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HORMONAL CONTROL OF GILL Na^+,K^+-ATPase AND CHLORIDE CELL FUNCTION

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I. INTRODUCTION

The secretion of excess sodium and chloride by teleosts in seawater is carried out by gill chloride cells. These highly specialized cells are characterized by a large, columnar appearance, numerous mitochondria, an extensive tubular system, an apical crypt, and mucosal-serosal exposure (see reviews by Evans *et al.*, 1982; Zadunaisky, 1984; Pisam and Rombourg, 1991). The tubular system is continuous with the basolateral membrane, resulting in a large surface area for the placement of transport

proteins. Perhaps the most important of these proteins is Na^+,K^+-ATPase. Also known as the sodium pump, Na^+,K^+-ATPase plays a central role in the salt-secretory function of chloride cells. Present in all animal cells, this energy-dependent, ion-translocating enzyme occurs in high concentrations in most transport epithelia; up to 10^8 sodium pumps may be present in a single chloride cell (Karnaky, 1986)! Na^+,K^+-ATPase participates in ion transport either directly through movement of sodium and potassium across the plasma membrane or indirectly through generation of ionic and electrical gradients.

Regulation of chloride cells and Na^+,K^+-ATPase is critical during movement of fish between fresh water and seawater and within estuaries, and is also important in stenohaline fish under several variable environmental conditions. Since the neuroendocrine system is the primary link between a changing environment and physiological adaptation, the hormonal control of chloride cells and Na^+,K^+-ATPase is critical to euryhalinity. Reviews in this general area have appeared previously (Foskett *et al.*, 1983; Mayer-Gostan *et al.*, 1987) though not recently, and this review will focus primarily on new approaches and recent evidence on the hormonal control of Na^+,K^+-ATPase and chloride cells in teleosts.

II. Na^+,K^+-ATPase AND CHLORIDE CELL FUNCTION

A. Seawater

In seawater the osmoregulatory function of the gill is to secrete excess monovalent ions. Although there are several models for ion movement across the gill (Payan *et al.*, 1984), the model proposed by Silva *et al.* (1977) has the most experimental support (Evans *et al.*, 1982). Na^+,K^+-ATPase, located on the basolateral membrane, creates low intracellular Na^+ and a highly negative charge within the cell. The Na^+ gradient is then used to transport Cl^- into the cell through a $Na^+/K^+/Cl^-$ cotransporter, then Cl^- leaves the cell "downhill" on an electrical gradient through an apical Cl^- channel. Na^+ is transported through a paracellular pathway down its electrical gradient (seawater being more negative than plasma).

In this model, Na^+,K^+-ATPase generates ionic and electrical gradients for use in excretion of Na^+ and Cl^-. Epstein *et al.* (1967) were the first to suggest a role for Na^+,K^+-ATPase in the salt excretory function of the gill, based on their observation of higher levels of gill Na^+,K^+-ATPase activity in seawater-adapted killifish (*Fundulus heteroclitus*). Ouabain, a

specific inhibitor of Na^+,K^+-ATPase that binds to the potassium-binding site, strongly inhibits Cl^- excretion and short-circuit current (Silva *et al.*, 1977; Karnaky *et al.*, 1977). The basal location of Na^+,K^+-ATPase was inferred from the greater efficacy of injected ouabain compared to external ouabain exposure (Silva *et al.*, 1977). Cell fractions of dispersed gill tissue that are rich in chloride cells have much higher Na^+,K^+-ATPase activity than other cell fractions (Kamiya, 1972; Sargent *et al.*, 1975). Karnaky *et al.* (1976) were able to visualize high concentration of Na^+,K^+-ATPase in chloride cells through the use of [^{3}H]ouabain autoradiography (Fig. 1). These results indicated that the sodium pump was not located on the apical surface, but rather throughout the cell on the extensive tubular system that is continuous with the basolateral membrane.

The model of Silva *et al.* (1977) offers several predictions of the biochemical properties of chloride cells that have yet to be verified. For example, a bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransporter should be located on the basolateral membrane of chloride cells, and an apical Cl^- channel should be present on the apical membrane of chloride cells. Molecular methods, along with more traditional immunological and pharmacological approaches, should allow exploration of these predictions.

B. Fresh Water

Our current understanding of chloride uptake by the gills in fresh water emphasize Cl^-–HCO_3^- exchange at the apical surface (Avella and Bornancin, 1990). Sodium uptake is accomplished either by apical Na^+–H^+/NH_4^+ exchange or by an apical Na^+ channel that is supported by an electrogenic, apical H^+-ATPase (see Lin and Randall, Chapter 9, this volume). In this model, gill Na^+,K^+-ATPase transports excess intracellular sodium into the blood, and may also provide an electrical gradient for apical transport of sodium and basolateral transport of chloride. However, the relative importance of Na^+,K^+-ATPase in sodium and chloride uptake is unclear. Ouabain has been reported to decrease ion uptake (Epstein *et al.*, 1967) or to have no effect (Kerstetter and Keeler, 1976) in rainbow trout (*Oncorhynchus gairdneri*). Increases in Na^+,K^+-ATPase in a few teleosts upon transfer from seawater to fresh water (discussed in the following) support a role for Na^+,K^+-ATPase in ion uptake. The role of the sodium pump in ion uptake is less certain than its role in ion excretion, and further studies that take account of potential differences among species are needed.

The site of gill ion uptake in teleosts is also uncertain. Foskett and Scheffey (1982) provided direct evidence for chloride cells as the site of chloride excretion in seawater teleosts, but similar direct evidence for

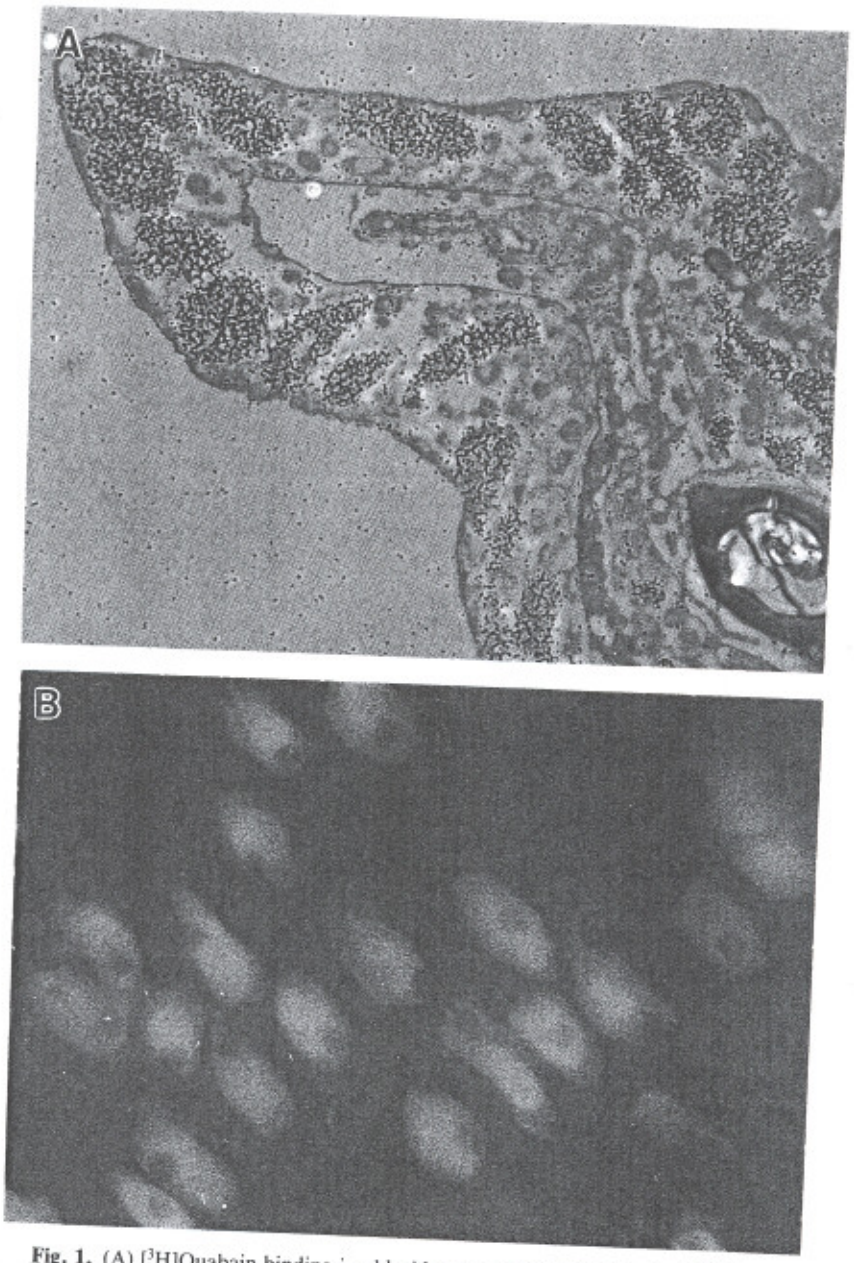


Fig. 1. (A) [^{3}H]Ouabain binding in chloride cells of seawater-adapted killifish. [Reproduced from the *Journal of Cell Biology*, Karnaky *et al.* (1976) **70,** pp. 157–177, by copyright permission of the Rockefeller University Press.] (B) Fluorescent staining of chloride cells in the jawskin of the long-jawed mudsucker following exposure to anthroylouabain. [Reproduced from McCormick (1990a) with permission from Springer-Verlag.] In both preparations, note the high concentration of ouabain binding throughout the cells (but not in the nucleus), indicative of its placement on the extensive tubular system of chloride cells.

chloride cells as the site of ion uptake is lacking. Proliferation in response to low ion content in fresh water is evidence for the role of chloride cells in ion uptake (Laurent and Dunel, 1980; Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989), but there is also evidence for involvement of other cells in the gill (see Goss *et al.*, Chapter 10, this volume). Although variation occurs among species, the appearance of chloride cells in fresh water is distinct from that of the same species in seawater (Pisam *et al.*, 1987; Laurent and Perry, 1991). In fresh water, chloride cells are generally smaller and less columnar, have a less pronounced tubular system, and often do not have apical and serosal contacts. Accessory cells, which interdigitate with seawater chloride cells to form "leaky" junctions for passive paracellular sodium efflux, are absent in fresh water (Laurent and Perry, 1991).

III. PROPERTIES OF Na^+,K^+-ATPase

A great deal is known about the structure and biochemistry of Na^+,K^+-ATPase, which is present in all animal cells (see reviews by De Renzis and Bornancin, 1984; Rossier *et al.* 1989; Skou and Esmann, 1992). The protein complex consists of a catalytic α-subunit and a β-subunit; the latter stabilizes folding of the α-subunit, confers K^+ affinities, and is involved in cell–cell interactions (Schmalzing and Gloor, 1994). Both subunits of Na^+,K^+-ATPase have been sequenced from one fish species to date, the Pacific electric ray (*Torpedo californica*), which contains an electric organ rich in Na^+,K^+-ATPase. The α-subunit consists of 1022 amino acids and has a calculated molecular mass of 112,000 and six to eight membrane-spanning domains (Kawakami *et al.*, 1985). The β-subunit is a 35-kDa glycoprotein of 300 amino acids with a single membrane-spanning domain (Noguchi *et al.*, 1986). Its amino acid sequence is less highly conserved among vertebrates (60%) than the α-subunit (Rossier *et al.*, 1989). The α-subunit has also been fully sequenced in one teleost, the white sucker (*Catostomus commersoni*), and consists of 1027 amino acids and a predicted eight membrane-spanning domains (Schörock *et al.*, 1991). There is substantial sequence similarity with other vertebrate Na^+,K^+-ATPase α-subunits (74–98%). A partial sequence (450 base pairs) of the gene encoding the rainbow trout α-subunit also shows high sequence similarity (Kisen *et al.*, 1994). Three isoforms of the α-subunit and four isoforms of the β-subunit have been isolated in mammals; these isoforms are present in varying proportions in different organs (Skou and Esmann, 1992).

The stoichiometry of Na^+,K^+-ATPase appears to be similar in all

animals investigated (Skou and Esmann, 1992). Three internal sodium ions are transported outward in exchange for two potassium ions. The sodium pump is therefore electrogenic, creating a potential difference across the cell membrane. Each translocation of ions requires the hydrolysis of ATP and is accompanied by a conformational change. Binding sites for ions, ATP, and inhibitors are all contained on the α-subunit.

IV. METHODS

A. Quantitation and Localization of Na^+,K^+-ATPase

Measurement of Na^+,K^+-ATPase is most frequently accomplished by measurement of V_{max} in a microsomal preparation or crude homogenate and expressed as activity per unit wet weight or protein content. The breakdown of ATP is detected (most often by measuring inorganic phosphate or ADP) in the presence and absence of ouabain. Such biochemical measurements are, by their nature, method dependent. Efforts should be made to optimize the conditions for expression of enzyme activity, including tissue preparation, ion concentrations, pH, ouabain, detergent, and temperature. Several methods specific for measurement of gill Na^+,K^+-ATPase activity of teleosts have been presented (Johnson *et al.*, 1977; Zaugg, 1982; Mayer-Gostan and Lemaire, 1991), including a "microassay" suitable for nonlethal biopsies and organ cultured tissues (McCormick, 1993). These biochemical assays are an approximation of the total number of sodium pumps and rapid activation or deactivation (e.g., phosphorylation) may not be detected by these methods.

[^{3}H]Ouabain binding to gill homogenates and whole tissue has also been used to measure the number of sodium pumps. In all cases examined to date, gill ouabain binding and Na^+,K^+-ATPase activity are strongly correlated (Sargent and Thomson, 1974; Stagg and Shuttleworth, 1982). B_{max} values for teleost gills range from 3 to 20 pmol/mg dry weight in the three species examined (Hossler *et al.*, 1979; Stagg and Shuttleworth, 1982; McCormick and Bern, 1989). Since chloride cells usually represent less than 10% of the gill, the sodium pump content of individual chloride cells is much higher (Karnaky, 1986). Hootman and Ernst (1988) outline a method for using [^{3}H]ouabain to measure turnover (*in situ* activity) and sodium pump numbers in intact cells.

Autoradiographic methods using [^{3}H]ouabain have localized Na^+,K^+-ATPase to chloride cells and provided information on their intracellular location (Fig. 1). A fluorescent ouabain analog, anthroylouabain, has been recently used to examine Na^+,K^+-ATPase-rich chloride cells in the oper-

cular membrane (Fig. 1). This probe has the advantage of ouabain specificity and increased fluorescence upon binding (Fortes, 1977). A major disadvantage is the high autofluorescence in the wavelengths necessary to view anthracene. Nonetheless, in tilapia this stain distinguishes the freshwater from the seawater form of the chloride cell. Histochemical methods have also been used to localize Na^+,K^+-ATPase in fish gills and are reviewed in De Renzis and Bornancin (1984).

Molecular methods have recently been applied to the measurement of Na^+,K^+-ATPase gene expression. Using a 450-base pair fragment of the α-subunit gene, Northern blot analysis was used by Kisen *et al.* (1994) to detect a prominent mRNA of 3.7 kilobases in rainbow trout gill. This probe was found to be useful for measuring gill Na^+,K^+-ATPase gene expression in several other teleosts. Northern blots can also be used to measure gill Na^+,K^+-ATPase mRNA in brown trout (*Salmo trutta*) using cDNA of the *Xenopus* α-subunit (S. Madsen, personal communication). These studies revealed that gill Na^+,K^+-ATPase α-subunit mRNA levels are higher in fish in seawater than in fish in fresh water; increases occur within 1 day of transfer to seawater in brown trout.

B. Morphology and Function of Chloride Cells

The distinguishing characteristics of chloride cells, especially numerous mitochondria and an extensive tubular system, permit their identification from other cells in the gill and opercular membrane at the light microscope level. The vital mitochondrial stain DASPEI (2-*p*-dimethylaminostyryl-ethylpyridiniumiodide) has been used widely on opercular membranes (Karnaky *et al.*, 1984). Such vital stains are rarely used on the gill because of tissue thickness and complex structure, although gill dispersions are possible (Perry and Walsh, 1989). Champy-Maillet's fixative (a 0.2% osmic acid with saturated zinc powder and 25% mg/ml iodine) stains plasma membranes and the extensive tubular system, rendering the entire chloride cell black (e.g., Avella *et al.*, 1987). Great care must be taken in the examination of chloride cells using postfixation staining methods such as hematoxylin and eosin, as positive identification of chloride cells can be problematic. Surprisingly, immunohistochemistry has not been widely used in studies of chloride cells. Currently available methods for protein isolation should permit production of antibodies specific for teleost Na^+,K^+-ATPase, transport proteins, or mitochondrial enzymes.

A variety of methods can be used to probe chloride cell function. Methods for direct and indirect measurement of ion movements have been widely used and are reviewed in detail elsewhere in this volume (see Marshall, Chapter 1). Methods for examining chloride cell function in skin

and opercular membranes, particularly the use of Ussing chambers to examine ion transport and electrophysiology, have been reviewed (McCormick, 1994). Although the vibrating probe has been used successfully to localize chloride secretion to chloride cells (Foskett and Scheffey, 1982), it has not yet been used to examine regulation of chloride cell function. Perhaps the most powerful new tools for exploring chloride cell physiology are ion-sensitive fluorescent dyes. These have already proven useful in basic studies of ion transport (see Chapters 1 and 8, this volume), and should be equally useful in regulatory (endocrine) studies of ion uptake and secretion. To date, these methods have been used only with chloride cells in the opercular membrane, though confocal laser microscopy may permit their use in gill tissue.

C. Organ and Cell Culture

In vitro methods are useful for determining the direct action of hormones and other agents on function of cells and tissues. Recent advances in gill respiratory cell culture (see Pärt and Bergstrom, Chapter 8, this volume) and rectal gland cell culture for elasmobranchs (see Valentich *et al.*, Chapter 7, this volume) illustrate the contributions that such methods can make to our understanding of transport physiology. Unfortunately, a method for long-term culture of chloride cells is not yet available. Isolated chloride cells have relatively poor viability in culture using methods suitable for other gill cells (Peter Pärt, personal communication). It is unclear whether chloride cell preparations would possess the transport capabilities of intact tissue, particularly if accessory cells are required for ion secretion. Nonetheless, chloride cell cultures would be very useful, if only to examine regulation of transport proteins such as Na^+,K^+-ATPase.

Long-term organ cultures of primary gill filaments and opercular membranes have been used in several studies of the hormonal regulation of Na^+,K^+-ATPase (McCormick and Bern, 1989; McCormick, 1990b; McCormick *et al.*, 1991a; Madsen and Bern, 1993; see McCormick, 1994, for detailed methods). Though more difficult to prepare, the opercular membrane culture has the advantage of being more accessible than gills for morphological and transport studies (e.g., Ussing chamber or ion-sensitive dye) following hormone exposure. In preparing culture media, physiological levels of pH, ions, and gases appropriate for the species being studied should be used. In many cases this means only a slight modification of existing commercial culture media. Minimal Essential Medium (MEM) has been widely used and apparently supports a variety of teleost cell types equally or better than media with more additives (Fernandez *et al.*, 1993). Gassing for physiological ranges of pH (7.7–8.0),

pCO_2 (1–4 mm Hg), and HCO_3^- (3–10 m*M*) (Heisler, 1993) can theoretically be achieved by using 0.3–0.5% CO_2 gas. One percent CO_2 is commercially available and has been used successfully in gill organ culture (S. D. McCormick, unpublished). Saturation with 5% CO_2, widely used in fish cell and organ culture, results in supraphysiological levels (for teleosts) of CO_2 and HCO_3^-, though this is apparently not detrimental to growth of cells or maintenance of tissue in culture (McCormick, 1990b; Fernandez *et al.*, 1993). The health and responsiveness of cells in culture are the most important criteria, and these can be judged through vital stains, histological appearance, and biochemical analyses.

V. ENVIRONMENTAL AND DEVELOPMENTAL REGULATION

An excellent review has been written on the environmental influences on chloride cells (Laurent and Perry, 1991), though a similar overview on gill Na^+,K^+-ATPase is lacking. Since space limitations preclude an exhaustive review, only heuristic and recent research will be presented here.

The most widely recognized and investigated environmental determinant of gill Na^+,K^+-ATPase and chloride cell development is salinity. For almost all teleosts examined to date, which includes several dozen species, increasing salinity results in higher levels of gill or opercular Na^+,K^+-ATPase activity (Kirschner, 1980; De Renzis and Bornancin, 1984). This is true both for comparisons within species and, generally, when comparing freshwater and marine forms. Despite this generalization, it is enlightening to examine the exceptions. No difference in gill Na^+,K^+-ATPase activity was found between freshwater- and seawater-adapted flounder (*Platichthys flesus;* Stagg and Shuttleworth, 1982) and striped bass (*Morone saxatilis;* Madsen *et al.*, 1994). Gill Na^+,K^+-ATPase activity has been found to be higher in fresh water in sea bass (*Dicentrarchus labrax*), thick-lipped mullet (*Chelon labrosus*), and Australian bass (*Macquaria novemaculeata;* Lasserre, 1971; Langdon, 1987). These fish are generally considered to be of marine origin. One possible explanation, which lacks experimental support, is that these fish maintain higher gill permeability and ion fluxes than other fish in fresh water and therefore require higher levels of gill Na^+,K^+-ATPase.

Exposure of fish to low ion content in fresh water results in a dramatic increase in chloride cell density, particularly on the secondary lamellae, which is presumably involved in ion uptake. Surprisingly, we know little of the impact of ion-poor water on gill Na^+,K^+-ATPase. In Atlantic salmon

(*Salmo salar*), gill Na^+,K^+-ATPase activity does not increase in ion-poor water (S. D. McCormick, unpublished).

Gill Na^+,K^+-ATPase activity generally increases following acclimation to low temperature, but there is contradictory evidence (even in the same species) that makes interpretations difficult (see De Renzis and Bornancin, 1984). In addition to methodological considerations, interpretation of temperature effects is made even more difficult by potential interaction of physiological demands on the gills, such as gas exchange and ion transport.

Although moderate changes in pressure have no effect on gill Na^+,K^+-ATPase activity *in vitro,* changes in pressure that might be experienced in the deep ocean result in rapid deactivation of gill Na^+,K^+-ATPase (Gibbs and Somero, 1989). This effect of pressure, which is presumably the result of changes in membrane fluidity and the structure of the enzyme, is less pronounced in fish living at greater depths. Deep-living species generally have lower gill Na^+,K^+-ATPase activity, which may reflect the lower activity and metabolic rate of these species (Gibbs and Somero, 1990).

Diet and nutrition may also play a role in regulation of chloride cell function. Food restriction in tilapia reduces chloride cell number and gill Na^+,K^+-ATPase activity, but this apparently has no effect on salinity tolerance (Kultz and Jurss, 1991). Diets high in NaCl can increase gill Na^+,K^+-ATPase activity and chloride cell numbers in rainbow trout, but this is not a universal finding among teleosts (Salman and Eddy, 1987).

Size has a potential but largely unexamined influence on chloride cell development. Gibbs and Somero (1990) found that total gill Na^+,K^+-ATPase (activity per fish) was related to size; this result was largely related to allometric growth of the gill rather than to changes within the gill. There is great variation of the scaling coefficient among species, indicating that gill Na^+,K^+-ATPase may increase or decrease with increasing size. No size relationship between protein-specific gill Na^+,K^+-ATPase activity and size was found in brook trout (*Salvelinus fontinalis*) weighing from 2 to 380 g (McCormick and Naiman, 1984a). Chloride cells on skin and yolk sac of small embryonic and larval fishes, present prior to gill development, may be important sites for ion exchange in fresh water and seawater (Ayson *et al.,* 1994).

Developmental events play a crucial role in regulating chloride cells in some diadromous fishes. In both downstream-migrating juvenile salmonids (known as smolts) and maturing "silver" European eels (*Anguilla anguilla*), chloride cell numbers and gill Na^+,K^+-ATPase activity increase prior to exposure to seawater (Zaugg and McLain, 1970; Thomson and Sargent, 1977; see reviews by McCormick and Saunders, 1987; Hoar, 1988). These preparatory adaptations result in increased salinity tolerance

of the migrants. In anadromous salmonids, this developmental event is size dependent and cued by increasing daylength (Saunders and Henderson, 1970; Duston and Saunders, 1990). Some of the endocrine factors discussed in Section VI are likely to be responsive not only to salinity but also to environmental and developmental influences.

VI. HORMONAL REGULATION

A. Cortisol

Cortisol was the first hormone shown to stimulate gill Na^+,K^+-ATPase activity (Pickford *et al.*, 1970b); following this work with killifish, American eel (*Anguilla rostrata*), tilapia (*Oreochromis mossambicus*), and several salmonids have been shown to respond similarly (Epstein *et al.*, 1971; Richman and Zaugg, 1987; Björnsson *et al.*, 1987; Dange, 1986; Madsen, 1990c; Bisbal and Specker, 1991). Stenohaline fish have not been sufficiently examined, and it would be of some interest to determine if stenohalinity is in part due to an inability to respond to cortisol (or other hormones). Hypophysectomy reduces gill Na^+,K^+-ATPase activity in American eel, killifish, and coho salmon (*Oncorhynchus kisutch*), which can be at least partially restored by cortisol treatment (Pickford *et al.*, 1970b; Butler and Carmichael, 1972; Björnsson *et al.*, 1987; Richman and Zaugg, 1987). This effect is presumably through the loss of pituitary ACTH (a cortisol secretagogue), though other pituitary hormones are involved in regulating circulating cortisol and gill Na^+,K^+-ATPase (see the following).

Cortisol treatment also affects the morphology and development of chloride cells (Fig. 2). Exogenous cortisol causes increased chloride cell numbers in American eel, tilapia, brown trout (*Salmo trutta*), rainbow trout, and coho salmon (Foskett *et al.*, 1981; Madsen, 1990a,c; Doyle and Epstein, 1993; Richman and Zaugg, 1987). Chloride cell size is less often measured, but was found to increase following cortisol treatment in brown trout (Madsen, 1990c). Doyle and Epstein (1972) reported increased development of the tubular system in chloride cells of the American eel after cortisol injection. As might be expected from these effects on gill Na^+,K^+-ATPase activity and chloride cells, cortisol treatment also increases salinity tolerance in eel, brown trout, and Atlantic salmon (Epstein *et al.*, 1971; Madsen, 1990c; Bisbal and Specker, 1991).

In vitro stimulation of gill and opercular membrane Na^+,K^+-ATPase by cortisol has been demonstrated in coho salmon and tilapia, respectively (Fig. 3; McCormick and Bern, 1989; McCormick, 1990b), indicating its direct effect on these tissues. Using anthroylouabain, increased gill

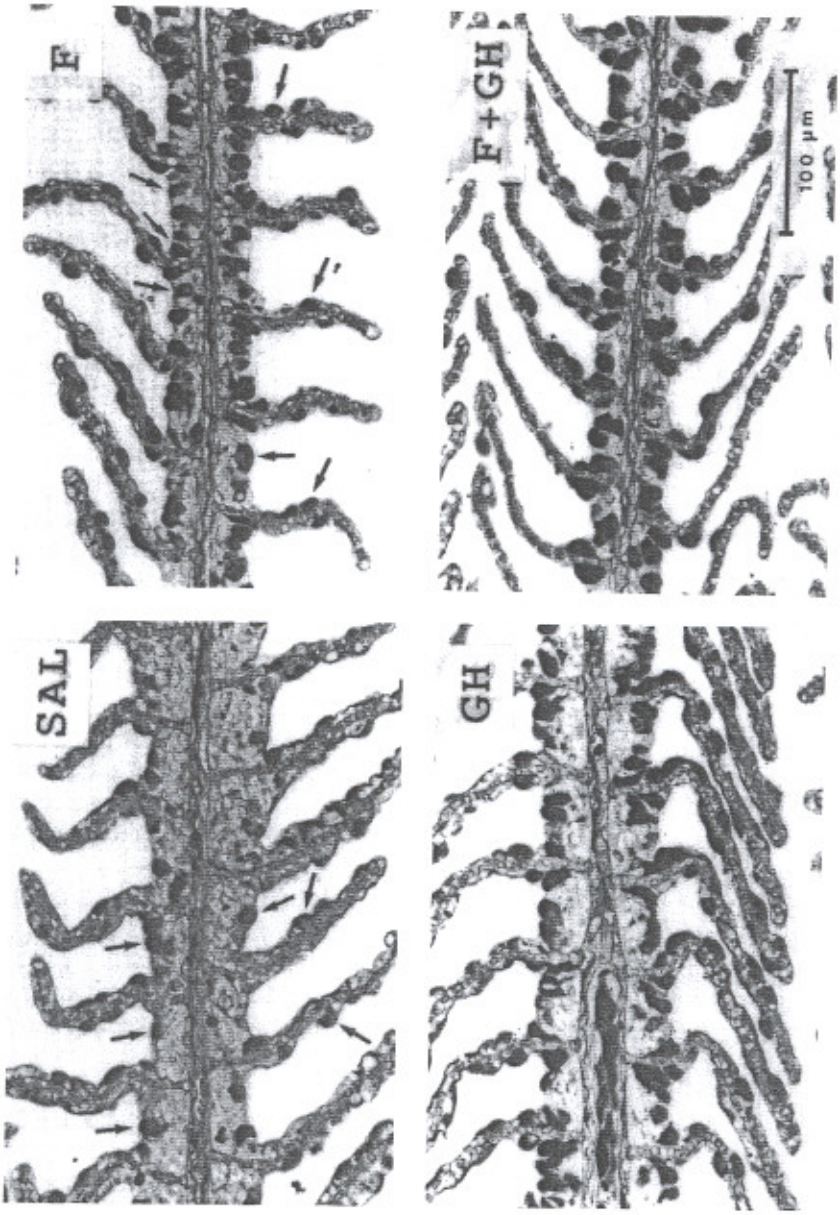


Fig. 2. The effect of cortisol (F) and growth hormone (GH) treatments on gill chloride cells of brown trout (SAL = saline-injected). Tissue was fixed in Champy-Maillet's fixative, which stains the extensive tubular system of chloride cells. Note that cortisol and growth hormone each increase cell number and cell size. When injected together, cell size is increased slightly (but not significantly) over treatment with either hormone alone. In the same experiment, cortisol and growth hormone acted additively to increase gill Na^+,K^+-ATPase activity. [Reproduced from Madsen (1990c) with permission from Academic Press.]

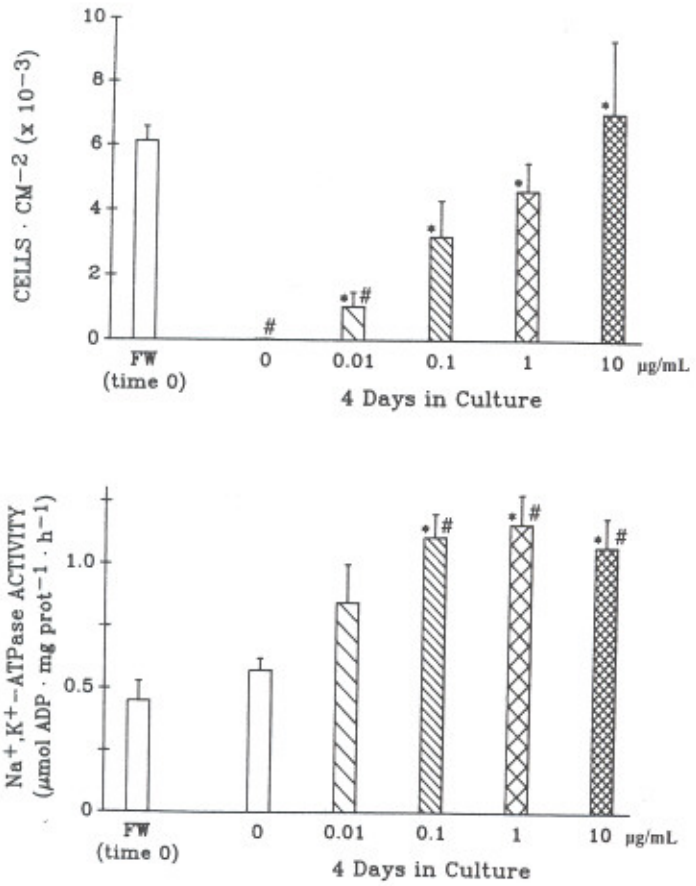


Fig. 3. Effect of cortisol *in vitro* on chloride cell number and Na^+,K^+-ATPase activity in opercular membrane of freshwater-adapted tilapia. Upper: In the absence of cortisol, chloride cell numbers decrease dramatically, and are increased in a dose-dependent manner by cortisol. Lower: Cortisol increases Na^+,K^+-ATPase activity to levels higher than seen initially. [*, indicates significant difference from control; #, indicates significant difference from initial (Time 0).] Cell height and the number of cells spanning the opercular membrane are also increased. [Reproduced from McCormick (1990b) with permission from The American Physiological Society.]

Na^+,K^+-ATPase was shown to be primarily a response of chloride cells. Cortisol also increased cell height and the proportion of chloride cells spanning the opercular membrane, but chloride cell numbers did not increase. From this evidence it was concluded that cortisol directly causes differentiation of the seawater form of the chloride cell. These *in vitro* findings contrast with the *in vivo* effect of cortisol in tilapia, where chloride cell density increases, but chloride cell size does not (Foskett *et al.*, 1981).

These differences suggest that other endocrine factors, stimulated by or acting with cortisol *in vivo*, are involved in chloride cell proliferation in tilapia. Electrophysiological properties of the opercular membrane are also not affected by cortisol *in vivo* (Foskett *et al.*, 1981), indicating that other hormones (or salinity itself) are required for activating sodium and chloride secretion.

Developmental differences in the responsiveness of gill Na^+,K^+-ATPase activity to cortisol *in vivo* and *in vitro* have been found in coho and Atlantic salmon (McCormick *et al.*, 1991a). Maximum responsiveness occurs when normal increases in gill Na^+,K^+-ATPase activity begin in early spring. As in the parr–smolt transformation itself, cortisol responsiveness can be altered by photoperiod. Changes in responsiveness may be due in large part to changes in cortisol receptors, which have been found in gill tissue of several euryhaline species (Sandor *et al.*, 1984; Chakraborti *et al.*, 1987; Maule and Schreck, 1990). In coho salmon and anadromous rainbow trout, gill cytosolic cortisol receptors are most numerous from January to March (Shrimpton *et al.*, 1994; McLeese *et al.*, 1994), the time of maximum *in vitro* responsiveness. Cortisol receptors may also be one avenue by which growth hormone interacts with cortisol (see the following). Weisbart *et al.* (1987) found that cytosolic cortisol receptors decrease and nuclear receptors increase following exposure of brook trout to seawater. This response is similar to that seen following injection of cortisol, suggesting that translocation of the cortisol receptor to the nucleus is involved in seawater acclimation. However, the location of cortisol receptors in gill tissue and their importance in chloride cell differentiation and/or proliferation have yet to be established. Corticoid receptor antagonists such as RU-486 (Baulieu, 1989) may prove to be useful in this regard.

Reported inabilities of cortisol treatment to stimulate gill Na^+,K^+-ATPase activity in salmonids and tilapia (Langdon *et al.*, 1984; Hegab and Hanke, 1984; Redding *et al.*, 1984) may in part relate to developmental differences in responsiveness to cortisol. The time course and method of administration of cortisol may also alter its effectiveness. Specker *et al.* (1994) have developed a vegetable oil-based implant that results in prolonged elevation of physiological levels of cortisol.

Often referred to as a "seawater-adapting hormone," it is interesting to find that cortisol may be more than permissively involved in ion uptake. Laurent and Perry (1990) have shown that cortisol treatment of freshwater rainbow trout results in increased influx of Na^+ and Cl^-, and increased number and apical surface area of gill chloride cells. Although it has yet to be demonstrated that cortisol increases net influx of Na^+ and Cl^-, this combination of ion transport and morphological evidence is particularly

powerful, and similar results were obtained for European eel, bullhead catfish (*Ictalurus nebulosus*), and tilapia (Perry *et al.*, 1992). Cortisol also increases gill H^+-ATPase, which has been implicated in Na^+ uptake in coho salmon in fresh water (Lin and Randall, 1993). This potential dual effect of cortisol raises several fundamental questions in our understanding of chloride cell function. Does cortisol simultaneously increase both freshwater and seawater forms of the chloride cell? Or are chloride cells "bipolar," with the same cells able to switch rapidly from ion uptake to ion secretion? Answers to these questions must await the development of methods that will permit the *functional* differentiation of chloride cells.

A possible rapid action (minutes to hours) of cortisol on ion transport should not be discounted. Cortisol increases quickly following exposure to seawater (and other stressors) (Schreck, 1981). Mineralocorticoids in amphibians and mammals rapidly increase sodium retention (Minuth *et al.*, 1987). Forrest *et al.* (1973) found increased sodium efflux in American eels treated for 2 days with cortisol and then transferred to seawater. This effect occurred prior to an increase in gill Na^+,K^+-ATPase activity, which occurred after 14 days of cortisol treatment.

B. Growth Hormone

Sakamoto *et al.* (1993) present evidence for the role of growth hormone (GH) in seawater acclimation of salmonids, including the effects of exogenous GH, changes in circulating GH, and metabolic clearance of GH and GH receptors. Exogenous GH increases salinity tolerance in Atlantic salmon, sockeye salmon (*Oncorhynchus nerka*), and rainbow and brown trout; this effect was found to be independent of the hormone's influence on body size (Komourdjian *et al.*, 1976; Clarke *et al.*, 1977; Bolton *et al.*, 1987; Madsen 1990c). Treatment with GH for more than 1 week results in increased gill Na^+,K^+-ATPase activity and chloride cells in all of these species (Boeuf *et al.*, 1990; Madsen, 1990b,c). Growth hormone partially restores the decrease in gill Na^+,K^+-ATPase activity and salinity tolerance following hypophysectomy of coho salmon (Björnsson *et al.*, 1987; Richman *et al.*, 1987). As with cortisol in eels, growth hormone treatment for 2 days can be shown to improve ion regulatory performance of rainbow trout and Atlantic salmon in seawater, prior to a detectable increase in gill Na^+,K^+-ATPase activity (Collie *et al.*, 1989; McCormick *et al.*, 1991b). The short-term and long-term effects of cortisol and growth hormone may involve different mechanisms (such as protein activation, protein synthesis, mitogenesis, and hemodynamics), though much remains to be investigated in this regard.

Almost all the published work on the role of growth hormone in teleost

hypoosmoregulation has been conducted with salmonids. However, Flik *et al.* (1993) found that GH treatment doubled chloride cells density in tilapia opercular membrane. GH also increases the salinity tolerance of hypophysectomized tilapia (T. Sakamoto and E. G. Grau, personal communication), suggesting that a significant role of GH in salt secretion may prove to be common among euryhaline fishes.

There is a strong interaction between growth hormone and cortisol in the regulation of salt secretion. GH and cortisol act in synergy to increase gill Na^+,K^+-ATPase activity and salinity tolerance in brown trout, rainbow trout, and Atlantic salmon (Madsen, 1990b,c). Findings of an increase in gill cortisol receptors following growth hormone treatment in coho salmon (Shrimpton *et al.*, 1995) suggest at least one pathway for this synergy. Although growth hormone receptors have been found in gill tissue of several salmonids (Gray *et al.*, 1990; Yao *et al.*, 1991; Sakamoto and Hirano, 1991), there is currently no evidence for a direct action of GH on Na^+,K^+-ATPase activity (McCormick *et al.*, 1991a). GH may also act through the interrenal to affect cortisol release. Young (1988) found that both *in vivo* and *in vitro* GH treatment increased responsiveness of the coho salmon interrenal to ACTH. However, an effect of GH on circulating levels of cortisol has yet to be demonstrated. In addition to its effect on cortisol receptors and cortisol secretion, at least one other avenue for the action of growth hormone is through its major influence on insulin-like growth factor I (IGF-I).

C. Insulin-like Growth Factor I

IGF-I is a 70-amino acid polypeptide that is produced primarily in the liver but also in several other tissues. Growth hormone is the most important secretogogue, and the physiological activity of IGF-I is controlled by several binding proteins, which like IGF-I itself are under complex endocrine and nutritional control. The cDNA of IGF-I has been isolated from five salmonids (Duan *et al.*, 1994), and the deduced amino acid sequence has an 80% similarity with mammalian IGF-I. Other aspects of the structure and actions of IGF-I in ectothermic vertebrates can be found in Bern *et al.* (1991).

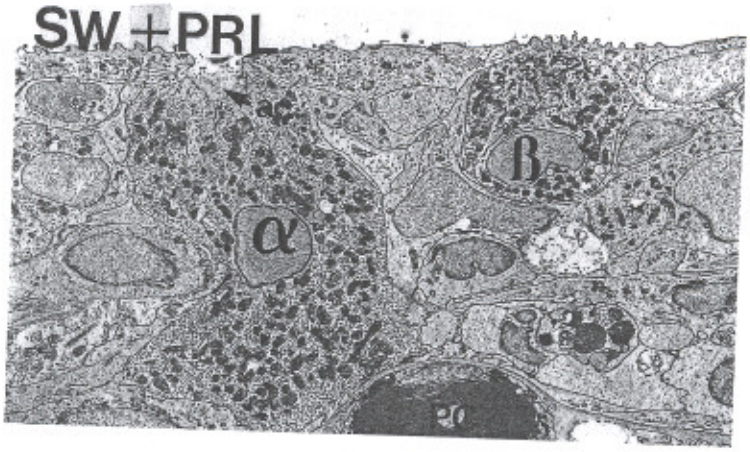
IGF-I mRNA increases in gill and kidney of rainbow trout following seawater exposure (Sakamoto and Hirano, 1993). As with growth hormone, increases in salinity tolerance have been observed within 48 hr of injection of IGF-I in rainbow trout and Atlantic salmon (McCormick *et al.*, 1991b, S. D. McCormick, unpublished). It is not known whether this short-term effect involves the well-known mitogenic activity of IGF-I or some other mechanism of action. In other vertebrates, IGF-I has varied

osmoregulatory actions, including rapid effects on glomerular filtration, direct stimulation of sodium transport in isolated epithelia, and mitogenesis of renal cells (Hammerman *et al.*, 1993).

IGF-I may also have a role in the long-term action of growth hormone and cortisol in stimulating gill Na^+,K^+-ATPase activity and chloride cells. Treatment of brown trout with cortisol, GH, or IGF-I increases gill Na^+,K^+-ATPase α-subunit mRNA (S. Madsen, personal communication). IGF-I treatment also increases responsiveness of gill Na^+,K^+-ATPase to cortisol in Atlantic salmon (S. D. McCormick, unpublished). IGF-I increases Na^+,K^+-ATPase in gill organ culture of coho salmon if the fish are pretreated with GH (Madsen and Bern, 1993). It appears, however, that IGF-I cannot carry out all the long-term effects of GH. Treatment of Atlantic salmon with GH for 2 weeks results in two-fold increases in gill Na^+,K^+-ATPase activity, but the same period of treatment with IGF-I has no effect (S. D. McCormick, unpublished). Although the mitogenic actions commonly associated with IGF-I suggest that this hormone may play a role in GH-mediated increases in numbers (or differentiation) of chloride cells, this remains to be established. Some of the action of IGF-I may be through paracrine or autocrine pathways (local hormone production); in rainbow trout, IGF-I mRNA levels in the gill are highest in chloride cells (T. Sakamoto and S. Hyodo, personal communication).

D. Prolactin

Since the seminal findings of Pickford and Phillips (1959) on the role of prolactin in maintaining ion homeostasis in freshwater killifish, prolactin has been shown to have a role in ion uptake in most, if not all, teleosts (see Hirano, 1986, for a review of prolactin in fish). In euryhaline fish, plasma prolactin levels decrease rapidly following transfer from fresh water to seawater, and increase upon "reverse" transfer. Surprisingly few investigations have examined the biochemical and morphological effects of prolactin on fish osmoregulatory organs. Prolactin decreases gill Na^+,K^+-ATPase activity and increases kidney Na^+,K^+-ATPase activity in hypophysectomized, freshwater killifish (Pickford *et al.*, 1970a). In seawater tilapia there was no effect of prolactin on gill Na^+,K^+-ATPase activity in spite of a profound effect on plasma ions and chloride cells (Herndon *et al.*, 1991). Madsen and Bern (1992) reported that prolactin injections reduce gill Na^+,K^+-ATPase activity in freshwater rainbow trout and antagonize the action of GH in increasing salinity tolerance and gill Na^+,K^+-ATPase activity. In contrast, Atlantic salmon implanted with prolactin show increased gill Na^+,K^+-ATPase activity, with no antagonism of the actions of GH (Boeuf *et al.*, 1994). This confusing picture of the effects of prolactin



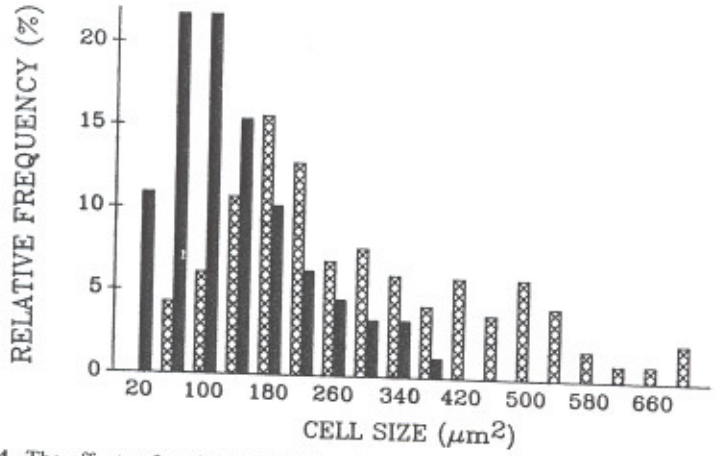


Fig. 4. The effects of prolactin (PRL) on chloride cell size and morphology in tilapia. Upper: α- and β-cells (the latter being the seawater form of the chloride cell) of the Nile tilapia (ac = apical crypt). Prolactin injection in seawater-adapted Nile tilapia caused the appearance of α-cells and decreased size of β-cells. [Reproduced from Pisam, M., Auperin, B., Prunet, P., Rentierdelrue, F., Martial, J., and Rambourg, A. (1993). Effects of prolactin on alpha and beta chloride cells in the gill epithelium of the saltwater adapted tilapia *Oreochromis niloticus*. *Anat. Rec.* **235,** 275–284. Copyright © (1993) by Wiley–Liss, Inc., a division of John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.] Lower: In Mozambique tilapia, prolactin (solid bars) treatment causes a dramatic shift in chloride cell size compared to saline (hatched bars) treatment. The vital mitochondrial stain DASPEI was used to identify chloride cells in this study. [Reproduced from Herndon *et al.* (1991) with permission from Academic Press.] The size distribution of chloride cells in these studies underscores the need to view them as a heterogeneous population of cells with varying degrees of development.

may stem from the use of heterologous hormones, species differences or developmental differences.

Although limited, current information indicates that prolactin strongly affects chloride cell morphology. Based on reductions in opercular membrane conductance and short-circuit current following prolactin treatment of seawater-adapted tilapia, Foskett *et al.* (1982) postulated that prolactin reduces chloride cell numbers and active transport in the remaining chloride cells. Herndon *et al.* (1991) found that prolactin treatment of seawater-adapted tilapia resulted in a dramatic reduction in chloride cell size without changing chloride cell density. Chloride cell height and proportion of cells spanning the opercular membrane were reduced, suggesting that these cells were effectively "removed" as chloride secretory cells (Fig. 4). Working with the closely related but stenohaline Nile tilapia (*Oreochromis niloticus*), Pisam *et al.* (1993) found that prolactin injection caused the appearance of the smaller "β-chloride cells" that were previously absent from these fish in seawater. The remaining "α-chloride cells" were also reduced in size and had shallower apical crypts and reduced tubular systems (Fig. 4).

These effects of prolactin on chloride cells raise several interesting questions. Is decreased cell size the result of prolactin acting on existing (secretory) chloride cells, or are undeveloped cells affected? How rapid do morphological changes occur and are they sufficient to explain the effects of prolactin on ion regulation? The possible mode(s) of action of prolactin have not been examined. Prolactin receptors have been found in gill tissue (Dauder *et al.*, 1990; Prunet and Auperin, 1994), but as yet there is no evidence for direct action of prolactin on the gills or opercular membrane and regulation of prolactin receptors has not been investigated. Investigations into other possible endocrine factors involved in the prolactin response and interaction of prolactin with other endocrine systems are required.

E. Thyroid Hormones

The reported effects of thyroid hormones in teleost hypoosmoregulation are contradictory. Thyroxine (T_4) or 3,5′,3′-triiodo-L-thyronine (T_3) incorporated in food can improve salinity tolerance of coho and Atlantic salmon (Fagerlund *et al.*, 1980; Refstie, 1982; Saunders *et al.*, 1985). It is unclear whether this is an effect on osmoregulation per se, since the effect apparently depends on prior increases in body size, and salinity tolerance in salmonids is size dependent (McCormick and Naiman, 1984b). Studies on chum and amago salmon have shown no effect of T_4 or thiourea, an inhibitor of T_4 production, on salinity tolerance (Miwa and Inui, 1983;

Iwata *et al.*, 1987) despite the fact that these treatments were effective in altering circulating levels of thyroid hormones and body silvering (a morphological change that is part of the parr–smolt transformation). The possible influence of thyroid hormones on salinity tolerance in nonsalmonids has not been examined.

Thyroxine has no apparent positive effect by itself on gill Na^+,K^+-ATPase activity in tilapia or amago, coho, and Atlantic salmon (Miwa and Inui, 1985; Saunders *et al.*, 1985; Björnsson *et al.*, 1987; Dange, 1986). In rainbow trout, T_4 injections had no effect on gill Na^+,K^+-ATPase activity (Madsen, 1990a), whereas T_3 immersion decreased activity (Omelanjiuk and Eales, 1986). In contrast to these studies, Madsen and Korsgaard (1989) found that multiple injections of thyroxine could advance increases in gill Na^+,K^+-ATPase activity and chloride cell numbers that occur during the parr–smolt transformation of Atlantic salmon.

Greater agreement in the literature is found regarding the interaction of thyroid hormones with other endocrine axes. Thyroxine increases the capacity of cortisol to increase gill Na^+,K^+-ATPase activity in tilapia (Dange, 1986). In amago salmon, T_4 and GH in combination were capable of elevating gill Na^+,K^+-ATPase, whereas each hormone alone had no effect. The most convincing evidence for a role of thyroid hormones in seawater acclimation utilized an inhibitor of 5′-monodeiodination of T_4 (Lebel and Leloup, 1992; Leloup and Lebel, 1993). In these studies, rainbow and brown trout have been found to require conversion of T_4 to T_3 for normal and GH-stimulated acclimation to seawater. From these results the authors suggest that the action of growth hormone on hypoosmoregulatory mechanisms is through its increase of T_4 to T_3 conversion (de Luze *et al.*, 1989). However, this seems to be an incomplete explanation given the equivocal effect of T_3 on salinity tolerance and its general inability to increase gill Na^+,K^+-ATPase activity. An alternative explanation is that T_3 is necessary for the peripheral action of GH (or IGF-I), possibly through regulation of receptors or binding proteins. Other indirect pathways for thyroid hormone action include their ability to increase pituitary GH production (Moav and McKeown, 1992) and interrenal sensitivity to ACTH (Young and Lin, 1988). Thyroid hormone receptors have been found in liver, kidney, and gill of rainbow trout, brown trout, and European eel (Bres and Eales, 1988; Lebel and Leloup, 1989), but their endocrine regulation and response to salinity have not been examined.

F. Sex Steroids

Normal sexual maturation and treatment with exogenous sex steroids have been shown to have a negative effect on the ability of several salmo-

nids to adapt to seawater (McCormick and Naiman, 1985; Ikuta *et al.*, 1987; Lundqvist *et al.*, 1989; Schmitz and Mayer, 1993). Repeated injections of 17β-estradiol result in decreased gill chloride cell density and Na^+,K^+-ATPase activity in Atlantic salmon (Madsen and Korsgaard, 1989). The mode of action of sex steroids, particularly whether they are acting directly on the gill or through other endocrine systems, is currently unknown. It is also unclear whether the action of sex steroids on hypoosmoregulation is peculiar to the anadromous life history of salmonids in which sexual maturation occurs only in fresh water; research on other fishes is clearly warranted.

G. Rapid Activation

Little work has been done on the rapid activation of Na^+,K^+-ATPase. Although it can be assumed that any of the hormones involved in rapid changes in ion efflux will involve stimulation of Na^+,K^+-ATPase, it is not clear whether these will be direct (stimulation of the pump itself) or indirect (through increased substrate availability provided by stimulation or inhibition of other transporters or channels). Important advances in this area will come from methods that permit rapid, simultaneous measurement of ion transport and morphological or biochemical changes in chloride cells. Ion-sensitive dyes and fluorescent probes may be particularly valuable in this area.

VII. SUMMARY AND PROSPECTUS

Any summary of the endocrine control of physiological function in fishes must confront the diversity and evolutionary history of this large group and the sometimes contradictory results in the literature. Nevertheless, a hypothetical model of endocrine regulation of Na^+,K^+-ATPase and chloride secretory cells will apply to some species (Fig. 5). Evidence to date indicates that cortisol (F), growth hormone (GH), insulin-like growth factor I (IGF-I), and thyroid hormones (T_4 and T_3) increase Na^+,K^+-ATPase and/or promote the differentiation of chloride secretory cells. Cortisol has been shown to have direct action on Na^+,K^+-ATPase and chloride cells, and cortisol receptors have been found in gill tissue. Growth hormone acts in several ways: by increasing gill cortisol receptors, by increasing the sensitivity of the interrenal to adrenocorticotropic hormone (ACTH), and by production of IGF-I. Growth hormone receptors have been found in both gill and kidney. Although IGF-I is known to increase salinity tolerance and Na^+,K^+-ATPase activity, its mode of action is

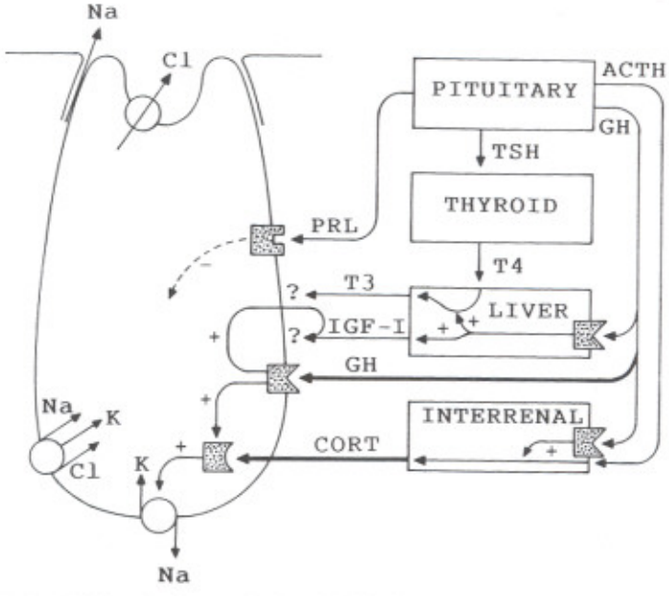


Fig. 5. Model of endocrine regulation of Na^+,K^+-ATPase and chloride secretory cells. Evidence to date indicates that cortisol (CORT), growth hormone (GH), insulin-like growth factor I (IGF-I), and thyroid hormones (T_4 and T_3) increase Na^+,K^+-ATPase and/or promote the differentiation of chloride secretory cells, whereas prolactin (PRL) is inhibitory. Adrenocorticotropic hormone (ACTH) and thyroid-stimulating hormone (TSH) are pituitary hormones involved in stimulating secretion of cortisol and thyroid hormones, respectively. See text for details.

unknown and receptors in the gill have not been demonstrated. The degree to which the GH/IGF-I axis is important in nonsalmonids requires clarification. Thyroid hormones promote chloride cell development, possibly through their interaction with the GH/IGF-I axis, though this has yet to be fully explored. Prolactin inhibits the development of the chloride secretory cell and promotes the development of the freshwater form of the chloride cell. Prolactin's effects on gill Na^+,K^+-ATPase are equivocal, and although prolactin receptors have been found in the gill, its mode of action is unclear. The universality of this model is suspect, and particularly may not apply to those species in which increased Na^+,K^+-ATPase is associated with exposure to fresh water rather than seawater.

Recent and upcoming advances promote optimism about the solution of the questions posed in this review. The advance of molecular methods will allow for the greater use of homologous hormones and radioimmunoas-

says and the detection of changes in receptor gene expression. Isolation of the cDNA for other ATPases and cotransport proteins of chloride cells (in fresh water and seawater) will be powerful tools for determining the functional attributes of chloride cells and their endocrine control. Continued development of methods for organ and cell culture will advance our understanding of direct hormone actions. Combining the new methods with classical physiology, histology, and endocrinology will be especially useful to our understanding of chloride cell regulation.

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