REGULATION OF INTESTINAL CHLORIDE SECRETION AND CLONING OF UROGUANYLIN IN THE KILLIFISH, *FUNDULUS HETEROCLITUS*

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Guanylin (G) peptides activate membrane guanylate cyclases (GC) and regulate intestinal secretion in mammals via cGMP. G-like peptides and/or GC-receptors (R-GC's) have been identified in all vertebrate classes including freshwater fish (Forte, L., *Reg. Peptides*. 81:25-39, 1999). We have recently reported that treatment of mucosa isolated from dogfish shark proximal intestine (Karnaky, K. J., Jr., *et al.*, Bull. MDIBL 41, 51-52, 2001; Karnaky K. J., Jr., *et al.*, Bull. MDIBL 42, 26-28, 2002) or from eel intestine (Karnaky K. J., Jr., *et al.*, Bull. MDIBL 42, 26-28, 2002) with *E. coli* heat-stable enterotoxin (ST) elicits significant increases in tissue cGMP. These results suggest that a guanylin-cGMP pathway is present in these tissues. Moreover, cDNAs encoding eel uroguanylin, zebrafish guanylin (Comrie *et al.*, Biochem. Biophys. Res. Comm. 281,1078-1085, 2001) and both eel and medaka forms of R-GCs have been identified (Mantoku *et al.*, *J.* Biochem. 125, 476-486, 1999; Comrie *et al.*, Comp. Biochem. Physiol. *B* 129:575-586, 2001). We continue to focus on the marine teleost intestine, most commonly thought of as a salt-absorbing tissue (Karnaky, K. J., Jr. in The Physiology of Fishes, 2nd edition. D. Evans, editor. CRC Press, pp. 157-176, 1998). Interestingly, Marshall *et al.* (J Exp Biol., 205, 745-58, 2002) recently demonstrated that the distal portion of the seawater-adapted killifish intestine secretes chloride and fluid when stimulated by three drugs: 1_M ionomycin, 0.5 mM 8-Br-cAMP, and 0.1mM 3-isobutyl-1-methylxanthine (IBMX). Thus, calcium and cAMP signaling mechanisms appear to regulate chloride secretion in the seawater-adapted killifish intestine, but the first messenger(s) is unknown. Many hormones are considered to regulate ion transport in the teleost intestine, including atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and vasoactive intestinal polypeptide (VIP) (reviewed in Marshall, W. S. *et al.*, *op. cit.*). This report describes our observations of the effects, or lack thereof, of these hormones on chloride secretion in the killifish intestine.

Intestines were obtained from killifish, *F. heteroclitus*, from Northeast Creek by minnow trap. Animals were maintained in running seawater aquaria and fed daily with commercial fish food (Hartz: Tetra Min). Animals were double pithed and sections of intestine were mounted in Ussing chambers using a 3mm aperture (Karnaky K. J., Jr. *et al.*, Science, 14,203-205, 1977). The voltage across the epithelial sheet was clamped to zero using a voltage clamp. The output was recorded using a strip chart recorder. After a stable short-circuit (Isc) was reached, ionomycin, cAMP, IBMX, atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), isoproterenol and/or vasoactive intestinal polypeptide (VIP) were added and effects on Isc and resistance were noted. Additionally, after a steady-state Isc was reached, we tested the effects of three chloride transport inhibitors, bumetanide, ouabain, or potassium cyanide. Intestines were also frozen on dry ice and used to isolate total RNA for homology cloning of cDNAs encoding uroguanylin and guanylin. Treatment of mucosa isolated from killifish proximal and distal

intestine with uroguanylin elicited 4 fold and 2 fold increases in cGMP levels, respectively indicating the presence of receptor-GCs for uroguanylin, guanylin and *E.coli* ST peptides in this animal.

Because Marshall, W. S., *et al.* (*op. cit.*) observed that chloride secretion did not occur until all three drugs, ionomycin, IBMX, and cAMP were added, test hormones were added after exposure to these drugs. On the other hand, since these three agents could maximally stimulate chloride secretion, we also performed experiments in which none, or only one or two of these drugs were added before the test hormones. Given in different combinations, ionomycin, IBMX, and cAMP stimulated Isc. Addition of 0.1mM IBMX and 0.5mM 8-Br-cAMP resulted in a 364% increase in Isc (Isc microamps/cm^2; n=4). Addition of 0.1mM IBMX resulted in a 75% increase in Isc (n=2). Addition of 0.4 microM ionomycin and 0.1 mM IBMX resulted in a 168% increase in Isc (n = 6). Addition of ionomycin, IBMX and 8-Br-cAMP resulted in a 112% increase in Isc (n=4).

Hormones were tested by their addition alone or after exposure of the killifish intestine to various combinations of the drugs. No change in Isc was observed with CNP alone (n=4), or CNP following IBMX (n=1), or CNP following IBMX and ionomycin (n=2), or CNP following IBMX, ionomycin, and cAMP (n=1). Addition of VIP after ionomycin (0.4 microM) and IBMX (0.1 mM) resulted in no change in Isc (n=4).

E. coli ST peptide, a molecular mimic of uroguanylin, was tested alone or after various combinations of drugs. ST appeared to cause a change in Isc in only a few cases. Addition of ST only to the apical side resulted in no change in Isc (n=4); addition of ST alone to the apical and serosal sides resulted in no change in Isc (n=1), in two cases resulted in a change of a declining slope to a slope of zero. In one case addition of ST to both sides following forskolin resulted in a 32.26% increase in Isc (n=1). Exposure to various combinations of IBMX, ionomycin, and cAMP prior to apical or serosal additions of ST did not elicit increases in Isc. In summary, ST caused an increase in Isc in one tissue, and stopped the decay in Isc in two others.

Total RNA from killifish intestine was used in a homology cloning method based on RT-PCR with degenerate oligonucleotide primers that were derived from our previous isolation of cDNAs encoding eel uroguanylin (Karnaky K. J., Jr., *et al.*, Bull. MDIBL 42, 26-28, 2002). Illustrated below are amino acid sequences of the killifish uroguanylin peptide obtained in this study compared with the primary structure of eel uroguanylin obtained previously.

Killifish uroguanylin: **S D P C E I C A N P S C F G C L D**

Eel uroguanylin: **P D P C E I C A N A A C T G C L**

These data confirms that Isc of killifish intestine is stimulated by the addition of ionomycin, IBMX, and cAMP (Marshall, W. S., *et al.*, *op. cit.*). There was no evidence that VIP or CNP modified Isc in this intestine, although these hormones have been reported to regulate ion transport across the intestines of various fish species (Marshall, W. S., *et al.*, *op. cit.*). Clearly, both proximal and distal intestine responds to uroguanylin *in vitro* with an increase in tissue cGMP. Moreover, we isolated cDNAs encoding the killifish form of uroguanylin from

intestinal RNA preparations. Thus, at least one of the enteric hormones and a receptor-GC is present in killifish intestine. Intriguingly, there were three positive responses to *E. coli* ST in short-circuited epithelia, which may indicate that a role for uroguanylin and/or guanylin in the cGMP-mediated regulation of chloride secretion in killifish intestine may exist *in vivo*. This tissue is easy to dissect and mount in Ussing chambers. Another value of this well-studied teleost species and intestinal preparation is that we have validated a method to verify whether the isolated epithelium is responsive to known chloride secretogogues after exposure to a primary messenger (hormone) that may not cause any change in electrical properties when administered alone. Moreover, this study has provided new information concerning the primary structure of killifish uroguanylin, which will allow us to synthesize a species-specific uroguanylin peptide for use in future experiments with killifish intestine. Such experiments should provide additional insights into a possible role for guanylin and uroguanylin in cGMP-dependent regulation of ion transport in the killifish intestine.

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