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Molecular characterization and transcriptional regulation of the Na^+/K^+ ATPase α subunit isoforms during development and salinity challenge in a teleost fish, the Senegalese sole (*Solea senegalensis*)

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ABSTRACT

In the present work, five genes encoding different Na^+,K^+ ATPase (NKA) α-isoforms in the teleost *Solea senegalensis* are described for the first time. Sequence analysis of predicted polypeptides revealed a high degree of conservation across teleosts and mammals. Phylogenetic analysis clustered the five genes into three main clades: α1 (designated *atp1a1a* and *atp1a1b*), α2 (designated *atp1a2*) and α3 (designated *atp1a3a* and *atp1a3b*) isoforms. Transcriptional analysis in larvae showed distinct expression profiles during development. In juvenile tissues, the *atp1a1a* gene was highly expressed in osmoregulatory organs, *atp1a2* in skeletal muscle, *atp1a1b* in brain and heart and *atp1a3a* and *atp1a3b* mainly in brain. Quantification of mRNA abundance after a salinity challenge showed that *atp1a1a* transcript levels increased significantly in the gill of soles transferred to high salinity water (60 ppt). In contrast, *atp1a3a* transcripts increased at low salinity (5 ppt). In situ hybridization (ISH) analysis revealed that the number of ionocytes expressing *atp1a1a* transcripts in the primary gill filaments was higher at 35 and 60 ppt than at 5 ppt and remained undetectable or at very low levels in the lamellae at 5 and 35 ppt but increased at 60 ppt. Immunohistochemistry showed a higher number of positive cells in the lamellae. Whole-mount analysis of *atp1a1a* mRNA in young sole larvae revealed that it was localized in gut, pronephric tubule, gill, otic vesicle, yolk sac ionocytes and chordacentrum. Moreover, *atp1a1a* mRNAs increased at mouth opening (3 DPH) in larvae incubated at 36 ppt with a greater signal in gills.

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1. Introduction

Sodium-Potassium ATPase (NKA) is an ubiquitously expressed integral membrane protein that couples the exchange of two extracellular K^+ ions for three intracellular Na^+ ions, a process that is linked to the hydrolysis of one molecule of ATP (Mobasheri et al., 2000). The Na^+, K^+ pump contains equimolar amounts of α and β subunits and belongs to the P-type class of ATPases (Lingrel and Kuntzweiler, 1994; Blanco and Mercer, 1998). The α subunit contains the catalytic site, which is phosphorylated in each pump cycle and its structure is stabilized by the β subunit, which also routes the αβ complex to the plasma membrane (Schoner and Scheiner-Bobis, 2007). A γ subunit has also been identified as a third putative subunit that in mammals is predominantly present in the kidney and is essential for blastocyst development (Jones et al., 1997). In eukaryote cells, Na+/K+ transport plays a key role in the maintenance of ionic homeostasis and it also provides a transmembrane Na^+ gradient for other cellular activities such as Na^+-dependent nutrients transport or signal transduction (Lai et al., 2013). Moreover, NKA can also display other non-ion transport functions (Krupinski and Beitel, 2009; Chen et al., 2011).

Four α isoforms (α1 to α4) have been identified in mammals that are encoded by a multigene family and despite sharing 90% sequence similarity they are under different transcriptional regulation and exhibit different tissue expression patterns (Lingrel and Kuntzweiler, 1994; Blanco and Mercer, 1998). The α1 isoform is ubiquitous and occurs in most cell types; α2 is the principal isoform of the skeletal muscle, but is also detected in brain and heart; the α3 isoform is mainly expressed in neural tissue; and the expression of α4 is restricted to the testes

Abbreviations: aa, amino acid; cDNA, CDS, coding sequence, DNA complementary to RNA; DPH, days post hatch; DEPC, diethyl pyrocarbonate; ECD, extracellular domain; IC, intracellular domain; ISH, *in situ* hibridization; IHC, immunohistochemistry; kb, kilobase(s); kDa, kiloDaltons; ML, maximum likelihood; NKA, Na+,K+ ATPase; nt, nucleotide(s); PBS, phosphate buffered saline; PK, protein kinase; ppt, parts per thousand; SEM, standard error of the mean, SSC, saline-sodium citrate; TMD, transmembrane domain, WISH, Whole mount ISH.

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germ cells (Lingrel and Kuntzweiler, 1994; Blanco and Mercer, 1998). The functional divergence of the different NKA α isoforms still remains to be fully characterized. In fish, only α1 to α3 subunit-encoding genes have been identified although according to species and subunit type there may be several paralogous genes. In zebrafish, nine distinct NKA α-subunit genes (six α1, one α2, and two α3) have been reported (Rajarao et al., 2001; Serluca et al., 2001; Blasiole et al., 2002; Canfield et al., 2002; Liao et al., 2009). In Atlantic salmon and rainbow trout, seven NKA α-subunit genes were identified (four α1, one α2, and two α3) (Richards et al., 2003; Gharbi et al., 2005). These NKA α subunits showed specific expression patterns in adult tissues (Rajarao et al., 2001; Richards et al., 2003), during embryogenesis (Canfield et al., 2002) and in response to salinity (Richards et al., 2003; McCormick et al., 2009) suggesting that a subfunctionalization process occurred during their evolution. The identification of the complete set of NKA α subunits and the evaluation of their expression patterns in embryos and juveniles is essential to understand the evolution of this complex gene family and their functional specialization.

Teleosts are exposed to a constant osmotic challenge from the surrounding water and osmoregulation maintains internal ion homeostasis when teleosts are in freshwater or seawater (Sakamoto et al., 2001; Marshall, 2002). Osmoregulatory plasticity varies between species with euryhaline teleosts being able to maintain almost constant blood osmolality even in the face of wide variations in external salinity. Most of these euryhaline teleosts exhibit higher NKA activity when they reside in seawater and are able to modulate NKA abundance when challenged by a salinity shift (Jensen et al., 1998; Marshall and Bryson, 1998; Lin et al., 2004; Laiz-Carrion et al., 2005). In teleost gills, NKA is located mainly in a specialized cell type known as mitochondrion-rich cells or ionocytes, the site of active ion transport. In the ionocytes, α subunits are differentially expressed and respond differently to osmotic challenges and acclimation to seawater and freshwater (Richards et al., 2003; Liao et al., 2009; Madsen et al., 2009). The Senegalese sole (*Solea senegalensis* Kaup 1858) is an euryhaline species and inhabits environments with wide seasonal fluctuations in salinity. In coastal marshes of the southern Iberian Peninsula salinity varies from 12.5 to 61 ppt in January and August respectively with significant salinity fluctuations throughout the year (Imsland et al., 2003; Arias and Drake, 2005; Yufera and Arias, 2010). Previous studies carried out in sole juveniles confirmed a high tolerance to a wide range of salinities (5–55 ppt). Soles are able to acclimate very quickly (~3 h) or over longer periods (17 days) by adjusting cortisol levels and energy allocation, with a linear relationship existing between environmental salinity and gill NKA activity (Arjona et al., 2007; Herrera et al., 2012). Curiously, Senegalese sole larvae incubated at low salinities (10 ppt) develop severe malformations including undeveloped jaws, abnormal lower jaw development, and absence of an upper jaw or gaping jaws (Salas-Leiton et al., 2012) suggesting their aetiology may somehow be linked to the larvae's osmoregulatory capacity. To evaluate the role of NKA α subunits on acclimation of sole to salinity, the aims of this work were: 1) identification and characterization of NKA α subunits in sole, 2) establishment of the relative abundance of NKA α subunits in juvenile tissues and during larval development, 3) transcriptional analysis of NKA α subunits responsiveness to salinity challenge in juveniles, and 4) mapping the change in the *atp1a1a* transcript amounts in developing larvae and in the gills of juveniles under a salinity challenge. The knowledge generated is the starting point to understand how this multigene family contributes to the variable osmoregulatory capacity of teleost fish. Moreover, since Senegalese sole is an economically important species both in fisheries and aquaculture, understanding the molecular mechanisms governing salinity adaptation will contribute to improvement of hatchery management techniques and growing procedures.

2. Materials and methods

2.1. Source of fish and experimental rearing conditions

Juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) samples to study tissue distribution of NKA α subunits were those reported previosly (Manchado et al., 2008b, 2009). Briefly, individuals (average mass = 23.2 ± 3.6 g; n = 3) were obtained from the facilities of IFAPA Centro *El Toruño* (El Puerto Santa María, Cádiz, Spain). They were sacrificed by immersion in 2-phenoxyetanol (300 ppm for 10 min). Liver, spleen, brain, gills, intestine, head kidney, heart, skeletal muscle, and skin were rapidly dissected out, frozen in liquid nitrogen, and stored at −80 °C.

To study the effects of osmotic conditions on mRNA abundance of the NKA α isoforms and their localization in the gill, we selected 150 juveniles (mean mass = 29.9 ± 0.8 g) that were acclimated in a tank at a temperature ranging from 17.6 to 18.3 ºC and a salinity that oscillated between 35.0 and 35.4 ppt. Ten days before experiment started, the temperature was reduced to 16.5 ºC (ranging 16–17 ºC) to fit better the environmental temperature conditions (January-February 2008) and to ensure the water temperature was maintained at 16–17 ºC during the experiment. During this acclimation period, animals were fed dry pellets (Skretting LE-2 Elite) provided by automatic feeders (approx. 1% biomass daily). Soles were maintained in a flow-through circuit under automatic control of temperature with 300% water renovation daily. Water supply was from the so-called “Rio San Pedro”. Before starting experiment, animals were distributed in six 100 L tanks (25 sole per tank) at a salinity of 35 ppt (35.0–35.3 ppt) under a natural photoperiod. After an acclimation period (24 h), water was completely replaced in less than half an hour establishing three different salinities in duplicate tanks: low-salinity water (5 ppt), control (35 ppt) and high-salinity water (60 ppt). Expected experimental salinities (low-salinity, control and high-salinity) were achieved by mixing seawater with dechlorinated tap water or marine salt depending on the condition. No food was provided during the experiment. Two individuals from each tank were initially sampled before salinity change (0 h); then three individuals per tank were sampled at 24, 48, 72, 96 and 168 h after salinity change. Animals were euthanized by phenoxyethanol overdose (300 ppm for 10 min). Gills, kidney and intestine were rapidly dissected out, frozen in liquid nitrogen and stored at −80 °C. Moreover, gill tissue from the 96 h sampling was fixed overnight in 4% paraformaldehyde at 4 ºC for ISH.

For larval studies, fertilized eggs from naturally spawning Senegalese sole broodstock (IFAPA Centro *El Toruño*) were collected in spring (April 2006) and separated by buoyancy. Water temperature for the broodstock tank (25 animals ratio 2 M:1 F) during spawning ranged between 17.5 and 20.2 ºC and salinity between 33.7 and 36.9 ppt. Breeders were fed polychaeta, mussels and squid. Eggs were incubated at a density of 2,000 eggs L^{-1} in 300 L cylindro-conical tanks in an open circuit containing gently aerated water at 20 ºC and a salinity of 36 ppt until 3 days post hatching (DPH). Larvae were then transferred to two 300 L tanks at an initial density of 45 to 50 larvae L^{-1} with a 16 L:8D photoperiod and light intensity of 300 lux. Larvae were fed rotifers (*Brachionus plicatilis*), previously enriched with *Isochrysis galbana* (T-ISO strain) cultivated at exponential phase, from 3 DPH until 9 DPH. T-ISO cells were also added (2 mg dry mass L^{-1} d^{-1}) directly to the larval culture tanks during the rotifer feeding stage. From 7 DPH up until the end of the experiment sole larvae were fed artemia metanauplii enriched with T-ISO. Larvae were sampled at 2, 3, 6, 7, 8, 9, 10, 11, 13, 15, 16, 19 and 22 DPH. In each sampling, three independent pools per tank were randomly collected. Each pool (from 10 to 100 larvae depending on the age) was collected using a 350-μm-mesh net, washed with DEPC water, placed in separate eppendorf tubes, frozen in liquid nitrogen and stored at −80 °C. To

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