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AVT is involved in the regulation of ion transport in the intestine of the sea bream (*Sparus aurata*)

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ABSTRACT

The intestine of marine fish plays a crucial role in ion homeostasis by selective processing of ingested fluid. Although arginine vasotocin (AVT) is suggested to play a role in ion regulation in fish, its action in the intestine has not been demonstrated. Thus, the present study investigated *in vitro* the putative role of AVT in intestinal ion transport in the sea bream (*Sparus aurata*). A cDNA encoding part of an AVT receptor was isolated and phylogenetic analysis revealed it clustered with the V1a2-type receptor clade. V1a2 transcripts were expressed throughout the gastrointestinal tract, from esophagus to rectum, and were most abundant in the rectum regardless of long-term exposure to external salinities of 12, 35 or 55 p.p.t. Basolateral addition of AVT (10^{-6} M) to the anterior intestine and rectum of sea bream adapted to 12, 35 or 55 p.p.t. mounted in Ussing chambers produced rapid salinity and region dependent responses in short circuit current (Isc), always in the absorptive direction. In addition, AVT stimulation of absorptive Isc conformed to a dose–response curve, with significant effects achieved at 10^{-8} M, which corresponds to physiological values of plasma AVT for this species. The effect of AVT on intestinal Isc was insensitive to the CFTR selective inhibitor NPPB (200 μM) applied apically, but was completely abolished in the presence of apical bumetanide (200 μM). We propose a role for AVT in the regulation of ion absorption in the intestine of the sea bream mediated by an absorptive bumetanide-sensitive mechanism, likely NKCC2.

1. Introduction

The intestine of marine fish plays a key role in ion regulation. The ionic disequilibrium of marine fish with their surrounding environment requires high rates of water ingestion (drinking) as part of the osmoregulatory process to compensate the dehydrating effect of seawater in the gills (Evans et al., 2005). In this context, ion assimilation from the ingested fluid is required to drive water absorption, making the role of the intestine vital to maintain extracellular homeostasis. Water absorption seems to rely on Cl^- uptake, which is mediated by an apical $Na^+/K^+/2Cl^-$ co-transporter (Musch et al., 1982) or apical Cl^-/HCO_3^- anion exchangers (Grosell, 2006, 2011), both mechanisms are active in the sea bream intestine (Carvalho et al., 2012; Gregorio et al., 2013). A basolateral Na^+,K^+-ATPase generates the electrogenic potential to facilitate apical Cl^- absorption (Ferlazzo et al., 2012). Accordingly, higher intestinal Na^+,K^+-ATPase activity is detected in seawater compared to freshwater trout (Fuentes et al., 1997) and is consistent with higher ATPase subunit expression in seawater compared to freshwater eels (Kalujnaia et al., 2007).

Arginine vasotocin (AVT) is a peptide secreted from the neurohypophysis of non-mammalian vertebrates, whereas arginine vasopressin (AVP) is the structural and functional counterpart in mammals (Acher, 1993). The AVT/AVP family exerts its endocrine action by binding specific plasma membrane receptors and has diverse physiological actions ranging from behavior, stress, reproduction, and vascular control to osmoregulation (Balment et al., 2006; Goodson, 2008; Kulczykowska, 2007; Mancera et al., 2008). Three AVP receptors (AVPRs) are described in mammals and retain a specific and well-established tissue distribution, e.g. the AVPR type V1a, is associated with vascular smooth muscle; the AVPR V1b, is in pituitary corticotrophs; and the AVPR V2 is in the kidney and linked to renal hydrosmotic actions (Mahlmann et al., 1994; Warne, 2001). In contrast, AVTRs in teleost fish have a broader tissue distribution. Thus, AVTR V1 or V2 subtypes have been described in the central nervous system and in tissues such as gill, kidney, gonads or the gastrointestinal tract (Konno et al., 2009; Lema, 2010).

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It seems likely that the prevailing action of the AVT/AVP system is directed to preservation of blood volume and osmolality (Warne et al., 2002). Thus, AVP stimulates for instance tubular Na^+ transport by activation of Na^+ channels present in the apical membranes of the kidney tubule (Mordasini et al., 2005; Schafer et al., 1990). Yet, in other epithelia, such as the bronchial epithelium, AVP stimulates Cl^- (and the resulting fluid) secretion via an NPPB-sensitive, likely CFTR dependent mechanism (Bernard et al., 2005). Other electrophysiological studies have demonstrated that AVP alters NaCl absorption with K^+ requirement in the mouse kidney through Na-K-2Cl (NKCC) co-transporter (Hebert and Andreoli, 1984; Sun et al., 1991), an action that seems to be mediated by recruitment of co-transporter to the apical membrane (Molony et al., 1987).

Several studies have demonstrated the importance of AVT in ion and water regulation in fish (Balment et al., 2006; Kulczykowska, 1997, 2001; Warne and Balment, 1995). Most of these studies focused on the effects of AVT in ion regulation and demonstrated it parallels AVP actions in mammals. For instance, in trunk kidney preparations of rainbow trout (Amer and Brown, 1995; Warne et al., 2002) and dogfish, *Scyliorhinus canicula* (Wells et al., 2002), AVT decreased urine output, an action probably linked to vascular effects. Additionally, a regulatory action of AVT on Cl^- secretion occurs in cultured branchial pavement cells from sea bass (*Dicentrarchus labrax)* (Avella et al., 1999; Guibbolini and Avella, 2003), and the effect is DPC-sensitive and likely mediated by CFTR. In fish, there is also indirect indication for a putative action of AVT in the intestine. Thus, intracerebroventricular injections of AVT caused a reduction in water intake in eels kept in seawater (Kozaka et al., 2003). Additionally, the presence of AVT receptors in the gastrointestinal tract of the rainbow trout was inferred from the action of AVT on contraction of intestinal strips *in vitro* (Conklin et al., 1999).

The physiological actions of AVT in the intestine of marine fish have received little attention despite its potential and intrinsic importance in ion uptake/secretion. Therefore, the present study determined AVT function in the gilthead sea bream (*Sparus aurata*) intestine by establishing: (i) the distribution of AVTR V1a2-type, (ii) the response of AVTR to modified external salinity and; (iii) the effect *in vitro* of AVT on ion absorption/secretion.

2. Materials and methods

2.1. Peptides and chemicals

Arginine vasotocin ([Arg8]-vasotocin acetate), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and bumetanide (Bum) of the highest grade were purchased from Sigma–Aldrich (Madrid, Spain).

2.2. Animals

Sea bream (*S. aurata*) juveniles were obtained from commercial sources (Cupimar S.A., Cádiz, Spain). Fish were quarantined for 60 days in Ramalhete Marine Station (University of Algarve, Faro, Portugal) in 1000 L tanks with running seawater at a density <5 kg/m^3 and handfed twice daily to a final ration of 2% body weight, with a commercial sea bream diet (Sorgal, Portugal). Fish were acclimated for at least 1 month before experimentation in flowing seawater (salinity 35 p.p.t.; water temperature 16–20 °C) under natural photoperiod for spring in the Algarve, Portugal. In all experiments, food was withheld for 36 h before sacrifice and tissue collection to ensure the absence of undigested food in the intestine.

For salinity adaptation juvenile sea bream (n = 90; 20–30 g body weight) were separated into three equal groups and transferred to 250 L tanks in three independent closed water circuits with biological filters maintained at final salinities of 12, 35 or 55 p.p.t., temperature of 21 °C and a 14:10 L:D photoperiod. Increase in salinity was achieved by adding Instant Ocean sea-salts to control seawater (35 p.p.t.), and decrease in salinity was achieved by dilution of full seawater with dechlorinated tap water. Fish were kept at different salinities for 2 months before tissue collection and were considered to be fully adapted (Laiz-Carrion et al., 2005). No mortality was registered during the trial and fish fed normally.

All animal manipulations were carried out in compliance with the Guidelines of the European Union Council (86/609/EU) and Portuguese and Spanish legislation for the use of laboratory animals. All animal protocols were performed under a "Group-I" licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas (Portugal).

2.3. Cloning of AVT receptor

For cloning of the AVT receptor degenerate primers were designed (Table 1) against highly conserved cDNA sequences of piscine species: *Platichthys flesus*: (GenBank accession number AF184966); *Astatotilapia burtoni*: (acc. No. AF517936); *Catostomus commersoni*: (acc. No. X76321); *Cyprinodon variegatus* (*V1a1*; acc. No. GU120189); *C. variegatus* (V1a2; acc. No. GU120190); *Cyprynodon nevadensis amargosae* (V1a1; acc. No. GU014233); *Cyprynodon nevadensis amargosae* (V1a2; acc. No. GQ981413). Tissues of unfed fish were collected into RNA Later (Sigma–Aldrich, Madrid, Spain) incubated for 24 h at 4 °C and stored at −20 °C until RNA extraction (within two weeks). Total RNA was isolated from brain, liver, kidney and gills using RNeasy® Plus MiniKit (Quiagen, Hidlen, Germany). Genomic DNA (gDNA) was eliminated with gDNA Eliminator spin columns (Quiagen, Hidlen, Germany). After reverse transcription of 500 ng total RNA (Super Script III, Invitrogen™, Paisley, UK) PCR amplifications were carried out with Platinum® Taq DNA Polymerase (Invitrogen) using the following PCR conditions: 94 °C, 1 min and 35 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min and finalized with 72 °C, 10 min. PCR products were cloned into TA Vectors (TOPO TA Cloning® Kit; Invitrogen™, Paisley, UK), sequenced and identity confirmed by interrogating NCBI databases using the blastn protocol (Altschul et al., 1990).

To establish receptor identity/subtype amino acid sequences were retrieved from the NCBI protein database (www.ncbi.nlm.nih.gov, accessed in January 2013) and phylogenetic analysis of AVTR/AVPR translated sequences was performed using MEGA5 software (Tamura et al., 2011) with the Close-Neighbor-Interchange algorithm, based on amino acid differences (p-distances) and pairwise deletions. Reliability of the phylogenetic tree was assessed using bootstrap values (1000 replicates).

2.4. AVTR V1a2 in the sea bream intestine

For AVTR V1a2-type expression analysis, specific primer pairs were designed using the software Primer3 (available in http://frod-

Table 1
Primers used for molecular identification of partial cDNA sequences of the AVT-R and for qPCR expression analysis.

	Nucleotide sequence
Degenerate primers	
AVT-RFw	5′-AGCGTSCTGCTGGCSATG-3′
AVT-RRv	5′-GCAKATRAASCCGTAGCA-3′
AVT-Rnested-Fw	5′-AARCACCTSAGCCTBGCBGA-3′
AVT-Rnested-Rv	5′-GTBATCCAGGTGATGTASGC-3′
qPCR primers	
AVTRFw	5′-TTATCTCAACGTGGATGTGCAG-3′
AVTRRv	5′-TTACCAGGTGATGTAGGCCTTG-3′
18sFw	5′-AACCAGACAAATCGCTCCAC-3′
18sRv	5′-CCTGCGGCTTAATTTGACTC-3′