RESEARCH ARTICLE

Renoguanylin stimulates apical CFTR translocation and decreases HCO3− secretion through PKA activity in the Gulf toadfish (Opsanus beta)

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ABSTRACT

The guanylin peptides – guanylin, uroguanylin and renoguanylin (RGN) – are endogenously produced hormones in teleost fish enterocytes that are activators of guanylyl cyclase-C (GC-C) and are potent modulators of intestinal physiology, particularly in seawater teleosts. Most notably, they reverse normal net ion-absorbing mechanisms that are vital to water absorption, an important process for seawater teleost survival. The role of guanylin-peptide stimulation of the intestine remains unclear, but it is hypothesized to facilitate the removal of solids from the intestine by providing fluid to enable their removal by peristalsis. The present study used one member of this group of peptides – RGN – to provide evidence for the prominent role that protein kinase A (PKA) plays in mediating the effects of guanylin-peptide stimulation in the posterior intestine of the Gulf toadfish (Opsanus beta). Protein kinase G was found to not mediate the intracellular effects of RGN, despite previous evidence showing that GC-C activation leads to higher cysenic monophosphate formation. RGN reversed the absorptive short-circuit current and increased conductance in the Gulf toadfish intestine. These effects are correlated to increased trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel to the apical membrane, which is negated by PKA inhibition. Moreover, RGN decreased HCO3− secretion, likely by limiting apical HCO3−/Cl− exchange (possibly by reducing SLC26a6 activity), a reduction that was enhanced by PKA inhibition. RGN seems to alter PKA activity in the posterior intestine to recruit CFTR to the apical membrane and reduce HCO3− secretion.

KEY WORDS: PKG, Cl− secretion, Guanylin peptides, Intestine, Seawater teleost

INTRODUCTION

Seawater presents a major osmoregulatory challenge for teleost fish, because of the direct interaction of their thin respiratory epithelia – the gills – with the environment, which causes unavoidable branchial fluid loss (Evans et al., 2005). To compensate, the seawater teleost constantly drinks water and absorbs it across the intestine. Imbibed seawater undergoes rapid desalination by the esophagus, so, by the time it enters the intestine, its osmolality is roughly that of blood plasma. In the intestine, precipitation of Ca2+ and Mg2+ by HCO3− to form CaCO3 and MgCO3 precipitates and titration of HCO3− by H+ facilitate further decreases in intestinal fluid osmolality by up 130 mOsmol kg−1 (Grosell et al., 2009a,b; Wilson et al., 2002). The combined effect of these processes, in addition to other ion-absorbing pathways, renders the fluid osmolality in the intestine similar to that of the extracellular fluids and allows for efficient water absorption that is coupled to Na+ and Cl− absorption (Skadhauge, 1969, 1974). Even though net water absorption and the mechanisms that facilitate it are vital to the survival of teleosts living in seawater, recent studies reveal region-specific areas of the intestine that display net secretory functions, at least periodically (Ruhr et al., 2014, 2016).

In the Gulf toadfish, Opsanus beta (Goode and Bean, 1880), members of the homologous guanylin family of intestinal peptides [guanylin, uroguanylin and renoguanylin (RGN)] reverse ion absorption in the posterior intestine, but not in the anterior region (Ruhr et al., 2014). This reversal also corresponds to RGN-stimulated water secretion in the posterior intestine and rectum, but not in the anterior intestine (Ruhr et al., 2014, 2016). The switch from net ion absorption to net ion secretion (from mucosa-to-serosa to serosa-to-mucosa) seems to be caused primarily by inhibiting ion absorption by the Na+/K+/Cl−–cotransporter type 2 (NKCC2) and limiting HCO3−/Cl− exchange activity, as well as activating Cl− secretion through the apical cystic fibrosis transmembrane conductance regulator (CFTR) channel (Ruhr et al., 2014, 2015, 2016). This response is also isolated to the mid and posterior intestine of the Japanese eel (Anguilla japonica) after stimulation by guanylin (Ando and Takei, 2015; Ando et al., 2014; Yuge and Takei, 2007). It is hypothesized that the inhibition of fluid absorption is required to facilitate the removal of solids, such as undigested food, fecal matter or carbonate precipitates. The presence of fluid has been shown to enhance the efficiency of peristalsis in mammals, thus making the movement of solids through the intestinal tract easier (Schulze, 2015).

A handful of studies on the intestinal physiology of seawater teleosts have demonstrated a reversal of ion absorption. In the winter flounder (Pseudopleuronectes americanus) intestine, atrial natriuretic factor, vasoactive intestinal peptide (VIP), cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) can alter NKCC2 activity and decrease the absorptive short-circuit current (Isc), which is a measure of total ion movement (O’Grady, 1989; O’Grady et al., 1988, 1985; Rao and Nash, 1988; Rao et al., 1984). These early studies demonstrated that the reversal of net ion absorption in seawater teleosts is largely driven by Cl− secretion, in addition to decreased activity of absorptive-type ion transporters (O’Grady and Wolters, 1990). A
more recent study on the killfish (Fundulus heteroclitus) similarly revealed that a combination of ionomycin, dibutyryl-cAMP and IBMX (a phosphodiesterase inhibitor) stimulates fluid secretion in the posterior intestine (Marshall et al., 2002). It is interesting that intestinal tissues from multiple species of seawater teleosts respond to the above-mentioned hormones, activators and inhibitors by decreasing ion-absorbing mechanisms that, in some cases, result in fluid secretion. The inhibition of ion transport and water absorption is widespread across seawater teleost species and might serve a beneficial purpose for life in seawater. In both mammalian and fish enterocytes, stimulation of the apical guanylin peptide receptor guanylyl cyclase-C (GC-C) leads to increased formation of cGMP (Chao et al., 1994; Kuhn et al., 1994; Schulz et al., 1990; Yuge and Takei, 2007), which activates protein kinase G (PKG) and increases cAMP formation [leading to protein kinase A (PKA) activation]

List of symbols and abbreviations

- cAMP: cyclic adenosine monophosphate
- CFTR: cystic fibrosis transmembrane conductance regulator
- cGMP: cyclic guanosine monophosphate
- GC-C: guanylyl cyclase-C
- GTe: transepithelial conductance
- H-89: PKA inhibitor
- fSC: short-circuit current
- KT8283: PKG inhibitor
- NKCC2: Na+/K+2Cl− symporter
- PKA: protein kinase A
- PKG: protein kinase G
- PKI: PKA inhibitor
- RGN: renoguanylin
- SLC26a6: solute carrier family 26 member 6 (HCO3−-antiporter)
- TEP: transepithelial potential
- VIP: vasoactive intestinal peptide

Composition of salines for short-circuit current, pH-stat titration and sac preparation experiments

| Compound                  | Mucosal | Serosal | HCO3−/CO2-free serosal |
|---------------------------|---------|---------|------------------------|
| NaCl (mmol l−1)           | 169.0   | 151.0   | 151.0                  |
| KCl (mmol l−1)            | 5.0     | 3.0     | 3.0                    |
| MgSO4 (mmol l−1)          | 77.5    | 0.88    | 0.88                   |
| MgCl2 (mmol l−1)          | 22.5    |         |                        |
| Na2HPO4 (mmol l−1)        | 0.5     | 0.5     |                        |
| KH2PO4 (mmol l−1)         | 0.5     | 0.5     |                        |
| CaCl2 (mmol l−1)          | 5.0     | 1.0     | 1.0                    |
| NaHCO3 (mmol l−1)         | 5.0     |         |                        |
| Hepes, free acid (mmol l−1) | 11.0   | 11.0    |                        |
| Hepes, Na+ salt (mmol l−1) |         | 11.0    | 11.0                   |
| Urea (mmol l−1)           | 4.5     | 4.5     |                        |
| Glucose (mmol l−1)        | 5.0     | 5.0     |                        |
| Osmolarity (mosmol kg−1 H2O) | 330   | 330     | 330                    |
| pH                        | 7.8     | 7.8     | 7.8                    |
| Gas formulations          |         | 100% O2 | 0.3% CO2 in O2 | 100% O2 |

*Activators and inhibitors (in final concentrations) were applied to the mucosal saline: renoguanylin (0.1 and 0.5 μmol l−1), KT8283 (2 μmol l−1), Rp-8-pCPT-cGMPS (25 μmol l−1), Na+/H+ exchanger (100 μmol l−1), H-89 (20 μmol l−1), and PKI (10 μmol l−1). The osmolarity of salines was adjusted with mannitol.

**When measuring HCO3− secretion, mucosal pH in Ussing chambers was maintained at 7.8 by pH-stat titration. **Salines were gassed for at least 1 h prior to experiments.

**MATERIALS AND METHODS**

**Experimental animals**

Gulf toadfish were caught by bycatch from Biscayne Bay, FL, USA. Upon arrival in the laboratory, ectoparasites were removed by a 3 min freshwater bath and treatment with Malachite Green in seawater. Fish were separated by size/mass classes and placed into 62 l aerated tanks (33–36 ppt, 20–26°C), with continuous flow-through of sand-filtered seawater from Biscayne Bay. Fish were fed weekly to satiation with squid, but fasted for at least 3 days prior to experimentation. Fish husbandry and experimental procedures were in accordance with the University of Miami Animal Care Protocol (IACUC no. 13-225, renewal 03, and 15-019, renewal 02). MS-222 (0.2 g l−1; Argent, Redmond, WA, USA) solution, buffered with 0.3 g l−1 NaHCO3− (Sigma-Aldrich, St Louis, MO, USA), was used to anesthetize fish; they were then killed by severing the spinal cord at the cervical vertebra and pithing of the brain.

**Composition of salines, hormones, activators and inhibitors**

Mucosal (pH 7.8) and serosal (pH 7.8) salines were prepared as described in Table 1 and used to bathe intestinal tissues. Eel RGN (Peptide Institute, Ibaraki-Shi, Osaka, Japan) was used in the present study as the activator and a tool to assess the function of activating GC-C in the posterior intestine. The purity of biologically active eel RGN was confirmed using reversed-phase, high-performance liquid chromatography (Yuge and Takei, 2007). The amino acid sequence of eel RGN (ADLCEICFAACTGCL, accession number: BAC76010.1) is nearly identical to that of Gulf toadfish guanylin (MDVCEICFAACTGCL, accession number: AIA09902.1) and stimulation of the Gulf toadfish posterior intestine with eel RGN has the greatest physiological effects compared with those of eel uroguanylin or eel guanylin (Ruhr et al., 2014).

Activators and inhibitors were dissolved in water, dimethylsulfoxide (DMSO; Sigma-Aldrich) or ethanol (EtOH; Sigma-Aldrich). The PKG inhibitors KT8283 and Rp-8-pCPT-cGMPS (Enzo Life Sciences, Farmingdale, NY, USA) were dissolved in DMSO and water, respectively. The cGMP analog/ PKG activator 8-bromo-cGMPS (Enzo) was dissolved in water. The PKA inhibitors H-89 (dihydrochloride; Cell Signaling Technologies, Beverly, MA, USA) and protein kinase inhibitor (PKI) (14-22, amide, myristoylated; Enzo) were dissolved in DMSO. Final concentrations were as follows: KT5823, 2 μmol l−1; Rp-8-pCPT-cGMPS, 25 μmol l−1; 8-bromo-cGMPS, 100 μmol l−1; H-89, 20 μmol l−1; and PKI, 10 μmol l−1. The concentrations for
these inhibitors were tested using the dose recommended by their respective manufacturers (and not surpassing the solubility that would exceed the ideal DMSO or EtOH content in the saline). Final solvent ratios in which inhibitors were dissolved were 1:1000 (0.1%) and 1:2000 (0.05%) for DMSO and EtOH, respectively. Vehicle controls for 0.1% DMSO and 0.05% EtOH did not affect the physiological parameters tested in the present (data not shown) and previous studies on Gulf toadfish (Ruhr et al., 2014, 2015, 2016).

**General experimental protocol: $I_{SC}$ and pH-stat titration**

$I_{SC}$, transepithelial conductance (TEP) and $G_{TE}$ of Gulf toadfish tissues were measured by Ussing chambers (model 2400; Physiologic Instruments, San Diego, CA, USA). The posterior intestine of a Gulf toadfish (15–30 g) was excised, cut open along the midline, mounted onto P2413 tissue holders (Physiologic Instruments) to expose 0.71 cm$^2$ of tissue, and placed between the two half-chambers of the Ussing apparatus. $I_{SC}$ and TEP were measured by silver (Ag) current and Ag/AgCl voltage electrodes, respectively, and were connected to an amplifier (model VCC600, Physiologic Instruments). $I_{SC}$ was measured using current electrodes under voltage-clamp conditions (0 mV), with 3 s of 2 mV pulses (mucosal-to-serosal) every 60 s. TEP was measured by voltage electrodes under current-clamp conditions (0 μA), with 3 s of 30 μA pulses (mucosal-to-serosal) every 60 s. $G_{TE}$ was calculated by Ohm’s law and was determined from the deflections in $I_{SC}$ and TEP during pulsing. Acqknowledge software (v.3.8.1, BIOPAC Systems, Goleta, CA, USA) recorded $I_{SC}$ and TEP onto a computer. The function and viability of Gulf toadfish intestinal tissues are stable in the Ussing chamber for more than 2 h (Ruhr et al., 2014).

$I_{SC}$ measurements were taken under symmetrical conditions using 2 ml of serosal saline (Table 1) in each half-chamber (the mucosal/apical/luminal and the serosal/basolateral/blood-side chambers). The salines were continually mixed by airlift gassing (0.3% CO$_2$/99.7% O$_2$) and maintained at 25°C by a recirculating chamber system (described by Grosell and Genz, 2006). TEP measurements were taken under asymmetrical conditions using 2 ml each of mucosal and HCO$_3^−$/CO$_2$-free serosal saline (Table 1), which were added to their respective half-chambers.

A previous study on the Gulf toadfish revealed that RGN might regulate the transport activity of apical HCO$_3^−$ transporters, such as SLC26a6 (Ruhr et al., 2016). This previous study used an HCO$_3^−$-free serosal saline to render the basolateral NBCe (an electrogenic Na$^+/HCO_3^−$-cotransporter) functionless and determined that the effects of RGN decreased apical HCO$_3^−$ secretion, independent of cytosolic carbonic anhydrase activity. Accordingly, the present study also used a HCO$_3^−$-free serosal saline to investigate the role of PKA and PKG activators and inhibitors on apical HCO$_3^−$ transport activity. The mucosal and serosal salines were continually mixed by airlift gassing (100% O$_2$) as described above and maintained at 25°C. HCO$_3^−$ secretion was measured using a pH-stat titrator (model TIM 854 or 856, Radiometer, Loveland, CO, USA) that was set up in tandem with an Ussing chamber system (described by Grosell and Genz, 2006). A mucosal pH of 7.8 was maintained using a pH electrode (Radiometer, model PHC4000.8) and an acid-delivering microburette tip (0.005 mol l$^{-1}$ HCl); both were immersed in the mucosal saline bath, allowing for symmetrical pH conditions on either side of the epithelium. The volume of acid titrant and mucosal pH was measured and recorded onto a computer using Titramaster software (Radiometer, v.5.1.0). The HCO$_3^−$ secretion rate from a tissue preparation was calculated from the rate of titrant secreted and its concentration (described by Grosell and Genz, 2006). RGN was added to mucosal half-chamber, to a concentration of 0.1 μmol l$^{-1}$.

**Intestinal sac preparations and immunohistochemistry**

Sac preparations from the posterior intestine were made and preserved for immunohistochemistry to determine the effects of RGN on CFTR translocation. The posterior intestine (1.5–3 cm) from Gulf toadfish (50–80 g) was excised as described above. Briefly, following a modified protocol (Ruhr et al., 2016), the flared end of a PE50 catheter was inserted into the posterior intestine and tied off with a silk suture. Mucosal saline (Table 1) was then injected into the catheter to rinse the tissue of any debris. The open end of the tissue was then tied off with a silk suture to form an intestinal sac.
The sac preparations were then injected with mucosal saline (control or containing 0.5 μmol l⁻¹ RGN). The catheter was sealed and placed in a scintillation vial containing serosal saline and continually gassed with 0.3% CO₂ (Table 1) for 1 h. At the end of the experimental period, the sac preparations were removed from the serosal saline. After the silk sutures were cut and the catheter removed, the tissue was drained and placed in Z-fix (Anatech, Hayward, CA, USA) for 48 h to fix the tissue. Following fixation, the tissue was immersed in 70% ethanol for 1 week. Then, ascending grades of ethanol washes (3 times in 95%, followed by 3 times in 100%) were used to dehydrate tissues. Tissues were then prepared for wax embedding by immersion in butanol, two washes in Histochrome (Amresco, Solon, OH, USA) and four washes in Paraplast Embedding Media (McCormick Scientific, St Louis, MO, USA), after which tissues were embedded in tissue holders containing Paraplast. A microtome (model 1512, Leitz, Grand Rapids, MI, USA) was used to slice sections of tissue (4 μm); the tissue sections from the posterior intestine were mounted onto poly-l-lysine-coated slides and dried for 24 h at 37°C.

Tissues were prepared for antibody treatment by washing slides twice in Histochrome (to remove the paraplast), followed by immersion in descending grades of ethanol (100%, 2×90%, 70% and 50%) and two washes in phosphate-buffered saline (PBS) (pH 7.3). To reveal antigenic sites, the following steps were taken. Slides were first incubated in 10 mmol l⁻¹ of citric acid solution (pH 6) and heated to boiling in a microwave (two 5-min incubations at 80% power). Next, slides were cooled to room temperature and were washed in the following order: 0.05% SDS-PBS for 5 min, solution A [PBS, NaCl (58.44 mmol l⁻¹), 0.2% Tween-20 (Sigma-Aldrich) and pH 7.3] for 5 min, and Image-iT (Life Technologies, Carlsbad, CA, USA) for 30 min. To block non-specific antigenic sites, slides were incubated for 1 h in PBS solution containing 5% skimmed milk, 1% bovine serum albumin (BSA), and 0.05% Tween-20. Slides were rinsed twice in PBS after each step.

Tissue sections from the posterior intestine were probed with fluorescent antibodies. The primary monoclonal mouse CFTR antibody [Human CFTR C-Terminus MAb (Clone 24-1), Mouse IgG2A, cat. no. MAB25031, R&D Systems, Minneapolis, MN, USA] was diluted to 10 μg ml⁻¹ in PBS solution containing 0.5% skimmed milk, 1% BSA and 0.05% Tween-20. This particular CFTR antibody was used previously by our group to reveal CFTR localization in the intestine of the Gulf toadfish (Rühr et al., 2014) and has been validated using western blot analysis of CFTR in the Gulf toadfish gill, with an approximate mass of 135 kDa (Zongli Yao, K.L.S., I.M.R., Edward M. Mager, Rachael M. Heuer and M.G., unpublished data). A hydrophobic pen was used to create a border around the tissues on a slide, inside of which primary antibody was deposited. Slides were placed in a humid chamber on an oscillating table at 5°C overnight, then rinsed twice in PBS containing 0.05% Tween-20 and once in normal PBS. The following steps were carried out in the absence of light. Donkey anti-mouse IgG secondary antibody (Alexa Fluor 488, cat. no. AB_2556542, Thermo Fisher Scientific, Waltham, MA, USA) was diluted to 10 μg ml⁻¹ in PBS solution containing 0.5% skimmed milk, 1% BSA and 0.05% Tween-20. The anti-mouse IgG was deposited inside the hydrophobic border surrounding the tissues. Slides were incubated in a humid chamber for 1 h at 37°C, after which they were rinsed twice in PBS containing 0.05% Tween-20 and once in normal PBS. ProLong Gold Antifade Reagent (Thermo Fisher Scientific) was used to adhere coverslips onto each slide. Control slides were treated the same as above, with the exception of
primary antibody treatment. Slides were observed with an Olympus fluorescence microscope (u-tvo.5xc-2), and images were taken with an attached QImaging camera (Retiga EXi, Fast 1394) using 40× and 60× magnification. Fiji (Schindelin et al., 2012), iVision and Gimp software were used to analyze the images. Three slides were analyzed for each individual (n=4 per treatment).

A modified procedure was used to determine corrected membrane fluorescence using ImageJ (Potapova et al., 2011). Briefly, a region was drawn around the apical membrane region for CFTR immunofluorescence (that excluded the subapical staining). The fluorescence intensity within a selected region was then calculated. On the same slide, a measurement of an area without fluorescent objects was used as the background subtraction. To calculate the corrected fluorescence, the area of the selected tissue was multiplied by the background intensity, which was then subtracted from the corrected fluorescence.

Whole-intestine, cytoplasm and cell membrane isolation
Sections of the posterior intestine were mounted onto Ussing chambers, as described above for measuring $I_{SC}$. and treated with RGN and H-89. The physiological data were used to correlate changes in CFTR trafficking to $I_{SC}$ and $G_{TE}$. Samples were prepared for western blot analysis following a modified protocol used previously in fish (Tresguerres et al., 2007). Briefly, tissues were snap frozen in liquid nitrogen and stored at −80°C. In order to achieve sufficient protein yield, tissues from each treatment were pooled in groups of three and were pulverized in a porcelain grinder and weighed, after which ice-cold homogenization buffer [250 mmol l⁻¹ sucrose, 1 mmol l⁻¹ EDTA, 1× HALT Protease Inhibitor Cocktail (Thermo Fisher Scientific) and 30 mmol l⁻¹ Tris pH 7.4] was added in a 1:10 w/v ratio. Samples were sonicated on ice for three 10-s pulses, and centrifuged (3000 g, 10 min at 4°C). The supernatant was collected; some was stored as the whole-intestine sample and the rest was centrifuged once more (20,800 g, 30 min at 4°C). The supernatant was collected (the cytoplasmic fraction) and the pellet re-suspended in homogenization buffer (the membrane fraction). Following this, protein concentration was determined via the bicinchoninic acid method (Thermo Fisher Scientific), according to the manufacturer’s instructions.

Western blot analysis
Samples were prepared in Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) at a concentration of 0.5–1.0 μg ml⁻¹ for western blot analysis. Proteins were separated on 4–15% SDS-PAGE gels (Bio-Rad) at 200 V and transferred to polyvinylidine fluoride (PVDF) membranes via wet transfer for 60 min at 90 V. The PVDF membrane was then blocked using Pierce™ Protein-Free (PBS) Blocking Buffer (Thermo Fisher Scientific) for 1 h and then incubated at 5°C overnight in primary mouse CFTR antibody (see ‘Intestinal sac preparations and immunohistochemistry’, above) or with primary polyclonal rabbit Na⁺/K⁺-ATPase [NKA; Na⁺/K⁺-ATPase-α Antibody (H-300), cat. no. SC-28800, Santa Cruz Biotechnology, Dallas, TX, USA] to confirm the purity of each isolated fraction (membrane, cytoplasm and whole-intestine
personal scanner, and total protein was quantified using Image Studio Lite. Western blot results were subsequently normalized to total protein in each lane to account for variations introduced during sample loading. Protein abundance was calculated relative to the appropriate control group.

Statistical analyses
The data are presented as means±s.e.m. and were evaluated using parametric and non-parametric statistical tests, as described in the figure legends. Means were considered significantly different at \( P \leq 0.05 \). Control measurements for \( I_{SC} \), \( TEP \) and \( HCO_3^- \) secretion were calculated from the final 30 min of the control fluxes and treatment values were calculated from the final 30 min of a 70 min treatment exposure. All \( P \)-values for the two-tailed Holm–Sidak tests are listed in Table 2.

RESULTS
Membrane isolation and confirmation of CFTR band
NKA bands (105 kDa) were clearly visible in the membrane fraction, less so in the whole-intestine fraction, and absent in the cytoplasmic samples (Fig. 1A). This confirmed that the membrane isolation protocol was successful, as NKA is well known to be confined to the enterocyte basolateral membrane (Loretz, 1995) and, in the Atlantic salmon (Salmo salar) gill, has a molecular mass of 89–94 kDa (McCormick et al., 2009). Subsequently, western blots for CFTR were conducted and confirmed the presence of a band corresponding to CFTR at slightly greater than 150 kDa in the membrane fraction (Fig. 1B). In agreement with the molecular mass of human CFTR, which varies between 142 and 168 kDa (GenBank: AAA51980.1).

Stimulation by RGN and inhibition of PKA and PKG
In isolated segments of the posterior intestine, RGN stimulation reversed the absorptive \( I_{SC} \) (from mucosa-to-serosa to serosa-to-mucosa) and increased \( G_{TE} \) (Fig. 2). The effect of RGN was not altered by the addition of a PKG inhibitor (KT5823; \( n=8 \), \( P = 0.642 \)) (Fig. 2).

The PKA inhibitor H-89 partially, yet significantly, inhibited the effects of RGN on the absorptive \( I_{SC} \) and \( G_{TE} \) (Fig. 3A,B). H-89 alone also decreased the absorptive \( I_{SC} \), which was fully reversed with the addition of RGN; furthermore, H-89 significantly decreased \( G_{TE} \), which was then increased by RGN to a value that surpassed the control (Fig. 3C,D). There was no effect on \( I_{SC} \) and \( G_{TE} \) of vehicle dosages of DMSO (in which H-89 stocks were prepared; Fig. 3E,F). (All activators and inhibitors were added to the mucosal side of the tissue.)

Translocation of CFTR to the apical membrane
Western blots for CFTR revealed that RGN stimulation of posterior intestinal tissues increased the intensity of the CFTR band in the membrane fraction, while pre-treatment with H-89 eliminated changes in band intensity (Fig. 4). In addition, immunohistochemistry showed that CFTR fluorescence increased significantly in the apical membrane of RGN-treated tissues, when compared with control (Fig. 5).

\[\text{HCO}_3^- \text{ secretion and TEP}\]
RGN stimulation alone reduced \( \text{HCO}_3^- \) secretion from 0.322±0.038 to 0.260±0.020 \( \mu \text{mol cm}^{-2} \text{h}^{-1} \) in the posterior intestine, and this was further reduced to 0.206±0.012 \( \mu \text{mol cm}^{-2} \text{h}^{-1} \) after the addition of H-89 (Fig. 6A). RGN also reversed the negative TEP, but had no effect on \( G_{TE} \); the subsequent addition of H-89 partially reversed the
The downstream effects of RGN on PKA

Guanylin, uroguanylin and RGN are endogenous activators of GC-C in vertebrates (Currie et al., 1992; Greenberg et al., 1997; Hamra et al., 1993; Yuge and Takei, 2007) and have been shown to regulate body electrolyte and fluid homeostasis by modulating the activity of various ion transporters (Arshad and Visweswariah, 2012; Forte, 1999; Forte and Hamra, 1996). In vertebrates, this occurs through elevated cGMP formation (Currie et al., 1992; Iio et al., 2005; Yuge et al., 2006), which can increase PKG activity, modulate cAMP elevations and regulate PKA activity (Arshad and Visweswariah, 2012; Forte, 2013). The results of the present study on the Gulf toadfish suggest a greater role for PKA, rather than PKG, in the downstream pathways activated by RGN. Indeed, inhibition of PKA (by H-89) significantly reversed the effect of RGN on the absorptive $I_{SC}$ and TEP, while inhibition of PKG did not. However, the inhibitory effect of PKA was partial and might be due to the fact that (i) PKA is involved in more than one signaling pathway, (ii) H-89 will target all PKA molecules within a cell and not just those involved in guanylin-peptide signaling and/or (iii) numerous cellular pathways are involved in the response, not just those regulated by PKA. Perhaps pre-treatment with H-89 prior to RGN addition results in the modulation of multiple intracellular pathways that relate to osmoregulation and cAMP/PKA. Consequently, the effects of H-89 might not be clear and could be masked once RGN is added, whereas this might not be the case when H-89 is added after RGN treatment.

PKA inhibition alone (in the absence of RGN) also decreased the absorptive $I_{SC}$ and TEP, a result that is not surprising, given the ubiquitous involvement of cAMP and PKA in cellular processes, including ion transport mechanisms. For example, cAMP/PKA has been implicated in NKCC2 stimulation in the Gulf toadfish and sea bass. The downstream effects of RGN on PKA seem to be primarily mediated by PKA, rather than PKG.

**DISCUSSION**

The present study confirmed the presence of CFTR in the plasma membrane of the posterior intestine of the Gulf toadfish by western blot analysis and apical localization by immunohistochemistry. There was increased translocation of CFTR to the apical membrane after stimulation of the enterocytes with RGN that coincided with a reversal of the absorptive $I_{SC}$, which was suppressed by PKA inhibition. RGN stimulation also led to inhibition of $HCO_3^-$ secretion (Fig. 5A). The results of the present study on the Gulf toadfish suggest a greater role for PKA, rather than PKG, in the downstream pathways activated by RGN. Indeed, inhibition of PKA (by H-89) significantly reversed the effect of RGN on the absorptive $I_{SC}$ and TEP, while inhibition of PKG did not. However, the inhibitory effect of PKA was partial and might be due to the fact that (i) PKA is involved in more than one signaling pathway, (ii) H-89 will target all PKA molecules within a cell and not just those involved in guanylin-peptide signaling and/or (iii) numerous cellular pathways are involved in the response, not just those regulated by PKA. Perhaps pre-treatment with H-89 prior to RGN addition results in the modulation of multiple intracellular pathways that relate to osmoregulation and cAMP/PKA. Consequently, the effects of H-89 might not be clear and could be masked once RGN is added, whereas this might not be the case when H-89 is added after RGN treatment.

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**Fig. 5. Immunolocalization of CFTR.** Immunohistochemistry revealed that RGN induces increased trafficking of the CFTR to the plasma membrane of enterocytes from the posterior intestine of the Gulf toadfish. Overlaid fluorescence images of 4 μm sections from the posterior intestine of the Gulf toadfish show CFTR (blue) and NKA (green). Sections from control (A,C) and RGN-treated tissues (B) reveal different CFTR fluorescence intensity. The brush border is more clearly seen in C, demonstrating the location of the CFTR immunofluorescence in apical and sub-apical areas. Images are representative of each treatment group ($n=4$ each) and are shown at $40\times$ (A,B) and $60\times$ (C) magnification. (D) Relative corrected apical fluorescence for the CFTR, calculated from fluorescence images of the posterior intestine of Gulf toadfish. Tissues treated with RGN display greater CFTR, fluorescence relative to control tissues. Values are means±s.e.m. and significant differences were revealed by a Student’s $t$-test (*$P<0.05$). A, apical membrane; B, basolateral membrane; L, lumen; BB, brush border.
bream (*Sparus aurata*) intestine (Carvalho et al., 2012; Tresguerres et al., 2010) and PKA has been shown to phosphorylate NKCC2 in the thick ascending limb of rats (Ares et al., 2011); both of these mechanisms stimulate ion absorption. Accordingly, their inhibition would reduce ion absorption, similar to what is observed in the posterior intestine of Gulf toadfish.

Although it is beyond the scope of the present study, RGN activation of GC-C (which has intrinsic GC activity) increases intracellular cGMP formation in fish (Yuge et al., 2006) and mammals (Forte et al., 1993), and the effect of cGMP in this signaling pathway seems to be mediated more by PKA than by PKG. Indeed, in T84 cells (which originate from human colon carcinomas), stimulation of GC-C by either STa (the heat-stable *Escherichia coli* enterotoxin that also activates GC-C) or guanylin elevates cGMP formation, leading to changes in cellular physiology, but these effects are negated by PKA inhibition (Chao et al., 1994). Previous research suggests that there are two possible pathways by which increased cGMP formation is primarily mediated downstream by PKA. The first is indirect activation by inhibition of phosphodiesterase 3 (which cleaves cAMP to its inactive, linear form, AMP), which allows the accumulation of cAMP (Arshad, and Visweswariah, 2013). The second is a putative pathway by which cGMP directly activates PKA, which is suggested to occur in the Japanese tree frog (*Hyla japonica*), after stimulation of urinary bladder cells by atrial natriuretic peptide (Yamada et al., 2006, 2007). Whatever the pathway might be, prior research on the winter flounder demonstrates that PKA plays a prominent role in intestinal physiology (using VIP and cAMP, both of which are upstream regulators of PKA) by inhibiting ion transport and producing secretory currents (O’Grady, 1989; O’Grady et al., 1988; Rao et al., 1984). Accordingly, PKA is a possible candidate for mediating the intracellular effects of the guanylin peptides in seawater teleosts.

**RGN stimulation leads to increased apical CFTR activation and trafficking**

This is the first study to reveal membrane trafficking of CFTR in the Gulf toadfish that is stimulated by RGN. Although it merits further study, RGN can lead to CFTR translocation (present study) or modulate CFTR ion transport (Ruhr et al., 2014). Both of these mechanisms are supported by mammalian and fish studies. In the villi of the rat jejunum, STa and cAMP induce CFTR translocation to the apical membrane and their effects are decreased by PKA inhibition (Golin-Bisello et al., 2005). Likewise, the regulation of CFTR by cAMP/PKA pathways has also been documented in several fish species. For example, in the opercular epithelium of killifish and in the rectal gland of spiny dogfish shark (*Squalus acanthias*), cAMP/PKA has been implicated in CFTR activation (de Jonge et al., 2014; Marshall et al., 2005). Correspondingly, the present study also shows that RGN stimulation increases CFTR translocation to the apical membrane and that the translocation is suppressed by PKA inhibition, similar to findings in the rat jejunum (Golin-Bisello et al., 2005). Additionally, stimulation of Gulf toadfish posterior intestinal tissues by RGN leads to Cl− efflux (Ruhr et al., 2015, 2016) and is mediated by CFTR (Ruhr et al., 2014). Moreover, because of the short duration of the experiments,
shown that blocking CFTR with an inhibitor prevents the reversal of mRNA expression after 24 h, but not before 12 h (Ruhr et al., 2015). Although the present study does not definitively show CFTR in the apical membrane of the Gulf toadfish intestine, we have previously shown that blocking CFTR with an inhibitor prevents the reversal of the absorptive I_sc by RGN when applied to the apical membrane (Ruhr et al., 2014), while its basolateral application has no effect (I.M.R., personal observations). Accordingly, the combined evidence using CFTR inhibition (Ruhr et al., 2014), apical immunofluorescence in the intestine [present and previous studies (Ruhr et al., 2014)] and western blots on membrane fragments (present study) is compelling in proposing an apical localization of CFTR in the Gulf toadfish intestine. Moreover, there exists the possibility of more than one CFTR isoform being expressed in the Gulf toadfish intestine, as occurs in the Japanese eel (Wong et al., 2016), which could be regulated differently by RGN. Taken together, the current and previous (Ruhr et al., 2014) studies on the Gulf toadfish posterior intestine support the idea that RGN both activates membrane-present CFTR and increases CFTR trafficking to the apical membrane. On a final note, as with many biological mechanisms, CFTR trafficking might be regulated by proteins other than cAMP/PKA. An example relevant to the present study is serum and glucocorticoid-regulated kinase 1 (SGK1), which is a well-known osmotic stress transcription factor found in many vertebrates, including the fish intestine (Wong et al., 2014). SGK1 protein abundance is elevated in the intestine of killifish upon transfer to seawater from freshwater, an increase that is absent following morpholino gene knockdown (Notch et al., 2011). When SGK1 protein synthesis is suppressed in this manner, there is also a corresponding suppression of CFTR insertion into the plasma membrane of the intestine during seawater exposure (Notch et al., 2011). These results are similar to observations in the present study, prompting the question: what other possible proteins, enzymes or intracellular pathways does guanylin-peptide stimulation modulate? Undoubtedly, more research on this topic must be carried out.

Does RGN affect apical HCO_3^- transport via PKA?

In the present and previous studies, posterior intestinal tissues from Gulf toadfish displayed decreased HCO_3^- secretion when exposed to RGN (Ruhr et al., 2014, 2015, 2016). However, the mechanism by which this occurs is unknown, although it has been suggested that it involves apical HCO_3^-/Cl^- exchangers, such as SLC26a6 (Ruhr et al., 2016), which is the major HCO_3^--secretory transporter in the Gulf toadfish intestine (Grosell, 2006). The present study looked at the possible effect of RGN on the relationship between PKA and HCO_3^- secretion. In the presence of RGN alone, the posterior intestine of Gulf toadfish displayed decreased HCO_3^- secretion. However, when tissues were pre-treated with the PKA inhibitor H-89 prior to RGN addition, the effect of RGN on HCO_3^- secretion was almost halved compared with when RGN was added alone (17.5 ± 3.9% decrease with RGN alone versus 9.6±3.3% with RGN+H-89). These data suggest that decreases in HCO_3^- secretion might be partially mediated by elevated PKA activity, as a result of RGN stimulation. This mechanism is similar to what has been proposed in mammalian models, in which the secondary messengers cAMP and PKC inhibit SLC26 transporters by internalization (Kato and Romero, 2011). Nevertheless, it is still unknown which HCO_3^- transporter is affected by the downstream effects of RGN, as there are additional bicarbonate transporters (SLC26a3 and SLC4a2), other than SLC26a6, present in the apical membrane (Grosell, 2011). Despite this gap in our knowledge, there exist further comparable mechanisms for decreasing HCO_3^- secretion in other animals. Indeed, in the sea bream, stimulation of putative soluble adenyl cyclase (sAC), which produces cAMP in intestinal tissues, results in increased HCO_3^- secretion (Carvalho et al., 2012), possibly mediated by PKA. For mammals, there is a larger body of work that corroborates what has been seen in fish [see reviews or studies on the pancreas (Ishiguro et al., 2012), exocrine glands (Hong et al., 2014) and intestine (Ko et al., 2002)]. In rat renal proximal tubular epithelial cells, both a cAMP analog and forskolin increase HCO_3^- secretion, which is believed to elevate the exchange activity of SLC26a6; these stimulatory effects are inhibited by PKA inhibition via H-89 (Simão et al., 2008). Conversely, to our knowledge, only one study has demonstrated the direct effects of phosphorylation on SLC26a6 exchange activity (Alvarez et al., 2005). This study revealed that PKC phosphorylates human SLC26a6, leading to the disruption of a metabolon between SLC26a6 and carbonic anhydrase II, which decreases HCO_3^- secretion (Alvarez et al., 2005). However, that study investigated the role of PKC, as opposed to the present study, which looked at the effects of PKG and PKA on HCO_3^- secretion. Overall, the previous studies and our present study support the hypothesis that RGN decreases HCO_3^- secretion in Gulf toadfish intestine,
possibly by stimulating PKA activity, but the exact intracellular mechanisms by which they occur are still unclear.

Conclusions

The present study revealed that RGN stimulation of the posterior intestine appears to elevate PKA activity (Fig. 7). Upon binding to its receptor, RGN could regulate HCO₃⁻ and Cl⁻ transport by modifying potential interactions between SLC26 transporters and CFTR in the Gulf toadfish, as described in the rat intestine. Briefly, the rat intestinal model proposes that SLC26 transporters and CFTR form complexes that interact with one another through binding proteins, and that SLC26 transporters regulate CFTR activity (Ko et al., 2004). However, there are two main differences between the rat and Gulf toadfish, with respect to HCO₃⁻ transport and the direction of Cl⁻ movement through CFTR. In the rat intestine, stimulation of HCO₃⁻ secretion by SLC26 transporters also stimulates Cl⁻ absorption by CFTR (Ko et al., 2004); conversely, in the Gulf toadfish intestine, inhibition of HCO₃⁻ secretion correlates with stimulated Cl⁻ secretion by CFTR in the Gulf toadfish (Ruh et al., 2014, 2015). Alternatively, the effect of RGN stimulation could be mediated by PKA acting within intracellular microdomains (co-assemblies of proteins), affecting local signaling. Although speculative in the present context (and requiring further study), the presence of microdomains would be advantageous, as they allow endogenous stimulators (e.g. hormones) to activate specific intracellular pathways that are targeted by cAMP and PKA, rather than all pathways involving these molecules (Cooper, 2003; Zaccolo et al., 2006).

Overall, the present study demonstrates that PKA potentially mediates the downstream effects of RGN in the Gulf toadfish intestine by decreasing HCO₃⁻ secretion, inhibiting absorptive ion transport, and increasing CFTR trafficking to the apical membrane. Although it is tempting to speculate that this response is associated with GC-Cs in Gulf toadfish intestinal tissues, RGN could also be operating via other receptors evolved to mediate RGN or other guanylin-like peptides. Regardless of which receptor mediates the guanylin-peptide response, previous research on the seawater teleost intestine shows that guanylin-peptide stimulation leads to a reversal of net ion flux (from absorption to secretion) and decreased HCO₃⁻ secretion, with the overall effect of inhibited (or reversed) water absorption by the intestine (Ando and Takei, 2015; Ando et al., 2014; Ruh et al., 2014, 2015; 2016; Yuge and Takei, 2007). These effects appear to occur by inhibited NKCC2 transport, activation of CFTR and Cl⁻ secretion, and inhibited HCO₃⁻/Cl⁻ exchange activity (Ruh et al., 2014, 2016). However, the present study reveals that these changes to ion flux might be a function of CFTR insertion into the apical membrane, which is mediated, in part, by PKA, as well as the direct effects on membrane-present CFTR function. Despite what is known about the guanylin peptides in seawater teleosts, their functional importance to whole-animal physiology is still unclear and merits further study.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: I.M.R., M.G.; Methodology: I.M.R., K.L.S., Y.T., M.G.; Validation: I.M.R., M.G.; Formal analysis: I.M.R., K.L.S., M.G.; Investigation: I.M.R., M.G.; Resources: M.G.; Data curation: I.M.R.; Writing - original draft: I.M.R.; Writing - review & editing: I.M.R., K.L.S., Y.T., M.G.; Visualization: I.M.R.; Supervision: M.G.; Project administration: M.G.; Funding acquisition: M.G.

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