

Water absorption and bicarbonate secretion in the intestine of the sea bream are regulated by transmembrane and soluble adenylyl cyclase stimulation

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Abstract In the marine fish intestine luminal, HCO_3^- can remove divalent ions (calcium and magnesium) by precipitation in the form of carbonate aggregates. The process of epithelial HCO_3^- secretion is under endocrine control, therefore, in this study we aimed to characterize the involvement of transmembrane (tmACs) and soluble (sACs) adenylyl cyclases on the regulation of bicarbonate secretion (BCS) and water absorption in the intestine of the sea bream (*Sparus aurata*). We observed that all sections of sea bream intestine are able to secrete bicarbonate as measured by pH-Stat in Ussing chambers. In addition, gut sac preparations reveal net water absorption in all segments of the intestine, with significantly higher absorption rates in the anterior intestine than in the rectum. BCS and water absorption are positively correlated in all regions of the sea bream intestinal tract. Furthermore, stimulation of tmACs (10 μM FK + 500 μM IBMX) causes a significant decrease in BCS, bulk water absorption and short circuit current (Isc) in a region dependent manner. In turn, stimulation of sACs with elevated HCO_3^- results in a significant increase in BCS, and bulk water absorption in the anterior intestine, an action completely reversed by the sAC inhibitor KH7 (200 μM). Overall, the results reveal a functional relationship between BCS and water absorption in marine fish intestine and modulation by tmACs and sAC. In light of the present observations, it is hypothesized that the endocrine effects on intestinal BCS and water

absorption mediated by tmACs are locally and reciprocally modulated by the action of sACs in the fish enterocyte, thus fine-tuning the process of carbonate aggregate production in the intestinal lumen.

Keywords Bicarbonate secretion · Intestinal tract · Marine fish · pH-Stat · Soluble adenylyl cyclase · cAMP · Transmembrane adenylyl cyclase · Ussing chamber · Water absorption

Introduction

The body fluids of teleost fish are hyperosmotic to fresh water (FW) and hypoosmotic to seawater (SW), and in these environments, fish tend to gain or lose water, respectively. The high osmolality of seawater tends to cause dehydration, as water is lost mainly through the gills. Thus, to maintain homeostasis in seawater (Fuentes and Eddy 1997b), high drinking rates are necessary and together with efficient water absorption in the intestine are essential for osmoregulation (Fuentes and Eddy 1997b; Whittamore et al. 2010). Imbibing high volumes of seawater leads to the exposure of the gastro-intestinal tract to high concentrations of Na^+ and Cl^- and large amounts of divalent ions i.e. Ca^{2+} , Mg^{2+} and SO_4^{2-} (Kurita et al. 2008; Whittamore et al. 2010).

A primary step of net ion assimilation from imbibed water takes place in the esophagus and is driven by NaCl via a $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (NKCC) co-transporter (Hirano and Mayer-Gostan 1976; Parmelee and Renfro 1983) and decreases fluid osmolality to facilitate water absorption in the intestine. Water absorption in seawater fish intestine is believed to take place by two distinct pathways, (1) a transcellular pathway that involves aquaporins and (2) a paracellular pathway that

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involves tight junctions (Cerdà and Finn 2010; Marshall and Grosell 2006; Martinez et al. 2005; Raldúa et al. 2008; Whittamore 2011; Wood and Grosell 2012).

Regardless of the route of water absorption it appears that Cl^- uptake is the driving force for the majority of water absorption in intestinal epithelia. The importance of a bumetanide-sensitive mechanism mediated by apical NKCC co-transporters has been established (Loretz 1995). However, recent studies indicate that apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers are also involved in chloride uptake (Grosell 2006; Grosell et al. 2005; Kurita et al. 2008; Taylor et al. 2010; Wilson et al. 2002). Moreover, apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers are responsible for the secretion of high amount of HCO_3^- into intestinal luminal fluid to raise the pH and promote the removal of divalent cations Ca^{2+} and Mg^{2+} by precipitation in the form of carbonate aggregates (Grosell 2011; Kurita et al. 2008; Wilson and Grosell 2003). The formation of carbonate aggregates reduces luminal fluid osmotic pressure (Kurita et al. 2008; Whittamore 2011; Wilson and Grosell 2003) and allows net water absorption. As a result, a strong relationship between bicarbonate secretion (BCS) and water absorption probably occurs in marine fish intestine (Grosell et al. 2005), although this depends on the composition of the external environment. Furthermore, at least in the European flounder, an elevated calcium concentration in the lumen fluid is a strong stimulator of BCS (Cooper et al. 2010; Wilson et al. 2002), although calcium concentration, in parallel with NaCl concentration, decreases along the length of the intestinal tract. Studies to establish if a water absorption gradient related to BCS also exists along the length of the intestine are lacking.

Apical BCS in the intestinal lumen has two origins (Whittamore et al. 2010; Wilson et al. 2002): intracellular resulting from the hydration of CO_2 and transcellular originating from plasma HCO_3^- that enters the cell via $\text{Na}^+/\text{HCO}_3^-$ co-transporters (Grosell 2006). In addition to luminal divalent aggregate formation, it appears that HCO_3^- has an important role in water absorption by activation of soluble adenylyl cyclase (sAC) (Tresguerres et al. 2010a). sAC activation has been suggested to induce, via the cAMP signaling pathway, recruitment to the membrane of transporters related to cellular acid–base regulation, such as the apical NKCC and the basolateral Na^+/K^+ ATPase, thus promoting NaCl and water absorption via cAMP stimulation. sACs belong to the adenylyl cyclase family which also comprises transmembrane adenylyl cyclases (tmAC) (Buck et al. 1999). sAC is suggested to be regulated by cytoplasmic HCO_3^- and Ca^{2+} and, unlike tmAC, is insensitive to forskolin (FK) (Litvin et al. 2003; Tresguerres et al. 2011). In higher vertebrates, sAC is suggested to locally modulate tmAC activation resulting from endocrine actions by regulation of functional signaling microdomains (Tresguerres et al. 2011). In marine fish sAC has

been reported in the intestine of the Gulf toadfish (*Opsanus beta*) and in dogfish (*Squalus acanthias*) gills (Tresguerres et al. 2010a, b).

In seawater fish drinking, water absorption and BCS are under endocrine control. For example, cortisol increases drinking in *Salmo salar* and *Ocorhynchus mykiss* upon transfer to seawater (Fuentes et al. 1996). Further, stimulation of the renin-angiotensin system induced higher drinking rates in *Salmo salar* (Fuentes and Eddy 1997b). In contrast, parathyroid hormone-related protein (PTHrP), a hypercalcemic factor, influences water balance by reducing drinking rate of sea bream larvae (Guerreiro et al. 2001). Moreover, a reciprocal negative interaction has been shown between PTHrP and cortisol in sea bream (Guerreiro et al. 2006). 17β -Estradiol (E_2) prevents water absorption, BCS and carbonate aggregate precipitation in rainbow trout intestine (Al-Jandal et al. 2011), but its action is likely indirect and through the action of PTHrP (Fuentes et al. 2007) on its receptor PTH3R (Rotllant et al. 2006). Additionally, PTHrP that uses cAMP as a secondary messenger in enterocytes (Rotllant et al. 2006), reduces BCS in fish intestine, while stanniocalcin-1 increases BCS and likely increases carbonate aggregate formation (Fuentes et al. 2010). The actions of PTHrP in drinking (Guerreiro et al. 2001) and bicarbonate secretion (Fuentes et al. 2010) suggest that calciotropic hormones may participate in the regulation of water balance, but their mode of action is not understood. Additionally, it has been demonstrated that both serotonin and vasoactive intestinal polypeptide reduce NaCl and water absorption via tmACs in marine fish intestine (Bakker et al. 1993; Trischitta et al. 1996, 1999). Although several studies have demonstrated the hormonal control of drinking and NaCl absorption in fish intestine, little information is available about the hormonal activation of tmACs and interaction with sACs in relation to BCS, ion, and water regulation. Therefore, the main objective of the present study was to characterize the role of tmACs and sAC cAMP-dependent mechanisms in BCS and water absorption in the intestine of the marine teleost, sea bream (*Sparus aurata*).

Materials and methods

Animals

Sea bream (*Sparus aurata*) juveniles were donated by CUPIMAR SA. (Cadiz, Spain) and transported to Ramalhete Marine Station (CCMAR, Faro, Portugal). Fish were maintained for 60 days in 1,000 l tanks with running seawater at a density $<5 \text{ kg/m}^3$ and fed 2 % ration (fish wet weight, Sorgal, S.A., Portugal; Balance 3) twice daily up until the experiments. For the experiments, $67.2 \pm 3.2 \text{ g}$ fish were transferred to 500 l tanks in a closed circuit

(35 ppt) with a biological filter, water temperature of 18–21 °C and 12 h light/dark photoperiod. Food was withheld for 36 h before sacrifice. No mortality was observed during the experiments.

The experiments conducted comply with the guidelines of the European Union Council (86/609/EU). All animal protocols were performed under a “Group-1” licence from the Direcção-Geral de Veterinária, Ministério da Agricultura, do Desenvolvimento Rural e das Pescas, Portugal.

Chemicals and reagents

All reagents were analytical grade and obtained from Sigma-Aldrich (Spain). In experiments, forskolin (FK) was used at 10 μM , 3-isobutyl-1-methylxanthine (IBMX) was used at 500 μM and (E)-2-(1H-benzo[d]imidazol-2-ylthio)-N'-(5-bromo-2-hydroxy benzylidene) propanehydrazide (KH7), a specific inhibitor of sAC, was used at a nominal concentration of 200 μM (Hallows et al. 2009; Schmid et al. 2007; Tresguerres et al. 2010a). However, the effective dose was likely much lower since, in agreement with the observation in toadfish experiments (Tresguerres et al. 2010a), immediate precipitation of KH7 was observed.

Bicarbonate secretion (BCS) in vitro pH-Stat

Fish were anesthetized in seawater containing 2-phenoxyethanol (1:2,000) and killed by decapitation. The abdominal region was exposed and the whole digestive tract removed and placed in pre-gassed serosal solution (160 mM NaCl, 1 mM MgSO_4 , 2 mM NaH_2PO_4 , 1.5 mM CaCl_2 , 5 mM NaHCO_3 , 3 mM KCl, 5.5 mM glucose and 5 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid); pH 7.800). The intestine was divided into 3 sections: the anterior intestine, which extends 3–4 cm caudal to the pyloric caeca; the middle intestine that is recognizable by its thinner musculature and terminates at the ileorectal sphincter; and the rectum, which is delimited by the ileorectal and anal sphincters and is 2–3 cm in length.

A segment of epithelium was excised from the region of interest and mounted on a tissue holder (model P2413, 0.71 cm^2 , Physiological Instruments, San Diego, US) and positioned between two half-chambers (P2400, Physiological Instruments, San Diego, US) containing 1.5 ml of physiological saline. The basolateral side contained pre-gassed serosal solution was continuously mixed by bubbling through 0.3 % CO_2 + 99.7 % O_2 . The apical side contained 88 mM NaCl, 9.5 mM MgCl_2 , 3 mM KCl, 7.5 mM CaCl_2 , 126.5 mM MgSO_4 and 1 mM Na_2HPO_4 ; pH 7.800 by pH-Stat; (Fuentes et al. 2006, 2010) and was continuously gassed with 100 % O_2 .

In experiments in which NaHCO_3 was omitted from the basolateral saline it was replaced with an equivalent

concentration of HEPES-Na (2-(4-(2-Hydroxyethyl) piperazinyl-1-ethanesulfonic acid sodium salt) and was mixed by bubbling through 100 % O_2 (160 mM NaCl, 1 mM MgSO_4 , 2 mM NaH_2PO_4 , 1.5 mM CaCl_2 , 5 mM HEPES-Na, 3 mM KCl, 5.5 mM glucose and 5 mM HEPES; pH 7.800). The temperature of the medium was maintained at 22 °C in all experiments. All bioelectrical variables were monitored by means of Ag/AgCl electrodes (with tip asymmetry <1 mV) connected to either side of the Ussing chamber with 3-mm-bore agar bridges (KCl, 1 M in 3 % agar). Voltage (mV) was monitored under current clamp of epithelia (0 μAmp). Epithelial resistance (R_t , Ωcm^2) or conductance (mS cm^{-2}) was calculated using the voltage deflections induced by a biphasic 2 s pulse of 10 $\mu\text{A cm}^{-2}$ every minute. Current injections were performed by means of a VCC 600 amplifier (Physiological Instruments, San Diego, US) and recorded onto a PC using a data acquisition system (LabTrax, WPI, Sarasota, US). For pH-Stat, a pH electrode (PHC 4000.8) and a microburette tip were immersed in the luminal saline and connected to a pH-Stat system (TIM 854, Radiometer, Copenhagen, Denmark) grounded to the amplifier to allow pulsing during titration. The characterization of bicarbonate secretion was performed on luminal saline at a physiological pH of 7.800 throughout the experiments. The pH values and the volume of acid titrant (HCl, 2.5 mM) were manually recorded. The total bicarbonate secreted was calculated from the titrant volume, the titrant concentration, and the surface area and is presented as $\text{nmol h}^{-1} \text{cm}^{-2}$ or $\text{nmol g}^{-1} \text{h}^{-1}$. The experimental protocol included 1 h of tissue stabilization, 30 min of stable control periods of bicarbonate secretion, when the bioelectric properties were monitored and a 30 min experimental period for FK + IBMX addition. Throughout the experimental procedures, basolateral saline was gassed 0.3 % CO_2 + 100 % O_2 in the basolateral side. However, when required for sAC activation and inhibition, tissue was stabilized for 1 h followed by 30 min of stable control periods of bicarbonate secretion (100 % O_2 in the basolateral side), when the bioelectric properties were monitored, plus 30 min experimental periods with 40 mM HCO_3^- basolateral followed by a 30 min experimental period with KH7 in the basolateral side (0.3 % CO_2 + 100 % O_2). FK + IBMX was used to directly stimulate tmACs. sAC is insensitive to FK, and stimulation was achieved by raising the basolateral HCO_3^- to 40 mM. sAC specific actions were exposed by the addition of its specific inhibitor KH7 in the presence of elevated HCO_3^- (Hallows et al. 2009; Schmid et al. 2007; Tresguerres et al. 2010a).

Voltage clamp in Ussing chambers

An intestinal segment of the region of interest was excised (anterior, middle and rectum in the case of FK + IBMX

experiments; only the anterior intestine in the case of sAC stimulation), mounted on a tissue holder (0.71 cm², model P2413, Physiological Instruments, US) and positioned between two half-chambers each containing 2 ml of pre-gassed serosal solution in which short circuit current (I_{sc} , $\mu\text{A cm}^{-2}$) was monitored by clamping epithelia to 0 mV. Epithelial resistance (R_t , $\Omega \text{ cm}^2$) and conductance (G_t , mS cm^{-2}) was manually calculated (Ohm's law) using the current deflection induced by a 2 mV pulse of 3 s every minute. Current injections were performed using a DVC-1,000 voltage clamp amplifier (WPI, Sarasota, US) or a VCC 600 amplifier (Physiological Instruments, San Diego, US). The apical side of the preparation was considered as the ground. Therefore, negative currents are absorptive, while secretory currents are positive.

The FK + IBMX experiments included bilateral addition, followed by monitoring for 40 min. To test the response to HCO_3^- increase, each concentration was added bilaterally and the bioelectric parameters monitored. After a steady state was achieved, usually within 25–30 min, a higher dose of bicarbonate was added (bilaterally).

Water absorption—gravimetric measurements

The intestinal tract was placed in oxygenated basolateral solution, flushed with saline, cleaned, and divided into three segments (anterior, middle and rectum) to form intestinal sacs. One of the edges of each segment was sealed with Teflon tape and the sac filled with 0.15–1 ml of pre-gassed serosal solution. Once filled, care was taken to remove gas bubbles and a second ligature was applied to create a watertight preparation with an internal pressure of 15 cm of water using PE 50 polythene tubing. In control experiments as well as for testing the effect of basolateral FK + IBMX addition, the sacs were maintained in pre-gassed serosal solution (0.3 % CO_2 + 99.7 % O_2) throughout the experiments.

To test the involvement of sAC in water absorption in the anterior intestine, NaHCO_3 was omitted from the saline and was replaced by HEPES-Na as described above. The sacs were maintained in oxygenated physiological saline bubbled with 100 % O_2 to measure water absorption (period 1). When NaHCO_3 (40 mM) was added to the medium, gassing was switched to 0.3 % CO_2 + 99.7 % O_2 , and maintained until the end of the experiments in the absence (period 2) or presence (period 3) of 200 μM KH7.

Water absorption was calculated by weighing the sacs at pre-determined intervals and registering weight loss to the nearest 0.1 mg. At the end of the experimental period, the sacs were opened, flattened, and laid out on millimetric paper to measure the surface area to the nearest 0.1 cm. Water absorption is presented as $\mu\text{l h}^{-1} \text{ cm}^{-2}$ or $\mu\text{l g}^{-1} \text{ h}^{-1}$.

Statistics

All results are shown as mean \pm standard error of the mean (mean \pm SEM). After assessing homogeneity of variance and normality, statistical analysis of the data was carried out using paired Student's *t* test, one-way analysis of variance or repeated measures analysis of variance as appropriate followed by the post hoc Bonferroni test (Prism 5.0, GraphPad Software for McIntosh). The level of significance was set at $p < 0.05$.

Results

Intestinal BCS and water absorption are region- and tmACs-dependent

BCS in discrete intestinal regions. The three regions of the sea bream intestine secrete HCO_3^- as measured in vitro by pH-Stat (Fig. 1). However, there was a gradual decrease in HCO_3^- secretion from the anterior intestine, which secretes the highest to the rectum, which secretes the lowest (Fig. 1a, $p < 0.05$, one-way ANOVA).

In a functional intestine, the surface area of the intestinal regions used in the study differs: anterior intestine $2.9 \pm 0.19 \text{ cm}^2$, middle intestine $6.4 \pm 0.46 \text{ cm}^2$, and rectum

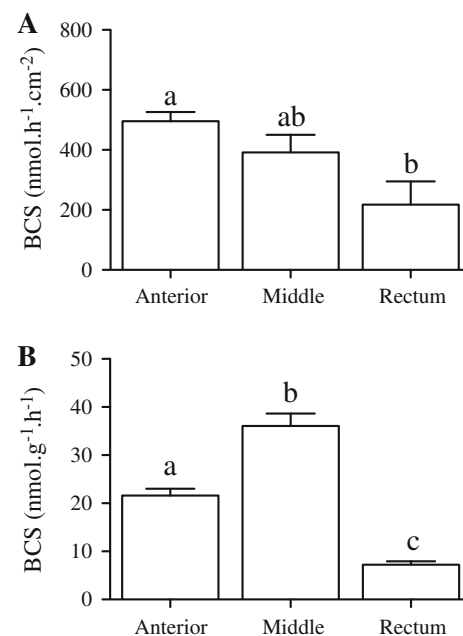
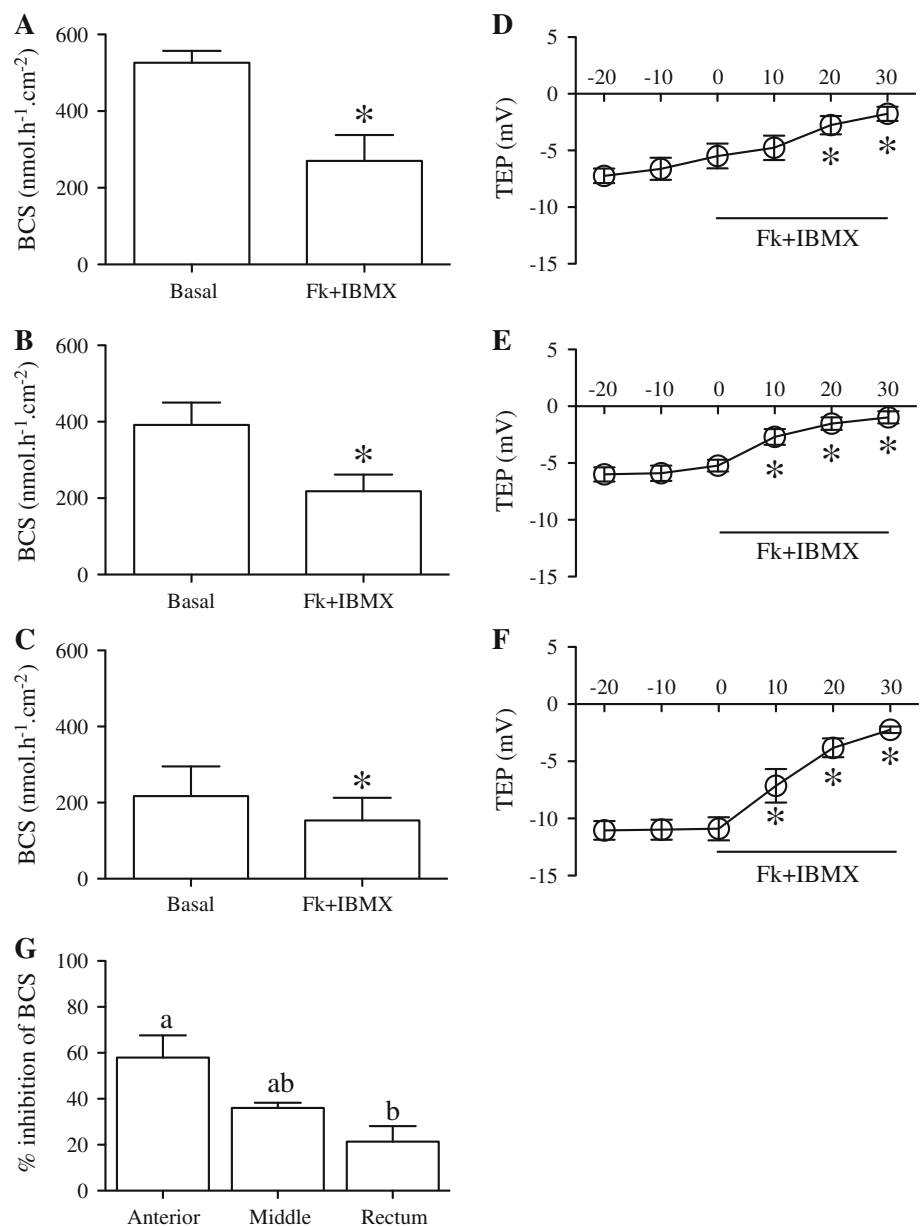


Fig. 1 Basal bicarbonate secretion (BCS, $\text{nmol h}^{-1} \text{ cm}^{-2}$) in, **a** discrete regions of the sea bream intestine (anterior, middle and rectum) mounted in Ussing chambers under current clamp ($I = 0 \mu\text{Amp}$) and measured by pH-Stat and **b** total BCS in each region per gram of animal ($\text{nmol g}^{-1} \text{ h}^{-1}$). Results are shown as mean \pm SEM ($n = 6$). Bars displaying different superscript letters are significantly different ($p < 0.05$, one-way ANOVA)

Fig. 2 Bicarbonate secretion (a–c; BCS, $\text{nmol h}^{-1} \text{cm}^{-2}$) and the corresponding transepithelial potentials (d–f; TEP, mV) in response to basolateral addition of 10 μM FK + 500 μM IBMX (FK + IBMX) to different regions of the sea bream intestine: a, c anterior intestine; b, d middle intestine; and c, e rectum. g Shows region-specific percentage inhibitions of basal BCS. Results are shown as mean \pm SEM ($n = 6$). Asterisks represent statistical differences ($p < 0.05$) from basal periods. In a–c paired Student's t test; in d–f one-way ANOVA repeated measures. In g groups displaying different superscript letters are significantly different ($p < 0.05$, one-way ANOVA)



$1.79 \pm 0.13 \text{ cm}^2$. After normalization for region-specific BCS rates using the total surface area of each intestinal region and fish mass, the contribution of each region to total intestinal HCO_3^- output differed. The middle intestine had the highest contribution to BCS, followed by the anterior region and the rectum (Fig. 1b; $p < 0.05$, one-way ANOVA).

Effect of FK + IBMX on intestinal BCS. Basolateral addition of FK + IBMX caused a significant decrease in BCS in all three regions of the intestine (Fig. 2a–c; $p < 0.05$, paired Student's t test). Additionally, TEP became less negative by 68 % in the anterior intestine, 83 % in the middle intestine, and 80 % in the rectum (Fig. 2d–f, $p < 0.05$, one-way ANOVA repeated measures).

The percentage inhibition of basal BCS in response to FK + IBMX (Fig. 2g) of the anterior and rectum intestinal region is significantly different ($p < 0.05$, one-way ANOVA repeated measures).

Water absorption in discrete intestinal regions. Negative values in water absorption rates indicate net absorption and are used for consistency with I_{sc} data. The anterior and middle intestine absorbs water at similar rates while the rate of water absorption in the rectum was significantly lower (Fig. 3a; $p < 0.05$, one-way ANOVA).

After normalization of specific water absorption rates using the total area of each intestinal region and fish mass, their contribution to total intestinal water absorption differed (Fig. 3b). The middle intestine has a significantly

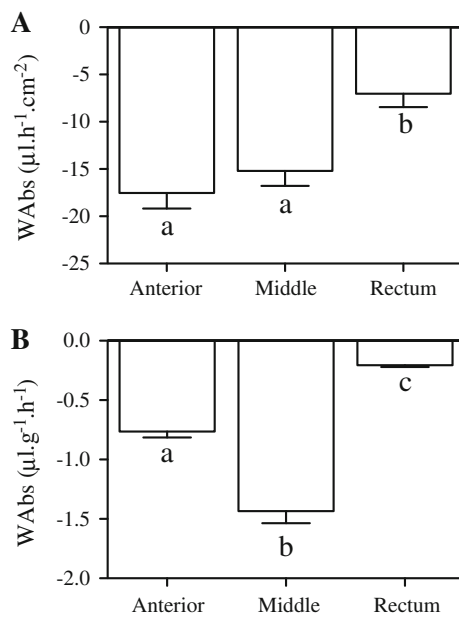


Fig. 3 Water absorption in the sea bream intestine. **a** Water absorption rates (WAbs, $\mu\text{l}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) in the intestine (anterior, middle and rectum) as measured in intestinal sacs by gravimetric methods and **b** total WAbs ($\mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) by the same regions normalized by contribution of specific regions to the whole body. Results are shown as mean \pm SEM ($n = 6\text{--}9$). Bars displaying different superscript letters are significantly different ($p < 0.05$, one-way ANOVA)

greater contribution to total intestinal water absorption ($59.6 \pm 4.3\%$), followed by the anterior intestine ($31.7 \pm 2.1\%$), and the rectum ($8.53 \pm 0.6\%$) ($p < 0.05$, one-way ANOVA). The water absorbed by the intestinal tract (from the point of insertion of pyloric caeca to rectum) per gram of fish was $-2.4 \pm 0.4\ \mu\text{l}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$.

Effect of FK + IBMX on intestinal water absorption. The basal water absorption in untreated intestinal sacs was constant in all regions analyzed over the duration of the experiments (Fig. 4a–c). Basolateral addition of FK + IBMX resulted in a significant 45–55 % inhibition in water absorption in the anterior and middle intestine with an effective response within 20 min after the onset of treatment (Fig. 4d–e; $p < 0.05$, one-way ANOVA repeated measures) but the rectum was not affected (Fig. 4f). The percentage inhibition of basal water absorption in response to FK + IBMX (Fig. 4g) is similar in the anterior and middle intestine and significantly lower in the rectum ($p < 0.05$, one-way ANOVA).

There is a strong positive relationship between specific rates (per cm^2) of basal intestinal water absorption and BCS through the intestinal regions ($R_{\text{basal}}^2 = 0.975$). FK + IBMX is able to decrease this relationship between water absorption and BCS ($R_{\text{FK+IBMX}}^2 = 0.421$).

Effect of FK + IBMX on short circuit current (Isc, $\mu\text{A}\cdot\text{cm}^{-2}$). Isc in the anterior intestine and the middle

intestine was absorptive with basal values of -9.2 ± 1.3 and $-6.9 \pm 1.3\ \mu\text{A}\cdot\text{cm}^{-2}$, respectively. In contrast, the rectum sustained a secretory current with a basal value of $+13.0 \pm 4.5\ \mu\text{A}\cdot\text{cm}^{-2}$ (Fig. 5). The application of bilateral FK + IBMX resulted in a significant decrease of Isc in the anterior intestine and middle intestine (Fig. 5a–b; $p < 0.05$, one-way ANOVA repeated measures), although it never became secretory. The rectum Isc was not modified in response to bilateral addition of FK + IBMX (Fig. 6c). Gt increased significantly in the anterior intestine and rectum after bilateral addition of FK + IBMX (Fig. 5d, f; $p < 0.05$, one-way ANOVA repeated measures) but was unchanged in the middle region (Fig. 5e).

Intestinal BCS and water absorption are sAC-dependent

HCO_3^- affects intestine short circuit current. Increasing bilateral concentrations of HCO_3^- by addition of NaHCO_3^- to 5, 10, 20 and 40 mM HCO_3^- tested the sensitivity of the intestinal epithelia to HCO_3^- , and is an indicator of sAC modulation of Isc and Gt. A significant increase of the Isc was observed at 20 and 40 mM HCO_3^- exposure (Fig. 6a; $p < 0.05$, one-way ANOVA repeated measures). Statistically significant increases of Gt were also observed at the same concentrations (Fig. 6b; $p < 0.05$, one-way ANOVA repeated measures).

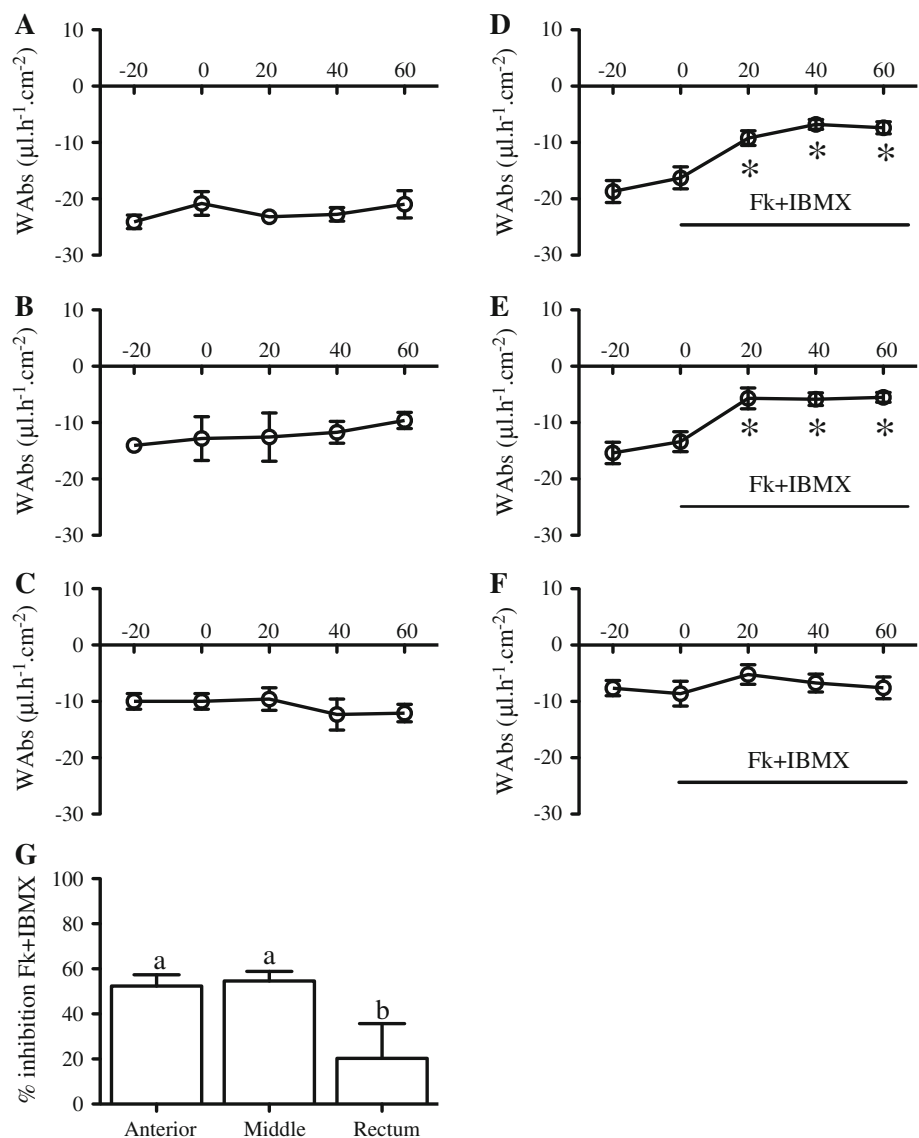
Effect of HCO_3^- and KH7 on apical BCS. Basolateral addition of 40 mM HCO_3^- caused a significant increase in apical BCS (Fig. 7a; $p < 0.05$, one-way ANOVA repeated measures). This effect was reversed by the sequential addition of 200 μM KH7. Gt increased significantly from basal only in the presence of both HCO_3^- and KH7 (Fig. 7b; $p < 0.05$, one-way ANOVA repeated measures).

Effect of KH7 on water absorption. Basolateral addition of 40 mM HCO_3^- caused a significant increase in bulk water absorption (Fig. 8; $p < 0.05$, one-way ANOVA repeated measures). Sequential addition of KH7 reversed the effect to levels significantly lower than control ($p < 0.05$, one-way ANOVA repeated measures).

Discussion

All regions of the sea bream intestine are able to absorb water and secrete HCO_3^- . They are also sensitive to regulation by transmembrane adenylyl cyclases (tmACs) and soluble adenylyl cyclases (sACs), which act in opposite directions. Stimulation of tmACs in vitro with a combination of FK + IBMX results in a reduction of both epithelial BCS and bulk water absorption. In contrast, stimulation of sACs with elevated bicarbonate induced increases of BCS and bulk water absorption, which could

Fig. 4 Basal (a–c) water absorption (WAbs, $\mu\text{l h}^{-1} \text{cm}^{-2}$) and in response to basolateral addition of 10 μM FK + 500 μM IBMX (FK + IBMX) (d–f) to discrete regions of the sea bream intestine: a, d anterior intestine, b,e middle intestine and c, f rectum. g Shows region-specific percentage inhibitions of basal WAbs. Results are shown as mean \pm SEM ($n = 4$ –9). In d–f asterisks represent statistical differences from basal periods ($p < 0.05$, one-way ANOVA repeated measures). In g groups displaying different superscript letters are significantly different ($p < 0.05$, one-way ANOVA)



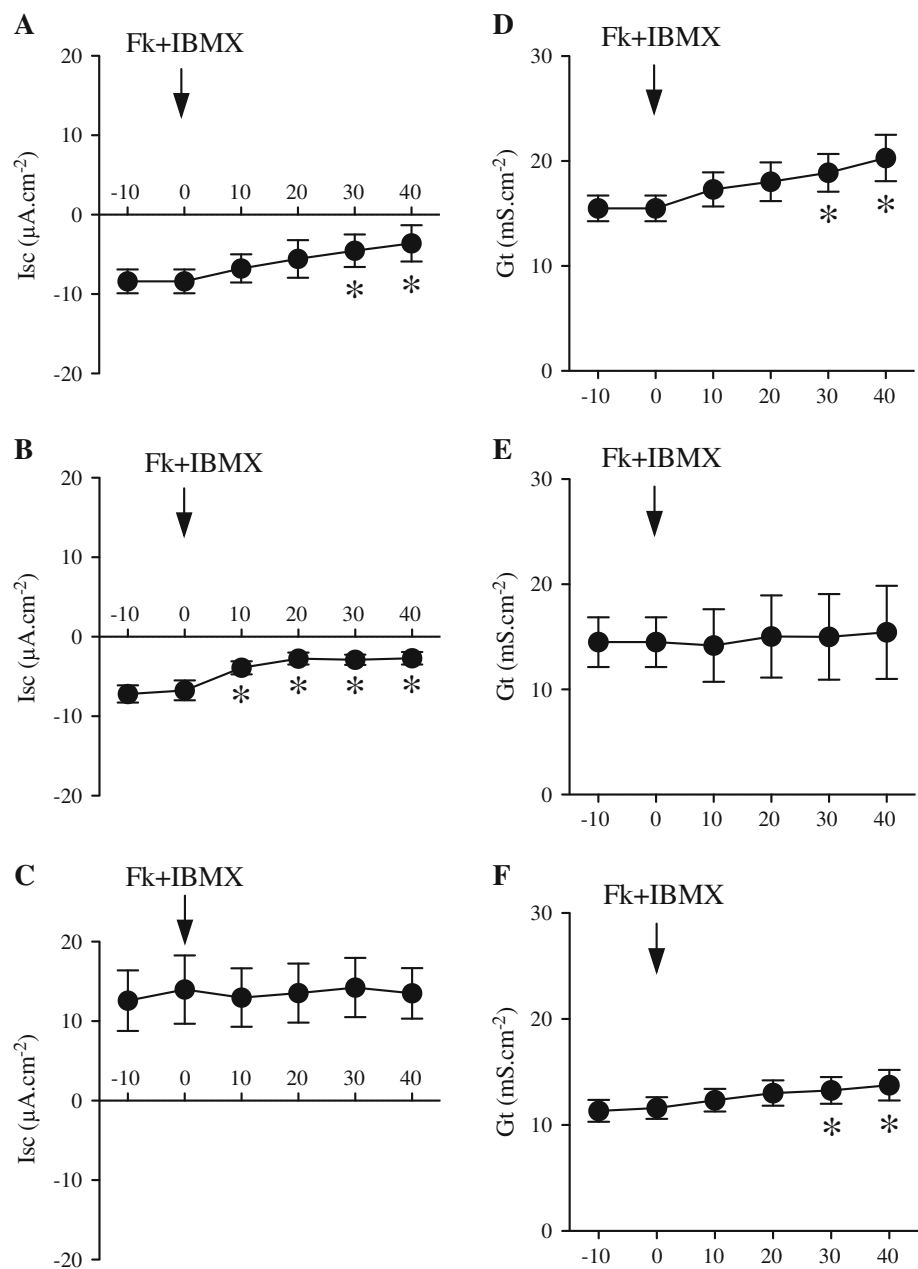
be abolished by the sAC specific inhibitor KH7. This demonstrates an integration of the endocrine effects on intestinal BCS and water absorption mediated by tmACs and the local chemical sensing mediated by the action of sACs in the fish enterocyte.

It is generally accepted that marine fish drink at rates between 2 and 7 $\text{ml kg}^{-1} \text{h}^{-1}$ (Fuentes and Eddy 1997a). The sea bream drinks 5 $\text{ml kg}^{-1} \text{h}^{-1}$ (Guerreiro et al. 2002), a rate slightly higher than those described for *Opsanus beta* (Genz et al. 2008; Grosell et al. 2004), *Salmo salar* (Fuentes et al. 1996) or *Oncorhynchus mykiss* in seawater (Fuentes et al. 1996; Wilson et al. 1996). In agreement with variable drinking rates, intestinal water absorption is also highly variable between species. Values in the range 38–85 % net absorption of imbibed water have been described (Genz et al. 2008; Wilson et al. 1996, 2002). In the present study, bulk water absorption by the

whole sea bream intestine was $2.4 \pm 0.4 \text{ ml kg}^{-1} \text{h}^{-1}$ which (Fig. 3), considering the published results for drinking in fish of similar size (Guerreiro et al. 2002), accounts for roughly 45 % of the imbibed water. However, while all intestinal regions are absorptive, different absorption rates are measured along the intestinal tract (Fig. 3), with higher absorption rates in the anterior and middle intestine, compared to the rectum. These results contrast with those reported in *Fundulus heteroclitus* where no difference in water absorption was reported between the anterior and posterior intestine using intestinal sac preparations (Marshall et al. 2002).

In keeping with the regionalization of water absorptive capacity in the intestinal tract of the sea bream, regionalization of BCS was also observed (Figs. 1 and 2), which is in good agreement with the observations in the Gulf toadfish (*Opsanus beta*), where higher rates of BCS were

Fig. 5 Short circuit current (a–c; I_{sc} , $\mu\text{A cm}^{-2}$) and the corresponding transepithelial conductances (d–f; G_t , mS cm^{-2}) in response to basolateral addition of 10 μM FK + 500 μM IBMX (FK + IBMX) to discrete regions of the sea bream intestine as measured in Ussing chambers under voltage clamp: **a, d** anterior intestine, **b, e** middle intestine and **c, f** rectum. Results are shown as mean \pm SEM ($n = 6$). Asterisks represent statistical differences ($p < 0.05$, one-way ANOVA repeated measures) from basal periods



described in the anterior intestine (Guffey et al. 2011). However, it contrasts with results from *Fundulus heteroclitus* (Genz and Grosell 2011), *Parophrys vetulus* and *Citharichthys sordidus* (Wilson et al. 2002) where similar BCS rates were shown for anterior and posterior intestine.

BCS and water absorption are FK-dependent

FK is a well-known activator of tmACs and IBMX is used to inhibit the action of phosphodiesterases that can decompose cAMP (Buck et al. 1999; Litvin et al. 2003; Tresguerres et al. 2010a, 2011). The use of FK in combination with IBMX is common practice to increase

intracellular cAMP. IBMX is a non-specific inhibitor of phosphodiesterases that could also increase intracellular cGMP. No studies have been performed in fish to substantiate isolated effect of IBMX in cGMP accumulation. However, IBMX alone was without effect in cGMP levels in cultured human airway smooth muscle cells (Hamad et al. 1997) or the human colonic adenocarcinoma cell line T84 (Sopory et al. 2004).

FK induces tmAC activation, coupled to G-proteins and intracellular cAMP signaling. A number of endocrine factors, such as serotonin and vasoactive intestinal polypeptide (Bakker et al. 1993; Trischitta et al. 1996, 1999) that are known to act at the level of the intestinal tract use

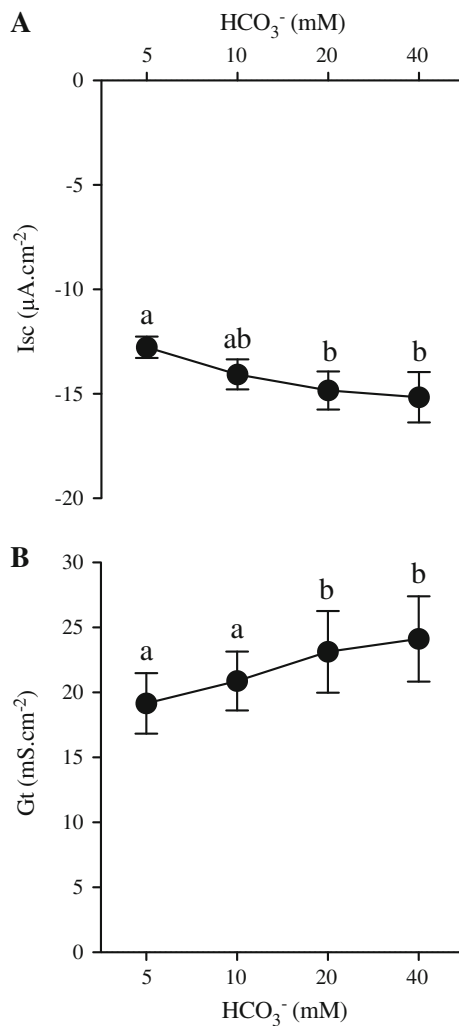


Fig. 6 **a** Short circuit current (Isc, μA cm⁻²) and **b** epithelial conductance (Gt, mS cm⁻²) in the anterior intestine of the sea bream in response to bilateral increases of HCO₃⁻ (5, 10, 20 and 40 mM), as measured in Ussing chambers under voltage clamp. Each point represents values obtained after consecutive 30 min stimulations for each HCO₃⁻ concentration (mean ± SEM, n = 3). Asterisks represent significant differences between concentrations (p < 0.05, one-way ANOVA repeated measures)

intracellular cAMP signaling. Of special interest is PTHrP that stimulates cAMP production in isolated sea bream enterocytes presumably via the PTH3R (Rotllant et al. 2006), and more importantly decreases BCS measured in Ussing chambers (Fuentes et al. 2010). The present results (Fig. 2) confirm our previous observations that treatment with FK + IBMX (tmACs activation) results in responses similar to those of PTHrP on BCS (Fuentes et al. 2010). Moreover, the preceding results are congruent with the intestinal effects of serotonin and vasoactive intestinal polypeptide (which act via tmACs) on NaCl and water absorption in the goldfish and eels (Bakker et al. 1993; Trischitta et al. 1996, 1999).

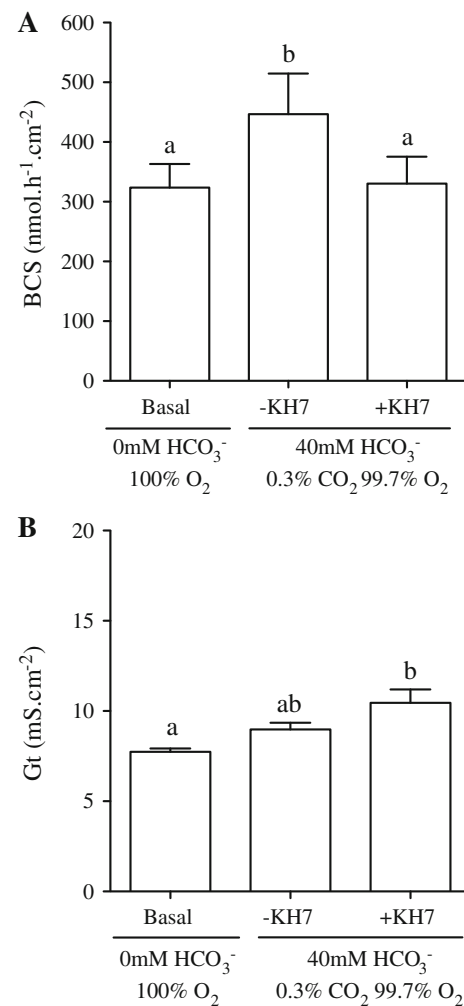


Fig. 7 **a** Bicarbonate secretion (BCS, nmol h⁻¹ cm⁻²) in the anterior intestine of the sea bream in 3 consecutive 30 min periods in basal control conditions (Basal, basolateral 0 mM HCO₃⁻, 100 % O₂), in response to basolateral addition of 40 mM HCO₃⁻ (0.3 % CO₂ in O₂) and subsequent basolateral addition of the sAC inhibitor KH7 (200 μM). **b** Corresponding transepithelial conductance (Gt, mS cm⁻²). Each column represents mean ± SEM (n = 6). Different subscript letters indicate significant differences between treatments (p < 0.05, one-way ANOVA repeated measures)

In the intestine of the killifish, *Fundulus heteroclitus* (Marshall et al. 2002), co-stimulation with the calcium ionophore ionomycin and a combination of the permeable cAMP analog dibutyryl-cyclic AMP + IBMX are required to make the epithelium secretory in relation to both fluid and NaCl. In the sea bream, activation of tmACs (ionomycin absent) with a combination of FK + IBMX does not evoke a shift from net absorption to water secretion, as shown by both the decrease of bulk water absorption in intestinal sacs (Fig. 4) and the decrease of short circuit current (Isc) in the absorptive epithelia anterior and middle intestine (Fig. 5), which never became secretory.

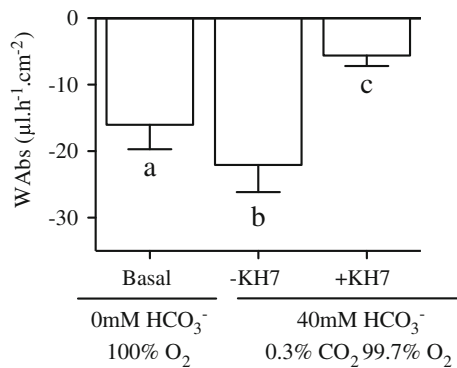


Fig. 8 Water absorption (WAbs, $\mu\text{l h}^{-1} \text{cm}^{-2}$) in the anterior intestine of the sea bream in 3 consecutive 30 min periods in basal control conditions (Basal, basolateral 0 mM HCO_3^- , 100 % O_2), in response to basolateral addition of 40 mM HCO_3^- (0.3 % CO_2 in O_2) and subsequent basolateral addition of the sAC inhibitor KH7 (200 μM). Each column represents mean \pm SEM ($n = 8$). Different subscript letters indicate significant differences between treatments ($p < 0.05$, one-way ANOVA repeated measures)

This study has not identified the specific molecular mechanisms that drive the decrease of BCS and water absorption in response to FK + IBMX. However, there is previous evidence of the contribution of apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers to Cl^- driven water absorption in the intestine of marine fish (Grosell et al. 2005). That would be in agreement with the following observations: (1) decreased TEP; (2) decreased BCS and (3) decreased water absorption observed in the sea bream intestine.

The regulatory actions of FK + IBMX in the sea bream intestine contrast with those in mammals where stimulation of different epithelia with FK + IBMX results either in stimulated water absorption or in apical secretion. For example in the renal thick ascending cell system, treatment with FK + IBMX stimulates recruitment of the NKCC2 co-transporter to the apical membrane to enhance NaCl absorption and subsequently water reabsorption (Caceres et al. 2009). In the mouse gallbladder (Martin et al. 1998), murine duodenum (Clarke and Harline 1998) and even the submucosal glands of the piglet esophagus, the combination stimulates apical secretion of HCO_3^- . In the airway epithelium (Smith and Welsh 1992), in mouse intestine (Seidler et al. 1997) and pancreas (Quinton 2010) with defective CFTR activity, FK + IBMX fails to stimulate HCO_3^- or Cl^- secretion. The different response of mammalian and fish intestinal epithelia to FK + IBMX may be explained by the putatively different function of BCS. While in terrestrial vertebrates BCS functions in protection and as a stabilizer of mucous secretion (Quinton 2010), in fish it serves, amongst other functions, as a facilitator of water absorption and calcium homeostasis (Fuentes et al. 2010; Grosell 2006; Wilson and Grosell 2003). The divergent role of BCS in mammals and fish may have led to

the evolution of different endocrine and intracellular control mechanism and may explain the differences identified in fish.

BCS and water absorption are KH7-dependent

The majority of intracellular cAMP is generated through the stimulation of tmACs, although cAMP can also be generated by tmAC-independent routes such as the acid–base sensor sAC (Buck et al. 1999; Litvin et al. 2003; Tresguerres et al. 2010a, 2011). sAC is activated by intracellular concentration of HCO_3^- and may modulate the recruitment of membrane transporters required in acid–base regulation (Tresguerres et al. 2010a, 2011). In the fish enterocytes, HCO_3^- is generated and transported for apical secretion at such high rates that it has been proposed to act as a potential stimulator of sAC activity (Tresguerres et al. 2011). For example, in the intestine of the Gulf toadfish a sAC system has been shown to serve as a modulator of absorptive short circuit current (Tresguerres et al. 2010a). In the present study in the sea bream, increased concentrations of HCO_3^- evoked increases of short circuit current in the anterior intestine that indicates sAC activation (Fig. 6). Further characterization demonstrated that raised HCO_3^- induced both an increase in BCS as measured by pH–Stat (Fig. 7) and bulk water absorption in intestinal sac preparations (Fig. 8). In addition, the specific sAC inhibitor KH7 abolished the increase of both BCS and water absorption. The role of sAC in water absorption in the Gulf toadfish shows activation of apical NKCC co-transporters (Tresguerres et al. 2010a). However, this mechanism may not totally explain the results of the present study with the intestine of the sea bream. KH7 in the presence of 40 mM HCO_3^- (0.3 % CO_2 in O_2) eliminated the increase in BCS evoked by raised HCO_3^- to pre-treatment levels. Nevertheless, KH7 reduced the water absorption to levels far lower than basal. It would be tempting to suggest that the effects of KH7 in water absorption are mediated possibly through additional effects on aquaporin inactivation.

The HCO_3^- dependent stimulation of absorptive short circuit current, apical BCS, water absorption, together with the specific inhibitory effects of KH7 point to the presence and functional importance of a sAC in the intestine of the sea bream. However, to establish the presence of a functional sAC in the intestine of marine fish further characterization with genomic/transcriptomic tools is required.

The proposed modes of water absorption in fish intestine involve aquaporins (transcellular pathway) or modification of tight junctions (paracellular permeability) (Cerdà and Finn 2010; Marshall and Grosell 2006; Martinez et al. 2005; Raldúa et al. 2008; Whittamore 2011). In the present study, we detect increases of epithelial conductance (Gt, electric expression of paracellular permeability) in voltage

clamp experiments that coexist with a decrease in water absorption as shown both by decreased short circuit current and by bulk water absorption in intestinal sacs. Our hypothesis is supported by a recent study in the *Fundulus heteroclitus* intestine (Wood and Grosell 2012) demonstrating that intestinal water absorption is HgCl_2 sensitive, thus likely aquaporin mediated. In functional terms, this implies a transcellular and not a paracellular pathway for the regulation of water absorption.

The present study demonstrates that all regions of the sea bream intestine are able to secrete bicarbonate and absorb water and that both processes are cAMP sensitive. However, the source of the enzyme generating cAMP in fish enterocytes is crucial and membrane-bound adenylyl cyclase (FK + IBMX-sensitive/ HCO_3^- insensitive) down regulates BCS and water absorption, while soluble adenylyl cyclases (FK + IBMX-insensitive/ HCO_3^- -sensitive) enhances BCS and water absorption. In a whole body context, these results demonstrate that the endocrine effects on intestinal BCS and water absorption evoked by endocrine stimulation via tmACs are locally modulated by the action of sACs in the fish enterocyte. sAC acts as a chemical sensor (to $\text{CO}_2/\text{HCO}_3^-$ and likely Ca^{2+}) to locally integrate the cellular response of chemical sensing and endocrine control (tmAC).

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