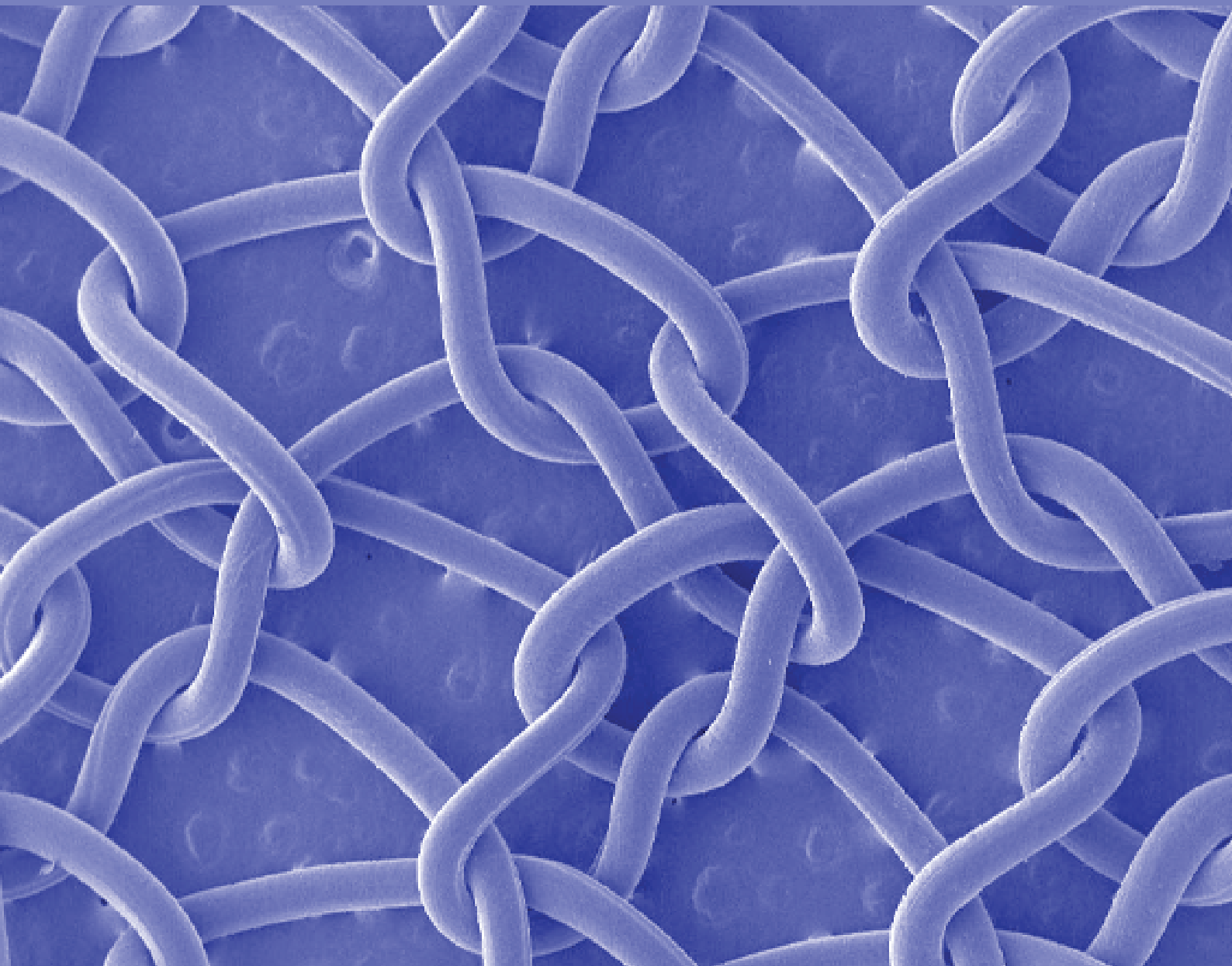


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ROLE OF ION CHANNELS IN SALT SECRETION BY ATLANTIC SALMON GILLS DURING ACCLIMATION TO SEAWATER

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ABSTRACT

Smoltification, also called parr-smolt transformation, is a complex developmental process that consists of a number of independent, but coordinated changes, in the biochemistry, physiology, morphology and behavior of juvenile salmon in their transition from freshwater to seawater life. A key component of smoltification is represented by the physiological adaptations that enable smolts to thrive in hyperosmotic environments. Instrumental to this process is the ability of smolt gills to gradually become capable of actively secreting salt through specialized cells known as mitochondria-rich (MR) cells, ionocytes or chloride cells. NaCl secretion by teleost gills is therefore accomplished via the secondary active transport of Cl⁻ and the passive transport of Na⁺. The driving force for active transport is provided by Na⁺/K⁺ ATPase, which maintains low intracellular Na⁺ and high intracellular K⁺ concentrations. However, this NaCl secretion mechanism needs at least two different ion channels: A CFTR type chloride channel for the passive exit of Cl⁻ and a potassium channel to recycle extracellular K⁺, which is a thermodynamic prerequisite to work under conditions imposed by high extracellular salinity in seawater. The characteristics of K⁺ channels required for NaCl secretion from MR cells into seawater are still unknown for *Salmo salar* and only recently have begun to be studied in other teleosts.

Keywords: smoltification, salt secretion, ion channels, atlantic salmon

1. GENERAL BACKGROUND

1.1. General summary of the salmon life cycle

Salmonids begin life in freshwater. After adult salmon spawn in freshwater, eggs hatch into alevins and begin their development into fry and parr. At this stage, environmental cues initiate the smoltification process, thus preparing the fish for downstream migration and entrance into seawater, where they will grow to become a marine, predatory species [1, 2]. This anadromous strategy confers reproductive and developmental advantages to salmon, because it enables them to utilize a relatively safe environment provided by freshwater for reproduction, whereas juvenile migration towards the ocean allows them to feed on a rich supply of fish and other marine organisms [3]. Hence, smoltification represents the key turning point in the anadromous life cycle of *Salmo salar* [2, 4].

1.2. Physiological changes during smoltification

Smoltification, is a complex process driven by the endocrine system which consists of a series of changes, from cellular physiology to morphology in the juvenile salmon, as an adaptive response in order to survive in the new seawater environment. All of these changes prepare the fish for downstream migration and transition to the marine life stage [2, 3]. Key elements of the parr-smolt transformation are: i) environmental cues, such as primarily photoperiod and temperature [1]; ii) endocrine control of smoltification [2]; and iii) the physiological changes in osmoregulation that allow the smolt to thrive in high-salt environments [5, 6].

1.3. Hormonal and environmental regulation of smoltification

It is well established that the photoperiod and seasonal temperature fluctuations are two important environmental signals that work together in the transformation of Atlantic salmon parrs into smolts [5]. Smoltification is complete by spring, when a rising temperature by around 8-10°C initiates the migration of wild smolts into seawater [5, 7]. The mechanism by which photoperiodic information is translated into a neuroendocrine response in teleosts has not been elucidated. Even so, the melatonin secretion by the pineal gland of salmonids can be directly entrained by a specific photoperiod [8] but the relationship between melatonin rhythm and the photoperiod in fish is unclear [9].

The Parr-smolt transformation involves several endocrine signaling systems. Some examples of well-studied endocrine mediators of smoltification are the growth hormone/insulin-like growth factor I system (GH-IGF-I), cortisol and thyroid hormones, all of them promote the smoltification process [10]. On the other hand, there is other hormones such as prolactin, which is considered to play an inhibitory function in the smoltification process [10, 11]. The light–brain–pituitary axis is stimulated by a size- or growth-related threshold, resulting in higher levels of GH, cortisol and thyroid hormones [8, 12]. GH, modulates the fish intermediary metabolism and osmoregulatory mechanisms by stimulating the activity of somatomedins, such as insulin-like growth factors IGF-1 and IGF-2 [13]. GH and cortisol interact to control hyperosmoregulatory mechanisms in gills, gut, and kidneys, promoting increased salinity tolerance as well as changes in growth and metabolism. In gills, cortisol and the GH/IGF-I axis promote the

differentiation of salt-secreting ionocytes (see section 3.2), which is a process that requires the up regulation of three major osmoregulatory membrane transporters: sodium-potassium ATPase (NKA), sodium-potassium-2 chloride cotransporter 1 (NKCC1), and the cystic fibrosis transmembrane conductance regulator (CFTR) [13]. Lastly, thyroid hormones regulate imprinting, metabolism, morphological changes such as silvering, and possibly behavior [8].

1.4. Osmoregulatory changes during smoltification

As was mentioned above, salmonids begin their life cycle in freshwater, where they present a hyperosmotic intracellular equilibrium respect to the external medium. In this situation, osmotic pressure favors the entry of water into the body and the loss of salt by diffusion across the gill. To compensate for this passive flow of water and ions, the fish eliminate excess water as diluted urine and obtain salts from food and by active uptake through the gill's epithelium [14]. However, when they move into seawater, the osmotic gradient is reversed and they become hyposmotic relative to the external medium (the internal fluids of salmonids are approximately one-third the osmolarity of seawater). Therefore, salmon lose water and gain salts by passive diffusion. As compensatory mechanisms, they drink seawater, reduce their urine production and actively secrete salts across the gill's epithelium through specialized type cell, the MR cells [5] (See Fig. 1).

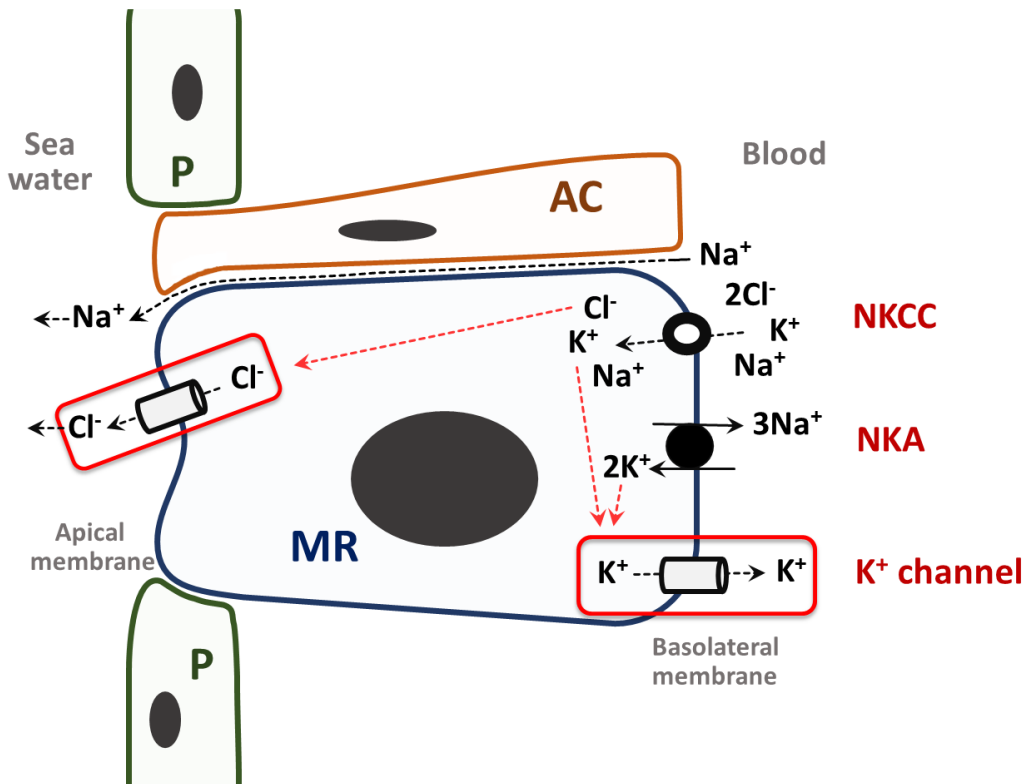


Fig. 1 Model of NaCl secretion by mitochondria-rich (MR) cells in gill epithelium. Cl⁻ enters MR cells via a Na⁺/K⁺/2Cl⁻ co-transporter driven by the Na⁺ gradient, which is maintained by Na⁺/K⁺ ATPase (NKA). Chloride ions accumulate intracellularly and exits at the apical membrane through CFTR-type anion channels. Na⁺ is secreted paracellularly. The red rectangle highlights the potassium channels required in this process. AC: accessory cell (Marshall and Grosell, 2006).

One of the key events in osmoregulatory changes during parr-smolt transformation involves the fine-tuning of the ion-transporting machinery in the gill epithelium [14]. Ion transport is primarily carried out by MR cells [3], and requires their rearrangement as well as changes in the expression of NKA, NKCC1 and other critical ion transport proteins at the cell surface [13]. A strong upregulation of gill NKA and NKCC1 is associated with seawater acclimation in most euryhaline teleosts [13, 15, 16].

1.5. Molecular mechanism of salt secretion by MR cells.

The NaCl secretion mechanism by MR cells in gills of teleost fish living in seawater has been studied extensively [for further reading see Evans et al., 2005 and Hiroi and McCormick, 2012], leading to a consensus model of NaCl transport (Fig. 1). This working model for NaCl secretion includes ouabain-sensitive NKA and bumetanide-sensitive NKCC1, both located in the basolateral membrane, as well as the anion channel CFTR in the apical membrane. NKCC1 mediates the entry of Na⁺ and Cl⁻ into the cellular compartment down the electrochemical gradient provided by NKA, followed by the passive exit of Cl⁻ and Na⁺ through the apical channel CFTR and the paracellular tight-junction pathway, respectively [8, 14, 16]. NaCl secretion by teleost gills is accomplished via secondary active transport of Cl⁻ and passive transport of Na⁺ (Fig. 1). The driving force for active transport is provided by NKA, which maintains intracellular Na⁺ at low levels and high intracellular K⁺ concentration [15]. However, this NaCl secretion mechanism needs an additional condition to work under seawater, namely a thermodynamic requirement to recycle K⁺ out, via conductive pathways mechanism (potassium channels, shown in Figure 1).

1.6. General principles of physiological osmoregulatory mechanisms in teleost fish

Ion and water homeostasis is an important challenge for fish, especially because they have to maintain their internal milieu in despite of the amount of salt present in the aqueous environment. In some cases, the water that fish are in contact with could vary in a range from freshwater (FW) with very few osmolytes (~0 mOsmol kg⁻¹) to approximately 1000 mOsmol kg⁻¹ in seawater (SW).

All FW fishes maintain their internal salt concentration at approximately 250–320 mOsmol kg⁻¹. They have to counter a diffusive ion loss and osmotic gain of water across the gill epithelium and other external surfaces. They solved this problem through two different process: a) to obtain ions via active transport of both Na⁺ and Cl⁻ across the gill epithelium from water and via the intestine from ingested food and b) the production of large volumes of dilute urine [17]. On the other hand, SW teleosts maintain their extracellular fluids at approximately one-third the osmotic concentration of SW. They suffer an osmotic loss of water across their gills and other epithelial surfaces. To counter this, they drink SW to maintain osmotic balance, absorbing water and salts but excreting the excess salts through ionocytes in the epithelium of the gills and skin. The transepithelial potential of the gill in SW is positive and allow the paracellular transport of Na⁺, whereas the transport of Cl⁻ is carried out by an active transcellular process. Any excess uptake of divalent ions is excreted by the kidney [17].

In general, different fishes have evolved and adapted to deal with both dehydrating and highly dilute environments. Most fishes are unable to move between freshwater and seawater (stenohaline), but around of 3–5% of fishes are capable of surviving in a wide range of salinities (euryhaline). Euryhaline fishes represent important model species for elucidating the physiological mechanisms of salt and water balance among vertebrates [18] and it is important to mention that some euryhaline fishes such as salmon, eels, and tilapia are economically important in worldwide fisheries and aquaculture.

2. STRUCTURAL INSIGHTS OF ION CHANNELS INVOLVED IN NaCl SECRETION IN THE GILL

2.1 CFTR.

Cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the ATP-binding cassette (ABC) transporter family. The members of this family is spread through the different branches among of life kingdoms (Higgins, 1992). The canonical architecture of ABC transporters consist of 2 transmembrane domains (TMDs).

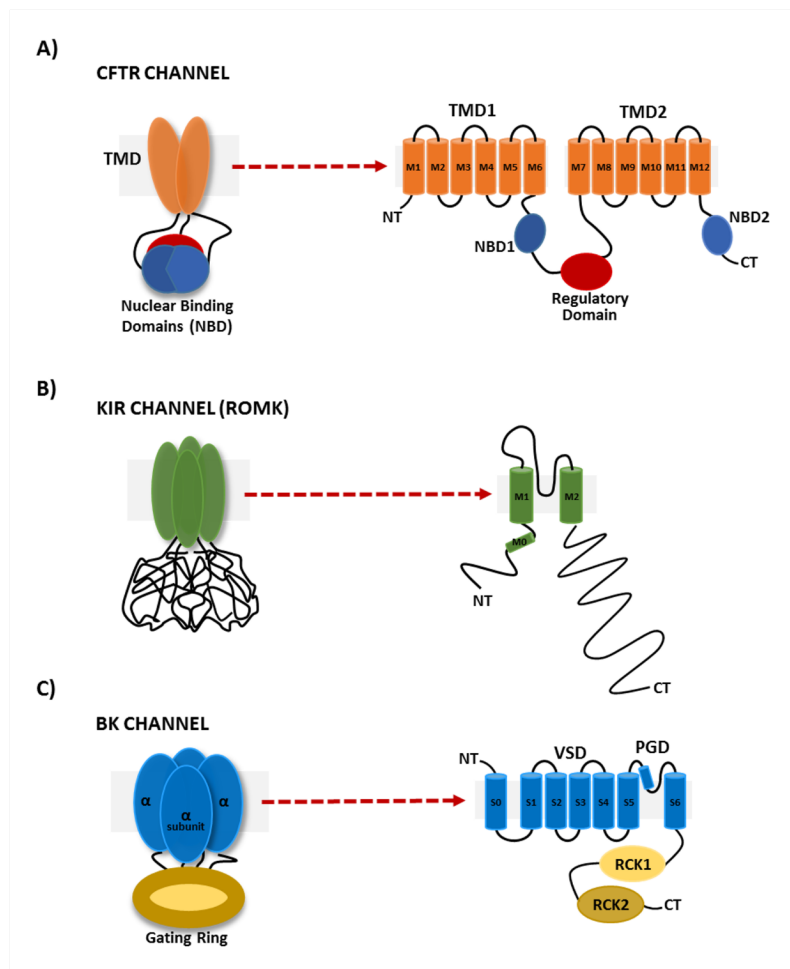


Fig. 2 Architecture of different ion channels involved in smoltification process. **A.** The CFTR chloride channel is divided in two transmembrane structural domains (TMD), each one contains six transmembrane segments (TM1-TM6 / TM7-TM12). It possess two intracellular nucleotides binding domains and a regulatory domain, which controls the activity of the channel. **B.** Kir channels present a tetrameric structure, where each monomer consist of two transmembrane domains (M1-M2) and intracellular helix (M0) in the N-terminal domain. The intracellular C-terminus harbors the pH regulation domain, and important structural determinants associated to gating and rectification. **C.** BK channel is a Ca^{2+} activated K^+ channel, in contrast to Kv channel it has an extra transmembrane segment domain (S0), and the N-

terminus facing the extracellular environment. BK channel activity is tightly regulated by intracellular Ca^{2+} concentrations, the molecular machinery that confers the Ca^{2+} sensitivity to BK is located in the C-terminus intracellular domain. This channel present two RCK domains, which conform a macro domain called gating-ring, which controls the BK channel activity to the local increases of intracellular Ca^{2+} .

Each one is formed by 6 transmembrane segments, S1-S6 harbored in the TMD1 and S7-S12 in the TMD2. In addition this protein presents two intracellular nucleotide-binding domains (NBDs) and a regulatory domain (Fig. 2A). This domains expressed as fusion protein do not prefer any order (TM1D/TMD2 or TMD2/TMD1) to produce fully functional channels [19]. Interestingly most of the member of ABC family function as classical transporters, CFTR is an anionic non-selective channel, with increased permeability to chloride and bicarbonate ions. Structural details and transport mechanism insights has been recently reviewed by Cant et al. (2014) [20].

2.2 Potassium channels

The hypothesis that K^+ ions entering the cell through NKA and NKCC1 are recycled via K^+ channels has been supported by experiments measuring epithelial short-circuit currents in the opercular epithelium from killifish. The different conductances present, whether in the apical or basolateral side of the cell, were revealing with an ussing-style membrane chamber, which works as a voltage-clamp for the whole epithelium. The addition of 2 mM Ba^{2+} (a non-specific blocker for potassium channels) to the basolateral side is able to inhibit short-circuit currents by 77%, suggesting that the function of K^+ channels in the basolateral membrane is to recycle K^+ ions, pumped into the cell by NKA (reviewed by Evans et al., 1999 and 2005). The molecular identity of this K^+ conductance is still unknown in salmonids, but there are several K^+ channels involved in MR cell function in other teleosts fish [15]. One plausible candidate for the K^+ recycling is the inward-rectifying K^+ channel (eKir), which is highly expressed in gills of the seawater-acclimated Japanese eel [21]. More recently, an inward-rectifying K^+ channel (Kir1.1 or ROMK for **R**enal **O**uter **M**edullary **K**⁺ channel) and a BK channel have been detected in MR cells from the *Mozambique tilapia* [22]. Another candidate is a large-conductance calcium-activated K^+ channel (called BK for “**B**ig **K**”) given its large unitary conductance compared to other K^+ channels), whose expression was recently detected in gills from the teleost fish *Porichthys notatus* [23] and recently in *Salmo salar* [24].

2.1.1. Kir and ROMK channels

Inward rectifying K^+ (Kir) channels are tetrameric complexes, assemble around its symmetry axis, which is the K^+ conductive pathway. Interestingly, Kir channel lacks the classical voltage sensor domain (VSD) present in other voltage-gated K^+ channel, each subunit is composed just by two transmembrane segments (M1 and M2) [25]. These transmembrane segments resemble the S5-S6 domain, present in voltage-gated K^+ channel, which confer the selectivity to K^+ ions over others [25]. In addition, each monomer presents short intracellular alpha-helix (M0), which precedes M1 and M2. This K^+ conductance is highly regulated by intracellular domains N and C-terminus [26] (Fig. 2B). Inward rectification refers to the ability of an ion channel to permeate ions in the inward direction rather than outward direction. The outcome is that Kir channel present robust inward currents at hyperpolarized potential, and there is no ionic current at positive voltages. There are several rectification mechanisms, being the particular one for Kir channel that given by a fast blockage of outward current by intracellular Mg^{2+} or polyamines [27]. In the case of smoltification, the Kir channel type involved in the process is Kir1.1 or ROMK, which among others Kir channels shows a relative weak

rectification. Kir1.1 presents a tight regulation by internal pH, at physiological range, which could be important for the smoltification process [28].

2.1.2. BK channels

The high conductance voltage- and Ca^{2+} -activated K^+ channel is one of the most broadly expressed channels in metazoans. The name 'Big K' stems from its single-channel conductance that can be as large as 250 pS under symmetrical 100 mM K^+ condition [29, 30]. BK channels are homotetramers formed by its α -subunit, encoded by the *slo-1* gene (KNCMA1), it is member of the voltage-gated potassium (Kv) channel superfamily [31]. BK channel architecture is different from typical Kv channel, it has an extra transmembrane segment (S0), thus each monomer presents seven transmembrane segments instead of six [32].

This channel presents an exquisite regulation by intracellular Ca^{2+} , achieving its maximum activity ($P_o \sim 1$) at 100 μM [33]. The structural motif that confers to BK Ca^{2+} sensitivity is the **R**egulator of **C**onductance of **K**⁺ (RCK) domain, which is part of the intracellular regulatory domain called gating ring conformed by the N and C-terminus (see part 2.1.3) (Fig. 2C) [34, 35]. BK channels have been implicated in a variety of physiological processes, from the regulation of smooth muscle tone [36] to the modulation of hormone and neurotransmitter release [37]. Interestingly, BK channels are also involved in modulating K^+ transport in the mammalian kidney [38, 39], pulmonary epithelium [40] and colon epithelium [41]. These channels have been characterized in different species, from *Drosophila* to humans, nonetheless detailed studies about their properties and functions in teleosts fish are lacking, with the exception of earlier work from Rohmann and colleagues, which identified BK currents as one of the major outward currents in teleost fish hair cells [23]. The genomic organization of *slo-1* has only recently been reported in the zebrafish (*Danio rerio*) [42]. BK channel transcripts have also been detected in the intestinal epithelium of the European eel (*Anguilla Anguilla*) [43], in the nervous system of different teleost fish and in gills of the midshipman fish (*Porichthys notatus*) [23] and the Mozambique tilapia (*Oreochromis mossambicus*) [22].

3. Coda

The smoltification process is the consequence of an adaptive response of the fish in order to survive the change of its environment, from freshwater to seawater. This tightly regulated process encompasses from huge morphological to subtle physiological changes in the fish. Within this changes, one of the most important is the change in the expression pattern of different set of membrane proteins and ion channels in specialized cells in the gills, called MR cells. The different biophysical properties of the channels mentioned above, help the fish to achieve the osmoregulation in different osmotic conditions (freshwater and seawater). Understanding the functioning of these channels is key to understand the smoltification process in salmonids, which is important for different productive processes, depending on the smoltification, like fish farming.

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