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Review

Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures

William S. Marshall*

Department of Biology, St. Francis Xavier University, P.O. Box 5000 Antigonish, Nova Scotia, Canada B2G 2W5

Abstract

This review summarizes the mechanism of Cl^- active secretion and its regulation in estuarine teleost fish. Small estuarine fish such as the killifish, *Fundulus heteroclitus*, forage in shallow water following advancing tides and are exposed regularly to very dilute microenvironments. Using the killifish opercular epithelium and related teleost membranes containing mitochondria-rich cells, the regulation includes a reduction of active Cl^- secretion and passive diffusive ion loss in a three-stage process spanning approximately 30 min. There is a combination of sympathetic neural reflex mediated by α_2-adrenoceptors operating via intracellular inositol tris phosphate and intracellular Ca^{2+} and a cellular hypotonic shock response, followed by covering over of ion-secreting cells by pavement cells. This effectively minimizes salt loss in dilute media. The upregulation of salt secretion on return to full strength seawater may be via hormones (arginine vasotocin and urotensin I) and neurotransmitter (vasoactive intestinal polypeptide) in combination with hypertonic shock. A hypothetical model includes involvement of protein kinase A and C and protein phosphatases 1 and 2A in regulation of the NKCC1 cotransporter on the basolateral side and protein kinase A regulation of the CFTR-like apical anion channel.

Keywords: Cotransport; Anion channel; Vasoactive intestinal polypeptide; Arginine vasotocin; Alpha-adrenoceptor; Cortisol; Gill epithelium

1. Introduction

1.1. Foraging by estuarine teleosts involves salinity change

Estuarine teleosts such as the common killifish (or mummichog), *Fundulus heteroclitus*, are frequently exposed to rapid changes in salinity, oxygen and temperature. This is especially true if the habits of the animals encourages foraging in specified microzones within an estuary. *F. heteroclitus* (killifish or mummichog) live in estuaries of the Eastern coast of North America and extensively form shoals or schools [1]. They were introduced to Europe [2] where they inhabit brackish estuaries, preferring salinities of 25 g/l. The shoals advance with the rising tide such that the lead fish are at the waters edge. Salt marsh margins in turn are rich in invertebrate infauna that serve as major food sources for nekton predators [3] especially during high spring tides when the marsh edge provides a rich foraging area [4]. The tidal margin shallows on spring tides also serve as the spawning location for *F. heteroclitus* [5]. Typically, the salinity in these shallows is low, ca. 3% seawater (1.0 g/l) or lower and the temperatures, especially in summer, can be extreme (25–35 °C). The killifish retreat to deeper parts of the estuary during ebb tides, where salinity approximates that of seawater (salinity 25–32 g/l) and temperatures are cooler. It is also common that the animals become stranded in puddles between high tides, thus forcing them to cope with low salinity for at least 12 h until the next high tide. The shoals reform with different individuals with preference for similar body size [6] and advance on the next rising tide. Hence, these 'sorties' to low salinities approximating freshwater are a regular feeding and reproductive strategy that exposes the fish to dilute media for several hours in each of two tidal cycles per day.

This paper attempts to address the expected coping strategy that killifish and other estuarine fish may use during short exposures to freshwater. Because killifish in brackish water retain chloride cell + accessory cell complexes in the opercular epithelium and gill at densities similar to animals in seawater (Fig. 1), this coping strategy primarily takes the form of shutdown of active chloride and passive sodium secretion at the gill in combination with reduction in passive

* Tel.: +1-902-867-2482; fax: +1-902-867-2389.
E-mail address: bmarshal@stfx.ca (W.S. Marshall).

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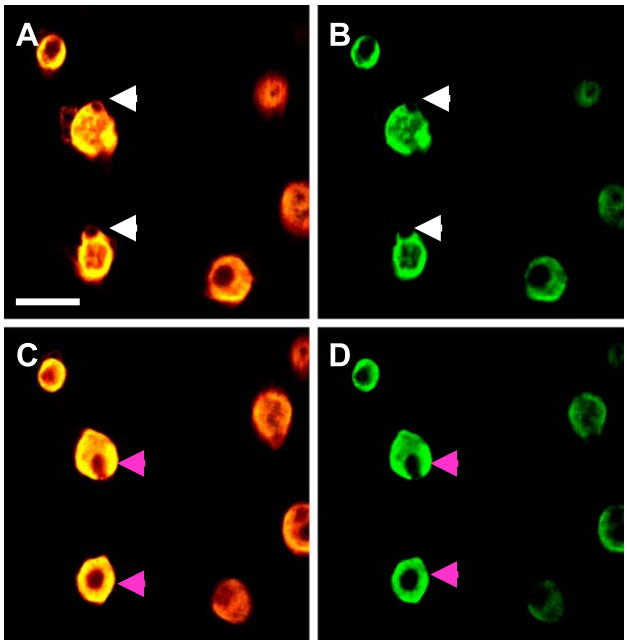

Fig. 1. Killifish opercular epithelium mitochondria-rich cells were fixed and stained for mitochondria with Mitotracker Red (red) and immunostained for Na^+,K^+-ATPase with mouse monoclonal anti chicken Na^+,K^+-ATPase alpha-5 subunit IGG (primary) and goat polyclonal anti mouse IGG Oregon Green 488 (secondary antibody). Methods as per Ref. [91]. A and B are optical sections of chloride cell complexes at the plane of the nuclei of the accessory cells (white arrows), while C and D are frames of the same cells four microns below, at the plane of the nuclei of the main chloride cells (purple arrows). Panels A and C show Mitotracker red and Oregon green with the yellow being positive for both. Panels B and D show only the Na^+,K^+-ATPase immunofluorescence and suggest that there is some unevenness in the enzyme distribution. A location above the nucleus has mitochondria but lacks Na^+,K^+-ATPase. Note that each chloride cell has a smaller accessory cell adjacent to it and that the accessory cells appear to have lower Na^+,K^+-ATPase immunofluorescence. Bar is 20 μM.

ion diffusion in the first hour in freshwater (review, Ref. [7]). Killifish are capable of permanent adaptation to freshwater (e.g. Ref. [8]) but have a strategy that includes active Na^+ uptake but a very low affinity Cl^- uptake (Refs. [7,9]; see Wood, this volume), which suggests a reliance on ions from food for survival in dilute media. Because the rapid inhibition of Cl^- secretion is short-lived (a few hours), voluntary (involving shoaling behavior) and mediated by autonomic reflex and neurohormones in combination with direct effects of blood tonicity on ion transporting cells, the concept of a *nonstressful* salinity change is put forward.

1.2. Mitochondria-rich chloride-secreting cells

Mitochondria-rich cells are present in large numbers in the gill and opercular epithelia of estuarine teleosts. The cell density is augmented in hypersaline conditions and moderates slightly in brackish water, but in the wild, these epithelia are capable of rapid chloride secretion. Typically, the cells appear with a smaller accessory cell entwined with the larger cell and between the accessory cells and chloride cells is a leaky paracellular pathway [10] that accommodates the transepithelial transport of sodium down its electrochemical gradient [11]. The opercular membrane of killifish (*F. heteroclitus*), of tilapia (*Oreochromis mossambicus*) and the skin of the euryhaline goby *Gillichthys mirabilis* provide convenient flat preparations of gill-like epithelia that actively secrete Cl^- at high rates [12–16]. The generally accepted chloride cell model (reviews, Refs. [17–19]; Fig. 2) comprises a basolateral Na^+,K^+,$2Cl^-$ symport (NKCC) or cotransporter of the NKCC1 type that carries Cl into the cell driven by the Na^+ transmembrane gradient in turn maintained by Na^+,K^+-ATPase (also on the basolateral membrane). Cl^- secretion is blocked by basolateral furosemide and bumetanide, loop diuretics that block NKCC type symports, but not by thiazide-type diuretics that block Na^+-Cl^- symports [17]. Once in the cytosol, the Cl^- diffuses to the apical membrane where anion channels conduct Cl^- across the epithelium and into the environment. The Cl^- transport generates a serosa-positive potential that is sufficient to drive Na^+ out via the paracellular pathway. Epithelial current and ion conductance are localized to chloride cells, measured by Foskett and Machen [20] using the vibrating microprobe. The anion

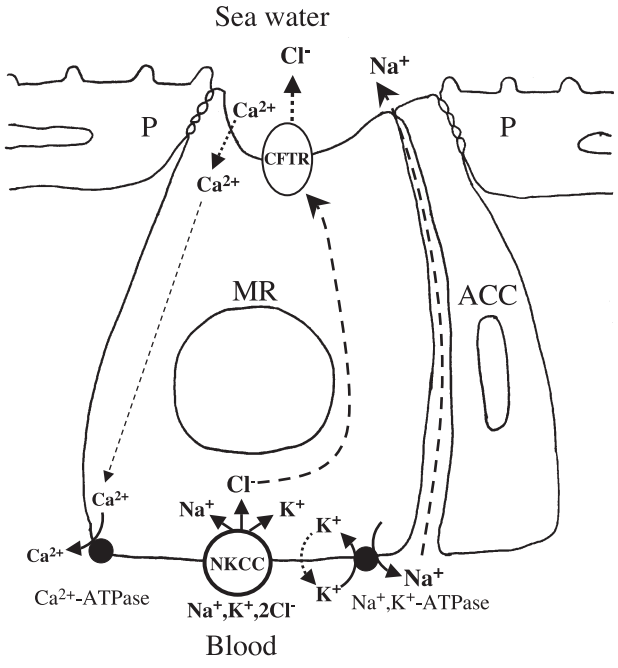

Fig. 2. Model of the seawater type mitochondria-rich cell complex with Na^+,K^+-ATPase, NKCC1 and K^+ channels on the basolateral membrane that accumulate Cl^- in the cytosol driven indirectly by the pump. Solid arrows and closed symbols indicate active transport; dashed lines and open symbols, passive processes. Cl^- exits through CFTR-like anion channels in the apical membrane that has a typical cup-shaped "apical crypt". Intercellular junctions between mitochondria-rich (MR) cells and pavement cells (P) are many-stranded and have low permeability, while junctions between accessory cells (ACC) and MR cells are single-stranded and provide a high conductance paracellular pathway that is cation (Na^+) selective. Shown also is the proposed uptake pathway for transepithelial Ca^{2+} uptake, via Ca^{2+} channels at the apical membrane and via Na/Ca^{2+} and Ca^{2+}-ATPase at the basolateral membrane. Acid/base and other solute transport pathways are not shown (adapted from Ref. [18]).

channel appears to be an 8 pS NPPB-sensitive, DIDS-insensitive, cAMP-activated channel [21] and has been cloned, sequenced and found by Singer et al. [22] to be homologous to hCFTR. It is the differential regulation of apical CFTR and basolateral transporters (NKCC, Na^+,K^+-ATPase and K^+ channels) that provide the present research challenge.

2. Control of Cl^- secretion

2.1. Overview

Recent reviews in the general area of hormonal control of osmoregulation in fish include McCormick [23] who reviewed salinity adaptation, and Evans [24] who examined cell signalling in fish gills. Many factors are known to affect chloride secretion in teleost fish pharmacologically (Table 1) and they include hormones and neurotransmitters that inhibit secretion (catecholamines, acetylcholine and urotensin II) and appear to do so via intracellular calcium, as the calcium ionophore ionomycin rapidly inhibits Cl^- secretion (measured as short-circuit current, SCC or I_{sc}) across the opercular epithelium [37]. There are also many hormones, neurotransmitters and drugs that stimulate chloride secretion (VIP, glucagon, AVT, urotensin I; Table 1) and in these cases, the common pathway appears to be an augmentation of intracellular cAMP, activation of protein kinase A (PKA). For instance, the β-adrenergic agonist isoproterenol at 1.0 mM causes rapid accumulation of cAMP in killifish opercular epithelia (Fig. 3).

Table 1
Summary of potential rapid secretagogues and inhibitory agents affecting Cl^- secretion by marine teleost chloride cells

Agent added	Effect on Cl^- secretion	System	References
Hormones			
Arginine vasotocin	+	CE	[18,25]
Urotensin I	+	OE	[26,27]
Urotensin II	−	OE	[26,27]
Epinephrine	−	CE, OE	[13,15,25,27]
Glucagon	+	OE	[29]
ANP	0?	OE	[24,30]
Neurotransmitters and parahormones			
Acetylcholine	−	OE	[31,32]
Norepinephrine	−	OE	[13,33]
Vasoactive intestinal peptide	+	OE	[29]
Serotonin	0	OE	this article
NO (sodium nitroprusside)	−	OE	[24]
Endothelin	−	OE	[24]
Prostaglandin (PGE_2)	−	OE	[34,35]
Agents (selected)			
Ionomycin	−	OE	[37,38]
A23187	0	OE	[13,37]
Furosemide, bumetanide	−	OE	[34,36]
DPC, NPPB (apical)	−	OE	[21,39]
Glibenclamide	−	OE	[39]
IBMX/db-cAMP	+	OE	[29,31,32,42]
Genistein	−	OE	[38]
Calyculin	+	OE	[39]
Treatments			
Hypotonic shock	−	OE	[38,70]
Hypertonic shock	+	OE	[39,40]

(+) Stimulation; (−) inhibition; (0) no effect; (NT) not tested; (OE) opercular epithelium or goby skin; (CE) cultured marine gill epithelium.

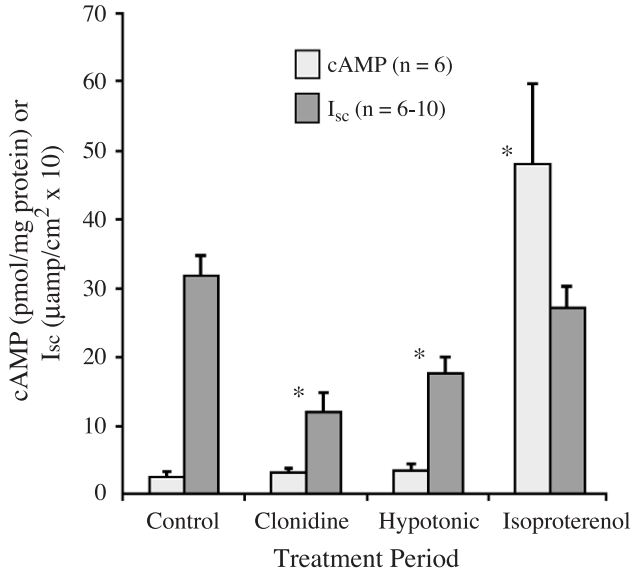

Fig. 3. Paired killifish opercular epithelia were either mounted in Ussing membrane chambers and treated as indicated while Cl^- secretion was measured (as short-circuit current, I_{sc}) or treated and assayed by RIA for cAMP. Whereas hypotonic shock (reduction from 300 to 240 mOsm/kg by dilution) and the α_2-adrenergic agonist clonidine (1.0 μM) markedly inhibited I_{sc}, there was no change in cAMP. The β-adrenergic agonist isoproterenol (5 μM) however, markedly stimulated I_{sc} and increased cAMP content more than 10-fold. Clonidine and hypotonic shock therefore do not act by decreasing tissue cAMP (adapted from Ref. [38]).

2.2. Catecholamines

2.2.1. α-Adrenoceptors

The most likely physiologically relevant response is the inhibition of Cl^- secretion by epinephrine and norepinephrine. The response is rapid, dose-dependent and occurs at catecholamine levels that are physiologically realistic, with ED_{50} of approximately 50 nM for epinephrine and 500 nM for norepinephrine. The effect is blocked by yohimbine, indicating an α-receptor type [32,33,37]. The α_2-receptor subtype was determined by comparing affinity for norepinephrine and epinephrine [37] by application of specific α_2-receptor agonist clonidine [32,37], by comparing α_1-adrenergic agonist phenylephrine with clonidine [37] and by use of α_2-receptor blockers to inhibit epinephrine action [32].

2.2.2. Physiological inhibition is via α_2-adrenoceptors

However marked the effect of β-adrenoceptor agonist isoproterenol might be in stimulating chloride secretion

(see below), the application of the natural nonspecific agonists (epinephrine and norepinephrine) invariably causes strong inhibition of the chloride secretion in tilapia opercular membranes [20,29], *Fundulus* opercular membranes [13,31,37] and goby (*G. mirabilis*) skin [27]. The β-adrenergic response therefore appears to be pharmacological. The ED_{50} for epinephrine is between 0.1 and 1.0 μM for opercular membrane of tilapia [29] and *Fundulus* [37] and *Gillichthys* skin [27]. Circulating catecholamine levels in resting, unstressed teleosts are below this range (0.05 μM), thus Cl^- secretion is not likely under tonic inhibition by the interrenal. In stressed teleosts, catecholamine levels are much higher (0.5–1.0 μM) and would inhibit Cl^- secretion by the gills. Spontaneous recovery of Cl^- secretion after epinephrine addition is observed in killifish and tilapia membranes and was initially ascribed to β-adrenergic activity, but was prevented by addition of antioxidants (ascorbate) [29], so oxidation of the catecholamine would appear to be the cause of the observed recovery.

The opercular epithelium was dissected with its nerve supply intact, mounted in an Ussing chamber and the nerve directly stimulated while measuring transepithelial Cl^- secretion [33]. Stimulation of the branch of the glossopharyngeal nerve that innervates the opercular epithelium in isolated epithelia illustrates that the natural sympathetic reflex response in the opercular epithelium (and likely also the gill chloride cells) is inhibition of chloride secretion. Autonomic nerves track alongside cranial nerves leading to the gill and head region of teleosts [41] so stimulation of the bundle would activate autonomic and motor neurons. Nerve stimulation induced rapid inhibition that was unaffected by cholinergic blockers (atropine and tubocurarine) but was blocked effectively by the α-adrenergic inhibitor yohimbine [33], demonstrating α-adrenergic mediation. Thus physiological activation of autonomic neurons innervating the opercular epithelium and gill produce rapid inhibition of Cl^- secretion.

Catecholamine effects on the gill vasculature are consistent with the abovementioned inhibition of epithelial Cl^- secretion. Epinephrine induces a reduction in perfusion of the ion transporting cells, in that epinephrine dilates the arterio-arterial perfusion of flounder gill lamellae at the expense of the arterio-venous flow that supplies the gill filament epithelium where the chloride cells reside [43]. In rainbow trout, epinephrine increases functional surface area for gas exchange, a combination of vasodilation via β-receptors on the afferent side and a balanced vasoconstriction on the efferent side mediated by α-receptors, reviewed by Sundin and Nilsson [41]. These changes are consistent with a shift away from perfusion of ion transporting cells more toward perfusion of the lamellae.

2.2.3. β-Adrenoceptors are also present

Isoproterenol stimulates Cl^- secretion in killifish opercular epithelium [32,33] and *Gillichthys* skin [27]. While specific β_1 and β_2 agonists were ineffective and thus did not identify the β-receptor subtype, β_1 antagonists (e.g. timolol) blocked the response to isoproterenol, whereas the β_2 antagonists were ineffective, indicating the presence of a β_1-receptor subtype [31]. The β-adrenoceptor second messenger is cAMP, as isoproterenol [38,42] and norepinephrine [42] significantly increase opercular epithelium tissue content of cAMP. It has been suggested that the β-adrenergic response might enhance recovery from an initial α-adrenergic inhibition.

2.3. Acetylcholine, serotonin, dopamine

Acetylcholine decreases Cl^- secretion rate in killifish opercular membranes via muscarinic receptors, based on blockade of the effect by homatropine [44]. The response is not mediated by cAMP decrease, as there was no change in cAMP levels after acetylcholine treatment, neither did acetylcholine decrease cAMP levels that had been stimulated by isoproterenol [31]. Because the α-adrenergic and cholinergic responses mirrored each other in a variety of pharmacological treatments, May and Degnan [31] proposed that the two responses converged on a common inhibitory pathway not involving cAMP lowering.

Serotonin is known to stimulate epithelial Cl^- secretion in dog tracheal epithelium [45] and rabbit corneal epithelium [46] and serotoninergic neurons have been identified in teleost gills (Ref. [47], review in Ref. [41]) particularly in proximity to smooth muscle. However, serotonin at low doses (1 and 10 μM) added to the killifish opercular epithelium has no effect, at high doses (50 μM) it is modestly inhibitory and appears to be acting through adrenergic receptors because the effect is blocked by yohimbine (Marshall, unpublished). Dopamine has not been tested in the opercular membrane system to see if this substance could potentially regulate Cl^- secretion.

2.4. Urotensin I and urotensin II

Urotensin I is a peptide resembling mammalian corticotrophin releasing factor (CRF) and amphibian sauvagine that is secreted by the caudal neurosecretory system of teleosts [48,49]. Urotensin I stimulates Cl^- secretion in *Gillichthys* skin chloride cells [26,28] and the effect is more apparent in epithelia that were previously inhibited by epinephrine. However, thus far, urotensin I receptors have not been localized in the gills of teleosts, while three different CRF type receptors have been localized in catfish to brain, heart, pituitary and urophysis [50]. The potent vasodilatory effects of urotensin I would suggest a role in these responses primarily [51].

Urotensin II is a somatostatin-like peptide that is secreted by the caudal neurosecretory system of teleost fish [48]. Urotensin II is most prevalent in the caudal neurosecretory system [48] but has now been identified in the brain of many vertebrates, suggesting an ancient neurosecretory role in vertebrates [52]. Urotensin II significantly inhibits Cl^-

secretion by the goby skin [26,28] and tilapia opercular membrane [53], an effect reversed by agents that augment tissue cAMP. Plasma levels measured by homologous RIA indicate that seawater adapted flounder (*Platichthys flesus*) had higher plasma urotensin II than their freshwater counterparts [54]. While the urotensins are associated with ion balance in fish, their release into the renal portal system by neurosecretory cells of the urophysis points toward renal and intestinal responses [48] rather than a more systemic effect on more remote osmoregulatory structures such as the gill and opercular membranes. Urotensin II levels in plasma of flounder transferred from SW to FW were significantly lower than SW–SW controls at 24 and 72 h after transfer [55], suggesting downregulation of urotensin II during FW acclimation. In both transfer groups, the disturbance appeared to inhibit urotensin II release compared to long-term acclimated FW or SW animals that had 20–25 fmol/ml urotensin II [55].

2.5. VIP, glucagon and atrial natriuretic peptide

Vasoactive intestinal polypeptide (VIP) rapidly increases Cl^- secretion by the tilapia opercular epithelium if the Cl^- secretion had been previously inhibited by epinephrine [29], while VIP alone applied to uninhibited epithelia had only minimal response. The response is consistent with a cAMP-mediated effect because addition of small amounts of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine augmented the VIP response. Similar results to VIP were obtained by Foskett et al. [29] using glucagon. In both cases, the doses necessary to produce marked stimulation of the Cl^- secretion were rather high (10^{-5} M). VIP (26–28 amino acids) belongs to the glucagon-like peptides and teleost VIP shares high similarity to human VIP (85% and 88% at the protein level for *Takifugu rubripes*, fugu and *Danio rerio*, zebrafish, respectively), while zebrafish and fugu VIP share 77% amino acid similarity (Table 2). However, the seawater teleosts (fugu and cod) have a phenylalanine in position 13 rather than leucine (mammal) and variations in the amino terminal amino acids (Table 2). These variations may explain the apparent insensitivity of the tilapia receptors for mammalian VIP [29]. The variation in VIP sequence suggests that human VIP could activate teleost VIP receptors but that glucagon could also be acting through the VIP receptors. VIP receptors have been identified in gill and other tissues of goldfish [56] and the EC_{50} for cod VIP on goldfish VIP receptor is 1 nM but that the receptor will also bind glucagon at a lower affinity [56]. The high doses of heterologous glucagon and VIP required in earlier work suggest that native VIP will be needed to examine truly physiological responses. In nature, VIP responses would most likely be mediated by VIP neurons rather than by circulating VIP. In Atlantic cod (*Gadus morhua*), there exist VIP immunoreactive cells on the gills [57] and in the goldfish gill there are immunoreactive VIP receptors in nerve cell bodies in the subepithelial space [58]. The position of these VIP neurons in the gill suggests a possible physiological response to VIP. VIP is vasodilatory in brown trout (*Salmo trutta*) gill, an effect blocked by indomethacin, hence suggesting a vasoactive response mediated by prostaglandins [59]. While it is not known specifically that neurally derived VIP stimulates Cl^- secretion by the gill, these results collectively point toward such a response.

Atriopeptin II (10^{-9}–10^{-7} M), an atrial natriuretic peptide (ANP), is reported to have a modest stimulatory effect on Cl^- secretion by the killifish opercular epithelium from seawater- and freshwater-adapted animals [30]. The effect was not mediated by neural activity, as the effect was not blocked by tetrodotoxin; likewise the effect was not mediated by adrenergic receptors, as blockade of β-adrenoceptors with propranolol did not affect the response. Recent re-examination however has failed to confirm the ANP response in this system [24].

2.6. Arginine vasotocin

The case for arginine vasotocin (AVT) regulation of Cl^- secretion is extensive but circumstantial. AVT-specific binding to eel gill cells is higher in seawater-adapted animals

Table 2
Sequence comparison for vertebrate VIP

Species	Amino acid																											
	1				5					10				15						20					25			
Dogfish	h	s	d	a	v	f	t	d	n	y	s	r	i	r	k	q	m	a	v	k	k	y	i	n	s	l	l	a
Cod	–	–	–	–	–	–	–	–	–	–	–	–	f	–	–	–	–	–	g	–	–	–	l	–	–			
Fugu	–	–	–	–	i	–	–	–	–	–	–	–	f	–	–	–	–	–	–	–	–	–	l	–	–			
Goldfish	–	–	–	–	–	–	–	–	–	–	–	–	y	–	–	–	–	–	a	–	–	–	l	–	–	v	h	
Zebrafish	–	–	–	g	–	–	–	–	s	–	–	–	y	–	–	–	–	–	–	–	–	–	l	a	t	v		
Chicken	–	–	–	–	–	–	–	–	–	–	–	–	f	–	–	–	–	–	–	–	–	–	l	–	–	v	–	t
Guinea pig	–	–	–	–	l	–	–	–	t	–	t	–	l	–	–	–	–	–	m	–	–	–	l	–	–	v	–	n
Rat/pig/cow/ mouse/human	–	–	–	–	–	–	–	–	–	–	t	–	l	–	–	–	–	–	–	–	–	–	l	–	–	i	–	n

Conserved amino acids (–). Sequences (carboxy terminus left, amino terminus right) derived from BLAST searches of fugu and zebrafish genomes; other sequences published with GenBank with accession numbers: dogfish A60303, cod PO9684, goldfish AAB47074, chicken CAA56867, guinea pig PO4566, rat PO1283, cow NP776395, mouse XP125478, human VRHU.

than FW eels [60], strong evidence for the existence of AVT receptors and a function in SW osmoregulation. The AVT receptor has been isolated from flounder gill, cloned and characterized by Warne et al. (GenBank AF184966; [61]). From Northern analysis, the AVT receptor is expressed in gill as well as brain, kidney and liver. Expression of AVT receptors in *Xenopus* oocytes results in dose–response stimulation of an inward current using native AVT and the mammalian V1 AVT agonist. In rainbow trout, there appears to be an increase in plasma AVT when the animals are moved to higher salinity [62]. Measurement of plasma AVT in flounder transferred from SW to FW revealed marked inhibition of AVT release compared to control fish that were transferred from SW to SW [55], indicating a possible role for AVT in SW. More directly, AVT plasma levels are correlated with plasma tonicity and rise significantly in flounder infused with NaCl [63], consistent with AVT release being stimulated by increases in plasma osmolality.

Whereas AVT has not been shown to stimulate Cl^- secretion by the opercular epithelium the cultured gill cell epithelium from sea bass (*Dicentrarchus labrax)* shows a modest increase in Cl^- secretion with 50 nM AVT [25]. Although the demonstrated effective AVT dose in vitro is much higher than measured plasma levels in vivo, it remains a possible upregulator that could restore SW level ion secretion.

2.7. Nitric oxide and endothelin

The recent observation that nitric oxide synthase co-localizes with mitochondria-rich cells in killifish opercular epithelium [24] invites consideration of this effector in regulation of Cl^- secretion. It is also possible that other agents may be acting through NO, here potentially acting in a paracrine/autocrine manner.

Big endothelin immunoreactivity is identified in mitochondria-rich cells of Atlantic stingray (*Dasyatis sabina*), suggesting presence of the peptide in gill ion transporting cells [24]. Addition of endothelin agonists to killifish opercular epithelia shows a concentration-dependent inhibition of Cl^- secretion with a threshold of 10^{-10} M [24]. There is growing evidence that NO and endothelin may interact to downregulate Cl^- secretion.

2.8. Prostaglandins and eicosanoids

The opercular epithelium and gills of killifish metabolize eicosanoids and produce prostaglandins and leukotrienes [35]. PGE_2 rapidly inhibits Cl^- secretion by the opercular epithelium [36,35], whereas several leukotrienes appear to stimulate Cl^- secretion [35]. As mentioned above, prostaglandins apparently mediate the VIP responses in gill vasculature, but thus far a prostaglandin that stimulates Cl^- secretion has not been identified, although such may exist because application of arachidonic acid alone produces a biphasic response where the first phase is stimulatory [35].

2.8.1. Mediation of Cl^- secretion stimulation

One locus for cAMP-mediated stimulation of Cl^- secretion appears to be an apically located CFTR-like anion channel. The channel was described in patch clamp experiments on killifish chloride cells to be a low conductance (8 pS), anion selective channel that is activated by cAMP [21]. The killifish CFTR gene, cloned and expressed in amphibian oocytes imparts a cAMP-activated current [22]. In excised patches from chloride cells, PKA and ATP addition activate CFTR-like channels in quiescent patches [64]. The beta adrenergic agonist isoproterenol [31,38] and forskolin [31] rapidly increase killifish opercular membrane cAMP levels accompanied by large increases in epithelial short-circuit current. It is thus clear that cAMP activates the apical membrane chloride conductance and transepithelial chloride secretion.

It is still possible for participation of NKCC activation at the basolateral membrane as well, but this has not been demonstrated in teleost preparations. Indeed, NKCC phosphorylation is a common yet complex regulatory mechanism in NKCC-containing systems (reviewed by Flatman [66]) and full activation of transepithelial transport would require activation of both components in a coordinated fashion.

In shark rectal gland, which secretes Cl^- in a manner similar to teleost chloride cells, isoproterenol activates secretion via cAMP and increases phosphorylation of NKCC threonine residues in the N-terminus [65]. This mode of NKCC activation seems widely distributed, as similar responses were observed in other NKCC secretory systems, rat parotid gland and tracheal epithelium [66]. The protein serine/threonine phosphatase (PP) PP1 and PP2A inhibitor calyculin A stimulates NKCC1 in several systems (review, Ref. [66]) and stimulates Cl^- secretion by the killifish opercular membrane [39], hence the model includes PP1 and PP2A as possible downregulators of NKCC1and activation of NKCC1 via cAMP and PKA.

Short-term activation of Na^+,K^+-ATPase and basolateral K^+ channels, could feasibly also contribute to upregulation of Cl^- secretion through enhancement of the Na^+ transmembrane gradient that drives NKCC1. Rapid activation of Na^+,K^+-ATPase, within hours of transfer to seawater, was observed in killifish gill [67,68]. Short-term activation of the enzyme in killifish gills can be as rapid 0.5 h [67] to 3.0 h [68] after transfer to seawater and the response is dependent on protein synthesis [68]. Upregulation of K^+ channels, in turn could increase turnover rate of Na^+,K^+-ATPase by enhancement of K^+ recycling across the basolateral membrane. Blockade of K^+ channels by barium [40,69] rapidly inhibits Cl^- secretion by the opercular epithelium, demonstrating the dependence on operational basolateral K^+ conductance.

2.8.2. Mediation of Cl^- transport inhibition

Much less is known regarding the downregulation of Cl^- secretion and the subsequent covering of inactive Cl^- cells by pavement cells. The calcium ionophore ionomycin

reduced Cl^- secretion with a similar time course to that of clonidine [37] but the less efficient calcium ionophore A23187 is ineffective [13,37,42], presumably because in some poikilothermic systems A23187 is not sufficiently active. The second messenger for the α_2-adrenergic response appears to be calcium, based on a blunting of the clonidine response in calcium-depleted media and the fact that clonidine did not decrease tissue levels of cAMP in killifish opercular epithelium [38]. Whereas calcium activation of Cl^- secretion is the norm for many otherwise similar systems from diverse origins, including shark rectal gland, colon, airway and avian salt gland, the effect of calcium in teleost chloride cells is inhibitory. The source of this regulatory "inversion" is unknown.

3. Hypotonic and hypertonic stress and Cl^- secretion

3.1. Hypotonic shock

Hypotonic shock and cell swelling inhibits Cl^- secretion by the killifish opercular epithelium in a dose-dependent fashion. The reduction in Cl^- secretion was approximately 60% for a 40 mOsm/kg reduction in basolateral osmolality [38]. The effect is freely reversible by reintroduction of isosmotic solutions, usually with an overshoot. Reduction in NaCl with added mannitol to maintain constant osmolality was a control and had no effect on Cl^- secretion rate, therefore the effect is purely osmotic. Hypotonic shock on the basolateral side produced the same effect as bilateral reductions in osmolality. Hypotonic shock had no effect on tissue cAMP levels and was not impeded by pretreatment of the tissue with the Ca^{2+} ionophore ionomycin or the calcium store depleting agent, thapsigargin [38]. The protein tyrosine kinase (PTK) inhibitor genistein mimicked the response and was not additive, suggesting that PTK inhibition may be involved. The inactive control molecule daidzein was ineffective by itself and did not affect the subsequent response to hypotonic shock. Taken together, these results imply that hypotonic shock inhibits Cl^- secretion in chloride cells by a mechanism involving PTK inhibition.

Hypotonic shock applied to the basolateral side of killifish opercular epithelia induces also a secondary decrease in transepithelial conductance that develops over an hour or so. Examination by SEM revealed significantly fewer exposed apical crypts in hypotonically stressed tissues and that pavement cells apparently close over chloride cells [70]. A similar reversible change in chloride cell density occurs in estuarine mudskippers, *Periophthalmus modestus* [71]. It would seem that regulation of the paracellular leak pathway, localized to leaky junctions between accessory cells and chloride cells [10] is accomplished at least in part by retraction of Cl^--secreting cells and their covering over by pavement cells that have tight intercellular junctions [10,70].

3.2. Hypertonic shock

Hypertonic shock has the opposite effect and can increase Cl^- secretion by 100% or more when NaCl or mannitol 50–100 mOsm/kg is added to the basolateral bathing medium [40]. In nature, blood plasma osmolality rises after seawater transfer to a peak at 8–16 h and the average increase for *Fundulus* is approximately 40 mOsm/kg [40]. Accompanying a smaller increase in Cl^- secretion, hypertonic stress of isolated opercular epithelia from freshwater-acclimated killifish significantly increases the density of exposed apical crypts [70], suggesting emergence of Cl^-secreting cells. The increase in Cl^- secretion in seawater-adapted opercular membranes could be blocked by basolaterally added bumetanide and by basolaterally added anion channel blockers, DPC [40], NPPB and glibenclamide [39]. Because glibenclamide blocks ATP-sensitive potassium channels in cardiac muscle of teleosts [72] it is possible that a secondary action via potassium channels in the tissue may impede the response to hypertonic shock. Curiously, large doses of amiloride (2 mM) on the basolateral side blocked the Cl^- transport stimulation by hypertonic shock [40], suggesting that the Na^+/H^+ exchange and/or intracellular pH may affect the hypertonic response.

3.3. Role of intracellular pH

Measurement of intracellular pH with cytoplasmically trapped fluorophore BCECF indicated no change in intracellular pH of chloride cells even after profound inhibition of Cl^- secretion by clonidine [37], suggesting that chloride secretion is not physiologically regulated by intracellular pH. However, pharmacological manipulation of intracellular pH has noticeable transport effects. Acidification of the cytosolic compartment in 5% CO_2 and addition of NH_4Cl to the basolateral but not the apical side of the killifish opercular membrane inhibits Cl^- secretion, apparently as a secondary effect of acidification of the cytosolic compartment by ammonium loading [40]. The recovery from this acidification is enhanced by phorbol ester (PMA) and inhibited partially by basolateral amiloride, suggesting that PKC activation may stimulate Na^+/H^+ exchange, raise intracellular pH and allow Cl^- secretion to return to normal rates. This implies intracellular pH may indirectly affect Cl^- secretion, with acidic stress being inhibitory. Meanwhile, blockage of the anion exchanger with DIDS has no effect on resting Cl^- secretion rate [21] and did not block the stimulation of Cl^- secretion by hypertonic shock [40], suggesting that Cl^-/HCO_3^- exchange is not involved in Cl^- secretion. However, 0.1 mM DIDS blocks the inhibition of Cl^- secretion in response to hypotonic shock [73], while amiloride was ineffective, again implicating the anion exchanger in mediation of the osmotic response. More direct evidence is needed to establish whether intracellular pH is involved in physiological regulation of Cl^- transport.

3.4. Cytoskeleton and transporters

Hypotonic and hypertonic shock may be mediated via the cytoskeleton in stretch-activated or -inactivated membrane elements. The possible connection of the CFTR-type anion channel and actin cytoskeleton arose from an association of cAMP-dependent phosphorylation by PKA blocked by the actin disrupter cytochalasin D [74]. The F-actin stabilizing agent phalloidin also inhibits generation of sustained Cl^- current in colonic T84 cells [75]. Partial disruption of F-actin activates, while more complete disruption inhibits CFTR function and renders CFTR refractory to cAMP/PKA activation [76]. Now it is known that CFTR is a nucleus for developing F-actin fibers, observed by confocal microscopy, and that a direct connection between the channel protein and actin exists [77]. Endocytic recycling and trafficking of CFTR seems to involve Golgi PDZ binding proteins [78] and the well-known PDZ binding site [79] on the carboxy terminus (-TRL) that is common to human and killifish [22] CFTR proteins. However, rapid inhibition of CFTR involves syntaxin 1A expressed in *Xenopus* oocytes [80] or in airway epithelia [81]. Current thinking has expanded to include syntaxin 1A/SNARE complex [82] or syntaxin 1A/SNAP-23 complex bound to the amino terminus of CFTR [83] where syntaxin 1A binds to both CFTR and the SNAP/SNARE proteins [82–84]. In this complex, binding of syntaxin 1A directly decreases channel open probability [84]. Meanwhile, activation of CFTR can occur via a complex at the carboxy terminus with the β-adrenoceptor, and ezrin/radixin/moesin binding phosphoprotein regulated by PKA [85]. It is tempting to extrapolate to the physiological downregulation of Cl^- secretion in teleost chloride cells by syntaxin 1A. Clearly, a demonstration of syntaxin 1A localization to chloride cells and binding to kfCFTR would be a first step to establishing this relationship. Important also is the identification by Kültz et al. [86] of a 14–3–3 gene in killifish that encodes for 14–3–3, a protein that is known to regulate ion channels, transporters and cytoskeleton for cells in changing environments. Protein 14–3–3.a is also upregulated in killifish gills on transfer of killifish from seawater to freshwater [86], implying the involvement of this protein in long-term freshwater adaptation. Finally, transfer of killifish to freshwater also activates three identified MAP-kinases: ERK1, SAPK1 (=Jun N-terminal kinase, JNK) and SAPK2 (=p38) kinase, as all three show enhanced phosphorylation by immunoblotting with antibodies specific for the phosphorylated form of the enzymes [87]. Thus, osmosensing in gill cells initiates MAP kinase transduced changes in cell physiology.

Whereas mammalian epithelial cells will not normally experience changes in basolateral osmolality of the magnitudes tested in cell volume research, gill epithelial cells of estuarine fish actually are osmotically stressed when these animals change salinity. Hence, the teleost epithelial cell responses could be more sensitive and amplified than in mammals, therefore easier to study.

3.5. Role of PKC isozymes

The association of PKCα and PKCε with actin cytoskeleton is demonstrated by the elegant work of Song et al. [88] where the PKC nonspecific agonist (PMA) in T84 epithelial cells was shown to activate first PKCα in actin disruption and stimulation of basolateral endocytosis, followed by a late effect involving activation of PKCα actin stabilization and inhibition of endocytosis. The isozymes of PKC that may be involved in Cl^- transport regulation in teleosts are unknown. Clearly, the actin–PKC relationship should be clarified for the teleost system before conclusive relationships between PKC, F-actin and intracellular Ca^{2+} can be realized. A parallel between the teleost system and T84 cells is the inhibitory phase of Cl^- transport regulation by acetylcholine in killifish [31,44] and in T84 cells [88]. The selective activation of PKCε by carbachol and the

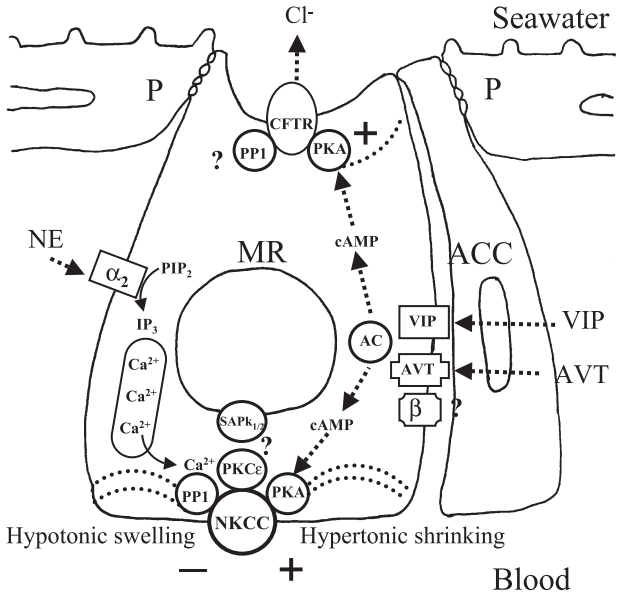

Fig. 4. Hypothetical model of rapid control of Cl^- secretion by seawater type mitochondria-rich cells of marine and estuarine teleosts. The basolateral NKCC (Na^+,K^+,2Cl^- cotransport, secretory type, NKCC1) may be activated by cell shrinkage linked via actin (beaded lines) to PKA or by cAMP that is in turn derived from VIP or AVT acting via their respective receptors in the basolateral membrane. NKCC may be downregulated by dephosphorylation by a protein phosphatase (PP1 or 2A) activated via actin and cell swelling. Norepinephrine (NE), derived from subjacent autonomic nerves, act via α_2-adrenergic receptors that are mediated through inositol tris phosphate (IP_3) from phosphoinositolbisphosphate (PIP_2). The IP_3 releases intracellular calcium that may activate a calcium-sensitive protein kinase C isoform (PKCε) that can inhibit NKCC indirectly by activating the PP1 (or 2A). This would account for the unusual inhibition of Cl^- secretion seen with calcium stimuli, whereas most other Cl^--secreting systems are activated by calcium. Hypotonic shock also activates MAP kinases, including stress-activated protein kinase (SAPK1) and SAPK2 and extracellular signal-regulated protein kinase (ERK1, not shown) but their role in transport regulation is unknown. At the apical membrane, CFTR anion channels are activated by cAMP via PKA and by hypertonic shock and cell shrinkage likely via actin downregulation of CFTR channels may be via a protein phosphatase. For details see text.

stimulation of endocytosis in the late inhibitory phase [88] invites speculation that the inhibitory action of acetylcholine and α-adrenoceptor agonists may be activation of a PKCε and endocytosis or dephosphorylation of NKCC1. Further, PKCα and PKCε are differentially translocated to the membrane fraction of cells subjected to hypotonic shock [89], implying that hypotonic shock effects could also be mediated via PKCε.

4. Nongenomic effect of cortisol

One possibility that has not been investigated for the gill and opercular epithelium is that of nongenomic responses to corticosteroids. There are well-established inhibitory roles for cortisol (distinct from other steroids) at physiological levels to depress prolactin release by cultured cells from the rostral pars distalis of tilapia [90]. These effects are not mediated by gene transcription or protein synthesis. The effects develop rapidly, in 10–20 min, and are reversible by removal of the hormone. Similar to somatostatin actions on prolactin cells, intracellular Ca^{2+} is decreased and adenylate cyclase and cAMP are reduced by cortisol [90]. Interestingly, the intracellular Ca^{2+} elevation produced by hypotonic shock in prolactin cells is reversed by 200 nM cortisol. Given that cortisol may be elevated in fish during a salinity change, the possible downregulation by decrease in adenylate cyclase or reduction of Ca^{2+}-mediated hypotonic response could either aid or exacerbate (respectively) ion regulation on entry into freshwater.

5. Proposed model for rapid regulation of chloride transport

Some of the mechanisms that enter into the hypothetical model here (Fig. 4) have been established in mammalian systems and appear with question marks and some others have been demonstrated to be present in teleosts. Killifish CFTR, because it shares the carboxy terminus PDZ binding domain in common with the human, may share some aspects of PDZ binding and regulation. All vertebrate CFTR proteins share the first two amino acids at the amino terminus and killifish shares the first six of ten with the human, suggesting the syntaxin 1A binding is also feasible. Whereas intracellular Ca^{2+} is normally associated with activation of Cl^- secretion, teleost Cl^- secreting gill and gill-like systems all have Ca^{2+}-mediated inhibition. The point of inversion in the regulatory pathway could be activation of a phosphatase such as Ca^{2+}-activated protein phosphatase (PP2B, calcineurin) but the PP2B antagonist FK506 is not effective in blocking clonidine action in the killifish opercular epithelium (Marshall, unpublished). Another possibility is the activation of a different PKC isozyme, such as PKCε that is linked to downregulation of membrane transport.

Thus, much needs to be done to establish the cellular mechanisms of Cl^- transport regulation, especially with regard to the phosphorylation/dephosphorylation of the key transport proteins, CFTR and NKCC1. Also, to establish that the voluntary salinity changes (as opposed to the obligatory transfers used in the past) are indeed nonstressful, salinity preference experiments and measures of stress-related variables such as plasma catecholamine and cortisol levels will be necessary.

The coping strategy of estuarine fish is hypothetical at present but it might operate in the following fashion. A possible scenario, one that includes major parts of the regulatory model discussed above, would start with entry of the fish to dilute FW at the edge of an advancing tide for the purpose of optimal foraging. Within minutes, reflex catecholamine release from the autonomic system would rapidly inhibit Cl^- secretion via α-adrenoceptors, hence conserving NaCl. Within 1–3 h, plasma hyposmolality would develop, a result of passive NaCl loss, in turn producing sustained inhibition of Cl^- secretion and retraction of Cl^- cells, thus reducing active and passive NaCl loss. Active foraging during this time would supplement body NaCl by intestinal absorption. As the tide turns to ebb, the fish retreat to deeper, higher saline microenvironments where stimulation from peptide hormone (AVT or urotensin I), neurotransmitter (VIP) and rising plasma osmolality would reinitiate NaCl secretion by emerging Cl^- secreting cells. In this way, the animal need not invoke permanent adaptation strategies, rather rapid acting, short-lived devices to cope with changing salinity.

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