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Graphical review

Dual skin functions in amphibian osmoregulation

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A B S T R A C T

August Krogh’s studies of the frog identified the respiratory function of the skin in 1904 and the osmoregulatory function of the skin in 1937. It is the thesis of my review that the osmoregulatory function of the skin has evolved for meeting quite different demands. In freshwater the body fluid homeostasis is challenged by loss of ions to the environment. This is compensated for by active ion uptake energized by the sodium-pump ATPase and the V-type proton pump ATPase. I conclude that Krogh’s astonishing observation of cutaneous chloride uptake from µM concentrations of NaCl is compatible with the free energy changes of ATP hydrolysis catalyzed by the sodium-potassium pump ATPase and the V-type proton pump ATPase operating in series, and in parallel with experimentally verified vanishingly small leak fluxes. On land the frog is challenged by evaporative water loss through the highly water permeable skin, similar to the water permeable conducting airways of terrestrial vertebrates including man. The epithelia serving respiratory gas exchanges are heterocellular and have molecular, structural and functional properties in common. The cutaneous surface liquid of amphibians evolved for protecting the skin epithelium from desiccation like the airway surface liquid of the lung. Published studies of ion transport mechanisms of acinar cells and the two types of epithelial cells, lead to the hypothesis that subepithelial gland secretion, evaporative water loss, and ion reabsorption by the epithelium regulate composition and volume of the cutaneous surface liquid.

1. Introduction

In studies of osmoregulation of the frog August Krogh (1937) demonstrated ion transport in a direction opposite to free diffusion. For this type of mass movement, Krogh introduced the concept of ‘active transport’ as opposed to diffusion, which was termed ‘passive transport’. By considering transport between cells and extracellular fluid and between the organism and its surroundings Krogh (1946) generalized the concept by emphasizing the energy requiring dynamic state of body fluids. Whether the frog is in freshwater or on dry land the skin is of decisive significance for whole body fluid homeostasis. It is the general thesis of my Graphical Review that the cutaneous ion transport mechanisms conveying adaptations to either environment have evolved for different purposes. In freshwater the epithelial ion transport serves to maintain extracellular fluid homeostasis of larval stages (Haugan et al., 2010; Møbjerg et al., 2000; Uchiyama et al., 2011) and of the metamorphosed amphibian. On land the skin is covered by a cutaneous surface liquid (CSL) from which water evaporates. The ion composition and volume of CSL are maintained by subepidermal gland secretion and reabsorption by the heterocellular epithelium in response to evaporation from this external compartment. The unavoidable evaporative water loss is compensated by cutaneous water uptake through a specialized abdominal region, stored in the urinary bladder and recycled into the body fluids, maintaining overall water balance on land (Hillman et al., 2009; Hillyard et al., 2007; Jørgensen, 1997).

This review investigates the cutaneous ion mechanisms evolved for supporting the life of metamorphosed amphibians, alternating between aquatic and terrestrial habitats. The issue of cutaneous water uptake was reviewed by Hillyard and Willumsen (2011) in a symposium paper celebrating the 90th anniversary of Krogh’s Nobel Prize in an issue of Acta Physiologica that also contained review by Hoffmann and Pedersen (2011) of their seminal work on the regulation of intracellular fluid volume and associated functions.

2. The frog in the pond

In frogs kept in distilled water for weeks, Krogh (1937) discovered an impressive capacity of anuran skin for taking up Cl⁻ from µM-solutions, either accompanied by Na⁺ or in exchange for HCO₃⁻ (Fig. 1A). The active uptake of Na⁺ is energized by the ouabain inhabitable sodium-potassium pump in series with the apical membrane furnished with ENaC (Fig. 1B). Under these conditions the paracellular junctions are tight to Na⁺ as indicated by the +135 mV skin depolarization in response to block of apical Na⁺ channels by amiloride (Fig. 1C). This maneuver held the cutaneous Cl⁻ uptake largely unaffected, JCl ≈ 13.9 ± 0.3 and JHCO₃ conr = 11.9 ± 0.6 pmol·s⁻¹·cm⁻², suggestive of an electroneutral apical mechanism (Jensen et al., 2003). Data shown in Fig. 1D from Ehrenfeld and Garcia-Romeu (1978) are in agreement with this suggestion by indicating linear dependence of chloride influx (JCl) and net base excretion (JHCO₃ conr). The regression line given by the
The equation, 
\[ J_{\text{HCO}_3}^{\text{out}} = -(0.50 \pm 0.06) \cdot J_{\text{Cl}}^{\text{in}} - (16.7 \pm 9.3), R = 0.78 \pm 0.15, P < 0.001, \]
of a numerical slope of 0.5 is compatible with an anion exchange mechanism that also mediates Cl\(^-\) self-exchange (Kristensen, 1985).

Toad skin (Bufo bufo) actively secretes protons (Fig. 1E), which may build up a [H\(^+\)]-gradient in the external unstirred layer here revealed by replacing external Cl\(^-\) for gluconate. Jensen et al. (1997) introduced a powerful technique using the external [H\(^+\)]-gradient in the unstirred surface layer for computing stationary excretion rates of protons, in this example \( J_{\text{H}^+}^{\text{active}} = 3.39 \text{ pmol} \cdot \text{s}^{-1} \cdot \text{cm}^{-2} \), which is a relatively small
cutaneous flux because the toad was in ion balance. Klein et al. (1997) showed that the active proton efflux of frog skin is generated by a V-type proton pump ATPase in mitochondria-rich (MR) cells, subsequently immuno-localized also to MR cells of toad skin (Fig. 1F). When the active uptake of Cl\(^{-}\) is stimulated by keeping the frog in a 50-μM NaCl-solution for 2–3 weeks, the proton efflux is also significantly stimulated. Because both the stimulated active uptake of Cl\(^{-}\) and the stimulated active secretion of H\(^{+}\) are inhibited by the V-type proton pump ATPase inhibitor concanamycin A (Fig. 1G), we can assume that the two fluxes are coupled via a cellular carbonic anhydrase according to the scheme indicated in Fig. 1H, which provides the necessary quantitative information for evaluating the metabolic energy requirement of the cutaneous ion uptake in Krogh’s study. With superscript (o) for freshwater, (e) for extracellular body fluids, and \(V_T\) indicating the skin potential, the electrochemical work done in transporting 1 mol of Na\(^{+}\) and 1 mol of Cl\(^{-}\) from freshwater to the extracellular fluid is given by,

\[
\frac{\Delta G}{\text{mol}} = \frac{zF T \ln a_{\text{e}}}{V_T}
\]

where \(z = \pm 1\) and \(a_{\text{e}}\) is the activity of the transporting ion in the extracellular fluid.

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**Fig. 2.** Skin functions of amphibians on land. (A) Krogh (1904) identified the pulmonary and cutaneous gas exchanges; rates in \(\text{cm}^3 \cdot \text{h}^{-1} \cdot \text{kg}^{-1}\). \(R.\) fusca is synonymous with \(R.\) temporaria. Krogh (1904) mentions: “I have only a few experiments on toads. In these animals, the larynx is so exceedingly narrow and deeply seated in the mucous membrane so as to forbid any fixation of cannula” (loc.cit page 363). Krogh’s experiment No. 31. 29./IX 1901 on a toad shows cutaneous and pulmonary exchange rates comparable to those in similar sized frogs. (B) Acinar epithelium of subepidermal glands constitutes a large area as compared to the absorbing pulmonary exchange rates. (C) Precise localization and identification of CFTR of the heterocellular epidermis were performed by two biophysical methods: (i) Single-channel patch clamping (Sorensen and Larsen, 1996), and (ii) non-evasive single-cell recordings of a polarized cell with the neck mounted in a glass pipette (Fig. 4E). With this method we proved coupling of apical CFTR to basolateral \(\beta\)-adrenergic receptors, that was stimulated by genistein and inhibited by glibenclamide (Larsen et al., 2003). Shown here, recording of single channel CFTR-currents of an inside-out patch of toad skin MR cell. [Cl\(^{-}\)]-bath = 125 mM. Left hand panel. [Cl\(^{-}\)]-bath = 125 mM, with linear \(i/V\)-relationship of slope indicating single channel conductance of, \(72 \pm 8 \text{ pS}\). Right hand panel. Recording of the same patch after substitution of the bath for a solution with \([\text{Cl}^{-}] = 25 \text{ mM}\) and \([\text{Ca}^{2+}] = 100 \text{ mM}\). The full line of the upward concave \(i/V\) relationship is best fit of the GHK-equation with \(P_{\text{GHK}} = 2.1 \times 10^{-14} \text{ cm}^2 \cdot \text{s}^{-1}\). The free single channel conductance of, \(71.25 \pm 10.0 \text{ pS}\). (F) Similar to upper airways (Boucher, 1999; Knowles et al., 1997; Luan et al., 2020) is the epidermis of \(R.\) esculenta covered by a thin film of hyposmotic liquid (Larsen and Ramlov, 2013). (F) Functional organization of amphibian skin. Height and composition of the cutaneous surface liquid are regulated by:

1. Secretion of near isosmotic fluid by subepidermal glands.
2. Reabsorption of ions by the heterocellular skin epithelium.
3. Unavoidable evaporation of water at a rate depending on temperature, air humidity, local airflow and animal activity. Volume turnover of CSL in mg \(\text{g}^{-1} \cdot \text{h}^{-1}\) can be estimated from the rate of the temperature dependent evaporative water loss, here given for \(B.\) boreas (Carey, 1970): 10.7 (10 °C), 24.5 (20 °C) and 36.6 (30 °C). To maintain hyposmotic conditions, the required reabsorption fluxes of NaCl would be (pmol·cm\(^{-2}\)·s\(^{-1}\)):
   - 41 (10 °C), 94 (20 °C) and 140 (30 °C) (Larsen, 2011), which are easily handled by the cutaneous transport mechanisms. The mechanism of potassium recovery is not clarified.
ΔμNa+ + ΔμCl− = RT ln(a(e)Na+/a(0)Na+) + FV
= RT ln(f(e)Na+/f(0)Na+) + RT ln(c(e)Cl−/c(0)Cl−) (1)

Krogh (1937) observed uptake of Cl− at an external concentration >10 μM. With the ratio of activity coefficients, f(e)/f(0) = 0.78 and extracellular concentrations of sodium ions and chloride of 106 and 84 mM, respectively (Jensen et al., 2003), the electrochemical work done in transepithelial uptake of 1 mol of NaCl amounts to <45 kJ. Considering the stoichiometry of ATP hydrolysis at the Na⁺–K⁺ pump and the proton pump, respectively (Fig. 1H), and a free energy change of, ΔGATP/3 = −60 kJ/mol-ATP (Eksensen and Ussing, 1985), the thermodynamic energy supply would be,

ΔGATP/3 + ΔGATP/2 = −50 kJ

which is compatible with the above electrochemical work of <45 kJ in Krogh’s, 1937-study.

3. Skin functions under terrestrial conditions

Using his own experimental setup in Christian Bohr’s laboratory in Copenhagen, Krogh (1904) separated pulmonary and cutaneous gas exchanges proving the respiratory function of the skin (Fig. 2A). Like the epithelium of upper airways, the cornified cell layer of anurans is freely permeable to water. If a Ringer’s solution is placed on the skin surface, the ions equilibrate with the water of cornified cells indicating that the stratum corneum of toads and frogs contains water that must considered an unstirred layer (Rick et al., 1980; Rick et al., 1978). The skin comprises numerous subepithelial glands in close vicinity to the heterocellular ion absorbing epithelium (Fig. 2B). With cytookeratin CK4 (Fig. 2C), CFTR (Fig. 2D) and the V-type proton ATPase (Fig. 1F) selectively expressed in the MR cell, this minority cell type of anuran skin is similar to a minority cell type named ionocyte of mammalian nasal
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and bronchial epithelia (Plasschaert et al., 2018). In addition to the wet cornified cells, the skin is covered by a cutaneous surface liquid (CSL), which constitutes the other component of the external unstirred layer. The CSL of frogs is hyposmotic (Fig. 2E) and characterized by a K\(^+\) concentration of 14.6 ± 1.9 mM, which is significantly above the [K\(^+\)] of the extracellular fluid (2–3 mM) (Larsen and Ramløv, 2013) as are secretions of the subepidermal exocrine glands (Watlington and Huf, 1971). A comparable K\(^+\) concentration of CSL was obtained in frogs stimulated to gland-secretion by raising the laboratory temperature to 30–34 °C (Larsen and Ramlov, 2013), and of the airway surface liquid (ASL) of conducting airways of mammalian lung (Boucher, 1999; Knowles et al., 1997; Luan et al., 2020). Additional similarities exist between mammalian airways and amphibian skin, which act as organs of immunological and microbiological barriers to pathogen invasion (Stanton, 2017; Varga et al., 2019).

Lissamphibians are challenged by evaporative water loss through the water permeable skin. As previously discussed (Larsen, 2011; Larsen and Ramløv, 2013), desiccation of the epithelium is prevented by the surface liquid of hyposmotic saline, because the direction of the diffusional flux of water is inward into the body fluids rather than outward to the CSL. Maintenance of CSL requires regulated balance between near-isosmotic subepithelial gland secretion and epithelial ion reabsorption. As this mechanism is of decisive importance for both respiration and osmoregulation it evolved with the lissamphibians’ life on land dated to be between 351 and 266 million years ago (Marjanovic and Laurin, 2007).

4. The formation of CSL: Ion transport by subepidermal glands

Fig. 3A summarizes ion channel studies of subepidermal glands of Rana esculenta, which express cAMP and Ca\(^{2+}\) activated channels in the luminal membrane of significance for exocrine secretion (Sørensen and Larsen, 1999). The overview indicates basolateral receptors that couple...
to Cl−, K+, and Na+ channels of the luminal membrane studied in micro-dissected subepithelial gland acini. The small conductance 6-pS linear channel of reversal potential at the Cl− equilibrium potential (Fig. 3B) is CFTR as judged by its regulation by protein kinase A and ATP (Sørensen and Larsen, 1998) via β-adrenergic receptors (Sørensen and Larsen, 1999). The large-conductance Ca2+- and depolarization activated K+ (BK) channel (Fig. 3C) is carrying the measured active efflux of K+ in stimulated cells (Sørensen et al., 2001), supposedly causing the relatively high K+ concentration of the secretion. The co-localization of apical BK and CFTR channels (Fig. 3C and Klein et al., 2016) implies that the simultaneous receptor stimulation of K+ and Cl− channels secures the demanded electrical driving force for luminal exit of Cl−. The luminal sodium channel of reversal potential, −Vr = 103 mV indicated in Fig. 3D is from a cell attached patch. With a membrane potential of −69.5 ± 0.7 mV (in unstimulated acinar cells, Sørensen and Larsen, 1999), according to physiological sign conventions, the reversal potential would be +33 mV, ie as expected for a Na+ channel. It is likely that this channel returns Na+ to the lateral space via the cell energized by the lateral Na-K pump hypothesized to be the mechanism regulating osmolality of the secretion (Ussing et al., 1996).

5. NaCl reabsorption by the epidermal epithelium

The epithelium of anuran skin (Fig. 2B) is heterocellular consisting of a syncytial multilayered Na+ transporting compartment (Rick et al., 1978), and a scattered distribution of MR cells in the uppermost layer facing the subcorneal space. It is physiologically important that the apical Cl− channels of MR cells are deactivated in freshwater and...
becoming activated in skins exposed on the outside to raised Cl− concentration (Fig. 4A). Under these conditions the Cl− flux through MR cells is driven by the skin potential energized by the active Na+ flux through principal cells (Larsen, 1991). The general theme of Fig. 4 concerns the cellular mechanisms evolved to maintain these functions. Studies on frogs (Kristensen, 1983) and toads (Harck and Larsen, 1986; Larsen and Rasmussen, 1982) show that the passive uptake of Cl− owes to a dynamic Cl− permeability of the apical membrane that is gated by outside chloride (Fig. 4A and B) and apical membrane potential (Fig. 4C and D). The flux-ratio analysis of stationary chloride fluxes (Fig. 4C) is accounting for the transition between active and passive transport, respectively, prevailing in the two habitats. It also is important that, in spite of their small size and low density, the sum of Cl− currents generated by single MR cells accounts quantitatively for the macroscopic transepithelial Cl− current (Fig. 4D, E). The power density spectrum of Fourier transformed V-activated stationary Cl− current fluctuations indicated a unitary Cl− conductance of 250 ± 18 pS (Larsen and Harvey, 1994). Our failing attempts at bridging the kinetics of macroscopic- and single channel currents (Fig. 4F) owe to insurmountable technical difficulties in recording currents through the “big” ~250-pS chloride channel in sealed patches of sufficient life time (Larsen, 2011).

6. Cross-talk between principal cell compartment and MR cells of the epidermis

According to the skin function of amphibians on land, the transport activities of the acinar cells and the surface epithelium must be coordinated for keeping the ion composition and the small volume of CSL within their respective physiological range. Cross-talk between the Na+ gradient for keeping the ion composition and the small volume of CSL and activities of the acinar cells and the surface epithelium must be coordinated (Larsen, 2011). Difficulties in recording currents through the “big” ~250-pS chloride channel in sealed patches of sufficient life time (Larsen, 2011).

Cross-talk between principal cell compartment and MR cells

The coupling for a large range of Na+ concentrations of NaCl is quantitatively compatible with the production of salivary gland secretion (Fig. 5A). This relationship makes physiological sense considering the dual function of transepithelial potential for the Cl− flux through MR cells: (i) Hyperpolarization of the skin is associated with depolarization of the apical membrane that drives the inward flux of Cl−, (ii) outward currents depicted in Fig. 4D and E), (ii) Depolarization of the apical membrane slowly activates the apical Cl− permeability of the MR cell (Fig. 5B). As a result, the steady-state Cl− currents become strongly rectified (Fig. 4E). The intriguing quantitative interactions between active Na+ currents through principal cells and voltage gated Cl− currents through the population of MR cells had to be studied by computational analysis of a mathematical model of the epithelium (Larsen and Rasmussen, 1985). As an example of interest for the discussion here, Fig. 5C shows how activation of the Na+ permeability of the apical membrane of principal cells (PNaCell) leads to activation of the Cl− permeability of MR cells (PCellClMR). As originally suggested by Koevoed-Johnsen and Ussing (1958), the sodium pump energizes the Na+ flux, while sodium pump generated skin potential energizes the passive transepithelial Cl− flux. We now see that this interplay is more sophisticated because the Cl− flux through V-gated Cl− channels in the apical membrane of MR cells secures tight functional coupling for a large range of Na+ fluxes through principal cells within a moderate range of skin potentials (Fig. 5C).

7. Conclusion

August Krogh’s observation in 1937 of uptake of chloride from micro-molar concentrations of NaCl is quantitatively compatible with energy supply by the sodium-potassium ATPase in series with the V-type proton-ATPase of mitochondria-rich cells of the skin and paralleled by vanishingly leak permeability. Ion transport by the skin of amphibians on land is associated with the function of a respiratory epithelium identified by August Krogh in 1904. The cutaneous surface liquid produced by subepithelial exocrine glands is hypsomotic with a K+ concentration above that of extracellular fluid. This is similar to the airway surface liquid of mammalian lung. The concentration- and voltage dependence of the Cl− channel of mitochondria-rich cells governs its closure in freshwater and - on land - the tight coupling of active Na+ and passive Cl− uptake from the cutaneous surface liquid. Future studies of the skin function would have to embrace the dynamic interplay between the heterocellular surface epithelium, the subepithelial exocrine glands, and the cutaneous surface liquid/wet stratum corneum evolved with amphibians’ life on dry land.

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Declaration of Competing Interest

None declared.

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