The differential role of renoguanylin in osmoregulation and apical Cl^-/HCO_3^- exchange activity in the posterior intestine of the Gulf toadfish (*Opsanus beta*)

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[1]*Department of Marine Biology and Ecology, The Rosenstiel School of Marine and Atmospheric Science, The University of Miami, Miami, Florida;* [2]*Department of Marine Bioscience, The Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwa, Chiba, Japan*

Ruhr IM, Mager EM, Takei Y, Grosell M. The differential role of renoguanylin in osmoregulation and apical Cl^-/HCO_3^- exchange activity in the posterior intestine of the Gulf toadfish (*Opsanus beta*). *Am J Physiol Regul Integr Comp Physiol* 309: R399–R409, 2015. First published May 27, 2015; doi:10.1152/ajpregu.00118.2015.— The guanylin family of peptides are effective regulators of intestinal physiology in marine teleosts. In the distal intestinal segments, they inhibit or reverse fluid absorption by inhibiting the absorptive short-circuit current (I_{sc}). The present findings demonstrate that mRNA from guanylin and uroguanylin, as well as at least one isoform of the guanylin peptide receptor, apical guanylyl cyclase-C (GC-C), was highly expressed in the intestine and rectum of the Gulf toadfish (*Opsanus beta*). In the posterior intestine, GC-C, as well as the cystic fibrosis transmembrane conductance regulator and basolateral $Na^+/K^+/2Cl^-$ cotransporter, which comprise a Cl^--secretory pathway, were transcriptionally upregulated in 60 parts per thousand (ppt). The present study also shows that, in intestinal tissues from Gulf toadfish held in 35 ppt, renoguanylin (RGN) expectedly causes net Cl^- secretion, inhibits both the absorptive I_{sc} and fluid absorption, and decreases HCO_3^- secretion. Likewise, in intestinal tissues from Gulf toadfish acclimated to 60 ppt, RGN also inhibits the absorptive I_{sc} and fluid absorption but to an even greater extent, corresponding with the mRNA expression data. In contrast, RGN does not alter Cl^- flux and, instead, elevates HCO_3^- secretion in the 60-ppt group, suggesting increased apical Cl^-/HCO_3^- exchange activity by SLC26a6. Overall, these findings reinforce the hypotheses that the guanylin peptide system is important for salinity acclimatization and that the secretory response could facilitate the removal of solids, such as $CaCO_3$ precipitates, from the intestine.

osmoregulation; HCO_3^- secretion; hypersalinity; marine teleost; fluid secretion

THE VERTEBRATE INTESTINE is a multifunctional organ whose role and function is controlled by various hormones. Among these is the guanylin family of peptides, whose members modify the intestinal physiology of terrestrial mammals and teleost fish. In mammals, the intestinal guanylin family consists of guanylin (GN) and uroguanylin (UGN), which are upstream regulators of the apical guanylyl cyclase-C (GC-C) in the intestine (6, 45, 46). Activation of GC-C results in direct cyclic guanosine monophosphate (cGMP) and indirect cyclic adenosine monophosphate (cAMP) production, and activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) by phosphorylation, as well as the modulation of phosphodiesterase (PDE) activity), all of which regulate the activities of a variety of cellular proteins and ion transporters (5, 6).

Accordingly, this control over cellular processes distinguishes GN and UGN as potent modulators of ion flux in the mammalian intestine. Through GC-C activation, GN and UGN activate an apical cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and stimulate Cl^- secretion, which drives fluid secretion into the intestinal lumen (15, 28, 31). In addition, they modulate transepithelial absorption of Na^+ and secretion of HCO_3^- in the intestine (5, 7, 10, 51). HCO_3^- secretion is upregulated to maintain a stable intestinal pH when acidic chyme enters the intestine from the stomach (7, 25), whereas fluid and net Cl^- secretion has been proposed to maintain osmotic homeostasis after the ingestion of a meal (30). Indeed, salt loading of rat intestinal tissues leads to increases in GN mRNA abundance and peptide secretion, leading to fluid secretion that seems to compensate for any increases in intestinal osmolality (8, 30, 33).

Teleost fish also express GN and UGN, as well as a third homologue, renoguanylin (RGN), in both the intestine and kidneys (48, 49). Although their physiological role is still uncertain, these peptides most likely play a part in salinity acclimatization because GN and UGN are transcriptionally upregulated in both the European eel (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*), following transfer from freshwater (FW) to seawater (SW) (11, 13, 29, 53). This response seems counterintuitive, because marine teleosts must continually absorb water across the intestinal epithelium to combat dehydration in the marine environment (20). However, the guanylin peptide system stimulate fluid secretion in the posterior intestine of the Gulf toadfish (*Opsanus beta*) (41) and, consequently, must serve an important function.

Interestingly, whereas GN and UGN have a physiological effect over the entire length of the mammalian intestinal tract (7, 14, 25, 31), their effects on the Japanese eel and Gulf toadfish are apparently restricted to the mid and posterior intestine, where they reverse the absorptive short-circuit current (I_{sc}) (4, 41, 54). It has been demonstrated that, similarly to mammals, a switch from net Cl^- absorption to net Cl^- secretion, associated with the reversal of the I_{sc} (from mucosa-to-serosa to serosa-to-mucosa), drives net fluid secretion in the Gulf toadfish posterior intestine (41) and coincides with the inhibition of fluid absorption in the Japanese eel mid and posterior intestine (3, 4). Surprisingly, unlike in mammals (7, 25), HCO_3^- secretion is partially inhibited by RGN in the Gulf toadfish posterior intestine (41). Although unexpected, the inhibition of HCO_3^- secretion in the Gulf toadfish might pro-

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mote increased fluid secretion into the intestinal lumen by decreasing Cl^- absorption and $CaCO_3$ precipitation the latter of which, which would normally reduce luminal osmolality by up 100 mosmol/kg H_2O (20). Moreover, the absorption of Cl^- and secretion of HCO_3^- are intimately associated, in the marine teleost, by SLC26a6, an electrogenic apical Cl^-/HCO_3^- antiporter (32) that exchanges up to 70% of intestinal Cl^- uptake for intracellular n HCO_3^- molecules (20). Conversely, there is also evidence for a Cl^--secretory pathway in the distal segments of the marine teleost intestine, composed of apical CFTR and a basolateral $Na^+/K^+/2Cl^-$ cotransporter, NKCC1 (SLC12a2) (4, 36, 41, 46), whose transport rates might be elevated by the guanylin peptides. Moreover, contrary to what might occur in mammals (45), there is no evidence that CFTR secretes HCO_3^- in the Gulf toadfish posterior intestine (41). Considering these effects, because one of the major Cl^- uptake pathways in marine teleosts is through SLC26a6 and the guanylin peptides stimulate net Cl^- secretion, it is likely that these peptides inhibit the exchange activity of SLC26a6, leading to the observed decreases in HCO_3^- secretion in the Gulf toadfish posterior intestine. Overall, the guanylin peptides either promote fluid secretion or hinder fluid absorption in the mid and posterior intestine of marine teleosts by possibly inhibiting NKCC2 (SLC12a1) and SLC26a6, and stimulating NKCC1 and apical CFTR, resulting in net Cl^- secretion.

However, it is still unclear what physiological role the guanylin peptides play in marine teleosts. It has been hypothesized that the guanylin peptides only affect Cl^- flux in the mid and posterior portions of the marine teleost intestine to fuel apical NKCC2 ion uptake and maintain fluid uptake (4, 54). In the marine teleost intestine, the concentration of Cl^- in the intestine decreases toward the rectum, while the concentration of divalent cations (Ca^{2+}, Mg^{2+}, and SO_4^{2-}) increases, making fluid absorption increasingly difficult (20, 35). However, this hypothesis may not apply universally, because some marine teleost species, including the Gulf toadfish, rely extensively on SLC26a6 as a major transporter of intestinal Cl^- uptake. Conversely, an alternative hypothesis proposes that the secretory response, initiated by the guanylin peptides, facilitates the removal of solids, such as undigested food or $CaCO_3$ precipitates, from the intestine (41). It is possible that, because of the narrower diameter of the posterior intestine relative to the anterior portion, a secretory response would be needed to promote solids to move more easily into the rectum.

To test the latter hypothesis, the present study examined Gulf toadfish in control (35 parts per thousand, ppt) and hypersaline (60 ppt) SW because hypersalinity leads to increased intestinal $CaCO_3$ precipitation (18). We hypothesized that acclimation to 60 ppt would lead to transcriptional upregulation of the components of the guanylin peptide system, made up of GN, UGN, GC-C, NKCC1, and CFTR, in the Gulf toadfish intestine. Greater expression of GN, UGN, and GC-C could lead to altered intracellular signaling (i.e., by stimulating or inhibiting ion-transporting mechanisms) with which to enhance the secretory response and, thus, elimination of $CaCO_3$ by increasing tissue responsiveness to RGN.

MATERIALS AND METHODS

Experimental animals. Gulf toadfish (*Opsanus beta*) were caught as bycatch from Biscayne Bay, FL, by fishermen. Upon arrival to the laboratory, Gulf toadfish were briefly placed in a FW bath (3 min) and

Table 1. *Composition of salines for short-circuit current, pH-stat titration, and intestinal sac preparation experiments*

Compound	Mucosal*	Serosal	HCO_3^-/CO_2 free
NaCl, mmol/l	69.0	151.0	151.0
KCl, mmol/l	5.0	3.0	3.0
MgSO₄, mmol/l	77.5	0.88	0.88
MgCl₂, mmol/l	22.5		
Na₂HPO₄, mmol/l		0.5	0.5
KH₂PO₄, mmol/l		0.5	0.5
CaCl₂, mmol/l	5.0	1.0	1.0
NaHCO₃, mmol/l		5.0	
HEPES, free acid, mmol/l		11.0	11.0
HEPES salt, mmol/l		11.0	11.0
Urea, mmol/l		4.5	4.5
Glucose, mmol/l		5.0	5.0
Osmolality, mosmol/kg H₂O§	325	325	325
pH	7.8†	7.8	7.8
Gas‡	100% O₂	0.3% CO₂ in O₂	100% O₂

*Mucosal application of renoguanylin: 10^{-9}-10^{-6} mol/l for short-circuit current and pH-stat experiments, 5×10^{-7} mol/l for intestinal sac preparations. †pH 7.80 was maintained by pH-stat titration. ‡Salines gassed for at least 1 h before experimentation. §Adjusted with mannitol to ensure transepithelial isosmotic conditions in all experiments.

then treated with malachite green to remove ectoparasites. Individuals were separated by size, and 8–10 were placed in aerated 62-l tanks, with continuous flow-through of sand-filtered SW from Biscayne Bay (34–36 ppt, 20–24°C). Gulf toadfish used for the present study were fed 5% of their body mass of squid 7 days before experimentation. Gulf toadfish held in experimental tanks were placed in groups of 6–8 and acclimated over 7 days to 35 ppt (control) or 60 ppt (hypersalinity), adjusted with Instant Ocean (SpectrumBrands). Each experimental tank was fitted with two recirculating pumps containing biological filters, and a water heater maintained temperature at $25.5 \pm 0.2°C$. Tanks were monitored daily for temperature and salinity. Fish husbandry and experimental procedures followed an approved University of Miami Animal Care Protocol (IACUC no. 13-225, renewal 02). All Gulf toadfish used for experimentation were killed using 0.2 g/l MS-222 (Argent) solution, buffered with 0.3 g/l $NaHCO_3^-$ (Sigma-Aldrich), followed by severing of the spinal cord at the cervical vertebra.

Composition of salines, hormones, and inhibitors. Japanese eel RGN was resuspended into 10^{-5} mol/l stock solutions in nano-pure water and stored in $-20°C$. Only eel RGN was used in the present study, because it elicits the strongest response from the Gulf toadfish posterior intestine compared to eel GN and UGN (41). Moreover, the amino acid sequence for eel RGN, ADLCEICAFAACTGCL (Gen-Bank accession number: BAC76010.1), is nearly identical to Gulf toadfish (tf) GN, MDVCEICAFAACTGC (accession number: AIA09902.1) (41). Mucosal (pH 7.8) and serosal (pH 7.8) salines were prepared as described in Table 1. These salines were used for intestinal tissues from Gulf toadfish exposed to both 35 and 60 ppt, because plasma and intestinal fluid osmolalities of Gulf toadfish at these two salinities are not significantly different (17, 37).

RNA isolation and molecular cloning. Total RNA from Gulf toadfish tissues (50–100 mg/ml) was extracted using RNA STAT-60 (Tel-Test) and an Ultra-Turrax T8 tissue homogenizer (Ika-Werke). Traces of genomic DNA were removed from each isolate (10 μg per sample) with DNase I (Turbo DNA-free kit, Ambion). Total RNA was then quantified using a spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific) at 260 nm. A spot check of samples underwent gel electrophoresis to confirm RNA integrity. Isolated RNA (1 μg) from each sample was reverse transcribed into cDNA using random hexamers and the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol.

Genes were amplified by PCR using Taq polymerase (Invitrogen). The partial cDNA sequence for GC-C was cloned by 3′- or 5′-RACE

Table 2. *Sequences of primers for cDNA cloning and qPCR*

Name	Sequence
tfGN-F	5′-AGCAAAGGGCAGCATCTGCA-3′
tfGN-R	5′-TGGCAAGATGTTTGTGGCTTTGC-3′
tfUGN-F*	5′-CCGACCCTCTCATGCCGCAGG-3′
tfUGN-R*	5′-TGCACGGAGGCATCGAGCTG-3′
GC-C-F†	5′-CACCACCTGTTGGCGTACCACC-3′
GC-C-R†	5′-GTCCAGCGCCATGCGGCAGATG-3′
tfGC-C-F	5′-CAGAGGCCACCATGCGGCGTCACCTA-3′
tfGC-C-R	5′-TTCACCAAGCGCTGCTCCGACCAA-3′
tfNKCC-F*	5′-TCCTGCAGCAGCTCGTTGAG-3′
tfNKCC-R*	5′-GAGCACGTGGCGGCCTTCGAGGAT-3′
tfCFTR-F*	5′-GTTTCATCACCGGCATGAACG-3′
tfCFTR-R*	5′-GTGCCTTTCTGTAGATGGCTCCAA-3′
EF1α-F	5′-AGGTCATCATCCTGAACCAC-3′
EF1α-R	5′-GTTGTCCTCAAGCTTCTTGC-3′
Universal primer‡	5′-CTAATACGACTCACTATAGGGCAAGCGTGGTATCAA CGCAGAGT-3′
	5′-CTAATACGACTCACTATAGGGC-3′

*Gene-specific primers designed from the Gulf toadfish (tf) annotated genome. †Designed sense primers. ‡Universal primer (Clontech SMARTer RACE cDNA Amplification Kit) consists of both a long and short sequence. F, forward; R, reverse; GN, guanylin; UGN, uroguanylin; GC-C, guanylyl cyclase-C; NKCC, $Na^+/K^+/2Cl^-$ cotransporter; CFTR, cystic fibrosis transmembrane conductance regulator; EF1α, elongation factor 1α.

using GC-C-F and -R and a universal primer (Table 2). Full-length sequences were then cloned using designed sense primers, tfGC-C-F and -R [tfGN and tfUGN were cloned previously (41)]. The following cycling parameters were used: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 3 min. Gene-specific primers for tfGN and tfUGN were designed from their respective full-length sequences, as described in Ruhr et al. (41), and those for tfCFTR and tfNKCC1 were designed from their respective partial sequences found in the Gulf toadfish transcriptome (unpublished data, C.M.R LeMoine, N. Corradi, P.J. Walsh).

Real-time PCR: hypersalinity. To test the effects of 60 ppt on the expression of tfGN, tfUGN, tfGC-C, tfNKCC1, and tfCFTR mRNA, the following procedure was used. First, the tissue distribution of tfGN, tfUGN, and tfGC-C was quantified by real-time qPCR. An acute salinity transfer (to 60 ppt) was then used to determine whether tfGN, tfUGN, tfGC-C, tfNKCC1, and tfCFTR transcription changed over time (0, 6, 12, 24, and 96 h), following the protocol by Taylor et al. (50). Gulf toadfish were held in SW aquaria as described above and were given a lethal dose of MS-222 anesthetic, followed by spinal cord severing at the cervical vertebrae. Tissues were collected by dissection and immediately snap frozen in liquid nitrogen. As described above, tissues were collected, RNA isolated, and cDNA synthesized. qPCR (Mx4000, Stratagene) was used to measure tfNKCC1, tfCFTR, tfGC-C, tfGN, and tfUGN expression using gene-specific primers (Table 2). Power SYBR Green (Applied BioSystems) was used as the reporter dye. Cycling parameters were as follows: 95°C for 10 min, 40–50 cycles of 95°C for 30 s, 55–60°C for 30 s, and 72°C for 30 s. The specificity for all PCR products was confirmed by observing a distinct, corresponding melting peak for each product following each qPCR run using a melting-curve analysis (GN: 79–85, UGN: 81–86, GC-C: 80–86, NKCC1: 81–86, and CFTR: 80–85, in °C). Calculations were performed based on the approach described by Pfaffl (40). Gene expression was normalized to elongation factor 1α (EF1α) and scaled relative to the tissue with the lowest gene expression for the tissue distribution study and to the 0-h control for the salinity challenge study.

Determining the effects of hypersalinity on I_{sc} and HCO_3^- secretion. Ussing chambers (model 2400, Physiologic Instruments) were used to determine differences in I_{sc}, transepithelial potential (TEP), and transepithelial conductance (G_{te}) in the posterior intestine

of 35- and 60-ppt-exposed Gulf toadfish treated with RGN. The posterior intestine of each individual Gulf toadfish (ranging from 15–30 g) was excised, cut open, and mounted onto tissues holders (model P2413, Physiologic Instruments). The tissue holders exposed 0.71 cm² of excised tissue and were placed between the two half-chambers of the Ussing apparatus.

Measurements of I_{sc} were performed under symmetrical conditions; both the mucosal (apical membrane/luminal side) and serosal (basolateral membrane/blood side) half-chambers of the Ussing apparatus were bathed in serosal saline (2 ml) and continually mixed by airlift gassing using 0.3% CO_2 in O_2 (Table 1) and maintained at 25°C by a recirculating bath (model 1160S, VWR), following a similar procedure by Grosell and Genz (21). To measure I_{sc} and TEP, current and voltage electrodes were connected to amplifiers (model VCC600, Physiologic Instruments). Current electrodes recorded differences in I_{sc} under voltage-clamp conditions at 0 mV, with 3 s of 2-mV pulses (mucosal-to-serosal) at 60-s intervals. Voltage electrodes recorded differences in TEP under current-clamp conditions at 0 μA with 3 s of 10-μA pulses (mucosal-to-serosal) at 60-s intervals. Ohm's law was used to calculate G_{te}, determined from the deflections in I_{sc} and TEP during pulsing. I_{sc} and TEP data were recorded by Acqknowledge software (v. 3.8.1, BIOPAC Systems) onto a computer.

To determine the dose response and effective concentration of RGN on the Gulf toadfish posterior intestine, RGN was added in increasing doses to the mucosal half-chamber, when I_{sc} values became stable (10^{-9}-10^{-6} mol/l), as described in Ruhr et al. (41). I_{sc} values were recorded as described above. For the data presented, negative and positive I_{sc} values refer to absorptive and secretory currents, respectively.

A pH-stat titration system (TIM 854 or 856 Titration Managers, Radiometer) set up in tandem with an Ussing chamber was used to measure HCO_3^- secretion on isolated posterior intestinal tissues from 35- and 60-ppt-exposed Gulf toadfish, as described in Grosell and Genz (21). Luminal-side tissues were bathed in mucosal saline (2 ml), and blood-side tissues were bathed in an equal volume of serosal saline (Table 1). The salines in each half-chamber were continually mixed by airlift gassing using 100% O_2 (mucosal half-chamber) or 0.3% CO_2 in O_2 (serosal half-chamber) (Table 1). In the mucosal half-chamber, a pH electrode (model PHC4000.8, Radiometer) and microburrette tip (which secreted acid into the half-chamber) were submersed to maintain a constant pH of 7.8, which allowed for symmetrical pH conditions on either side of the intestinal epithelium. The amount of acid titrant (0.005 mol/l HCl) and pH values were recorded onto computers using the Titramaster software (v. 5.1.0). Epithelial HCO_3^- secretion rates were calculated from both the rate of titrant secreted and its concentration, as described in Grosell and Genz (21).

To assess whether the increased HCO_3^- secretion in the posterior intestine of 60-ppt-acclimated Gulf toadfish after RGN treatment was derived from endogenous CO_2 production or was dependent on serosal HCO_3^-/CO_2 absorption, a HCO_3^-/CO_2-free serosal saline (Table 1) was used to compare secretion rates with the rates obtained using serosal saline containing 5 mmol/l HCO_3^- (Table 1). Similarly to a study by Ruhr et al. (41), posterior intestinal sections from both control and 60-ppt-acclimated Gulf toadfish were exposed to 100 min in the presence of serosal HCO_3^- (including a 30-min control, followed by a 70-min flux, in which the mucosal saline had been spiked with 10^{-7} mol/l RGN). Immediately after this 100-min flux, the serosal saline was replaced with a serosal saline devoid of HCO_3^- and gassed with O_2 for an additional 70 min. A reverse experiment was then conducted in which the intestinal segments were first exposed and maintained for 30 min in the absence of serosal HCO_3^-, followed by 70 min with RGN in the mucosal half-chamber (in the absence of serosal HCO_3^-), and then 70 min in the presence of serosal HCO_3^-. Note that the tissues were continually in the presence of RGN after the control flux because the mucosal saline was never exchanged throughout an individual experiment.
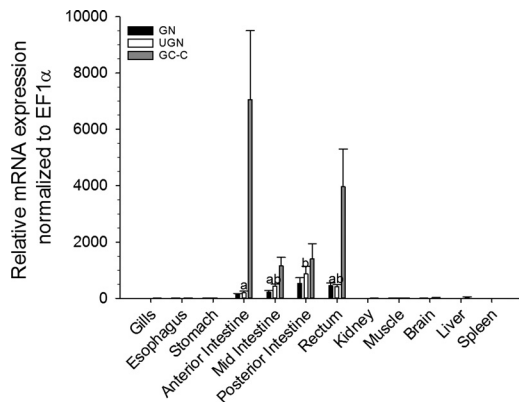
Fig. 1. Relative transcription levels of guanylin (GN), uroguanylin (UGN), and guanylyl cyclase-C (GC-C) in various organs from Gulf toadfish held in 35 parts per thousand (ppt). Expression levels for an individual gene are normalized to elongation factor 1α (EF1α) and scaled relative to the tissue with the lowest gene expression (kidney for GN, spleen for UGN, and stomach for GC-C), which was assigned an expression value of 1. Significant differences in UGN mRNA expression were revealed by a 2-way ANOVA followed by a Tukey's test ([a,b]$P \leq 0.05$).

Intestinal sac preparations. Intestinal sac preparations were used to examine fluid, Cl^-, and Na^+ fluxes across the posterior intestinal epithelium, following a similar protocol by Ruhr et al. (41). The intestine of an adult Gulf toadfish was cut once at its midway point and once proximally to the rectal sphincter. A PE50 catheter was inserted into the posterior end of the excised intestine and tied off with a silk suture. Mucosal saline (Table 1) was then injected into the catheter to rinse the intestine of any debris, precipitates, and intestinal fluids. The intestine was then closed with a silk suture, ~1.5 cm away from the first suture, to form an intestinal sac and was blot dried and weighed. Intestinal sacs from 35- and 60-ppt-acclimated Gulf toadfish were filled with either mucosal saline containing no RGN or 5×10^{-7} mol/l RGN (Table 1). After an intestinal sac was filled, a subsample of mucosal saline was taken to measure the initial Cl^- and Na^+ concentrations. The catheter was sealed, and the volume of injected mucosal saline was determined by calculating the difference in mass between a filled and unfilled intestinal sac preparation. The intestinal sacs were immediately placed in scintillation vials filled with serosal saline, gassed with 0.3% CO_2 (Table 1), and underwent a 2-h flux period, after which the sacs were removed, blot dried, and weighed for final masses. The mucosal saline from each sac was then collected to measure final Cl^- and Na^+ concentrations. Emptied intestinal sacs were cut in half and placed onto tracing paper for surface area measurements. Changes in volume were calculated by taking the difference in mass between the filled intestinal sacs at the beginning and end of the 2-h flux period. Na^+ concentrations were measured using flame spectrometry (Varian 220FS), and Cl^- concentrations were measured using anion chromatography (DIONEX 120). Fluid, Cl^-, and Na^+ fluxes were determined by the difference between initial and final intestinal sac mass or ion concentration, divided by flux period and tissue surface area.

Statistical analyses. Data are presented as absolute means ± SE. To compare means, one- and two-way ANOVAs, one- and two-way repeated-measures ANOVAs, and Student's t-tests were used for parametric data. Kruskal-Wallis one-way and Friedman repeated-measures ANOVAs on ranks, and the Mann-Whitney rank sum test were used for nonparametric data. Appropriate post hoc tests (Dunn's, Tukey's, and Holm-Sidak tests) were used to reveal specific differences between groups. SigmaStat 3.5 and SigmaPlot 11.0 were used for statistical analyses to calculate EC_{50} values and to plot the data. Means were considered significantly different when $P \leq 0.05$. Additionally, for the pH-stat titration experiments, the average of the last 30 min of each exposure flux (i.e., 70–100 and 140–170 min) were compared with the average control flux (0–30 min).

RESULTS

Gene distribution of GN, UGN, and GC-C. A distinct, partial GC-C-like molecule was cloned from Gulf toadfish intestinal cDNA, revealing a protein of 252 amino acids, encoded by 756 base pairs (GenBank accession number: KP844723). The small, partial nucleotide sequences of putative NKCC1 and CFTR were found in the Gulf toadfish intestinal transcriptome, coding for proteins of 117 and 95 amino acids, respectively. A basic local alignment search tool (BLAST), using both nucleotide and amino acid queries, confirmed that these molecules were partial sequences for a tfGC-C isoform, tfNKCC1 (accession number: KR360748), and tfCFTR (accession number: KR360747).

The intestinal segments and the rectum expressed high levels of GN and UGN mRNA ($n = 8$ for all tissues), whereas the other tissues analyzed displayed very low expression levels (Fig. 1). Analyzing only the intestinal segments and the rectum, we discovered that the anterior intestine expressed UGN mRNA at significantly lower levels than the posterior intestine (Fig. 1). As with the GN and UGN, GC-C mRNA was highly expressed in the intestinal segments and in the rectum (Fig. 1).

Gene expression of intestinal tissues exposed to hypersalinity. For the 60-ppt-acclimated Gulf toadfish, posterior intestinal tissues revealed significant increases in mRNA expression, relative to the control (0 h), of the following genes: GC-C at 96 h, NKCC1 at 96 h, and CFTR at 24 and 96 h (Fig. 2). On the basis of these data, only the posterior intestine of the Gulf toadfish was subjected to further physiological study, following hypersalinity challenge.

Effects of RGN on I_{sc} of tissues exposed to hypersalinity. For Gulf toadfish exposed to 35 ppt, mean I_{sc} values were significantly lower when compared with the control (no dose) when tissues were exposed to $\geq 2.5 \times 10^{-8}$ mol/l RGN and reversed at concentrations of $\geq 10^{-7}$ mol/l RGN (the point at which
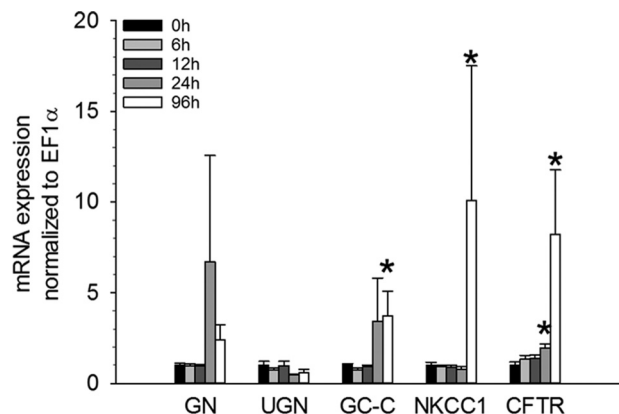

Fig. 2. Relative transcription levels of guanylin (GN), uroguanylin (UGN), guanylyl cyclase-C (GC-C), $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), and cystic fibrosis transmembrane conductance regulator (CFTR) in the posterior intestine of Gulf toadfish exposed to 0, 6, 12, 24, and 96 h in 60-ppt saltwater (SW). Expression levels are normalized to EF1α and scaled relative to the tissue with the lowest gene expression (0-h control), which was assigned an expression value of 1. Values are means ± SE ($n = 8$). *Statistically significant difference ($P \leq 0.05$) from the 0-h control, revealed by Kruskal-Wallis 1-way ANOVAs, followed by Dunn's tests.

tissues switch from absorptive/negative I_{sc} values to secretory/positive) (Fig. 3A). For Gulf toadfish exposed to 60 ppt, mean I_{sc} values were significantly lower when compared with the control when tissues were exposed to $\geq 10^{-8}$ mol/l RGN and reversed at concentrations $\geq 10^{-7}$ mol/l RGN (Fig. 3A). Furthermore, the mean I_{sc} of the posterior intestine of Gulf toadfish exposed to 60 ppt was greater than tissues exposed to 35 ppt at 0 and 10^{-9} mol/l RGN (Fig. 3A). Comparatively, the ΔI_{sc} of the posterior intestine of Gulf toadfish exposed to 60 ppt was greater than from those exposed to 35 ppt at concen-

trations $\geq 2.5 \times 10^{-8}$ mol/l RGN. The mean pooled EC$_{50}$ value was $8.04 \pm 10.3 \times 10^{-8}$ mol/l RGN (Fig. 3A). For the tissues from Gulf toadfish held in 35 and 60 ppt, significant differences in G_{te} occurred at concentrations $\geq 10^{-7}$ and 5×10^{-8} mol/l RGN, respectively (Fig. 3C).

All posterior intestinal tissues for subsequent experiments were dosed with 10^{-7} mol/l RGN, with the exception of the intestinal sac preparations, which were fluxed with 5×10^{-7} mol/l. At these concentrations, the means I_{sc} of both the 35- and 60-ppt-exposed tissues were reversed (mean I_{sc} values > 0 μA/cm^2; Fig. 3A) and demonstrate maximal ΔI_{sc} readings (Fig. 3B).

Intestinal sac preparation experiments. Corresponding with a previous study on the Gulf toadfish (41), posterior intestinal tissues from fish exposed to 35 ppt and treated with RGN displayed net Cl$^-$ secretion compared with untreated tissues (Fig. 4A). Additionally, tissues from the 60-ppt-acclimated Gulf toadfish untreated with RGN had significantly greater net absorptive Cl$^-$ rates than tissues from untreated fish held in 35 ppt (Fig. 4A). In parallel to the Cl$^-$ measurements, tissues from both the 35- and 60-ppt-exposed Gulf toadfish treated with RGN displayed net Na$^+$ secretion, whereas untreated tissues were net Na$^+$ absorptive (Fig. 4B). In the posterior intestinal sac preparations of Gulf toadfish exposed to 35 ppt, tissues treated with RGN absorbed significantly less fluid than untreated intestinal sac preparations (Fig. 4C). Intestinal sac preparations from Gulf toadfish exposed to 60 ppt and treated with RGN also absorbed less fluid than untreated intestinal sac preparations from fish held in 60 ppt and from fish held in 35 ppt and treated with RGN (Fig. 4C). There were no differences in absorption between the untreated intestinal sac preparations from fish held in 35 and 60 ppt.

Effects of RGN on HCO$_3^-$ secretion. For tissues from Gulf toadfish acclimated to 35 ppt, treatment with RGN resulted in a significantly lower HCO$_3^-$ secretion rate compared with the control flux, and this rate was further decreased when tissues were simultaneously treated with RGN and the absence of serosal HCO$_3^-$ (Fig. 5A). In contrast, for tissues from the 60-ppt-acclimated Gulf toadfish, RGN significantly increased the HCO$_3^-$ secretion rate compared with the control flux, although the absence of serosal HCO$_3^-$ significantly decreased this rate (Fig. 5C). The TEP of tissues from the 35- and 60-ppt-acclimated fish was significantly decreased, and even reversed, by RGN, and this change was magnified by simultaneous treatment with RGN and the absence of serosal HCO$_3^-$
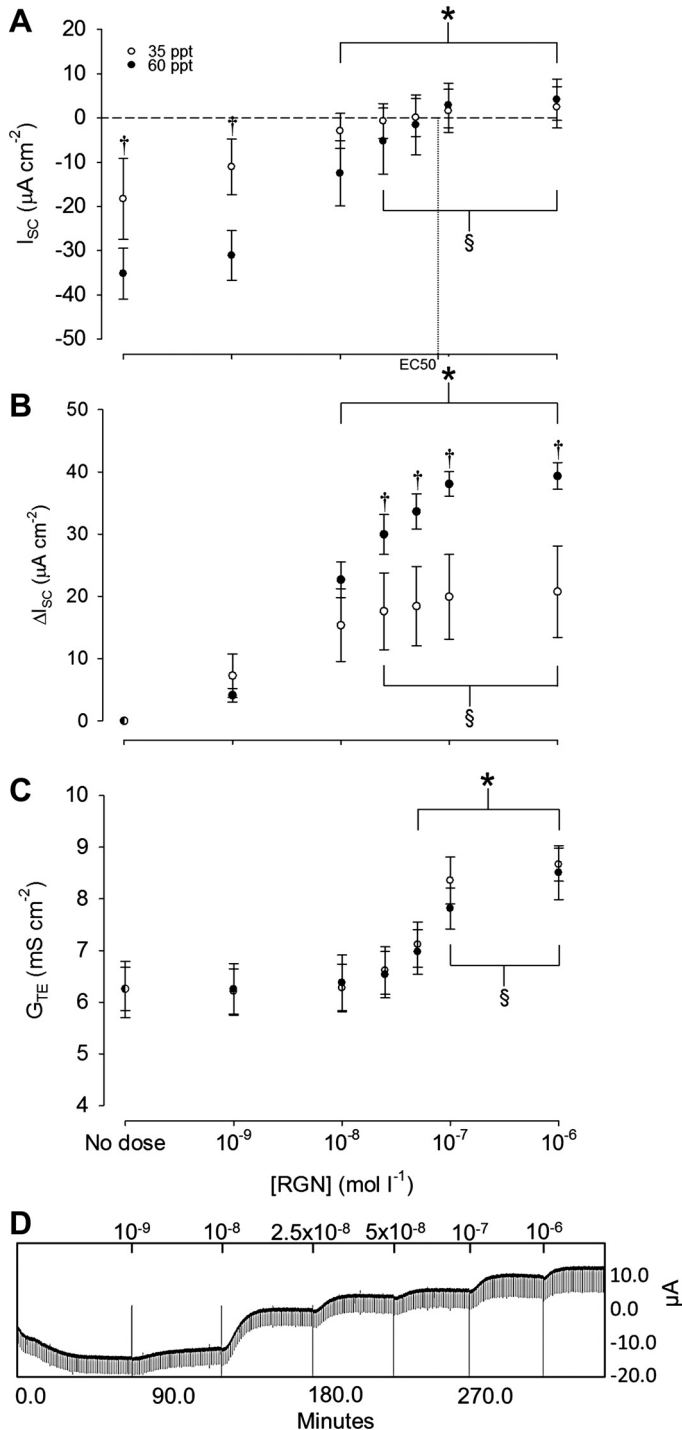

Fig. 3. Log scale of the dose-dependent effects of mucosal application of renoguanylin (RGN) on the mean short-circuit current (I_{sc}) (A), ΔI_{sc} (B), and transepithelial conductance (G_{te}) (C) of the posterior intestine from Gulf toadfish held in 35 ppt (○) or 60 ppt (●). ΔI_{sc} values were calculated as the difference in I_{sc} from the control (no dose), as a function of peptide concentration. D: representative trace of I_{sc} from the posterior intestine of a Gulf toadfish exposed to 60 ppt. The concentration of RGN was increased once I_{sc} values reached a stable reading (denoted by the vertical lines in the representative trace), resulting in a range of time-course lengths: 35 ppt: 248.63–459.23 min, 60 ppt: 334.59–465.62 min. Positive and negative values on the mean I_{sc} y-axis indicate secretory and absorptive currents, respectively. Values are means ± SE ($n = 5$). Significant differences ($P \leq 0.05$) for the parameters I_{sc}, ΔI_{sc}, and G_{te} were revealed by a Friedman repeated-measures ANOVA on ranks, followed by a Dunn's test (§35-ppt group) and a 1-way repeated-measures ANOVA, followed by a Holm-Sidak test (*60-ppt group). †Differences between the 35- and 60-ppt groups were revealed by a Student's t-test ($P \leq 0.05$).
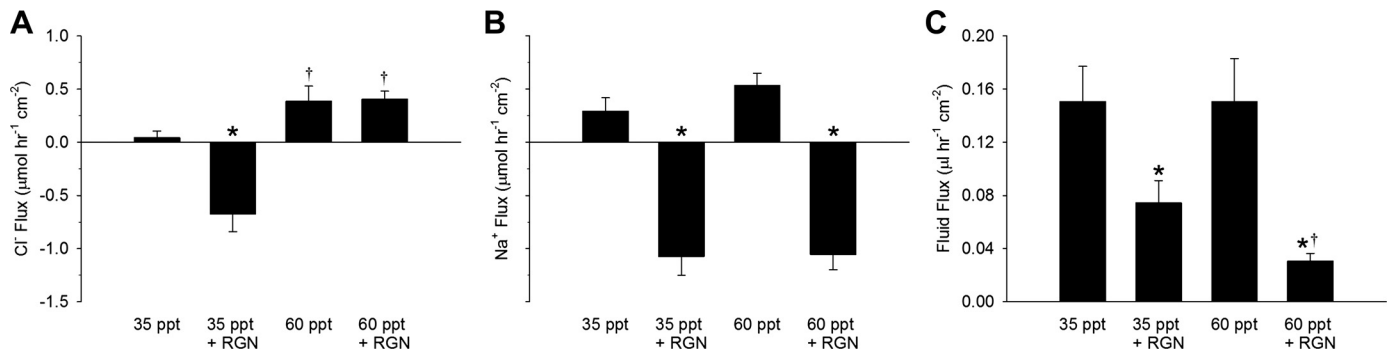
Fig. 4. Cl$^-$ (*A*), Na$^+$ (*B*), and fluid (*C*) fluxes obtained from posterior intestinal sac preparations of Gulf toadfish held in 35- and 60-ppt SW, with and without luminal addition of 5×10^{-7} mol/l RGN. Negative and positive values indicate secretion and absorption, respectively. Values are means $\pm$ SE ($n = 6-8$). Significant differences in Cl$^-$, Na$^+$, and fluid fluxes were revealed by Student's *t*-tests (*$P \leq 0.05$). Significant differences between the 35- and 60-ppt groups were revealed by a Mann-Whitney rank sum test (†$P \leq 0.05$).

(Fig. 5, *B* and *D*). In tissues from Gulf toadfish acclimated to 60 ppt, the conductance during the control flux was significantly lower than when tissues were simultaneously exposed to RGN and the absence of serosal HCO$_3^-$ (Table 3). In contrast, the tissue conductance from the 60-ppt-acclimated fish was significantly greater than those exposed to 35 ppt (Table 3). Control HCO$_3^-$ secretion rates of tissues from the 60-ppt-acclimated fish were greater than those exposed to 35 ppt (Fig. 5, *A* and *C*).

When Gulf toadfish posterior intestinal tissues were alternatively exposed to 0 mmol/l HCO$_3^-$ during the control flux, then to RGN, and subsequently to 5 mmol/l serosal HCO$_3^-$, the tissues from the 35-ppt-acclimated Gulf toadfish treated with

RGN displayed significantly lower HCO$_3^-$ secretion rates than in the presence of serosal HCO$_3^-$ (Fig. 6*A*). In consideration of the part of the experiment without serosal HCO$_3^-$, RGN treatment resulted in a significant decrease in HCO$_3^-$ secretion as expected. The HCO$_3^-$ secretion rate of the control flux was significantly greater for tissues from fish acclimated to 60-ppt SW than from fish acclimated to 35-ppt SW. The control TEP of tissues from Gulf toadfish acclimated to 35 ppt was significantly lower than when the tissues were simultaneously treated with RGN and the presence of serosal HCO$_3^-$ (Fig. 6*B*). Similarly, the TEP of tissues from the 60-ppt-acclimated fish was significantly less than in the RGN flux, and the TEP values of these two treatments were significantly less than when the
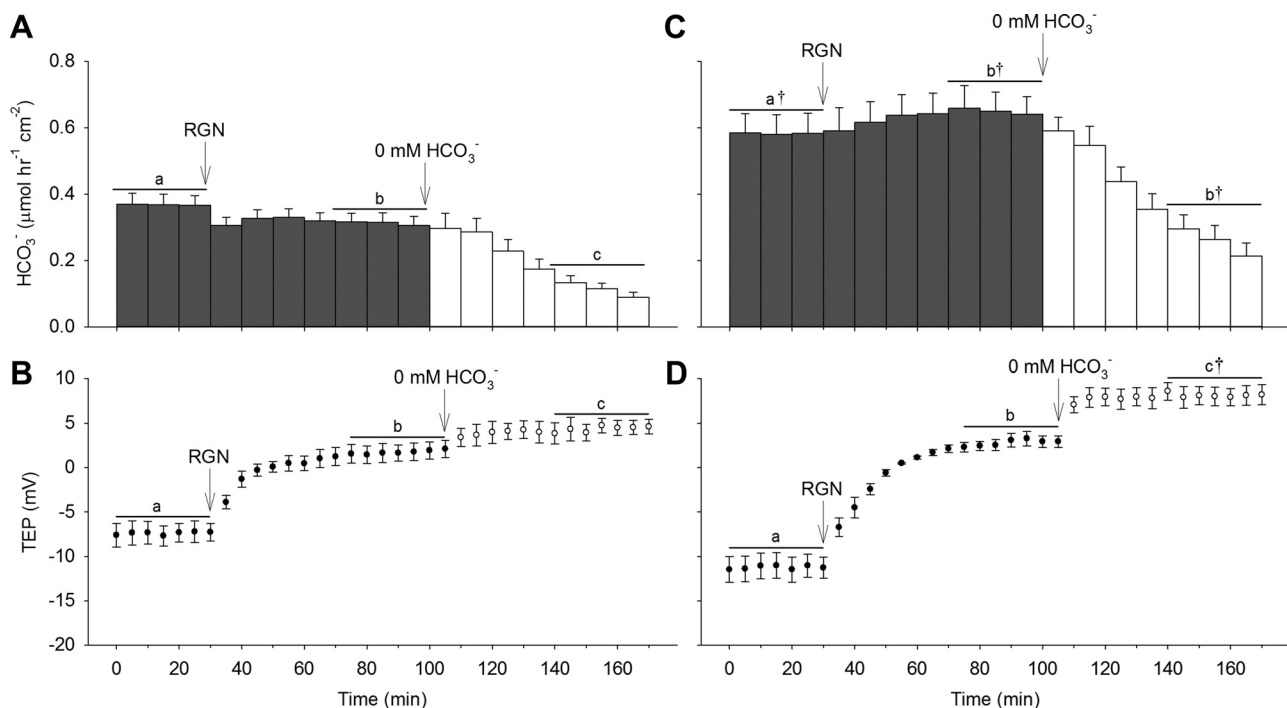

Fig. 5. *A* and *B*: HCO$_3^-$ secretion and transepithelial potential (TEP) of posterior intestinal tissues from Gulf toadfish held in 35 ppt. *C* and *D*: HCO$_3^-$ secretion and TEP of posterior intestinal tissues from Gulf toadfish acclimated to 60 ppt. After a 30-min control flux in the presence of 5 mmol/l serosal HCO$_3^-$, RGN (10^{-7} mol/l) was added to the mucosal half-chamber of an Ussing chamber. The serosal saline was exchanged for a serosal saline containing 0 mmol/l HCO$_3^-$ after a combined 100 min. Solid bars and ● indicate the presence of serosal HCO$_3^-$; open bars and ○ indicate the absence of serosal HCO$_3^-$. Values are means $\pm$ SE ($n = 9$). Horizontal lines represent 30-min mean values. [a,b,c]Significant differences ($P \leq 0.05$) in HCO$_3^-$ secretion and TEP within the 35- or 60-ppt groups were revealed by 1-way repeated-measures ANOVAs, followed by Holm-Sidak tests. †Significant differences between the 35- and 60-ppt groups were revealed by a Mann-Whitney rank sum test.

Table 3. *Transepithelial conductance of the posterior intestine in response to RGN and HCO$_3^-$/CO$_2$-free serosal saline*

| Corresponding Figure | G_{te}, mS/cm^2 | | Saline | | Time, min |
	35 ppt	60 ppt	Mucosal	Serosal	
6	3.9 ± 0.4	2.7 ± 0.2*†		+	0–30
	3.9 ± 0.3	3.4 ± 0.5†‡	RGN	+	70–100
	3.7 ± 0.2	4.2 ± 0.5‡	RGN	−	140–170
7	3.1 ± 0.3†	3.3 ± 0.2*†‡		−	0–30
	2.9 ± 0.2†	2.9 ± 0.3†	RGN	−	70–100
	5.4 ± 0.8‡	6.0 ± 0.4‡	RGN	+	140–170

Values are means ± SE (n = 5–9) for the response of the posterior intestine to HCO$_3^-$/CO$_2$-free serosal saline (0 mmol/l HCO$_3^-$) and/or renoguanylin (RGN) (10^{-7} mol/l). RGN was applied to the mucosal half-chamber (2-ml volume) of an Ussing chamber. The presence or absence of HCO$_3^-$/CO$_2$ is indicated by + or −, respectively. G_{te}, transepithelial conductance. *Significant differences ($P \le 0.05$) between the 35- and 60-parts per thousand (ppt) exposures; †significant effects within the 35-ppt exposure; ‡significant effects within the 60-ppt exposure.

tissues were simultaneously treated with RGN and the presence of serosal HCO$_3^-$ (Fig. 6D). In tissues from Gulf toadfish acclimated to 35 ppt, the conductances of both the control and RGN fluxes were significantly lower than when tissues were simultaneously treated with RGN and in the presence of serosal HCO$_3^-$ (Table 3). For tissues from Gulf toadfish acclimated to 60 ppt, the conductance was significantly increased compared with the control, when tissues were treated with RGN and the presence of serosal HCO$_3^-$ (Table 3). As was the case in the presence of serosal HCO$_3^-$ (Fig. 5, *A* and *B*), HCO$_3^-$ secretion rates (Fig. 6, *A* and *B*) and conductance (Table 3) of the control

flux from the tissues obtained from 60-ppt-acclimated Gulf toadfish were significantly greater in tissues from those acclimated to 35 ppt.

DISCUSSION

The present study demonstrates transcriptional upregulation of tfGC-C, tfNKCC1, and tfCFTR in the posterior intestine of Gulf toadfish acclimated to 60 ppt and a parallel increase in the secretory response of this tissue to RGN. Moreover, GN and UGN are shown to be present in all the intestinal segments and in the rectum of the Gulf toadfish, as well as in European and Japanese eels (29, 53), despite the fact that only the mid and posterior intestine appear to respond to these peptides (4, 41, 54). The present study also confirms the expression of at least one isoform of GC-C in all the intestinal segments and in the rectum of the Gulf toadfish. The existence of two or more GC-C isoforms is likely, as is the case with other teleosts (12, 27, 55). The partial clone of tfGC-C sequenced in the present study spans a highly conserved region of vertebrate GC-C, which does not allow for differentiation between possible tfGC-C isoforms that might otherwise be evident from less conserved regions found in the full-length sequences. It is perhaps surprising that tfGC-C is expressed roughly equally throughout the intestine, because the anterior intestine lacks a physiological response to guanylin peptide stimulation, at least for the parameters measured in the present and a previous study (41). This implies the possibility that GC-C and the guanylin peptides play a different, untested role in the anterior intestine. Moreover, the absence of apical CFTR in the anterior intestine most likely prevents the switch from net Cl$^-$ absorption to
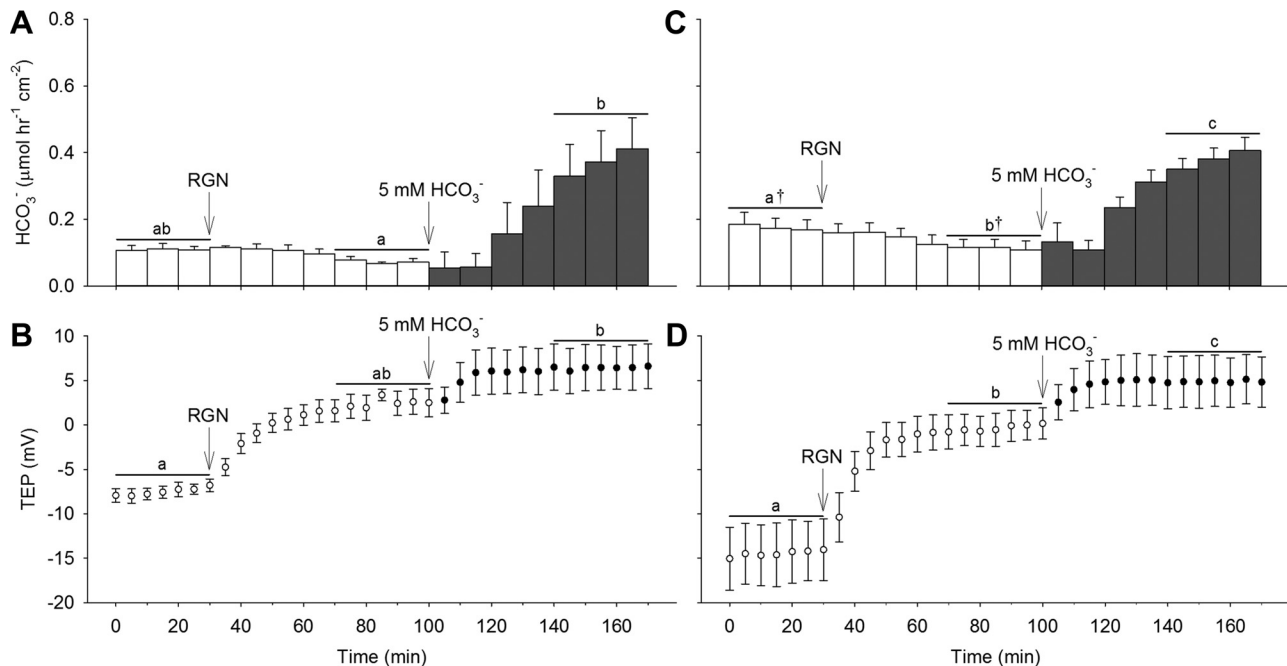

Fig. 6. *A* and *B*: HCO$_3^-$ secretion and TEP of posterior intestinal tissues from Gulf toadfish held in 35 ppt. *C* and *D*: HCO$_3^-$ secretion and TEP of posterior intestinal tissues from Gulf toadfish acclimated to 60 ppt. After a 30-min control flux in the absence of serosal HCO$_3^-$ (0 mmol/l), RGN (10^{-7} mol/l) was added to the mucosal half-chamber of an Ussing chamber. The serosal saline was exchanged for a serosal saline containing 5 mmol/l HCO$_3^-$ after a combined 100 min. Solid bars and ● indicate the presence of serosal HCO$_3^-$; open bars and ○ indicate the absence of serosal HCO$_3^-$. Values are means ± SE (n = 5). Horizontal lines represent 30-min mean values. [a,b]Significant differences ($P \le 0.05$) in HCO$_3^-$ secretion and TEP within the 35-ppt groups were revealed by a Friedman repeated-measures ANOVA on ranks, followed by a Tukey test. [a,b,c]For the 60-ppt group, significant differences were revealed by a 1-way repeated-measures ANOVA, followed by Holm-Sidak test. †Significant differences between the 35- and 60-ppt groups were revealed by a Mann-Whitney rank sum test.

secretion. This contrasts with the posterior intestine, in which RGN stimulation leads to activation of apical CFTR and, subsequently, Cl^- secretion into the intestine (41).

In support of our hypothesis, that the guanylin-induced Cl^--secretory response might facilitate the removal of $CaCO_3$ precipitates from the intestine, the present study demonstrates that tfGC-C, tfNKCC1, and tfCFTR are transcriptionally upregulated in the posterior intestine of Gulf toadfish after 96 h in 60 ppt. These observations suggest the potential of a greater secretory response at 60 ppt than at 35 ppt. Similar changes in NKCC1 and CFTR mRNA expression have also been shown in the intestine of hypersaline-acclimated Mozambique tilapia (*Oreochromis mossambicus*) (34) and sea bream (*Sparus aurata* L.) (19). The potential for a larger secretory response is important when considering that Gulf toadfish transferred from 35 to 50 ppt increase the formation of $CaCO_3$ by 2.3-fold (18). Consequently, a greater secretory response by intestinal tissues from the 60-ppt-acclimated Gulf toadfish, mediated by transcriptional upregulation of the Cl^--secretory pathway (apical CFTR and basolateral NKCC1), would be expected to facilitate

the removal of the increased production of $CaCO_3$ precipitates. The lack of transcriptional upregulation of GN and UGN in the posterior intestine suggests that these peptides do not limit the intracellular guanylin-stimulated signaling pathway. The absence of transcriptional upregulation of GN and UGN (8) and their secretion (30) has also been observed in the intestine of salt-loaded rats. Accordingly, it is likely that some combination of GC-C, NKCC1, and apical CFTR limits the tissue response to guanylin peptide stimulation (Fig. 7).

Correlating with increases in tfGC-C, tfNKCC1, and tf-CFTR mRNA expression in the posterior intestine is the roughly twofold larger ΔI_{sc} of RGN-treated tissues from Gulf toadfish acclimated to 60 ppt. This would indicate that the posterior intestine from Gulf toadfish held in 60 ppt, relative to those held in 35 ppt, could produce a greater secretory response after RGN stimulation. When guanylin peptides inhibit the absorptive I_{sc}, either net fluid absorption should be inhibited, or net fluid secretion should be stimulated (4, 16, 41). In the present study, the absorptive I_{sc} of the tissues from the 35- and 60-ppt-acclimated Gulf toadfish was modestly, but signifi-
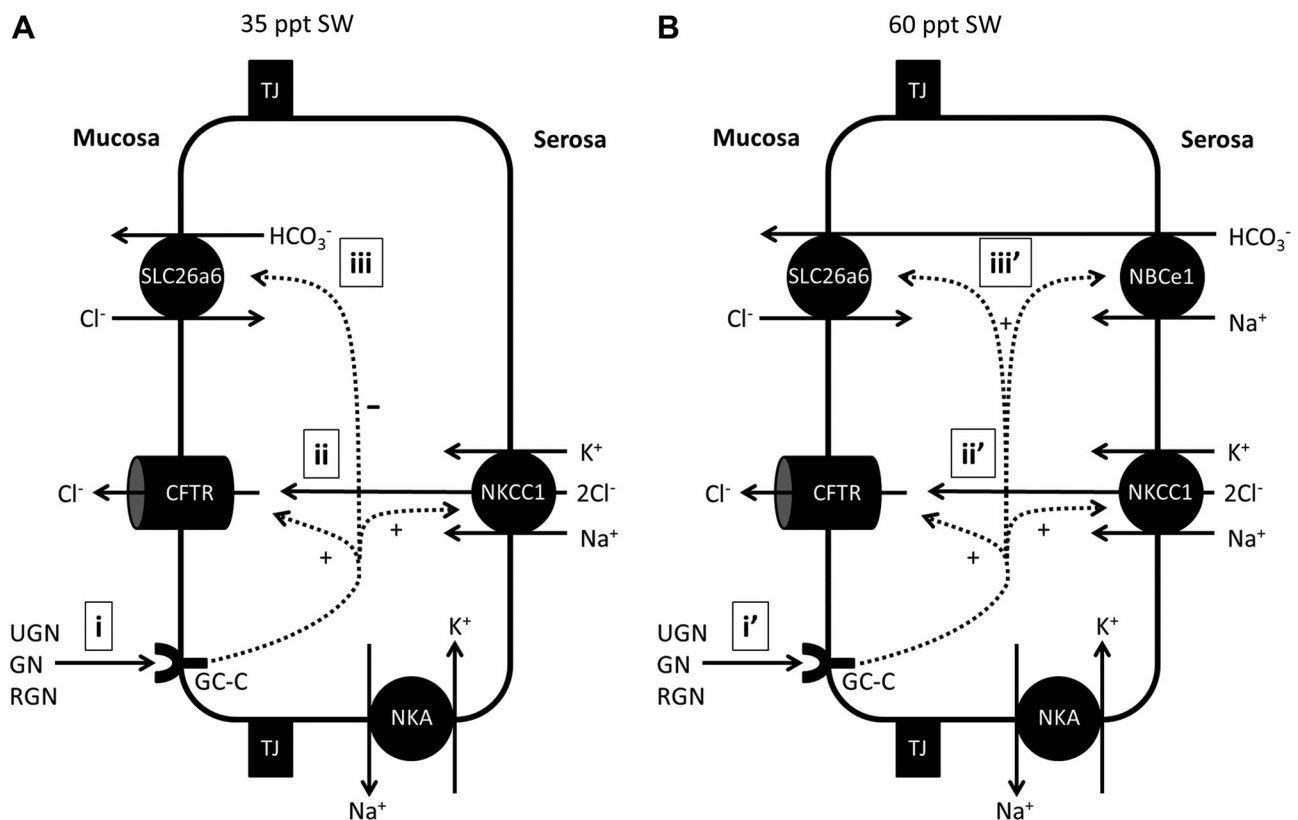

Fig. 7. Proposed effects of guanylin peptides in the posterior intestinal epithelia of Gulf toadfish (*Opsanus beta*). *A*: in tissues from 35-ppt-acclimated fish, guanylin (GN), uroguanylin (UGN), and renoguanylin (RGN) bind to an apical GC-C receptor (*i*). GC-C initiates an intracellular transduction cascade that stimulates the opening and/or increased insertion of apical CFTR Cl^- channels and upregulates the transport activity of NKCC1 (*ii*). Net ion absorption is inhibited by the activation of GC-C by the guanylin peptides, leading to either decreased fluid absorption or net fluid secretion. An unknown intracellular pathway is modulated and decreases the activity of the apical SLC26a6 anion exchanger, limiting the ability of the enterocyte to secrete HCO_3^- (*iii*). *B*: in tissues from 60-ppt-acclimated fish, GN, UGN, or RGN bind to GC-C (*i'*) and initiate the intracellular signals described above, including upregulation in the activity of the Cl^--secretory pathway (*ii'*). In 60 ppt, the posterior intestine has both an elevated HCO_3^- secretion rate and a stronger response to the guanylin peptides (leading to greater Cl^- secretion via CFTR), relative to 35 ppt. Increased Cl^- secretion in 60 ppt leads to greater cell depolarization than what occurs in 35 ppt, leading to a more favorable gradient for the influx of negative charges (i.e., increased basolateral HCO_3^- uptake). Combined, the guanylin peptides further elevate HCO_3^- secretion in 60 ppt by elevating the transport activity of both SLC26a6 and electrogenic Na^+-HCO cotransporter (NBCe1) (*iii'*) (see text for the role of NBCe1). In the present study, Japanese eel RGN was used as a research tool, but the presence of toadfish (tf)RGN, in the Gulf toadfish intestine, remains to be established. +, stimulatory effects; -, inhibitory effects; NKA, Na^+/K^+-ATPase; TJ, tight junctions. [Cell diagrams modified from Grosell (20) and Ruhr et al. (41)].

cantly, reversed, which led to inhibited fluid absorption. Despite this inhibition of net ion absorption, the conductance of these tissues increased in the presence of RGN, possibly attributable to the opening of apical CFTR channels, as has been observed previously in marine teleosts (4, 41, 54). In a previous study on the Gulf toadfish posterior intestine from fish held in 35 ppt (41), GN, UGN, and RGN reversed the absorptive I_{sc}, which corresponded to net Cl^- and fluid secretion. In contrast, RGN in the present study and GN in the Japanese eel posterior intestine (4) also reversed the absorptive I_{sc}, with fluid absorption being inhibited rather than reversed to secretory conditions. In the case of the wild-caught Gulf toadfish, these contrasts could reflect seasonal differences in transport physiology. Indeed, the previous study on the Gulf toadfish was performed in the spring and early summer, whereas the present study was conducted in late summer and in early autumn. Nevertheless, it is the presence of fluid in the intestinal lumen that would be necessary to help move solids from the intestine during peristalsis. Increased fluid volume, in combination with mucus lining the intestinal wall and the mechanical motions that help grind solids, would facilitate the passage of a solid through the intestine (44). In any case, it should be noted that net fluid movement of the intestinal tissues is the sum of simultaneous secretory and absorptive processes and that the reduced absorption, seen in the present study, is likely attributable to the stimulation by RGN of the Cl^--secretory pathway, comprised of basolateral NKCC1 and apical CFTR.

The observed inhibition of Na^+ absorption by RGN might possibly be attributable to the inhibition of the apical NKCC2 cotransporter that seems to occur in the Japanese eel (3, 4, 54). Inhibition of NKCC2 would facilitate a secretory response by the tissues, as occurs in mammals (6). However, paracellular secretion of Na^+ is entirely possible, as suggested to occur in the GN-treated Japanese eel intestine (3). The fact that RGN produces a larger secretory I_{sc} and the observation of transcriptional upregulation of tfGC-C, tfCFTR, and tfNKCC1 would suggest that RGN-treated tissues from fish acclimated to 60 ppt should secrete more Cl^- than RGN-treated tissues held in 35 ppt. Unexpectedly, RGN-treated and -untreated sac preparations from Gulf toadfish acclimated to 60 ppt did not differ in Cl^- flux. This unanticipated result might be explained by elevated HCO_3^- secretion, via anion exchange, of tissues from Gulf toadfish acclimated to 60 ppt (and treated with RGN). Greater HCO_3^- secretion would result from increased apical Cl^-/HCO_3^- exchange activity and not from transport via CFTR, as has been previously shown in the Gulf toadfish (41), leading to complete reabsorption of the Cl^- secreted as part of the RGN-induced response of the tissues. The complete reabsorption of Cl^- could be useful for maintaining the drinking rate for two important reasons, first, to compensate for increased branchial fluid loss in hypersalinity and, second, because elevated intestinal Cl^- concentration has been shown to reduce drinking behavior (1).

The apical membrane of the marine teleost intestine expresses the SLC26a6 antiporter, a Cl^-/HCO_3^- exchanger (20, 23, 32), and its transporting activity is limited by HCO_3^- (24, 52) and not by Cl^- (22, 41). In hypersalinity, the Gulf toadfish and sea bream intestine display the tendency to secrete HCO_3^- at a higher rate than in 35 ppt (19, 26). In the Gulf toadfish, it does so, to a certain extent, by increasing the expression of the electrogenic Na^+-HCO cotransporter (NBCe1, SLC4a4), a

basolateral Na^+/HCO_3^- symporter (50), and cytosolic carbonic anhydrase (CA_C) (43), but not of SLC26a6 (23). The exchange activity of SLC26a6 is limited, in part, by the availability of intracellular HCO_3^-, which is limited by the protein expression of NBCe1 and CA_C. However, the expression of NBCe1 and CA_C is not elevated in the posterior intestine of the 60-ppt-exposed Gulf toadfish (43, 50), suggesting that any contribution of NBCe1 and CA_C is posttranslational and that intracellular regulation by RGN might mediate the increased HCO_3^- secretion rate. Indeed, certain regions of mammalian tissues, such as in the kidneys and pancreas, also express isoforms of basolateral NBCs to reabsorb or secrete HCO_3^-, respectively (42). In the pancreas, for example, activation of a transmembrane adenylyl cyclase (tmAC) leads to intracellular increases in cAMP formation and PKA phosphorylation, which leads to the opening of apical CFTR (42). Activation of CFTR increases Cl^- secretion across the apical membrane and depolarizes both apical and basolateral membranes, resulting in a favorable gradient for HCO_3^- influx across the basolateral membrane via NBC (5, 42). A similar situation may also occur in the posterior intestine of Gulf toadfish acclimated to 60 ppt and treated with RGN, in which activation of apical CFTR leads to the depolarization of enterocytes, as Cl^- is excreted across the apical membrane. Concurrently, acclimation to 60 ppt leads to increased HCO_3^- secretion by the enterocytes; combined with depolarization by the guanylin peptides, this could produce a more favorable gradient that would increase the influx of negatively charged ions, namely HCO_3^-, through the basolateral NBCe1, resulting in the increased HCO_3^- secretion rate observed. As stated above, the exchange activity of SLC26a6 is limited by HCO_3^-; thus, it is plausible that increased HCO_3^- secretion in 60 ppt and in the presence of RGN could enhance Cl^- absorption via SLC26a6, which results in the absence of Cl^- flux differences in tissues from 60-ppt-acclimated Gulf toadfish, despite clear differences in ΔI_{sc} and fluid and Na^+ fluxes.

One objective of the present study was to determine how RGN might inhibit HCO_3^- secretion in the posterior intestine of Gulf toadfish held in 35 ppt. The reduction in HCO_3^- secretion occurs when either endogenous CO_2 is solely responsible for intracellular HCO_3^- or when both endogenous CO_2 as well as serosal HCO_3^- contribute to HCO_3^- secretion. The most parsimonious interpretation of these observations is that RGN downregulates the exchange activity of SLC26a6 through some unknown intracellular mechanism. Conversely, posterior intestinal tissues from Gulf toadfish acclimated to 60 ppt and in the presence of both mucosal RGN and serosal HCO_3^- show elevated HCO_3^- secretion, indicating that RGN enhances the transport of HCO_3^- by NCBe1 to provide additional substrate for apical anion exchange by SLC26a6. Moreover, it is unlikely that apical CFTR contributes to HCO_3^- secretion and is primarily responsible for Cl^- secretion, which is the primary cause of the I_{sc} reversal (41). In mammals, the guanylin peptide activation of GC-C can lead to increases in cAMP formation and PKA phosphorylation that stimulate apical CFTR channels (5). In the mammalian intestine, GN, UGN, and forskolin (which also elevates cAMP levels) act to increase HCO_3^- secretion, supporting the above suggestion. In contrast, forskolin and 3-isobutyl-1-methylxanthine, a PDE inhibitor, decrease HCO_3^- secretion, fluid absorption, and the absorptive I_{sc} in the intestine and rectum of the sea bream attributable to

tmAC stimulation or inhibition of soluble adenylyl cyclase (sAC) (9). This response appears analogous to what is observed when the posterior intestine of Gulf toadfish held in 35 ppt is treated with RGN. It is clear that the transfer of the Gulf toadfish from 35 to 60 ppt modifies the intracellular pathways that are regulated by the guanylin peptides.

From the studies mentioned above, future investigations on the intracellular regulation of the posterior intestine of the Gulf toadfish (from fish held in both 35 and 60 ppt) by the guanylin peptides should focus on the secondary messengers mentioned above to elucidate their roles in HCO$_3^-$ flux. Indeed, there is evidence that PKC regulates the transport activity of SLC26a6 in mammals by altering its interactions with CA (1). It is plausible that guanylin peptide-induced activation of PKG and PKA could have similar effects on SLC26a6. Moreover, the present findings from the Gulf toadfish also demonstrate significantly reduced fluid absorption by the posterior intestine from fish acclimated to 35 and 60 ppt, likely attributable to Cl$^-$ secretion by SLC26a6 and NKCC2. However, it remains to be seen whether these observations apply to marine teleosts in general.

Significance and perspectives. The transcriptional upregulation of tfGC-C, tfCFTR, and tfNKCC1 in the posterior intestine of Gulf toadfish exposed to 60 ppt, and the corresponding enhanced secretory response, further demonstrates the important function the guanylin peptide system has in osmoregulation. The present study demonstrates that fluid absorption and the absorptive I_{sc} are inhibited, or even reversed, by RGN in the posterior intestine of Gulf toadfish from 35 ppt (the latter to a greater extent than the former) and that these parameters are more impacted in posterior intestinal tissues from fish acclimated to 60 ppt. These observations support the hypothesis that the RGN-stimulated secretory response may serve the role of eliminating CaCO$_3$ precipitates from the intestine. In 35 ppt, Cl$^-$ secretion by the posterior intestine treated with RGN correlated with a modest, but significant, reversal of the absorptive I_{sc} (from mucosa-to-serosa to serosa-to-mucosa). However, in 60 ppt, there was no net change in Cl$^-$ absorption in the presence of RGN despite a greater inhibition of the absorptive I_{sc} and fluid absorption, relative to that in 35 ppt. This is explained by possible membrane depolarization initiated by RGN, in combination with increased baseline HCO$_3^-$ secretion that occurs in 60 ppt. Studies on the Japanese eel suggest that the Cl$^-$ concentration in the intestinal lumen modulates drinking rate; when Cl$^-$ levels in the lumen become elevated, drinking rate is reduced (2). In the context of the present study, it appears that the posterior intestine of Gulf toadfish held in 35 ppt and treated with RGN secretes Cl$^-$ and reduces HCO$_3^-$ secretion to limit the formation of CaCO$_3$ precipitates and to promote their removal from the intestine. In contrast, Cl$^-$ secreted by intestinal tissues from Gulf toadfish held in 60 ppt, attributable to RGN stimulation, is completely reabsorbed by elevated apical Cl$^-$/HCO$_3^-$ exchange activity, perhaps serving to preserve the drinking behavior of the fish. Increased drinking rate by marine teleosts held in hypersalinity is important for counteracting increased fluid loss through the gills. GN and UGN have also been linked to relaxation of the smooth muscles surrounding the guinea pig cecum (38) and bronchioles (39) and those of the human corpora cavernosa (47). If this smooth muscle relaxation also occurs in the Gulf toadfish intestine, it may serve as an important adaptation in preventing intestinal blockages that would inhibit the drinking behavior of a marine teleost. Increased fluid secretion into the intestine could work synergistically with smooth muscle relaxation to allow solids to be more easily removed from the intestine.

The present study employed in vitro techniques, but nothing is known about the effects of the guanylin peptides in situ in teleost fish. Furthermore, the present study raises the intriguing questions about the intracellular control that the guanylin peptides have in the Gulf toadfish intestine. Further studies should be carried out on the possible roles secondary messengers play in intestinal physiology, specifically PKG, PKA, and PDEs (which break down cAMP and cGMP), whose phosphorylation levels are regulated by GC-C activity, and that of sAC, a HCO$_3^-$-sensing protein, which produces cAMP and is suggested to regulate NKCC2 in marine teleosts (9, 52).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: I.M.R. and M.G. conception and design of research; I.M.R. and E.M.M. performed experiments; I.M.R., E.M.M., and M.G. analyzed data; I.M.R. and M.G. interpreted results of experiments; I.M.R. prepared figures; I.M.R. drafted manuscript; I.M.R., Y.T., and M.G. edited and revised manuscript; M.G. approved final version of manuscript.

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