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Review

Proton pump-driven cutaneous chloride uptake in anuran amphibia

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Abstract

Krogh introduced the concept of active ion uptake across surface epithelia of freshwater animals, and proved independent transports of Na^+ and Cl^- in anuran skin and fish gill. He suggested that the fluxes of Na^+ and Cl^- involve exchanges with ions of similar charge. In the so-called Krogh model, Cl^-/HCO_3^- and Na^+/H^+ antiporters are located in the apical membrane of the osmoregulatory epithelium. More recent studies have shown that H^+ excretion in anuran skin is due to a V-ATPase in mitochondria-rich (MR) cells. The pump has been localized by immunostaining and H^+ fluxes estimated by pH-stat titration and mathematical modelling of pH-profiles in the unstirred layer on the external side of the epithelium. H^+ secretion is voltage-dependent, sensitive to carbonic-anhydrase inhibitors, and rheogenic with a charge/ion-flux ratio of unity. Cl^- uptake from freshwater is saturating, voltage independent, and sensitive to DIDS and carbonic-anhydrase inhibitors. Depending on anuran species and probably on acid/base balance of the animal, apical exit of protons is coupled to an exchange of Cl^- with base (HCO_3^-) either in the apical membrane (γ-type of MR cell) or in the basolateral membrane (α-type MR cell). The γ-cell model accounts for the rheogenic active uptake of Cl^- observed in several anuran species. There is indirect evidence also for non-rheogenic active uptake accomplished by a β-type MR cell with apical base secretion and basolateral proton pumping. Several studies have indicated that the transport modes of MR cells are regulated via ion- and acid/base balance of the animal, but the signalling mechanisms have not been investigated. Estimates of energy consumption by the H^+-ATPase and the Na^+/K^+-ATPase indicate that the γ-cell accomplishes uptake of NaCl in normal and diluted freshwater. Under common freshwater conditions with serosa-positive or zero V_t, the K^+ conductance of the basolateral membrane would have to maintain the inward driving force for Na^+ uptake across the apical membrane. With the K^+ equilibrium potential across the basolateral membrane estimated to -105 mV, this would apply to external Na^+ concentrations down to 40–120 μmol/l. NaCl uptake from concentrations down to 10 μmol/l, as observed by Krogh, presupposes that the H^+ pump hyperpolarizes the apical membrane, which would then have to be associated with serosa-negative V_t. In diluted freshwater, exchange of cellular HCO_3^- with external Cl^- seems to be possible only if the proton pump has the additional function of keeping the external concentration of HCO_3^- low. Quantitative considerations also lead to the conclusion that with the above extreme demand, at physiological intracellular pH of 7.2, the influx of Cl^- via the apical antiporter and the passive exit of Cl^- via basolateral channels would be possible within a common range of intracellular Cl^- concentrations.

Keywords: Osmoregulation in freshwater; Krogh model; frog and toad skin; Mitochondria-rich cell; H^+-pump V-ATPase; Cl^-/HCO_3^- exchange

1. Introduction

Extracellular ion balance in freshwater animals is energized by the Na^+/K^+-pump P-ATPase and the H^+-pump V-ATPase in osmoregulatory epithelia of skin, gill, operculum, gastro-intestinal tract, and kidney. These ion pump ATPases mediate the conversion of chemical energy stored in ATP to electrochemical energy stored in the concentration difference between the extracellular fluid and the external bath for small diffusible ions like Na^+ and Cl^-. At steady state, ion pumping balances the dissipative fluxes via the permeable renal and extrarenal epithelia. Fairly early it was established that epithelial Na^+ uptake is due to a sodium pump [1]. In the first model of a transporting epithelium suggested for frog skin [2], Na^+ enters the cell by passive transport through a Na^+-permeable apical plasma membrane and exits by active transport via the Na^+/K^+-pump through a K^+-permeable basolateral plasma membrane. In this model the uptake of Cl^- is passive and driven by the transepithelial electrical potential difference. It accounts for Cl^- uptake from solutions of [NaCl]>10 mmol/l, but fails when applied

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to Cl^- uptake from freshwater both in amphibians and teleosts [3–5]. In this environment, active uptake of small diffusible ions and respiratory gas exchange are interdependent and coupled to regulation of whole body acid/base balance [6–8] with a proton pump providing additional energy for transepithelial ion fluxes between freshwater and body fluids [9–12]. The osmoregulatory epithelia in teleosts and anuran skin are heterocellular with functions and ion pathways associated with highly specialized cell types [13–15]. Recent reviews cover function of individual cell types [16–19] and genes and isoforms of ion transporters [20]. The evolution, functions, and biochemistry of V-ATPases have been reviewed in Refs. [21,22].

This review is focused on functions of a proton pump ATPase in mitochondria-rich (MR) cells of anuran skin epithelium with a discussion of energy expenditure of uptake of ions in freshwater.

2. In vivo studies

2.1. Krogh: 'active chloride uptake'

Metabolically dependent epithelial ion transport was first demonstrated by Huf [23,24]. In bags of isolated frog skin exposed on either side to a Ringer's solution, he demonstrated transport of Cl^- towards the serosal side that could be abolished by cyanide. The concept of active transport was analysed by Krogh in a paper from 1937 [25] in which he raised two fundamental questions that turned out to become major themes in general and comparative physiology. The first one was whether transport of ions across biological membranes may take place against a prevailing concentration difference. Krogh studied uptake of ions by the semi-aquatic frog, *Rana esculenta*, as he expected that active ion transport might be disclosed in the surface epithelium of a freshwater animal that maintains extracellular ion concentrations above that of the environment. The other question raised concerned the biological function of active transport, in casu, the significance of active uptake for ion balance in freshwater animals. In his 'Croonean Lecture' [26] Krogh developed the concept further by including a comprehensive discussion of the significance of active and passive mechanisms for maintaining steady-state ion concentrations of body cells of eukaryotic organisms. Initially, the studies focused on Cl^-. By titration of relatively large samples of the bathing solution taken at intervals of several hours, Krogh discovered a powerful Cl^- uptake mechanism in frog skin as shown in Fig. 1. These results would be equally consistent with active uptake of Na^+, active uptake of Cl^-, or with both ions being submitted to active transport. For deciding between these possibilities, Krogh applied ion substitution protocols, and summarized his findings as follows: (i) Na^+ and Cl^- are absorbed together from sodium chloride solutions. (ii) From solutions of potassium chloride, Cl^- and K^+ can also be absorbed together, but absorption is limited to a small amount of the two ions. (iii) Little or no Ca^{2+} is absorbed from a $CaCl_2$ solution, but a limited amount of Cl^- is taken up in exchange of HCO_3^-. From these finding Krogh concluded, "that Cl^- is the ion always actively absorbed". With respect to the second major question raised, the biological significance of active transport, it was found that the Cl^- mechanism could be demonstrated only in non-fed animals forced into negative ion balance by being kept for several weeks in running distilled water. This treatment induced the active cutaneous uptake together with a very significant reduction of cutaneous and renal loss of Cl^- to the surroundings. Krogh suggested that normally frogs get enough ions from food and that the cutaneous active uptake of NaCl probably would be of major significance only during hibernation at the bottom of ponds where the animal does not feed [25].

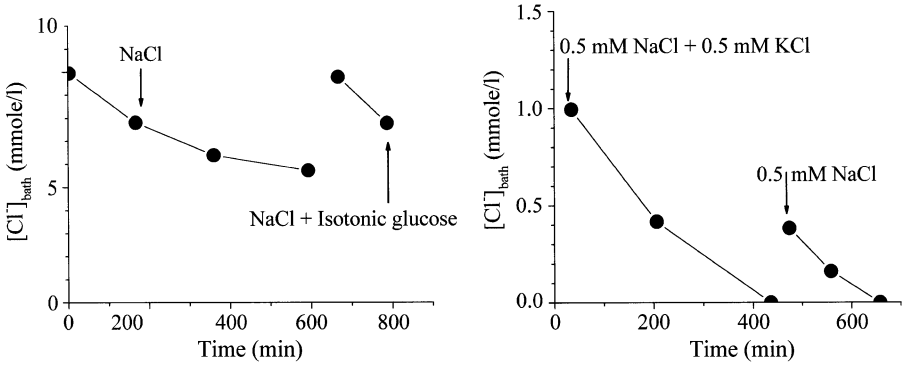

Fig. 1. In experiments with the frog, *R. esculenta*, August Krogh discovered a powerful active Cl^- uptake mechanism in anuran skin. Kept in a small celluloid box, the frog remains motionless for days with breathing movements stirring the bath. The cloaca was cannulated for separating voided urine from bath. The resolution of his Cl^- titration method corresponds to a bath-[Cl^-] of 10 μmol/l. Left: Uptake of Cl^- from a solution that initially was 1/10 Ringer's. After about 10 h, the bath was replaced by fresh 1/10 Ringer's made isotonic by sucrose. The continued uptake of Cl^- shows that it is not coupled to an osmotic uptake of water. The frog was kept in running distilled water for 11 days prior to the experiment. Data from Table 3 of Ref. [25]. Right: Uptake of Cl^- from a solution with [Cl^-] of 1/100 of that of Ringer's solution and Na^+ and K^+ as cations and glucose added to isotonicity. After about 7 h, the bath was replaced with a NaCl solution that was diluted × 500 as compared to Ringer's but with glucose added to isotonicity. The frog was kept in running distilled water for 3 weeks prior to the experiment. Data from Table 4 of Ref. [25].

The analysis was extended to other freshwater animals and ions. In salt-depleted teleosts, active uptake of Cl^- was observed in gills of bullhead (*Ameiurus nebulosus*), salmon (*Salmo irideus*), roach (*Leusiscus rutilus*), goldfish (*Carassius auratus*), horke (*Acerina cernua*), perch (*Perca fluviatilis*), and in two species of sticklebacks (*Gasterosteus aculeatus*, *G. pungitius*). As the only freshwater species studied by the above technique, eels (*Anguilla vulgaris*) did not show active uptake of Cl^- [27]. In a more detailed investigation of goldfish and mitten crab (*Eriocheir sinensis*), Krogh found independent and selective active transports of cations and anions to be developed in the gills and he also discovered excretion of NH_4^+ across the gill epithelium [28].

In Krogh's terminology, active transport of an ion was demonstrated if the ion is transported against a concentration gradient. In the above experiments with the frog, where active transport of Cl^- was demonstrated, only the bath-concentration of this ion was measured, so it "was provisionally assumed that cations were attracted electrostatically" (quoted from Ref. [28]). In this study [28] the hypothesis was tested in experiments in which also the Na^+ concentration was measured. It was now directly shown that Na^+ and Cl^- are taken up together from millimolar solutions and found that the rate of uptake of the two ions could vary considerably. A major conclusion of this paper was that the two ions, Na^+ and Cl^-, are submitted to active transport by independent mechanisms. Krogh did not consider the possible existence of an electrical potential difference across plasma membranes or epithelia, but he was aware of the large electrostatic forces involved in separating a charged ion from its environment. So he assumed that tight coupling of ions moving across the epithelium would be a necessity for electroneutral charge transfer in epithelial cells. In one of his summary statements, he therefore writes: "The transport is supposed to involve an exchange with an ion of the same sign". And further: "The CO_2 produced by metabolism and excreted through the skin or gills is probably sufficient to serve in exchange for Cl^- absorbed" [28]. In subsequent investigations, these considerations have been referred to as the 'Krogh model' of ion uptake from freshwater with an epithelial Cl^-/HCO_3^- exchange mechanism and an epithelial Na^+/H^+ exchange mechanism, respectively.

2.2. Flux-ratio analysis confirmed active transport of Cl^-

While the above investigations demonstrated ion uptake by frogs from very diluted solutions, in a subsequent study of the mechanism of cutaneous Cl^- transport, the trans-epithelial electrical potential difference was also taken into account. With radioisotopes as tracers, Jørgensen et al. [3] showed that the fluxes of Cl^- fulfill the strict criterion for active transport as defined by Ussing's flux-ratio equation for electrodiffusion across a composite membrane [29]. While in animals exposed to 3 mmol/l NaCl the potential difference varied between 50 and 120 mV (inside of the skin positive), uptake of Cl^- from a 3 mmol/l KCl solution could take place at a potential difference of reversed polarity, e.g., in the range of -20 to -50 mV. It was shown that the sodium and the chloride uptake mechanisms could be stimulated separately.

2.3. Quantitative studies of chloride/base- and sodium/proton exchange

The considerations of Krogh and Jørgensen et al. discussed above were applied and further developed by Garcia Romeu et al. [30] in a study of ion exchange across the skin of the South American frog *Calyptocephalella gayi*. With salt-depleted animals, they obtained results confirming uptake of Na^+ and Cl^- in a near 1:1 proportion from dilute concentrations of NaCl (<2 mmol/l). In experiments where frogs individually were selectively depleted from Cl^-, or Na^+, they confirmed that the skin has capacity for steady-state net uptake of Cl^- during a long period of time without a concomitant uptake of Na^+, and vice versa. A major result of this [30] and subsequent studies with other amphibian species [4,31,32] was the demonstration of a near 1:1 transcutaneous exchange of external Na^+ for H^+ and a near 1:1 transcutaneous exchange of external Cl^- for base, probably HCO_3^-. They were both depressed by the carbonic anhydrase inhibitor diamox [32]. It was hypothesized that at low external concentrations, the exchange of Cl^- with HCO_3^-, and Na^+ with H^+, respectively, is obligatory and suggested that in experiments in which both Na^+ and Cl^- are taken up at about similar rates, elimination of protons and bicarbonate ions would be masked by them forming H_2O and CO_2 in the external bath. In concordance with results of a previous investigation [33] no evidence was obtained for NH_4^+ excretion by frog skin [30,32].

2.4. Energy expenditure of NaCl uptake from freshwater

Extracellular concentrations of Cl^- and Na^+ of anurans are fairly low as compared to those of other vertebrates (Table 1). The $[HCO_3^-]/pCO_2$ system is the major buffer of extracellular pH and both on land and in freshwater at 20 °C is plasma-pH maintained at about 7.8. With pulmonary gas exchange, $[HCO_3^-]$ is about 22 mmol/l (*B. marinus*, Ref. [35]), which is compatible with the estimated difference of $[Na^+]_{lymph}$ and $[Cl^-]_{lymph}$ of *Bufo bufo* (20.0 ± 2.7 mmol/l,

Table 1
Extracellular concentrations of sodium and chloride of the European toad, *B. bufo*, in ion balance (unpublished)

$N=10$	Lymph $[Na^+]$ (mmol/l)	Lymph $[Cl^-]$ (mmol/l)	Difference (mmol/l)
Mean ± S.E.M.	105.5 ± 1.0	85.4 ± 2.6	20.0 ± 2.7

The toads were kept in a terrarium with free access to tap water and mealworms. Fluid samples (40–50 μl) were taken from the dorsal lymph sac as described in Ref. [34]. Chloride and sodium were measured by titration (CMT10 Radiometer, Copenhagen, Denmark) and photometry (FLM3 Radiometer), respectively.

Table 1). With no access to food, during the first week in running distilled water, toads experience a decrease of extracellular [Cl^-], on average for seven animals, from 87.0 ± 1.1 (control) to 74.4 ± 1.0 mmol/l (Fig. 2). During the following 2 weeks, the decrease was relatively smaller (day 21, $[Cl^-]_{lymph} = 70.1 \pm 1.9$ mmol/l). All of the seven animals lost body mass (Fig. 2). For the purpose of illustration, if this loss is assumed associated with the reduced extracellular water volume, with an initial extracellular space of 30% of the body mass [36] the extracellular Cl^- pool would have been reduced from 2611 ± 33 to 1414 ± 124 μmol/100 g body weight, corresponding to a loss of 46% of the initial steady-state pool. Even if this is an overestimation, it is indicated that the above mentioned in vivo studies have been carried out with animals that had experienced a fairly large perturbation of their ion balance. Toads recover fully from this treatment when transferred to a terrarium with free access to mealworms and a pool of tap water.

The expenditure of metabolic energy associated with active uptake of Na^+ and Cl^- must exceed the sum of the two ions' transcutaneous electrochemical potential difference, which is given by:

$$\Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{Cl} = RT\ln\frac{(Na)_i}{(Na)_o} + FV_t + RT\ln\frac{(C1)_i}{(C1)_o} - FV_t$$

$$= RT\ln\frac{f_i^{Na}[Na]_i}{f_o^{Na}[Na]_o} + RT\ln\frac{f_i^{Cl}[Cl]_i}{f_o^{Cl}[Cl]_o} \quad (1)$$

Here, parentheses indicate activity, brackets concentration, f activity coefficient, and V_t the transcutaneous electrical potential difference. Krogh observed that salt-depleted frogs could take up Cl^- from the bath with a NaCl concentration as low as about 0.01 mmol/l, which was the resolution of his method. With the ratio of activity coefficients of plasma and external bath being ~ 0.76, $[Cl^-]_i = 70$ mmol/l (Fig. 2), and $[Na^+]_i = 90$ mmol/l, from Eq. (1) at 20 °C the limiting thermodynamic work would have been, $\Delta\tilde{\mu}_{Na} + \Delta\tilde{\mu}_{Cl} = 42$ kJ/mol NaCl transported. In Ussing and coworker's studies of NaCl transport by frog skin it was shown that the net inward flux of chloride in the isolated preparation exposed to an external $[Cl^-]$>10 mmol/l is driven by the transepithelial electrical potential difference generated by the sodium pump [37]. For uptake of Na^+ energized by the pump with a stoichiometry $3Na^+$:$2K^+$:1ATP, the Cl^- uptake driven by V_t, and a ΔG_{ATP} of about − 60 kJ/mol, the useful work of the sodium pump is less than 20 kJ/mol NaCl transported (i.e., $< -\Delta G_{ATP}/3$). Thus, we have to assume that in Krogh's experiments another pump, in addition to the sodium pump, was in operation. The above-mentioned efflux of protons would indicate that a proton exit mechanism provided the required additional coupling to cellular energy metabolism. An apical Na^+/H^+ exchange mechanism can account for the kinetic relationship between sodium uptake and proton excretion. However, according to this hypothesis discussed in Ref. [5], it is still the sodium pump that is doing the entire work. From this point of view, the epithelial H^+-pump discovered in Steinmetz' studies of turtle urinary bladder [38,39], which was concluded to be present also in the skin of several anuran species [40–43], would be a more likely candidate for supplying metabolic energy to the cutaneous uptake of NaCl in freshwater. In what follows is a discussion of the function of the proton excretion mechanism in anuran skin, which has been hypothesized to energize apical Na^+ uptake [43] and apical active uptake of Cl^- [44].

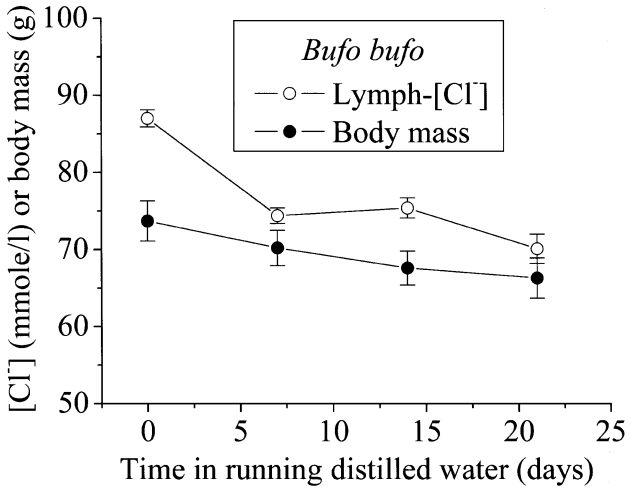

Fig. 2. Body mass and concentration of Cl^- in samples from the dorsal lymph sac of $N = 7$ toads (*B. bufo*) transferred to running distilled water at time zero. Prior to the experiments, the animals had free access to mealworms and tap water. Paired t-test with {null-hypothesis}: Body mass, {Day0 − Day7 ≤ 0} $P < 0.001$; {Day7 − Day14 ≤ 0} $P < 0.001$; {Day14 − Day21 ≤ 0} $P > 0.1$; {Day0 − Day21 ≤ 0} $P < 0.01$; chloride concentration, {Day0 − Day7 ≤ 0} $P < 0.001$; {Day7 − Day14 ≤ 0} $P > 0.5$; {Day14 − Day21 ≤ 0} $P > 0.5$; {Day0 − Day21 ≤ 0} $P < 0.001$. (Unpublished).

3. In vitro studies

3.1. Interdependence of proton secretion and sodium uptake

Already in the early studies, it was observed that isolated frog skin has capacity for acidifying the external solution [45–47]. Acidification was found to take place at transepithelial equilibrium, in the absence of external CO_2, being sensitive to carbonic anhydrase- and metabolic inhibitors as well as being depressed in the absence of O_2 [40,41]. It was concluded that proton secretion is active and depends on metabolically produced CO_2. It is noteworthy that species differences were observed in regards to dependence on external anions [42]. Thus, with preparations of *Rana ridibunda*, the efflux of H^+ was independent of whether Cl^- or SO_4^{2-} was present in the external bath. In contrast, the H^+ efflux in skins of *B. bufo* could not be detected if external SO_4^{2-} was replaced by Cl^-. Also another difference was noted between the two anuran

species. Whreas proton secretion by short-circuited frog skin was unaffected by Na^+ uptake perturbation (amiloride inhibition, antidiuretic hormone stimulation, external Na^+/Mg^+ substitutions), with a similar protocol perturbations of the active Na^+ influx in toad skin resulted in parallel changes of proton secretion [42]. Furthermore, it was noted that the stoichiometric relationship between the H^+ efflux and Na^+ influx that was described at low external $NaCl^-$ concentrations vanishes at high external [NaCl]. Thus, at an external [NaCl] = 2 mmol/l, diamox inhibited both of these fluxes [43], whereas the proton efflux, only, was inhibited by diamox if the skin was exposed to Ringer's solution on the outside [41,43]. From these findings and further studies on the kinetics of Na^+-uptake, Ehrenfeld and Garcia-Romeu suggested that two uptake mechanisms prevail. The first one would be dominating at concentrations above 2 mmol/l and is not coupled to an efflux of protons. The other one would be dominating at low external NaCl concentrations and is coupled to an obligatory exchange with metabolically produced H^+ [48]. More recently, Harvey and Ehrenfeld [49] suggested that the two pathways for Na^+ entrance are located in principal cells and MR cells, respectively. Like principal cells, also MR cells express amiloride blockable Na^+ channels in the apical membrane [50–52], although not all cells may exhibit this feature [52]. Thus, they hypothesized [11,49] that at low concentrations a rheogenic apical proton pump by hyperpolarizing the apical membrane forces the flux of Na^+ from the external bath into the MR cells. Such a mechanism would be operating independently of the external anion, and, in agreement with their experimental findings, a 1:1 Na^+/H^+ stoichiometry would be expected if Na^+ were the only ion that is transported across the epithelium. In a subsequent paper, Harvey [11] suggested that HCO_3^- exits across the basolateral membrane in exchange for serosal Cl^-, a model similar to the A-type (= α-type) intercalated cells of vertebrate distal renal epithelia [53–56]. This interpretation would be consistent with proton efflux being independent of the external anion (Cl^- or SO_4^{2-}) as observed in frog skin [41] as well as the observed exit of base towards the serosal bath [57]. By immunofluorescence microscopy, polarized expression of the targeted epitope of a V-ATPase was localised to the apical region of the MR cells in the skin epithelium [58].

3.2. Interdependence of proton secretion and chloride uptake

The active inward chloride flux can be studied in the isolated skin, and in some species is the flux of a magnitude that compares with the active sodium flux. The advantage of using isolated preparations is that the active component is well defined and can be measured accurately. Table 2 lists Cl^- fluxes obtained under transepithelial thermodynamic equilibrium conditions in which the net flux is a measure of the active component. The indicated agreement between the observed transepithelial electric current and the sum of ion currents calculated from the isotope tracer fluxes is a strong argument in favour of a rheogenic inwardly directed active transport of chloride. The above chloride current as well as the component of the chloride flux that exhibits saturation kinetics at low external concentrations are sensitive to carbonic anhydrase inhibitors [60,62–65]. As mentioned above, the proton pump is also depressed by application of inhibitors of carbonic anhydrase, whose activity in the skin seems to be confined to MR cells [66]. As a testable working hypothesis, it was suggested, therefore, that a primary function of the proton pump in toad skin epithelium would be to energize the transepithelial active uptake of Cl^- [13,44]. With the proton pump and the Cl^-/HCO_3^- exchanger in the apical membrane of MR cells, this hypothesis would also account for the active Cl^- influx being rheogenic.

3.2.1. The proton pump of toad skin epithelium

We have tested experimentally a number of other predictions that follow from the hypothesis. With a double-barreled proton-sensitive microelectrode, pH was recorded in the external unstirred layer as function of distance from the cornified cells [67]. This is shown in Fig. 3 depicting the recorded pH-profile above the outer surface of a preparation with the stirred bulk solution buffered to 7.4 by 0.1 mM Tris. It was verified that with gluconate as the major external anion, the epithelium makes the apical unstirred layer acidic, that this acidification was abolished after depression of cellular energy metabolism, and that it was strongly dependent on serosal pCO_2. These observations were taken to indicate that an apical proton pumping mechanism generated the measured pH-gradient. A theoretical analysis of $[H^+]$-

Table 2
The active flux of Cl^- in anuran skin is associated with an outward current that contributes to the short circuit current

Species	$I_{Cl}=-FJ^{net}_{Cl^-}$ ($\mu A/cm^2$)	$I_{Na^+}=FJ^{net}_{Na^+}$ ($\mu A/cm^2$)	$I_{Cl^-}+I_{Na^+}$ ($\mu A/cm^2$)	I_{sc} ($\mu A/cm^2$)	Reference
Leptodactyllus ocellatus	−19 ± 3	76 ± 12	57	53	[59]
B. bufo	−5 ± 1	30 ± 2	25	24 ± 2	[60]
B. arenarium[a]	−6.9 ± 1.7	0	–	−10.0 ± 1.3	[61]

Steady-state unidirectional fluxes of Cl^- and Na^+ were measured in short-circuited preparations. The net flux multiplied by the Faraday (F) provides the estimate of the associated electrical current (second column).

[a] The active Na^+ flux was blocked by amiloride in the solution bathing the apical side of the epithelium. With this protocol, the short circuit current reversed direction and became outward (negative).

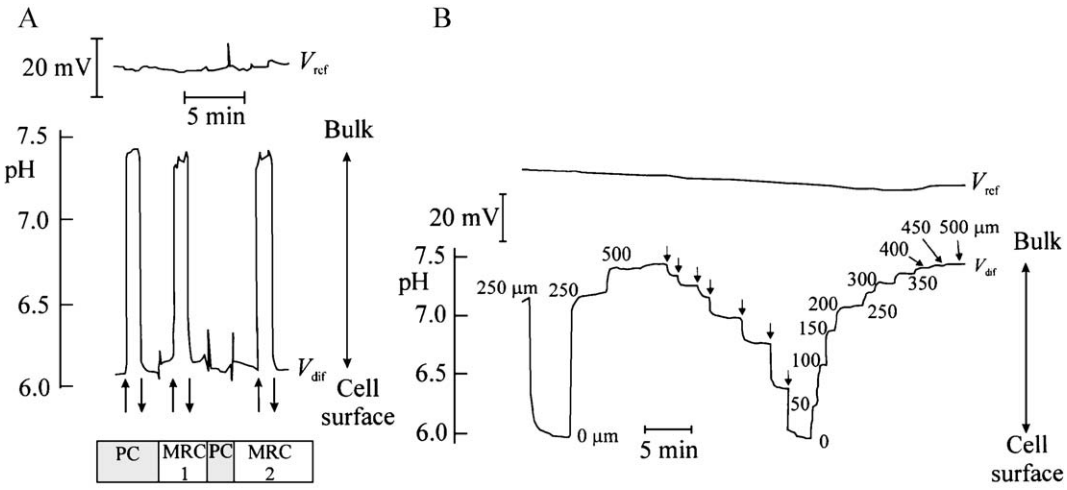

Fig. 3. Proton profile in unstirred layer above epithelium of toad skin. (A) The double barreled ion selective microelectrode was moved between bulk solution (pH = 7.4) and surface of the epithelium with a pH about 6.1 (vertical arrows). pH is independent of the position of the tip of the electrode whether it being above a principal cell ('PC') or an MR cell (MRC1 and MRC2). (B) Estimation of pH as a function of distance from the surface of the epithelium. The tip of the electrode was moved in steps of 50 µm from the cornified layer (0 µm) to bulk solution (500 µm) (Redrawn from Ref. [67]).

gradients above an epithelium with the individual MR cells treated as point sources showed that the generally relatively high density of the cells, together with the fact that the tip of the electrode cannot come closer to the apical membrane than about 5–10 µm (the thickness of cornified cell layer), prevents resolutions of the individual point source [67]. Thus, the spatial resolution turned out to be insufficient for an unequivocal localization of the pumps to the population of MR cells. With an antibody raised against the catalytic 70-kDa A-subunit of bovine V-type H^+-ATPase, positive reaction was seen exclusively in MR cells (Fig. 4). This, together with the above measured apical pH-profile, indicates that a functional H^+-pump V-ATPase is expressed in the apical membrane of MR cells of the toad skin epithelium.

Since the diffusion of protons in the unstirred layer can be treated as diffusion from a plate source, the active flux of protons, J_H, can be estimated from one-dimensional diffusion equations relating the proton concentration ($[H^+]$) to distance (y) in the stagnant layer above the preparation. From Fick's law and taking into account diffusion of free protons and buffer molecule-associated protons (BH), the differential- and buffer equation read:

$$J_H = -D_H \cdot \frac{d[H^+]}{dy} - D_{BH} \cdot \frac{d[BH]}{dy} \quad (2)$$

$$[BH] = \frac{[H^+] \cdot [B]_{TOT}}{[H^+] + K_a} \quad (3)$$

where D_H and D_{BH} are the respective diffusion coefficients, $[B]_{TOT}$ the total buffer concentration, and K_a the dissociation constant of the buffer. Eq. (2) is valid in the unstirred

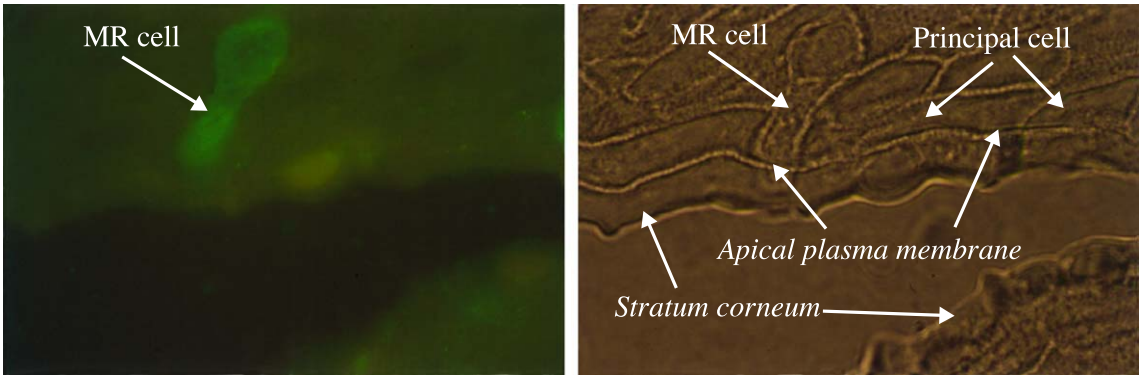

Fig. 4. Left-hand panel: Immunolocalization of H^+-pump V-ATPase in MR cells of toad skin epithelium using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to a sequence from the catalytic 70-kDa A-subunit of the bovine V-type H^+-ATPase complex (SHITGGDIYGIVNEN). Toad skin was embedded in paraffin and sectioned into 5-µm slices. After rehydration, nonspecific binding was blocked with 10% normal goat serum for 15 min. The sections were then incubated O/N at 4 °C with 1° antibody (1:500 in PBS + 0.25% BSA + 0.1% Triton X-100), washed in PBS, and Alexa 488 conjugated goat-anti-rabbit antibody was added for 40 min. The slices were then washed in PBS and mounted. Right-hand panel: DIC image of the same preparation showing details and position of the different cell types of the heterocellular epithelium. The antibody was a gift from Jonathan M. Wilson. (Unpublished).

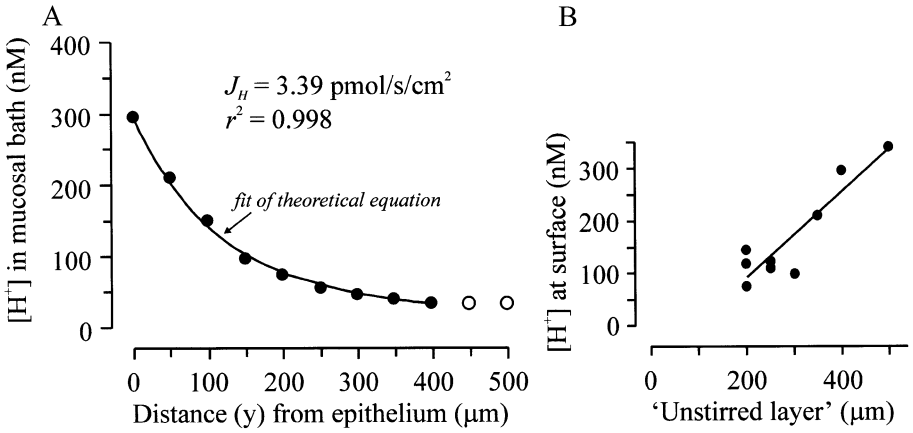

Fig. 5. (A) The measured proton concentration profile in the unstirred layer can be described by equations derived from diffusion theory applied to a plate source taking into account diffusion of both free H^+ and buffer-associated H^+. The active flux of protons into the layer was estimated by a curve fitting procedure as described. Open symbols were not included in the fitting procedure (see text and Ref. [67] for details). (B) In preparations generating proton fluxes of similar magnitude, there is an inverse relationship between thickness of unstirred layer and proton concentration at the surface of the epithelium. As shown here, this fundamental feature of the diffusion model was verified in experiments with toad skin epithelium. The proton concentration in the bulk solution was clamped at 40 nM by a 0.1 mM Tris buffer (redrawn from Ref. [67]).

layer where convection can be disregarded. With the thickness of unstirred layer, Δ, and the proton concentration at $y=0$ and $y=\Delta$ denoted $[H^+]_0$ and $[H^+]_\Delta$, respectively, the solution to the above set of equations reads:

$$J_H = D_H \frac{[H^+]_0 - [H^+]_\Delta}{\Delta} + \frac{D_{BH} \cdot [B]_{TOT} \cdot K_a}{([H^+]_0 + K_a) \cdot ([H^+]_\Delta + K_a)} \cdot \frac{[H^+]_0 - [H^+]_\Delta}{\Delta} \quad (4)$$

It is a prerequisite for calculating the proton flux that the thickness of the unstirred layer is known. As it turned out that Δ varied a great deal among preparations and could not be estimated by any direct method, we developed a curve fitting procedure to the type of data shown in Fig. 3. With an expression developed from Eqs. (2) and (3), J_H and Δ were varied until the best fit was obtained[3]. An example of this type of analysis is shown in Fig. 5A. Fast stirring of the outer solution will reduce the unstirred layer and the concentration of protons just outside the epithelium. In the limit with no unstirred layer, $[H^+]_0$ would be equal to that of the bulk solution. The data verified this expected reverse relationship between unstirred layer thickness and the proton concentration at $y=0$ for preparations generating proton fluxes of similar magnitude (Fig. 5B). Likewise, as predicted, the free proton concentration at the surface of the epithelium was significantly depressed if the buffer concentration was increased [67]. The above analysis showed that the measured pH-profile is correctly accounted for by our diffusion theory. Thus, the study could be taken a step further. With the isolated epithelium mounted in an Ussing-chamber, the active proton flux was calculated from the mucosal pH-gradient and compared to the associated short-circuit current in the absence of Na^+ transport. The result of the analysis shown in Fig. 6 provides the evidence that the proton pump is rheogenic with a ratio of unity between charge flow and proton flux [69].

3.2.2. The apical uptake mechanism for chloride

As illustrated in Fig. 7A, mucosal acidification also depends on the external anion. The reversible reduction of

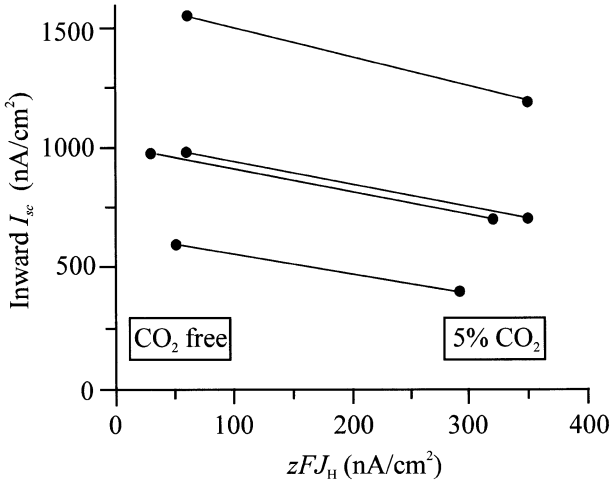

Fig. 6. Response of the inward short-circuit current (I_{sc}) and the transepithelial outward proton current, $I_H = z_H F J_H$, to pulsing exogenous CO_2/HCO_3^- to 5%/20 mM. Amiloride (100 μmol/l) added to the external bath. The proton flux was calculated from the mucosal pH gradient as discussed in the text. The ratio, $-\Delta I_{sc}/\Delta I_H = 0.96 \pm 0.07$ ($N=4$), is not significantly different from unity ($P=0.67$, one population t-test). (Redrawn from Ref. [69]).

[3] We applied a pragmatic definition of unstirred layer thickness. The two free parameters, J_H and Δ, were varied until the best fit of the theoretical equation to paired $[H^+]$–y data was obtained. The range $y=\Delta$ was chosen as the point from which there was a systematic deviation between theory and observation (for detailed discussion see Ref. [67]).

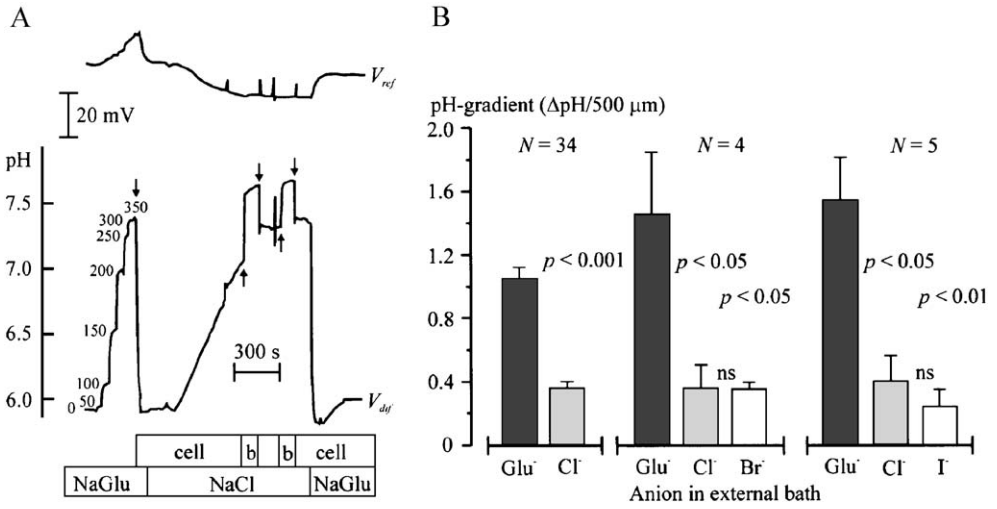

Fig. 7. The apical proton pump in toad skin epithelium is expressed in parallel with a Cl^-/HCO_3^- exchange mechanism. (A) With gluconate-Ringers as mucosal bath, the tip of a double-barreled proton-sensitive microelectrode was moved in 50-μm steps from the surface of the skin (pH about 6) to the buffered bulk solution (pH = 7.4). Mucosal acidification was reduced significantly, but not fully, during the period external gluconate was replaced mole for mole by Cl^-. "cell": tip of the electrode just above the cell (position zero). "b": tip of electrode withdrawn to bulk solution (position>350 μm). (B) The three halides tested, Cl^-, Br^-, and I^-, reduced mucosal acidification, indicating that they all exchange with cellular HCO_3^-. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; ns: difference not significant. Data from Ref. [67].

the pH-gradient in response to a transient exposure of the apical side of the epithelium to a Cl^- containing Ringer's is in agreement with the predicted exchange of external Cl^- with HCO_3^- across the apical membrane, and the observation that Br^- and I^- produce similar effects (Fig. 7B) agrees with previous observations that both of these halide ions are submitted to active transport [25,68]. A more recent study [69] of unidirectional $^{36}Cl^-$ fluxes in paired preparations exposed to low concentration of NaCl (< 3 mmol/l) verified active and exchange diffusion of Cl^-, sensitivity of the influx to DIDS and ethoxzolamide, and interaction between Cl^- and its tracer ($^{36}Cl^-$). These observations are in agreement with carrier-mediated apical uptake at low external [Cl^-] via an apical Cl^-/HCO_3^- exchange mechanism. The voltage independence of the apical anion exchanger was tested by comparing the unidirectional $^{36}Cl^-$ influx under open circuit conditions prior to and after adding amiloride to the apical solution. With this protocol, we obtained large voltage perturbations, in some preparations of more than 100 mV, which did not affect significantly the influx (see Fig. 8). In the skin of *R. esculenta* exposed to freshwater on the outside, the V-ATPase inhibitor concanamycin A depressed the efflux of H^+ and the influx of Cl^- in similar proportions [69], providing the evidence that also in frog skin is the active uptake of Cl^- from low concentrations of its salt energized by the proton pump.

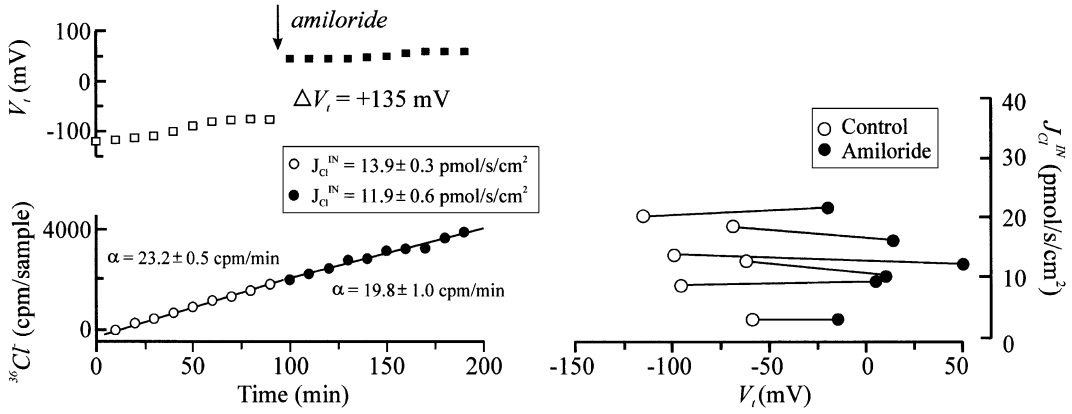

Fig. 8. Unidirectional influx of Cl^- in toad skin epithelium (measured with $^{36}Cl^-$) from a 2.2 mM NaCl solution is independent of transepithelial potential difference (V_t). Left: After an initial control period with V_t varying from −120 to −95 mV (mucosal side negative with respect to the grounded serosal solution), amiloride was added to the mucosal solution. This resulted in a change of V_t of about +135 mV. With a fractional resistance of the low conductance apical membrane near unity, ΔV_t is dissipated across the apical membrane. It can be concluded that uptake of Cl^- is mediated by a voltage independent mechanism in the apical membrane. Right: With the above protocol, in none of the six preparations tested did the Cl^- influx respond to the change of V_t as expected for channel-mediated transport.

However clear-cut the results of the testing of the model may seem, we also obtained results that are not in full agreement with the above interpretations. In 11 out of a total of 45 preparations tested with the protocol of Fig. 7A, mucosal acidification did not respond as shown in this figure [67]. This finding would indicate heterogeneity in the polarization of the acid–base secretory mechanisms among toads (see Section 4.1). In a series of experiments with frog skin and toad skin producing fairly large proton fluxes, concanamycin A was without effect [69]. This would indicate that a type of proton-pump ATPase, other than the V-ATPase, might also be expressed in anuran skin epithelium. Further studies are needed to characterize putative concanamycin A insensitive H^+-fluxes.

4. Hypotheses and models

4.1. MR cells of anuran skin

Fig. 9 summarizes models of anuran MR cells. The functional organization of acid/base transporters of the α- and β-types is similar to the intercalated cells of turtle urinary bladder and cortical collecting duct, which serve elimination of acid and base loads, respectively (reviewed in Refs. [70,71]). There is good evidence that cell types with these functions are represented among the population of MR cells of anuran skin [72,73]. Sodium uptake via α-cells was discussed by Harvey [11] who suggested that the α-cell is the pathway for an obligatory coupling of active Na^+ uptake and H^+-excretion under conditions of no other transcutaneous ion movements. The γ-MR cell of Fig. 9C is depicted with pumps and ion channels as discussed above and elsewhere [13,19]. With a major function being uptake of Cl^-, the cell belongs to the class of epithelial cells that is also referred to as 'chloride cells' [14,16], although 'chloride cells' of teleosts and amphibians are not configured in a similar way. As compared to the principal cells of the epithelium, the MR cell is distinguished also by its expression profile of cytokeratins [74], by dense tubules in the neck region, and by actin filaments being confined to the sub-apical domain that contrasts a general sub-membrane distribution of actin in principal cells [75].

The one of the two apical Cl^- channels of Fig. 9C is a CFTR-like small-conductance cAMP and forskolin-activated Cl^- channel, which is regulated via a β-adrenergic receptor on the basolateral membrane [76,77]. CFTR of toad skin has been cloned, and immunostaining with antibodies raised against human CFTR verified selective expression of the epitopes in MR cells [78,93]. The other apical Cl^- channel is carrying the Cl^- current that is activated by transepithelial hyperpolarization [79,80] and, therefore, coupled to the active inward flux of Na^+ through principal and MR cells [81]. A 200–300-pS depolarization-activated apical channel identified by whole-cell current fluctuation analysis [82] and single-channel recordings [77] is the likely candidate channel. Accordingly, the same cell is pathway for Cl^- uptake from low and high external NaCl concentrations, respectively, via two quite different mechanisms. This is accomplished by down-regulation at low external [Cl^-] of the Cl^- conductance [2,4] via an apical receptor with affinity for Cl^- [83]. When the receptor is occupied by Cl^- (or Br^-, but not I^-), the Cl^- conductance can be activated by membrane depolarization [68]. In freshwater with [Cl^-] < 2 mmol/l, few channels are activated and the major influx of Cl^- goes via the apical anion exchanger [69]. The experimental evidence for this intriguing regulation of the MR-cell Cl^- conductance was reviewed recently [19].

4.2. Is proton pumping energizing apical sodium uptake?

As discussed above, it has been hypothesized that the major function of the proton pump is to hyperpolarize the

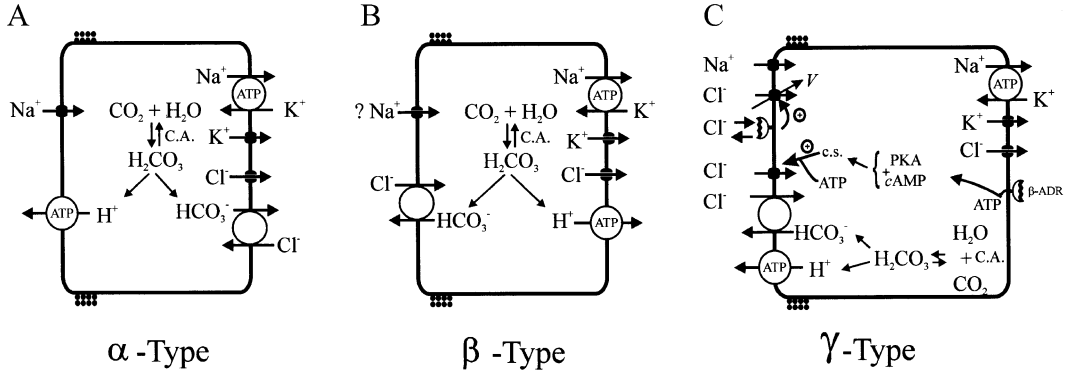

Fig. 9. Three types of MR cells in anuran skin. (A) The α-type MR cell corresponds to the A-type intercalated cell of vertebrate distal renal epithelia, conf. e.g. Refs. [11,39]. It is likely that this type is dominating in skin preparations that generate net proton fluxes, or acidify the mucosal solution, even with a mucosal Cl^- concentration of 100 mmol/l. There is evidence that it is being stimulated in acidosis. (B) The β-type MR cell is similar to mammalian B-type intercalated cell and seems to be stimulated in alkalosis. This cell generates a non-rheogenic active uptake of chloride and accounts for uptake of Cl^- in exchange with cellular HCO_3^- under conditions of no other transepithelial ion flows. (C) The γ-type MR cell mediates active transport of Cl^- in a rheogenic fashion. This cell is pathway also for the passive uptake of Cl^- through apical Cl^- channels, which are closed in freshwater (modified from Refs. [13,44]; see text for discussion).

apical membrane for generating a sufficient driving force for uptake of Na^+ from freshwater. It is unlikely that proton pumping is driving the cell potential to a value that is numerically larger than the K^+-equilibrium potential across the basolateral membrane. Otherwise, potassium ions flow from the interstitial fluid into the cell both via basolateral K^+ channels and Na^+/K^+-pumps. In a skin with dominating sodium current, the inside of the epithelium would be relatively positive ([3], Fig. 4). Thus, the apical membrane potential is at most −105 mV (E_K of the inner membrane [12], with $V_t = 0$ mV), and with an intracellular $[Na^+]$ of, e.g., 3–10 mmol/l, the passive flux of Na^+ would reverse at an external concentration of 35–118 μmol/l (20 °C, $f_{cell}^{Na}/f_o^{Na} = 0.76$). This indicates that even at an external Na^+ concentration that is smaller than in most lakes and creeks, a relatively large K^+ conductance of the basolateral membrane is sufficient for establishing the driving force for the net flux of Na^+ from the environment into the cell.

The above example also illustrates that uptake of Na^+ and Cl^- from 10 μmol/l of the salt (Fig. 1) can be accomplished only if proton pumping reverses the transepithelial potential difference as has been observed in vivo [3] and in the isolated skin [69]. Due to the small concentrations of Na^+ in cell and freshwater, even with large apical sodium permeability, the conductance of the apical membrane would be very small as compared to that of the basolateral membrane. Thus, within the range observed, $-20\ \text{mV} \le V_t \le -50\ \text{mV}$ (serosal side of the skin negative), and a fractional resistance of the apical membrane close to unity, the apical membrane potential would be between, e.g., −120 and −145 mV, which probably is a sufficient electrical driving force for generating a net influx of Na^+ through the apical channels at 10 μmol/l external $[Na^+]$. In connection with this hypothesis, it is interesting that in zebrafish (*Danio rerio*) bafilomycin (1 μmol/l) depressed Na^+ uptake at an environmental $[Na^+]$ of 35 μmol/l, but was without effect on the Na^+ uptake from a $[Na^+]$ of 1.5 mmol/l [92].

4.3. Proton pumping and chloride/bicarbonate exchange

Uptake of both ions from 10 μmol/l NaCl solution was calculated to cost 42 kJ/mol of NaCl transported (Section 2.4). With the energy input of the sodium pump of 20 kJ/mol, that of the proton pump would have to be larger than 22 kJ/mol. With a stoichiometry of the V-ATPase of 2 H^+ pumped per ATP hydrolyzed [84] and $\Delta G_{ATP} = -60$ kJ/mol (as above), the useful work of the proton pump is 30 kJ/mol of Cl^- transported. In this estimate, we assume that all of the HCO_3^- formed leaves the MR cell through the apical Cl^-/HCO_3^- exchange mechanism. Thus, from the point of view of energy conservation, our γ-MR cell model would accomplish the uptake of NaCl from a 10 μmol/l NaCl solution.

The coupling of Cl^- influx and HCO_3^- efflux in the apical membrane requires that cell-to-bath concentration gradient of HCO_3^- be larger than that of Cl^-. A quantitative evaluation is a fairly complicated matter and cannot, as yet, lead to a definitive conclusion, but is useful for elucidating the nature of the problem. The cellular $[HCO_3^-]$ depends on pCO_2 in the epithelial cell, which would have to be lower than that of arterial pCO_2 and larger than that of bath-pCO_2. With an intracellular pH of ~7.2 [85,86] and pulmonary gas exchange, arterial pCO_2 is ~10 mm Hg [35]. Then, from the Henderson–Hasselbalch equation $[HCO_3^-]_{cell} \le 4.5$ mmol/l.[4] With cutaneous gas exchange arterial pCO_2 is smaller, e.g., $pCO_2 = 3–5$ mm Hg (teleost fish [87,88]). Accordingly, at a $pH_{cell} = 7.2$, as an upper-limit estimate we obtain, $[HCO_3^-]_{cell} \le 2.3$ mmol/l. With these estimates and $[Cl^-]_{out} = 10$ μmol/l, uptake of Cl^- would be possible if $[Cl^-]_{cell}$ is less than 4.5 and 2.3 mmol/l for the two types of breathing, respectively, and providing the acid secretion maintains $[HCO_3^-]_{out} < 10$ μmol/l in the external unstirred layer. With Ringer's solution on the outside $[Cl^-]_{cell}$ is 30–45 mM [50,52,77]. Since this reflects a passive distribution [50,77], with freshwater outside, the concentration of Cl^- in the MR cell would probably be much smaller. The exit across the basolateral membrane through anion selective channels [89] requires that intracellular Cl^- be above equilibrium across this membrane. With a membrane potential close to −105 mV (E_K, conf. above) and $[Cl^-]_{ext} \approx 70$ mmol/l (Fig. 2), this would be fulfilled for $[Cl^-]_{cell} > 1$ mmol/l. This is interesting because it shows that with extreme demands on the uptake mechanism, influx of Cl^- via the apical exchange mechanism and passive exit of Cl^- across the basolateral membrane would be possible within a common range of intracellular Cl^- concentrations. The conclusion is that as a prerequisite for the γ-cell model (Fig. 9C) to be compatible with Krogh's observations (Fig. 1), the apical proton pump would have to have the additional function of maintaining the bicarbonate concentration low in the fluid layer outside the skin.

There is another problem that also would have to be considered. Frogs that were selectively depleted of Cl^- took up Cl^- in exchange for HCO_3^- apparently with no other transcutaneous ion movements [30]. This would indicate non-rheogenic active uptake of Cl^-, which is incompatible with the γ-type MR-cell of Fig. 9C, where a positive charge moves in the outward direction across the apical and basolateral membrane for each chloride ion entering the animal from the bath. Cells configured according to the β-type MR-cell of Fig. 9B accomplishes this type of anion uptake, which results in acidification of body fluids. Tadpoles that experience acidosis in response to external hypercapnia compensate by mobilizing a labile pool of $CaCO_3$ [86]. It is an interesting problem whether the predicted acidification of body fluids in frogs with Cl^- uptake via β-MR cells in a similar way is being compensated by mobilizing buffer base from the paraventral and subcutaneous lime sacs that are developed in the metamorphosed anuran.

[4] In the calculation, a solubility coefficient of $CO_2 = 0.06$ mmol/mm Hg and a $pK = 6.3$ were used together with a $pCO_2 = 9.8$ mm Hg (20 °C).

5. Concluding remarks

Considering the thermodynamic work with conservative assumptions, the γ-type model of the MR cells (Fig. 9C) seems to account for the findings Krogh published in 1937 [25]. In the most extreme situation of low external [NaCl], hyperpolarization of the apical membrane by proton pumping is required for inward transport of Na^+ across the apical membrane. There is no evidence of a similar demand on the proton pump under common freshwater conditions. A large number of recent experimental observations are also fully compatible with this model, which accounts for passive as well as active uptake of Cl^- across anuran skin.

Krogh observed that the 'leak' permeability of the skin is down-regulated while the active mechanism is up-regulated in animals forced into negative Cl^- balance [25]. The passive mechanisms are submitted to fast and slow regulations that involve cellular signalling and proliferation and growth in number of MR cells [90,91]. Undoubtedly, difficulties in reproducing observations on anuran skin function in freshwater are due to a gap in our knowledge about the regulation of the active Cl^- mechanism, the capacity of which varies between species and with acclimation of the animals in study. This point was emphasized recently also in a study comparing individuals of zebrafish (*D. rerio*) acclimated to soft water and hard water, respectively. It was found that the first group up-regulated the maximum flux of Cl^- as well the uptake affinity for external Cl^- [92]. Thus, the plasticity of osmoregulatory mechanisms in freshwater may also involve regulation of affinity of transporters via cellular co-factors or shift between different membrane transporters for the same ion. Added to this, there is good evidence that anuran acid- and base-secreting mechanisms serve three functions, i.e., restoration of an acid load, restoration of a base load, and whole body Cl^- balance. It is unknown how these specialized functions are regulated. In Fig. 9, tentatively three different cell types are depicted for each of these functions. This is in analogy with mammalian kidney in which the α-cell and the β-cell have been discovered and studied in details. Studies on anurans acid/base balance are too few to decide whether similar heterocellular differentiations characterize the anuran skin epithelium or whether a single cell type shifts functional polarity according to acid/base balance.

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References

[1] H.H. Ussing, K. Zerahn, Active transport of sodium as the source of electric current in the short-circuited isolated frog skin, Acta Physiol. Scand. 23 (1951) 110–127.
[2] V. Koefoed-Johnsen, H.H. Ussing, The nature of the frog skin potential, Acta Physiol. Scand. 42 (1958) 298–308.
[3] C.B. Jørgensen, H. Levi, K. Zerahn, On active uptake of sodium and chloride ions in anurans, Acta Physiol. Scand. 30 (1954) 178–190.
[4] L.B. Kirschner, The study of NaCl in aquatic animals, Am. Zool. 10 (1970) 365–376.
[5] R. Motais, F. Garcia-Romeu, Transport mechanisms in the teleostean gill and amphibian skin, Annu. Rev. Physiol. 34 (1972) 141–176.
[6] D.G. McDonald, Y. Tang, R.G. Boutilier, Acid and ion transfer across the gills of fish: mechanisms and regulation, Can. J. Zool. 67 (1989) 3046–3054.
[7] G.G. Goss, S.F. Perry, J.N. Fry, P. Laurent, Gill morphology and acid base regulation in freshwater fish, Comp. Biochem. Physiol. 119A (1998) 107–115.
[8] M. Busk, F.B. Jensen, T. Wang, Effects of feeding on metabolism, gas transport, and acid–base status in the bullfrog *Rana catesbeiana*, Am. J. Physiol. 278 (2000) R185–R195.
[9] H. Lin, D.J. Randal, Evidence for the presence of an electrogenic proton pump on the trout gill epithelium, J. Exp. Biol. 161 (1991) 119–134.
[10] H. Lin, D.C. Pfeiffer, A.W. Vogl, J. Pan, D.J. Randall, Immunolocalization of H^+ ATPase in the gill epithelia of rainbow trout, J. Exp. Biol. 195 (1994) 16–183.
[11] B.J. Harvey, Energization of sodium absorption by the H^+-ATPase pump in mitochondria-rich cells of frog skin, J. Exp. Biol. 172 (1992) 289–309.
[12] E.H. Larsen, N.J. Willumsen, B.C. Christoffersen, Role of proton pump of mitochondria-rich cells for active transport of chloride ions in toad skin epithelium, J. Physiol. 450 (1992) 203–216.
[13] E.H. Larsen, Chloride transport by high-resistance heterocellular epithelia, Physiol. Rev. 71 (1991) 235–283.
[14] S.F. Perry, The chloride cell: structure and function in the gills of freshwater fishes, Annu. Rev. Physiol. 59 (1997) 325–347.
[15] J.M. Wilson, P. Laurent, B.L. Tufts, D.J. Benos, M. Donowitz, A.W. Vogl, D.J. Randal, NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization, J. Exp. Biol. 203 (2000) 2279–2296.
[16] W.S. Marshall, Na^+, Cl^-, Ca^{2+} and Zn^+ transport by fish gills: retrospective review and prospective synthesis, J. Exp. Zool. 293 (2002) 264–283.
[17] W.S. Marshall, T.D. Singer, Cystic fibrosis transmembrane conductance regulator in teleost fish, Biochim. Biophys. Acta 1566 (2002) 16–27.
[18] C.M. Wood, S.P. Kelly, B. Zhou, M. Fletcher, M. O'Donnell, B. Eletti, P. Pärt, Cultured gill epithelia as models for freshwater fish gill, Biochim. Biophys. Acta 1566 (2002) 72–83.
[19] N.J. Willumsen, J. Amstrup, N. Møbjerg, Å. Jespersen, P. Kristensen, E.H. Larsen, Mitochondria-rich cells as experimental model in studies of epithelial chloride channels, Biochim. Biophys. Acta 1566 (2002) 28–43.
[20] C.P. Cutler, G. Cramb, Molecular physiology of osmoregulation in eels and teleosts: the role of transport isoforms and gene duplication, Comp. Biochem. Physiol. 130A (2001) 551–564.
[21] S.L. Gluck, D.M. Underhill, M. Iyori, L.S. Holliday, T.Y. Kostrominova, B.S. Lee, Physiology and biochemistry of the kidney vacuolar H^+-ATPase, Annu. Rev. Physiol. 58 (1996) 427–445.

[22] N. Nelson, W.R. Harvey, Vacuolar and plasma membrane proton-adenosinetriphosphatases, Physiol. Rev. 79 (1999) 361–385.
[23] E.G. Huf, Versuche über den Zusammenhang zwischen Stoffwechel, Potentialbildung und Funktion des Froschhaut, Pfluegers Arch. 235 (1935) 655–673.
[24] E.G. Huf, Über activen Wasser-und Saltztransport durch die Froschhaut, Pflügers Arch. 237 (1936) 143–166.
[25] A. Krogh, Osmotic regulation in the frog (*R. esculenta*) by active absorption of chloride ions, Skand. Arch. Physiol. 76 (1937) 60–74.
[26] A. Krogh, The active and passive exchange of inorganic ions through the surface of living cells and through living membranes generally, Proc. Royal Soc., B 133 (1946) 140–200.
[27] A. Krogh, Osmotic regulation in fresh water fishes by active absorption of chloride ions, Z. vgl. Physiol. 24 (1937) 656–666.
[28] A. Krogh, The active absorption of ions in some freshwater animals, Z. vgl. Physiol. 25 (1938) 335–350.
[29] H.H. Ussing, The distinction by means of tracers between active transport and diffusion, Acta Physiol. Scand. 19 (1949) 43–56.
[30] F. Garcia Romeu, A. Salibián, S. Pezzani-Hernández, The nature of in vivo sodium and chloride uptake mechanism through the epithelium of the Chilean frog *Calyptocephalella gayi* (Dum. et Bibr., 1841). Exchanges of hydrogen against sodium and of bicarbonate against chloride, J. Gen. Physiol. 53 (1969) 816–835.
[31] L.B. Kirschner, L. Greenwald, T.H. Kerstetter, Effect of amiloride on sodium transport across body surfaces of freshwater animals, Am. J. Physiol. 224 (1973) 832–837.
[32] F. Garcia-Romeu, J. Ehrenfeld, In vivo Na^+ and Cl^- independent transport across the skin of *Rana esculenta*, Am. J. Physiol. 228 (1975) 839–844.
[33] F. Garcia-Romeu, A. Salibián, Sodium uptake and ammonia excretion through the in vivo skin of the South American frog *Leptodactylus ocellatus* (L. 1758), Life Sci. 7 (1968) 465.
[34] S.D. Hillyard, E.H. Larsen, Lymph osmolality and rehydration from NaCl solutions by toads, *Bufo marinus*, J. Comp. Physiol., B. 171 (2001) 283–292.
[35] R.G. Boutilier, D.J. Randal, G. Shelton, D.P. Toews, Acid–base relationships in the blood of the toad, *Bufo marinus*: I. The effects of environmental CO_2, J. Exp. Biol. 82 (1979) 331–334.
[36] C.B. Jørgensen, K. Brems, P. Geckler, Volume and osmotic regulation in the toad *Bufo bufo bufo* (L.) at low temperature, with special reference to amphibian hibernation, in: C.B. Jørgensen, E. Skadhauge (Eds.), Proc. Alfred Benzon Symposium XI, Osmotic and Volume Regulation, Munksgaard, Copenhagen, 1978, pp. 62–79.
[37] V. Koefoed-Johnsen, H. Levi, H.H. Ussing, The mode of passage of chloride ions through the isolated frog skin, Acta Physiol. Scand. 25 (1952) 150–163.
[38] P.R. Steinmetz, Characteristics of hydrogen ion transport in urinary bladder of water turtle, J. Clin. Invest. 46 (1967) 1531–1540.
[39] P.R. Steinmetz, Cellular mechanism of urinary acidification, Physiol. Rev. 54 (1974) 890–956.
[40] M.G. Emilio, M.M. Machado, H.P. Menano, The production of a hydrogen ion gradient across the isolated frog skin. Quantitative aspects and the effect of acetazolamide, Biochim. Biophys. Acta 203 (1970) 394–409.
[41] T. Machen, D. Erlij, Some features of hydrogen (ion) secretion by the frog skin, Biochim. Biophys. Acta 406 (1975) 120–130.
[42] M.G. Emilio, H.P. Menano, The excretion of hydrogen ion by the isolated amphibian skin: effects of antidiuretic hormone and amiloride, Biochim. Biophys. Acta 382 (1975) 344–352.
[43] J. Ehrenfeld, F. Garcia-Romeu, Active hydrogen excretion and sodium absorption through isolated frog skin, Am. J. Physiol. 233 (1977) F46–F54.
[44] E.H. Larsen, NaCl transport by amphibian skin, in: R. Greger (Ed.), NaCl Transport in Epithelia, Adv. Comp. Environ. Physiology, vol. 1, Springer-Verlag, Berlin, 1988, pp. 189–248.
[45] H.H. Ussing, The active transport through the isolated frog skin in the light of tracer studies, Acta Physiol. Scand., (1949) 1–37.
[46] E.G. Huf, J. Parish, C. Weatherford, Active salt and water uptake by isolated frog skin, Am. J. Physiol. 164 (1951) 137–142.
[47] W.R. Fleming, On the role of hydrogen ion and potassium ion in the active transport of sodium across the isolated frog skin, J. Cell. Comp. Physiol. 49 (1957) 129–152.
[48] J. Ehrenfeld, F. Garcia-Romeu, Kinetics of ionic transport across frog skin: two concentration dependent processes, J. Membr. Biol. 56 (1980) 139–147.
[49] B.J. Harvey, J. Ehrenfeld, Epithelial pH and ion transport regulation by proton pumps and exchangers, Proton Passage Across Cell Membranes, Ciba Foundation Symposium 139, Wileym, Chichester, 1988, pp. 139–164.
[50] E.H. Larsen, H.H. Ussing, K.R. Spring, Ion transport by mitochondria-rich cells in toad skin, J. Membr. Biol. 99 (1987) 25–40.
[51] B.J. Harvey, E.H. Larsen, Sodium and chloride currents in single mitochondria-rich cells of amphibian skin, J. Physiol. 459 (1993) 241 (abstract).
[52] R. Rick, Intracellular ion concentrations in the isolated frog skin epithelium: evidence for different types of mitochondria-rich cells, J. Membr. Biol. 127 (1992) 227–236.
[53] D.L. Stetson, P.R. Steinmetz, α- and β-types of carbonic anhydrase-rich cells in turtle bladder, Am. J. Physiol. 249 (1985) F553–F565.
[54] P.R. Steinmetz, Cellular mechanisms of urinary acidification, Am. J. Physiol. 251 (1986) F173–F187.
[55] D. Brown, S. Hirsch, S. Gluck, Localization of proton-pumping ATPase in rat kidney, J. Clin. Invest. 82 (1988) 2114–2126.
[56] D. Brown, S. Breton, Mitochondria-rich, proton-secreting epithelial cells, J. Exp. Biol. 199 (1996) 2345–2358.
[57] E. Duranti, J. Ehrenfeld, B.J. Harvey, Acid secretion through the *Rana esculenta* skin: involvement of anion-exchange mechanism at the basolateral membrane, J. Physiol. 378 (1986) 195–211.
[58] U. Klein, M. Timme, W. Zeiske, J. Ehrenfeld, The H^+ pump in frog skin (*Rana esculenta*): identification and localization of a V-ATPase, J. Membr. Biol. 157 (1997) 117–126.
[59] J.A. Zadunaisky, O.A. Candia, D.J. Chiarandini, The origin of short-circuit current in the isolated skin of the South American frog *Leptodactyllus ocellatus*, J. Gen. Physiol. 47 (1963) 393–402.
[60] K. Bruus, P. Kristensen, E.H. Larsen, Pathways for chloride and sodium transport across toad skin, Acta Physiol. Scand. 97 (1976) 31–47.
[61] D.M. Berman, O.M. Soria, A. Coviello, Reversed short-circuit current across isolated skin of the toad *Bufo arenarium*, Pflügers Arch. 409 (1987) 616–619.
[62] D. Erlij, Salt transport across isolated frog skin, Philos. Trans. R. Soc., B 262 (1971) 153–161.
[63] P. Kristensen, Chloride transport across frog skin, Acta Physiol. Scand. 84 (1972) 338–346.
[64] F. Garcia-Romeu, J. Ehrenfeld, Chloride transport through the non-short-circuited isolated skin of *Rana esculenta*, Am. J. Physiol. 228 (1975) 845–849.
[65] J. Ehrenfeld, F. Garcia-Romeu, Coupling between chloride absorption and base excretion in isolated skin of *Rana esculenta*, Am. J. Physiol. 235 (1978) F33–F39.
[66] S. Rosen, N.J. Friedley, Carbonic anhydrase activity in *Rana pipiens* skin: biochemical and histochemical analysis, Histochemistry 36 (1973) 1–4.
[67] L.J. Jensen, J.N. Sørensen, E.H. Larsen, N.J. Willumsen, Proton pump activity of mitochondria-rich cells. The interpretation of external proton-concentration gradients, J. Gen. Physiol. 109 (1997) 73–91.
[68] A.F. Harck, E.H. Larsen, Concentration dependence of halide fluxes and selectivity of the anion pathway in toad skin, Acta Physiol. Scand. 128 (1986) 289–304.
[69] L.J. Jensen, N.J. Willumsen, E.H. Larsen, Proton pump activity is required for active uptake of chloride in isolated amphibian skin exposed to freshwater, J. Comp. Physiol., B. 172 (2002) 503–511.
[70] S.L. Gluck, M. Iyori, L.S. Holliday, T.Y. Kostrominova, B.S. Lee, Distal urinary acidification from Homer Smith to the present, Kidney Int. 49 (1996) 1160–1664.

[71] S.L. Gluck, B.S. Lee, S.-P. Wang, D. Underhill, J. Nemoto, L.S. Holliday, Plasma membrane V-ATPase in proton-secreting cells of the mammalian kidney and osteoclast, Acta Physiol. Scand. 163 (Suppl. 643) (1998) 203–212.
[72] J.C. Vanatta, L.W. Frazier, The epithelium of *Rana pipiens* excretes H^+ and NH_4^+ in acidosis and HCO_3^- in alkalosis, Comp. Biochem. Physiol. 68A (1981) 511–513.
[73] R.D. Page, L.W. Frazier, Morphological changes in the skin of *Rana pipiens* in response to metabolic acidosis, Proc. Soc. Exp. Biol. Med. 184 (1987) 416–422.
[74] I. Spies, Immunolocation of mitochondria-rich cells in epidermis of the common toad, *Bufo bufo* (L.), Comp. Biochem. Physiol. 118B (1997) 285–291.
[75] N.J. Willumsen, J.W. Mills, Distribution of cytoskeletal proteins in toad skin epithelium, FASEB J. 12 (1998) A729.
[76] N.J. Willumsen, L. Vestergaard, E.H. Larsen, Cyclic-AMP and β-agonist activated chloride conductance of a toad skin epithelium, J. Physiol. 449 (1992) 641–653.
[77] J.B. Sørensen, E.H. Larsen, Heterogeneity of chloride channels in the apical membrane if isolated mitochondria-rich cells from toad skin, J. Gen. Physiol. 108 (1996) 421–433.
[78] J. Amstrup, J. Frøslev, N.J. Willumsen, N. Møbjerg, Å. Jespersen, E.H. Larsen, Expression of cystic fibrosis transmembrane conductance regulator in the skin of the toad, *Bufo bufo*-possible role for Cl^- transport across the heterocellular epithelium, Comp. Biochem. Physiol., Part A 130 (2001) 539–550.
[79] E.H. Larsen, P. Kristensen, S. Nedergaard, N.J. Willumsen, Role of mitochondria-rich cells for passive chloride transport–with a discussion of Ussing's contributions to our understanding of shunt pathways in epithelia, J. Membr. Biol. 184 (2001) 247–254.
[80] W. Nagel, P. Somiesky, U. Katz, The route of passive movement across amphibian skin: localization and regulatory mechanisms, Biochim. Biophys. Acta 1566 (2002) 44–54.
[81] E.H. Larsen, B.E. Rasmussen, A mathematical model of amphibian skin epithelium with two types of transporting cellular units, Pflügers Arch. 401 (1984) S50–S58.
[82] E.H. Larsen, B.J. Harvey, Chloride currents of single mitochondria-rich cells of toad skin epithelium, J. Physiol. 478.1 (1994) 7–15.
[83] P. Kristensen, Chloride transport in frog skin, in: J.A. Zadunaisky (Ed.), Transport Mechanisms in Epithelia, Academic Press, London, New York, 1982, pp. 310–332.
[84] H. Kibak, L. Taiz, T. Starke, P. Bernasconi, J.P. Gogarten, Evolution of structure and function of V-ATPases, J. Bioenerg. Biomembranes 24 (1992) 415–424.
[85] B.J. Harvey, S.R. Thomas, J. Ehrenfeld, Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium, J. Gen. Physiol. 92 (1988) 767–791.
[86] M. Busk, E.H. Larsen, F.B. Jensen, Acid–base regulation in tadpoles of *Rana catesbeiana* exposed to environmental hypercapnia, J. Exp. Biol. 200 (1997) 2507–2512.
[87] F.B. Jensen, R.E. Weber, Kinetics of the acclimational responses of tench to combined hypoxia and hypercapnia, J. Comp. Physiol. 156B (1985) 205–211.
[88] M. Nikinmaa, F.B. Jensen, Blood oxygen transport and acid–base status of stressed trout (Salmo gairdnerii): pre- and postbranchial values in winter fish, Comp. Biochem. Physiol. 84A (1986) 391–396.
[89] N.J. Willumsen, E.H. Larsen, Identification of anion-selective channels in the basolateral membrane of mitochondria-rich cells, J. Membr. Biol. 157 (1997) 255–269.
[90] P.E. Budtz, B.C. Christoffersen, J.S. Johansen, I. Spies, N.J. Willumsen, Tissue kinetics, ion transport, and recruitment of mitochondria-rich cells in the skin of the toad (*Bufo bufo*) in response to exposure to distilled water, Cell Tissue Res. 280 (1994) 65–75.
[91] U. Katz, S. Gabay, Dynamics and density of mitochondria-rich cells in toad skin epithelium, Biol. Cell 85 (1995) 185–190.
[92] A.M.Z. Boisen, J. Amstrup, I. Novak, M. Grosell, Transport in soft water and hard water acclimated zebrafish (*Danio rerio*), Comp. Biochem. Physiol. 134A (2003) S83.
[93] E.H. Larsen, J. Amstrup, N.J. Willumsen, β-Adrenergic receptors couple to CFTR chloride channels of intercalated mitochondria-rich cells in the heterocellular toad skin epithelium. Biochim Biophys Acta (this issue).