to weeks depending on species, season, and temperature (Larsen, 1976). Due to a particular abundance of mucous glands in the abdominal seat patch of terrestrial toads, Hillyard suggested that mucous glands of this region are of significance for validating the quality of a hydration source prior to cutaneous drinking (Hillyard et al., 2009). Recently, it was argued that formation of CSF by submucosal glands has an osmoregulatory function in amphibians on land (Larsen, 2011).

We wanted to investigate whether EWL stems from the fluid generated by submucosal glands rather than from water diffusing through the skin. For distinguishing between these two mechanisms, we measured the osmolality and cation concentrations of the CSF of frogs in vivo stimulated to gland secretion by a β-adrenergic agonist and by elevating the environmental temperature. Our study leads to the conclusion that CSF constitutes an external physiological compartment the volume and ion composition of which are regulated by epidermal transport mechanisms.

Abbreviations: P: Na^+/K^+ pump; tj: tight junction; PKA: Protein kinase A; c.s.: catalytic subunit; G: G-protein; AC: adenylyl cyclase; β-adr.: β-adrenergic receptor.

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2. Methods

2.1. Experimental animals

European green frogs (*Rana esculenta; Pelophylax* kl. *esculentus*) were kept in a laboratory terrarium with free access to mealworms and a pool of tap water. The frogs are easily stressed by handling which induces mucous secretion. To secure that gland secretion was stimulated by laboratory protocols, prior to experiments the frogs were kept individually in a dry plastic box measuring 12 cm × 8 cm × 3 cm. The box was covered by a transparent lid furnished with drilled holes through which CSF-sampling pads could be placed and withdrawn with a minimum disturbance of the animal. The size of the frogs prevented them from moving around in the box during the period of observation.

2.2. Sampling of CSF and lymph

For measuring the osmolality and cation concentrations of the cutaneous surface fluid, a dry solute free filter paper of an area of 0.31 cm^2 (SS-033 Sample Discs, Wescor Inc., USA) and of known mass was placed on the skin surface. After a few minutes, the moist disc was withdrawn and weighed immediately on a Mettler AT261 fine-balance for determining the volume of the sampled fluid to the nearest 10 nL. Clear samples of lymph were obtained with a syringe from a dorso-lateral lymph vessel. A lymph volume of 10 μL was placed on a sample disc for immediate measurement of osmolality. To ensure that gland secretion was evoked by the chosen protocols rather than by stress imposed by handling, lymph- and CSF concentrations, respectively, were not always obtained the same day and not always on the same animal.

2.3. Osmolality of collected fluids

The osmolality (mosm/kg) of sample-disc fluid was measured in a Wescor Vapro model 5520 vapor pressure osmometer (Wescor Inc., USA) calibrated by the manufacturer' Optimol osmolality standard solutions.

2.4. Concentrations of Na^+ and K^+

The sample discs were immersed in 200 μL MillQ-H_2O. The concentrations of Na^+ and K^+ were measured on a Metrohm ion chromatography system of the following specification: 830 IC interface, 818 IC pump, 819 IC conductivity detector and fitted with a Metrosep C 4 150/4.0 cation IC column (Metrohm AG, Herisau, Switzerland). The eluent was prepared according to the manufacturer's instructions: 0.7 mmol/L dipicolinic acid (Merck, cas# [499-83-2]) + 1.7 mmol/L HNO_3 (65%) in MillQ-H_2O (Merck). The instrument was calibrated with a Multielement ion chromatography standard (Fluka 89316-50 ml-F, lot# BCBD9892). The calibration was rechecked after every 6 measurements with a Multielement cation IC standard in water (Reagecon, ICC-DX-611, lot#: 4947). Standard curves for Na^+ and K^+ were produced from NaCl and KCl, respectively (Merck). The cation concentration of the sample was brought into the linear range by diluting to 1:1 or 1:10 with MillQ-H_2O as required. The injection volume was 60 μL for both standards and samples and each sample was measured in replicates of 3.

2.5. Data presentation

For illustrating the variation of osmolality among the samples of the same animal and among different animals, individual measurements are given as well as mean ± s.e.m. *N* is the number of animals studied, and *n* is the number of individual CSF samples.

3. Results

3.1. Osmolality of lymph

Individual osmolalities of lymph samples collected from ten frogs of a mass, 87.2 ± 2.8 g (range 70.7–104.0 g) are listed in Table 1. The lymph osmolality of frog I - frog IX, was 240.4 ± 3.6 mosm/kg (mean ± s.e.m., $N = 9$). The osmolality of 333 mosm/kg of frog X deviated markedly from this mean, which is further discussed below. The individual measurements reflect the fact that anurans do not regulate their extracellular osmolality and ion concentrations as precisely as other vertebrates. All values of Table 1 are within those reported for anurans kept under similar laboratory conditions or studied in the field (Hillyard et al., 2009).

3.2. Isoproterenol treated animals

Isoproterenol was given subcutaneously at a concentration of 5 μmol per kg-body mass and sampling of CSF began after about 5 min. Since bladder urine is important for the frog's osmoregulation and the study had to be carried out on undisturbed animals, the urinary bladder was not emptied before hormone treatment. Since 1–2 μM isoproterenol provides a substantial secretory response of the submucosal glands of frogs in vitro (Mills et al., 1985; Sørensen and Larsen, 1999), the above concentration of 5 μmol per kg-body mass would have a sufficient stimulatory effect even if the bladder contained a urine volume of 30% of the body mass (which is highly unlikely). The skin of *R. esculenta* is moist and slippery when kept outside the pool of water. Although the whole body surface was covered by a mucous layer, both prior to and after isoproterenol injection, as well as during heat treatment (see below), it was difficult within reasonable time to obtain an amount of fluid that could be measured in the osmometer. Keeping the sample-disc for a longer period time for sampling a sufficient volume might have resulted in evaporation from the disc surface that would increase the solute concentrations of the remaining fluid. We therefore decided to place the disk on different skin areas between the shin and the femur or between femur and the lateral skin surface on both left and right side of the animal. In this way, it was not too difficult to obtain from a single animal several samples of fluid suited for analysis.

The osmolalities of CSF samples of four isoproterenol treated frogs each studied during a period of about 2 h are shown in Fig. 1. It is striking that the osmolality of CSF samples of the same animal varied a good deal, e.g., in frog 1 from 188 to 287 mosm/kg, and in frog 4 from 131 to 177 mosm/kg. The mean values of the three frogs kept at 23 °C are similar; 144 ± 9.0, 153 ± 6.0, 156 ± 7.4 mosm/kg, respectively. The osmolality of CSF of the frog kept in a box cooled by ice to 15 ± 0.3 °C was significantly higher than those of frogs kept at room temperature; 244 ± 9 mosm/kg. This was also the frog with the highest lymph concentration, i.e., 333 mosm/kg (Table 1), measured after completion of the experiment but on the same day.

3.3. Osmolality of the cutaneous surface fluid of frogs at 30 °C–34 °C

In studies of the effect of a raised environmental temperature, the experimental chamber was exposed to a 60 W heating lamp while

Table 1
Osmolality of lymph samples of ten *R. esculenta* kept in the laboratory as indicated in Methods.

I	II	III	IV	V	VI	VII	VIII	IX	X
mOsm/kg									
249	228	247	254	232	237	232	232	253	333

The lymph osmolality of 333 mosm/kg of frog X deviated markedly from those of the other nine frogs, which was 240.4 ± 3.6 mosm/kg (mean ± s.e.m., $N = 9$). See text for further explanation.