Am J Physiol Regul Integr Comp Physiol 286: R560–R568, 2004.
First published November 20, 2003; 10.1152/ajpregu.00281.2003.

NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis

David H. Evans,[1,2] Rachel E. Rose,[1,2] Jennifer M. Roeser,[1,2] and James D. Stidham[2,3]

[1]*Department of Zoology, University of Florida, Gainesville, Florida 32611;* [3]*Department of Biology, Presbyterian College, Clinton, South Carolina 29325; and* [2]*Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine 04672*

Submitted 22 May 2003; accepted in final form 17 November 2003

Evans, David H., Rachel E. Rose, Jennifer M. Roeser, and James D. Stidham. NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am J Physiol Regul Integr Comp Physiol* 286: R560–R568, 2004. First published November 20, 2003; 10.1152/ajpregu.00281.2003.—Recent evidence suggests that paracrine signaling agents, such as endothelin (ET), nitric oxide (NO), superoxide (O_2^-), and prostanoids can modulate mammalian renal function by affecting both hemodynamic and epithelial ionic transport pathways. Since these signaling pathways have been described in fish blood vessels, we hypothesized that they may control salt transport across the gill epithelium—the primary site of ion excretion in marine teleost fishes. We found that ET, the NO donors sodium nitroprusside and spermine NONOate, and the prostanoid PGE_2 each can produce a concentration-dependent reduction in the short circuit current (I_{sc}) across the isolated opercular epithelium of the killifish (*Fundulus heteroclitus*), the generally accepted model for the marine teleost gill epithelium. Sarafotoxin S6c was equipotent to ET-1, suggesting that ET_B receptors are involved. Incubation with N^G-nitro-L-arginine methyl ester (L-NAME) or indomethacin reduced the effect of subsequent addition of SRXS6c by 17 and 89%, respectively, suggesting the presence of an ET to NO and PGE axis. The effects of L-NAME and indomethacin were not additive, but the superoxide dismutase mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) reduced the effect of SRXS6c by 34% and preincubation with L-NAME, indomethacin, and TEMPOL reduced the SRXS6c response to zero. This suggests a direct role for O_2^- in this axis. COX-2 appears to be the major enzyme involved in this axis because the specific COX-2 inhibitor NS-398 was twice as effective as the COX-1 inhibitor SC560 in inhibiting the SRXS6c effect. The I_{sc} was stimulated by the EP_2 agonist butaprost and inhibited by the $EP_{1,3}$ agonist sulprostone, suggesting both stimulatory and inhibitory PGE receptors in this tissue. Carbaprostacyclin (PGI_2 analog), thromboxane A_2, $PGF_{2\alpha}$, and PGD_2 did not affect the I_{sc}. Our data are the first to suggest the importance of an ET-stimulated and NO-, O_2^--, and PGE_2-mediated signaling axis that can modify active extrusion of NaCl across the killifish opercular epithelium and, by inference, the marine teleost gill epithelium.

fish; gill transport; paracrine signaling

SINCE THE DISCOVERY of the first endothelin (ET) in 1988 (61), it has become clear that this family of paracrine signaling peptides (ET-1, ET-2, ET-3) plays a significant role in mammalian physiology and pathophysiology (e.g., 28). The cardiovascular effects of ETs are mediated by two receptors, termed ET_A and ET_B (e.g., 31), which are expressed in both vascular smooth muscle and endothelial cells. The two receptors can be discriminated by radioligand displacement and/or relative agonist sensitivity, because the agonist sarafotoxin S6c (SRXS6c) shows very high specificity for ET_B receptors (e.g., 36). ET produces potent constriction in a variety of vessels via activation of either ET_A or ET_B receptors (e.g., 54). ET binding to the endothelial ET_B receptors stimulates the release of the endothelium-derived relaxing factors (EDRF) nitric oxide (NO) and prostacyclin (PGI_2) (8). Indeed, infusion of ET into mammals produces a biphasic response: transient hypotension followed by a more significant and prolonged hypertension (e.g., 7), and the initial hypotension can be blocked by inhibition of NO synthase (NOS) (9), suggesting that NO is the primary EDRF.

ET-like immunoreactivity also has been found in gill, heart, or neural tissue in all major groups of fishes, including agnathans, elasmobranchs, and teleosts (e.g., 27, 57), and a trout ET recently has been purified, sequenced, and synthesized (60). Infusion of either this peptide or human ET-1 produces a complex suite of cardiovascular responses in the trout, including a triphasic pressure change (pressor-depressor-pressor) in the dorsal aorta produced by changes in cardiac output, gill resistance, systemic resistance, and venous compliance (22). In vitro, ET-1 produces substantial constriction in blood vessels isolated from a variety of fish species (e.g., 16, 18, 55, 60). Pharmacological and physiological protocols have delineated both ET_A and ET_B receptors in fish blood vessels and gill tissue (e.g., 15, 16, 18).

NOS has been identified in central and peripheral neural tissue in a variety of fish species (e.g., 6, 20, 48–50) and also in neurons and epithelial cells in the gill of a catfish (e.g., 63) and the killifish (12). Prostanoids are produced in various tissues (including the gill, see below) in a variety of fish groups (e.g., 5, 29). NO (or an NO donor) is dilatory in teleost vessels (e.g., 39, 53) but constrictory in elasmobranch and hagfish vessels (e.g., 17, 18) and produces a biphasic response (constriction followed by dilation) in the lamprey ventral aorta (18). Prostanoids, however, are dilatory in vessels isolated from a variety of fish species (e.g., 14, 17–19), although it is unclear if the effector is PGI_2 or PGE_2, because of potential cross-reactivity with receptors. Despite these uncertainties, it is clear that modern representatives of the most ancient vertebrate lineages express the components of the paracrine signaling system that involves ET, NO, and prostanoids.

Both ET_A and ET_B receptors also have been identified in the mammalian kidney (e.g., 4) and ET produces natriuresis and diuresis at concentrations too low to affect systemic or renal hemodynamics (e.g., 47). These data suggest that ET can affect tubular transport as well as perfusion, and various studies have shown that ET inhibits salt and water reabsorption in the cortical and medullary thick ascending limbs (THAL) (e.g.,

Address for reprint requests and other correspondence: D. H. Evans, Department of Zoology, University of Florida, Gainesville, FL 32611 (E-mail: devans@zoo.ufl.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

45), as well as the cortical and medullary collecting ducts (56). Like ET, NO can produce natriuresis and diuresis without concomitant changes in either glomerular filtration or renal blood flow, and the tubular effects appear to be primarily in the proximal tubule, THAL, and collecting duct (reviewed in 43). Moreover, NOS is expressed in renal tissue (e.g., 23, 35). Recent evidence suggests that superoxide (O_2^-) also may have measurable effects on THAL transport, either directly or via interactions with NO (e.g., 44). Prostanoids (generated by cyclooxygenase; COX) also can have epithelial as well as vascular effects in the kidney, inhibiting salt uptake in the THAL and collecting duct. The major effector appears to be PGE_2, acting via EP_1 and/or EP_2 receptors, although receptors for other prostanoids (e.g., thromboxane A_2, PGI_2, $PGF_{2\alpha}$, and perhaps, PGD_2) are also expressed in renal tissue (reviewed in 3). In addition, both COX-1 and COX-2 are expressed in renal tubules (e.g., 59). We are unaware of any published studies suggesting that the renal effects of ET can be linked to prostanoid production.

The gill is the primary site of osmoregulation in fishes, and the gill epithelium expresses many of the transport proteins found in the mammalian nephron. These transporters mediate NaCl excretion or uptake, depending on osmoregulatory needs of the fish (e.g., 32). For example, marine teleosts are hypoosmotic to their environment and use the gill epithelium to excrete the excess NaCl that diffuses inward across the gill, as well as that absorbed to drive intestinal uptake of water after ingestion of seawater to offset the osmotic loss of water across the gill epithelium (e.g., 24). There is good evidence that this NaCl transport system can be modulated by circulating hormones, such as cortisol, prolactin, growth hormone, and IGF, thyroid hormones, and arginine vasotocin (fish equivalent of vasopressin) and adrenergic neurotransmitters (e.g., 12). The role(s) of paracrine agents such as ET, NO, O_2^-, and prostanoids on teleost fish gill transport remain largely unexamined, however. The extant literature consists of two early studies (11, 58) that showed that prostanoids inhibit the short circuit current (I_{sc}) across the opercular epithelium of the killifish (*Fundulus heteroclitus*). This tissue is the standard model of the branchial epithelium of marine teleost fishes because both tissues express the same cell types (e.g., mitochondrion-rich cells, pavement cells, and mucous cells), and the opercular epithelium can generate an I_{sc} that is the result of net transport of Cl^- across the epithelium (from basolateral to apical), with Na^+ following passively via paracellular channels (26).

In the present study we examined the effects of ET, NO, and prostanoids on the I_{sc} across the killifish opercular epithelium. Our data provide the first evidence for an ET to NO-O_2^--PGE_2 axis of inhibition involving ET_B receptors, NOS and COX-2. PGE_2 appears to be the major effector in this axis, not NO, and the effect is mediated via EP_1 and/or EP_3 receptors. In addition, there is some evidence for a stimulatory, EP_2-mediated receptor system. We found no evidence that other prostanoids (e.g., PGI_2, thromboxane A_2, $PGF_{2\alpha}$, or PGD_2) are involved.

MATERIALS AND METHODS

Adult killifish (*Fundulus heteroclitus*, ~5 g; both sexes) were collected by minnow trap in North East Creek, Mt. Desert Island, ME, during the summers of 1999–2002 and maintained in running seawater aquariums (16–20°C) for at least 24 h before death by cervical section and pithing. Gill operculae were removed, and the inner epithelium was removed by dissection under a microscope. The tissue was mounted over a 3 mm diameter aperture recessed in a Lucite plate and held in place by a small amount of silicone grease and a Lucite ring to minimize edge damage. The plate was inserted into a Lucite Ussing Chamber (Jim's Instruments) so that the 3 mm epithelial circle separated two chambers, each containing 2 ml of killifish Ringer (26) bubbled with 100% oxygen. Each chamber had ports to allow for aeration, solution addition, and removal and the insertion of Ag-$AgCl_2$ electrodes to measure transepithelial electrical potential and produce an I_{sc}. The output from these electrodes was monitored by a University of Iowa model 742C dual voltage clamp and recorded and saved by a Biopac MP100 data-acquisition system, using AcqKnowledge software on a Macintosh computer. Experiments were initiated only if and when a stable I_{sc} ($I_{sc} > 50$ $\mu A/cm^2$) and resistance (>35 ohms/cm^2) were reached, usually within 30 min. During the course of each experiment, electrical resistance was monitored by the automatic generation of a ±1 mV pulse every minute by the voltage clamp and recording the I_{sc} deflection. No systematic changes in tissue resistance were observed in the course of any of the experiments. In all experiments, equal volumes of solution (agonist/inhibitor vs. carrier) were added to both sides of the tissues (experimental and control) to avoid volume and osmotic effects, because our initial studies confirmed that even a 2% osmolarity differential across this epithelium can affect the I_{sc} (33).

Experimental chemicals were solubilized according to manufacturer's instructions, and the final concentrations used were determined from our earlier studies with these agonists/inhibitors or from published studies from other laboratories. In each case, the substance was dissolved in the appropriate solvent, subdivided, stored either frozen (−70 or −20°C) or at 4°C, and made up to the desired concentration by further dilution in the solvent and/or in the experimental medium (killifish Ringer) the day of the experiment. The final volume of agonist/inhibitor added ranged from 0.1 to 4% of the initial volume of the experimental medium. ET-1 (human) and SRXS6c (American Peptide, Sunnyvale, CA) were dissolved in 1% acetic acid and 50% DMSO, respectively, lyophilized (ET-1 only), and stored at −20°C. Sodium nitroprusside (Sigma, St. Louis, MO), TEMPOL (4-hydroxy-2, 2, 6, 6-tetramethylpiperidine 1-oxyl; Sigma), and L-NAME (N^G-nitro-L-arginine methyl ester; Cayman Chemical, Ann Arbor, MI) were dissolved in killifish Ringer and stored at 4°C; 1-benzylimidazole [1-(phenylmethyl)-1H-imadazole; Cayman] was dissolved in killifish Ringer and stored at −20°C. Indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid; Sigma] was dissolved in 100 mM $NaHCO_3^-$-ethanol (3:1) and stored at 4°C. PGE_2, carbaprostacyclin (6,9α-methylene-11α,15S-dihydroxy-prosta-5E,13E-dien-1-oic acid), PGD_2, $PGF_{2\alpha}$, U-46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z, 13E-dien-1-oic acid), I-BOP {1S-[1α-α(Z),3β(1E,3S*),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)]-1-butenyl]-7-oxabicyclo[2.2.1]hept-yl]-5-heptenoic acid}, SC550 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole], NS-398 {N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide}, butaprost (9-oxo-11α,16R-dihydroxy-17-cyclobutyl-prost-13E-en-1-oic acid, methyl ester), and sulprostone [N-(methylsulfonyl)-9oxo-11α,15R-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide] (all from Cayman Chemical) were dissolved in DMSO and stored at −20°C. Spermine NONOate {(Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate} was dissolved in 0.01 N NaOH on ice, purged with N_2 and stored at −70°C.

The research protocols in this study were approved by IACUC committees at the University of Florida and the Mt. Desert Island Biological Laboratory.

All data are expressed as means ± SE. *P* values for statistical differences were calculated by the appropriate, two-tailed, paired or unpaired Student's *t*-tests, and concentration-dependence data were analyzed using repeated-measures ANOVA and the appropriate post-tests. In the data analysis of all putative inhibitor experiments, each tissue served as its own control when testing the effect of the

inhibitor(s), but paired tissues (experimental vs. control) were compared when determining the effect of most of the putative inhibitors on the SRXS6c-mediated inhibition of the I_{sc}. The exception was the COX-1 vs. COX-2 inhibitor study, where SC560 and NS-398 were applied to paired epithelia before SRXS6c was added, and the effect of either on the SRXS6c-mediated inhibition of the I_{sc} was compared with the sum of the control effects of SRXS6c in the previous experiments (L-NAME, indomethacin, etc.). In this case, unpaired statistical analyses were used. In all cases, $P < 0.05$ was taken as significant. Specific statistical analyses were performed using Prism (GraphPad Software, San Diego, CA) and are indicated in the text and figure legends.

RESULTS

Preliminary experiments determined that the cumulative addition of ET, sodium nitroprusside (SNP; NO donor), and PGE_2 to both sides of the isolated tissue inhibited the I_{sc} across the operculum, so the ET receptor distribution was examined by comparing the effect of basolateral vs. apical addition of 10^{-7} M ET-1. Addition of ET-1 to the basolateral side of the opercular epithelium inhibited the I_{sc} to the same extent (31.5 ± 3.3%; $n = 6$) as addition to both sides of the tissue (37.3 ± 8.6%; $n = 5$; $P = 0.52$; unpaired *t*-test, 2 tailed), but addition to the apical side did not produce a significant inhibition of the I_{sc} (8.0 ± 3.9%; $n = 7$; $P = 0.09$, *t*-test vs. zero), suggesting that the endothelin receptors are located on the basolateral surface. Because the effect was maximal after basolateral addition, all subsequent experiments tested the effects of putative agonists or inhibitors after addition to the basolateral solution.

To confirm that the response of the I_{sc} to ET-1 was concentration dependent, and to attempt to delineate the role of ET_A vs. ET_B receptors, the effects of the cumulative addition of ET-1 (agonist for both ET_A and ET_B receptors) or SRXS6c (ET_B specific) were monitored. Both agonists produced a concentration-dependent inhibition of the I_{sc}, becoming significant at 10^{-8} M and reaching 30–40% at 10^{-7} M in each case (Figs. 1*B* and 2). ET-1 and SRXS6c were equipotent at all concentrations tested. Because SRXS6c produced significant responses (suggesting the presence of ET_B receptors), all subsequent experiments used SRXS6c as an ET agonist, to constrain the study to ET_B-mediated effects.

Similar experiments determined the concentration dependence of the response of the I_{sc} to either of two NO donors, SNP (Fig. 1*C*) and spermine NONOate (SPNO) or the prostanoids PGE_2 (Fig. 1*D*) and carbaprostacyclin (CPR; stable analog of PGI_2). Both NO donors (SNP and SPNO) produced a small, concentration-dependent inhibition of the I_{sc}, reaching significance at 10^{-7} and 10^{-6} M, respectively (Fig. 3). The efficacy of both donors was the same at all concentrations. Both PGE_2 and CPR also produced what appeared to be a concentration-dependent inhibition of the I_{sc}, but only the effect of PGE_2 reached statistical significance (Fig. 4). PGE_2 was somewhat less effective than either ET or SRXS6c at equivalent concentrations.

Because these experiments determined that ET-1, PGE_2, and NO donors produced concentration-dependent inhibition of the I_{sc}, interactions between these putative signaling agents were examined by determining the effect of inhibition of either NOS by L-NAME or COX by indomethacin on the baseline (unstimulated) I_{sc} as well as the SRXS6c-mediated inhibition of the I_{sc}. These experiments used paired epithelia from the same animal, and either 10^{-4} M L-NAME or 10^{-5} M indomethacin was added to the experimental tissue, and the same volume of carrier was added to the control tissue. Initial studies determined that any response was relatively rapid, so after 15 min, 10^{-7} M SRXS6c was added to experimental tissue, and an equal volume of the carrier was added to the control tissue, and the I_{sc} was recorded for an additional 30–60 min to equilibrium. Addition of L-NAME to the opercular epithelium stimulated the I_{sc} slightly but significantly (experimental vs. control: 130 ± 10.2 vs. 113 ± 7.99 $\mu A/cm^2$; $n = 25$; $P < 0.01$, paired *t*-test, 2 tailed). Subsequent addition of SRXS6c inhibited the I_{sc}, but this inhibition was reduced by 17% in those tissues that had been pretreated with the NOS inhibitor (Table 1). Inhibition of COX by indomethacin did not affect the initial I_{sc} (186 ± 37.5 vs. 191 ± 43.6 $\mu A/cm^2$; $n = 8$; $P = 0.79$, paired *t*-test, 2 tailed), but it reduced the SRXS6c-mediated inhibition by nearly 90% (Table 1).

To determine if the effect of inhibition of NOS and COX (on baseline and SRXS6c inhibition) was additive, in another series of experiments both inhibitors were added to the tissue before the addition of SRXS6c. When L-NAME and indomethacin were added simultaneously, the initial I_{sc} did not change (124 ± 21 vs. 124 ± 26 $\mu A/cm^2$; $n = 6$; $P = 0.99$, paired *t*-test, 2 tailed), demonstrating that the inhibition of COX abolishes the stimulation of the I_{sc} seen when L-NAME is added alone. The presence of both L-NAME and indomethacin inhibited the effect of subsequent addition of SRXS6c by 83% (Table 1), but the net effect was no greater than the effect of indomethacin alone.

Because NOS-mediated effects may be secondary to chemical interactions of NO with O_2^-, another series of experiments examined the effects of the spin trap TEMPOL (5×10^{-3} M; a superoxide dismutase mimetic) on the baseline and SRXS6c-mediated inhibition of the I_{sc}, as well as the effect of the simultaneous addition of L-NAME, indomethacin, and TEMPOL on these parameters. Addition of TEMPOL had no effect on the initial I_{sc} across the epithelium (256 ± 47.7 vs. 260 ± 47.9 $\mu A/cm^2$; $n = 7$; $P = 0.39$, paired *t*-test, 2 tailed), but it did reduce the SRXS6c-mediated inhibition by 34% (Table 1), twice the effect of inhibition of NOS by L-NAME (Table 1). Simultaneous addition of L-NAME, indomethacin, and TEMPOL did not affect the I_{sc} (186 ± 53.4 vs.185 ± 49.2 $\mu A/cm^2$; $n = 6$; $P = 0.90$, paired *t*-test, 2 tailed), but it completely inhibited the effect of subsequent addition of SRXS6c (Table 1; percent reduction by SRXS6c not different from zero; $P = 0.14$).

To attempt to differentiate between COX-1- and COX-2-mediated responses, the effects of SC560 (10^{-6} M; COX-1-specific inhibitor) and NS-398 (10^{-6} M; COX-2-specific inhibitor) on baseline I_{sc} and SRXS6c-mediated inhibition were studied in another series of experiments. Addition of SC560 had no effect on the initial I_{sc} (139 ± 25.5 vs. 138 ± 24.8 $\mu A/cm^2$; $n = 5$; $P = 0.89$, paired *t*-test, 2 tailed), but the addition of NS-398 stimulated the I_{sc} by 16% (150 ± 23.6 vs. 129 ± 20.8 $\mu A/cm^2$; $n = 5$; $P < 0.01$; paired *t*-test, 2 tailed). Subsequent addition of SRXS6c inhibited the I_{sc}, but previous inhibition of COX-1 reduced this inhibition by 46%, and inhibition of COX-2 reduced the effect by 90% (Table 1).

Because PGE_2 can bind to any of four receptors (termed EP_{1-4}), another series of experiments tested the effects of cumulative addition of either butaprost (EP_2 specific) or sul-

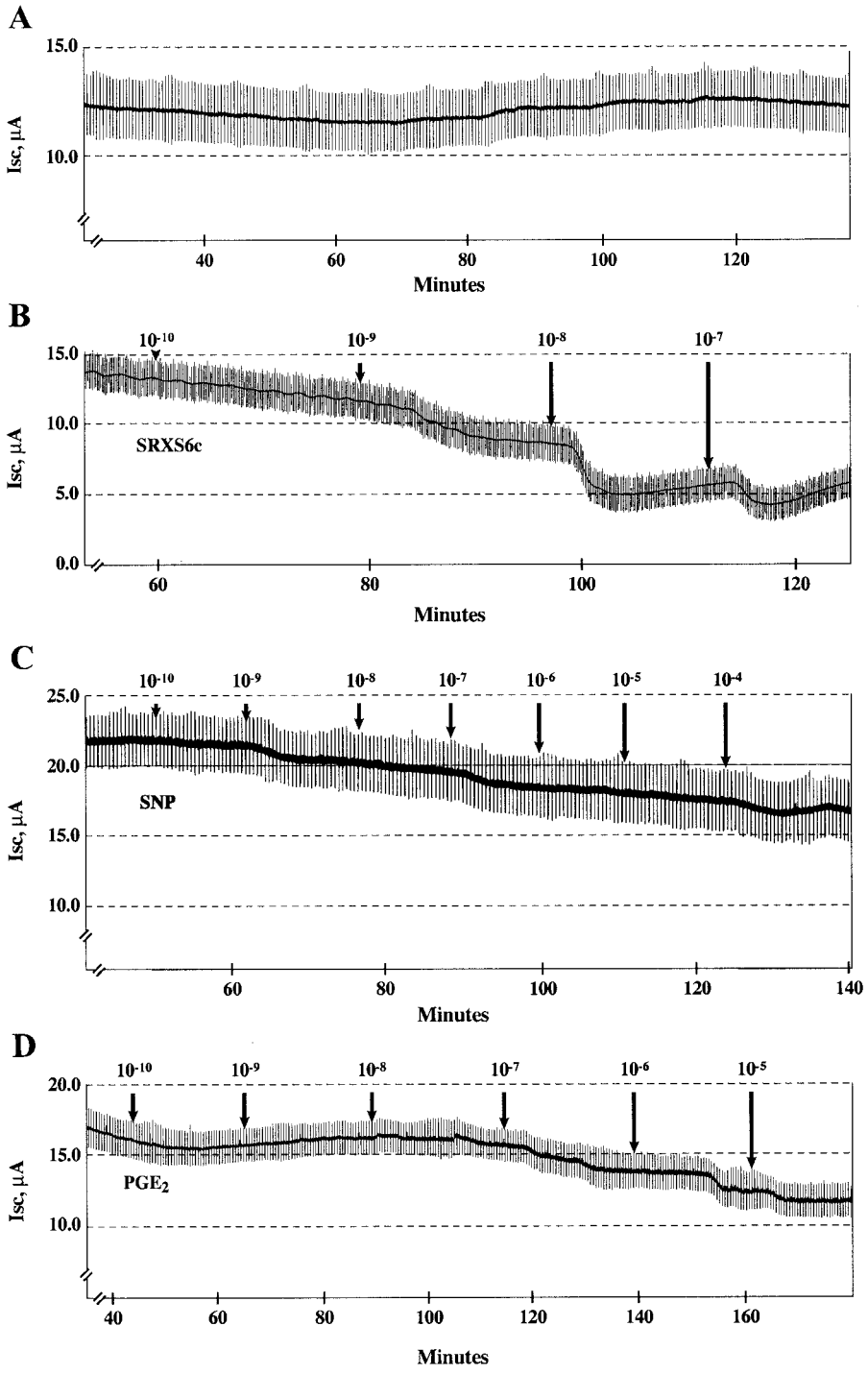

Fig. 1. Representative tracings of a time course control experiment (*A*) vs. the effect SRXS6c (*B*), SNP (*C*), and PGE_2 (*D*) on the short-circuit current (I_{sc}) across opercular epithelium of *Fundulus heteroclitus*. Cumulative additions of the agonists to produce the specific concentration (molar) are marked by vertical arrows. Continuous, vertical deflections in the I_{sc} were produced by automatic, ±1-mV pulses in the circuit to monitor tissue resistance, which did not change during the experiments. Note scale differences in the time course.

prostone ($EP_{1/3}$ specific; e.g., 3) on the I_{sc} across paired opercular epithelia. Butaprost stimulated the I_{sc} across the opercular epithelium in a concentration-dependent manner (reaching significance at 10^{-6} M); sulprostone produced the opposite effect on the I_{sc}, reaching significance at 10^{-7} M (Fig. 5).

To test the efficacy of other prostanoids, putative agonists (carbaprostacyclin for PGI_2, U-46619 and I-BOP for thromboxane A_2, $PGF_{2\alpha}$, or PGD) were applied to opercular tissues using the same protocol as described for the PGE_2-mediated concentration-dependence experiments. In addition, the ability of the thromboxane synthase inhibitor 1-benzylimidazole (10^{-5} M) to affect either baseline I_{sc} or SRXS6c-mediated inhibition of the I_{sc} was tested, using the protocol described for L-NAME, indomethacin, and TEMPOL. Neither of the putative TXA_2 agonists (10^{-10}-10^{-6} M U-46619 or I-BOP; $n = 4$) nor $PGF_{2\alpha}$ or PGD_2 (10^{-10}-10^{-6} M; $n = 5$) produced any change in the I_{sc} across the opercular epithelium (data not shown). Moreover, pretreatment with 1-benzylimidazole had no effect on the unstimulated I_{sc} and did not blunt the SRXS6c-mediated

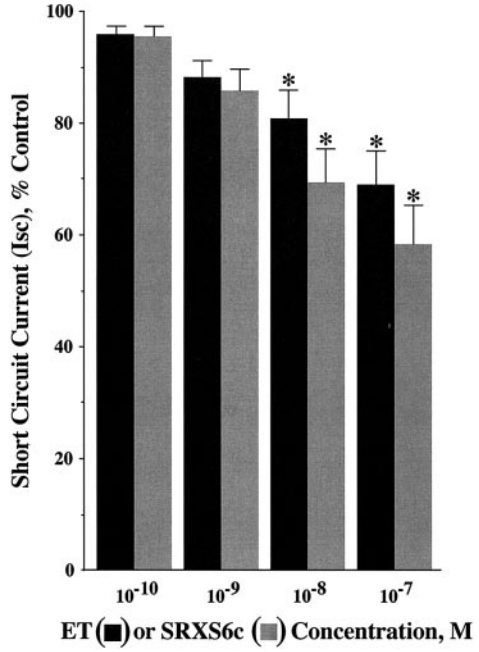

Fig. 2. Concentration-dependence of the effect of endothelin (ET; black) or SRXS6c (shaded) on the I_{sc} across paired opercular epithelia. Both agonists ($n = 8$) produced inhibition of the I_{sc} that showed a linear trend ($P < 0.0001$; repeated-measures ANOVA, posttest for linear trend), reaching a significant difference from the initial I_{sc} at concentrations indicated by an asterisk ($P < 0.05$; Dunnett's posttest). Effects of ET vs. SRXS6c were not different at any concentration (Bonferroni posttest of all pairs of columns).

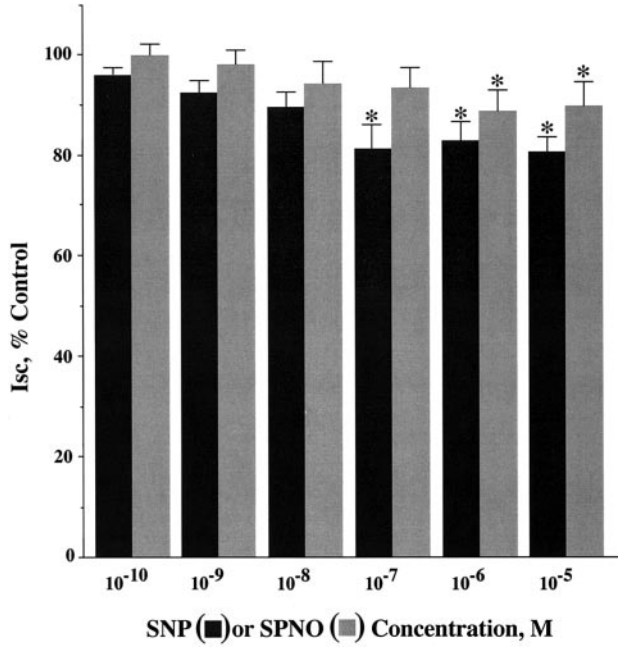

Fig. 3. Concentration-dependence of the effect of sodium nitroprusside (SNP) and spermine NONOate (SPNO) on the I_{sc} across the opercular epithelium ($n = 4$). Both produced inhibition of the I_{sc} that showed a linear trend ($P < 0.1$; repeated-measures ANOVA, posttest for linear trend), reaching a significant difference from the initial I_{sc} at concentrations indicated by an asterisk ($P < 0.05$; Dunnett's posttest). Effects of SNP vs. SPNO were not different at any concentration (Bonferroni posttest of all pairs of columns).

inhibition of the I_{sc} ($P = 0.81$, paired *t*-test, 2 tailed; $n = 4$; data not shown).

DISCUSSION

Our data show that ET-1 and SRXS6c, two NO donors (SNP and SPNO), and PGE_2 each can inhibit the I_{sc} across the opercular epithelium of the killifish *Fundulus heteroclitus* in a concentration-dependent manner (Figs. 1–4). This is the first demonstration of a putative role for ET and NO in modulating salt transport across this epithelium, which models the gill epithelium of marine teleost fishes (e.g., 26). Our finding that PGE_2 is inhibitory corroborates earlier studies using the same epithelial preparation (11, 58). The response to ET is mediated by basolateral receptors, as one might expect; the slight (and statistically insignificant) apical response may be due to leakage of the applied ET from the basolateral to apical surface, or a smaller population of apical receptors may be present, as has been described for other epithelia (e.g., 25). Since the ET_B-specific agonist SRXS6c was as effective as ET-1 in inhibiting the I_{sc} (Figs. 1 and 2), we conclude that stimulation of ET_B-like receptors mediate this response to ET. Our data do not preclude the presence of ET_A receptors in the opercular epithelium, however, but the response of the I_{sc} to ET-1 appears to be wholly via ET_B receptors.

The fact that incubation with 10^{-4} M L-NAME produced a small, but significant, stimulation of the I_{sc} suggests that tonic release of NO inhibits the I_{sc} in the unstimulated tissue. However, if COX is also inhibited by the simultaneous addition of 10^{-5} M indomethacin and 10^{-4} M L-NAME, this stimulation is lost (Table 1). Thus another explanation of the L-NAME-mediated stimulation is that the unstimulated I_{sc} is actually set by the sum of tonic, COX-mediated stimulation and NOS-mediated inhibition. However, inhibition of COX alone by preincubation with 10^{-5} indomethacin did not change the unstimulated I_{sc}, contrary to the inhibition of the I_{sc} one might expect if this model were correct. Moreover, inhibition of COX-2 alone, using the specific inhibitor NS-398, actually

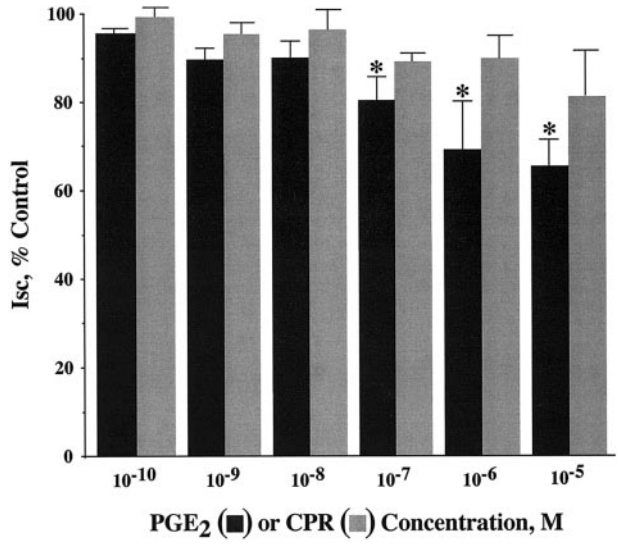

Fig. 4. Concentration-dependence of the effect of PGE_2 and carbaprostacyclin (CPR; stable PGI_2 analog) on the I_{sc} across the opercular epithelium ($n = 6$). Both prostanoids produced inhibition of the I_{sc} that showed a linear trend ($P < 0.0001$; repeated-measures ANOVA, posttest for linear trend), but only the effect of PGE_2 reached a significant difference from the initial I_{sc} at concentrations indicated by an asterisk ($P < 0.05$; Dunnett's posttest).

Table 1. *Effect of various inhibitors on the SRXS6c-mediated reduction of the I_{sc} across the opercular epithelium of Fundulus heteroclitus*

Inhibitor	%Reduction in I_{sc}: SRXS6c Only (*n*)	%Reduction in I_{sc}: SRXS6c After Drug Pretreatment (n)	*P* Value; Control vs. Experimental
10^{-4} M L-NAME	63.9±5.64(14)	53.0±6.19(14)	<0.01
10^{-5} M Indomethacin	46.4±9.07(8)	5.15±1.33(8)	<0.01
10^{-4} M L-NAME + 10^{-5} M indomethacin	34.4±4.93(6)	5.78±1.78(6)	<0.01
5×10^{-3} M TEMPOL	28.4±6.25(7)	18.6±4.77(7)	<0.01
5×10^{-3} M TEMPOL + 10^{-4} M L-NAME + 10^{-5} M indomethacin	34.7±3.60(6)	4.58±2.59(6)	<0.001
10^{-6} M SC560	45.8±3.61(41)	24.4±3.65(5)	<0.05
10^{-6} M NS-398	45.8±3.61(41)	4.76±1.15(5)	<0.001

Values are means ± SE. *n* values in parentheses. I_{sc}, short-circuit current; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; L-NAME, N^G-nitro-L-arginine methyl ester.

stimulated the I_{sc} across the unstimulated tissue, suggesting the presence of a tonic, COX-mediated inhibition of the unstimulated I_{sc}. Thus the current data suggest that both NO and a prostanoid may be tonically controlling the I_{sc} across the unstimulated opercular epithelium, but the net roles of either signaling system cannot be determined from our data.

Despite our uncertainty about the relative roles of NO and prostanoids in tonic control, it is clear that both NO and prostanoids play a role in the response to stimulation of ET_B receptors by SRXS6c, but prostanoids are obviously of greater importance (Table 1). Interestingly, published data suggest that prostanoids, not NO, are also the dominant endothelium-derived relaxing factor in the few fish species that have been studied (e.g., 17, 19, 41). Inhibition of either NOS or COX did not blunt the SRXS6c effect completely, and addition of both inhibitors simultaneously did not produce an additive response (Table 1), suggesting some interaction between the putative ET-NO and ET-PG axes and/or the presence of other components in the inhibition produced by activation of the ET_B receptor by SRXS6c.

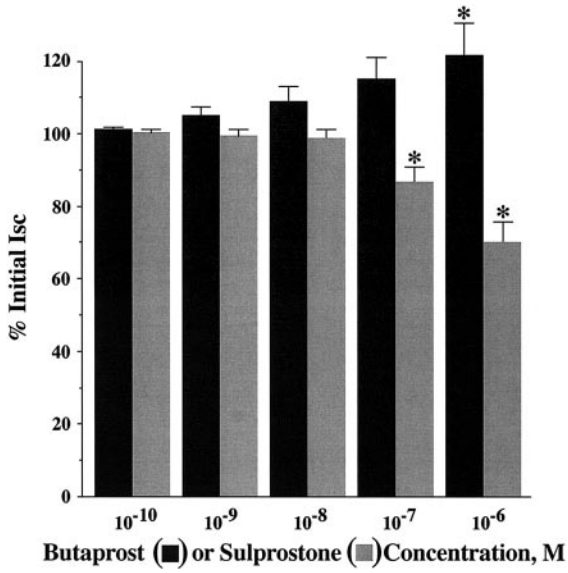

Fig. 5. Concentration-dependence of the effect of butaprost ($n = 6$) vs. sulprostone ($n = 10$) on the I_{sc} across the opercular epithelium. Butaprost stimulation of the I_{sc} showed a linear trend ($P < 0.001$; repeated-measures ANOVA, posttest for linear trend), reaching a significant difference from the initial I_{sc} at the concentrations indicated by an asterisk ($P < 0.05$; Dunnett's posttest). Sulprostone inhibition of the I_{sc} showed a linear trend ($P < 0.0001$; repeated-measures ANOVA, posttest for linear trend), reaching a significant difference from the initial I_{sc} at the concentrations indicated by an asterisk ($P < 0.05$; Dunnett's posttest).

It has become clear that the role of NO in a variety of signaling pathways is at least partially controlled by its effectively instantaneous reaction ($K \approx 7 \times 10^9$ mol·l^{-1}·s^{-1}) with superoxide ions (O_2^-) to form peroxynitrite ($OONO^-$), an especially toxic molecule (e.g., 30). These three molecules have been termed "the good, the bad, and the ugly" (e.g., 2) because the highly oxidative O_2^- and $OONO^-$ could produce physiological or pathophysiological responses directly, or merely because O_2^- removes NO from the system. The fact that the addition of the NO scavenger TEMPOL (5×10^{-3} M) blunted the effect of subsequent addition of SRXS6c by 34%, twice the effect of the addition of L-NAME, suggests that O_2^- itself plays a role in this signaling axis in the opercular epithelium (Table 1). Indeed, if TEMPOL, L-NAME, and indomethacin were added simultaneously to unstimulated tissue, the effect of subsequent addition of SRXS6c was completely inhibited (Table 1). Because TEMPOL reduced the effect of SRXS6c significantly more than L-NAME (the NO-dependent component), we hypothesize that O_2^- itself is inhibitory and can be produced by a pathway that is stimulated by ET/SRXS6c. In fact, a recent study demonstrated that ET generated O_2^- in a COX-dependent pathway after brain injury in newborn pigs (1), so it could be that the TEMPOL-dependent effect on the opercular epithelium is actually the sum of reduction of NO-O_2^- interactions and reduction of COX-generated O_2^-. It is notable that indomethacin alone reduced the SRXS6c effect to the same degree as indomethacin plus L-NAME or indomethacin plus L-NAME and TEMPOL, despite the fact that only the three inhibitors together produce inhibition that is statistically 100% (Table 1). This suggests that the COX-mediated prostanoid and O_2^- production is by far the dominant pathway in the operculum. It is clear that O_2^- production is not tonic, because TEMPOL did not affect the unstimulated I_{sc}. Garvin's group (42) recently showed that addition of TEMPOL increases the inhibition of Cl^- transport across the THAL of the rat loop of Henle produced by NO. The effect, however, appears to be via inhibition of a stimulatory response to O_2^- (rather than by removal of O_2^- and the subsequent stimulation of an NO-mediated inhibition), because exogenous production of O_2^- by the addition of xanthine oxidase/hypoxanthine stimulated Cl^- transport across the THAL. They concluded, therefore, that O_2^- itself is stimulatory and not just a modulator of NO concentration (44).

The fact that pretreatment with NS-398, but not SC560, inhibited the I_{sc} suggests that there may be a COX-2-mediated, tonic inhibition of the I_{sc}, as is the case with NO (see above), in contrast to the indomethacin experiments that suggested that there is COX-mediated, tonic stimulation of the I_{sc}. At this point, we cannot differentiate between these two alternatives, but these experiments do demonstrate that COX-mediated, tonic production of prostanoids may have effects on salt transport across the epithelium, and they also suggest that there may be different roles played by COX-1 vs. COX-2. Because inhibition of COX-2 was twice as effective in attenuating the SRXS6c-mediated inhibition of the I_{sc} as inhibition of COX-1 (Table 1), it appears that both COX-1 and -2 are involved in the production of prostanoids after ET_B stimulation in this tissue but that COX-2 is the major effector. The physiological importance of COX-1 and COX-2 has become appreciated in the past few years, and both are expressed in the rat nephron. For instance, a recent study has shown that COX-1 mRNA predominates in the glomerulus, distal tubule, and collecting duct, whereas COX-2 message is localized in the glomerulus and medullary THAL (59). The genes for the homologues of COX-1 and/or COX-2 have been cloned for the zebrafish (21), two trout species (46, 51), and dogfish shark (62), where the clone was amplified from the rectal gland, a functional analog of both the marine teleost gill and THAL of the mammalian nephron (e.g., 40, 52). The latter study found that the specific COX-2 inhibitor, NS-398, reduced the Cl^- secretion rate of the shark rectal gland. This study suggests that prostanoids are stimulatory, rather than inhibitory, contrary to the present findings and what has been published in the renal literature (e.g., 3). On the other hand, stimulation of salt secretion by the shark rectal gland has the same effect as inhibition of salt uptake by the mammalian nephron: increased salt excretion.

Because the PGE_2 receptors EP_1 and EP_3 predominate in mammalian renal tubules (e.g., 3), we attempted to differentiate between putative receptors in the killifish operculum by comparing the efficacy of the relatively specific agonists, butaprost (EP_2), and sulprostone ($EP_{1/3}$) in this system. The fact that butaprost produced a concentration-dependent stimulation and sulprostone produced a concentration-dependent inhibition (Fig. 5) suggests that EP_2 and EP_1 (and/or EP_3) receptors are present and that release of PGE_2 in the opercular epithelium can produce stimulation or inhibition, depending on the distribution of the respective receptors. Our data suggest that inhibitory receptors (EP_1 and/or EP_3) predominate, but the finding that the L-NAME-induced stimulation of the I_{sc} is inhibited by the simultaneous addition of indomethacin suggests the presence of at least some stimulatory receptors. The actual receptors present could be identified by immunological or molecular techniques, as well as measurement of intracellular second messengers in the future. It is important to note, however, that early work on the killifish operculum demonstrated that the I_{sc} could be stimulated by isoproterenol and inhibited by epinephrine and arterenol (10) and subsequent studies showed that the stimulation vs. inhibition is mediated by β-adrenergic and α-adrenergic receptors, respectively (37). In the killifish operculum, β-adrenergic receptors stimulate intracellular cAMP (38) and α-adrenergic receptors stimulate intracellular inositol triphosphate (34), parallel to at least EP_2 and EP_1, respectively. Thus the intracellular second messengers for the putative EP receptors that our data suggest appear to be present in the opercular epithelium.

The inability of thromboxane A_2 agonists or $PGF_{2\alpha}$ and PGD_2 to elicit a change in the I_{sc} across opercular skin suggests that, if receptors for these other prostanoids are present in this tissue, they play a minor role in modulating the transport of NaCl across this tissue. The inability of a TXA_2 synthase inhibitor to reduce the SRXS6c-mediated reduction in the I_{sc} supports this conclusion. The putative PGI_2 agonist CPR was not as effective as PGE_2 in inhibiting the I_{sc} and the effect was not statistically significant (Fig. 4), which suggests that IP receptors are not important in this system. CPR, on the other hand, can also bind to the EP_1 receptor (e.g., 3), and the PGE_2 and the sulprostone data suggest that the EP_1 or EP_3 receptor mediates the majority of the inhibition seen after SRXS6c addition.

Our data are the first to suggest that ET can modulate salt transport across the killifish opercular epithelium. It is unclear what stimulus activates the ET receptor (presumably ET_B); changes in plasma osmolarity or cardiovascular parameters, or other signaling agents, might be suggested to be the putative stimuli. Nevertheless, our data suggest that activation of the ET_B receptor stimulates the production of NO, O_2^-, and prostanoids (probably PGE_2), each of which inhibit the transport of NaCl. Prostanoids probably account for 70–90% of the inhibition, even when accounting for what appears to be a significant role for O_2^- in this system. It is not clear if NO plays a direct role or merely modifies O_2^- concentrations. COX-2 and COX-1 are both involved in the synthesis of the active prostanoid (as well as O_2^-), although COX-2 appears to play a much bigger role. Both stimulatory (EP_2) and inhibitory

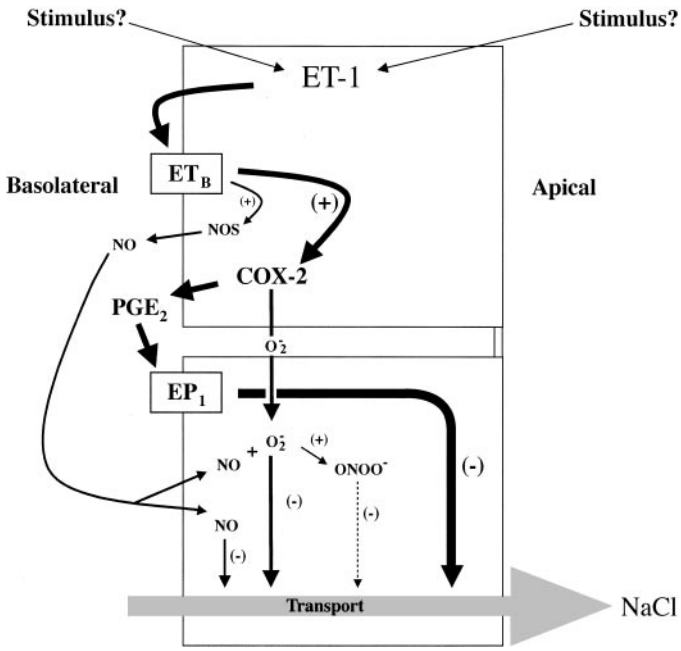

Fig. 6. Working hypothesis for the putative pathways of ET-inhibited NaCl transport across the fish gill. Two cells are diagramed, but the system may be expressed within a single cell. Width of the arrows is proportional to the presumed importance of the specific pathway in the axis. See text for details. ET-1, endothelin; ET_B, endothelin B receptor; NOS, nitric oxide synthase; COX-2, cyclooxygenase-2; NO, nitric oxide; EP_1, PGE_2 receptor; O_2^-, superoxide ion; $ONOO^-$, peroxynitrite ion.

($EP_{1/3}$) PGE receptors appear to be involved, although inhibition appears to be the most significant response. Our data do not allow a definitive model for the roles of NO and prostanoids in maintaining the unstimulated salt transport across this epithelium, but some aspects of this work provide evidence for some role for both effectors without any stimulation of the ET receptor. Figure 6 summarizes our current working hypothesis for the interactions between ET, NO, O_2^-, and PGE_2 in inhibiting salt extrusion by the marine teleost gill epithelium, as modeled by the killifish opercular epithelium.

Perspectives

Because the opercular epithelium is the generally accepted model for the marine teleost fish branchial epithelium (see introduction), our data provide the first evidence that salt extrusion by the gill can be modulated by release of paracrine agents, in this case: ET, NO, O_2^-, and PGE_2. Although we provide some evidence for a stimulatory prostanoid pathway, the bulk of the data suggest ET-stimulated and NO-O_2^--PGE_2-mediated inhibition of salt transport. The result would be salt retention, because the gill is the dominant site of salt secretion in marine teleosts (e.g., 24). This signaling pathway, therefore, has the opposite final result from that found in the mammalian kidney, where ET, NO, and prostanoids are predominantly natriuretic because of inhibition of uptake in the renal tubules (see introduction). Because salt retention is important in fish in hypoosmotic environments (e.g., 13), we hypothesize that this paracrine modulating system is most important in freshwater species or euryhaline species as they enter freshwater. Indeed, gill tissue from the eel produces significantly more prostanoids (actually, PGD_2 and 6-keto-$F_{1\alpha}$) after acclimation to freshwater (5). Interestingly, PGE_2 concentrations were very low in this tissue, as well as trout gill, in this study, and no significant changes in PGE_2 concentrations were seen in either species after acclimation to freshwater vs. seawater (5).

The cellular site of ET, NO, and prostanoid production (as well as cellular receptors) in the teleost gill is currently under investigation. Zaccone's group (64) demonstrated immunoreactivity for big-ET (ET prohormone) and NOS in what they term "neuroendocrine" cells in the branchial epithelium from a variety of teleosts and elasmobranchs. Our preliminary studies (12; and K. A. Hyndman, P. M. Piermarini, and D. H. Evans, unpublished observations) have localized immunoreactive NOS in cells distinct from the mitochondrion-rich cells (MRC; Cl^- transporting) in both the killifish opercular epithelium and gill epithelium, but it is not clear if these are "neuroendocrine," mucous, or pavement cells. In the stingray gill, we find immunoreactive big-ET is in the MRC and COX is expressed in the filamental central venous sinus, but in the killifish gill big-ET can be localized to epithelial cells distinct from the MRC, and COX is seen in the MRC. Thus it is clear that species differences may exist; nevertheless, localization of the effectors and receptors for this new signaling axis is of great interest for comparative vertebrate physiology and may provide new insights into paracrine control axes in the mammalian kidney.

ACKNOWLEDGMENTS

Appreciation is expressed to Drs. K. Karnaky, D. Dawson, and D. Petzel for many discussions about epithelial tissue voltage clamping in the early stages of this study. M. S. Kozlowski assisted in some of these studies.

GRANTS

This study was supported by National Science Foundation grant IBN-0089943 to D. H. Evans.

REFERENCES

1. **Armstead WM.** Endothelin-induced cyclooxygenase-dependent superoxide generation contributes to K^+ channel functional impairment after brain injury. *J Neurotrauma* 18: 1039–1048, 2001.
2. **Beckman JS and Koppenol WH.** Nitric oxide superoxide and peroxynitrite: the good, the bad, and ugly. *Am J Physiol Cell Physiol* 271: C1424–C1437, 1996.
3. **Breyer MD and Breyer RM.** G protein-coupled prostanoid receptors and the kidney. *Annu Rev Physiol* 63: 579–605, 2001.
4. **Brooks DP, DePalma PD, Pullen M, Gellai M, and Nambi P.** Identification and function of putative ET_B receptor subtypes in the dog kidney. *J Cardiovasc Pharmacol* 26: S322–325, 1995.
5. **Brown JA, Gray CJ, Hattersley G, and Robinson J.** Prostaglandins in the kidney, urinary bladder and gills of the rainbow trout and European eel adapted to fresh water and seawater. *Gen Comp Endocrinol* 84: 328–335, 1991.
6. **Cioni C, Francia N, Fabrizi C, Colasanti M, and Venturini G.** Partial biochemical characterization of nitric oxide synthase in the caudal spinal cord of the teleost *Oreochromis niloticus*. *Neurosci Lett* 253: 68–70, 1998.
7. **Cristol JP, Warner TD, Thiemermann C, and Vane JR.** Mediation via different receptors of the vasoconstrictor effects of endothelins and sarafotoxins in the systemic circulation and renal vasculature of the anaesthetized rat. *Br J Pharmacol* 108: 776–779, 1993.
8. **De Nucci G, Gryglewski RJ, Warner TD, and Vane JR.** Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc Natl Acad Sci USA* 85: 2334–2338, 1988.
9. **De Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, and Vane JR.** Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* 85: 9797–9800, 1988.
10. **Degnan KJ and Zadunaisky J.** Open-circuit sodium and chloride fluxes across isolated opercular epithelia from the teleost *Fundulus heteroclitus*. *J Physiol* 294: 483–495, 1979.
11. **Eriksson O, Mayer-Gostan N, and Wistrand PJ.** The use of isolated fish opercular epithelium as a model tissue for studying intrinsic activities of loop diuretics. *Acta Physiol Scand* 125: 55–66, 1985.
12. **Evans DH.** Cell signaling and ion transport across the fish gill epithelium. *J Exp Zool* 293: 336–347, 2002.
13. **Evans DH.** Osmotic and ionic regulation. In: *The Physiology of Fishes*, edited by Evans DH. Boca Raton, FL: CRC, 1993, p. 315–341.
14. **Evans DH.** Vasoactive receptors in abdominal blood vessels of the dogfish shark, *Squalus acanthias*. *Physiol Biochem Zool* 74: 120–126, 2001.
15. **Evans DH, Gunderson M, and Cegelis C.** ET_B-type receptors mediate endothelin-stimulated contraction in the aortic vascular smooth muscle of the spiny dogfish shark, *Squalus acanthias*. *J Comp Physiol [A]* 165: 659–664, 1996.
16. **Evans DH and Gunderson MP.** Characterization of an endothelin ET_B receptor in the gill of the dogfish shark *Squalus acanthias*. *J Exp Biol* 202: 3605–3610, 1999.
17. **Evans DH and Gunderson MP.** A prostaglandin, not NO, mediates endothelium-dependent dilation in ventral aorta of shark (*Squalus acanthias*). *Am J Physiol Regul Integr Comp Physiol* 274: R1050–R1057, 1998.
18. **Evans DH and Harrie AC.** Vasoactivity of the ventral aorta of the American eel (*Anguilla rostrata*), Atlantic hagfish (*Myxine glutinosa*), and sea lamprey (*Petromyzon marinus*). *J Exp Zool* 289: 273–284, 2001.
19. **Farrell AP and Johansen JA.** Vasoactivity of the coronary artery of rainbow trout, steelhead trout, and dogfish: lack of support for non prostanoid endothelium-derived relaxation factors. *Can J Zool* 73: 1899–1911, 1995.
20. **Funakoshi K, Kadota T, Atobe Y, Goris RC, and Kishida R.** NADPH-diaphorase activity in the vagal afferent pathway of the dogfish, *Triakis scyllia*. *Neurosci Lett* 237: 129–132, 1997.
21. **Grosser T, Yusuff S, Cheskis E, Pack MA, and FitzGerald GA.** Developmental expression of functional cyclooxygenases in zebrafish. *Proc Natl Acad Sci USA* 99: 8418–8423, 2002.

22. **Hoagland TM, Weaver L Jr, Conlon JM, Wang Y, and Olson KR.** Effects of endothelin-1 and homologous trout endothelin on cardiovascular function in rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 278: R460–R468, 2000.
23. **Ishii N, Fujiwara K, Lane PH, Patel KP, and Carmines PK.** Renal cortical nitric oxide synthase activity during maturational growth in the rat. *Pediatr Nephrol* 17: 591–596, 2002.
24. **Karnaky KJ Jr.** Osmotic and ionic regulation. In: *The Physiology of Fishes* (2nd ed.), edited by Evans DH. Boca Raton, FL: CRC, 1998, p. 157–176.
25. **Karnaky KJ Jr.** Regulating epithelia from the apical side: new insights. Focus on "Differential signaling and regulation of apical vs. basolateral EGFR in polarized epithelial cells." *Am J Physiol Cell Physiol* 275: C1417–C1418, 1998.
26. **Karnaky KJ Jr, Degnan KJ, and Zadunaisky JA.** Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science* 195: 203–205, 1977.
27. **Kasuya Y, Kobayashi H, and Uemura H.** Endothelin-like immunoreactivity in the nervous system of invertebrates and fish. *J Cardiovasc Pharmacol* 17: S463–466, 1991.
28. **Kedzierski RM and Yanagisawa M.** Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* 41: 851–876, 2001.
29. **Knight J, Holland JW, Bowden LA, Halliday K, and Rowley AF.** Eicosanoid generating capacities of different tissues from the rainbow trout, *Oncorhynchus mykiss*. *Lipids* 30: 451–458, 1995.
30. **Koppenol WH.** The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic Biol Med* 25: 385–391, 1998.
31. **Lee JA, Ohlstein EH, Peishoff CE, and Elliott JD.** Molecular biology of the endothelin receptors. In: *Endothelin: Molecular Biology, Physiology, and Pathology*, edited by Highsmith RF. Totowa, NJ: Humana, 1998, p. 31–73.
32. **Marshall WS.** Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J Exp Zool* 293: 264–283, 2002.
33. **Marshall WS, Bryson SE, and Luby T.** Control of epithelial Cl^- secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. *J Exp Biol* 203: 1897–1905, 2000.
34. **Marshall WS, Duquesnay RM, Gillis JM, Bryson SE, and Liedtke CM.** Neural modulation of salt secretion in teleost opercular epithelium by α-adrenergic receptors and inositol 1,4,5-trisphosphate. *J Exp Biol* 201: 1959–1965, 1998.
35. **Martin PY, Bianchi M, Roger F, Niksic L, and Feraille E.** Arginine vasopressin modulates expression of neuronal NOS in rat renal medulla. *Am J Physiol Renal Physiol* 283: F559–F568, 2002.
36. **Masaki T.** The endothelin family: an overview. *J Cardiovasc Pharmacol* 35: S3–S5, 2000.
37. **May SA, Baratz KH, Key SZ, and Degnan KJ.** Characterization of the adrenergic receptors regulating chloride secretion by the opercular epithelium. *J Comp Physiol [B]* 154: 343–348, 1984.
38. **Mendelsohn SA, Cherksey BD, and Degnan KJ.** Adrenergic regulation of chloride secretion across the opercular epithelium: the role of cyclic AMP. *J Comp Physiol [A]* 145: 29–35, 1981.
39. **Mustafa T, Agnisola C, and Hansen JK.** Evidence for NO-dependent vasodilation in the trout (*Oncorhynchus mykiss*) coronary system. *J Comp Physiol [A]* 167: 98–104, 1997.
40. **Olson KR.** Rectal gland and volume homeostasis. In: *Sharks, Skates, and Rays*, edited by Hamlett WC. Baltimore, MD: Johns Hopkins University Press, 1999, p. 329–352.
41. **Olson KR and Villa J.** Evidence against nonprostanoid endothelium-derived relaxing factor(s) in trout vessels. *Am J Physiol Regul Integr Comp Physiol* 260: R925–R933, 1991.
42. **Ortiz PA and Garvin JL.** Interaction of O_2^- and NO in the thick ascending limb. *Hypertension* 39: 591–596, 2002.
43. **Ortiz PA and Garvin JL.** Role of nitric oxide in the regulation of nephron transport. *Am J Physiol Renal Physiol* 282: F777–F784, 2002.
44. **Ortiz PA and Garvin JL.** Superoxide stimulates NaCl absorption by the thick ascending limb. *Am J Physiol Renal Physiol* 283: F957–F962, 2002.
45. **Plato CF, Pollock DM, and Garvin JL.** Endothelin inhibits thick ascending limb chloride flux via ET_B receptor-mediated NO release. *Am J Physiol Renal Physiol* 279: F326–F333, 2000.
46. **Roberts SB, Langenau DM, and Goetz FW.** Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. *Mol Cell Endocrinol* 160: 89–97, 2000.
47. **Schnermann J, Lorenz JN, Briggs JP, and Keiser JA.** Induction of water diuresis by endothelin in rats. *Am J Physiol Renal Fluid Electrolyte Physiol* 263: F516–F526, 1992.
48. **Schober A, Malz CR, and Meyer DL.** Enzyme histochemical demonstration of nitric oxide synthase in the diencephalon of the rainbow trout (*Oncorhynchus mickiss*). *Neurosci Lett* 151: 67–70, 1993.
49. **Schober A, Malz CR, Schober W, and Meyer DL.** NADPH-diaphorase in the central nervous system of the larval lamprey (*Lampetra planeri*). *J Comp Neurol* 345: 94–104, 1994.
50. **Schober A, Meyer DL, and Von Bartheld CS.** Central projections of the nervus terminalis and the nervus praeopticus in the lungfish brain revealed by nitric oxide synthase. *J Comp Neurol* 349: 1–19, 1994.
51. **Secombes C, Zou J, Daniels G, Cunningham C, Koussounadis A, and Kemp G.** Rainbow trout cytokine and cytokine receptor genes. *Immunol Rev* 166: 333–340, 1998.
52. **Silva P, Solomon RJ, and Epstein FH.** The rectal gland of *Squalus acanthias*: a model for the transport of chloride. *Kidney Int* 49: 1552–1556, 1996.
53. **Small SA, MacDonald C, and Farrell AP.** Vascular reactivity of the coronary artery in rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 258: R1402–R1410, 1990.
54. **Stjernquist M.** Endothelins—vasoactive peptides and growth factors. *Cell Tissue Res* 292: 1–9, 1998.
55. **Sverdrup A, Krüger PG, and Helle KB.** Role of the endothelium in regulation of vascular functions in two teleosts. *Acta Physiol Scand* 152: 219–233, 1994.
56. **Tomita K, Nonoguchi H, Terada Y, and Marumo F.** Effects of ET-1 on water and chloride transport in cortical collecting ducts of the rat. *Am J Physiol Renal Fluid Electrolyte Physiol* 264: F690–F696, 1993.
57. **Uemura H, Naruse M, Naruse K, Hirohama T, Demura H, and Kasuya Y.** Immunoreactive endothelin in plasma of nonmammalian vertebrates. *J Cardiovasc Pharmacol* 17: S414–416, 1991.
58. **Van Praag D, Farber SJ, Minkin E, and Primor N.** Production of eicosanoids by the killifish gills and opercular epithelia and their effect on active transport of ions. *Gen Comp Endocrinol* 67: 50–57, 1987.
59. **Vitzthum H, Abt I, Einhellig S, and Kurtz A.** Gene expression of prostanoid forming enzymes along the rat nephron. *Kidney Int* 62: 1570–1581, 2002.
60. **Wang Y, Olson KR, Smith MP, Russell MJ, and Conlon JM.** Purification, structural characterization, and myotropic activity of endothelin from trout, *Oncorhynchus mykiss*. *Am J Physiol Regul Integr Comp Physiol* 277: R1605–R1611, 1999.
61. **Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, and Masaki T.** A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415, 1988.
62. **Yang T, Forrest SJ, Stine N, Endo Y, Pasumarthy A, Castrop H, Aller S, Forrest JN Jr, Schnermann J, and Briggs J.** Cyclooxygenase cloning in dogfish shark, *Squalus acanthias*, and its role in rectal gland Cl secretion. *Am J Physiol Regul Integr Comp Physiol* 283: R631–R637, 2002.
63. **Zaccone G, Ainis L, Mauceri A, Lo Cascio P, Lo Giudice F, and Fasulo S.** NANC nerves in the respiratory air sac and branchial vasculature of the Indian catfish, *Heteropneustes fossilis*. *Acta Histochem* 105: 151–163, 2003.
64. **Zaccone G, Fasulo S, Ainis L, and Licata A.** Paraneurons in the gills and airways of fishes. *Microsc Res Tech* 37: 4–12, 1997.