

# Structure-Function Relations in Amphibian Skin Epithelium: Mitochondria-Rich Cells and Graphic Models

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## Abstract

Mitochondria-rich cells in the skin epithelium of Amphibia are individually separated with their apical pole aligned towards the outer side of the epithelium. They are common in all Amphibian species, they are rich with mitochondria and carbonic anhydrase and in addition to H<sup>+</sup> secretion they participate mainly in chloride conductance across the epithelium. These cells carry out various functions except sodium transport that is carried out by the principal cells' compartment. Application of agonists and amiloride affected separately the two ionic pathways. MR cells distribute unevenness over the body surface, apparently in relation to water conservation. Studies in two species during ontogenetic development have revealed the gradual appearance of MR cells, and that Cl<sup>-</sup> and Na<sup>+</sup> transports developed separately and independent of each other. It is not clear how the principal cells compartment and MR cells are related in relation to their transport functions.

**Keywords:** Chloride; Development; Heterocellularity; KCl; Proton ATPase

## Introduction

The amphibian skin epithelium plays protective and respiratory functions and is a primary osmoregulatory organ, in addition to the kidneys and urinary bladder. The physiological and biophysical transport properties of the epithelium have been studied extensively [1-6] and there is a fair description of its histology and cellular composition [7,8].

The skin epithelium is multilayered and hetero cellular, a common characteristic of tight epithelia in general. It is comprised of Principal (Pr) Cells' compartment which is engaged typically in the active uptake of Na<sup>+</sup>. Other transport pathways are localized in the intercalated Mitochondria-Rich (MR) cells that are assumed to be the site for anion uptake and H<sup>+</sup> secretion [5,6,9,10]. A separate, NaCl secretory mechanism, based on secondary active Cl<sup>-</sup> transport, is located in the dermal mucus skin glands [5,11]. The functional relationships and precise role(s) of the amphibian skin MR cells are not yet settled [1,5,10,12]. There are also similarly analogous intercalated or carbonic anhydrase-rich cells in the mammalian kidney and the turtle urinary bladder which are the sites of H<sup>+</sup> and

HCO<sub>3</sub><sup>-</sup> secretion that are ouabain insensitive [13].

The study of amphibian skin MR cells has progressed considerably in the past years [5,6,12] both biophysically and morphologically. It includes the use of specific molecular probes, antibodies and lectins, allowing for a more faithful address of structure- function relation in the epithelium [10]. The purpose of the present appraisal is 1. A critical account of the structure of the amphibian skin, both anurans and urodeles, 2. Examine the structure and composition of the MR cells and their diverse functions and, 3. Analyze the individual transport functions of the skin epithelium and integrate them into a rationally consistent picture. As will come up in the following, all species examined contain MR cells in the skin, which in certain species contain a selective Cl<sup>-</sup> conductance (GCl). Mostly, the skin of mature and adult forms will be considered, but ontogenetic and developmental aspects will also be discussed [14-17]. It is remarkable that despite the presence of MR cells in all species, activated GCl is found mostly in toads' skins and only rarely in those of frogs (i.e. *Rana pipiens*; [18]

## Epithelial Structure and Cell Asymmetry

The amphibian skin epithelium contains 3-5 cell layers. The first layer at the bottom, the stratum germinativum lays on the

basement membrane that rests on the dermal supporting connective tissue that contains blood vessels and the exocrine glands. The cells of the epithelial layer divide at a constant rate [19], and the cells move towards the outer surface of the epithelium. As the cells approach their position at the outer surface of the epithelium, they acquire their potential asymmetry [20], becoming the stratum granulosum cells [21]. These cells, denote also first reacting cell layer (RCL; [22], are connected through Tight Junctions (TJ) at their apical pole and form the outer, polar integumental barrier of the skin epithelium. Moulting in the amphibian skin epithelium is particular. The outer cell layer is sloughed as a single layer at regular intervals (3-5 days). This process was studied in depth in Copenhagen (19, etc.). It turned out as an intensive dynamics that is hormonally controlled [23]. It involves continual differentiation of specific channels (particularly Na channels at the apical face and Na /K ATPase in the basolateral membranes) as was revealed naturally in moulting *Bufo viridis* [21]. Gland tubes and a few MR cells are lost at each cycle of sloughing [24]. The epithelium appears then as a dynamic tissue, and its cells undergo extensive turnover [7]

The principal cells intercommunicate through gap junctions, and form a functional syncytium [4] The Mitochondria-Rich (MR) cells are excluded from this syncytium and are singly and separately dispersed among the principal cells, at the outer surface of the epithelium.

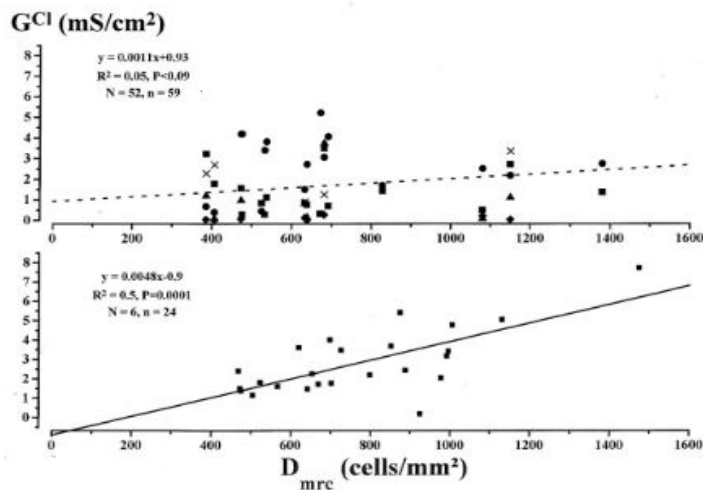
### Morphology and Histology

Intercalated Mitochondria-Rich (MR) cells occupy variable percentage of the outer surface of the epithelium, amounting some 3-10% of the total epithelial cells surface [19,25]. The cells are flask shaped, asymmetric, and must differentiate before they approach their position among the outer epithelial cells. Recent studies have begun to shed light on aspects of cell biology and dynamics of these cells [12,26,27]. A few Merkel and sensory cells are also found in the epithelium [28], but they do not participate in transport processes. The MR cells contain a great variety of components, i.e. they are rich in mitochondria and Carbonic Anhydrase (CA [29] and react apically with anti H<sup>+</sup>-ATPase and antiband 3 antibodies. They are characteristically stained by silver, and accumulate methylene blue from the serosal side. Different enzyme pictures were found in MR cells upon acclimation (*Xenopus*, in 3 environmental conditions - NaCl, KCl and distilled water, DW). Carbonic anhydrase, alkaline phosphatase and malic dehydrogenase increased in NaCl acclimation and H<sup>+</sup>-ATPase increased in KCl acclimation. It did not have a transport correlate; it is not universal and differs among the various amphibian species [30]. In *Bufo viridis*, higher NaCl acclimation caused a great decrease of MR cells' density, CA and a dramatic decrease of both I<sub>sc</sub> and GCl [12]. The high density of mitochondria specifically in MR cells indicates high energy demands of the activities in these cells, which could be related to the H<sup>+</sup>-ATPase, and possibly other routes in these cells.

It was not possible to find concrete difference at the EM level (X 12 000), between MR and other cells, following acclimation in NaCl compared with NaNO<sub>3</sub> (100 mmol/l), whilst there is a great difference in skin conductance<sup>3</sup> (GCl) between these conditions. It is hard to distinguish morphologically between the skin epithelium undergoing various acclimations and treatments. Yet, the apical interdigitated folding could be more developed in Cl<sup>-</sup> free conditions. There seems to be a larger space between the RCL and str. corneum in the Cl<sup>-</sup> free acclimated skins. On the whole, shape and state of cells look quiet comparable.

### MR Cell Density and Regional Distribution over the Body Surface

Density of Mitochondria-Rich Cells (DMRC) was associated with Cl<sup>-</sup> conductance reversibly in a number of species [6,30] under various conditions. This is illustrated in Figure 1 that shows the relationship of Dmrc and Cl<sup>-</sup> conductance in the skin of *Bufo viridis*. High environmental chloride brought to a substantial decrease in MR cells density, except for KCl, acclimation. In the latter conditions Dmrc remains as high as before or even increased (13, in frogs; 20, in toads), which was attributed to blood acidification. A clue to the mechanism of this ontogenetic adaptation may come from the work of Al-Awqati [31] in the mammalian collecting duct, where the protein Hensin is involved in the transformation of β to α type of intercalated cells upon acidosis. The universality of this mechanism remains to be found out [32].



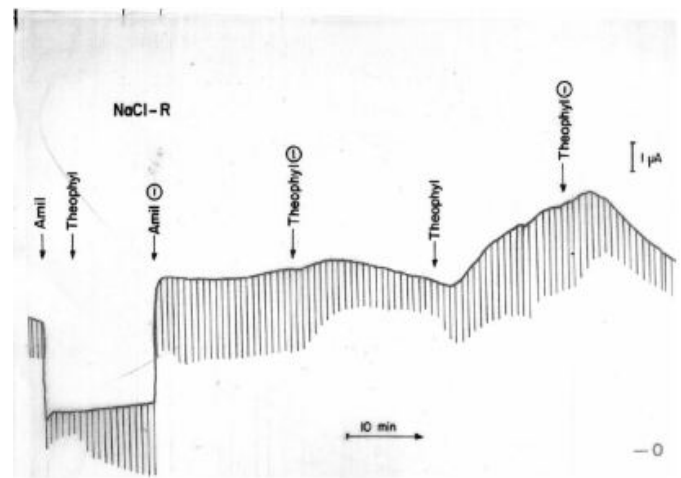
**Figure 1:** Relationship of MR density and Cl<sup>-</sup> conductance across the skin of *Bufo viridis*. The upper panel shows there is no relation in control skins and the lower one shows the relationship following voltage activation at 80 mV. NaCl ringer on both sides. N=number of animals used. n=number of skin pieces used.

The density of mitochondria-rich cells varies over the body surface and among species. This was studied in anurans, revealing a spatial distribution among species, characterized by a dorsoventral unevenness that is more pronounced in the terrestrial

species [15,31,33]. At the extreme we find the terrestrial *Hyla* sp, where no MR cells are found on the back side, while more or less even distribution of MR cells over the whole body surface is found in the fossorial *Pelobates syriacus* and the semi-aquatic *Xenopus laevis* [15,31,33]. It was suggested that the specific unevenness dorso-ventral distribution of the MR cells is related to defense against excessive evaporation, most pronounced in species that are greatly exposed to air, i.e., *Hyla* sp. Furthermore, examination of skin pieces from opposite sides of *Hyla arborea* showed that only the abdominal piece that contains MR cells responded on the application of theophylline, a phosphodiesterase inhibitor, while the back side skin that lacks MR cells was neutral to the addition of the drug [15]. The later proofs explicitly the associations between MR cells and GCl [34].

### Transport Functions

The two major epithelial cells' types, i.e. Pr and MR cells, are set apart from each other and carry out singular functions separately. The mechanism of sodium transport across frog skin was modeled and has been firmly established, culminating in the now classical two membranes model with general acclaim to all epithelia [4]. Unilateral chloride transport was localized to the mitochondria-rich cells, but the mechanisms have not been established. Technically, this route is confined to singly dispersed cells that are hard to be punctured continually and hard to follow separately on either of their sides. They were studied directly using external electric probe in toto while Na<sup>+</sup> transport is eliminated completely, either by replacement of apical Na<sup>+</sup> or by the application of the specific inhibitor amiloride. Selective Cl<sup>-</sup> conductance is thus localized exclusively to MR cells [10,35]. H<sup>+</sup>-ATPase was demonstrated in the MR cells [29], but several attempts to verification of H<sup>+</sup> secretion from these cells, did not succeed [2,36]. These cells also contain carbonic anhydrase [29,37] which is involved in Na<sup>+</sup>/H<sup>+</sup> exchange across the skin [38]. Cell density, CA content and both I<sub>sc</sub> and Cl<sup>-</sup> conductance dropped dramatically upon long term acclimation in higher NaCl [29]. However, Katz and Gabbay, [12] have demonstrated immunohistochemically, that the presence of various components in these cells is not universal among species, and could not be correlated with the measured transport properties of the epithelium. Any interrelation between the two pathways has not been settled [39]. The two cells' compartments respond independently on the application of stimulators (hormons and agonists) and inhibitors (such as amiloride). This is illustrated in Figure 2, where amiloride inhibits the short circuit current (Na transport, I<sub>sc</sub>) and has only a negligible effect on the conductance. Theophylline, in the presence of amiloride, elevated the conductance, which enhances Cl<sup>-</sup> transport. In the absence of amiloride, addition of theophylline enhances both the current (I<sub>sc</sub>) and Cl<sup>-</sup> conductance.

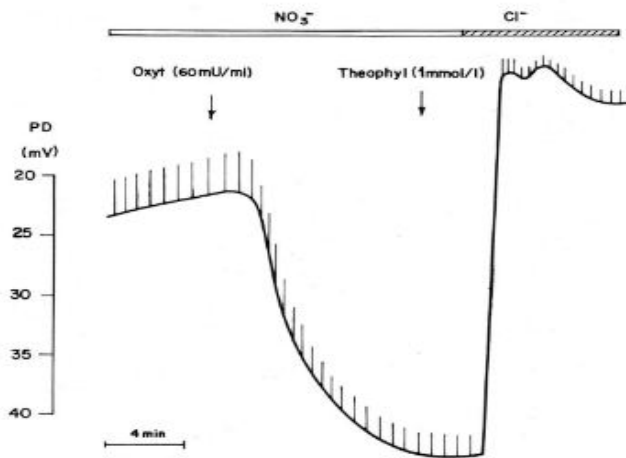


**Figure 2:** A short circuited toad skin (*Bufo viridis*) responding to amiloride and theophylline. Application of amiloride blocked Na<sup>+</sup> transport; addition of theophylline enhanced (more than doubled), the skin conductance with no effect on the current. Removal of amiloride reversed the current and improved the conductance, while theophylline had a combined effect in the absence of amiloride. Bars are the response to intermittent 3 mV voltage pulses. The skin was bathed with NaCl on both sides.

Studies on skin transport of *Xenopus laevis* represent a particular and opposite case [32,33,15]. This African species lives mostly in water sources, it accumulates urea when acclimated to hypertonic solutions (22 and others), has a poor Na<sup>+</sup> transport capacity and no finite anion (Cl<sup>-</sup>) conductance. MR cells on the other hand, are contained in the skin epithelium; they distribute equally over the body surface and are comparable to other species. Yet, the density of these cells decreased upon acclimation to high NaCl solutions [32]. The aligned functions in this and other species all containing MR cells remain to be resolved.

Skin MR cells carry out particular transport functions including Cl<sup>-</sup>, H<sup>+</sup>, HCO<sub>3</sub><sup>-</sup>, and organic acid, except for Na<sup>+</sup>. Amiloride had no effect on electrolyte concentrations in frog skin MR cells [40]; however, a low level of basolateral Na<sup>+</sup>/K<sup>+</sup> ATPase was discovered in intercalated cells of mouse kidney [35], functioning apparently at housekeeping of the cells. In amphibian skin epithelium passive Cl<sup>-</sup> conductance is thus the primary species transported through MR cells.

Figure 3 shows the electrical properties of toad skin in open circuit condition. The effects of oxytocin, theophylline and the presence of external Cl<sup>-</sup> across the skin are recorded. It shows that in the absence of chloride, serosal oxytocin hyperpolarizes greatly the skin electrical potential. Addition of theophylline hardly had any effect, but upon replacement of chloride the potential is depolarized greatly and immediately.



**Figure 3:** Effects of oxytocin and theophylline on open circuit trans epithelial potential of a skin from *Bufo viridis* in the absence ( $\text{NO}_3^-$ ) and presence of extracellular chloride. At first, oxytocin was added in  $\text{Cl}^-$ -free external solution, leading to a large increase in the trans epithelial potential. Application of theophylline did not have further effect. However, replacing the external solution with  $\text{Cl}^-$  led to prompt relax of the potential and decreased conductance.

**Regulations - Hormonal and External Agents**

Both  $\text{Na}^+$  and  $\text{Cl}^-$  pathways in the skin are greatly affected by environmental salinity and are regulated hormonally in diverse ways. The effect of cyclic AMP on  $\text{Cl}^-$  conductance in the skin epithelium was studied by Willumsen et al. [35] Katz and Nagel, [41] and others strengthen the notion that  $\text{Cl}^-$  pathway should contain two components: an anion passive path which is regulated by a unilateral voltage sensitive component.

Adrenergic receptors turned to be effective on the chloride pathway. Thus, activation of  $\beta$ -adrenoceptors applied from the basolateral side increased  $\text{GCl}$ , which must due to elevated cellular cAMP. Epinephrine antagonized this effect, and exerted effective inhibition by the  $\alpha_1$ -adrenoceptor also applied from the serosal side [42]. The effect of epinephrine did not overcome cAMP induced  $\text{GCl}$ , suggesting it affects the regulatory component of the pathway. A distinct difference was observed between direct application of cAMP and the use of agents thought to elevate cellular cAMP. Theophylline and forskolin which are supposed to elevate cellular cAMP facilitated a great increase in  $\text{GCl}$ . However, the time dependent increase of voltage conductance was eliminated upon direct application of cAMP [the non hydrolysable analogues dibutyryl and 8-(4-Chlorophenylthio) cAMP], and had somewhat stronger effect [35,40]. Mucosal Application of N-ethylmaleimide (NEM) potently inhibited the chloride channel [43]. Trypsin from the serosal side inhibited  $\text{GCl}$  reversibly by some 40%, which is due to its effect on the regulatory component of the pathway.

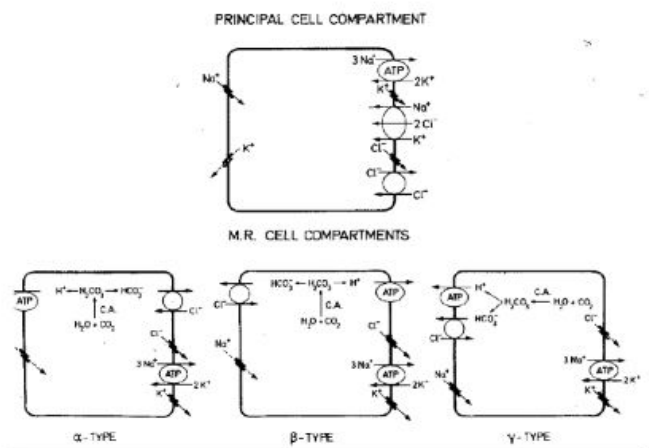
**Ontogenetic Development**

Structural and physiological features of development were investigated in two species, an anuran (*Pelobates*, [14,15] and

urodele (Salamander, [16]. In both species primordial cells were identified in the early developmental stages, while mature, characteristic MR cells were revealed in the adult forms.  $\text{Na}^+$  transport and  $\text{Cl}^-$  conductance emerge at maturity, and they are cellularly autonomous and morphologically separate from one another.  $\text{Na}^+$  transport measured as amiloride sensitive ISC occurred in both species associated with electrical tightening of the epithelium. However, even that at metamorphosis MR cells appeared in both  $\text{Cl}^-$  conductance was evident only in *Pelobates*, and not in Salamander. It indicates that even though MR cells are a specific route for  $\text{Cl}^-$ , they may fulfill other functions unrelated to that of  $\text{Cl}^-$  transport, a question that remains to be answered.

**Modelling**

Graphic presentation is inherently limited. It helps conceiving the cellular situation and used to test for alternatives, but is limited for the actual and unknown functional roles played by these cells. It rests on experimental evidence, and yet a passion for completeness. Models should therefore be taken cautiously before recognized for reality. The two membranes model is a good example case where a minimal graphic model manifested itself as a general one applicable to all known epithelia. This has been used ever since in numerous examples of epithelial transport in many animals and for numerous specific tissues and solutes. Skin MR cell is another example, where the two membrane model was implicated. Here the models are based on those of intercalated kidney tubules, i.e.,  $\alpha$  and  $\beta$  cells type, footing for  $\text{H}^+$  or  $\text{HCO}_3^-$  secretion, while in the skin they are a major pathway for chloride conductance and proton secretion. This led to a proposal of another  $\gamma$  type model for MR cell in amphibian skin epithelium Figure 4 [6]. This model adds up our present and more knowledge on the activities of this cell, but its reality remains vague.



**Figure 4:** Graphic presentation of functional epithelial cells. Alpha and betha types play a role in  $\text{H}^+$  and  $\text{HCO}_3^-$  transport in various epithelia; the gamma type depicts a possible situation in amphibians' skin epithelium, and includes a number of transporters and channels in the same cell (from Larsen, 1991).

In conclusion, mitochondria rich cells in the skin epithelium of amphibians are individually separated and are commonly present in all species that were tested. They are silver stained, and are particularly rich with mitochondria and carbonic anhydrase. Some other characteristics are also noted, such as apical band 3 identified immunohistochemically. These cells are the major site of unilateral chloride conductance and H<sup>+</sup> secretion, but despite their common presence, these functions are not frequent in all species.

The emergence of MR cells, jointly with functional Na<sup>+</sup> transport and Cl<sup>-</sup> conductance were followed in two species during ontogenesis. MR cells occurred in both species, but Cl<sup>-</sup> conductance appeared only in the skin of *Pelobates*, not in that of the Urodele, Salamander. Amiloride sensitive Na<sup>+</sup> transport assigned to principal cells occurred in both. [44-52]

Based on the use of specific inhibitors and stimulators, a simple model composed of a passive anion pathway controlled by a regulatory component governed through cAMP is proposed for the chloride conductance. Other functions of these cells in all species are not resolved.

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