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CHARACTERIZATION OF AN ENDOTHELIN ET_B RECEPTOR IN THE GILL OF THE DOGFISH SHARK *SQUALUS ACANTHIAS*

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Summary

Endothelins (ETs) are potent vasoconstrictive peptides that are secreted by the vascular endothelium and other tissues in vertebrates. Previous studies have demonstrated that ETs are expressed in a variety of fish tissues and contract various blood vessels. In order to determine if receptors for ET are expressed in fish gill tissue, we examined the binding kinetics of ^{125}I-labeled, human ET-1 to membrane fragments isolated from the gill of the dogfish shark, *Squalus acanthias*. ^{125}I-ET-1 bound at a single site, with a dissociation constant (K_d) and binding site number (B_{max}) very similar to those described in a variety of mammalian blood vessels. ET-1 and ET-3 competed equally with ^{125}I-ET-1, suggesting that the receptor was ET_B, which has been shown in mammalian systems to bind to both ligands equally. The ET_B-specific agonists sarafotoxin S6c, IRL-1620, and BQ-3020 also competed against ^{125}I-ET-1 at a single site, supporting this hypothesis. We conclude that the shark gill expresses an ET_B receptor with substantial homology to the mammalian receptor and that ET may play an important role in modulating such vital gill functions as gas exchange, ion regulation, acid–base balance, and excretion of nitrogen.

Key words: endothelin, receptor, *Squalus acanthias*, gill.

Introduction

The endothelins (ETs) are considered to be the most potent vasoconstrictive agents in animals (e.g. Tamirisa et al., 1995). In addition to cardiovascular effects, it is now clear that ETs play a role in the function of many organs, including the pituitary, kidney and adrenal gland (e.g. Kennedy et al., 1993; Masaki, 1993). ET-1 was first isolated from endothelial cells, but ET-1 and the other members of the peptide family, ET-2 and ET-3, have been described from many tissues including the intestine, stomach, heart, kidney and brain of mammals (e.g. Masaki, 1993; Stjernquist, 1998).

The effects of the endothelins are mediated through at least two receptors, termed ET_A and ET_B, differentiated by their relative specificity for agonists and antagonists. ET_A is much more sensitive to ET-1 than to either ET-2 or ET-3 and it is blocked by the inhibitor BQ-123. ET_B is equally sensitive to all three ETs, and to the analogue sarafotoxin S6c (SRX S6c; originally isolated from the venom of the snake *Atractaspis engaddensis*) and various linear analogues of ET-1 (such as Ac-[$Ala^{11,15}$]endothelin-1 (6-21), termed BQ-3020; and Suc-[Glu^{9}, $Ala^{11,15}$]endothelin-1 (8-21), termed IRL-1620) (e.g. Huggins et al., 1993; Hiley, 1995). In mammals, ET_A receptors mediate the constrictory effect of ET on the vascular smooth muscle, although in some vessels, especially veins but also various arteries, constriction may be mediated *via* the ET_B receptors (e.g. Sumner et al., 1992; Pollock and Opgenorth, 1993; Warner et al., 1993; Sudjarwo et al., 1994; Teerlink et al., 1994; White et al., 1994). More commonly, ET_B receptors mediate the initial, transitory dilation produced by infusion of ET-1 (Spokes et al., 1989), which is now known to involve an autocrine action of ET on endothelial cells, stimulating the release of the endothelium-derived relaxing factor, nitric oxide (De Nucci et al., 1988; Rubanyi and Polokoff, 1994). In fact, there is some suggestion that distinct ET_B receptors, termed ET_{B1} and ET_{B2}, may mediate dilation *versus* constriction, respectively (e.g. Warner et al., 1993; Bax and Saxena, 1994; Sudjarwo et al., 1994; Teerlink et al., 1994; Zuccarello et al., 1998a), although this hypothesis is not universally accepted (e.g. Clozel and Gray, 1995).

There is an emerging literature demonstrating that ET and its receptors are present in fish tissues, but little is known about the physiology of endothelin in this group. Immunoreactive ET has been localized in the brain of the lamprey, *Lampetra japonica*, and the caudal neurosecretory system, gill and kidney of the teleost, *Oryzias latipes* (medaka) (Kasuya et al., 1991) as well as the gills of the Indian catfish (*Heteropneustes fossilis*), brown trout (*Salmo trutta*), electric ray (*Torpedo marmorata*), dogfish shark (*Scyliorhinus canicula*) and bowfin (*Amia calva*) (Zaccone et al., 1996). In addition, ET-1 constricted various blood vessels (e.g. ventral aorta, coronary artery, mesenteric artery, posterior cardinal vein or

celiacomesenteric artery) from the catfish (*Amiurus melas*) (Poder et al., 1991), rainbow trout (*Oncorhynchus mykiss*) (Olson et al., 1991), Atlantic salmon (*Salmo salar*) and cod (*Gadus morhua*) (Sverdrup et al., 1994) and *S. acanthias* (Evans et al., 1996). We have also recently determined that the ventral aortae of the lamprey (*Petromyzon marinus*), Atlantic hagfish (*Myxine glutinosa*), Florida gar (*Lepisosteus platyrhynchus*), gulf toadfish (*Opsanus beta*), American eel (*Anguilla rostrata*) and African lungfish (*Protopterus aethiopicus*) constrict when ET-1 is applied (D. H. Evans, unpublished). It is clear, therefore, that at least vascular receptors for ET arose before the earliest vertebrates. Indeed, immunoreactive ET has been described in the nervous system of invertebrates (Kasuya et al., 1991). In all but one of the functional studies in fishes, the specific ET receptor was not delineated; however, we determined that an ET_B receptor mediates the constrictory response in the ventral aorta of *S. acanthias* (Evans et al., 1996).

The fish gill is a structurally complex organ that plays a vital role in such important processes as gas exchange, ion regulation, acid–base regulation and excretion of nitrogenous waste products (e.g. Evans, 1998). We have previously shown that the gills of various species (*M. glutinosa*, *S. acanthias* and *O. beta*) express receptors for natriuretic peptides (Donald et al., 1994, 1997; Toop et al., 1995), although the precise location of the receptors (vascular *versus* epithelial) is still unknown. To begin to access the role of endothelins in fish gill function, we have characterized ET binding to membrane fragments isolated from the gill of the dogfish shark, *S. acanthias*.

Materials and methods

Adult spiny dogfish *Squalus acanthias* (L.) (3–5 kg) were netted in Frenchman Bay, Maine, USA and maintained in live cars with running sea water (12–15 °C) at the Mount Desert Island Biological Laboratory (Salsbury Cove, ME, USA) for at least 24 h before use. Fish were killed by pithing through the snout, and gills were removed and frozen immediately in liquid N_2. The tissue was stored at –70 °C before shipment to the University of Florida on dry ice.

Membrane fragments were prepared as described previously (e.g. Donald et al., 1997). Gill tissue was scraped off the gill arch and homogenized in 3–5 volumes of buffer (50 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} $NaHCO_3$, pH 7.4 at room temperature (RT); 24 °C) using a Tissue-Tearor (Biospec, Bartlesville, OK, USA). The homogenate was diluted 1:1 with a buffer solution containing 50 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} $MgCl_2$ (pH 7.4), and centrifuged at 800 ***g*** for 15 min at 4 °C. The supernatant was poured through three layers of cheesecloth into a second tube and spun at 30,000 ***g*** for 20 min at 4 °C. This supernatant was discarded, and the pellet was washed with a solution of 50 mmol l^{-1} Tris-HCl and 250 mmol l^{-1} sucrose (pH 7.4 at RT) and resuspended in the same solution (250–500 µl). A subsample was taken for protein analysis (BCA Protein Assay Kit, Pierce), the rest subdivided and stored at –70 °C until use.

For the kinetic analysis of the binding of ^{125}I-ET-1 to a putative membrane receptor, 50 µg of gill protein was incubated at RT in 250 µl of a buffer solution [50 mmol l^{-1} Tris-HCl, 100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 0.25 % bovine serum albumin, 0.05 % Bacitracin, 4 µg ml^{-1} Leupeptin, 2 µg ml^{-1} Chymostatin, 2 µg ml^{-1} Pepstatin and 1 µg ml^{-1} phenylmethyl-sulfonyl fluoride (PMFS) and 10^{-12}–10^{-6} mol l^{-1} ^{125}I-ET-1 (Peninsula Laboratories, Belmont, CA, USA)]. Preliminary experiments determined that 90 min incubation was sufficient for maximal binding. Non-specific binding was measured by including 10^{-6} mol l^{-1} ET-1 in a separate set of incubation tubes containing the incubation buffer, gill protein and concentration range of radiolabeled ET-1. For competition assays, the same protocol was used, except that each reaction tube contained 30 pmol l^{-1} ^{125}I-ET-1 and specific concentrations of either unlabelled ET-1 or putative competitive agonists or antagonists for either ET_A or ET_B receptors. In all experiments, binding was terminated by addition of 2 ml of ice-cold 50 mmol l^{-1} Tris-HCl and 100 mmol l^{-1} NaCl (pH 7.4), and bound and free ligand were separated by vacuum filtration through a Whatman GF/C filter, pretreated with 1 % polyethylenimine. The filters were then washed with 5 ml of the ice-cold washing buffer, placed in glass vials and assayed on a Beckman gamma counter with 78 % efficiency.

ET-1 (human) and ET-3 (human) were purchased from American Peptide (Sunnyvale, CA) and dissolved in 1 % acetic acid, divided into portions, evaporated to dryness (SpeedVac; Savant, Framingdale, NY, USA) and stored at –70 °C until use. SRX S6c, BQ-3020, IRL-1620 and RES-701-1 (Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp) were also purchased from American Peptide, dissolved in 5 % dimethylsulfoxide (DMSO) and portions stored at –70 °C until use. Maximal DMSO concentration in the final reaction solution was 0.05 %. All data analysis (non-linear regression for saturation and competitive binding, and Student's *t*-test)

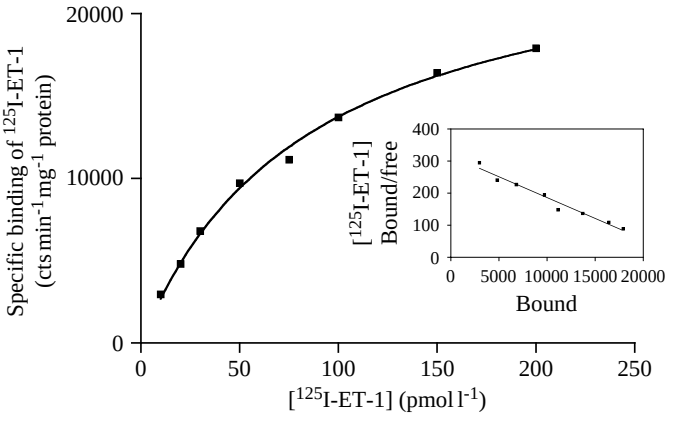

Fig. 1. Representative saturation binding curve of ^{125}I-ET-1 to gill membranes from *Squalus acanthias*. Inset: Scatchard analysis of the binding data.

used Prism (GraphPad, Inc., San Diego, CA, USA). Data are expressed as mean ± S.E.M.

Results

^{125}I-ET-1 binding to shark gill membranes was saturable, with an apparent dissociation constant K_d of 68.8±10.9 pmol l^{-1} and a binding site number B_{max} of 780±59.7 fmol mg^{-1} protein (N=3), calculated by non-linear regression (Fig. 1). In all three binding experiments, non-linear regression equations for one-*versus* two-site models were tested using Prism; in every case, the single-site equation provided the best fit. The Scatchard analysis (Fig. 1) also indicated a single binding site.

Unlabeled ET-1 competed with ^{125}I-ET-1 at a single site with a 50% inhibition constant IC_{50} of 5.11±0.99 nmol l^{-1}

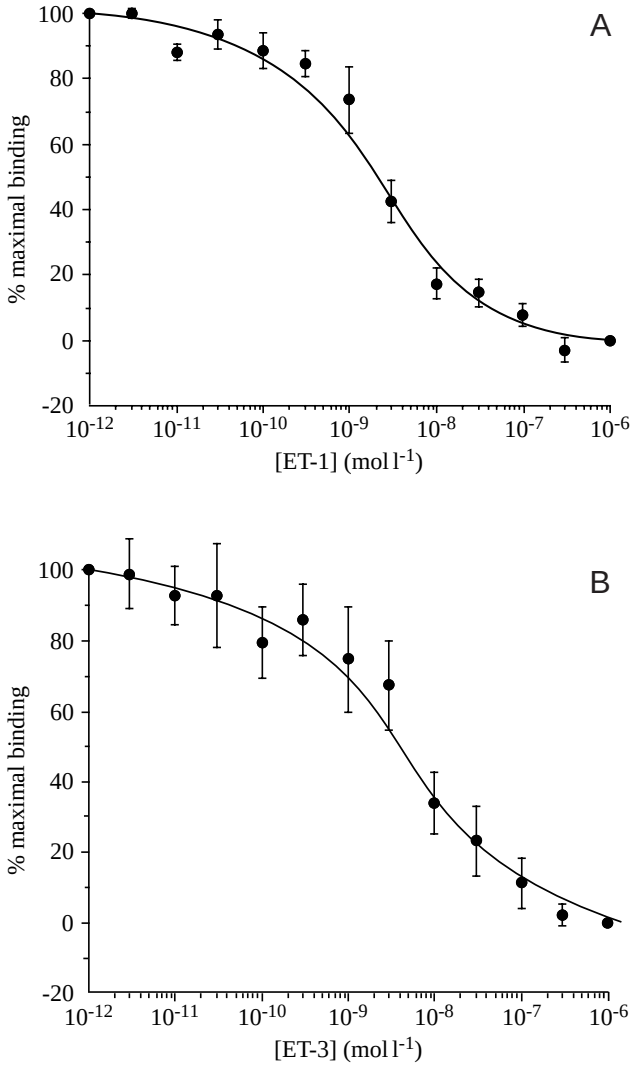

Fig. 2. Plot of competition between unlabeled human ET-1 (A) or ET-3 (B) and ^{125}I-ET-1 for binding to gill membranes. Data were normalized so that maximal competition (zero binding of ^{125}I-ET-1) was assumed to be at 10^{-6} mol l^{-1} unlabelled ET-1 or ET-3. Values are means ± S.E.M.; N=7–11 for ET-1 and 6–7 for ET-3; the curves were fitted by eye.

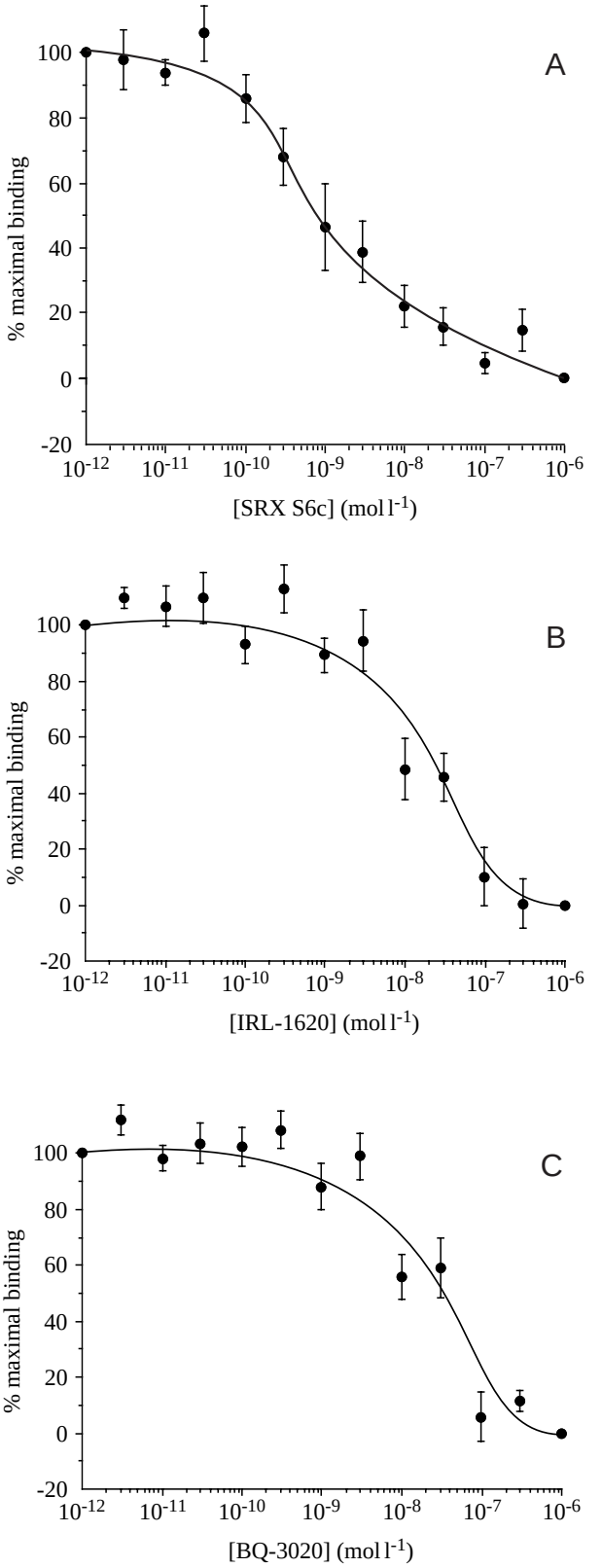

Fig. 3. Plot of competition between SRX S6c (A, N=8-9), IRL 1620 (B, N=7) and BQ-3020 (C, N=6-7) and ^{125}I-ET-1 for binding to gill membranes. Data were normalized so that maximal competition (zero binding of ^{125}I-ET-1) was assumed to be at 10^{-6} mol l^{-1} for each agonist. Values are meams ± S.E.M.

Table 1. *Published kinetic characteristics of endothelin receptors in mammalian blood vessels*

Species	Tissue	K_d (pmol l^{-1})	B_{max} (fmol mg^{-1} protein)	Reference
Dogfish shark	Gill	68.8	780	Present study
Rat	Renal microvessels	20.1	1343	Edwards and Trizna (1995)
Rabbit	Renal microvessels	21.5	810	Edwards and Trizna (1995)
Rat	Preglomerular vessels	310	1336	De Leon (1995)
Human	Cerebral resistance vessels	800	690	Yu et al. (1995)
Human	Pulmonary artery	850	15.2	Davenport (1995)
Human	Coronary artery	140	71.0	Davenport (1995)
Human	Aorta	510	9.4	Davenport (1995)

K_d, dissociation constant; B_{max}, binding site number.

(N=10; Fig. 2A). The inhibitor constant K_i was calculated to be 3.57±0.69 nmol l^{-1}, using the method of Cheng and Prusoff (1973). ET-3 also competed with ^{125}I-ET-1 at a single site with an IC_{50} of 8.93±4.90 nmol l^{-1} (N=6; Fig. 2B), not significantly different from the IC_{50} of ET-1 (P=0.4). The ET_B-specific agonists SRX S6c, IRL-1620 and BQ-3020 also displaced the radiolabeled ET-1 (Fig. 3A–C) with high efficacy: IC_{50}=1.77±1.31 nmol l^{-1} (N=8), 32.8±18.7 nmol l^{-1} (N=7) and 37.2±13.5 nmol l^{-1} (N=7), respectively. All competitions were at a single site. Only the IC_{50} of BQ-3020 was signficantly above (P<0.001) that of ET-1. The ET_B-specific antagonist RES-701-1 did not compete with ^{125}I-ET-1 until relatively high concentrations (producing a 15 % reduction in specific binding at 3×10^{-7} mol l^{-1} and 50 % reduction at 10^{-6} mol l^{-1} RES-701-1; data not shown).

Discussion

These data provide the first pharmacological characterization of an endothelin receptor in a fish gill, or any tissue in a fish. They suggest that there is a single endothelin receptor expressed (Fig. 1), with kinetic characteristics (K_d, B_{max}) quite similar to those described for the binding of ET-1 to mammalian vascular endothelin receptors (Table 1). Since a heterologous ligand was used (human ET-1), it is clear that the fish gill ET receptor must share significant homology with the mammalian receptor. The fact that ET-3 is as competitive as ET-1 at the single site (Fig. 2) suggests that the gill receptor is an ET_B, because that is the promiscous receptor that binds to all native ligands equally (e.g. Huggins et al., 1993; Masaki et al., 1994). This hypothesis is supported by our finding that three ET_B-specific receptor agonists (SRX S6c, IRL-1620 and BQ-3020; e.g. Masaki et al., 1994) each competed with ^{125}I-ET-1 at a single site with a relatively low IC_{50}, equivalent to that for ET-1 in each case except for BQ-3020 (Fig. 3). In the rat aorta and atrium (in which ET_A is the predominant receptor expressed), ET-1 is 50 000 times more potent than SRX S6c in displacing ^{125}I-ET-1 (Williams et al., 1991). IRL-1620 has an ET_B/ET_A selectivity of 120 000 in binding experiments (Takai et al., 1992), but BQ-3020 has a lower ratio of selectivity (4700; Saeki et al., 1991). Our data are also consistent with our previous study (Evans et al., 1996), which demonstrated that the ET-1-induced contraction of the ventral aorta from *S. acanthias* was mediated by a single receptor with nearly equivalent sensitivity to ET-1, ET-3 and SRX S6c and no sensitivity to BQ-123, the well-described ET_A-specific antagonist (e.g. Ihara et al., 1992).

Although the hypothesis that the ET_B receptors which mediate dilation (termed ET_{B1}) are distinct from the ET_B receptors that mediate contraction (termed ET_{B2}; e.g. Stjernquist, 1998) is not universally accepted (e.g. Clozel and Gray, 1995), many studies have demonstrated differences in the sensitivity of ET_B receptors to specific agonists and antagonists. It has been suggested that the putative ET_{B1} receptor is sensitive to the agonist IRL-1620 and the antagonist RES-701-1 (e.g. Sudjarwo et al., 1994; Brooks et al., 1995; Zuccarello et al., 1998b), while the ET_{B2} receptor is insensitive to either substance. The receptor in the shark gill was extremely sensitive to IRL-1620 (Fig. 3), but the putative ET_{B1} receptor antagonist, RES-701-1, showed little or no competition with ^{125}I-ET-1. In addition, our earlier physiological studies demonstrated that RES-701-1 did not inhibit the ET-1 stimulated contraction of aortic rings from this species (D. H. Evans and M. P. Gunderson, unpublished data). These data suggest that the shark ET_B receptor shares characteristics with both the putative ET_{B1} and ET_{B2} receptors that have been proposed for mammals (e.g. Stjernquist, 1998).

Our data do not allow us to delineate specific sites for the ET_B receptor in the *S. acanthias* gill, but two recent studies (Sundin and Nilsson, 1998; Stenslokken et al., 1999) have demonstrated that ET receptors may produce a redistribution of blood flow within the gill lamellae of the rainbow trout (*O. mykiss)* and Atlantic cod (*G. morhua*) by constriction of pillar cells. In their experiments, ET-1 increased blood flow to the periphery of the lamellae, which presumably alters the epithelial surface area for gas exchange or passive movements of ions. Whether ET receptors are expressed on pillar cells or in microvessels in the shark gill remains to be determined, as does its putative role in gill hemodynamics.

Gill ET receptors also may play a role in controlling other gill functions, such as osmoregulation, acid–base regulation and nitrogen excretion. ET-1 has been shown to stimulate

Na^+-K^+-activated ATPase in both the rat adrenal zona glomerulosa (Pecci et al., 1994) and cerebral capillary endothelium (Kawai et al., 1995); but it inhibited this enzyme in inner medullary collecting duct cells in the kidney of the rabbit (Zeidel et al., 1989). However, ET-1 did not affect the activity of Na^+-K^+-activated ATPase in rabbit colonic mucosa (Roden et al., 1992). ET-1 stimulated Na^+-K^+-Cl^- transport (Vigne, 1994; Kawai et al., 1995) and Na^+/H^+ exchange (Vigne et al., 1991) in the rat cerebral capillary endothelium as well as a Cl^- channel in the rat colonic mucosa (Hosokawa et al., 1995). It also inhibited influx of Na^+ through an apical, amiloride-sensitive channel in the colonic mucosa (Hosokawa et al., 1995). ET-1, however, inhibited both Na^+/H^+ exchange and V-type H^+ pump activity in cultured bovine corneal epithelial cells (Wu et al., 1998). The transport mechanisms whereby Na^+ and Cl^- cross the epithelium of the fish gill are well characterized, especially in the teleosts, and there is functional evidence that all of these proteins that have been shown to be affected by ET-1 are expressed and mediate various pathways for NaCl extrusion or uptake (depending on the salinity) as well as acid–base regulation (see Evans et al., 1999). It is likely, therefore, that ET may play a role in gill solute transport as well as gill hemodynamics. In fact, our preliminary data (D. H. Evans and K. J. Karnaky, unpublished) suggest that both ET-1 and SRX S6c inhibit the short-circuit current, dose-dependently, across the opercular skin from the killifish, *Fundulus heteroclitus*, which is the standard model for salt extrusion by the marine teleost gill (e.g. Karnaky, 1998).

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