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Cell Signaling and Ion Transport Across the Fish Gill Epithelium

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ABSTRACT A large array of circulating and local signaling agents modulate transport of ions across the gill epithelium of fishes by either affecting transport directly or by altering the size and distribution of transporting cells in the epithelium. In some cases, these transport effects are in addition to cardiovascular effects of the same agents, which may affect the perfusion pathways in the gill vasculature and, in turn, affect epithelial transport indirectly. Prolactin is generally considered to function in freshwater, because it is the only agent that allows survival of some hypophysectomized fish species in freshwater. It appears to function by either reducing branchial permeability, Na,K-activated ATPase activity, or reducing the density of chloride cells. Cortisol was initially considered to produce virtually opposite effects (e.g., stimulation of Na,K-activated ATPase and of chloride cell size and density), but more recent studies have found that this steroid stimulates ionic uptake in freshwater fishes, as well as the activity of H-ATPase, an enzyme thought to be central to ionic uptake. Thus, cortisol may function in both high and low salinities. Growth hormone and insulin-like growth factor appear to act synergistically to affect ion regulation in seawater fishes, stimulating both Na,K-activated ATPase and Na-K-2Cl co-transporter activity, and chloride cell size, independent of their effects on growth. Some of the effects of the GH–IGF axis may be via stimulation of the number of cortisol receptors. Thyroid hormones appear to affect seawater ion regulation indirectly, by stimulating the GH–IGF axis. Natriuretic peptides were initially thought to stimulate gill ionic extrusion, but recent studies have not corroborated this finding, so it appears that the major mode of action of these peptides may be reduction of salt loading by inhibition of oral ingestion and intestinal ionic uptake. Receptors for both arginine vasotocin and angiotensin have been described in the gill epithelium, but their respective roles and importance in fish ion regulation remains unknown. The gill epithelium may be affected by both circulating and local adrenergic agents, and a variety of studies have demonstrated that stimulation of α-adrenergic versus β-adrenergic receptors produces inhibition or stimulation of active salt extrusion, respectively. Local effectors, such as prostaglandins, nitric oxide, and endothelin, may affect active salt extrusion as well as gill perfusion. Recent studies have suggested that the endothelin inhibition of salt extrusion is actually mediated by the release of both NO and prostaglandins. It is hoped that modern molecular techniques, combined with physiological measurements, will allow the dissection of the relative roles in ion transport across the fish gill epithelium of this surprisingly large array of putative signaling agents. *J. Exp. Zool. 293:336–347, 2002.*

A myriad of signaling agents, including many of the vasoactive hormones described in Olson (2002a, this issue), appears to control the movement of ions across the gill epithelium, which is critical for the osmoregulation described by Marshall (2002, this issue). These modulations of ion transport may involve morphological changes in the cellular architecture and/or stimulation or inhibition of transport proteins. Excellent reviews on this subject have appeared in the last decade (e.g., Wendelaar Bonga, '93; McCormick, '95; Hazon and Balment, '98; McCormick, 2002).

1. CIRCULATING EFFECTORS

Prolactin

The importance of prolactin in osmoregulation of freshwater fishes was first demonstrated by Pickford and Phillips (Pickford and Phillips, '59) who showed that hypophysectomized *Fundulus*

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heteroclitus (killifish) could only survive in freshwater if injected with prolactin. Two subsequent studies found that this prolactin therapy was associated with a reduction in ionic efflux rather than an increase of ionic uptake, suggesting that prolactin reduces branchial permeability rather than stimulates active transport (Maetz et al., '67; Potts and Evans, '67), but hemodynamic changes could have produced the same effects. However, Pickford's group subsequently showed that prolactin injections inhibited branchial Na,K-activated ATPase in hypophysectomized killifish in freshwater (Pickford et al., '70). Prolactin also reduced Na,K-activated ATPase activities in the gill of the euryhaline *Chelon labrosus* (Gallis et al., '79) as well as *Oncorhynchus mykiss* (rainbow trout; Madsen and Bern, '92) but had no effect on either the activity or molecular expression of Na,K-activated ATPase in the gill of *Salmo trutta* (brown trout; Madsen et al., '95). Prolactin also did not affect the activity of the enzyme in the gills (and opercular skin) of *Oreochromis mossambicus* (tilapia) acclimated to seawater (Herndon et al., '91), or chloride cell density, but it did reduce the size of the individual cells significantly. Similar, prolactin-induced reductions in chloride cell size were also demonstrated in *Oreochromis niloticus* (Nile tilapia; Pisam et al., '93), and a recent study found that prolactin reduced the chloride cell density in *Sparus sarba* (sea bream; Kelly et al., '99). Foskett et al. ('82) had suggested earlier that (in addition to direct inhibition of ionic extrusion) branchial remodeling may be one mode of action of prolactin. This conclusion was based upon parallel changes in active transport and conductance across the killifish opercular skin.

The suggestion that prolactin is involved with freshwater, rather than seawater, osmoregulation is supported by a few studies that have demonstrated that plasma levels of prolactin decreased when fish were transferred to seawater (e.g., Morgan et al., '97) or increased when fish were transferred to freshwater (e.g., Yamauchi et al., '91).

The complete cDNA sequence of the gene for the fish prolactin receptor has been determined for three species: *O. mossambicus* (Sandra et al., '95), *Sparus aurata* (sea bream) (Santos et al., '99), and *Carassius auratus* (goldfish; Tse et al., 2000). Expression of a prolactin receptor has been demonstrated in the gills of goldfish, using RT-PCR/Southern and Northern analyses (Tse et al., 2000) and, more specifically, in the chloride cells of both *S. aurata* (Santos et al., '99) and *O. mossambicus* (Weng et al., '97), using homologous and heterologous (mouse) antibodies, respectively. Paradoxically, the prolactin receptors were most abundant (Western Blot) in the gills of *O. mossambicus* acclimated to seawater, corroborating earlier data (radioreceptor binding analyses) in *O. niloticus* after acclimation to brackish water (Auperin et al., '95). Clearly, more studies are necessary before we can be sure of this apparent up-regulation of the prolactin receptor in high salinities, in direct contrast to the plasma hormone levels. It does highlight, however, the caution that the role of a specific signaling agent in a physiological response cannot be ascribed unless levels of expression of the receptor(s) are also known.

Cortisol

This steroid's role in acclimation to seawater is well established, but more recent evidence suggests that it also plays a role in fish osmoregulation in freshwater. Since the initial finding that injection of cortisol reversed the reduction gill Na,K-activated ATPase activity produced by hypophysectomy (Pickford et al., '70), it has been shown that cortisol therapy stimulates Na,K-activated ATPase activity in a variety of fish species, and also increases tolerance to high salinities (Madsen et al., '95; McCormick, '95). Moreover, cortisol increased expression of the Na-K-2Cl co-transporter in the gills of *Salmo salar* (Atlantic salmon) acclimated to freshwater (Pelis and McCormick, 2001). Cortisol treatment also increases branchial chloride cell size and density in salmonids (Madsen, '90a,b). The biochemical and morphological effects are at least partially direct, because they occurred in *O. mossambicus* opercular skin stimulated with cortisol in vitro (McCormick, '90). Interestingly, cortisol does not stimulate active transport across the tilapia opercular skin (Foskett et al., '81), suggesting that other factors (e.g., salinity, photoperiod, developmental stage, other hormones) must be involved in facilitating salt extrusion in addition to cortisol-stimulated cell differentiation and up-regulation of Na,K-activated ATPase.

Cortisol receptors have been identified in gill tissue from *Anguilla rostrata* (American eel), *O. mykiss*, *Salvelinus fontinalis* (brook trout), and *Oncorhynchus kisutch* (coho salmon), using radioligand protocols (Sandor et al., '84; Chakraborti et al., '87; Maule and Schreck, '90), and localized in gill chloride (and pavement) cells from chum

salmon fry (*Oncorhynchu keta*), using molecular and immunological techniques (Uchida et al., '98). In chum salmon, acclimation to seawater was associated with an increase in the expression of cortisol receptors in the gills (Uchida et al., '98); however, in the coho salmon, cortisol treatment lowered both the affinity and number of gill receptors, just as chronic stress did, presumably via the secretion of cortisol (Maule and Schreck, '91). This suggests, once again, that factors in addition to cortisol must be involved in tolerance of seawater. The cortisol receptor has been cloned from *O. mykiss*, and its amino acid sequence shares much closer homology with the mammalian glucocorticoid receptor than the mineralocorticoid receptor (Ducouret et al., '95). However, spironolactone, a mineralocorticoid receptor antagonist, inhibited the cortisol-induced proliferation of chloride cells in the gill of rainbow trout, but RU486 (which antagonizes glucocorticoid receptors) did not (Sloman et al., 2001).

On the basis of these physiological studies, one might expect that plasma cortisol levels would generally increase during acclimation to seawater. This was found in early studies on the eel and tilapia (Ball et al., '71; Assem and Hanke, '81), but more recent studies have demonstrated that plasma cortisol levels increase significantly when a variety of fish species (e.g., eel, tilapia, killifish, mullet, flounder, and sea bass) are transferred to low salinities or freshwater (see McCormick, 2002). This finding suggests that cortisol also may play an important role in osmoregulation in freshwater. Early work had shown that cortisol treatment stimulated sodium uptake by intact or inter-renalectomized eels and intact goldfish in freshwater and also increased the plasma osmolality of hypophysectomized eels or goldfish in freshwater (see McCormick, 2002). Treatment with ACTH (presumed to stimulated cortisol production) increased survival of hypophysectomized *Gambusia* sp. (mosquito fish) and *Amia calva* (bowfin) and increased plasma sodium levels in intact *F. kansae* (killifish) in freshwater (see McCormick, 2002). It appears that both cortisol and prolactin may be necessary for survival in low salinities, because hypophysectomized *Ictalurus melas* (black bullhead) and *Heteropneustes fossilis* (catfish) need both prolactin and cortisol to maintain normal ion homeostasis in isosmotic or freshwater salinities, respectively (Fortner and Pickford, '82; Parwez and Goswami, '85).

More recently, it has been shown that cortisol injection increased the chloride cell surface area, as well as the Na and Cl influx in four freshwater species: rainbow trout, European eel (*Anguilla anguilla*), tilapia, and catfish (*Ictalurus nebulosus*; Laurent and Perry, '90; Perry et al., '92). Cortisol-induced changes in chloride cell morphology (proliferation and differentiation) have also been demonstrated in *Anguilla japonica* (Japanese eel) acclimated to freshwater, although the response was greater in seawater-acclimated eels (Wong and Chan, 2001). Chloride cell density in both the gills and opercular skin of freshwater-acclimated *O. mossambicus* was increased by cortisol treatment, as was the volume of the intracellular tubular system as well as the immunoreactive Na,K-activated ATPase density in the system (Dang et al., 2000). Using cultured pavement cells from freshwater-acclimated *O. mykiss*, Kelly and Wood (2001) demonstrated that cortisol induced an increase in transepithelial resistance and stimulated active uptake of both Na and Cl; however, tissue Na,K-activated ATPase levels were unaltered. This stimulation of ionic uptake corroborates earlier work that found that cortisol stimulates H-ATPase in the gills of trout (Lin and Randall, '93), the enzyme generally considered to be crucial for Na uptake by the freshwater gill epithelium (Marshall, 2002, this issue).

In summary, it appears that cortisol is involved in osmoregulation by the fish gill in both high and low salinities, playing roles in both morphological changes and stimulation of transport protein activity. There is some indication that cortisol and prolactin are both necessary for survival in low salinities, and the following section will demonstrate that cortisol also may synergize with another hormonal axis in seawater; namely, growth hormone and insulin-like growth factor.

Growth hormone and insulin-like growth factor

It is well established that injection of growth hormone (GH) increases salinity tolerance in a variety of salmonid species, that this is associated with increased gill Na,K-activated ATPase activity (or mRNA levels) and chloride cell size and density, and that these effects are independent of growth stimulation (e.g., McCormick, '95, 2002). It is now clear that that GH's role in seawater osmoregulation is not limited to the salmonids, because similar effects have been demonstrated in two tilapias, *O. niloticus* and *O. mossambicus* (Sakamoto et al., '97; Xu et al., '97), killifish (Mancera and McCormick, '98), and *S. sarba*

(Kelly et al., '99). In most of these species, injection of insulin-like growth factor (IGF-1) produces the same responses (McCormick, 2002). Recent immunocytochemical studies have demonstrated that IGF-enhanced Na,K-activated ATPase expression and GH-enhanced Na-K-2Cl co-transporter expression can be localized to chloride cells on the gill filament of *S. trutta* and *S. salar*, respectively (Seidelin et al., '99; Pelis and McCormick, 2001).

Exposure to seawater increased the plasma concentration of GH in four species of salmonids (Sakamoto et al., '93), and plasma concentration of IGF-1 increased when *S. salar* was exposed to seawater (McCormick, 2002). In addition, transfer of smolting *O. kisutch* to seawater was associated with increased gill IGF-1 mRNA (Sakamoto et al., '95). The tilapia IGF-1 gene has been cloned and transcripts and gene products localized in chloride cells by Southern blots and immunohistochemistry, respectively (Reinecke et al., '97). GH receptors are present in gill tissue from *O. kisutch* (Gray et al., '90) and IGF-1 receptors have been sequenced in various species of fishes, but not yet localized to the gill epithelium (McCormick, 2002). Because IGF-1 can stimulate gill Na,K-ATPase in vitro (Madsen and Bern, '93), but GH apparently cannot, and GH regulates the response to IGF-1 in vitro and stimulates the expression of IGF-1 mRNA in the gill (Sakamoto and Hirano, '93), the physiological response to GH may be indirect, via the production of the local effector, IGF-1 (McCormick, 2002).

It appears that the actions of the GH–IGF axis and cortisol are synergistic. For instance, injection of GH and cortisol together had a greater effect on both Na,K-activated ATPase activity and high salinity tolerance than either hormone in several salmonid species, as well as *F. heteroclitus* (McCormick, 2002). The same synergy has been described for the GH- and cortisol-stimulated expression of the Na-K-2Cl co-transporter in the gill of *S. salar* (Pelis and McCormick, 2001). In addition, Seidelin et al. ('99) demonstrated that IGF-1 and cortisol have an additive effect on Na,K-activated ATPase activity and expression, as well as the number of chloride cells in the gill of *S. trutta*. This positive interaction may be via regulation of receptor density, because it has been shown that GH injection increased the number of cortisol receptors in the gill of two species of salmonids (Shrimpton et al., '95; Shrimpton and McCormick, '98).

Thyroid hormones

The roles of thyroid hormones in gill transport are unclear and may be indirect. Early demonstrations that T_4 or T_3 injection into salmonids increased salinity tolerance were probably due to effects on growth rather than transport per se, because no increase in gill Na,K-activated ATPase was generally seen (e.g., McCormick, '95). However, one study showed that T_4-induced an increase in gill enzyme activity and chloride cell number in *S. salar* (Madsen and Korsgaard, '89), and Trombetti et al. ('96) found that dietary T_3 increased the number of chloride cells in *O. mykiss* gill but did not change the Na,K-activated ATPase activity. A more recent study by the same group, however, demonstrated that T_3 treatment did increase enzyme activity in *O. mykiss* (Ventrella et al., 2001). In addition, treatment of *O. mossambicus* with T_4 increased immunoreactive Na,K-activated ATPase expression, as well as chloride cell size (but not density; Peter et al., 2000).

It appears that thyroid hormones probably work indirectly, through the cortisol and/or GH and IGF axes (see McCormick, 2002). For instance, an early study found that T_4 treatment alone did not affect Na,K-activated ATPase activity in the gill of *O. mossambicus*, but it did increase the stimulatory effect of cortisol on the enzyme (Dange, '86). T_4 treatment also potentiated the stimulation of gill enzyme activity by GH in the amago salmon (*O. rhodurus*; Miwa and Inui, '85). T_3 treatment increased the number of cortisol receptors in the gills of *O. mykiss* and increased the ability of cortisol to stimulate gill Na,K-activated ATPase activity in vitro (Shrimpton and McCormick, '99). T_3 administration also increased the number of cortisol receptors in *S. salar*, and this effect was potentiated when GH was added (Shrimpton and McCormick, '98).

Natriuretic peptides

The idea that members of this family of peptides, so important in cardiovascular control (including fishes, see Olson, 2002, this issue), may play a central role in fish osmoregulation (especially in seawater) emerged over a decade ago (Evans, '90; Evans and Takei, '92) and recently has been reviewed quite extensively (Loretz and Pollina, 2000; Takei, 2000). The only direct evidence that a natriuretic peptide (NP) can affect gill transport directly is the study by Scheide and Zadunaisky ('88) that found that a slightly truncated, mammalian peptide (atriopeptin II)

stimulated NaCl transport across the isolated killifish opercular skin. The stimulation was dose-dependent and reached 19% at 10^{-7} M peptide. This finding was intuitively pleasing, since the natriuretic peptides, as their name stipulates, stimulate renal salt loss in mammals. However, recent experiments in our laboratory (Evans et al., 2002a) have been unable to corroborate these results. We applied concentrations of eel ANP and porcine CNP between 10^{-10} and 2×10^{-7} M to killifish opercular skin and could not demonstrate any statistically significant alteration in the short-circuit current (I_{sc}) by either peptide; however, subsequent addition of the endothelin agonist sarafotoxin S6c produced significant inhibition of the I_{sc} (48%). In addition, this tissue is exquisitely sensitive to a variety of other putative signaling agents (see below). Our data corroborate unpublished results from the laboratory of K.L. Karnaky (personal communication), which has had extensive experience with this system (e.g., Karnaky et al., '77). This lack of an effect on salt extrusion across the fish gill is logical when one considers that the ionic transport effects of natriuretic peptides in the mammalian kidney are generally inhibitory (of renal salt reabsorption), rather than stimulatory (e.g., Jamison et al., '92). Moreover, NPs inhibit fish drinking and intestinal salt uptake (Loretz, '96), so it appears that the osmoregulatory importance of NPs in marine fishes is inhibition of salt loading, rather than stimulation of salt loss. Of course, the cardiovascular effects of NPs (Olson, 2002, this issue) may also affect the pattern of gill blood flow, which might produce indirect effects on passive and active salt movements across the gill epithelium.

Arginine vasotocin and angiotensin

Both vasopressin and angiotensin have well-known affects on salt transport in the mammalian kidney (e.g., Guyton and Hall, 2000), so, intuitively, one might propose that their fish equivalents are important in controlling salt movements across the gill epithelium, exclusive of their potential effects on blood flow, as well as on the kidney. Plasma concentrations of both arginine vasotocin and isotocin did not change when *O. mykiss* were acclimated to either freshwater or seawater, but did fall in isotonic solutions (Pierson et al., '95), and plasma AVT levels were not correlated with salinity in *A. anguilla* (Balment et al., '93). On the other hand, AVT concentrations rose when the flounder, *Platichthyes flesus*, was acclimated to freshwater (Balment et al., '93), and another study found that AVT concentrations were higher when trout were acclimated to freshwater (Kulczykowska, '97).

A receptor for AVT has been described in gill tissue from *O. mykiss*, but it has been designated NH_F, rather than V_1 or V_2, based upon agonist and antagonist sensitivities (Guibbolini and Lahlou, '90). This receptor was sensitive to AVT in the range of 10^{-12} to 10^{-10} M, which is equivalent to measured plasma levels (Guibbolini and Lahlou, '87). However, contrary to expectations, gill tissue response was characterized by inhibition of cAMP activity, rather than stimulation, which is commonly seen in mammalian renal epithelia responding via V_2 receptors (e.g., Peter et al., '95). To date, we have no data on any biochemical or transport effects of AVT treatment, so the role of neurohypophyseal hormones in gill transport remains unknown.

Despite some initial uncertainty, it is now clear that the renin–angiotensin cascade exists throughout the vertebrates (Nishimura, 2001). Relatively little data exists on circulating levels of angiotensin, but it was shown recently that plasma ANG II levels increased when the river lamprey (*Lampetra fluviatilis*) was acclimated to seawater (Rankin et al., 2001). These data corroborate earlier studies that found that plasma renin activities and ANG II levels were significantly higher in *A. anguilla* acclimated to seawater (Henderson et al., '76; Nishimura et al., '76; Tierney et al., '95). A similar correlation between salinity and plasma renin activity has been described for both *O. mykiss* and *S. salar* (Smith et al., '91).

Receptors for angiotensin II have also been described in fish gills, specifically in *A. anguilla*, the icefish (*Chionodraco hamatus*), and the Japanese dogfish (elasmobranch; *Triakis scyllia*) (Marsigliante et al., '96, '97; Tierney et al., '97). In both teleost species, chloride cells were immunoreactive; at least in the icefish, reactivity was also described for pavement cells. Higher expression of receptor protein was seen in gills from seawater acclimated eels than freshwater, which correlated with the dose-dependent increase in Na,K-activated ATPase seen in eel gills perfused with ANG II (Marsigliante et al., '97). An earlier study had demonstrated that ANG II could partially inhibit the transepithelial electrical potential across the perfused flounder gill (Lyndon, '93), but the gill TEP is largely due to differential ionic permeabilities, rather than active transport (e.g., Evans

et al., '99), so these results do not allow a very definitive conclusion.

Thus, both AVT and ANG II are putative agents controlling gill transport in fishes, but the lack of biochemical, physiological, or molecular data precludes any specific hypotheses about mode of action or importance.

2. LOCAL EFFECTORS

In addition to circulating peptide and steroid hormones, the fish gill epithelium is exposed to a suite of local signaling agents: neurotransmitters secreted by resident neurons (see Sundin and Nilsson, 2002, this issue) and paracrines from adjacent cells. Recent evidence suggests that these substances are quite effective in modulating gill transport, in addition to perfusion (see Olson, 2002, this issue).

Epinephrine/norepinephrine

Soon after the killifish opercular skin was suggested as a model for NaCl extrusion by the teleost gill (Degnan et al., '77), it was shown that the isotopic effluxes and short-circuit current across this epithelium were stimulated by activation of β-adrenergic receptors, but inhibited by activation of α-adrenergic receptors, suggesting dual adrenergic control (Degnan and Zadunaisky, '79). Subsequent studies have found that the β-mediated stimulation is associated with an increase in intracellular cAMP levels (Mendelsohn et al., '81) and the α-mediated inhibition is via elevation of intracellular IP_3 through α_2 receptors (Marshall et al., '98), rather than through inhibition of intracellular cAMP (May and Degnan, '84). The specific, intracellular site of cAMP-mediated stimulation (or IP_3-mediated inhibition) is unknown in the gill chloride cell, but the apical Cl channel is stimulated by cAMP in the shark

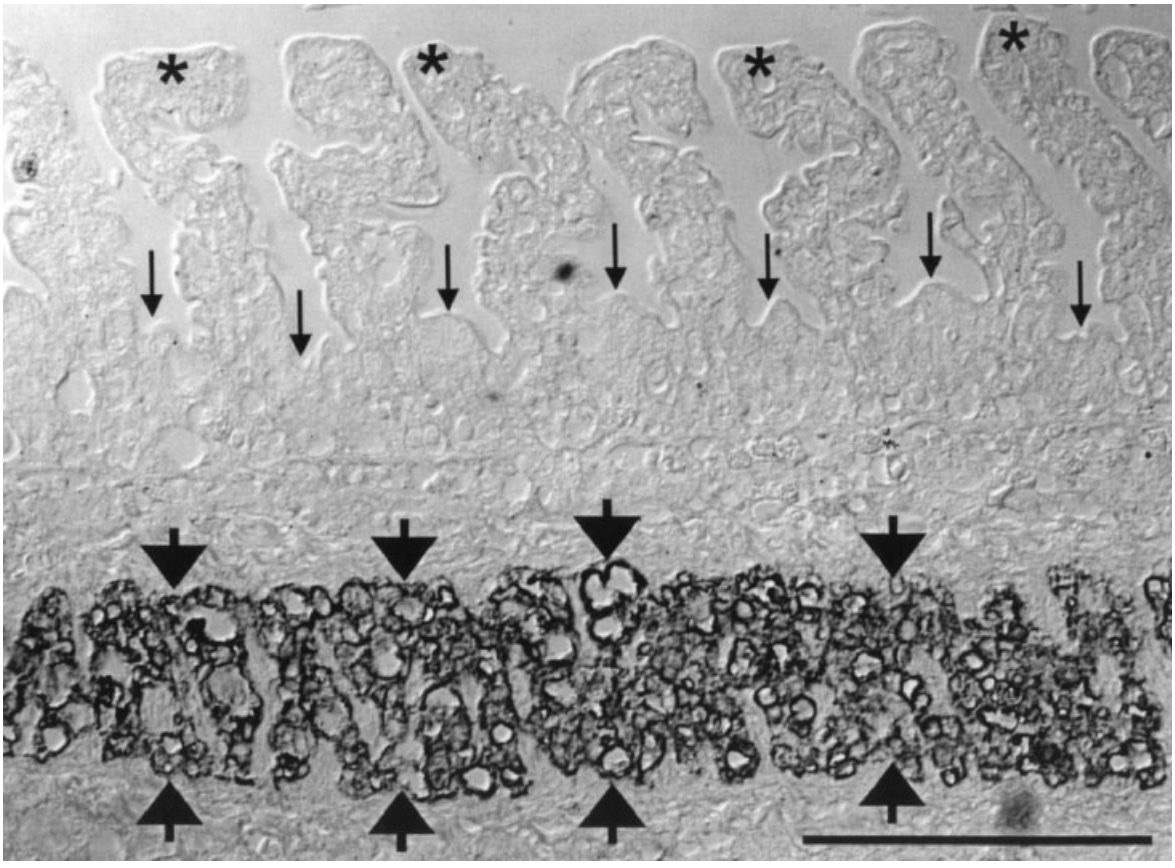

Fig. 1. Representative photomicrograph of cyclooxygenase (COX) immunoreactivity (between the wide arrows) in the gills of a seawater Atlantic stingray (*Dasyatis sabina*) Immunostaining occurs in the arteriovenous interlamellar network (Olson, 02b, this issue) and not in the epithelium where BET staining occurred (Fig. 3; thin arrows). Asterisks indicate lamellae. Gill tissue was fixed in 3% paraformaldehyde for 24 hr at 4°C, embedded and sectioned in paraffin, and stained with a rabbit polyclonal antibody to sheep COX (Cayman Chemical) using DAB. Scale bar = 100 μm.

rectal gland, which excretes salt via what are essentially chloride cells (Devor et al., '95). The respective roles of resident nerves versus circulating epinephrine (from heart or chromaffin tissue in the fish interrenals) as the source of the adrenergic signals is unclear at this point.

Prostaglandins

Fish gill tissue produces prostaglandins (e.g., Brown et al., '91; Knight et al., '95), as does the killifish opercular skin (Van Praag et al., '87), and the concentration of PGE_1 in the gill tissue fell when *A. anguilla* was acclimated to seawater (Brown et al., '91). Early work on the killifish opercular skin established that PGE_2 inhibits the I_{sc} across this tissue (Eriksson et al., '85; Van Praag et al., '87), and our recent data have confirmed this. In our hands, the inhibition of the I_{sc} by PGE_2 was concentration dependent, statistically significant at all concentrations at or above 10^{-10} M and reached 40% at 10^{-5} M (Evans et al., 2002a). In addition, our preliminary experiments using PGE receptor (designated EP) agonists suggest that two to three receptors are present, which can produce either inhibition (EP_1 or EP_3) or stimulation (EP_2). Our evidence also suggests that PGs are part of a signaling axis that includes both nitric oxide and endothelin (see following). Specific cellular sites of production of prostaglandins are lacking, but our preliminary evidence suggests that, at least in *Dasyatis sabina* (Atlantic stingray), PGs are produced by cyclooxygenase in the arteriovenous interlamellar network (Olson, 2002b, this issue), rather than in surface epithelial cells (Fig. 1).

Nitric oxide

There are no published measurements of gill production of the gas nitric oxide, but we have localized nitric oxide synthase (neuronal NOS) in the killifish opercular skin in cells that are adjacent to chloride cells (Fig. 2). Earlier studies have localized nNOS in branchial nerves of the cod, *Gadus morhus* (Gibbins et al., '95), as well as ''neuroendocrine cells'' in the gills of the catfish *H. fossilis* (Mauceri et al., '99). A partial clone of a gene encoding nNOS is now available from the gill of the spiny dogfish, *Squalus acanthias* (Genbank accession number AF232227; Evans, Catches, Claiborne, and Morrison-Shetlar, unpublished data), as is a partial sequence of the inducible NOS from the gill *O. mykiss* (accession number AJ300555; Grabowski et al., '96).

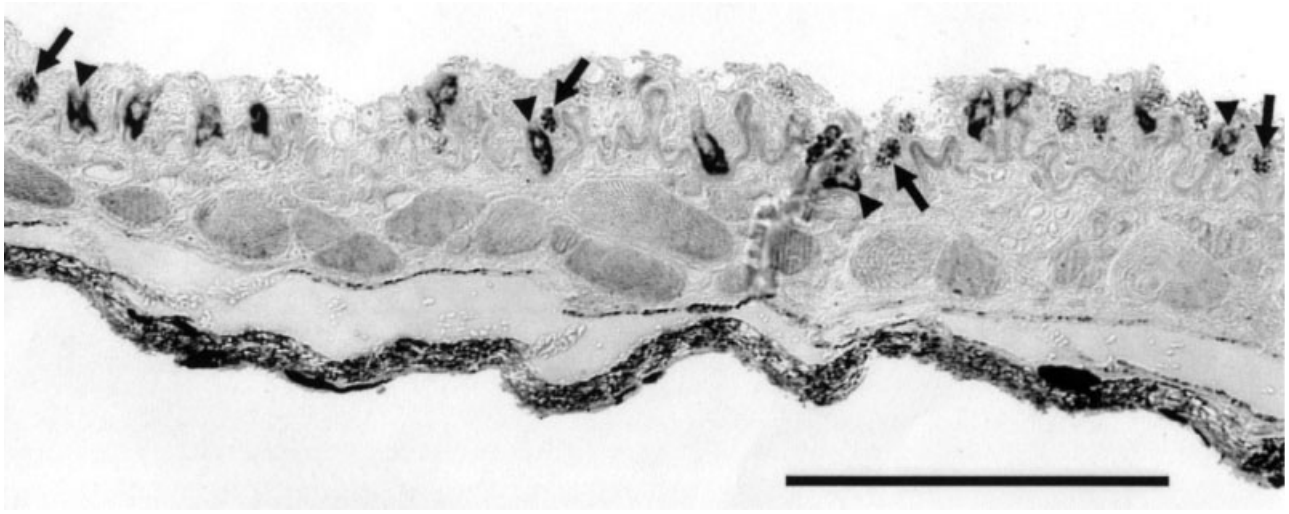

Fig. 2. Representative photomicrograph of neuronal nitric oxide synthase (nNOS) immunoreactivity (arrows) colocalized with Na,K-ATPase (arrowheads) in the opercular epithelium of seawater *Fundulus heteroclitus*. Immunostaining for nNOS is found in relatively large cells of the operculum, but does not occur in the Na,K-ATPase-rich chloride cells (arrowheads). In addition, the punctate appearance of the nNOS staining may indicate that this enzyme is stored in cytoplasmic vesicles. Operculae were fixed in Bouin's fixative for 24 hr at 4°C, embedded and sectioned in paraffin, and stained with a mouse monoclonal antibody specific for the α-subunit of avian Na,K-ATPase (Developmental Studies Hybridoma Bank, University of Iowa) using Vector SG and a rabbit polyclonal antibody to human nNOS (Chemicon International) using DAB (brown stain). For a full-color image of this figure please visit http://www.zoo.ufl.edu/dhefish/OpercNOS.jpeg. Scale bar = 100 μm.

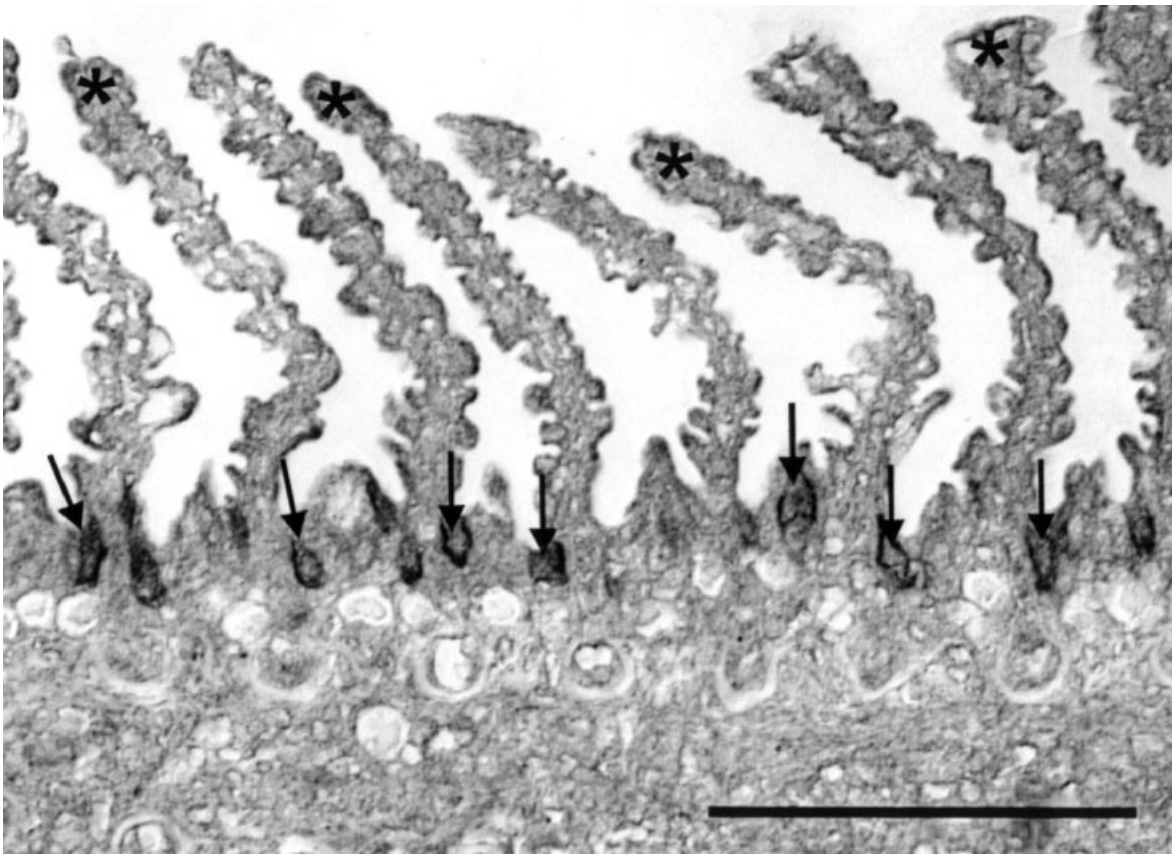

Fig. 3. Representative photomicrograph of big endothelin (BET) immunoreactivity (arrows) in the gills of a seawater Atlantic stingray (*Dasyatis sabina*). Immunostaining occurs throughout the cytoplasm of relatively large cells in the gill epithelium that are basal to and between lamellae (asterisks). Colocalization studies demonstrated that BET-positive cells are also Na,K-ATPase-rich cells (data not shown). Gill tissue was fixed in 3% paraformaldehyde for 24 hr at 4°C, embedded and sectioned in paraffin, and stained with a rabbit polyclonal antibody to human BET (Peninsula Laboratories, Inc.) using DAB. Scale bar = 100 μm.

Our preliminary studies found that the nitric oxide donor sodium nitroprusside (SNP) inhibited the I_{sc} across the killifish opercular skin, dependent upon concentration, statistically significant above 10^{-8} M, and reaching 20% at 10^{-4} M (Evans et al., 2002b). Interestingly, if NOS was inhibited in this preparation by the addition of 10^{-4} M L-NAME, the I_{sc} increased by 15%, suggesting that active transport across the opercular skin is under tonic inhibition by resident NO production. Moreover, this inhibitory pathway appears to be part of the inhibition produced by endothelin (see below).

Endothelin

Endothelin (ET) production has not been measured by the fish gill, but recent studies have localized ET (actually its precursor, big ET) in the gills of *H. fossilis* and the eel, *Conger conger* (Zaccone et al., '96), and our recent study has localized big ET in the chloride cells in the gills of the Atlantic stingray (Fig. 3). Both ET-1 and the ET_B-receptor specific agonist sarafotoxin S6c (SRX) inhibit the I_{sc} across the killifish operculum. The inhibition was dependent upon concentration, significant at 10^{-10} M for both agonists, and reached 30% and 40% at 10^{-7} M ET-1 and SRX, respectively (Evans et al., 2002c). The sensitivity to SRX suggests that the dominant receptor is the ET_B- type, which corroborates our earlier, pharmacological study of the *S. acanthias* gill tissue (Evans and Gunderson, '99). In mammalian systems, the effect (vascular or transport) of stimulation of ET_B receptors is often mediated by the release of NO (e.g., Plato et al., 2000), and this appears to be the case in the operculum. Preincubation of the skin in 10^{-4} M L-NAME decreased the SRX-induced inhibition of the I_{sc} by 15%; but inhibition of the synthesis of prostaglandins (indomethacin, COX inhibitor) reduced the effect of SRX by 90% (Evans, Roeser, and Kozlowski,

unpublished). Thus, it appears that an ET–NO–PG axis may be a major modulator of fish gill ionic transport. The intracellular site of action of this axis is unknown, but the NO inhibition of transport across the mammalian thick ascending limb of the loop of Henle is mediated through the Na-K-2Cl co-transporter (Ortiz et al., 2001), which is certainly in the fish chloride cell (Marshall, 2002, this issue).

SUMMARY AND FUTURE DIRECTIONS

It is clear that NaCl transport across the fish gill is under the control of an array of circulating and local signaling agents, including proteins and amino acid derivatives, steroids, prostaglandins, and even a gas. It is of especial interest that many of these agents also affect vascular diameter, so they can potentially modulate both gill perfusion and salt transport. This economy of action is consistent with the recent genomic data that, at least in the human, indicates far fewer genes than expected. Future research in this area of cell signaling must include the recognition that signaling axes often interact in both negative and positive ways, that they may be systemic or local, and that they may affect a variety of functions. Clearly the tools of molecular biology (cloning, expression, in situ hybridization, immunohistochemistry, differential gene expression, etc.) are beginning to allow discrimination of processes and interactions that were unknown a few years ago. Such approaches, applied to a variety of species (rather than a few model organisms) can allow study of the evolution of gill ionic transport process in the early vertebrates.

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