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The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste

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Evans, David H., Peter M. Piermarini, and Keith P. Choe. The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste. *Physiol Rev* 85: 97–177, 2005; doi:10.1152/physrev.00050.2003.—The fish gill is a multipurpose organ that, in addition to providing for aquatic gas exchange, plays dominant roles in osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes. Thus, despite the fact that all fish groups have functional kidneys, the gill epithelium is the site of many processes that are mediated by renal epithelia in terrestrial vertebrates. Indeed, many of the pathways that

mediate these processes in mammalian renal epithelial are expressed in the gill, and many of the extrinsic and intrinsic modulators of these processes are also found in fish endocrine tissues and the gill itself. The basic patterns of gill physiology were outlined over a half century ago, but modern immunological and molecular techniques are bringing new insights into this complicated system. Nevertheless, substantial questions about the evolution of these mechanisms and control remain.

I. INTRODUCTION

Fishes are aquatic vertebrates that are members of the largest and most diverse vertebrate taxon (~25,000 species), that dates back over 500 million years. They have evolved into three major lineages: Agnatha (hagfish and lampreys), Chondrichthyes (sharks, skates, and rays; usually referred to as elasmobranchs), and Actinopterygii (bony fishes, with teleosts being the most prevalent). Regardless of lineage, the majority of fish species uses the gill as the primary site of aquatic respiration. Aerial-breathing species may use the gill, swim bladder, or other accessory breathing organs (including the skin). The fish gill evolved into the first vertebrate gas exchange organ and is essentially composed of a highly complex vasculature, surrounded by a high surface area epithelium that provides a thin barrier between a fish's blood and aquatic environment (Fig. 1). The entire cardiac output perfuses the branchial vasculature before entering the dorsal aorta and the systemic circulation. The characteristics of the gill that make it an exceptional gas exchanger are not without trade-offs. For example, the high surface area of the gills that enhances gas exchange between the blood and environment can exacerbate water and ion fluxes that may occur due to gradients between the fish's extracellular fluids and the aquatic environment. In the past 50 years, it has become clear that the branchial epithelium is the primary site of transport processes that counter the effects of osmotic and ionic gradients, as well as the principal site of body fluid pH regulation and nitrogenous waste excretion. Thus the branchial epithelium in fishes is a multipurpose organ that plays a central role in a suite of physiological responses to environmental and internal changes. Despite the fact that fishes do have kidneys, the gill actually performs most of the functions that are controlled by pulmonary and renal processes in mammals. The purpose of this review is to integrate the latest morphological, biochemical, and molecular data (with appropriate references to historical studies) in an effort to delimit what is known and unknown about this interesting organ.

II. EVOLUTIONARY ORIGIN AND EXTERNAL STRUCTURE

A. Gills of Protovertebrates and Modern Fishes

One of the defining characteristics of vertebrates is the presence of pharyngeal gill slits during some life stage (e.g., embryo, juvenile, or adult). In protovertebrates (i.e., urochordates, cephalochordates), the gills are relatively simple structures compared with those of extant fishes. For example, in amphioxus (a cephalochordate), the gill slits are formed by numerous, vertically oriented gill bars in the pharynx. These gill bars contain an internal support rod (made of collagen), blood vessels, and neuronal processes (33); externally they possess a ciliated epithelium. Despite the presence of branchial blood vessels that connect the ventral aorta to the dorsal aorta (502), the gill bars of amphioxus do not have a large diffusing capacity for oxygen uptake and are not a likely site of respiratory

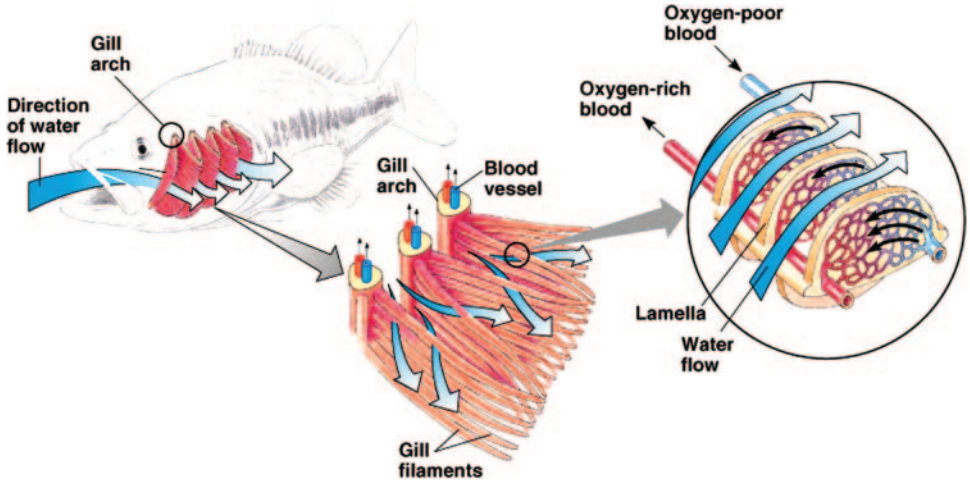

FIG. 1. Schematic of the teleost fish gill. See text for details. [From Campbell and Reece (81).]

gas exchange (669). Instead, the gill bars play a prominent role in feeding by filtering food particles from water that is passed through the gill slits by the ciliated gill bar epithelium.

In contrast to the specialized feeding device of protovertebrates, the gills of modern fishes have evolved into an anatomically complex, multifunctional tissue with discrete external (i.e., epithelial) and internal (i.e., circulatory and neural) elements. The gills of fishes are located near the head region and are composed of several paired gill arches on the pharynx (Figs. 1 and 2). Anchored to the gill arches is a complex arrangement of epithelial, circulatory, and neural tissues, which will be described in the next two sections.

B. External Structure of Fish Gills

1. Gross anatomy

The general anatomy of the gills varies among the three extant evolutionary lineages of fishes, but a simplified arrangement can be generalized for elasmobranchs and teleosts (agnathans will be described separately). Typically, cartilaginous or bony support rods (gill rays) radiate laterally from the medial (internal) base of each gill arch, and connective tissue between the gill rays forms an interbranchial septum. The interbranchial septum supports several rows of fleshy gill filaments (termed hemibranchs) that run parallel to the gill rays on both the cranial and caudal sides of a gill arch (Fig. 2). Gill filaments are the basic functional unit of gill tissue, and their structure is described in section II*B2*.

A set of cranial and caudal hemibranchs from the same arch is referred to as a holobranch. In most elasmobranchs, four pairs of holobranchs are present in the branchial chamber, with only a pair of caudal hemibranchs on the first gill arch (Fig. 2*C*). The interbranchial septum of elasmobranch holobranchs extends from the base of each respective gill arch to the skin and forms distinct external gill slits. The typical path of water flow through the elasmobranch gills is for water to enter the pharynx via the mouth or spiracles (cranial valves for water entry), then pass over the gill filaments and follow the interbranchial septum until the water exits via the gill slits (Fig. 2, *C* and *G*).

In teleosts, only four pairs of holobranchs are present, and the interbranchial septum is much reduced compared with elasmobranchs. The septum usually only extends to the base of the filaments (Fig. 2*H*), and thus the filaments of teleosts are much more freely moving than those of elasmobranchs. No distinct external gill slits are found in teleosts, but a thin, bony flap called the operculum externally protects the branchial chamber (Figs. 1 and 2*D*). In teleosts, water enters the pharynx from the mouth, then passes over the filaments and follows the inner wall of the operculum until it exits via a caudal opening of the operculum (Figs. 1 and 2*H*).

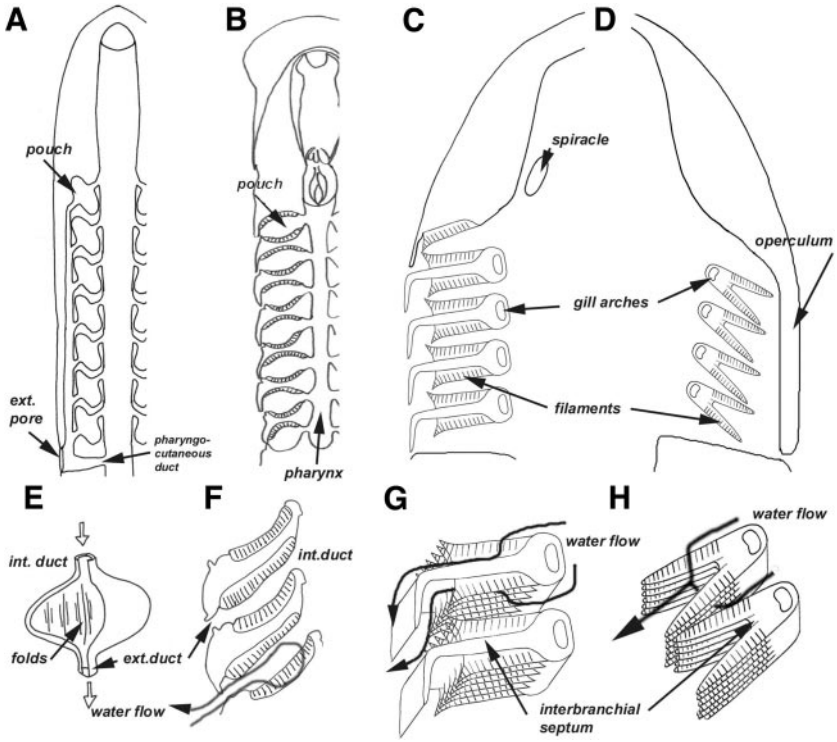

FIG. 2. Generalized schematics of gills and associated pouches/arches in hagfishes (*A* and *E*), lampreys (*B* and *F*), elasmobranchs (*C* and *G*), and teleosts (*D* and *H*). All schematics are oriented anterior to posterior, with oral opening at top. [Modified from Wilson and Laurent (805).]

Among agnathans, the gills of adult, parasitic lampreys have a similar overall organization to elasmobranch gills, except no gill rays are present in lampreys, and the skeletal base of the gill arch in lampreys is situated external (lateral) to the hemibranchs; in elasmobranchs (and teleosts), the base of the gill arch is medial to the hemibranchs. In addition, the interbranchial septa of lampreys are slightly concave in shape, which results in the cranial and caudal hemibranchs of adjacent holobranchs forming pouchlike structures with discrete internal and external branchiopores or ducts that open to the pharynx and environment, respectively (Fig. 2, *B* and *F*). Lampreys have six pairs of holobranchs, with only a caudal and cranial hemibranch on the first and last gill arch, respectively; this results in a total of seven paired gill pouches (Fig. 2*B*). Water flow through lamprey gills is rather diverse compared with other fishes, because, during feeding, lampreys attach to their prey by oral suction, and therefore the typical mouth to gill water path is not available. Through contractions of the extensive musculature that surrounds the pouches, water is moved into and out of the external ducts in a tidal manner to irrigate the gill filaments (e.g., Fig. 2*F*), which allows branchial respiration to occur while feeding. The flow of water through lamprey gills can also be modified to pump water from the external ducts, through the gills, and into the pharynx via the internal ducts. This water path may be used to clean the pharynx (see Ref. 632), or it may assist in the removal of the lamprey from its prey.

The gills of hagfish (the other agnathan lineage) have a rather unique gill anatomy and organization compared with those of other fishes. The gills are composed of 5–14 pairs of lens-shaped pouches that have discrete incurrent and excurrent ducts that connect to the pharynx and the environment (either directly or indirectly via a common pore, depending on species), respectively (Figs. 2*A* and 3*A* and Ref. 25). Unlike other fishes, no well-developed skeletal structures (e.g., arches) are associated with the pouches. From the internal wall of each pouch, several extensive epithelial folds arise that span the lateral dimension of the gill pouch and extend towards the center of the pouch (Fig. 3*A*). The folds are radially arranged around the mediolateral axis of the pouch. Despite their atypical arrangement and morphology, these folds are considered

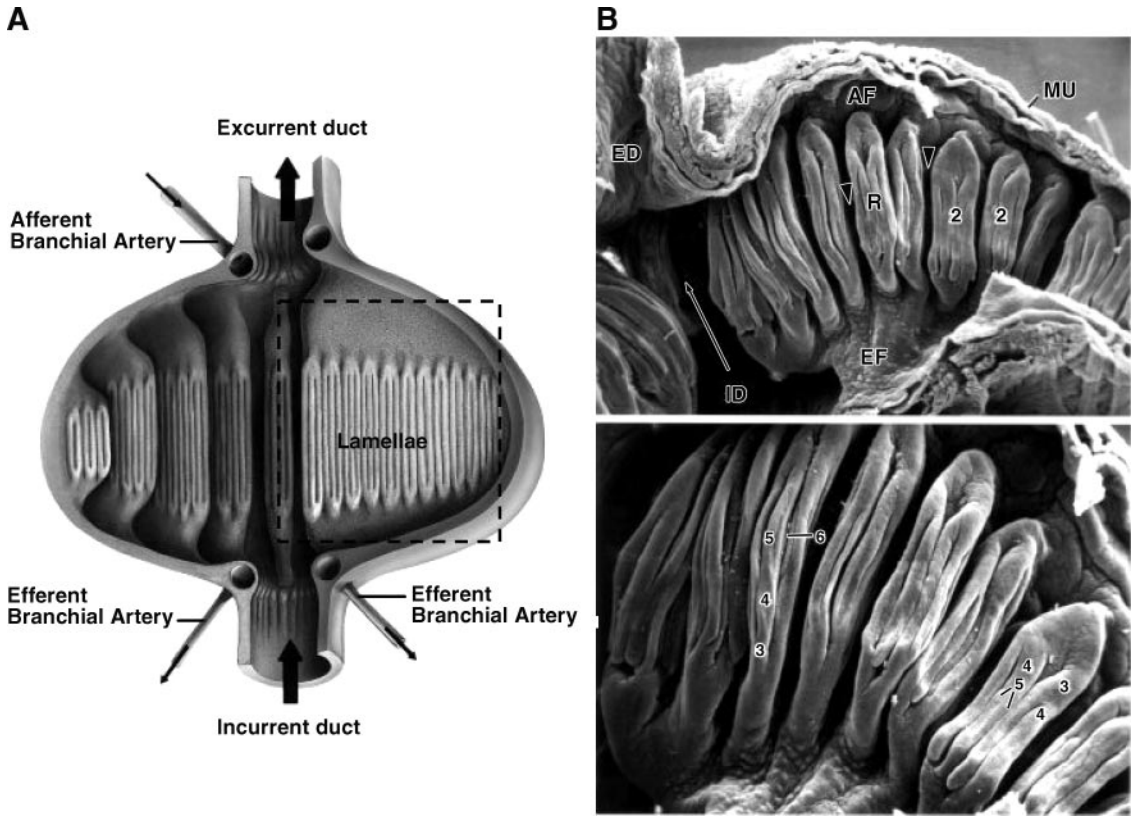

FIG. 3. Anatomy of hagfish gills. *A*: schematic of a longitudinal cut through a gill pouch from the Atlantic hagfish, with a lateral perspective of a primary gill fold (filament) and its lamellae (boxed area). Note radial arrangement of additional filaments around the pouch. Large arrows indicate direction of water flow; small arrows indicate direction of blood flow. [Modified from Elger (169).] *B*: scanning electron micrographs of a gill filament from the Pacific hagfish, comparable to boxed area in *A*. Top micrograph (×30) shows an overview of a filament, with afferent (AF) and efferent (EF) regions, and respiratory lamellae (R) with second-order folds (2). MU indicates muscular layer around the pouch. Arrow indicates flow of water through pouch from incurrent duct (ID) to excurrent duct (ED); arrowheads indicate flow of blood across filament. Bottom panel (×70) reveals higher order folds of the lamellae, i.e., third- (3), fourth- (4), fifth- (5), and sixth-order (6) folds. [Modified from Mallatt and Paulsen (432).]

to be comparable to the gill filaments of other fishes (25, 432). The path of water through hagfish gills resembles that of other fishes, except that water enters the pharynx through a nasal opening instead of the mouth. Water then enters the pouches via their incurrent ducts, passes the gill filaments, and exits the pouches via excurrent ducts (Fig. 2*E*). In addition to this path, hagfish can irrigate the pharynx via a pharyngocutaneous duct on the left side of the animal that is caudal to the last gill pouch and connects the pharynx directly to the environment (Fig. 2*A*). This unique duct may be used to irrigate the pharynx and gill pouches while the animal is feeding, which usually involves burying the head region within a prey item (see Ref. 805).

2. Gill filament anatomy

In most fishes, gill filaments are long and narrow projections lateral to the gill arch that taper at their distal end (Fig. 4). Each filament is supplied with blood from an afferent filamental artery (AFA) that extends along the filament. Blood in this vessel also travels across the filament's breadth through numerous folds on the dorsal and ventral surfaces of the filament-termed lamellae, which are perpendicular to the filament's long axis (Figs. 1 and 4). Blood that crosses the lamellae drains into an efferent filamental artery (EFA) that runs along the length of the filament and carries blood in the opposite direction to that in the AFA. Blood flow through the gills and gill filaments is described in more detail in section III*A*.

Lamellae are evenly distributed along a filament's length, and the spaces between lamellae are channels through which water flows. A closer look at an individual lamella reveals that it is essentially composed of two epithelial sheets, held apart by a series of individual cells, termed pillar cells (Fig. 5). The spaces around the pillar cells and between the two epithelial layers are perfused with blood, flowing as a sheet, not through vessels per se (Fig. 5). Lamellae dramatically increase the surface area of the gill filament epithelium and result in a small diffusion distance between the blood that perfuses each lamella and the respiratory water. Moreover, blood flow through the lamellae is countercurrent to water flow between them (Fig. 1). Therefore, the lamellae are well-suited for gas exchange, but are also well-suited for diffusive losses or gains of ions and water to/from the environment (see sects. IV and V).

The above description of the gill filament (and lamellae) is applicable to most fish groups (i.e., elasmobranchs, teleosts, and lampreys), but not hagfish. As with their gill gross anatomy, the gill filament anatomy of hagfish is unique compared with other fishes. Each filament is supplied with blood from an afferent radial artery that begins near the excurrent duct of a pouch. Blood traverses medially across the filament where it encounters lamellae

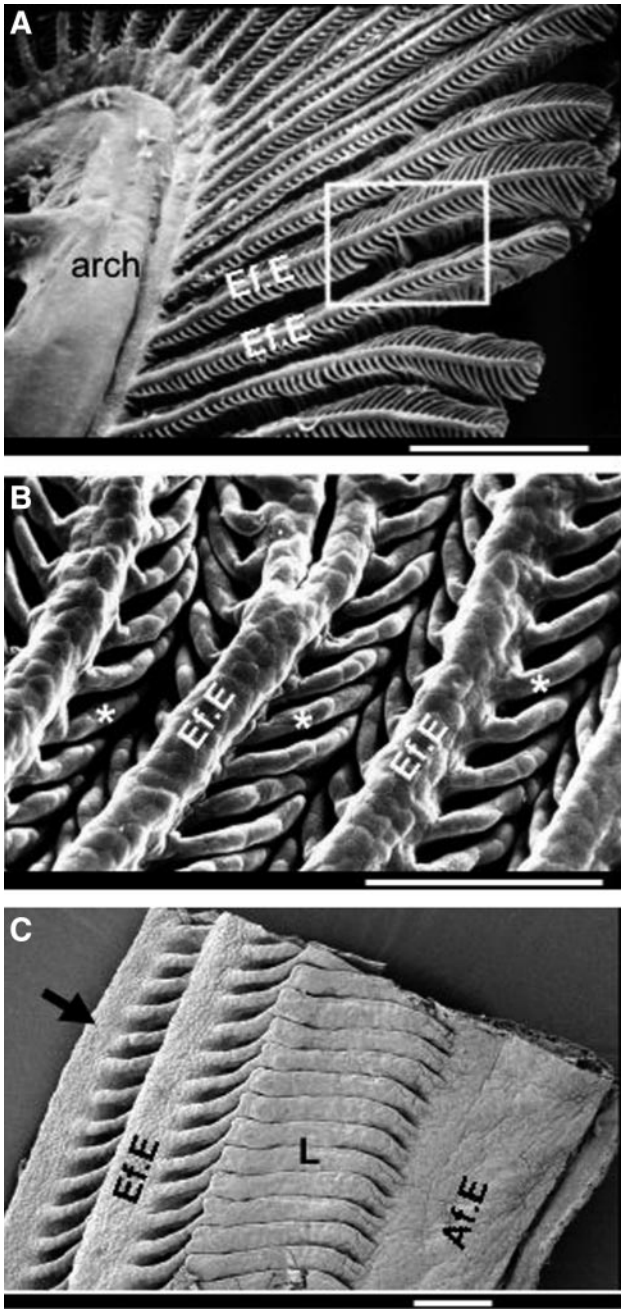

FIG. 4. Scanning electron micrographs of gill filaments. *A*: filaments from a teleost (gulf toadfish, *Opsanus beta*), which radiate off a gill arch. Leaflike lamellae branch off the filaments (e.g., boxed area). Note the efferent edge (Ef.E) of the filaments, which is facing up; the afferent edge is in the depth of the figure. Bar = 100 μm. [From Evans (181).] *B*: high magnification view of filaments from an agnathan (pouched lamprey, *Geotria australis*), with the Ef.E of the filaments facing up. An asterisk indicates a lamella on each filament. Bar = 100 μm. [From Bartels et al. (29).] *C*: side profile of a filament from an elasmobranch (ocellated river stingray, *Potamotrygon motoro*) showing the afferent edge (Af.E), Ef.E, and lamellae (L). Arrow indicates the Ef.E of an underlying filament. Bar = 100 μm. (From P. M. Piermarini and D. H. Evans, unpublished micrograph.)

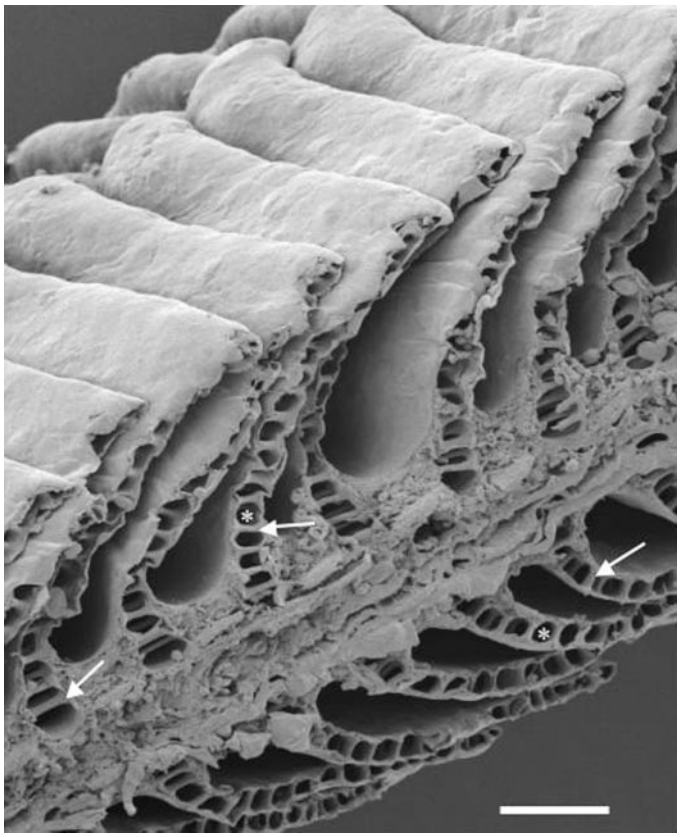

FIG. 5. Scanning electron micrograph of a longitudinal cut through a gill filament from an elasmobranch (Atlantic stingray, *Dasyatis sabina*). Asterisks indicate lamellar blood spaces formed by pillar cells (arrows). Bar = 50 μm. (From Piermarini and Evans, unpublished micrograph.)

(Fig. 3) that are oriented in the same plane as the filamental blood flow; lamellae are oriented perpendicular to filamental blood flow in other fishes. Blood that flows through the lamellae is collected by an efferent radial artery near the incurrent duct of a pouch. Blood flow through hagfish gills and gill filaments is described in more detail in section III*A*. Lamellae of hagfish gills are much more complex than those of other fishes. For example, lamellae begin as secondary folds of the gill filament and continue to fold to form several higher order folds (Fig. 3*B*). These folds increase the surface area of the filament and, similar to lamellae in other fishes, the folds are relatively thin structures composed of epithelial sheets separated by pillar cells. Regardless of the degree of folding, blood flow through the lamellae remains in the same direction (i.e., lateral to medial), which is countercurrent to water flow through the pouch. Thus, like other fishes, the lamellae of hagfish gills are well-suited for gas exchange.

In all fishes, the region of the filament that contains the afferent blood supply is commonly referred to as the afferent edge, whereas the region that collects efferent blood is referred to as the efferent edge. These two terms are synonymous with trailing edge and leading edge, respectively, relative to water flow across the filament. In hagfish, the additional term of *respiratory region* or *respiratory zone* is sometimes given to the lamellae and their higher order folds.

3. The gill epithelium

The epithelium that covers the gill filaments and lamellae provides a distinct boundary between a fish's external environment and extracellular fluids and also plays a critical role in the physiological function of the fish gill. The gill epithelium is composed of several distinct cell types (reviewed in Refs. 386, 805), but primarily consists of pavement cells (PVCs) and mitochondrion-rich cells (MRCs), which comprise >90% and <10% of the epithelial surface area, respectively.

A) PAVEMENT CELLS. Although PVCs cover the vast majority of the gill filament surface area, they are largely considered to play a passive role in the gill physiology of most fishes (see below for exceptions). PVCs are assumed to be important for gas exchange because they are thin squamous, or cuboidal, cells with an extensive apical (mucosal) surface area and are usually the primary cell type that covers the sites of branchial gas exchange, i.e., the lamellae (386, 390, 805).

The apical membrane of PVCs is characterized by the presence of microvilli and/or microplicae (microridges), which often have elaborate arrangements that vary between species (e.g., Figs. 9, 11, and 13*A*). These apical projections likely increase the functional surface area of the epithelium and may also play a role in anchoring mucous to the surface. Typically, PVCs do not contain many mitochondria but may be rich in cytoplasmic vesicles or have a distinct Golgi apparatus (see Refs. 386, 390). In agnathans and elasmobranchs, PVCs possess subapical secretory granules or vesicles that contain mucous and fuse with the apical membrane (24, 25, 169, 432, 805) (Fig. 13*A*). The intercellular junctions between PVCs and adjoining cells are extensive or multistranded (e.g., Figs. 7*B*, 10*B*, 12*B*, and 13*A*), which makes the junctions "tight" and presumably relatively impermeable to ions (26, 31, 344, 664).

In freshwater teleost gills, evidence suggests that some PVCs may play an active role in ion uptake and acid-base transport by the gills. For example, an ultrastructural study of the fresh water, brown bullhead catfish (*Ictalurus nebulosus*) gill epithelium demonstrated the presence of studded vesicles near the apical membrane of PVCs that resembled vacuolar-proton-ATPase (V-ATPase)-rich vesicles found in other ion regulatory epithelia (e.g., turtle urinary bladder and mammalian renal tubules) (391). Subsequent immunohistochemical (405, 716, 806) and biochemical (378) studies in rainbow trout (*Onchorynchus mykiss*) and tilapia (*Oreochromis mossambicus*) have verified the presence of V-ATPase in

PVCs (see sects. v*B1* and vi*D1*). Moreover, recent studies have isolated a subpopulation of mitochondrion-rich PVCs (MR-PVCs) from rainbow trout gills that are V-ATPase-rich (248, 262). These MR-PVCs appear to play an active role in sodium uptake and acid extrusion (248, 639) (see sect. v*B1*).

B) MITOCHONDRION-RICH CELLS. In contrast to PVCs, MRCs occupy a much smaller fraction of the branchial epithelial surface area, but they are considered to be the primary sites of active physiological processes in the gills. Whereas PVCs are found in all regions of gill filaments, MRCs are usually more common on the afferent (trailing) edge of filaments, as well as the regions that run between individual lamellae, termed the interlamellar region (Fig. 6). Also, MRCs are usually not found on the epithelium covering the lamellae; however, certain environmental conditions are associated with the presence of lamellar MRCs in some species (see below).

The ultrastructure and function of MRCs are highly variable among the three extant lineages of fishes, and therefore specific details on MRCs in agnathans, elasmobranchs, and teleosts will be described separately. However, a few general trends exist that are applicable to all groups. For example, MRCs are large ovoid-shaped cells, and as their name suggests, they have high densities of mitochondria in their cytoplasm, relative to PVCs. In addition, MRCs are highly polarized cells, with their apical and basolateral cell membranes having distinct morphologies (see below) and transport-protein expression profiles (see sects. v, *B* and *C*, vi*D*, and vii*B*).

1) Teleosts. The ultrastructure of teleost MRCs has been studied extensively in several species, and numerous, extensive reviews exist on the subject (e.g., Refs. 343, 386, 390, 586, 617, 805). Teleost MRCs are often referred to as "chloride cells" in the literature, which is attributed to their NaCl secretory function in seawater teleosts. In freshwater teleosts, MRCs are also present, but they are characterized by different forms and functions than MRCs of seawater teleosts, and will be described separately.

One of the most striking characteristics of MRCs in seawater teleosts is the presence of an intricate tubular system that is formed by extensive invaginations of the basolateral membrane (601). This tubular/membranous labyrinth extends throughout most of the cytoplasm, where it closely associates with mitochondria (see Refs. 386, 390, 601, 805) (Fig. 7*A*) and is the site of expression for the active transport enzyme Na^+-K^+-ATPase, which indirectly energizes NaCl secretion by these cells (349) (see sects. v*B4* and v*C2*A). The apical membrane of MRCs is concave and recessed below the surface of surrounding PVCs to form an apical pore or crypt that is shared with other MRCs (Fig. 7). These crypts (e.g., Fig. 9*A*) have been described as "potholes" in a "cobblestone street" of PVCs that expose the apical surface of MRCs to the ambient environment (343). The apical membrane itself is sparsely

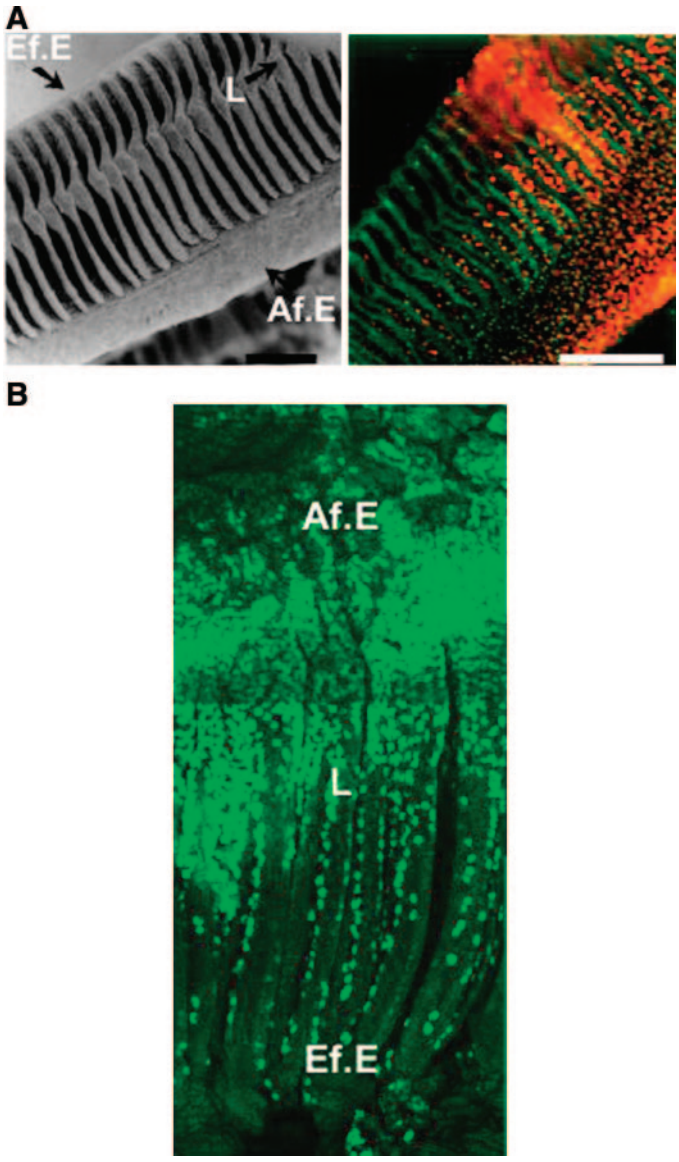

FIG. 6. Distribution of mitochondrion-rich cells (MRCs) in gill filaments. *A*: scanning electron micrograph (*left*) and a corresponding confocal laser scanning micrograph (*right*) of a filament from a teleost (tilapia, *Oreochromis mossambicus*). The filament in the confocal micrograph is stained with DASPMI, a vital mitochondrial fluorescent dye. In this micrograph, the MRCs are stained red; note their increased abundance between the lamellae, which are stained green, and on the Af.E, compared with the Ef.E. Bar in scanning electron and confocal micrographs = 100 and 250 μm, respectively. [Modified from Van Der Heijden et al. (769).] *B*: confocal laser scanning micrograph of a filament from an agnathan (Atlantic hagfish) stained with DASPEI (another vital mitochondrial fluorescent dye). Filament is in similar orientation as the one shown in Figure 3*B*. Note similar distribution of MRCs as found in the tilapia, i.e., increase in MRC abundance from Ef.E to Af.E. [Modified from Choe et al. (94).]

populated with short microvilli (387), and overall is morphologically unspecialized. However, the subapical cytoplasm contains a tubulovesicular system comprised of numerous vesicles and tubules that shuttle to and fuse with the apical membrane (Fig. 7*A*) (393, 805); these

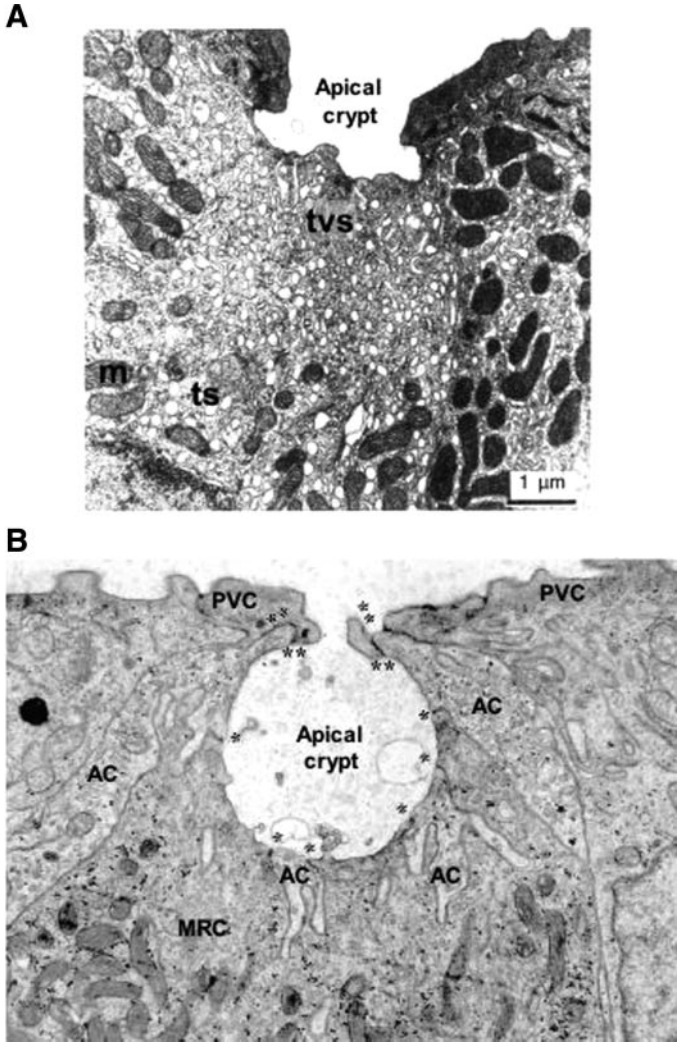

FIG. 7. Transmission electron micrographs of MRCs from the gills of seawater teleosts. *A*: MRC from the sole (*Solea solea*) containing numerous mitochondria (m), a tubular system (ts), a subapical tubulovesicular system (tvs), and an apical crypt. [From Evans (185).] *B*: apical region of a MRC from the killifish (*Fundulus heteroclitus*). This MRC forms deep tight junctions (**) with surrounding PVCs and shallow tight junctions (*) with surrounding accessory cells (ACs), which share an apical crypt with the MRC. [Modified from Evans et al. (202).]

vesicles may be responsible for trafficking of the cystic fibrosis transmembrane regulator (CFTR; see sect. v*C3*) chloride channel to the apical membrane of MRCs, as seen by Marshall et al. (453). Intercellular junctions between MRCs and adjacent PVCs are extensive (multistranded) and are considered to be "tight," forming a relatively impermeable barrier to ions (344, 664) (Fig. 7*B*).

In seawater teleosts, MRCs exist in multicellular complexes with other MRCs and accessory cells (ACs). Similar to MRCs, ACs contain numerous mitochondria, but ACs are much smaller (e.g., Fig. 9*C*), with a less-developed tubular system and lower expression of Na^+-K^+-ATPase, relative to MRCs (307). The true nature of ACs is debatable; for example, in some species (e.g., brown trout; *Salmo trutta*) ACs appear to be a well-defined, discrete cell type (615), whereas in others (e.g., tilapia) evidence suggests that ACs are just a developmental stage of MRCs (796, 799). Regardless, in seawater teleosts, the multicellular complexes form an apical crypt shared by the apical membranes of ACs and MRCs (Fig. 7*C*). Cytoplasmic processes of ACs extend into the apical cytoplasm of MRCs to form complex interdigitations (387, 805). Importantly, these interdigitations form junctions between MRCs and accessory cells that are not extensive (Fig. 7*B*) and are considered to be leaky to ions (307, 344, 664), thus providing a paracellular route for Na^+ extrusion (see sect. v*C*).

It should be noted that high densities of MRCs that are ultrastructurally and functionally identical to MRCs of the seawater teleost gill epithelium are found in the opercular epithelium of killifish (*Fundulus heteroclitus*) (350) and tilapia (232) and the jawskin epithelium of the longjaw mudsucker (*Gillichthys mirabilis*; Ref. 454). These flat epithelial sheets have been extremely valuable model systems for deciphering the mechanisms of ion transport in seawater teleost MRCs (reviewed by 343, 441) (see sect. v*C*).

In freshwater teleosts, MRCs are primarily found on the afferent edge and interlamellar region of the gill filament, but lamellar MRCs are also present in some species (e.g., Refs. 682, 764). In general, the MRCs of freshwater teleosts share some ultrastructural characteristics with those from seawater teleosts, such as the extensive basolateral membrane infoldings that form a tubular system associated with mitochondria, and the subapical tubulovesicular system (see Refs. 393, 586, 590). However, important differences exist. For example, the ACs and multicellular complexes associated with MRCs in seawater teleosts are not common in freshwater teleosts; the MRCs often occur singly in freshwater teleost gill epithelium, intercalated between PVCs. Also, freshwater teleost MRCs form extensive, multistranded intercellular junctions with surrounding PVCs or other MRCs to form a relatively impermeable barrier to ions. The apical membrane of freshwater MRCs is usually flush with or noticeably protruded above adjacent PVCs (e.g., Ref. 353) and contains distinct patterns of microvilli or microridges (586, 590). However, in some species (e.g., tilapia, mangrove killifish, *Rivulus marmoratus*) apical crypts still exist (361, 586, 590, 614). Although the basolateral tubular system still occurs in MRCs of freshwater teleosts, this structure appears to be less developed compared with seawater MRCs (e.g., Ref. 610).

In several species of freshwater teleosts, distinct morphological MRC subtypes have been detected in the gills (e.g., Atlantic salmon, *Salmo salar*; brown trout; guppies, *Poecilia reticulata*; loaches, *Cobitis taenia*; grudgeon, *Gobio gobio*; and Nile tilapia, *O. niloticus*). Pisam and colleagues (612–616) have described two MRC

subtypes (α and β) based on the degree of osmium staining (dark or light) in the cytoplasm, apical membrane morphology and associated subapical structures, extent of tubular system, cell shape, and anatomic location on the filament. The α-MRCs (Fig. 8*A*) have light cytoplasmic staining, usually a smooth apical membrane associated with numerous subapical vesicles, a well-developed tubular system, an elongated shape, and are found at the base of lamellae where they may associate with an accessory cell (e.g., in Atlantic salmon and tilapia). In contrast, β-MRCs (Fig. 8*B*) have dark cytoplasmic staining, usually have complex apical membrane projections associated with an extensive subapical tubulovesicular network, a less-developed tubular system, an ovoid shape, and are found in the interlamellar region. In the brown trout, β-MRCs are associated with an accessory cell (615). Upon acclimation to seawater, the α-MRCs proliferate into the typical seawater teleost MRC, whereas the β-MRCs degenerate. Specific functional roles of α- and β-MRCs in freshwater teleosts have yet to be identified.

Recent studies using immunohistochemistry and cell isolation techniques have corroborated and extended some of Pisam and colleagues' findings. For example, immunohistochemical studies of Na^+-K^+-ATPase in guppy and chum salmon (*Oncorhynchus keta*) gills (682, 680) corroborated the proliferation of α-MRCs upon sea-

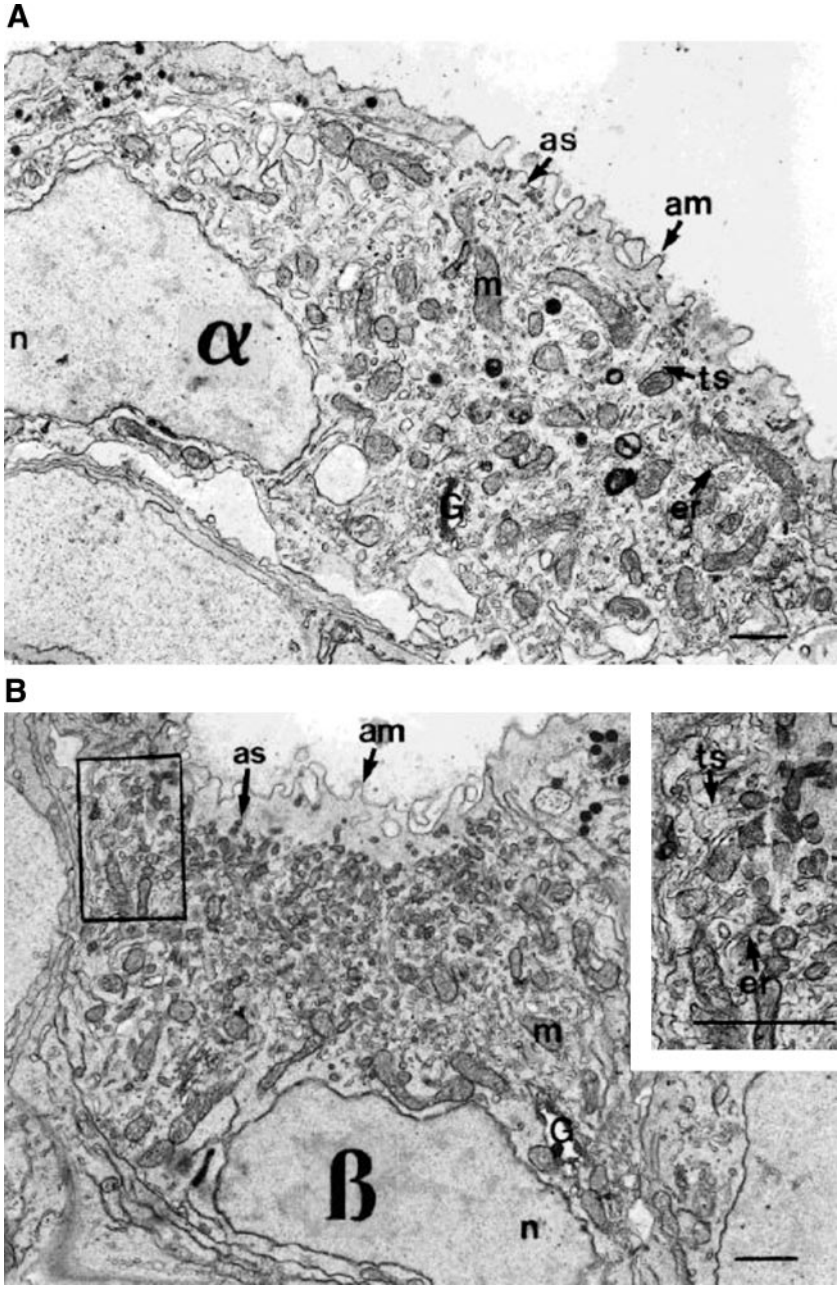

FIG. 8. Transmission electron micrographs of α- and β-MRCs from a freshwater teleost (brown trout, *Salmo trutta*). *A*: α-MRC with few, small apical structures (as) that are primarily located below the apical membrane (am) and a well-developed tubular system (ts) that associates with parts of the endoplasmic reticulum (er). *B*: β-MRC with many, large apical structures and a less-developed tubular system (see *inset*), compared with the α-MRC. In both panels, also note mitochondria (m), nuclei (n), and Golgi bodies (G). Bar = 1 μm. [Modified from Pisam et al. (615).]

water acclimation and also demonstrated that the α- and β-MRCs are rich and poor in Na^+-K^+-ATPase, respectively, which is consistent with the less-developed tubular system of β-MRCs. With the use of a Percoll density gradient and subsequent cell sorting to isolate MRCs from gill suspensions in Japanese eels (*Anguilla japonica*), two MRC populations have been identified in freshwater eels that correspond to the α- and β-MRCs, and a decreased percentage of β-MRCs (with a corresponding increased percentage of α-MRCs) has been described upon seawater acclimation (816, 817).

This cell sorting technique has been used more recently to isolate, purify, and characterize two populations of MRCs (based on binding to peanut lectin agglutinin) in the gill epithelium of freshwater rainbow trout (248, 262). The lectin-binding cells (PNA^+) have ultrastructural characteristics typical of teleost MRCs (e.g., a tubular system and tubulovesicular system), and the cells that did not bind lectin (PNA^-) are actually MR-PVCs (see above). Reid et al. (639) have referred to the PNA^+ MRCs and MR-PVCs as β- and α-MRCs, respectively, based on functional analogy to type B and type A intercalated cells of the mammalian nephron, respectively. However, it is not known if either of the above MRCs in the rainbow trout directly correspond to the morphological β- and α-MRCs described by Pisam and colleagues in other teleosts.

A recent ultrastructural and immunohistochemical study on the gills of a euryhaline killifish (*F. heteroclitus*) (353) has detailed the acute and chronic changes in MRCs associated with transfer from seawater to freshwater environments (Fig. 9). In this study, it was found that MRCs from seawater killifish first morphologically and immunochemically convert to freshwater MRCs and then are gradually replaced by newly generated freshwater MRCs. Among the acute changes noted were *1*) a transformation of a recessed, concave apical membrane with few microvilli to a protrusive, convex apical membrane with extensive microvilli, within 7 days of freshwater exposure; *2*) degeneration of ACs and multicellular complexes within 12 h of exposure to fresh water; and *3*) loss of apical CFTR immunoreactivity within 24 h of exposure to freshwater.

II) Elasmobranchs. Despite a few recent studies in the spiny dogfish (*Squalus acanthias*) (807, 809), the ultrastructure of elasmobranch MRCs in general has been little studied. All ultrastructural descriptions of elasmobranch MRCs have been conducted on primarily marine species, and the ultrastructure of MRCs in the gills of freshwater elasmobranchs has not been examined to date. Elasmobranch MRCs are usually found on the afferent edge of the filament and between lamellae and exist singly in the epithelium. However, numerous MRCs have been identified on gill lamellae of an elasmobranch living in fresh water (Atlantic stingray, *Dasyatis sabina*) (604, 605). The tight junctions between elasmobranch MRCs and adjacent PVCs are multistranded (Fig. 10), which suggests that the junctions are relatively impermeable to ions (807, 809). In elasmobranch MRCs, the tortuous basolateral tubular system seen in marine teleost MRCs is lacking. However, the basolateral membrane of elasmobranch MRCs does have moderate infoldings (Fig. 10), and they are likely the site of Na^+-K^+-ATPase expression (604, 807) in some MRCs and V-ATPase expression in other MRCs (605) (see sect. v*B1*).

The subapical cytoplasm of elasmobranch MRCs is characterized by a tubulovesicular system that contains

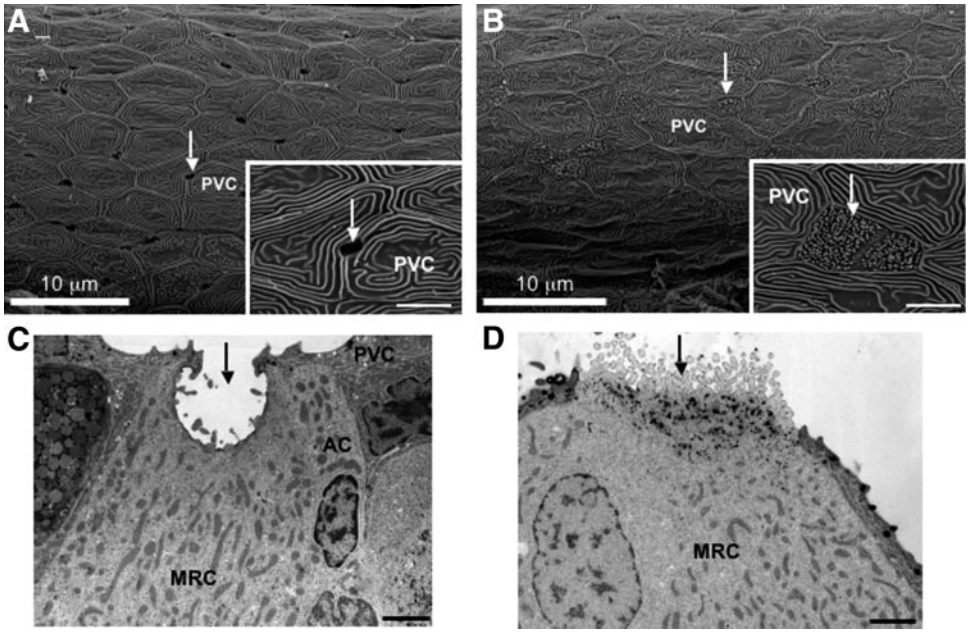

FIG. 9. Scanning electron and transmission electron micrographs of gill filaments from killifish in seawater (*A* and *C*) and killifish transferred from seawater to fresh water after 30 days (*B* and *D*). Note transformation of the apical region of MRCs (arrows) from a smooth, concave crypt that is recessed below the pavement cells (PVCs) (*A* and *C*) to a convex surface studded with microvilli that extend above the surrounding PVCs (*B* and *D*). Also note that an accessory cell (AC) is not associated with the MRC from fresh water-acclimated killifish (*C* and *D*) and that the distinct, whorl-like microridges on the surfaces of PVCs do not change with salinity (*A* and *C*). Bar = 1 μm, except where noted. [Modified from Katoh and Kaneko (352).]

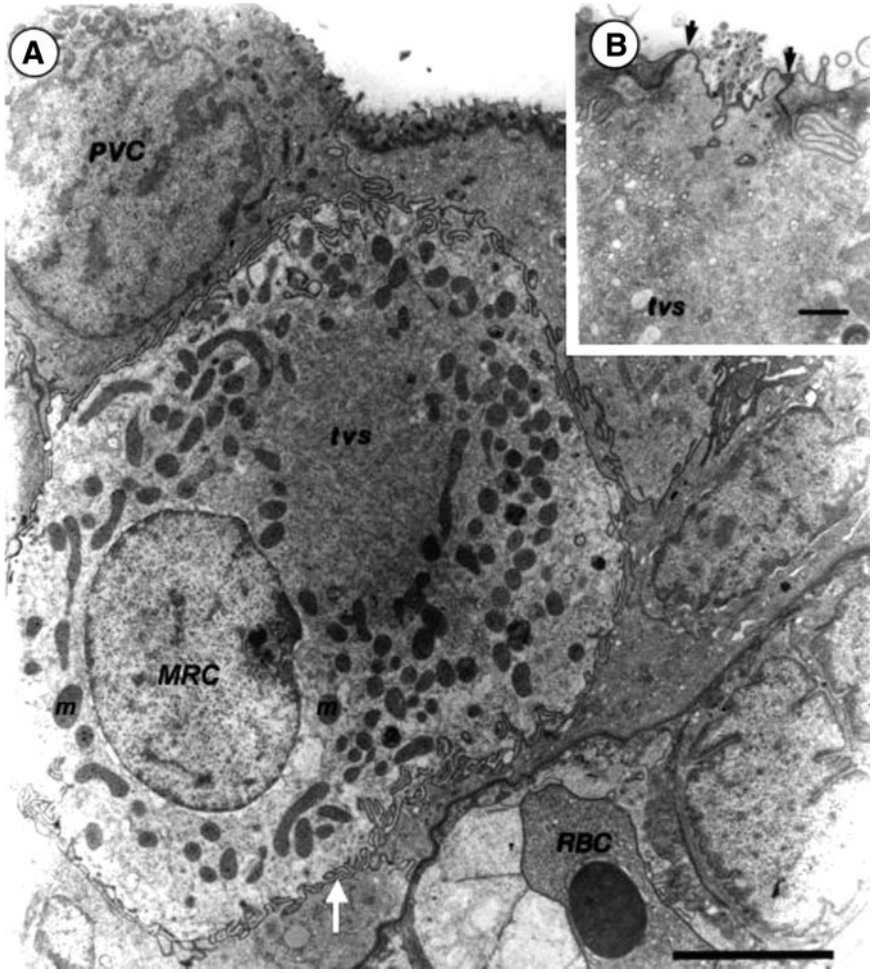

FIG. 10. Transmission electron micrographs of the gill epithelium from a seawater elasmobranch (spiny dogfish). In *A*, note the large size of the MRC with its numerous mitochondria (m), complex basolateral membrane infoldings (white arrow), and extensive tubulovesicular system (tvs), relative to the neighboring PVC. The apical membrane of this MRC is not visible, but *B* shows the apical surface of another MRC, which forms deep intercellular junctions (black arrows) with adjacent PVCs, and has an extensive subapical tubulovesicular system. In *A*, bar = 5 μm and RBC indicates a red blood cell. In *B*, bar = 0.5 μm. [Modified from Wilson et al. (809).]

numerous vesicles (Fig. 10), and the apical membrane is characterized by dense clusters of microvilli that can exist in different morphologies (127, 387, 807) (Piermarini and Evans, unpublished data) (Fig. 11). This may suggest that different functional MRC subtypes are present in the elasmobranch gill epithelium, and recent data suggest that at least two immunohistochemically distinct populations of MRCs exist in the Atlantic stingray (605, 607) and spiny

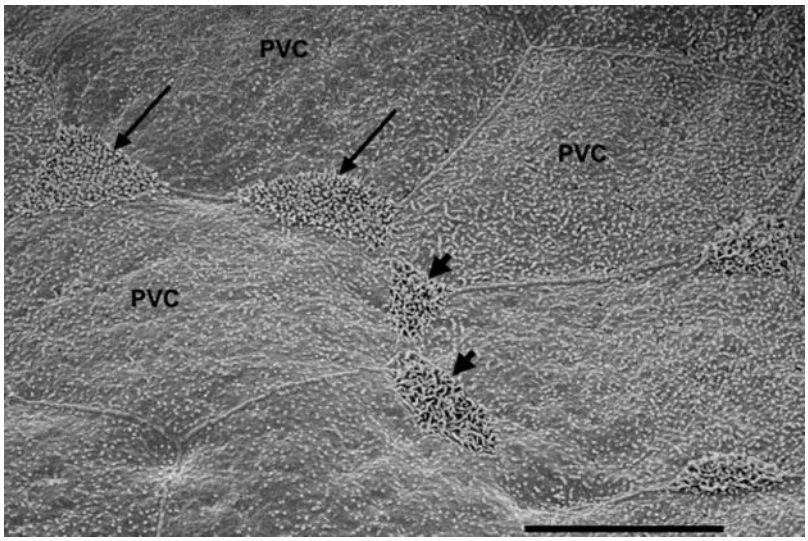

FIG. 11. Scanning electron micrograph of the gill epithelium from a freshwater elasmobranch (Atlantic stingray). Note the expansive, flat apical surface of PVCs that is sparsely populated with microvilli, relative to the constricted, uneven surfaces of MRCs that are characterized by dense clusters of microvilli (long arrows) and microplicae (short arrows). Bar = 10 μm. (From Piermarini and Evans, unpublished micrograph.)

dogfish (201) gills and that these cells are likely involved with distinct ion regulatory functions (see sects. v*B4* and v*C2*).

III) Agnathans. The ultrastructure of MRCs in agnathans varies considerably between hagfish and lampreys. In two hagfish species that have been examined (Atlantic hagfish, *Myxine glutinosa*; Pacific hagfish, *Eptatretus stouti*), MRCs are primarily found on the afferent edge of gill filaments (25, 94, 169, 432) (e.g., Fig. 6*B*). In contrast to marine teleost MRCs, hagfish MRCs exist singly in the epithelium and the tight junctions between MRCs and adjacent PVCs are extensive, which suggests they are impermeable to ions (26) (Fig. 12). Similar to marine teleost MRCs, hagfish MRCs have an extensive intracellular tubular system that is continuous with the basolateral membrane and is closely associated with mitochondria (25, 169, 432) (Fig. 12*B*). This tubular system is likely the site of Na^+-K^+-ATPase expression (see Refs. 32, 94), but high-resolution localization data are still lacking. The apical membrane of hagfish MRCs contains microvilli, and the subapical region of the cytoplasm has a tubulovesicular system with densely packed vesicles that fuse with the apical membrane (25, 169, 432) (Fig. 12*B*).

In the gills of adult, anadromous lamprey species (e.g., pouched lamprey, *Geotria australis*; sea lamprey, *Petromyzon marinus*; river lamprey, *Lampetra fluviatilis*) ultrastructural studies have identified two distinct types of MRCs that are associated with freshwater and seawater life-stages. When in fresh water, lamprey gills possess a MRC subtype that is only found in freshwater individuals (intercalated MRC or FW-MRCs) and another MRC subtype that is found in both freshwater and seawater individuals (chloride cells or SW-MRCs) (Fig. 13). The FW-MRCs are found in the interlamellar region and the afferent edge of filaments where they occur singly in the epithelium, intercalated between PVCs and SW-MRCs (29, 32). The basolateral membrane of FW-MRCs is not highly convoluted and is not associated with an elaborate tubular system (32, 507). The FW-MRC apical membrane is characterized by numerous microridges (515) that form distinct patterns (29, 32), and the subapical cytoplasm is rich in vesicles that can fuse with the apical membrane (32, 507, 515) (Fig. 13*A*). Additionally, freeze-fracture studies have found particles in the apical membrane of FW-MRCs that are associated with the presence of V-ATPase in other epithelia (32). Given these morphological properties and the occurrence of FW-MRCs only in freshwater lampreys, it has been suggested that this cell is involved with active ion uptake (32, 507).

The SW-MRCs (or chloride cells) of lampreys are primarily found in the interlamellar region, but also occur on the afferent edge of the filament. They are laterally compressed in the longitudinal plane of the filament and line up next to one another for almost the entire length of the interlamellar region (29, 31) (Figs. 13*B* and 14). Ultrastructurally, SW-MRCs share more similarities to marine teleost MRCs than to FW-MRCs of lampreys. For example, SW-MRCs have extensive basolateral membrane infoldings that form an intracellular tubular system, which closely associates with mitochondria (29, 31, 507, 847). Additionally, SW-MRCs have a subapical tubulovesicular system composed of vesicles. In contrast to marine teleost MRCs, the apical membrane of lamprey SW-MRCs does not form an apical pit, but it does have relatively dense or sparse clusters of microvilli compared with surrounding cells, depending on salinity (see below).

Ultrastructural differences exist between the SW-MRCs of freshwater and seawater lampreys. For example, the tight junctions between SW-MRCs in freshwater lampreys are extensive, but upon acclimation to seawater the junctions become less extensive and are presumably leaky to ions. The apical membrane morphology of SW-MRCs also varies with salinity (29). In freshwater lampreys the apical membrane surface area is restricted due to surrounding pavement cells that cover much of SW-MRC's apical surface, which results in a dense packing of apical microvilli on SW-MRCs (Fig. 14*A*). However, in seawater lampreys or freshwater lampreys recently accli-

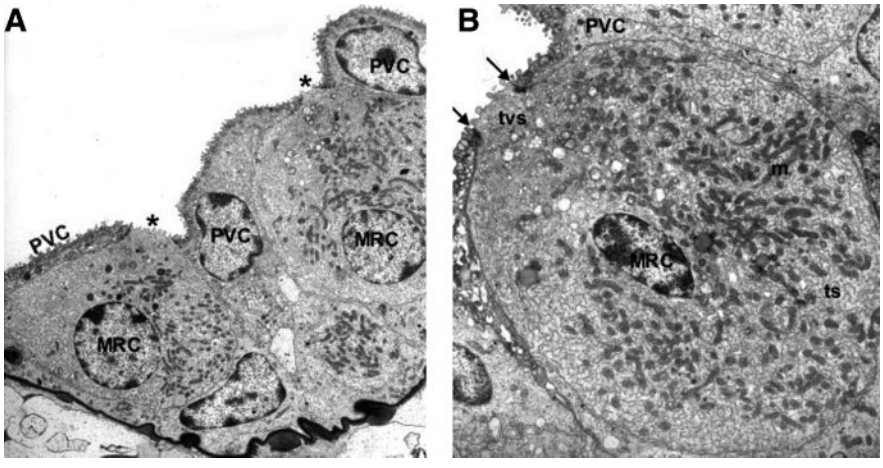

FIG. 12. Transmission electron micrographs of the gill epithelium from a hagfish (Atlantic hagfish). In *A* (×3,740), note the large MRCs intercalated between PVCs. Asterisks indicate exposed apical membranes of MRCs. (Unpublished micrograph generously provided by Dr. Helmut Bartels, München.) *B* (×6,630) shows a higher magnification micrograph of a MRC, with a basolateral tubular system (ts) that closely associates with mitochondria (m). Also note the subapical tubulovesicular system (tvs) and the deep intercellular junctions (black arrows) between the MRC and neighboring PVCs. [Modified from Bartels (25).]

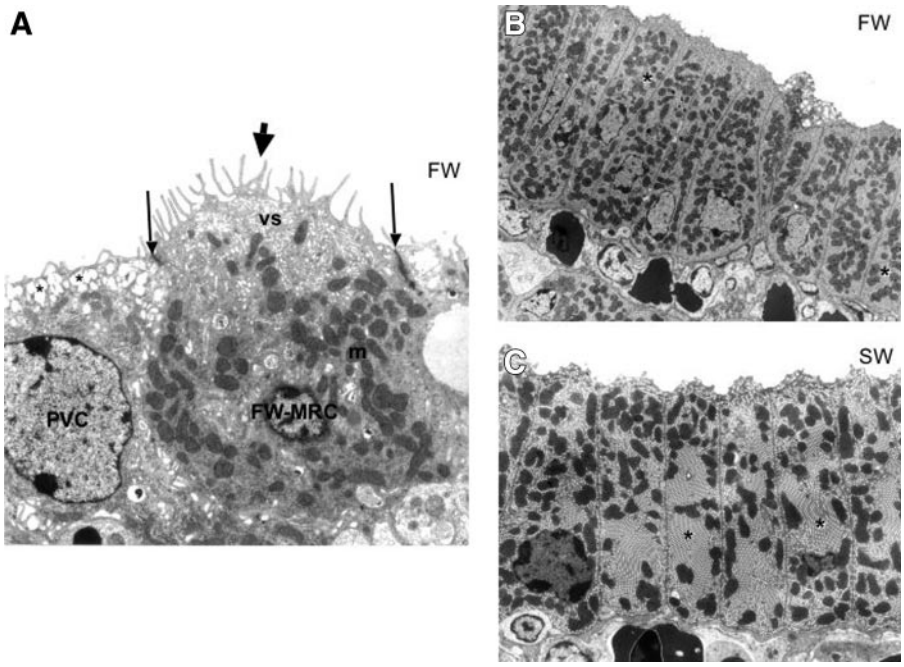

FIG. 13. Transmission electron micrographs of the gill epithelium from freshwater (FW) and seawater (SW) lampreys. *A* (×3,740) shows a PVC and a FW-MRC from a FW brook lamprey (*Lampetra appendix*). In the PVC, note the subapical secretory vesicles (asterisks) and relatively flat apical membrane. In the FW-MRC, note the numerous mitochondria (m), subapical vesicular system (vs), and extensive apical membrane microprojections (short arrow). Intercellular junctions between PVCs and FW-MRCs are extensive (long arrows). [Unpublished micrograph generously provided by Drs. Helmut Bartels and John Youson (Univ. of Toronto).] *B* shows cross sections through the gill filament of FW (×2,750) and SW (×5,000) pouched lampreys to show the SW-MRCs lined up next to one another. Note the more extensive and organized tubular system (asterisks) between mitochondria in the SW-MRCs of SW lampreys, relative to FW lampreys. (Unpublished micrograph generously provided by Dr. Helmut Bartels.)

mated to seawater, the PVCs appear to retract and expose more of the SW-MRCs apical surface, which becomes relatively flat with few microvilli (Fig. 14*B*). Finally, the tubular system of SW-MRCs appears to be more organized and robust in seawater lampreys compared with freshwater lampreys (31) (Fig. 13*B*). All of these differences are consistent with a more prominent and active SW-MRC in the gills of seawater lampreys, which suggests they are involved with active NaCl secretion.

III. INTERNAL STRUCTURE: VASCULAR AND NEURAL

A. Vascular

Blood enters the gills through afferent branchial arteries (ABAs), which receive the entire cardiac output via the ventral aorta. The blood that flows through an ABA feeds the two hemibranchs of an arch, where the blood is oxygenated at the lamellae of the filaments. Oxygenated blood from the filaments of an arch is collected by an efferent branchial artery (EBA), which directs blood to the dorsal aorta for systemic distribution. Upon closer examination of blood flow through the filaments, two distinct, yet interconnected, circulatory systems are apparent: the arterio-arterial and arteriovenous vasculature. Because of the large variations in the anatomy of these systems among species (especially in the arteriovenous vasculature), it is difficult to make generalizations. Therefore, the description that follows is not intended to be an all-encompassing description of the gill circulatory pathways, but aims to introduce some of the more important anatomical features of the vasculature that relate to functional aspects of the physiological processes discussed later in this review. For more thorough accounts of gill vascular anatomy, see References 386, 542, 547.

1. Arterio-arterial vasculature

The arterio-arterial vasculature is often referred to as the respiratory pathway, because it is responsible for the exchange of gases between a fish's blood and its environment. Because of the general similarities in this circulatory system among elasmobranchs, lampreys, and teleosts, these groups will be described together; the respiratory pathway of hagfishes will be described separately. In the arterio-arterial system, blood from an ABA feeds filaments on the hemibranchs of an arch via afferent filamental arteries (AFAs), which travel along the length of a filament (Fig. 15). In elasmobranchs and lampreys, the AFA regularly feeds the corpus cavernosum (CC) or cavernous body (Fig. 16), which is an extensive network of interconnected vascular sinuses in the afferent portion of the filament, e.g., the spiny dogfish (149a, 556); little skate, *Raja erinacea* (556); lesser-spotted dogfish, *Scyliorhinus canicula* (487); Endeavour dogfish, *Centrophorus scalpratus* (123); sparsely spotted stingaree, *Urolophus paucimaculatus* (153); Western shovelnose stingaree, *Trygonoptera mucosa* (153); and Arctic lamprey, *Lampetra japonica* (516, 517). Possible functions of the CC are as a hydrostatic support device for the gill filament

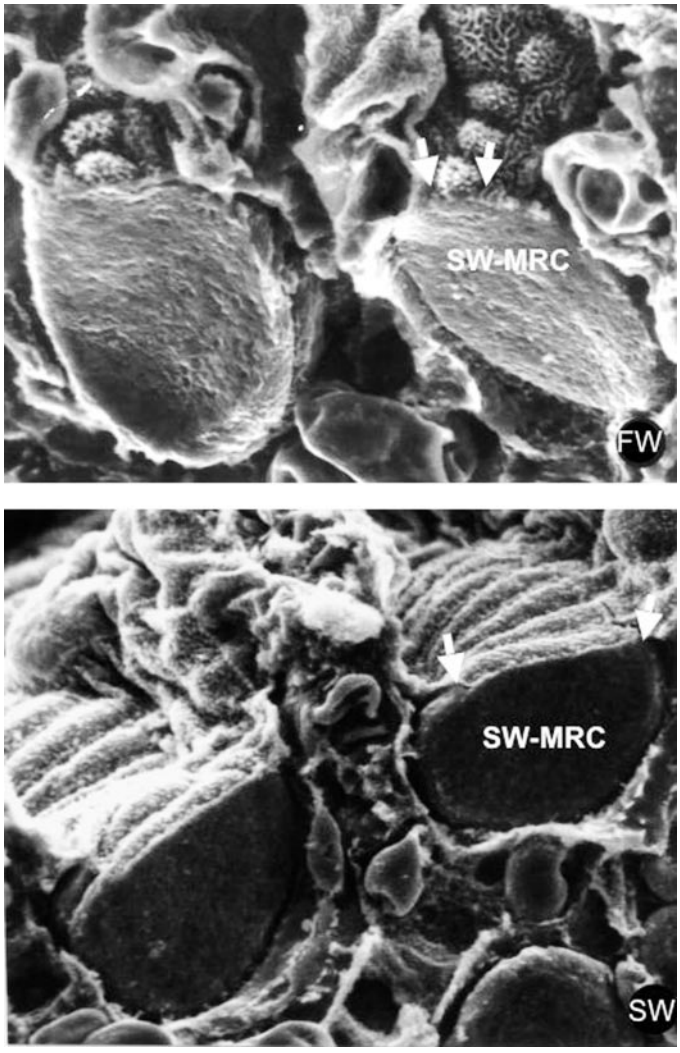

FIG. 14. Scanning electron micrographs of a fracture through the interlamellar region of a FW (×2,625) and SW (×1,950) pouched lamprey gill epithelium. Note the SW-MRCs stacked along the interlamellar regions and the constricted apical surface (between arrows) of SW-MRCs from the FW lamprey, relative to the expansive apical surfaces (between arrows) of SW-MRCs from the SW lamprey. [Modified from Bartels et al. (29).]

(123), a blood pressure regulator (516, 835), and a site of erythrocyte phagocytosis (516, 835).

In teleosts, a CC is not present, but in some species the AFAs contain blebs or dilations, e.g., the channel catfish, *Ictalurus punctatus* (51); European perch, *Perca fluviatilis* (244, 389); rainbow trout (389); and skipjack tuna, *Katsuwonus pelamis* (552). It has been hypothesized that these blebs are vestigial structures derived from the CC of elasmobranchs and agnathans (386) or are functional structures that play a role in dampening pulsatile blood flow (244).

Regularly spaced along the length of the CC (elasmobranchs and lampreys) or AFA (teleosts) are afferent lamellar arterioles (ALAs) that feed one or more lamella(e) (Figs. 15 and 17). Blood flow through the lamellae is consistent with "sheet flow" theory (208) and occurs through narrow vascular spaces delineated by pillar cells, termed the lamellar sinusoids (see sect. II*B2* and Figs. 5, 18, and 19). Pillar cells are composed of a central trunk that houses the main body of the cell and thin cytoplasmic extensions (flanges) that spread from the trunk and connect to adjacent pillar cells (see Refs. 547, 805), thereby lining the lamellar blood space (Fig. 19). In rainbow trout and another teleost, the roach (*Rutilus rutilus*), the lamellar sinusoids lead to deformation, deceleration, and redirection of erythrocytes during their passage through lamellae. This phenomenon is hypothesized to enhance lamellar gas exchange (528).

Contractile microfilaments are found in pillar cells of several teleosts (43, 523), lesser-spotted dogfish (835), and sea lampreys (847), and evidence for smooth muscle myosin has been detected in pillar cells from the Australian snapper (*Chrysophrys auratus*), a teleost (699). More recently, FHL5, a novel actin fiber-binding protein, has been localized in pillar cells in the Japanese eel, and its expression increased subsequent to acclimation to fresh water or volume expansion (499). These findings are suggestive of a contractile nature of pillar cells to possibly regulate blood flow through the lamellae (see Ref. 386). Recently, in the rainbow trout (724) and Atlantic cod (*Gadus morhua*; Ref. 711), in vivo videomicroscopy has provided direct evidence for regulation of blood flow through the lamellar sinusoids via pillar cell contraction (see sect. VII*B*).

In teleosts (e.g., Refs. 312, 523), elasmobranchs (e.g., Ref. 835), and lampreys (e.g., Refs. 517, 847), pillar cells also envelop extracellular collagen bundles, which connect to collagen fibrils of the basement membranes that underlie the lamellar epithelial sheets (Fig. 19). The collagen bundles may play a role in anchoring the opposing epithelial sheets to one another, and in maintaining structural integrity of a lamella during increases of blood pressure.

In addition to the lamellar sinusoids, blood flow across the lamellae may take a less convoluted route via the inner marginal channel (IMC) and outer marginal channel (OMC) (Figs. 18–20). The IMC is a discontinuous channel at the base of lamellae that is interrupted by pillar cells (Fig. 20*A*). This structure is embedded within the body of the filament body, which suggests it does not contribute to gas exchange. Moreover, evidence suggests that the IMC may allow blood to cross lamellae without exchanging gases with the respiratory medium, thus acting as a nonrespiratory shunt (571, 762). The IMC may also be involved with the collection and distribution of blood within the lamellar sinusoids (see Ref. 547).

In contrast to the IMC, the OMC is a low-resistance, continuous blood space at the periphery of lamellae (Fig. 20), lined by endothelial and pillar cells on its outer and

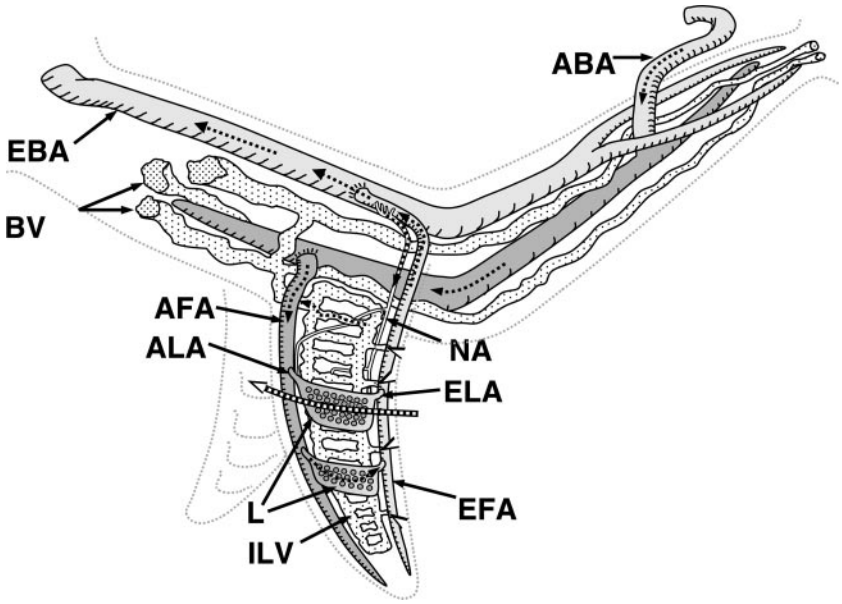

FIG. 15. Generalized schematic of blood flow through the major vessels of a gill arch and filament. *Arterio-arterial pathway*: blood travels (thin dotted arrows) from the afferent branchial artery (ABA) to an afferent filamental artery (AFA), which runs along the length of a filament. The AFA distributes blood to lamellae (L) via afferent lamellar arterioles (ALAs), and the lamellar blood is received by an efferent filamental artery (EFA) via efferent lamellar arterioles (ELAs). Blood flow through the lamellae is countercurrent to water flow across the lamellae (large white-on-black dotted arrow). The EFA returns blood from the filament to the efferent branchial artery (EBA), which distributes blood to the dorsal aorta for systemic circulation. *Arteriovenous pathway*: blood in the EFA can be distributed to the arteriovenous circulation, i.e., interlamellar vessels (ILVs), via postlamellar arteriovenous anastomoses (arrowheads) or by nutrient arteries (NA), which arise from the EFA or EBA. The ILVs are presumably drained by branchial veins (BV), which return blood to the heart. [Modified from Olson (547).]

inner boundaries, respectively (Fig. 19). Compared with the lamellar sinusoids, the OMC has a larger diameter, lower resistance, more erythrocytes, and greater surface area in contact with the ambient water (see Ref. 542); thus the OMC is hypothesized to be a preferential route of blood flow for shunting large volumes of blood around the lamellar sinusoids to maintain dorsal aortic blood pressure (see Ref. 516), and for possibly enhancing gas exchange (see Ref. 547). The latter hypothesis is supported by the recent data that demonstrate that the lamellae of skipjack tuna, a teleost with a high demand for oxygen, have multiple OMCs per lamella and a direct delivery of blood to the OMCs from the ALAs (552). In rainbow trout and Atlantic cod, the peptide hormone endothelin can redistribute lamellar blood flow to and from the OMC (711, 724) (see sect. VIII*B7*), which may allow these teleosts to alter the functional surface area of their lamellae. This may affect gas exchange as well as the diffusive gains and/or losses of water and osmolytes between a fish's blood and its environment.

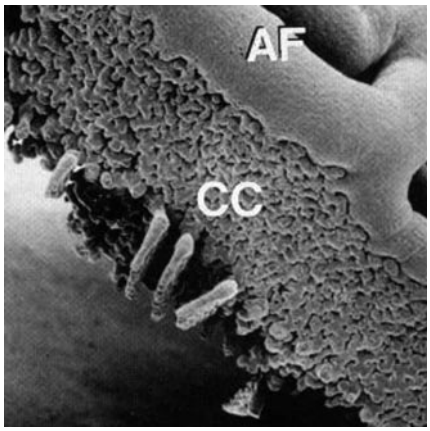

FIG. 16. Scanning electron micrograph (×45) of a vascular cast from the gills of an elasmobranch (Western shovelnose stingaree, *Trygonoptera mucosa*), showing the afferent filamental artery (AF) and corpus cavernosum (CC). [Modified from Donald (153).]

Blood is collected from the lamellae by efferent lamellar arterioles (ELAs), which are short vessels that drain into the efferent filamental artery (EFA) (Figs. 15 and 20*B*). The EFA travels along the entire length of a filament's efferent side, and blood flow through this vessel is counter to that of the AFA (i.e., toward the gill arch). The EFA also contains vessels that connect to the arteriovenous vasculature (see sect. III*A2*). Near the filament's origin on the arch, the EFA contains a distinctive muscular sphincter before it joins the EBA. This sphincter is highly innervated and may play a role in regulating lamellar blood flow and branchial resistance (see sect. VIII*B1*). The EBA collects oxygenated blood from the filaments and distributes the blood to the dorsal aorta for systemic circulation.

In the Atlantic and Pacific hagfishes, each gill pouch is supplied with blood by an ABA, which connects to an afferent circular artery (ACA) that encircles the excurrent duct of a pouch (25, 169, 432, 621) (Figs. 3*A* and 21). Branching off the ACA are afferent radial arteries (ARAs) that feed each gill fold (i.e., filament) in a pouch. An ARA connects to cavernous tissue that permeates the afferent portion of the filament and is comparable to the CC of elasmobranchs and lampreys. At the efferent edge of the cavernous tissue, ALAs arise that provide blood to the highly folded gill lamellae. Interestingly, the complex folding pattern of the hagfish lamellae results in numerous OMCs (see Ref. 169).

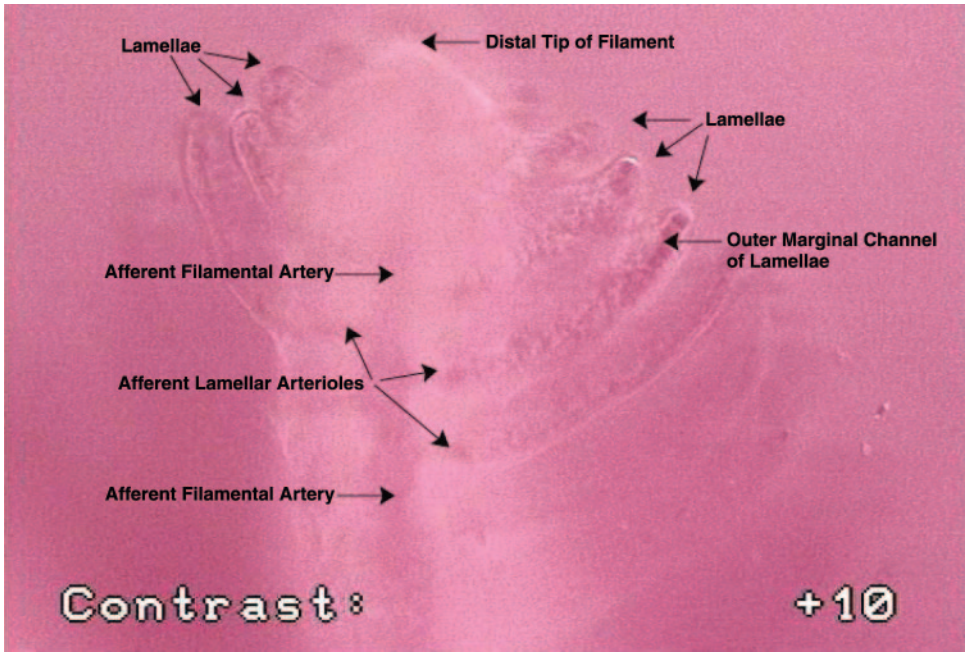

FIG. 17. Video of blood flow through the afferent filamental artery and a series of afferent lamellar arterioles leading into lamellae of a single filament in the gill of the American eel, *Anguilla rostrata*. Go to http://physrev.physiology.org/cgi/content/full/00050.2003/DC1.

Similar to other fishes, pillar cells within the lamellar folds of hagfish establish the lamellar sinusoids, and, like other fishes, the pillar cells contain microfilaments (169, 432). Hagfish pillar cells can exist singly and envelope extracellular collagen bundles in a similar manner to other fishes (169), or pillar cells can occur in clusters of two or more cells that encircle extracellular collagen bundles (169, 432). Intercellular junctions connect adjacent pillar cells in the Atlantic hagfish, which may suggest a degree of functional synchronization between the cells, possibly to influence lamellar perfusion (27, 169). These intercellular junctions are also found in the pillar cells of lampreys (30) but have not been found in any other fishes.

Blood in the lamellar blood space of hagfishes is drained by ELAs that empty into an efferent cavernous tissue. This cavernous tissue is less complex than its afferent counterpart and feeds an efferent radial artery (ERA). The ERA then joins with an efferent circular artery (ECA) that encircles the pouch's incurrent duct. Blood

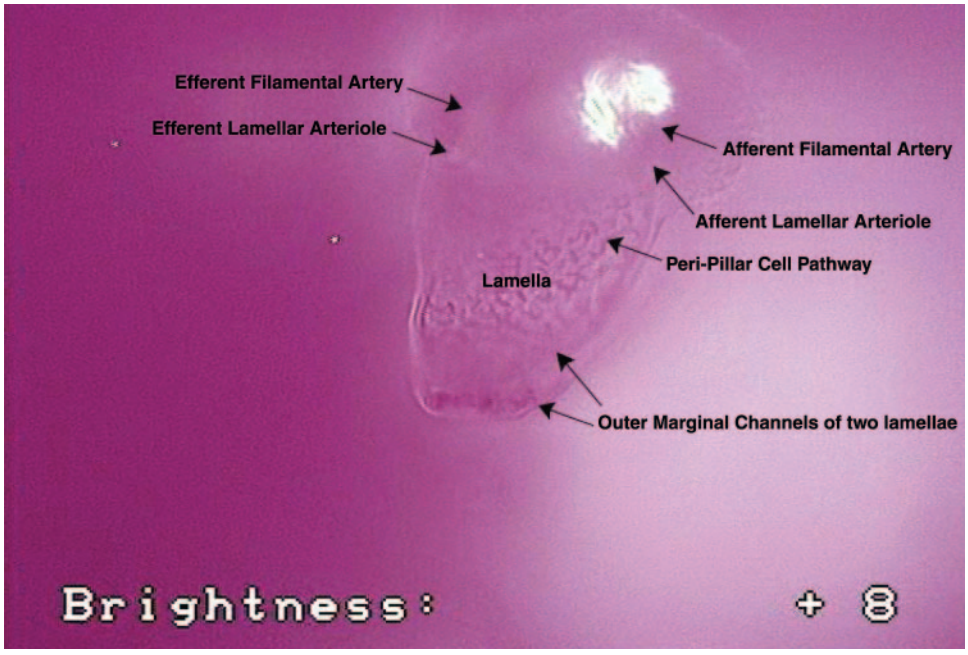

FIG. 18. Video of flow of blood through a single lamella in the gill of the American eel, *Anguilla rostrata*. Go to http://physrev.physiology.org/cgi/content/full/00050.2003/DC1.

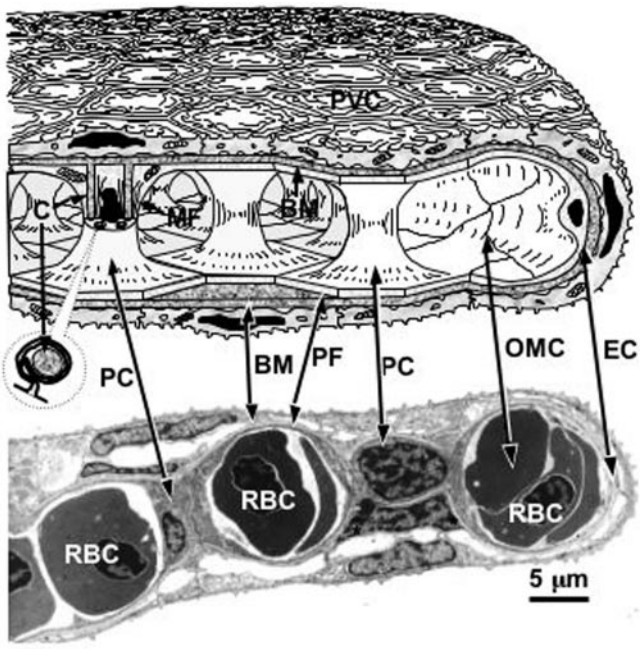

FIG. 19. Schematic and transmission electron micrograph of a cross section through an outer portion of a teleost (rainbow trout) lamella. Note the spool-shaped pillar cells (PC) with their cytoplasmic flanges (PF) that line the lamellar blood spaces, which are filled with red blood cells (RBC) in the micrograph. The pillar cells envelope extracellular bundles of collagen (C), which connect to the basement membrane (BM) that underlies the lamellar epithelium (e.g., PVC). Within the pillar cells are microfilaments (MF) that may be involved with pillar cell contraction (see text). The outer marginal channel (OMC) is formed by pillar cells on its inner boundary and endothelial cells (ECs) on its outer boundary. [Modified from Olson (547).]

from the ECA is returned to systemic circulation by two EBAs (25, 169, 432, 621) (Figs. 3 and 21).

2. Arteriovenous vasculature

The arteriovenous vasculature is often referred to as the nonrespiratory pathway. Although the exact function of this system is not known, it is likely involved with providing nutrients to the filament epithelium and the underlying supportive tissues of the gill filaments and may also provide a means for filamental blood to enter the venous circulation without traversing lamellae (see below). The arteriovenous pathway may be considered a component of the systemic circulation (e.g., Refs. 487, 547) because it is primarily supplied with postlamellar blood. Due to general similarities in this circulatory system among elasmobranchs and teleosts, these groups will be described together; the arteriovenous pathway of lampreys and hagfishes are each unique and thus are described separately.

The core of the arteriovenous network in elasmobranchs and teleosts is composed of a highly ordered series of saclike vessels arranged like a ladder. The "rungs" of the ladder are termed the interlamellar vessels (ILVs), which lie underneath the interlamellar epithelium and run parallel to the lamellae (Figs. 20*A* and 22). The "legs" of the ladder are termed the collateral vessels or sinuses, which flank and connect to the afferent and efferent boundaries of the ILVs, and run parallel to the length of the filament (Fig. 22). The ILVs and their collateral vessels connect to other venous and sinus systems within the filament that return blood to the base of the filament and eventually to the heart (386, 547).

In the literature, the ILVs are often collectively referred to as the central venous sinus (CVS), because descriptions of the ILVs morphologies vary among fishes and are conflicting. For example, vascular casts by some researchers in teleost and elasmobranchs found that the ILVs primarily appeared to be a continuous, singular, and amorphous sinus below the interlamellar epithelium that ran along the length of the filament, with little secondary

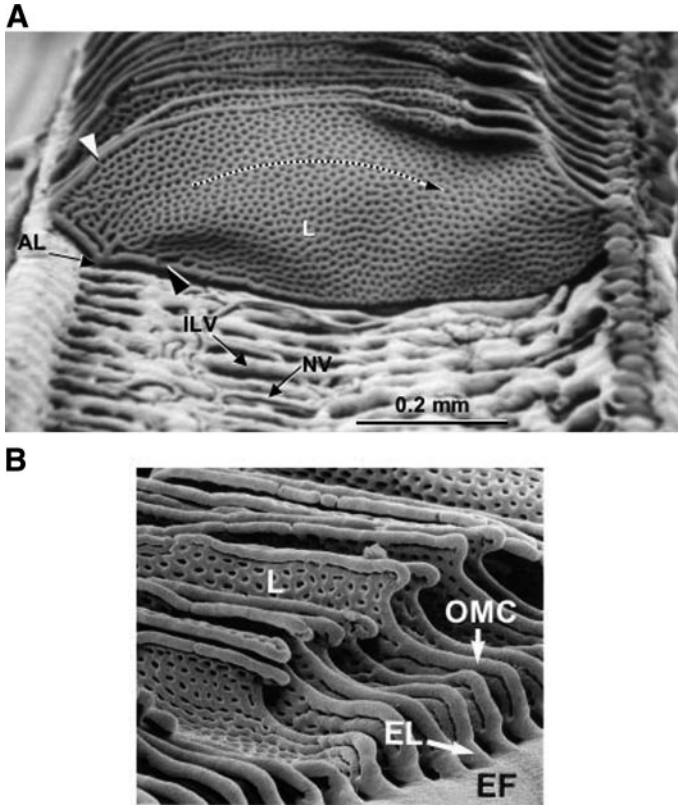

FIG. 20. Scanning electron micrographs of vascular casts from gill filaments of a teleost and an elasmobranch. *A*: a cast of a filament from a teleost (rainbow trout) with some lamellae (L) removed to show the underlying arteriovenous vasculature. Note the afferent lamellar arteriole (AL), inner marginal channel (black arrowhead), outer marginal channel (white arrowhead), an interlamellar vessel (ILV), and a nutrient vessel (NV). Dotted arrow indicates direction of lamellar blood flow. Holes in the lamellae correspond to the position of pillar cells. [Modified from Olson (547).] *B* (×125): a cast of a filament from an elasmobranch (sparsely spotted stingaree, *Urolophus paucimaculatus*) to show the efferent portion of lamellar blood flow. Note the outer marginal channel (OMC), efferent lamellar arteriole (EL), and efferent filamental artery (EF). [Modified from Donald (153).]

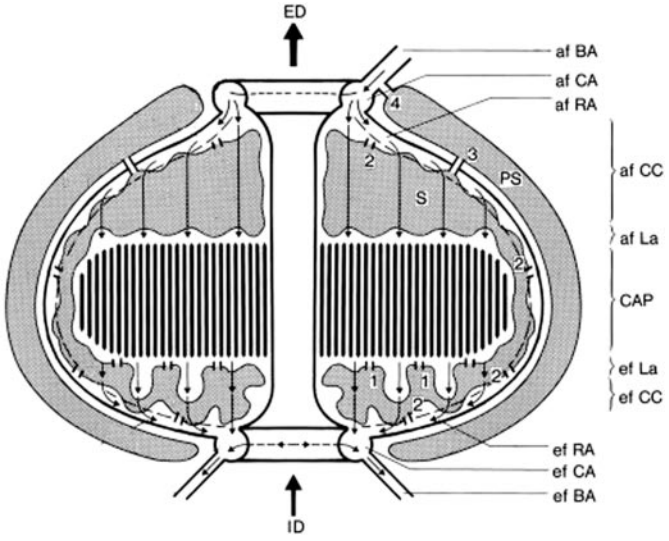

FIG. 21. Schematic of arterio-arterial and arteriovenous vasculature in the gill pouch and filament of hagfishes. The arterio-arterial vasculature includes afferent (af) and efferent (ef) branchial arteries (BA), circular arteries (CA), radial arteries (RA), cavernous tissue (CC), and lamellar arterioles (La). Also noted are the lamellar sinusoids or capillaries (CAP). The arteriovenous vasculature includes the peribranchial sinus (PS) and a sinusoid system (S). The numbers *1*, *2*, and *4* indicate sites of arteriovenous anastomoses. Number *3* marks an anastomosis between the venous sinusoid system and the venous peribranchial sinus. Thin arrows indicate direction of blood flow, and thick arrows indicate direction of water flow. [Modified from Elger (169).]

structure, e.g., the rainbow trout (389); European perch (389); European eel, *Anguilla anguilla* (389); smooth toadfish, *Tetractenos glaber* (124); spiny dogfish (149a); and Endeavour dogfish (123). In contrast, vascular casts by other researchers in some of the same and other teleost and elasmobranch species determined that the ILVs were primarily arranged like rungs on a ladder (see above), e.g., the channel catfish (51); spiny dogfish (556); skipjack tuna (552); walking catfish, *Clarias batrachus* (555); Asian catfish, *Heteropneustes fossilis* (561); climbing perch, *Anabas testudineus* (560); lesser-spotted dogfish (487); spiny dogfish (556); little skate (556); sparsely spotted stingaree (153); and Western shovelnose stingaree (153).

Because the morphology of these vessels in the rainbow trout is sensitive to the perfusion pressure of the vascular casting agent, the latter description is likely the true structure of the ILVs (540). Therefore, the continuous, amorphous morphology of the ILVs described by some researchers was probably an artifact of superphysiological perfusion pressures, which greatly distend the vessels and create the appearance of a singular sinus. Regardless, the findings of the above-mentioned studies suggest that the ILVs are pliable vessels with an ability to change their structure, and possibly their function, in response to changes in blood pressure.

Blood is supplied to the arteriovenous vasculature by at least three sources (543, 547) (Fig. 22): *1*) prelamellar arteriovenous anastomoses (AVAs), *2*) postlamellar AVAs, and *3*) filamental nutrient vessels (see below). Prelamellar AVAs connect the AFA, ALA, or CC to the ILVs. These AVAs are not universal among teleosts and elasmobranchs but have been detected in several species, e.g., channel catfish (51), smooth toadfish (124), European eel (389), Endeavour dogfish (123), and spiny dogfish (149a, 556). In contrast, postlamellar AVAs (also referred to as feeder vessels) that connect the EFA and/or ELA to the ILVs occur much more frequently than prelamellar AVAs and have been detected in the vast majority of teleost and elasmobranch species examined to date (see Refs. 386, 542, 547). It is important to note that blood in the ILVs of teleosts is often characterized by a lower hematocrit than blood found in the arterio-arterial circulation [e.g., the rainbow trout (322, 541), channel catfish (541), and black bullhead (*Ictalurus melasis*; Ref. 541)]. These findings suggest that the prelamellar and postlamellar AVAs may be sites of plasma skimming (322, 541), which emphasizes the nonrespiratory function of the ILVs.

The demonstration of both prelamellar and postlamellar AVAs in some species was consistent with early experimental data (644, 708) that suggested the ILVs may function as a putative lamellar bypass for blood, i.e., a pathway for blood to pass from the AFA (or CC) to the EFA without traversing the lamellar sinusoids. However, this hypothesis was largely refuted (see Refs. 124, 310, 487), and it is more likely that prelamellar AVAs act as a

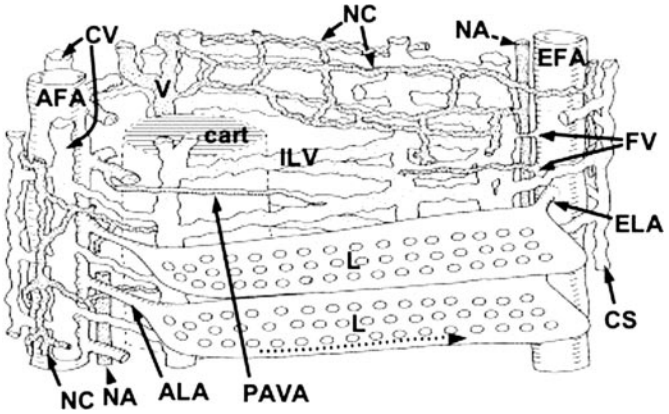

FIG. 22. Schematic of arterio-arterial and arteriovenous vasculature in the gill filament of teleosts. Nonstippled vessels represent the arterio-arterial vasculature, i.e., afferent filamental artery (AFA), afferent lamellar arterioles (ALA), lamella (L), efferent lamellar arterioles (ELA), and efferent filamental artery (EFA). Dotted arrow indicates direction of lamellar blood flow. Stippled vessels represent the arteriovenous vasculature, i.e., interlamellar vessels (ILV), collateral vessels (CV) and sinuses (CS), prelamellar arteriovenous anastomoses (PAVA), postlamellar arteriovenous anastomoses or feeder vessels (FV), nutrient arteries (NA), nutrient capillaries (NC), and filament veins (V). Also note filamental cartilage (cart) that runs along the length of the filament on the afferent edge. [Modified from Olson (547).]

lamellar shunt (310, 556), i.e., a pathway for blood to pass from the AFA (or CC) to the ILVs for venous return to the heart without traversing the lamellar sinusoids.

Another source of blood to the ILVs is through nutrient vessels, which compose another meshwork of vasculature within the core of the gill filament termed the nutrient system (NS). It is unclear, however, whether the NS represents a distinct subsystem of the arteriovenous vasculature (see Refs. 543, 547) or is simply another source of blood to the ILVs, like AVAs (see Ref. 386). Given the infrequent observations of nutrient vessels connecting to the ILVs (see Ref. 543), it is more likely that the NS is in fact a discrete component of the arteriovenous vasculature that supplies nutrients to the underlying tissues of the filaments and arches (543, 547) and is not just a supplier of blood to the ILVs.

Arterioles and arteries that bud off the EFA and EBA primarily supply blood to the NS. In teleosts, it is common for short, but often tortuous, small-diameter arterioles to branch off the EFA and EBA, and then anastomose into larger diameter nutrient arteries, e.g., rainbow trout (389), European perch (389), climbing perch (560), walking catfish (555), and skipjack tuna (552). In elasmobranchs and some teleosts, large-diameter nutrient arteries arise directly from the EFA, e.g., spiny dogfish (556), little skate (556), smooth toadfish (124), and channel catfish (51). Regardless of how nutrient arteries originate, they travel along the filament's length alongside the EFA or may bifurcate and traverse towards the afferent side of the filament (e.g., Fig. 20*A*) where they branch into nutrient arteries that run medially along the filament's long axis, or into nutrient arteries that run adjacent to the AFA (Fig. 22). Additionally, smaller vessels may branch off the nutrient arteries towards the center of the filament and anastomose with one another to form a weblike arrangement of nutrient arterioles and capillaries that surround, and may anastomose with, the ILVs (547) (Fig. 22).

Given the similar location and trajectories of the ILVs and some vessels of the NS, it is often difficult to discern one system from the other with vascular casts, especially in cases when the ILVs are distended and envelop parts of the NS (see Ref. 547). However, there are unique morphological features of the vessels that identify these two systems. For example, the vessels of the NS tend to have typical artery/arteriole morphology with a robust endothelium and many erythrocytes, whereas vessels of the ILVs have a more pliable venouslike morphology with a meager endothelium and few erythrocytes (542, 543, 547) (Fig. 23). Regardless, both the ILVs and NS are drained by venous systems within the filament that return blood to the base of the filament and eventually to the heart (386, 547).

In lampreys, the arteriovenous vasculature has not been extensively studied. However, a structure in a similar anatomical location to teleost and elasmobranch ILVs, termed the "axial plate lacunae," has been described in the Arctic lamprey (517). This structure is openly connected to the CC and EFA without AVAs (517) and appears to function more like an IMC of a teleost lamella (see above) than an ILV (see Ref. 517). The major venous drainage structures in lamprey filaments are the peribranchial sinuses (PBS), which are located at the basal portion of hemibranchs, on both sides of an interbranchial septum. Blood is supplied to the PBS by filament veins, which run parallel to and alongside the AFA. These veins receive blood from the AFA via AVAs that are analogous to prelamellar AVAs of elasmobranchs and teleosts; filament veins then feed the PBS via anastomosing channels along the vein's length (517). The PBS is also supplied with postlamellar blood via AVAs in the EFA that are analogous to postlamellar AVAs of elasmobranchs and teleosts. From the PBS, blood eventually returns to the heart via other sinuses and veins (517). It is not known if nutrient vessels are found in the arteriovenous vasculature of lampreys.

In hagfishes, the arteriovenous vasculature also has not been extensively studied, but evidence suggests it

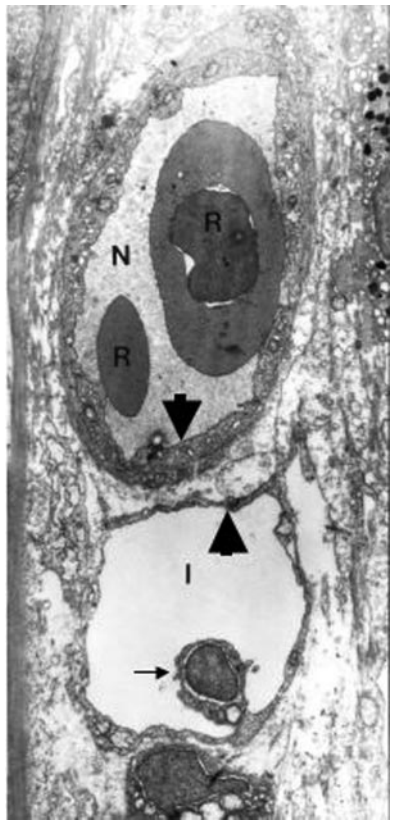

FIG. 23. Transmission electron micrograph (×4,400) of a section through a nutrient (N) and interlamellar (I) vessel. Note the thicker endothelium (large arrows) and occurrence of red blood cells (R) in the nutrient vessel, compared with the interlamellar vessel. Small arrow indicates a nucleus bulging from an endothelial cell. [Modified from Olson (543).]

shares some similarities with that of the other fish groups. Similar to lampreys, a PBS is present, which surrounds the gill pouch (25, 169, 621) (Fig. 21). In the Atlantic hagfish, a sinusoid system, which is analogous to the ILVs of elasmobranchs and teleosts, surrounds the afferent and efferent portions of the filaments, but not the lamellae (169) (Fig. 21). In the afferent and efferent cavernous tissues, AVAs supply prelamellar and postlamellar blood, respectively, to the sinusoid system (169) (Fig. 21). Other AVAs also have been noted between the sinusoids and the radial vessels, but it is not clear if nutrient vessels are present (169, 621).

B. Neural

Branchial nerves in fishes are derived from the VIIth (facial), IXth (glossopharyngeal), and Xth (vagus) cranial nerves, each divided into posttrematic, motor rami, and pre- and posttrematic, sensory rami. In a given gill arch, posttrematic rami from one nerve may mix with pretrematic rami from an adjacent nerve (see Fig. 24 and Ref. 728). Gill arches other than the first are generally innervated by the vagus nerve (e.g., Ref. 529). There are substantial data supporting sensory and motor function for the teleost branchial autonomic innervation (see below); however, similar functional data for elasmobranchs and agnatha have not been published, despite the fact that at least adrenergic and cholinergic receptors have been functionally described in the branchial vasculature of both elasmobranchs (e.g., Refs. 99, 152, 486) and hagfish (16, 228, 720).

Histochemical studies have described catecholamine-containing fibers associated with AFAs and ALAs in a variety of teleost species, and similar fibers associated with ELAs and EFAs in some species (e.g., Ref. 155), but the lamellae do not appear to be innervated (e.g., Ref. 151). On the other hand, catecholamine-containing fibers have been described around the ILV in a number of teleost species (e.g., Refs. 150, 151, 161, 163). Cholinergic-type fibers have been described in the gills but are only associated with the efferent vasculature, specifically with a prominent sphincter in the basal third of the efferent filamental artery (18), which also appears to contain serotonergic fibers (19). Serotonergic neuroepithelial cells (NECs) have also been described on the gill filament in a variety of species (e.g., Ref. 853), as have fibers and NECs that contain immunoreactive nitric oxide synthase (e.g., Ref. 851). Specific roles for the neurosecretory products of these fibers and cells are discussed in sections IV*D* and VIII*B*.

IV. GAS EXCHANGE AND GAS SENSING

A. Introduction

Because the branchial epithelium is essentially a monolayer of mixed cells (mostly squamous PVCs on the lamellar surface), and the medium irrigates the external surface countercurrent to the internal perfusion (see sect. II), the fish gills appear to be ideal for gas exchange. The ratio of irrigation (ca. 5,000–20,000 ml $\cdot$ kg^{-1} $\cdot$ h^{-1}) to perfusion ($\dot{V}_g/\dot{Q}$) is generally of the order of 1–8 but may approach 50 in very active, pelagic fishes such as tuna (by substantial increases in $\dot{V}_g$), and oxygen extraction efficiencies vary from 50 to 90% (69), higher than those described for mammals. The metabolic cost of gill gas exchange is substantial, however, because of the low solubility of oxygen in aqueous solutions (~7 ml/l; 3% that in air) and the high viscosity (800 times air) and density (60 times air) of the medium. Thus the cost of routine ventilation may be 10% of the oxygen uptake ($\dot{M}O_2$), and it may approach 70% of $\dot{M}O_2$ during exercise, when ven-

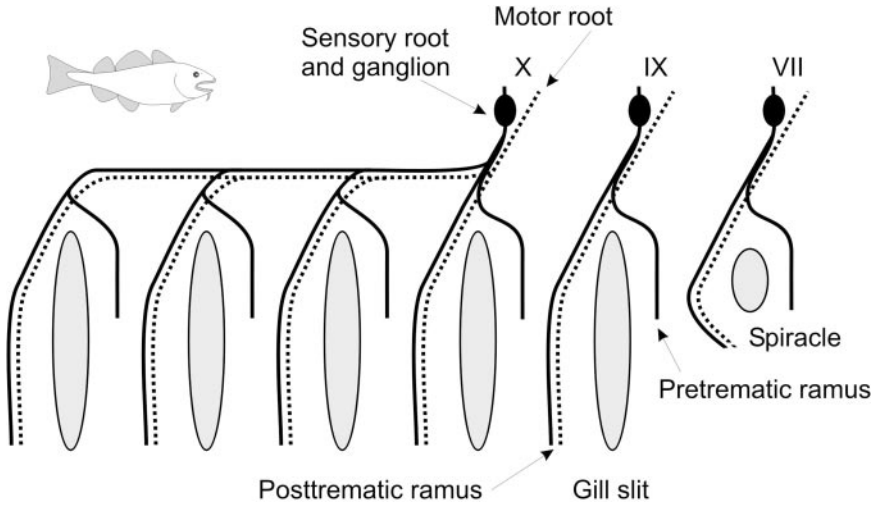

FIG. 24. Outline of the components of the branchial nerves of fish, showing facial (VII), glossopharyngeal (IX), and vagal (X) rami in relation to the gill slits. The orientation is cephalic to the right, as indicated by the outline of the cod. [From Sundin and Nilsson (728).]

tilation volumes may triple (reviewed in Ref. 593). Pelagic fish species reduce the cost of ventilation by utilizing forward movement and open mouths to drive water across the gills (termed ram ventilation; Ref. 648).

B. Lamellar Gas Exchange

The actual site of gas exchange is assumed to be the lamellar epithelium, but no direct measurements of lamellar versus filamental gas permeabilities have been published. The lamellar surface is likely the primary site, because various studies have shown a correlation between lamellar surface area and respiratory needs (e.g., benthic vs. pelagic) or measured rates of oxygen uptake (reviewed in Ref. 593). In fact, recruitment of distal lamellae is generally considered to be a means of increasing gas exchange, although this hypothesis is largely based on two studies that demonstrated that only 60–70% of lamellae in rainbow trout and lingcod (*Ophiodon elongatus*) were perfused at rest (54, 207). In the trout, lamellar perfusion was measured by the distribution of vitally stained erythrocytes after infusion into trout via a cannula in the ventral aorta; in the lingcod, the distribution was measured by perfusion of isolated branchial arches with saline containing Prussian Blue dye. In both cases, it is unclear if lamellar perfusion mimicked the in vivo condition. Our own videography of blood flow through the gills of American eels (*Anguilla rostrata*) or longhorn sculpin (*Myoxocephalus octodecimspinosus*) indicates that even distal lamellae are perfused in anesthetized individuals, so lamellar recruitment may not be a general phenomenon (e.g., Fig. 17). The respiratory (lamellar) surface area is generally 0.1–0.4 m^2/kg but may be as high as 1.3 m^2/kg in tuna (equivalent to human) or as low as $<$0.1 m^2/kg in species that utilize the swim bladder (or other accessory air-breathing organs) for oxygen uptake (593). The respiratory medium (water) to blood diffusion distance (i.e., the thickness of lamellar epithelium) also varies with life-style, ranging from 10 μm in aerial breathers to $<$1 μm in tuna (equivalent to human; Ref. 593). It is not clear if this dimension can be dynamically regulated, but thinning of the epithelium might possibly occur if an increase in cardiac output distends lamellae. In addition, alteration in the distribution of gill cells (MRC vs. PVC) under hypoxic or hypercapnic conditions may alter the diffusion distance and thereby impact gas exchange (reviewed in Ref. 589).

C. Perfusion Versus Diffusion Limitations

The uptake of oxygen across the fish gills appears to be perfusion limited, but all data published to date are from a single species, the rainbow trout, so more data are needed to support the general statement (589). Nevertheless, the fact that rainbow trout arterial oxygen content (Pa_{O_2}) did not vary appreciably with changes in perfusion produced by alteration of blood volume argues for perfusion limitation (149), as does the lack of any alteration in Pa_{O_2} subsequent to the ligation of two gill arches (producing a 30% reduction in gill surface area; Ref. 333). In contrast, excretion of CO_2 by the rainbow trout gill epithelium appears to be diffusion limited, at least in teleosts (e.g., Ref. 255) (Fig. 25). Thus alterations in blood volume in the trout were correlated with alterations in blood CO_2 (Pa_{CO_2}; Ref. 149), and in rainbow trout a 30% reduction in gill surface area was accompanied by a significant elevation of Pa_{CO_2} (333). For a more complete discussion of the data supporting perfusion limitation for O_2 uptake and diffusion limitation for CO_2 excretion across the fish gills, see Reference 589. The diffusion limitation to CO_2 excretion in teleosts is apparently secondary to the need for erythrocyte Cl^-/HCO_3^- exchange (via band 3) at the gills, the only means of CO_2 production at the gas exchanger because of the lack of endothelial carbonic anhydrase (CA), as is found in the mammalian lung alveoli (e.g., Ref. 591). The recent findings that injection of CA offset the increased Pa_{CO_2} seen in the rainbow trout after an increase in cardiac output (149) or decrease in gill surface area (333) support this conclusion. It is unclear if the constraints of CO_2 excretion are the same in most fishes, but it is apparent that the dynamics of CO_2 excretion across the gills in at least three other fish taxa may be different. For instance, elasmobranchs do have vascular CA (253, 256), hemoglobin-less ice fishes lack erythrocytes and therefore band 3 (430), and lampreys are unique among vertebrates in the lack of a functional anion exchanger in the erythrocyte (761). One might expect CO_2 excretion in these three groups to be perfusion limited, but this hypothesis has not been tested to date. Despite these theoretical constraints on CO_2 excretion, the fish gills appear to be hyperventilated with regard to CO_2, because the blood Pv_{CO_2} (~1–3 mmHg in teleosts, elasmobranchs, and agnatha; Ref. 761), and bicarbonate concentration (~4 mM) is far below that found in mammals, producing a plasma pH (~7.8) that is substantially higher than that measured in mammalian plasma. The impact of these values on acid-base regulation is discussed in section VI*B*.

D. Chemoreceptors

1. O_2 sensors

Because the branchial arch arteries of fishes are the evolutionary precursors of the carotid and pumonary arteries in mammals (402), one might suspect that the gills would be the site of afferent sensory neurons whose efferent motor output may control cardiovascular and respiratory responses to environmental and/or systemic

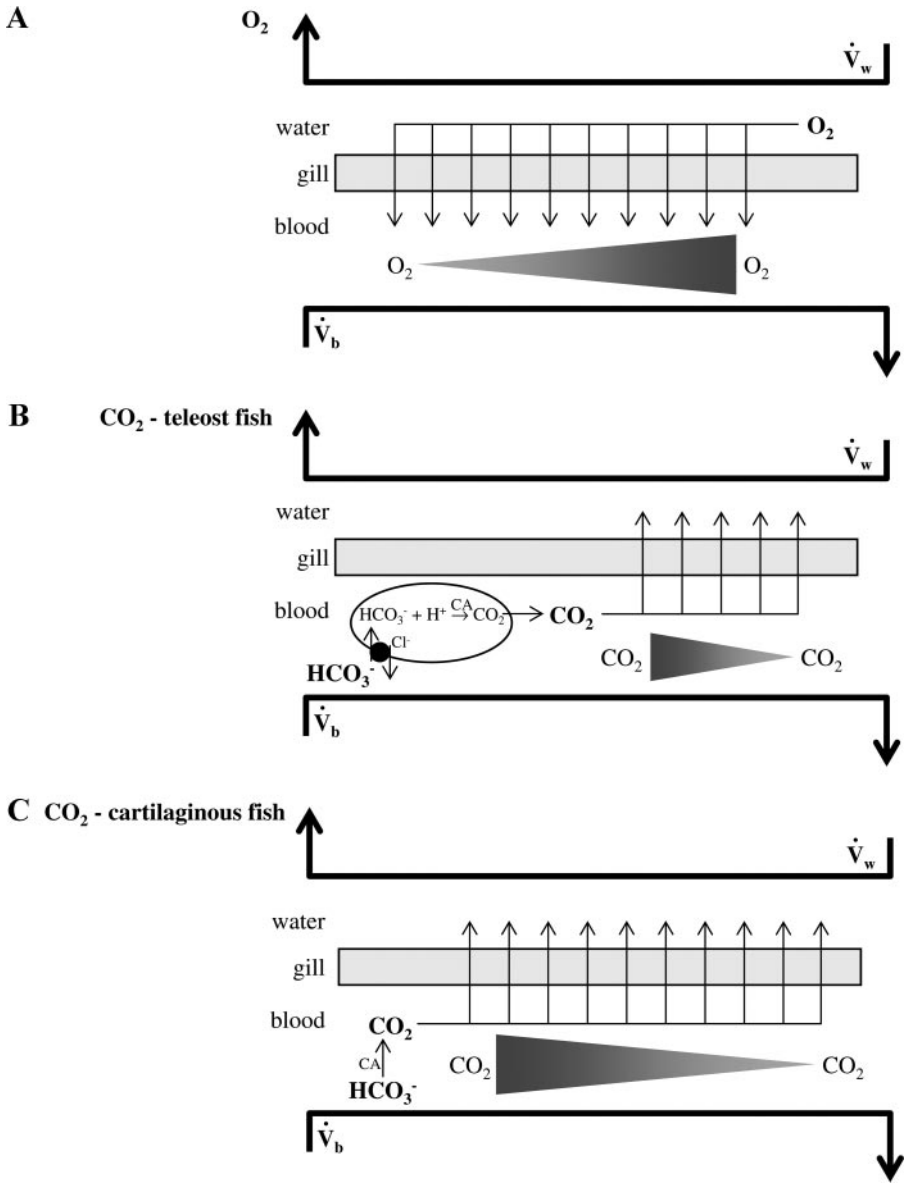

FIG. 25. Schematic models of O_2 (*A*) and CO_2 transfer across the gills of typical teleost (*B*) and cartilaginous (*C*) fish. In theory, any area of the gill that is both ventilated and perfused is available for O_2 diffusion, and O_2 transfer is limited primarily by perfusion. For CO_2 transfer, the conversion of plasma HCO_3^- to molecular CO_2 is limited by the slow rate of entry of HCO_3^- into the red blood cell in teleost fish. Thus the surface area available for CO_2 diffusion out of the blood is limited, i.e., the functional surface area for CO_2 diffusion is less than the total surface area. Because of these chemical equilibrium limitations, branchial CO_2 transfer in teleost fish behaves as a diffusion-limited system. In contrast, the availability of carbonic anhydrase to catalyze HCO_3^- dehydration in the plasma of cartilaginous fish permits the entire surface area to be used for CO_2 diffusion, and branchial CO_2 transfer is predicted to be perfusion limited. CA, carbonic anhydrase; $\dot{V}b$, blood flow; $\dot{V}w$, water flow. [From Perry and Gilmour (589).]

changes in dissolved gas concentrations. This is, in fact, the case. As is found in other vertebrates, changes in environmental or blood gas tensions elicit a suite of ventilatory and cardiovascular responses in fishes. The primacy of O_2 as the proximate signal is well established, but recent studies have demonstrated that CO_2/pH also may be a relevant signal (reviewed in Refs. 253, 493, 589). Environmental hypoxia elicits hyperventilation in a variety of species, and the response is generally larger changes in stroke volume than rate, thereby reducing the energetic effects of irrigating the gills with a relatively dense and viscous medium; hyperoxia elicits the opposite response (see Table 1 in Ref. 253). The responses occur within seconds of the exposure, suggesting external receptors, and this hypothesis is supported by the finding that externally applied cyanide elicits a hyperventilatory response (e.g., Ref. 729). The receptors are distributed throughout the gill arches and are innervated by the 9th and/or 10th cranial nerves (reviewed in Ref. 67). There are also internally oriented, oxygen chemoreceptors in the gill vasculature that respond to either hypoxemia (in the absence of external hypoxia) or injection of cyanide (reviewed in Ref. 64). Such receptors would be advantageous during exercise or other changes in metabolic rate. It is not clear whether the external or internal chemoreceptors respond to Pa_{O_2} or oxygen content, although it is more likely that tension is the proximate signal since vagus nerve recordings from isolated gill arches from both tuna and rainbow trout showed responses to reductions in perfusate Pa_{O_2} even when total O_2 concentration was unchanged (65, 494). Extrabranchial, buccal O_2-sensitive chemoreceptors have also been described, in par-

ticular in air-breathing fishes (e.g., Refs. 495, 729), but there is no convincing evidence to date for O_2 receptors in the central nervous system of fish (e.g., Refs. 287, 651). The neuroendocrine physiology of gill oxygen chemoreceptors is relatively unstudied, although current evidence suggests that neuroepithelial cells (NEC) are involved, as they are in the lungs of terrestrial vertebrates (e.g., Ref. 846). Dunel-Erb et al. (162) described NEC on the gill filaments (near the efferent filamental artery, facing the afferent respiratory water flow) of a variety of teleosts and one elasmobranch, that degranulated under hypoxic conditions, and serotonergic NEC have been confirmed in other species (e.g., Ref. 852). More recently, it has been shown that zebrafish (*Danio rerio*) respond to hypoxia (23), and the filaments and lamellae of all four gill arches of the developing embryo contain both serotonergic and nonserotonergic NEC (332). Innervation patterns in this preparation suggest that the serotonergic NEC may be generating signals back to the central nervous system (to modulate respiratory and cardiovascular parameters) as well as releasing paracrines to affect local perfusion patterns within the filament and lamellae. The nonserotonergic NEC are immunopositive for the purinergic $P2X_3$ receptor, not innervated, and often associated with the pillar cells of the lamellae, suggesting a paracrine role for these NEC (332).

2. CO_2 sensors

The general finding that ventilation is matched to oxygen demands, even at the expense of blood pH (e.g., hyperventilation in hypoxic conditions is accompanied by respiratory alkalosis), suggests that O_2 demand overrides acid-base disturbances (253). This does not mean, however, that a CO_2/pH ventilatory drive does not exist. For instance, increasing the Pa_{CO_2} of a hyperoxic medium abolishes the usual hypoventilatory response in rainbow trout (362) or may even produce hyperventilation in the channel catfish (*Ictalurus punctatus*; Ref. 66). Indeed, the physiology of putative CO_2/pH-sensitive receptors in the gill epithelium has generated much recent interest (reviewed in Refs. 253, 493, 589). Fishes from all major taxonomic groups respond to environmental hypercapnia by a significant increase in gill irrigation and bradycardia (reviewed in Ref. 589). Until recently, it was assumed that the responses were indirect, the result of hypoxemia produced by the effect of blood hypercapnia on hemoglobin oxygen affinity (Bohr effect) and carrying capacity (Root effect; e.g., Ref. 700). More recent studies, however, have demonstrated a direct effect of CO_2/pH on cardiorespiratory parameters, under normoxemic conditions (see Table 2 in Ref. 253). The CO_2/pH-sensitive chemoreceptors appear to be primarily on the first gill arch in some species (e.g., Refs. 596, 729) but may be more generally distributed in other species, because the responses are abolished only when all gill arches are denervated (e.g., Ref. 640). The receptors appear to respond to external rather than internal changes in CO_2/pH, because injection of acetazolamide (which raises blood Pv_{CO_2}) did not alter ventilatory frequency or amplitude in the rainbow trout, catfish, and spiny dogfish (Table 3 in Ref. 253), nor did a bolus infusion of CO_2-enriched saline into the posterior caudal vein of the spiny dogfish (594). On the other hand, the fact that postexercise hyperventilation in the rainbow trout was reduced by injection of CA (which reduced the attending respiratory acidosis) suggests that internal receptors may be present (819).

In all tetrapod vertebrates, receptors in the central nervous system respond to changes in perfusate CO_2/pH, but it is unclear if such receptors are present in the central nervous system of water-breathing fishes (reviewed in Ref. 493). Forty years ago, an experiment on a single tench (*Tinca tinca*) found that injection of slightly acidic saline into the posterior medulla increased the amplitude of electrical discharges that were correlated with respiratory movements (reported in Ref. 311), and a subsequent study (651) demonstrated an inverse correlation between the bicarbonate concentration of the saline perfusing the isolated brain of the lamprey (*Lampetra fluviatilus*) and the frequency of putative respiratory motor output. Recent studies of water-breathing fishes have not corroborated these findings. For instance, hypercapnia-induced hyperventilation in the skate (*Raja ocellata*) was correlated with the pH of the arterial blood, not the cerebrospinal pH or intracellular pH of brain tissue (834), and similar findings have now been reported for other species (e.g., Ref. 495). On the other hand, aerial gas exchange has arisen multiple times in the piscine lineage (e.g., gars, eels, tarpon, lungfishes), and it appears that central CO_2/pH chemoreceptors have evolved in at least some of these groups (495). For example, reduction of the pH of the solution perfusing the brain stem-spinal cord preparation of the gar (*Lepisosteus osseus*) was correlated with a significant increase in the putative air-breathing motor output, but not the motor output thought to be correlated with gill ventilation (811). Similar results have been described for the South American lungfish (*Lepidosiren paradoxa*), where lowering the pH of the perfusate in the IVth ventricle was associated with an increase in lung ventilation frequency (659). Thus the extant data do not allow us to stipulate exactly when central CO_2/pH receptors evolved in fishes, but most likely it was associated with either the independent evolution of aerial respiration in early fish lineages (represented by gar) or with the lineage that gave rise to the amphibia (represented by lungfishes) (493). Clearly more data are needed on this interesting question.

A fall in intracellular pH is generally considered to be the proximate stimulus in the mammalian carotid chemoreceptor (e.g., Ref. 261), and it is likely that this is also the

intracellular stimulus in the fish chemoreceptor (589). Recent evidence suggests that the proximate, extracellular stimulus in the fish gill chemoreceptor is actually a change in extracellular CO_2 rather than pH. For instance, changes in medium pH did not elicit the usual cardiorespiratory responses of two tropical teleosts if the Pv_{CO_2} was unaltered (640, 729). In the Atlantic salmon and spiny dogfish, CO_2-enriched water produced the expected bradycardia and hyperventilation, but acidified, CO_2-free water did not elicit cardiorespiratory changes (594).

In summary, respiratory function in fishes (using the gills) is similar to that in tetrapod vertebrates (using lungs), but medium-constrained differences do exist. There is a need for more studies in a variety of fish groups before some of the generalities presented above can be verified.

V. OSMOREGULATION AND ION BALANCE

A. Introduction

The structure and function of the branchial epithelium as a site of gas exchange dictates that it is also the site of osmosis and ionic diffusion if gradients exist. The marine, invertebrate ancestors of the vertebrates (i.e., sister taxa to modern echinoderms and tunicates) were and are isosmotic (but not always isoionic) to seawater. In contrast to an early hypothesis of freshwater origin (703), it is now generally accepted that the first vertebrates evolved in seawater (e.g., Refs. 305, 536), entered brackish and fresh water (one lineage giving rise to the Amphibia), and then reentered the marine environment in some cases (e.g., Ref. 84). The suggestion that the presence of a renal glomerulus in nearly all vertebrates (there are aglomerular marine teleosts, e.g., Ref. 185) is the result of origin in fresh water (703) is invalidated by the presence of functional glomeruli in the totally marine hagfish (e.g., Ref. 646), modern members of the earliest fish lineage, which do not have a history of freshwater ancestry. The scenario of marine origin followed by entry into fresh water also is supported by the extant fossil record (e.g., Ref. 305) and is the most parsimonious explanation for the isotonicity of hagfish to seawater and the general finding that the NaCl concentration of the blood and extracellular fluids of all the other fish taxa (indeed, all other vertebrates) is ~30% that found in seawater, but distinctly above the NaCl content of fresh water (e.g., Ref. 176). Table 1 presents representative plasma solute data, and it is clear that despite a general pattern, systematic differences do exist between fish groups, in particular those in the marine environment. The maintenance of blood and intracellular tonicity in the face of these osmotic and ionic gradients across the fish branchial epithelium takes energy, but the most recent calculations suggest that it is only of the order of 2–4% of the resting oxygen consumption (e.g., Ref. 505), far below the cost of ventilation (see sect. IV*A*).

B. Osmoregulation in Fresh Water

Like all other freshwater animals, modern fishes (and, presumably, their evolutionary precursors as they entered brackish and fresh water) balance the osmotic uptake of water by having substantial glomerular filtration rates and urine flows and minimize renal salt loss by a significant tubular reabsorption of necessary ions. Net ionic losses in the urine, and by diffusional outflux across the gill, is balanced by active uptake mechanisms in the gill epithelium plus any ionic gain from food. For general

TABLE 1. *Blood osmolarity and major solutes of representative fishes*

Medium	Species	Osmolarity, mosM	Na^+, mM	Cl^-, mM	K^+, mM	Ca^{2+}, mM	Mg^{2+}, mM	SO_4^{2+}, mM	Urea, mM	TMAO, mM
Seawater		1,050	439	513	9.3	9.6	50	26	0	0
	Hagfish	1,035	486	508	8.2	5.1	12	3.0	NPD	NPD
	Lamprey	333	156	159	32	3.5	7.0	NPD	NPD	NPD
	Shark	1,118	255	241	6.0	5.0	3.0	0.5	441	72
	Stingray	1,034	310	300	NPD	NPD	NPD	NPD	394	NPD
	Teleost	452	180	196	5.1	2.8	2.5	2.7	NPD	NPD
	Flounder	297	142	168	3.4	3.3	NPD	NPD	NPD	NPD
Fresh water		1	0.25	0.23	0.005	0.07	0.04	0.05	0	0
	Lamprey	272	120	104	3.9	2.5	2.0	NPD	NPD	NPD
	Stingray I	621	212	208	NPD	NPD	NPD	NPD	196	NPD
	Stingray II	320	178	146	NPD	NPD	NPD	NPD	1.2	NPD
	Teleost	274	130	125	2.9	2.1	1.2	NPD	NPD	NPD
	Flounder	240	124	132	2.9	2.7	NPD	NPD	NPD	NPD

Fresh water refers to "soft" fresh water. TMAO, trimethylamine oxide; NPD, no known published data. For seawater: hagfish, *Myxine glutinosa;* lamprey, *Petromyzon marinus;* shark, *Squalus acanthias* and *Scyliorhinus canicula* combined data; stingray, curyhaline stingray (*Dasyatis sabina*); teleost, stenohaline marine teleost (*Lophius piscatorius*); flounder, euryhaline flounder (*Pleuronectes flesus*). For fresh water: lamprey, *Lampetra fluviatilis;* stingray I, euryhaline stingray (*Dasyatis sabina*); stingray II, stenohaline, Amazonian stingray (*Potamotrygon sp.*); teleost, carp (*Cyprinus carpio*); flounder, euryhaline flounder (*Pleuronectes flesus*). [Redrawn from Evans (185) and 277 Evans et al. (201).]

reviews of freshwater fish osmoregulation, see References 176, 185, 345; for specific reviews on mechanisms of gill NaCl transport, see References 202, 298, 364, 442, 597.

1. Apical Na^+ uptake

The mechanisms of ionic uptake were first suggested by August Krogh, who found that Na^+ and Cl^- were extracted from fresh water independently by the goldfish (*Carassius auratus*) and suggested that blood ions such as NH_4^+, H^+, and HCO_3^- could function as counterions to maintain electroneutrality across the epithelium (373). Subsequent studies appeared to support this hypothesis (reviewed in Refs. 176, 480), but uptake of Na^+ via passive exchange with either NH_4^+ or H^+ has been questioned on thermodynamic grounds (14, 364, 363, 623). For instance, gill epithelium intracellular Na^+ concentrations have been reported in the range of 50–90 mM in two species of trout (504, 830) and ~10 mM in tilapia (400), far above the common freshwater concentration of <1 mM Na^+. In addition, it has been calculated that a pH gradient of 0.3 units would be necessary to drive H^+ outward (in exchange for external Na^+) (839), but rainbow trout are able to excrete protons into water with a pH of 6 (406) despite the fact that the gill epithelial cell pH in this species is ~7.4 (823), and blood pH is even higher (see sect. IV*C*). In addition, many of the early studies that appeared to support the model of Na^+/H^+ or Na^+/NH_4^+ exchange by demonstrating sensitivity of Na^+ uptake to amiloride used concentrations (0.1–1 mM) that could not distinguish among inhibition of ionic exchange, blockade of an apical Na^+ channel, or even inhibition of basolateral Na^+-$K^+(NH_4^+)$-ATPase (e.g., Refs. 189, 366). Moreover, the Na^+/H^+ exchanger (NHE)-specific inhibitor dimethylamiloride did not inhibit Na^+ uptake by the isolated European flounder (*Platichthys flesus*) gill filament (111).

Recent data suggest that the uptake of Na^+ by the fish gill epithelium may be electrically coupled to proton efflux, rather than directly coupled by a single exchanger (i.e., NHE) to proton excretion (see Refs. 408, 442 for reviews). For instance, it was found that the HCO_3^- concentration in the external water (at a neutral pH) actually fell as the medium flowed past the rainbow trout gills (407), contrary to what one might expect if excreted CO_2 was hydrated in the unstirred layer on the water side of the gill epithelium. Moreover, the fall in HCO_3^- concentration was not altered by the disulfonic stilbene SITS, so it was probably not due to reversed Cl^-/HCO_3^- exchange. These data suggest that secreted protons dehydrate the HCO_3^- in the unstirred layer. Addition of vanadate to the external medium at a concentration (0.1 mM) that does not inhibit basolateral Na^+-K^+-ATPase in the frog skin inhibited 50% of the putative proton secretion by the rainbow trout gills, suggesting that the excretion was via an apical, P-type proton pump (407). Other studies described a vesicular fraction isolated from rainbow trout gills that transported protons and expressed a Mg^{2+}-dependent ATPase (367) that was sensitive to *N*-ethylmaleimide (NEM; Refs. 334, 409), a known inhibitor of both V-type and P-type H^+-ATPases, but with a 10- to 100-fold selectivity for the former (226). Moreover, one study (409) was in the presence of EGTA, azide, and ouabain, so activity of Ca^{2+}-activated ATPase, mitochondrial H^+-ATPase, and Na^+-K^+-ATPase was assumed to be minimal. In addition, the NEM-sensitive ATPase activity was also inhibited by other H^+-ATPase inhibitors, such as dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES), *p*-chloromercuribenzenesulfonate (PCMBS), and bafilomycin. The inhibitor profile did not allow a definitive differentiation between P- and V-type proton pumps, but subsequent physiological and immunological studies have made a V-type proton pump (V-H^+-ATPase) the most likely. For example, the V-H^+-ATPase specific inhibitor bafilomycin (1–10 μM in the freshwater medium) inhibited 60–90% of Na^+ uptake by tilapia, carp (*Cyprinus carpio*), and zebrafish (50, 212). These results corroborate molecular studies that showed that heterologous antibodies raised against various subunits of V-H^+-ATPase localized expression to the branchial epithelium of rainbow trout (405, 716), coho salmon (*Oncorhyncus kisutch*; Ref. 810), tilapia (806), zebrafish (50), and two elasmobranchs: the spiny dogfish shark (809) and Atlantic stingray (*Dasyatis sabina*; Refs. 605, 607). Homologous antibodies raised against the zebrafish isoforms demonstrated expression in gill tissue from that species (49). In addition, the mRNA for V-H^+-ATPase has been localized in the branchial epithelium of the rainbow trout by Northern blot, using a probe complementary to the cloned B-subunit of the rainbow trout gene (587) as well as by in situ hybridization using probes complementary to the bovine renal proton pump (717). The amino acid sequence for this rainbow trout gill V-H^+-ATPase subunit shares >95% identity with the vatB1 isoform of the eel swim bladder gas gland V-H^+-ATPase and zebrafish gill vatB1, as well as the vatB1 isoform from humans ("kidney isoform," Ref. 49). Similar amino acid sequence identities have been shown for the vatB2 isoform from the European eel gas gland (526) and zebrafish gills compared with the human sequence ("brain isoform," Ref. 49), but the gene for the vatB2 isoform has not been cloned from the rainbow trout gill. A partial sequence from the β-subunit from the gill of the Osorezan dace (*Tribolodon hakonensis*) has been reported (296), as well as a full-length clone of the α-subunit of V-H^+-ATPase from the killifish gill (352).

The presence of V-H^+-ATPase in the fish branchial epithelium supports the hypothesis that Na^+ uptake by this tissue may be coupled to electrogenic proton extrusion (e.g., Ref. 14), as has been described for tight epithelia such as frog skin (e.g., Ref. 281) and distal portions of the mammalian nephron (reviewed in Ref. 519). The mul-

tistranded junctional complexes between MRC and PVC cell in the branchial epithelium of freshwater fishes (see sect. II*B3*) suggest an electrically tight epithelium, and the extremely high resistance of the cultured, freshwater rainbow trout gill epithelium (3.5 $k\Omega \cdot cm^2$) supports this proposition (818). If the "tight epithelium" model of electrical coupling between proton extrusion and Na^+ uptake is correct, then V-H^+-ATPase and a Na^+ channel should be colocalized to the apical membrane, and Na^+-K^+-ATPase should be localized to the basolateral membrane of the same cell. The initial immunolocalization of V-H^+-ATPase suggested that both MRC and PVC in the rainbow trout gill expressed the proton pump (405), and the labeling in both cells now has been shown to be apical (806). In the coho salmon, V-H^+-ATPase was localized to MRC (810). Another study on the rainbow trout, however, has localized the proton pump in the apical region of only PVCs (716), and this PVC-only localization has now been extended to tilapia (806). This localization of the proton pump to the PVC was also supported by PVC versus MRC distribution changes under acidotic conditions (see below and Ref. 269), high-magnification transmission electron microscopy (391), and X-ray microanalysis studies (504; for reviews, see Refs. 202, 586). Whether these differences in the cellular localization of V-H^+-ATPase are antibody specific or due to differences in rainbow trout populations, holding conditions, or species, remains to be seen, but current data do support an apical position for the proton pump in one or more types of epithelial cells in the freshwater fish gill.

An amiloride-sensitive Na^+ channel has not been cloned from fish, but benzamil (an amiloride analog with a high affinity for ENaC) inhibited Na^+ uptake by freshwater European flounder gills (111). In addition, a recent study demonstrated that an ENaC polyclonal antibody (raised against a synthetic peptide corresponding to residues 411–420 of the β-subunit of the human epithelial Na^+ channel) colocalized with the V-H^+-ATPase, in only PVC in tilapia and in both PVC and MRC in the rainbow trout (806).[1] In all of these studies, MRC cells were identified either by their morphology (and/or position in the epithelium) or by immunological staining with heterologous antibodies raised against Na^+-K^+-ATPase, which is assumed to have high expression in the MRC (see sect. V*B4*). Recent cell separation techniques may offer an avenue for better delineation of specific cellular function. It has been demonstrated that PVC and MRC from the rainbow trout gill can be separated by Percoll density gradient, combined with peanut lectin agglutinin (PNA) binding and magnetic bead separation (248, 262). With the use of this technique, MRC can be separated into two subpopulations, one of which (PNA negative; termed α-MRC) displays bafilomycin-sensitive, acid-activated ^{22}Na uptake, which also is sensitive to the amiloride analog phenamil, an ENaC inhibitor (639). Importantly, in this study, the PVC (defined by position in the Percoll gradient) displayed very low rates of Na^+ uptake that were not sensitive to bafilomycin. This suggests that the acid-secreting cells in the rainbow trout gill are a subpopulation of MRCs, not PVCs. Thus data supporting the tight epithelium model for Na^+ uptake (and H^+ extrusion) exist for the branchial epithelium of freshwater teleosts, but the data are restricted to two species (rainbow trout and tilapia) and must be extended to a wider array of species (Fig. 26).

This model for Na^+ uptake across the apical membrane of the freshwater fish gill epithelium may apply to Na^+ uptake from very dilute solutions in some teleosts. But passive, chemically coupled Na^+/H^+ exchange could mediate Na^+ uptake if electrochemical gradients for either Na^+ or H^+ are favorable (e.g., in solutions with a [Na^+] higher than a few mM and/or pH >7.6, which may be found in some fresh water). Under these circumstances, one might propose apical Na^+/H^+ and Cl^-/HCO_3^- exchangers coupled to basolateral Na^+-K^+-ATPase. These are the apical transporters responsible for Na^+ absorption in mammalian intestinal and renal proximal tubule cells (850) and therefore would be expected to be the isoforms if apical NHEs function in freshwater fishes. Antibodies raised against human proteins have been used to localize NHE3 immunoreactivity in the branchial epi-

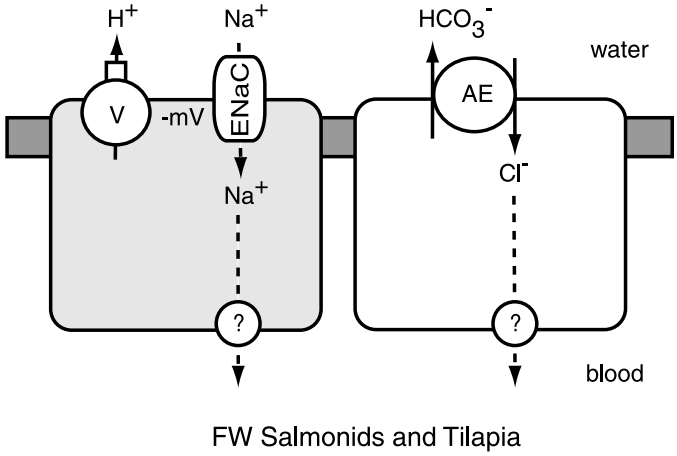

FIG. 26. Working model of NaCl uptake mechanisms proposed for freshwater salmonids and tilapia. Apical proton extrusion by V-H^+-ATPase (V) provides the electrical gradient to draw in Na^+ across the apical surface via an ENaC-like channel, and Cl^- enters via AE1. The basolateral exit step for Cl^- is unknown and the expected role of Na^+-K^+-ATPase in basolateral Na^+ is unclear. Whether these proteins are expressed in a single cell or two is still undetermined and may be species specific; however, adjacent cells in the freshwater gill epithelium generally have multistranded, tight junctions. See text for details.

[1] The use of heterologous antibody and the fact that sequence for ENaC does not exist in the zebrafish or fugu genomes do not allow for a definitive conclusion that the gill Na^+ channel is ENaC; hence, it will be designated ENaC-like in this review.

thelium of freshwater rainbow trout (168) and NHE2 immunoreactivity in the branchial epithelium of freshwater tilapia (806). Neither study specifically localized Na^+-K^+-ATPase to differentiate between MRC and PVC, but morphology and position of the stained cells suggested that the NHEs were only in PVC in the rainbow trout and in both PVC and MRC in tilapia. The most convincing evidence that an apical NHE is expressed in gills in freshwater fishes comes from a recent study that cloned a full-length transcript homologous to NHE3 from the gills of the Osorezan dace and used a homologous antibody to localize expression to the apical surface of MRC (296).

Data from another species, the killifish, suggest a third alternative for the distribution of the pumps and channels necessary for at least Na^+ uptake by the fish branchial epithelium. V-H^+-ATPase has been localized to the basolateral membrane of cells that also express Na^+-K^+-ATPase, using homologous antibodies (raised against a peptide fragment coded by cloned, killifish V-H^+-ATPase). This suggests that basolateral cation extrusion by both pumps may produce an electrochemical gradient across the apical membrane that is sufficient to drive Na^+ into the cell from the external fresh water (352).

It is not clear if environmental or species differences can account for these disparate models for Na^+ uptake across the freshwater fish gill epithelium, but it is clear that molecular techniques, applied to a variety of species and conditions, are needed to provide a better delineation of the mechanisms of Na^+ uptake.

2. Apical Cl^- uptake

Chloride uptake by the freshwater fish gill generally is considered to be via Cl^-/HCO_3^- exchange, but only a few studies have examined this putative pathway directly. Inhibitors of this anionic exchange reduce Cl^- uptake and produce metabolic alkalosis in freshwater fishes (reviewed in Refs. 268, 586), and polyclonal antibodies raised against rainbow trout AE1 (anion exchanger, isoform 1) localized to the apical surface of gill cells in tilapia (806) and coho salmon (*Oncorhynchus kisutch*; Ref. 810) that also were immunopositive for Na^+-K^+-ATPase. In addition, an oligonucleotide probe, complementary to rat AE1 cDNA, hybridized to RNA in cells in both the filament and lamellae of the rainbow trout, suggesting that both PVCs and MRCs contain the message for AE1 (717).

Recently, an alternative mode for Cl^-/HCO_3^- exchange across the apical membrane of the elasmobranch gill epithelium has been proposed (Fig. 27). With the use of a heterologous antibody to human pendrin (a non-AE1 anion exchanger), pendrin-like immunoreactivity has been localized to the apical region of V-H-ATPase-rich, but not Na^+-K^+-ATPase-rich, cells in the branchial epithelium of the Atlantic stingray (607) and spiny dogfish (201). This colocalization is remarkably similar to V-H-ATPase-rich, HCO_3^--secreting intercalated cells in the mammalian collecting duct (652).

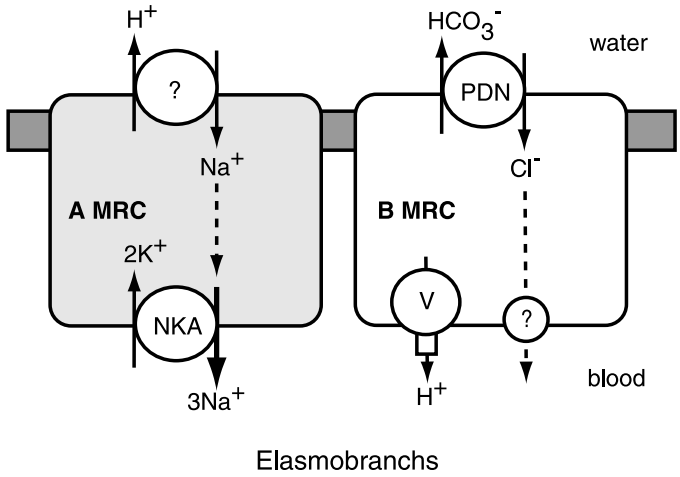

FIG. 27. Working model of NaCl uptake mechanisms proposed for freshwater Atlantic stingrays. One type of MRC (A MRC) expresses Na^+-K^+-ATPase on its basolateral membrane and is hypothesized to draw in Na^+ across the apical surface in exchange for cytoplasmic H^+. The other MRC (B MRC) expresses V-H^+-ATPase (V) on its basolateral membrane and draws Cl^- into the cell via pendrin (PDN). The pathway for basolateral Cl^- movement is unknown. See text for details. [Redrawn from Piermarini et al. (607).]

3. Intracellular CA

The branchial epithelium also contains CA, which is generally thought to be necessary for the production of H^+ and HCO_3^- for the apical exchangers, pumps, and channels that are present (see Ref. 292 for a recent review). Histological studies (using the cobalt phosphate/cobalt sulfate vital stain) demonstrated the presence of CA in the apical region of both MRCs and PVCs in the branchial epithelia of a variety of fish species (e.g., Refs. 117, 225, 379), and immunohistochemical studies (using homologous antibodies raised against purified enzyme) have confirmed this localization to the apical region of MRCs and PVCs in both the rainbow trout and European flounder (629, 678). A CAII-like gene has been sequenced from cDNA from the gills of the Osorezan dace, and the product has been localized to the MRC by homologous immunohistochemistry (296). In both teleosts and elasmobranchs, this intracellular CA represents the majority of CA activity in gill homogenates (256). A role for CA in at least Na^+ uptake had been proposed, because injection of acetazolamiode into either the rainbow trout or small spotted dogfish shark inhibited gill Na^+ uptake (358, 578), and it has been demonstrated that exposure of zebrafish to ethoxzolamide (another CA inhibitor) reduced both Na^+ and Cl^- uptake (50).

4. Basolateral Na^+ and Cl^- exit

As indicated above, there is clear evidence that Na^+-K^+-ATPase is expressed in MRCs in the branchial epithe-

lium of freshwater fishes or euryhaline (wide salinity tolerance) fishes in fresh water (teleosts and elasmobranchs) (e.g., Refs. 136, 296, 352, 403, 476, 582, 604, 771, 806). In addition, immunocytochemical studies of Na^+-K^+-ATPase (using heterologous antibodies) localized expression of the transporter to basolateral and/or tubulovesicular components of the MRCs (136, 604). Partial and full-length Na^+-K^+-ATPase α-subunits have been cloned from a variety of freshwater species (133, 134, 209, 296, 313, 365, 396, 670, 677), and many of these studies have identified multiple isoforms (e.g., Ref. 645). The basolateral expression of Na^+-K^+-ATPase is assumed to provide an exit step for the Na^+ from the branchial epithelial cell into the extracellular fluids. There is some evidence that one form of a Na^+-HCO_3^- cotransporter (NBC1) may be expressed in a subpopulation of MRC (defined by Na^+-K^+-ATPase immunolocalization) in the acid-stressed Osorezan dace (296) and the rainbow trout (597) which could mediate both Na^+ and HCO_3^- exit to the blood. The basolateral exit step for Cl^- has not been studied directly, but an antibody raised against human CFTR localized to the basolateral regions of both MRC and PVCs in the freshwater killifish opercular epithelium (which models the gill epithelium, see sect. v*C*) (453), suggesting that this Cl^- channel may be involved.

5. Basolateral K^+ recycling

The addition of 2 mM Ba^{2+} to the basolateral side of the killifish opercular epithelium inhibited the short-circuit current by 77%, suggesting the presence of K^+ channels to recycle the K^+ entering the cell via Na^+-K^+-ATPase (146). More recently, a full-length sequence for an inward rectifier K^+ channel (eKir) has been reported from Japanese eel gills, and immunogold staining with homologous antibodies has localized expression to the basolateral, tubular system in the MRC (736).

It is not clear if the uncertainty about the mode(s) of NaCl uptake is secondary to species differences, salinity differences (see below), or the use of heterologous versus homologous antibodies, but more species need to be examined before a general model can be proposed. However, there is a distinct possibility that species differences do exist. In any case, the attending extrusion of H^+ (or NH_4^+) and HCO_3^- provides potential pathways for acid-base regulation and excretion of nitrogen. This is discussed further in section vi*D*.

6. Divalent ion uptake

Because freshwater concentrations of divalent ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} are far below plasma levels, fish must extract the needed ions from either food or freshwater itself. Fish (at least teleost) bone is acellular (e.g., Ref. 402), so it does not provide a pool for Ca^{2+} as it does in terrestrial vertebrates. Recent evidence suggests that branchial uptake mechanisms exist for all three ions (44, 300), but Ca^{2+} is the best studied (reviewed in Refs. 218, 222, 442, 446).

Because the intracellular Ca^{2+} concentration of gill cells is presumably <1 μM, it is assumed that apical uptake is diffusional even from soft fresh water (10 μM Ca^{2+}; Ref. 442), and partial or full-length sequences for a putative ECaC have now been reported from tilapia (B.-K. Liao, C.-H. Yang, T.-C. Pan, and P.-P. Hwang, unpublished data), fugu (GenBank accession no. AY232821), and rainbow trout (AY256348; Ref. 597). In both species, the putative ECaC is expressed in the gill, and in tilapia the expression increased fourfold when the fish were acclimated to low-Ca^{2+} fresh water, a condition where Ca^{2+} uptake increased (Liao et al., unpublished data). Presumed electrochemical gradients at the basolateral membrane suggest that active transport mechanisms must be involved for Ca^{2+} exit to the blood. Ca^{2+} transport across isolated membrane vesicles (217) and the killifish opercular epithelium (774) is Na^+ dependent, so Na^+/Ca^{2+} exchange may be involved. The complete sequence for a putative Na^+/Ca^{2+} exchanger (NCX) has been reported from tilapia (AY283779), but its expression in the gill of this species did not change under low Ca^{2+} conditions (T.-C. Pan, C.-H. Yang, C.-J. Huang, and P.-P. Hwang, unpublished data). Ca^{2+}-dependent ATP hydrolysis can also be measured in gill vesicle preparations, with a $K_{1/2}$ of 63 nM, so a high-affinity Ca^{2+}-activated ATPase is assumed to play a role (223). A partial sequence for a putative Ca^{2+}-activated ATPase (PMCA) has been reported (AF236669), but its expression in the gill did not change under low Ca^{2+} conditions (Liao et al., unpublished data). Taken together, these results suggest that apical uptake, rather than basolateral extrusion, is the limiting factor in Ca^{2+} balance, at least in tilapia. The fact that Ca^{2+}-activated ATPase activity covaries with Na^+-K^+-ATPase activity in the European eel (223) and rainbow trout (217), both are stimulated by prolactin injection in the eel (224), and Ca^{2+} uptake is proportional to the density of MRC in tilapia (475), rainbow trout (450), and killifish (447) suggest that MRCs are the site of Ca^{2+} uptake by these putative transport processes (Fig. 28).

7. Water permeability and aquaporins

Early isotopic and physiological measurements of diffusional and osmotic water permeabilities suggested that both were higher in the branchial epithelium of freshwater than that of seawater fishes (174, 508), and the substantial differential between the osmotic and diffusional permeabilities suggested the presence of "water-filled pores" in the freshwater, but not seawater, gill epithelium (508). Aquaporin-3 (AQP-3) now has been cloned from the gills of the eel and found to be 67–69% homologous to AQP-3 from rat, mouse, and human (131). The

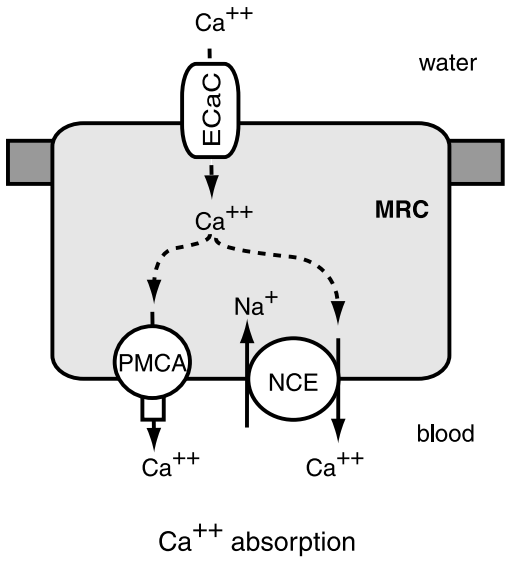

FIG. 28. Working model for the Ca^{2+} uptake mechanisms across the freshwater teleost gill epithelium. Ca^{2+} is drawn in via a Ca^{2+} channel (ECaC) on the apical surface of the MRC and extruded across the basolateral membrane via a Ca^{2+}-ATPase (PMCA) or a Na^+/Ca^{2+} exchanger (NCE). See text for details.

expression of AQP-3 declined significantly when eels were acclimated to seawater, and a homologous antibody localized AQP-3 immunoreactivity to both the apical and basolateral aspects of branchial MRC, as well as other branchial cells (PVCs not specified) (403). AQP-3 also has been cloned from the gills of the acid-stressed, Osorezan dace, and homologous antibodies localized expression to the basolateral aspect of the MRC (296). What role AQP-3 plays in osmoregulation is unknown (131), although it may interact with CFTR (672). Moreover, another aquaporin (AQP-1) has been shown to transport both ammonia (520) and CO_2 (518), both of which traverse the fish branchial epithelium.

8. Lampreys

There is little known about the specifics of gill ion transport by lampreys in fresh water (and seawater, see below), but their plasma is hypotonic to fresh water (see Table 1), and more than one type of MRC is present in the gill epithelium of various lamprey species when acclimated to freshwater (see sect. II*B3*B and Refs. 24, 32, 507, 515, 580). A recent study has demonstrated two types of MRC in the Australian pouched lamprey: one that expresses Na^+-K^+-ATPase on the basolateral aspect and another that expresses CAII in the apical area and V-ATPase in the cytoplasm (96). The authors propose a model for parallel pathways for Na^+ and Cl^- uptake identical to that proposed for the elasmobranch gills (605, 607) and similar to that found in the proximal tubule cells (for Na^+ uptake) and the type B intercalated cells (for Cl^- uptake) of the mammalian kidney. These data are all consistent with the generally accepted hypothesis that these primitive agnathan fishes possess the same gill epithelial ionic transport mechanisms as those described for freshwater teleosts, suggesting that the origin of these pathways was of the order of 500 million years ago. For more general discussions of lamprey osmoregulation in fresh water, see References 185, 345.

C. Osmoregulation in Seawater

The majority of fish species live in the marine environment and thereby face the reverse osmotic and ionic gradients across the branchial epithelium to those found in the freshwater environment (Table 1). Interestingly, the two major groups of modern, marine fishes (teleosts and elasmobranchs) have evolved different strategies to balance the potential osmotic loss of water and diffusional gain of NaCl. In teleosts, which are distinctly hyposmotic to seawater, the osmotic loss of water is balanced by ingestion of seawater and subsequent intestinal uptake of NaCl to withdraw needed water from the lumen contents (e.g., Ref. 294). The resulting salt load adds to that produced by the diffusional uptake of NaCl across the gills. The sum is balanced by gill epithelial NaCl extrusion, because the lack of a loop of Henle precludes the production of urine that is hyperosmotic to the plasma. In elasmobranchs, the retention of urea at extraordinary levels (Table 1) brings the plasma osmolarity hypertonic to seawater, so oral ingestion of seawater is not necessary. Nevertheless, sharks, skates, and rays must offset the diffusional uptake of NaCl across the gill epithelium by excretion of a hypersaline fluid by the rectal gland, derived from the posterior intestine (for recent reviews, see Refs. 647, 689, 690). However, various studies have shown that the rectal gland is not a vital organ because fish were able to osmoregulate after removal of the rectal gland (e.g., Refs. 63, 200), despite producing urine that is isotonic to the plasma. Hagfish are isotonic to seawater (Table 1), and the ionic differentials (especially for divalent ions) are probably maintained by renal and mucous secretion. Lampreys, on the other hand, maintain substantial ionic gradients across the gill epithelium, and it appears that their osmoregulatory strategies are similar to those found in marine teleosts. For general reviews of marine fish osmoregulation, see References 176, 185, 345; for specific reviews on mechanisms of gill NaCl transport, see References 202, 298, 442.

1. NaCl secretion in marine teleosts

Net salt extrusion by the fish gill epithelium was first described over 70 years ago in a classic paper on Cl^- transport across the perfused gills of the eel by Ancel Keys (359), in which he stated: "These experiments dem-

onstrated beyond doubt that a concentrated chloride solution is secreted by the gills in opposition to a large concentration gradient." In a subsequent paper, Keys stated that "chloride secretion exhibited by the gills of the eel in seawater is an active process requiring the expenditure of large amounts of energy," surely one of the earliest suggestions that an epithelium could actively transport ions (360).[2] This latter paper is also a classic in the field, because it was the first description of a specific cell involved in active transport: the "Cl^- secreting cell," which is now known to be a type of teleost MRC (see sect. II*B3*B).

The mechanisms of NaCl secretion by at least the teleost gill epithelium are more clear than the mechanisms of NaCl uptake by the same tissue (see above). As the importance of Na^+-K^+-ATPase in a variety of cells and epithelia (including the fish gills; e.g., Ref. 170) became known 35 years ago, it was proposed that apical Na^+-K^+-ATPase-mediated Na^+ extrusion across the branchial epithelium, because, for example, removal of external K^+ or external application of ouabain inhibited Na^+ efflux, measured radioisotopically (e.g., Refs. 190, 197, 424). This proposition of apical Na^+-K^+-ATPase extruding cellular Na^+ was rendered untenable when Karnaky demonstrated unequivocally that Na^+-K^+-ATPase was restricted to the basolateral membrane of the MRC in the branchial epithelium of the killifish (349), and this localization has now been confirmed for a variety of species, including elasmobranchs (e.g., Refs. 296, 582, 604, 806–808). The importance of basolateral Na^+-K^+-ATPase in both Na^+ and Cl^- extrusion was demonstrated by the fact that injection of ouabain into the eel completely inhibited both Na^+-K^+-ATPase activity and the efflux of both ions (688). It was proposed that NaCl extrusion was mediated by a basolateral cotransport of NaCl down the electrochemical gradient produced by Na^+-K^+-ATPase, coupled with an apical extrusion of Cl^- via a channel and paracellular extrusion of Na^+, both down their respective electrochemical gradients (688). This model was consistent with work on other epithelia (reviewed in Ref. 243) and was corroborated by concurrent, electrophysiological studies of the epithelial sheets from the operculum of the killifish (147, 347) and the lower jaw of the longjaw mudsucker (440). The killifish opercular epithelium contained ~60% MRCs, maintained an open-circuit electrical potential of 19 mV (serosal side positive) and an electrical resistance of 174 $\Omega \cdot cm^2$, and produced a net short-circuit current (I_{sc}; 137 $\mu A/cm^2$) that was equivalent to the net Cl^- efflux, measured isotopically. Addition of ouabain or furosemide, or ionic substitutions to the basolateral side, inhibited the I_{sc} and effluxes of Na^+ and Cl^-, consistent with the model of basolateral Na^+-K^+-ATPase and NaCl cotransport (147, 440, 446). Studies showing a direct correlation between MRC number and Cl^- currents in the opercular and jaw skin epithelia (346, 454), combined with salinity-induced changes in structure and number of MRCs in fish gills (e.g., Refs. 159, 348, 351, 580, 664, 683, 768, 859), suggested the primacy of the MRC in NaCl extrusion. This was proven directly when Cl^- currents were localized to the MRC of the killifish operculum, using a vibrating probe technique (233). Figure 29 diagrams the generally accepted model for NaCl extrusion by the teleost MRC, and supporting evidence for specific pathways follows.[3]

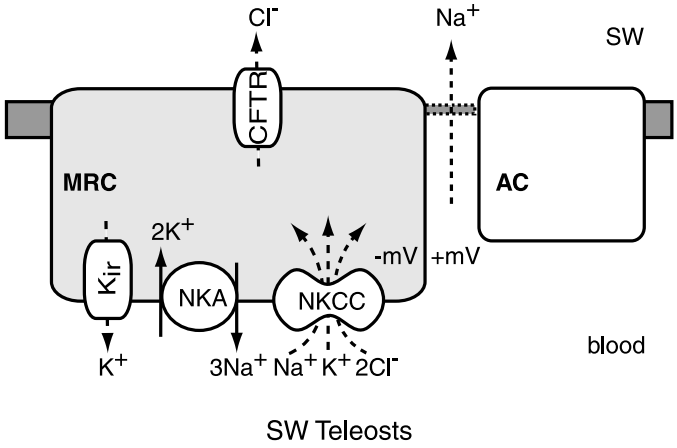

FIG. 29. Working model for the extrusion of NaCl by the marine teleost gill epithelium. Plasma Na^+, K^+, and Cl^- enter the cell via basolateral NKCC; Na^+ is recycled back to the plasma via Na^+-K^+-ATPase and K^+ via a K^+ channel (K_{ir}). Cl^- is extruded across the apical membrane via a Cl^- channel (CFTR). The transepithelial electrical potential across the gill epithelium (plasma positive to seawater) drives Na^+ across the leaky tight junctions between the MRC and the AC. See text for details.

2. Basolateral Na^+ and Cl^- uptake

A) NA^+-K^+-ATPASE. Complete or partial sequences for Na^+-K^+-ATPase now have been reported from a variety of freshwater and euryhaline teleosts (see sect. V*B4*), as well as stenohaline marine species from the Antarctic and New Zealand, where multiple isoforms were identified (277). Na^+-K^+-ATPase expression can be localized to the MRCs in the gill epithelium in marine species (including the dogfish shark) or euryhaline species in seawater (including the stingray) using nucleotide probes and antibodies

[2] We are indebted to Dr. L. B. Kirschner, who brought these quotes to our attention.

[3] This model assumes that the transepithelial potential (TEP) across the marine teleost gills is of the order of +25 mV (plasma relative to SW) so that Na^+ is in electrochemical equilibrium. Measurement of a gills-only TEP in vivo has not been reported, but 50% of the published whole body TEPs (salt bridge in peritoneal fluids versus salt bridge in SW) are <20 mV, including six species that have an inside-negative TEP (177, 622). Thus it is not clear if the model for NaCl extrusion in Figure 29 is applicable to all marine teleosts.

(e.g., Refs. 133, 209, 476, 604, 681, 682, 808, 810). In some cases, Na^+-K^+-ATPase has been specifically localized to the basolateral aspect of the MRC (130, 396, 604, 771, 807, 808). Expression and activity of Na^+-K^+-ATPase is often correlated directly with salinity (e.g., Refs. 133, 145, 170, 209, 283, 314, 331, 404, 421, 436, 645, 675, 692, 748, 755, 810). Moreover, relative Na^+-K^+-ATPase activity in gill biopsies can actually predict the future downstream migratory behavior in freshwater brown trout (527). On the other hand, Na^+-K^+-ATPase activity and/or expression actually decreases when some euryhaline species are acclimated to seawater (reviewed in Ref. 446; see also Refs. 410, 604). The expression of Na^+-K^+-ATPase increased (compared with seawater controls) when the sea bass (*Dicentrarchus labrax*) is acclimated to either fresh water or 200% seawater (771), which suggests that any osmotic stress might affect the expression of this important transport enzyme. Some of these discrepancies may be due to differential expression of Na^+-K^+-ATPase isoforms, because a recent study has demonstrated that, in the rainbow trout gills, Na^+-K^+-ATPase α1b is upregulated in seawater, but Na^+-K^+-ATPase α1a is downregulated (645).

B) NA-K-2CL COTRANSPORT. Fragments of the NKCC gene have been cloned from the European eel (homologous to NKCC1 and NKCC2; Ref. 132), the brown trout and Atlantic salmon (NKCC1; Ref. 750), and the striped bass (*Morone saxatilis*; Ref. 748). In all cases, transcripts were detected in gill tissue (by Northern blots), and expression was highest when the fishes were in seawater. The T_4 antibody raised against human colonic NKCC1 (416) has localized expression of the cotransporter to the basolateral aspect of MRC in the gill epithelium of the giant mudskipper (*Periophthalmodon schlosseri*; Ref. 808) and Hawaiian goby (*Stenogobius hawaiiensis*; Ref. 476), in MRC in the Atlantic salmon (colocalized with Na^+-K^+-ATPase; Ref. 582) (Fig. 30) and killifish (453), and in what appear to be MRC in the brown trout (750). In the latter three studies, the protein expression was directly correlated with salinity, as it was (measured by Western blots)

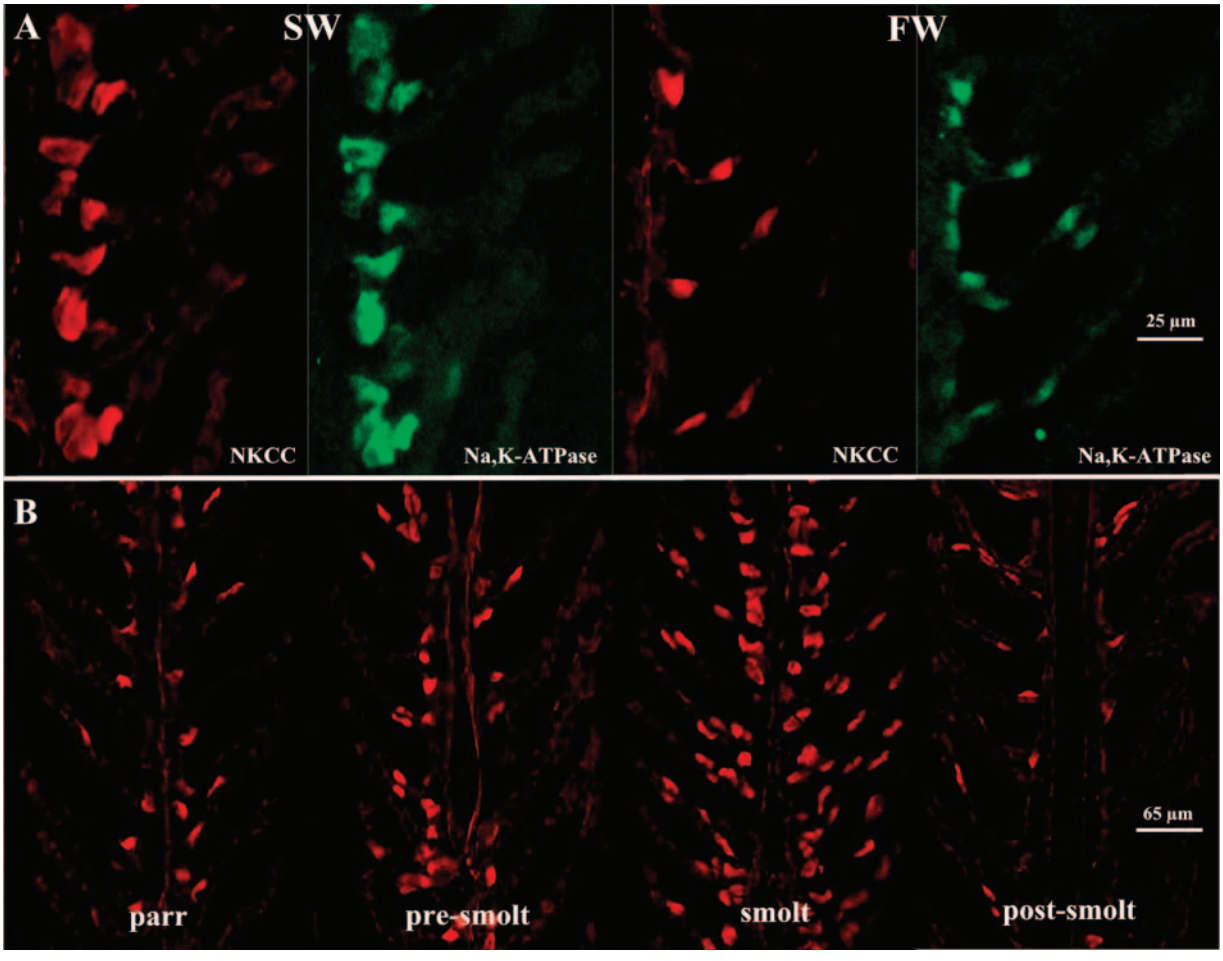

FIG. 30. Colocalization of the Na^+-K^+-$2Cl^-$ cotransporter (NKCC; red) and Na^+-K^+-ATPase (green) to gill chloride cells of FW and SW Atlantic salmon parr (*A*). Na^+-K^+-$2Cl^-$ cotransporter immunoreactivity in the gill during the parr-smolt transformation (*B*; parr in May, presmolts in February, smolts in May, and postsmolts in June). [From Pelis et al. (582).]

in gill extracts from striped bass in seawater versus fresh water (748).

C) BASOLATERAL K^+ RECYCLING. The hypothesis that K^+ that enters the cell associated with Na^+-K^+-ATPase and NKCC is recycled through the K^+ channel is suggested by the effect of Ba^{2+} on the I_{sc} across the killifish operculum (146) and the cloning of an inward rectifier K^+ channel in the Japanese eel (736). Acclimation of the eel to seawater increased the expression of the gene product significantly, as measured by RNase protection assay (736).

3. Apical salt extrusion

Marshall's group has identified a low-conductance (8 pS) anion channel in a primary culture of cells from the killifish opercular epithelium (which retained MRC) that was stimulated by cAMP and inhibited by two anion channel blockers, diphenyl-amine-2-carboxylic acid (DPC) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (449). A full-length clone of a CFTR gene was sequenced from a killifish gill cDNA library that encoded a protein (59% identical to human CFTR amino acid sequence) and that produced a cAMP-activated anion conductance when its mRNA was expressed in *Xenopus* oocytes (693). Importantly, expression of the product in the gills increased severalfold after transfer of killifish to seawater. Full-length clones of CFTR are now available from the Atlantic salmon (95% identical to killifish amino acid sequence; Ref. 91) and fugu (*Fugu rubripes*, 84% identical to killifish amino acid sequence; Ref. 144). Antibodies directed against hCFTR have localized expression to the apical region of MRC in the giant mudskipper (808) and Hawaiian goby (476), and a monoclonal antibody directed against the shared carboxy terminus of hCFTR and kCFTR confirmed this location in the killifish gill epithelium (453). In the latter study, the immunoreactive CFTR moved from the central part of the cytosol to the apical surface of the MRC when the killifish were acclimated to seawater (Fig. 31). In the Hawaiian goby, CFTR-positive cells increased fivefold in seawater-acclimated fish (476). For an excellent review of the role of CFTR in the seawater teleost gill, see Reference 455.

D. Does the Elasmobranch Gill Excrete NaCl?

The fact that removal of the rectal gland from at least *Squalus acanthias* does not result in significant hypernatremia suggests that other tissues can secrete salt when necessary. The elasmobranch gill epithelium does contain MRCs, which express immunoreactive Na^+-K^+-ATPase (e.g., Refs. 604, 807), and NKCC expression has been noted in the dogfish gills (841). However, neither MRC number or size nor Na^+-K^+-ATPase activity increased after the removal of the rectal gland in the dogfish (807), counter to what one would expect if branchial extrusion mechanisms were upregulated in response to the loss of the rectal gland. Moreover, acclimation of freshwater Atlantic stingrays to seawater was associated with a decrease in branchial Na^+-K^+-ATPase activity and Na^+-K^+-ATPase immunoreactivity (604). More studies are warranted, but it is possible that increased renal Na^+ and Cl^- excretion could offset diffusional salt uptake in the absence of the rectal gland, because the elasmobranch gains water by osmosis and, therefore, has high urine flow rates. As long as the rate of water absorption via osmosis is greater than or equal to diffusional salt uptake, blood osmolarity can be maintained by excretion of urine that is isotonic or even hypotonic to the plasma. Indeed, Burger (62) found increased urinary salt loss after removal of the rectal gland in the spiny dogfish (62).

E. Divalent Ion Excretion

Although the Ca^{2+} and Mg^{2+} concentrations of the plasma of marine fishes are below that of seawater, it is unlikely that diffusional influx is significant because of the serosal positive electrical potential across the gills (see above and Ref. 222). However, the oral ingestion of seawater by at least marine teleosts presents a divalent ion load to the fish (to the degree that intestinal uptake exists), which must be excreted by renal and branchial mechanisms. For instance, Hickman (294) calculated that only ~15% of the ingested Mg^{2+} (and SO_4^{2-}) was absorbed, but 100% of that was excreted renally by the southern flounder (*Paralichthys lethostigma*). In contrast, 68% of the ingested Ca^{2+} was absorbed, and only 11% of that could be accounted for by renal loss. Thus the remaining 89% of the Ca^{2+} removed from the intestine must be excreted by the gills (294). This putative branchial extrusion of Ca^{2+} could be passive because the calculated Nernst equilibrium electrical potential for Ca^{2+} is of the order of 10–15 mV (inside positive), which is probably below that across the marine fish gill epithelium (see above). More data are needed.

F. Hagfish and Lampreys

Despite the lack of significant osmotic or NaCl gradients across their gills (Table 1), the hagfish branchial epithelium does contain cells that share many of the morphological characteristics that define the MRC of teleosts and elasmobranchs (see sect. II*B3*B). Moreover, Na^+-K^+-ATPase can be immunolocalized to MRC on the filaments and lamellae of the gill epithelium of the Atlantic hagfish (94), and NHE-1 expression was detected in gill extracts by Western blots in the same species (97). Intracellular distribution was not determined, but expression of these two proteins suggests that the MRC in the gills of

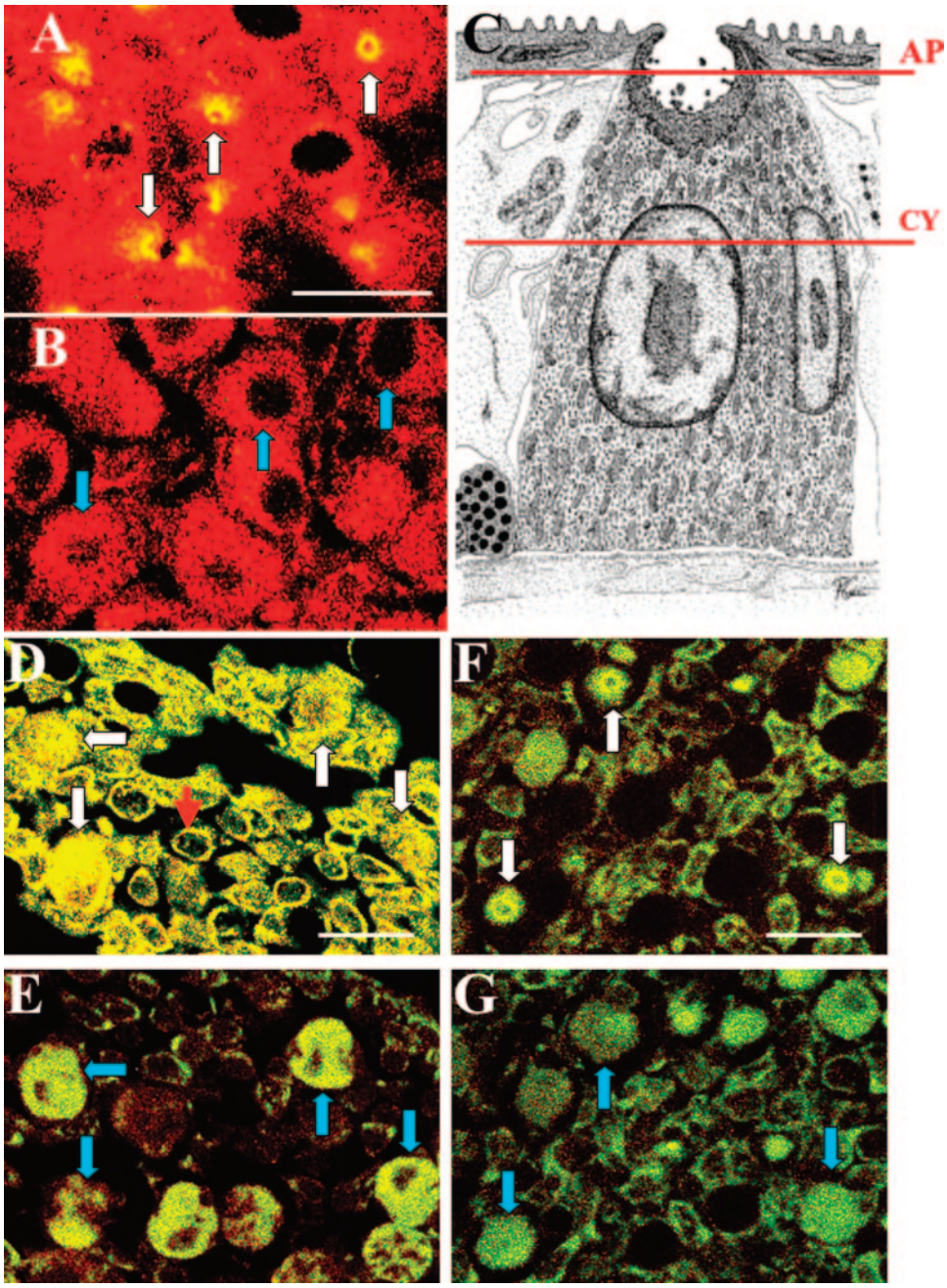

FIG. 31. Confocal laser scanning images of kfCFTR immunofluorescence of mouse anti-human CFTR and goat anti-mouse IgG Oregon Green 488 in killifish opercular epithelium show the distribution of kfCFTR (green fluorescence + red yields yellow) in mitochondria-rich (MR) chloride cells counterstained for mitochondria with Mitotracker Red. *A*: membrane from a SW-adapted animal has kfCFTR immunofluorescence at 4.5 μm from the surface of the chloride cells is localized to the apical crypt (white arrows). The asterisk indicates a gap in the pavement cell layer (*B*). The same frame as for *A* but at a depth of 7.5 μm at the plane of the nucleus of the MR cell (see drawing, *C*, for approximate depths of the optical sections; AP, apical crypt level; CY, cytosol level). Blue arrows in *B* indicate the mitochondria-rich chloride cells below the apical crypt. *D* and *E*: similar kfCFTR immunostaining, but in MR cells from fish acclimated to FW. An optical section (*D*) at 4.5 μm from the surface has positive kfCFTR immunofluorescence evenly distributed in the chloride cells (white arrows) as well as in the pavement cells at the plane of the pavement cell nuclei (orange arrow). Optical section (*E*) is same frame as for *D* but 10.5 μm into the tissue at the level of the MR cell nuclei. Blue arrows indicate the MR cells with diffusely distributed positive immunofluorescence for kfCFTR. *F* and *G*: similar immunostaining but for a fish transferred from FW to SW for 48 h. *F*: an optical section 9.0 μm from the surface with ring-shaped kfCFTR positive immunofluorescence (white arrows). *G*: 13.5 μm from the surface with kfCFTR immunofluorescence diffusely distributed in MR cells (blue arrows). Scale bars in *A*, *D*, and *F* are 20 μm. [From Marshall et al. (453).]

the hagfish may be involved in acid-base regulation, as it is in teleosts and elasmobranchs (see sect. VI*D*).

All lampreys breed in fresh water, and one group, the petromyzonitids, enter seawater after metamorphosis. The marine lampreys (e.g., *P. marinus*) migrate back to fresh water as breeding adults, and one population is actually resident in the Great Lakes of North America. Little is known about osmoregulation in the marine populations because of the difficulty of capturing animals, but MRCs in the gill epithelium of marine lampreys lose basolateral invaginations after acclimation to fresh water (28), and the activity of gill Na^+-K^+-ATPase is much higher in seawater versus freshwater individuals (36), suggesting a role for Na^+-K^+-ATPase in MRCs in seawater osmoregulation. More data are needed, but it is generally assumed that the gill mechanisms for osmoregulation in seawater lampreys parallel those in the marine teleost gills. Modern molecular techniques should allow confirmation of this assumption.

G. Euryhalinity

The vast majority of fishes only can tolerate salinities near to either fresh water or seawater (termed stenoha-

line), but a large number of species (in all major fish groups) are able to tolerate a much broader range of salinities, including some that are fully euryhaline (e.g., lampreys, Atlantic stingrays, bull sharks, sturgeon, eels, salmon, shad, striped bass, mullet, flounders, killifish, etc.; Ref. 179). Recent evidence suggests that the specific molecular or biochemical events that accompany acute salinity transfers may be species specific, but they generally involve reciprocal regulation of expression and/or activity of Na^+-K^+-ATPase, NKCC, CFTR, V-ATPase, and possibly NHE (e.g., Refs. 605, 673, 748–750). One might propose that stenohaline species lack the requisite, branchial salt transport mechanisms and/or are unable to make the necessary quantitative changes in renal or intestinal function for osmoregulation in seawater versus fresh water. However, there is now clear evidence that the putative branchial salt absorption mechanisms (NHEs, and/or V-ATPase electrically balanced by an ENaC-like channel) are present in marine species, presumably for acid-base regulation (see sect. VI, *D* and *E*). This was originally suggested as an evolutionary "holdover" from the ancestors of present-day marine gnathostomes that inhabited fresh water (175, 578), but this idea was challenged when Na^+ and Cl^- substitutions were shown to also inhibit acid and base excretion, respectively, from the Atlantic hagfish (178), an osmoconforming agnathan that is physiologically limited to seawater. Hagfish are believed to lack a period of freshwater existence in their evolutionary history, suggesting that Na^+- and Cl^--linked acid and base secretory mechanisms originally evolved for acid-base regulation in seawater, and not for ion absorption in fresh water (178).

If the ionic transport mechanisms that mediate ion absorption in modern freshwater fishes arose in the earliest vertebrates for acid-base regulation and persist today in modern marine fishes (including relatively stenohaline marine teleosts, elasmobranchs, and hagfish), one might ask why all fishes are not euryhaline? Presumably, an inability to alter the secretion of hormones and paracrines (see sect. VIII) that control the branchial processes of osmoregulation (in addition to intestinal and renal processes) may be a factor, but it seems likely that the interplay between branchial ionic permeability and the efficacy of ionic transport may be a major limiting factor in at least the transition between seawater and brackish or fresh water (179). The importance of permeability is best seen in the role of Ca^{2+} in freshwater survival of various species of inshore fishes that are normally stenohaline (56), as well as in the Na^+ balance and survival of the marine pinfish (*Lagodon rhomboides*) in low salinities (83). The inability of most freshwater fish species to enter hyperosmotic salinities simply may be caused by the lack of the requisite extrusion proteins in their MRC, or more subtle hormonal, renal, or intestinal deficiencies may be present (e.g., no neuroendocrine axis to stimulate oral ingestion). Nevertheless, there is a substantial number of fish families that have euryhaline members, including such relatively primitive fish species as lampreys, sturgeons, eels, tarpon, and rainbow trout, suggesting that euryhalinity evolved several times in the early vertebrate lineage (179).

H. Osmoreceptors

The ability of some euryhaline species to enter lower salinities during seasonal migration (e.g., eels, salmon) or daily movements in the inshore environment (e.g., killifish) suggests that relatively rapid changes in transport can take place, and that osmo- or ionoreceptors may be present in the branchial epithelium, vasculature, or central nervous system. There is now good evidence that the gill epithelium itself can respond to changes in the external salinity and/or composition. It was found that a 50 mosM increase in the Ringer solution bathing the basolateral (but not apical) side of the killifish opercular epithelium produced a significant decline in the volume of individual MRC, which was associated with a doubling of the I_{sc}. The increased I_{sc} could be inhibited by the addition of bumetanide, suggesting that activation of NKCC was involved in the response to basolateral hypertonicity (855). More recently, another study demonstrated that a 40 mosM decrease in the basolateral solution inhibited the I_{sc} by 58% (448), which corroborates the hypothesis that plasma osmolarity changes can elicit very rapid changes in at least active NaCl extrusion by the killifish gill epithelium.

The intracellular messengers that elicit these responses are relatively unstudied, but there is now evidence that protein kinase C (PKC) and myosin light-chain kinase (MLCK), but not protein kinase A (PKA), are activated by increased osmolarity, and that a "heavily serine phosphorylated protein of about 190 kDa" is also in higher concentrations is seawater- but not fresh water-acclimated killifish, suggesting that upregulation of a serine/threonine kinase may be involved (299). On the other hand, the activity of three mitogen-activated protein kinases (SAPK2, SAPK1, and ERK1) all increased significantly in the gill epithelium during hyposmotic stress of the killifish (376). Kültz et al. (377) have cloned a 14.3.3 protein from the killifish that is expressed in high concentrations in the gill epithelium, and which increases two- to fourfold within 24 h of transfer of the fish into seawater. This suggests a role for this putative cofactor for protein kinases, phosphatases, and other phosphoproteins (e.g., Ref. 245) in structural or functional changes in the epithelium during at least short-term salinity changes. Longer term morphological and physiological changes associated with euryhalinity are discussed in section VIII.

Recent studies suggest that polyvalent cation receptors (CaR) in the gill epithelium also may stimulate osmo-

regulatory changes by responding to changes in internal or external [Ca^{2+}]. Full-length cDNA sequences of CaR have now been reported from both the gilthead sea bream (*Sparus aurata*; 214) and spiny dogfish (521), and nucleotide probes have localized expression in branchial MRCs in both species, as well as the winter flounder (*Pleuronectes americanus*) and Atlantic salmon (521). Moreover, the CaR also may be a salinity receptor, because (after expression in human embryonic kidney cells) its sensitivity to Ca^{2+} or Mg^{2+} was inversely related to the Na^+ concentration of the incubation medium (521). Thus the MRC itself may be a salinity sensor, responding to changes in volume and/or changes in stimulation of Ca^{2+} receptors.

VI. pH REGULATION

A. Introduction

Fishes, like all vertebrates, have three compensatory mechanisms to regulate the acid-base status of their extracellular body fluids: *1*) instantaneous physiochemical buffering with bicarbonate and nonbicarbonate buffers, *2*) respiratory adjustments of the "open" CO_2-HCO_3^- buffer system within minutes, and *3*) net transport of acid-base relevant molecules between the animal and environment within minutes to hours. As discussed in section IV*A*, using an aqueous medium for respiration limits the first two mechanisms relative to air-breathing vertebrates, but enhances the third mechanism. For general reviews of fish acid-base regulation, see References 100, 101, 290, 288.

B. Respiratory Compensation

In well-aerated water, the obligatorily high gill ventilation rates and high capacitance of CO_2 allows metabolically generated CO_2 to readily leave through the gill without a substantial buildup in the tissues of fishes, as it may in air-breathing vertebrates. This results in an arterial P_{CO_2} that is never more than a few mmHg above the water irrigating the gills (Pa_{CO_2} ~1 mmHg in normocapnic water), a minute CO_2 gradient compared with air-breathing vertebrates such as mammals (~40 mmHg, see Table 3.1 of Ref. 758 for comparisons to other animals). Accordingly, the blood bicarbonate concentration of fishes is also low (~4 mM in normocapnic water compared with 24 mM in humans, Ref. 758). The low steady-state Pa_{CO_2} and bicarbonate concentration minimize the ability of fishes to "blow-off" buffered metabolic acid loads via hyperventilation as occurs in mammals and reduces the buffering capacity of the extracellular compartment during metabolic acid-base disturbances. For example, data on longhorn sculpin, lemon sole (*Parophrys vetulus*), and rainbow trout show that acute mineral acid infusions actually cause a slight respiratory acidosis (in addition to a severe metabolic acidosis) (108, 481, 744), suggesting a lack of any respiratory compensation. The same was shown for several species when lacticacidosis was induced by strenuous exercise; a slight respiratory acidosis (Pa_{CO_2} increase of 2–3 mmHg) developed that lasted for 3–10 h even though hyperventilation occurred in response to internal hypoxia (reviewed in Refs. 288, 822).

Although respiratory compensation for metabolic acidosis does not appear to exist in fishes, several elasmobranch and teleost species have been shown to hyperventilate in response to environmental hypercapnia (see sect. IV*D2* and Table 2 of Ref. 253). However, this mechanism is not an effective compensation for respiratory acidosis for three reasons: *1*) it only responds to hypercapnia of external origin, and not to hypercapnia of endogenous origin (253); *2*) even if hyperventilation was pronounced enough to abolish the Pv_{CO_2} gradient between blood and environmental water completely, the blood pH would only be raised by one- or two-tenths of a pH unit (calculated from Henderson-Hasselbalch equations) above what it would be in the absence of hyperventilation because of the small steady-state P_{CO_2} gradient at the gills (589); and *3*) hyperventilation can never fully compensate for an environmental hypercapnia, because the source of the acidosis is the respiratory medium. Therefore, the P_{CO_2}-sensitive hyperventilation is more likely to have evolved as a "back-up" of P_{O_2}-sensitive hyperventilation to maintain O_2 uptake at the gills, because hypoxia and hypercapnia usually occur simultaneously in natural waters (253, 589).

C. Metabolic Compensation

The limitations that water-breathing imposes on bicarbonate buffering and respiratory compensations make fishes heavily dependent on net exchange of nonvolatile acid-base equivalents with their environment, a task well suited to gills. As noted earlier, fish gills are covered with an epithelium that is in direct contact with an aqueous external medium that is usually large enough to act as an infinite source and sink of solutes (i.e., acid-base equivalents and ions with which they are exchanged). Assuming an appropriate ionic environment (e.g., available counter ions and near neutral pH), this is an advantage over internal transport tissues such as the kidneys, which could build-up opposing gradients because of the limited volume of the urine. For example, resting water flow rates at the gills are between 5,000 and 20,000 ml · kg^{-1} · h^{-1} (e.g., Refs. 633, 832), which dwarfs the urine flow rates of fishes (~1–10 ml · kg^{-1} · h^{-1}; e.g., Refs. 95, 129, 438, 737, 833) and other vertebrates (e.g., humans, ~1 ml · kg^{-1} · h^{-1}; Ref. 643) by several orders of magnitude. These high

flow rates presumably minimize the creation of large pH gradients across the gills during times of branchial net-acid excretion and usually obviate the need for complementary secretion of buffers such as phosphate and ammonia, which are required by kidneys (370). Accordingly, although the skin, rectal glands, and kidneys of fishes have all been implicated in net acid-base transport, the gills usually account for over 90% of compensatory net acid-base transfers in fishes (see Tables 6 and 7.3 in Refs. 288, 758).

Data indicate that most fishes secrete a nonvolatile, net acid flux of 10–100 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} under control steady-state acid-base conditions, with a few exceptions where a net base flux of similar magnitude is observed (95, 302). This "typical" control net acid flux is comparable to that of mammals such as humans ($\sim$50 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}) (see Tables 1 and 7.2 in Refs. 288, 758), and presumably reflects a net production of nonvolatile acidic equivalents from several metabolic processes. In nature, fishes experience large deviations from these control values caused by either external stressors, such as changes in respiratory gas partial pressures (e.g., hypercapnia, hypoxia, and hyperoxia), pH, salinity, and temperature, or by internal stressors such as lacticacidosis (e.g., during prey capture, predator avoidance, and migrations). By fitting fish with indwelling blood catheters, and placing them in recycling water containers of fixed volume, the internal acid-base responses and whole animal compensatory net acid fluxes to these stressors have been studied extensively in teleosts (75, 106, 107, 304, 592, 751, 831) and elasmobranchs (95, 102, 128, 172, 291, 302, 511), and to a lesser degree in lampreys and hagfish (479, 803). For details on the blood acid-base status, and time courses of compensatory transport, see the following excellent reviews (100, 288–290, 758).

In general, regardless of the fish taxon or the source of the acid-base stress, the return of blood toward control pH is primarily due to adjustments of blood bicarbonate concentrations via exchange of acid-base equivalents at the gills. Although variable with the type and extent of acid-base disturbance, compensatory transport is usually activated within 20–30 min of the disturbance and can reach net-acid excretion rates of 1,000 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} within a few hours of inducing acidosis (e.g., Refs. 95, 106, 109, 303, 820, 822) and net-base excretion rates as high as 1,000 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} within a few hours of inducing alkalosis (e.g., Ref. 820). Therefore, fish gills respond to acid-base disturbances rapidly and efficiently, demonstrating well-developed mechanisms of acid-base transport that are under tight regulation.

The mechanisms of ion and acid-base transport across the gill epithelium have been the subject of many studies in the past 70 years, beginning with the first studies by Homer Smith (701, 702) and August Krogh (373). Early in vivo experiments correlated isotopic fluxes of Na^+ and Cl^- to fluxes of net acid and ammonia across the gills of freshwater teleosts and demonstrated that secretion of endogenous acid (H^+ and/or NH_4^+) is linked to transepithelial absorption of Na^+ and that secretion of endogenous base (HCO_3^- and/or OH^-) is independent of acid secretion and linked to transepithelial absorption of Cl^- (250, 358, 428). Although the goal of these early studies was to determine the mechanism of ion absorption in freshwater fishes (see above), the model also provided mechanisms for secreting ammonia (see below) and regulating the metabolic component of extracellular acid-base status (i.e., strong ion difference). It was originally assumed that seawater fishes would not possess these transport mechanisms, because of the obvious osmotic and ionic burden that active Na^+ and Cl^- absorption would impose on the active NaCl secretion mechanisms (see above). However, in vivo studies on seawater fishes demonstrated similar links between Na^+ and Cl^- absorption and acid and base secretion (reviewed in Ref. 180). These include a demonstration of complete inhibition of net-acid excretion from two marine elasmobranchs [spiny dogfish and little skates (*Raja erinacea*)] and two marine teleosts (toadfish and longhorn sculpin) when external Na^+ was substituted with the organic ions choline or *N*-methyl-D-glucamine (108, 172, 196), and an increased net-acid excretion from longhorn sculpin (presumably due to decreased HCO_3^- secretion) when external Cl^- was substituted by isothyanate (108). The presence of external Na^+- and Cl^--linked acid and base secretory mechanisms in seawater fishes is now generally accepted, despite the incurred ionic load (100, 101, 180, 290, 597) (see also sect. v*G*).

Regardless of the mechanisms, these Na^+- and Cl^--linked acid-base transport mechanisms make acid-base regulation sensitive to external salinity. For example, seawater teleosts generally compensate for acidosis faster than freshwater teleosts when presented with similar pH disturbances (103, 324, 585, 743, 751). Recently, we examined the effect of salinity on compensation from hypercapnia in the euryhaline Atlantic stingray and demonstrated that salinity has the same effect on rate of recovery in elasmobranchs (95). In two of these studies, [HCO_3^-] in the water was held constant, ensuring that the effect of salinity was due to the availability of Na^+ and Cl^- counterions, and not bicarbonate in the water at the surface of the gills (95, 324). The universal finding that acid secretion is linked to Na^+ absorption, and that base secretion is linked to Cl^- absorption, does not mean that all fishes possess the same arrangement of acid-base transport proteins in their gills. In fact, its now clear that the type of acid-base transport proteins expressed, and their locations (cellular and subcellular), vary with species and with external water conditions, such as salinity and pH (101, 442). Many studies have focused on the identification and location of proteins involved in acid

secretion (e.g., V-ATPase, Na^+-K^+-ATPase, NHEs, anion exchangers, CA, NBC) with pharmacological, immunological, and molecular techniques (see sect. v*B*, *1* and *2*). These studies support the presence of two acid secretion mechanisms in fishes, which are also the two proposed mechanisms of Na^+ absorption: *1*) an apical V-ATPase electrically linked to Na^+ absorption via ENaC-like channels, and *2*) electroneutral exchange of Na^+ and $H^+(NH_4^+)$ via proteins of the NHE family. Fewer studies have focused on the identification and location of proteins involved in base secretion, but they suggest the presence of two apical Cl^-/HCO_3^- exchangers in fishes: *1*) AE1 and *2*) pendrin.

D. Acid Secretion

1. V-ATPase

Pharmacological agents have been used to determine the possible mechanisms of acid secretion by the gills of several fish species. In addition to blocking Na^+ absorption (see sect. v*B1*), amiloride has been shown to inhibit net-acid excretion when placed in water irrigating the gills of rainbow trout, brown trout, European flounder, killifish, little skate, and longhorn sculpin (74, 108, 111, 173, 407, 522, 575, 576). However, these results do not distinguish between the two acid secretion mechanisms, because amiloride inhibits Na^+ transport by NHEs and ENaC (39). For example, amiloride would be expected to inhibit acid secretion from an NHE by blocking the exchanger directly, but could also theoretically inhibit acid secretion by inhibiting an ENaC-like channel and thereby altering the apical electrochemical gradient (623). However, three studies were able to eliminate NHE as the predominant acid transporter in three freshwater teleost species. In rainbow trout, 0.1 mM amiloride had no effect on net acid excretion (407), a concentration that inhibits >80% of Na^+ absorption in this species (595, 838). Similarly, 1 mM amiloride had no effect on net acid excretion from brown trout but did inhibit ~70% of Na^+ absorption (522). Finally, 5 mM 5-(*N*,*N*-dimethyl)amiloride (HMA), a specific inhibitor of NHEs (485), had no effect on net acid excretion (or Na^+ absorption) from isolated gill filaments of freshwater European flounder (111). These demonstrations of decoupled acid secretion and Na^+ absorption are strong arguments against a direct role for apical NHEs in freshwater teleosts, and together with the apical localization of V-ATPase in rainbow trout, coho salmon, and tilapia (see sect. iv*B1*), help support the role of V-ATPase as the predominant mechanism of acid secretion in freshwater teleosts.

Several studies have attempted to determine if the quantity of V-ATPase expression in freshwater teleost gills is increased during acidosis, which would be expected if this proton pump were responsible for the majority of acid secretion. In support of this hypothesis, a recent study used real-time PCR (RT-PCR) on rainbow trout gills to demonstrate large, sustained increases (4- to 70-fold increases) in V-ATPase expression during intravascular infusions of HCl (597). Unfortunately, the results for hypercapnia are conflicting, even though almost all of them were conducted on a single family of fishes (Salmonidae, mainly rainbow trout). For example, Northern blots and RT-PCR detected a transient 1.5- to 2.0-fold increase in B subunit expression in the first 2 h of chronic hypercapnia (587, 597), but this increase subsided to control levels by 4 h and was never again elevated for the rest of the experiment (24 h total). Other studies, using the same species and subunit, suggested increased mRNA and protein expression after 18 h of hypercapnia, a time frame that is out of phase with the RT-PCR and Northern blot results (587, 716, 717). In contrast, a preliminary study on rainbow trout failed to detect any change in A subunit protein levels after 40 h of hypercapnia (405), and a study on another salmonid (Atlantic salmon) actually measured a small, but consistent, decrease in B subunit mRNA expression after 24 h of 2% hypercapnia (676). Unfortunately, the reasons for these inconsistent results are unknown, but they do suggest that large changes in the expression of V-ATPase probably are not an important mechanism of regulating net acid secretion during hypercapnia. Interestingly, analogous tissues (such as mammalian renal tubule collecting ducts) appear to use rapid changes in the location (vesicular vs. apical) and not the total expression of V-ATPase pumps, as mechanisms of regulating acid secretion (34).

Prior to studies that localized V-ATPase and measured changes in the enzyme's expression, a series of ultrastructural studies were conducted to determine if morphological changes to PVC and/or MRC occur in response to acid-base disturbances, as they do in mammalian renal tubule intercalated cells (reviewed in Refs. 269, 393). Scanning electron micrographs demonstrated a 30 and 85% decrease in apical MRC fractional area (MRCFA) during hypercapnia in rainbow trout (267) and brown bullhead catfish (264), respectively (Fig. 32). They were also used to measure a 50% increase in MRCFA during metabolic alkalosis in rainbow trout (266, 270). These changes in MRCFA were directly proportional to changes in unidirectional Cl^- uptake and inversely proportional to changes in net acid secretion and unidirectional Na^+ uptake, suggesting that MRCs are responsible for apical Cl^-/HCO_3^- exchanges and not for acid secretion or Na^+ absorption (265). Alternatively, a 100% increase in the density of PVC microvilli was measured in response to hypercapnia in brown bullhead catfish (263) (Fig. 33), suggestive of an increase in apical cell area of this cell type (264). It was proposed that net acid secretion was increased by removal of Cl^-/HCO_3^- exchangers from the apical membrane of MRC and/or insertion of proton

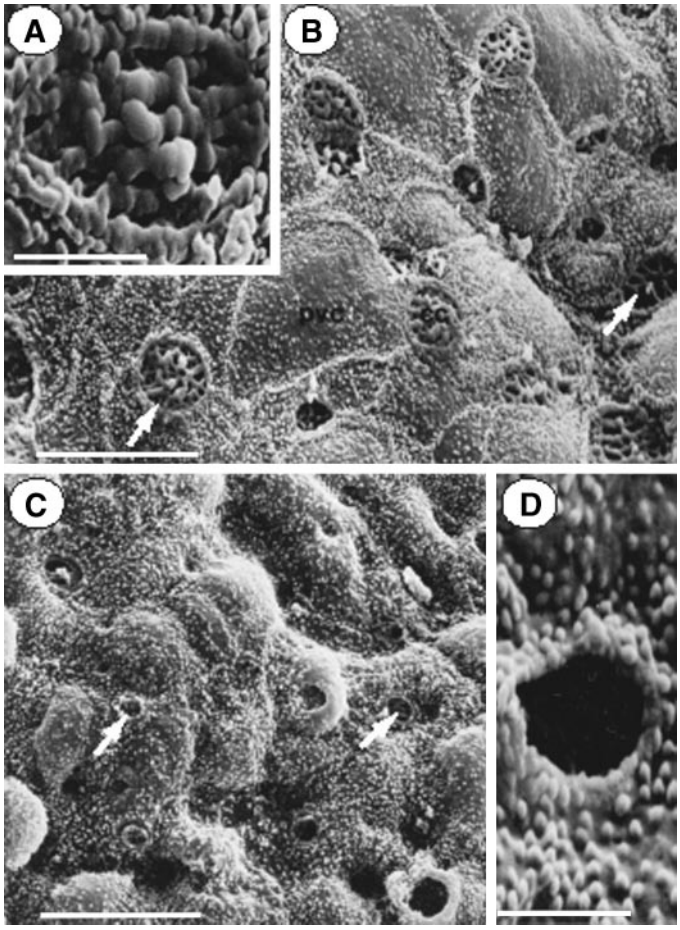

FIG. 32. Representative low- and high-magnification scanning electron micrographs of the filamental epithelium from brown bullhead catfish (*Ictalurus nebulosus*) during normocapnia (*A* and *B*) and after 6 h of environmental hypercapnia (*C* and *D*). Hypercapnia caused a reduction in apical mitochondrion-rich cell fractional area (i.e., MRC fractional area) and a change in the appearance of the MRCs. *A* and *D* are high magnifications of the cells marked with white arrows in *B* and *C*, respectively. Bars: 2 μm in *A* and *D*; 20 μm in *B* and *C*. [From Goss et al. (263).]

pumps into the apical membrane of PVCs (265), a model that is consistent with the expression of apical Cl^-/HCO_3^- exchangers in MRCs and V-ATPase in PVCs. However, one ultrastructural result contradicted this model: a metabolic acidosis was correlated with a 135% increase in MRCFA of rainbow trout (270). One explanation raised for this conflicting result was the possibility of two or more MRC subpopulations, one with apical Cl^-/HCO_3^- exchangers and one with apical proton pumps, which is consistent with studies that have localized V-ATPase in MRCs and PVCs (see sect. v*B1*). Therefore, although more work is needed to confirm the specific roles of MRCs and PVCs, opposing changes in the location of acid and base transporters (via altered apical membrane morphologies) may be important mechanisms for regulating rates of net acid excretion from the gills of freshwater teleosts.

It is important to note that with the exception of tilapia, all of the immunological evidence for apical V-ATPase is limited to salmonids, and therefore, it is premature to propose that V-ATPase is the sole route of acid secretion in all freshwater fishes. Moreover, with the exception of European flounder (111), all of the evidence against a direct coupling between Na^+ and H^+ are from salmonids, and therefore, it is premature to propose that apical NHEs do not function in any freshwater fishes. In fact, recent studies (see sect. v*B1*) on killifish, Osorezan dace, and Atlantic stingrays suggest that V-ATPase is not an important route of apical acid secretion in all fishes. Moreover, a homologous antibody for V-ATPase was used to exclusively localize the proton pump to the basolateral membranes of Na^+-K^+-ATPase-rich MRCs in the gills of freshwater acclimated killifish (352). Although mechanisms of acid-base transport were not proposed by the authors, it is clear that V-ATPase cannot be the direct route of acid secretion from this species. Previous pharmacological studies on killifish demonstrated a strong link between Na^+ absorption and net acid excretion when 0.1 mM amiloride was applied, which is more consistent

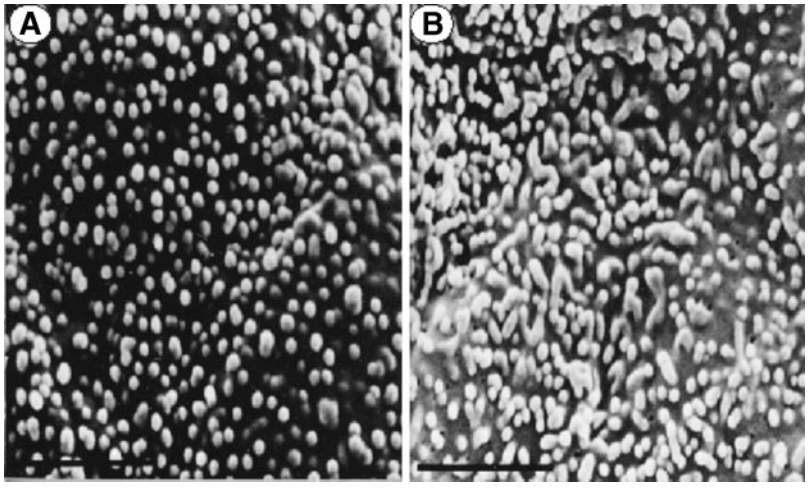

FIG. 33. Representative high-magnification scanning electron micrographs of pavement cells (PVCs) on the filamental epithelium of brown bullhead catfish (*Ictalurus nebulosus*) during normocapnia (*A*) and after 6 h of environmental hypercapnia (*B*). Hypercapnia caused an increase in microvilli density. Bars: 2 μm. [From Goss et al. (263).]

with an apical NHE than V-ATPase and an ENaC-like channel (576). In a molecular and immunological study by Hirata et al. (296), control Osorezan dace were shown to have low levels of V-ATPase B subunit expression, which increased only slightly when exposed to low pH water (3.5) for up to 5 days. In light of much larger increases in other acid transporters, it was suggested that V-ATPase does not play a large role in acclimation to low pH (a condition that causes metabolic acidosis; Refs. 100, 290), and an alternative model was presented (see below). Finally, V-ATPase may have roles in base secretion and Cl^- absorption, as opposed to acid secretion, in nonteleost fishes. As mentioned in section v*B1*, V-ATPase has been localized to the basolateral region of MRCs that contain apical immunoreactivity for pendrin in fresh and seawater elasmobranchs (201, 605, 607). Similarly, V-ATPase is basolateral in type B intercalated cells of mammalian renal tubule collecting ducts and therefore provides a route of acid exit from the serosal side of the cells, in addition to generating base for pendrin (the apical base secreting anion exchanger) (652). This function is also presumed for V-ATPase and pendrin-rich cells of elasmobranchs (606).

2. NHE

Intuitively, NHEs might be proposed as the dominant route of acid secretion from fishes in seawater, where the high Na^+ concentration favors Na^+ entry across the apical membrane (see reviews in Refs. 100, 101). HMA (0.1 mM) was shown to inhibit 75% of net acid excretion from longhorn sculpin in brackish water, suggesting that NHEs are responsible for the majority of acid secretion from this seawater teleost (108). This result and the finding of reduced or absent immunoreactivity for V-ATPase in seawater rainbow trout and coho salmon (405, 810) have led the general idea that NHEs are the proteins responsible for acid secretion at the apical side of seawater fish gills (100, 101). Unfortunately, the effects of specific NHE inhibitors on acid secretion from seawater fishes have only been reported for longhorn sculpin, so it is not known if this generality can be applied to all seawater fishes.

Despite the relatively few pharmacological data that support a direct role in acid excretion, NHEs are clearly expressed in the gills of many fishes, and recent studies have demonstrated large increases in expression during treatments that cause acidosis. For example, full-length transcripts homologous to NHE2 have been cloned from longhorn sculpin, killifish, and spiny dogfish (101), and a full-length transcript homologous to NHE3 has been reported from freshwater Osorezan dace (296). In addition to their previously mentioned roles in Na^+ absorption, these isoforms are responsible for acid secretion from mammalian intestinal and renal proximal tubule cells, and therefore are predicted to facilitate Na^+/H^+ exchange at the apical membrane of seawater fishes (101). Before the availability of these full-length fish sequences, heterologous antibodies were used to localize NHE2 and -3 immunoreactivity in many species of seawater teleosts and elasmobranchs (101, 167, 168, 810). In general, the immunoreactivity was located in MRCs, and in some cases colocalized with Na^+-K^+-ATPase. This is consistent with models that predict that basolateral Na^+-K^+-ATPase creates a low intracellular [Na^+] to drive apical Na^+/H^+ exchange (97). On the other hand, a homologous NHE antibody was made recently for NHE2 of the spiny dogfish, and NHE2 immunoreactivity was localized to a population of MRCs that were not rich in Na^+-K^+-ATPase (793). The membrane location of this shark NHE2 was not obvious in light or confocal micrographs, so its function(s) in this cell type remain(s) to be determined. Future immunohistochemcal studies with homologous antibodies to the recently sequenced NHEs will help determine if these localization discrepancies are due to isoform, antibody, and/or species differences.

The gill of hagfish also has been shown to express an NHE, but a 1,047-bp transcript was found to be only 38–50% homologous to NHEs in GenBank (166). Despite this low homology, a hydropathy plot of the hagfish NHE suggested a similar topology to mammalian NHEs (166). In addition, relative RT-PCR was used to demonstrate an increase in expression following an injection of H_2SO_4, a treatment that causes metabolic acidosis. The increased expression was highest 2 h after the injection (166), a time that is consistent with the maximum rates of net-acid excretion (479), suggesting a direct role for this putative agnathan NHE in systemic acid-base regulation.

A recent study used homologous antibodies and molecular probes to support the hypothesis that NHE3 is the route of acid secretion from a unique freshwater teleost, the Osorezan dace (296). This is a freshwater teleost that lives in the extremely acidic (pH 3.4–3.8) Lake Osorezan of Japan. In this acid-tolerant fish, NHE3 immunoreactivity was localized to the apical side of MRCs that also expressed CAII, Na^+-K^+-ATPase, and NBC1. Northern blots were also used to demonstrate that the mRNA expression of NHE and other transport related enzymes (CAII and NBC1) increased greatly when dace were transferred from water of neutral to highly acidic pH (3.5) (296), a condition that causes metabolic acidosis. Interestingly, NHE3 is responsible for most of the acid secretion from brush-border membranes of mammalian renal proximal tubule cells where its expression also increases during metabolic acidosis (2). Mammalian renal proximal tubules also express CAII, Na^+-K^+-ATPase, and NBC1, making a compelling argument that the dace NHE3-rich cells function in a similar manner (see model in Fig. 34). The most interesting characteristic of this study is that it suggests that an NHE-dependent mechanism may be func-

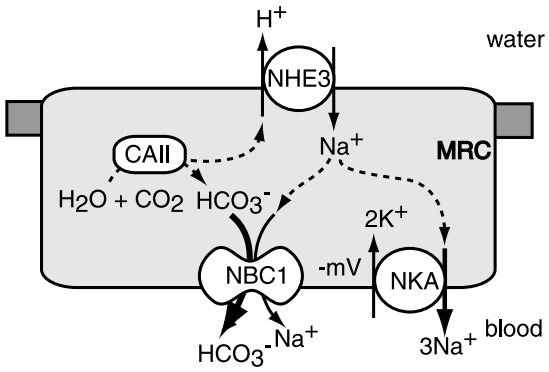

FIG. 34. Model of acid secretion and Na^+ absorptive mechanisms in gill MRCs of FW Osorezan dace. Expression of transcripts for all four proteins were shown to increase during exposure to acidic water (pH 3.5) and were localized to a single cell type with dace-specific antibodies (296). In the model, acid secretion and Na^+ absorption are initiated by Na^+-K^+-ATPase, which produces a low intracellular [Na^+] and a negative inside membrane potential. These conditions then favor Na^+ absorption, in exchange for acid secretion through an apical NHE3, which increases the intracellular pH. The higher pH increases intracellular [HCO_3^-] via CO_2 hydration by carbonic anhydrase II. Finally, the increased intracellular [HCO_3^-] and negative potential drive electrogenic efflux of Na^+ and HCO_3^- across the basolateral membrane through NBC1. Electrogenic transport is indicated with unequal arrow weights. Solid arrows indicate facilitated transport, and broken arrows indicate diffusion. See text for abbreviations and details. [Modified from Hirata et al. (296).]

tioning in fresh waters with a Na^+ concentration of ~1 mM and a pH below 4.0 (296), concentrations that should make an apical NHE thermodynamically unfavorable (14). Although CAII, Na^+-K^+-ATPase, and NBC1 activity in the same cells would be expected to help make H^+ and Na^+ gradients more favorable by lowering intracellular [Na^+] and pH, it is hard to imagine how a >1,000-fold uphill H^+ gradient could be overcome. However, two other strategies might be involved: *1*) the expression of glutamine dehydrogenase (GDH) increased markedly in all tissues in acidic water, and *2*) the NHE3-rich cells formed a follicle in acidic water (296). GDH is a mitochondrial enzyme that catalyzes NH_4^+ and HCO_3^- production from the substrate glutamine, and the increase in its expression suggests large increases in NH_4^+ and HCO_3^- synthesis in low pH water. Presumably, the increased NH_4^+ synthesis would be secreted from the gills (see below), leaving a net base HCO_3^- gain in the fish. In mammals, acidosis causes this to happen predominantly in the kidneys, where NH_4^+ is secreted by NHE3 in the proximal tubule (370). Therefore, as suggested by Hirata et al. (296), the large proton gradient in dace may be overcome by secreting NH_4^+ via NHE3, instead of H^+; a demonstration of higher affinity for NH_4^+ than H^+ would help support this model. Alternatively, ammonia may leave the gills in its basic gas form (NH_3) by simple diffusion, as it does in most fishes (see below). This would buffer the water adjacent to the gills and therefore decrease the proton gradient inhibiting acid secretion. The follicular structure might help by partially sealing the NHE3-rich cells from the low pH of the bulk water and preventing immediate loss of buffers from its lumen. Clearly, more studies like these are needed with fish-specific immunological and molecular probes to determine if NHE3 and/or NHE2 expression are regulated during acid-base disturbances in other fishes, and where they are located in the gills.

3. H^+-K^+-ATPase

Although NHEs and V-ATPase are apical acid transporters in fish gills (and most models predict that one or the other functions in a given species), it is possible that other acid transporters also may be expressed in the gill epithelium. For instance, mammalian renal epithelial cells have H^+-K^+-ATPases, as well as NHEs, V-ATPases, along their luminal membranes. These transporters are divided further into isoforms that are thought to function in different nephron segments and may be stimulated by different pH and/or electrolyte imbalances (257, 567, 691, 813, 814). It has recently been demonstrated that a transcript homologous to an H^+-K^+-ATPase is expressed in the gills of elasmobranchs. RT-PCR was used with mRNA from spiny dogfish and Atlantic stingrays to obtain sequences that are over 80% identical to HKα1 sequences at the amino acid level. In addition, two antibodies made for different parts of mammalian HKα1 stain the gills of marine Atlantic stingrays (98). However, the subcellular location of this staining is not clear, and RT-PCR comparisons of expression between control and hypercapnic stingrays failed to detect a difference, making further expression and localization studies necessary to understand HKα1's role in gill acid secretion.

4. NBC

Base efflux across the basolateral membrane must compliment acid secretion at the apical membrane for net systemic acid excretion to occur in epithelial cells. The complete cDNA sequence of a homolog of a base transporter, NBC1, recently has been reported from the gills of rainbow trout (597) and Osorezan dace (296), and a partial sequence from the gill of Atlantic stingrays is also known (Choe and Evans, GenBank accession no. AY652419). NBC1 facilitates Na^+ and HCO_3^- efflux across the basolateral membrane of mammalian proximal tubules in a 1:3 Na^+-to-HCO_3^- ratio (649, 706) and has been hypothesized to have a similar function in fish gills (296). In support of this proposed role in systemic acid excretion, the dace NBC1 homolog was localized to the basolateral membranes of Na^+-K^+-ATPase-rich MRCs and its expression increased during exposure to low pH water (296). Similarly, expression of the rainbow trout gill NBC1 homolog increased during hypercapnia (597). As dis-

cussed by Perry et al. (597), it is also possible that the putative fish NBC1 homologs function in an influx mode, as occurs in mammalian pancreas cells (706). This would alkalize cells and would still be consistent with an increased expression during acidosis as a mechanism of intracellular pH regulation. However, at least for Osorezan dace, NBC1 was specifically localized in MRCs that were also shown to have apical NHE3 and basolateral Na^+-K^+-ATPase (296). Because the activities of these two highly expressed transporters would alkalize and hyperpolarize the cell, it is more likely that dace NBC1 functions in an efflux mode. Further studies are clearly needed to determine the role of NBC1 in fish gills.

E. Base Secretion

As mentioned above, AE1 has been localized to the apical side of a subpopulation of Na^+-K^+-ATPase-rich MRCs in the gills of two freshwater teleosts (coho salmon and tilapia) with an antibody raised against the rainbow trout erythrocyte band 3 protein. Interestingly, this finding of two or more subpopulations of freshwater MRCs agrees with some of the previously mentioned morphological and immunolocalization results for freshwater salmonids and suggests that there may be base- and acid-secreting freshwater MRCs (405, 806, 810). Simultaneous localization of AE1, V-ATPase, and Na^+-K^+-ATPase in the same sections would be needed to verify this possibility. Only one study has examined the effects of an acid-base stress on the expression of AE1 in the gills. This study demonstrated an increased number of cells expressing AE1 during chronic $NaHCO_3$ infusions (rainbow trout; Ref. 717), which suggests a role for this exchanger in base secretion. Although AE1 is predicted to also secrete base in seawater teleosts, evidence is still lacking. Wilson et al. (810) demonstrated a loss of immunoreactivity for AE1 when coho salmon were acclimated from fresh water to seawater. This could mean that a different base transporter was activated or that AE1 expression was diminished below detectable levels (but still functional with the higher external [Cl^-]). Clearly, more immunological and molecular data are needed before the model of apical AE1 in Na^+-K^+-ATPase-rich MRCs is applied to all freshwater and seawater teleosts.

The use of AE1 in the apical membrane of Na^+-K^+-ATPase-rich cells to secrete base and absorb Cl^- in the fish gill is different from intercalated cells of mammalian renal collecting ducts in a two important ways. For example, in B type cells, the recently discovered anion exchanger pendrin is thought to be the apical acid exchanger instead of AE1 (652), and basolateral V-ATPase is the route of acid exit across the basolateral membrane (257). Alternatively, with the exception of killifish (352), V-ATPase is only associated with the apical membrane in freshwater teleosts and therefore is probably not the basolateral route of acid exit. It is still unclear how acid exits the basolateral membrane of the presumptive teleost base-secreting cells, and what role, if any, basolateral Na^+-K^+-ATPase plays in apical Cl^-/HCO_3^- exchange.

The recently developed model of base secretion and Cl^- absorption for the elasmobranch gill epithelium is much more similar to mammalian renal type B intercalated cells than the teleost model. For example, apical pendrin and basolateral V-ATPase immunoreactivity occur in a subpopulation of MRCs (607). Although immunoreactivity for pendrin and V-ATPase was inversely correlated with acclimation salinity (607), suggesting a direct role in Cl^- absorption for ion regulation, similar experiments are needed in relation to acid-base disturbances to verify a role in base secretion. In addition, the antibody used was raised against human pendrin, and no molecular data have been obtained for this putative anion exchanger in elasmobranchs. Therefore, molecular sequence for this putative exchanger is needed to determine its evolutionary affiliation within the anion exchanger families.

VII. NITROGEN BALANCE

A. Introduction

Unlike biomolecules such as fatty acids and sugars, excess amino acids are not stored or excreted. Their carbon skeletons are instead converted into intermediate metabolic fuel sources after removing the α-amino group (714). While the carbon backbone is converted into fatty acids, ketone bodies, and carbohydrates, the excess nitrogen is usually excreted. This net gain in excess nitrogen is magnified in the high-protein diets of many fishes where most of their dietary carbon is extracted from amino acids (825). In addition, fasting fishes can use their own muscle proteins as a source of amino acids, which are deaminated to produce ATP for maintenance, or converted to glucose by the liver (309). Excess nitrogen is not usually stored in tissues because it is either too toxic (i.e., ammonia) or too energetically costly to convert to less toxic forms (i.e., uric acid and urea). For example, ammonia, the most useful form of nitrogen for synthesizing amino acids, is highly toxic (see below) and therefore cannot be sequestered at any significant level. Although less toxic than ammonia, urea and uric acid require ATP consumption for their synthesis, counteracting the benefits of catabolizing proteins for energy. In addition, uric acid precipitates out of solution and is produced by terrestrial animals that need to conserve water (e.g., birds and reptiles) (836), obviously not a problem for at least freshwater fishes. Some fishes (elasmobranchs and coelacanths) do synthesize and sequester nitrogen as urea but for osmoregulation (see sect. v*C*), not nitrogen storage. In

fact, eukaryotes lack ureases, and therefore chondrichthian fishes cannot reincorporate the urea they synthesize unless they harbor urease-expressing bacteria (369). Although an apparently successful strategy (>1,000 extant species), the high urea concentrations of ureosmotic fishes (>300 mM) are toxic and must be balanced with stabilizing molecules such as trimethylamine oxide (844). Therefore, net production of nitrogenous waste occurs in most fishes, under most conditions, and because of toxicity and energetic costs of storage, most fishes deal with waste nitrogen by excreting it immediately. Nitrogenous molecules other than ammonia and urea are excreted by fishes (e.g., taurine, creatine, creatinine, purines, and methylamines), but their quantitative contributions are small and mechanisms of excretion poorly understood (785). Mechanisms of ammonia and urea metabolism and excretion have been studied extensively in fishes, and several excellent reviews are available (6, 77, 79, 189, 201, 258, 260, 579, 785, 788, 801, 802, 824). Here, we focus on mechanisms of excretion by the gills.

The gills are the primary site of ammonia and urea excretion from fishes for many of the same reasons that they are the primary site of gas exchange and systemic ion and acid-base transport (e.g., large surface area, perfusion by 100% of cardiac output, large ventilation rates, small diffusion distances, and contact with a voluminous mucosal medium). Branchial and renal nitrogen excretion rates have been compared in several species, and >80% of the total nitrogen (ammonia plus urea) was excreted from the gills of most species (see review in Ref. 824). The vast majority of fishes, including almost all actinopterygians and agnathans, excrete the majority of their nitrogenous waste as ammonia and are referred to as ammonotelic (see Table 1 in Ref. 824). This appears to be the default condition for fishes, reflecting the ease with which NH_3 can diffuse through tissues and into a large external aqueous environment (see below). Alternatively, coelacanths, most elasmobranchs, and a few teleosts excrete most of their nitrogenous waste as urea and are referred to as ureotelic (785, 836). Ureotelism appears to have evolved either as a mechanism of detoxifying ammonia in environments that inhibit ammonia excretion (e.g., air exposure and high pH) or as a mechanism of osmoregulation in seawater (coelacanths and elasmobranchs).

B. Ammonia

Ammonia is a weak base and occurs as both a gas, ammonia (NH_3), and an ion, ammonium (NH_4^+), in aqueous solutions; the sum of both forms is referred to as total ammonia (T_{amm}). Because the pH of fish blood and intracellular fluid is over one unit below the p*K* of ammonia (~9–10) (76), ~95% of T_{amm} exists as NH_4^+ in fish tissues. Although the specific mechanisms of T_{amm} toxicity in animals are not completely understood, high micromolar concentrations are clearly incompatible with many tissue functions (80). The most acute effects of ammonia are probably related to the ability of its ionic form, NH_4^+, to substitute for K^+ in ion transporters and disrupt electrochemical gradients in central nervous systems (125). This may explain why fishes, which have less advanced central nervous systems, are more tolerant of T_{amm} than mammals. For instance, T_{amm} concentrations in human blood are kept below 40 μM by hepatic urea synthesis, and even small increases (50%) cause neural pathologies. Control T_{amm} concentrations reported for fish blood are up to an order of magnitude higher and more variable (between species and study) (see Table 2 in Ref. 824). Wood (824) attributed much of the variability to different blood collection and analysis techniques and estimated that the "true" control arterial T_{amm} concentrations of fishes are <500 μM and typically between 100 and 200 μM. Although fishes are more tolerant of T_{amm} than mammals, they are susceptible to the same toxic effects at higher concentrations (634) and therefore must excrete T_{amm} at the same rate it is synthesized.

Unlike in mammals, where ammonia is synthesized at the site of excretion, i.e., kidneys (368), <25% of excreted ammonia is synthesized at the gills of fishes. Most of the ammonia excreted by the gills is produced in other tissues and cleared from the blood. During control conditions, the liver appears to synthesize the majority of ammonia in fishes, with skeletal muscle, kidneys, and gills contributing in descending quantitative order (579, 824). However, skeletal muscle can dominate ammonia synthesis during glycolytic exercise or hypoxia via adenylate deamination (reviewed in Ref. 824). Whole body T_{amm} excretion rates have been measured for many species, including teleosts, elasmobranchs, and agnathans. Under control conditions, most ammonotelic fishes have excretion rates between 100 and 350 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, and ureotelic elasmobranchs have excretion rates below 50 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (reviewed in Ref. 824). However, T_{amm} excretion rates over 1,000 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ have been measured after feeding (825) and NH_4Cl infusion (78, 479, 820), demonstrating a high capacity for T_{amm} transport across the gill epithelium.

Despite being studied exhaustively over the past 30 years, the exact mechanisms of ammonia transport across the branchial epithelium of fishes remain somewhat controversial, because it is difficult to measure and/or control transepithelial gradients of pH, P_{NH_3}, NH_4^+, and electrical potential simultaneously in whole fish or whole tissue experiments (785). Although at least five potential mechanisms have been hypothesized, with some data supporting each (189), the majority of current experimental data support three pathways of ammonia excretion from the gills of fishes (see model in Fig. 35). These include *1*) NH_3 diffusion, *2*) NH_3 diffusion trapping, *3*) Na^+/NH_4^+ ex-

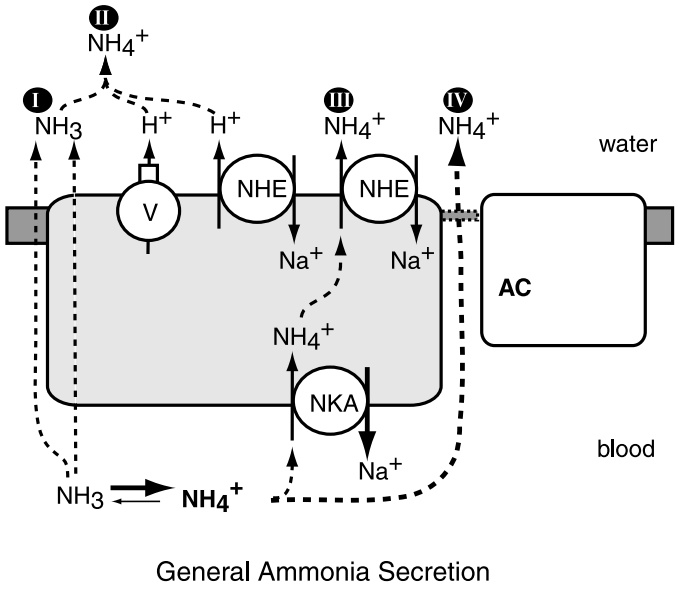

FIG. 35. Composite model of ammonia secretion pathways in the gills of fishes (e.g., agnathans, elasmobranchs, and teleosts), numbered in decreasing order of occurrence (Roman numerals). *I*, diffusion of NH_3 either through or between epithelial cells down favorable blood-to-water diffusion gradients. *II*, diffusion of NH_3 that relies on proton secretion to "trap" ammonia as NH_4^+. *III*, transport of ammonia through NHE and/or Na^+-K^+-ATPase. *IV*, diffusion of NH_4^+ through the leaky junctions that occur between MRC and AC in seawater teleosts. *Pathway I* can occur for any cell type, *pathways II* and *III* can only occur in acid secretion cells, and *pathway IV* can only occur with salt secretory cells of teleosts. See text for abbreviations and details. [Modified from Wilke (802).]

change, and *4*) NH_4^+ diffusion. Each mechanism is not exclusive, and their quantitative contributions are determined by conditions such as external water, salinity, pH, and buffer capacity.

1. NH_3 diffusion

Experiments on an agnathan, an elasmobranch, and a number of freshwater teleost species suggest that most T_{amm} crosses the branchial epithelium as NH_3, down favorable blood-to-water diffusion gradients (teleost data reviewed extensively by Refs. 801, 802) (Fig. 35, *pathway I*). Three lines of evidence support this conclusion. *1*) Moderate decreases in external water pH, which decreases [NH_3] in the external water via protonation, increased the rate of T_{amm} excretion from freshwater rainbow trout, carp, and goldfish (14, 107, 425, 426, 838). *2*) Increases in external water pH, which increases [NH_3] in the external water via deprotonation, decreased the rate of T_{amm} from freshwater rainbow trout (482, 804, 838, 845). *3*) Ammonium injections [NH_4Cl or $(NH_4)_2SO_4$] caused an initial increase in T_{amm} excretion, in excess of net acid excretion, from Atlantic hagfish, spiny dogfish sharks, rainbow trout, and channel catfish, suggesting that at least some of the infused ammonium dissociated and crossed the gills as NH_3 (76, 78, 479, 820). These ammonium injections also caused a metabolic acidosis, which presumably reflects residual H^+, left in the blood after dissociation of NH_4^+ and excretion of NH_3.

2. NH_3 diffusion trapping versus Na^+/NH_4^+ exchange

As discussed in sections VI*B1* and VI*D*, early models of gill Na^+ absorption suggested the presence of a Na^+/NH_4^+ exchange system in freshwater teleosts. This hypothesis was formed from observations that amiloride addition to and Na^+ removal from external water decreased net ammonia excretion rates and that ammonium salt injections increased Na^+ absorption in freshwater teleosts (reviewed in Ref. 801). However, as pointed out by Avella and Bornancin (14), all these results can also be explained by diffusion trapping of NH_3 via H^+ or CO_2 secretion into an unstirred layer of water on the apical surface of the gills (Fig. 35, *pathway II*). For instance, amiloride addition and Na^+ removal could inhibit T_{amm} excretion by inhibiting acid secretion from either NHEs or the V-ATPase/ENaC-like system (see sect. VI*D1*). A test of this hypothesis was conducted on rainbow trout by adding HEPES to the water (812), which should make diffusion trapping of NH_3 negligible, because the external buffer would bind with any secreted acid at the apical surface of the gills, minimizing acidification of the unstirred layer (801, 802). Despite a 90% inhibition of Na^+ uptake by amiloride in the HEPES-buffered fresh water, T_{amm} excretion was unaffected. Similar results were observed for Lahontan cutthroat trout (*Oncorhychus clarki henshawi*), which live in highly buffered, alkaline water of Pyramid lake (837). This lack of Na^+/NH_4^+ exchange in freshwater trout probably reflects the use of apical V-ATPase and an ENaC-like channel for acid secretion and Na^+ absorption (instead of apical NHEs), as is hypothesized for freshwater salmonids, tilapia, and flounder (see sect. VI*D1*).

Most seawater and some freshwater fishes, however, are thought to use apical NHEs for acid secretion and Na^+ absorption, and therefore at least have the potential for apical Na^+/NH_4^+ exchange (see sect. VI*D2* and Ref. 101). Nevertheless, most of the data for seawater fishes under normal, low external T_{amm} conditions suggest that little to no ammonia excretion is via Na^+/NH_4^+ exchange (802). For example, Na^+ removal from and amiloride addition to external water inhibited net T_{amm} excretion from intact spiny dogfish sharks and Gulf toadfish (*Opsanus beta*) by $<$50% (172). In addition, similar treatments had no effect on net T_{amm} excretion from intact Atlantic hagfish (178) and little skates (196), and amiloride, in the presence of oubain, had no effect on net T_{amm} excretion from isolated perfused heads of spiny dogfish sharks and toadfish (198, 199). Therefore, under normal conditions when the T_{amm} in external water is low, diffusion (NH_3 and/or NH_4^+)

probably dominates. However, even if we assume that seawater fishes have apical NHEs that can substitute NH_4^+ for H^+, Na^+/NH_4^+ exchange might not be needed in most cases because of favorable diffusion gradients for NH_3 and NH_4^+. Alternatively, in crowded or confined waters, the T_{amm} can rise rapidly, eliminating the favorable diffusion gradients for NH_3 and NH_4^+ (634), requiring active secretion of ammonia against a gradient.

For fishes, the only unequivocal demonstration of T_{amm} excretion against a gradient is from the giant mudskipper, an amphibious, air-breathing teleost that lives in brackish-water swamps of Southeast Asia (635, 808). Mudskippers regularly leave water and have apparently evolved a mechanism of active ammonia excretion in the absence of favorable diffusion gradients. Randall et al. (635) demonstrated that T_{amm} excretion from this species was maintained in water of 30 mM T_{amm}, without any increase in blood concentration from its normal value of ~150 μM. T_{amm} excretion was not affected by HEPES, suggesting that diffusion trapping via boundary layer acidification did not play an important role in ammonia secretion (808). Alternatively, it was inhibited by amiloride and ouabain, suggesting the use of NHEs and Na^+-K^+-ATPase for active NH_4^+ secretion. Wilson et al. (808) used heterologous antibodies to demonstrate immunolocalization for NHE2 and NHE3 in the apical area of MRCs that also expressed high levels of basolateral Na^+-K^+-ATPase (808). Therefore, it was hypothesized that active branchial ammonia excretion from these mudskippers takes place by a mechanism very similar to mammalian renal proximal tubules, in which NH_4^+ is secreted across the apical membrane by substituting for H^+ in NHE (368, 567). Na^+-K^+-ATPase in the basolateral membrane assists by creating a low intracellular [Na^+] that drives NHE, and by directly facilitating NH_4^+ entry into the cell (via substitution for K^+ on Na^+-K^+-ATPase) (Fig. 35, *pathway III*). More recent experiments suggest that the gills of this species also secrete acid to trap any excreted NH_3 as NH_4^+ and maintain low skin NH_3 permeability by a combination of high cholesterol and fatty acid content (320).

As discussed above, freshwater Osorezan dace may also be capable of apical Na^+/NH_4^+ exchange to allow acid secretion and Na^+ absorption in a highly acidic environment (see sect. VI*D2*). In addition, hagfish, elasmobranchs, and marine teleosts are all thought to have a similar arrangement of transporters for acid secretion in MRCs, and therefore also may be able to secrete ammonia actively when external T_{amm} increases so that diffusion gradients are reversed from normal. Future studies should measure expression levels of these potential NH_4^+ transporters during high external ammonia (40) to test this possibility. In addition, heterologous expression systems should be used to determine if the recently cloned fish NHE2- and NHE3-like transcripts (see sect. VI*D*) can transport NH_4^+ when it is present at physiological concentrations.

3. NH_4^+ diffusion

NH_4^+ diffusion is probably minimal in all fishes except seawater teleosts and lampreys, because of low permeability to cations by the branchial epithelium (see sect. II*B3*). Seawater teleosts and lampreys have shallow tight junctions between MRCs, which increase cation permeability for Na^+ secretion (see sect. II*B3*) and therefore are probably more permeable to NH_4^+ than other fishes (199) (Fig. 35, *pathway IV*). This was suggested by experiments on seawater longhorn sculpin, which did not develop a blood alkalosis when exposed to high environmental T_{amm} concentrations, suggesting that ammonia loading occurred as the acid-base neutral form, NH_4^+, and not the basic form, NH_3 (104). In addition, Goldstein et al. (259) demonstrated that net ammonia excretion rates from seawater longhorn sculpin and toadfish were sensitive to changes in external NH_4^+ concentrations but not external NH_3 concentrations.

C. Urea

Blood urea concentrations have been measured in many fish species and appear to vary by taxonomic group and by conditions that inhibit passive ammonia excretion via diffusion (reviewed in Ref. 824). Ammonotelic teleosts and agnathans, which include the majority of their respective groups, typically have blood plasma urea concentrations below 2–3 mM, reflecting their low urea synthesis rates and dependence on ammonia excretion to remove nitrogenous waste. Higher plasma urea concentrations have been measured for teleosts that live in environments that are unfavorable for ammonia excretion. For example, the Lahontan cutthroat trout of Pyramid Lake, a cyprinid of Lake Van, Turkey (*Chalcalburnus tarichi*), and a tilapia of Lake Magadi, Kenya (*Alcolapia grahami*) have plasma urea concentrations of 8.15, 36.18, and 10.52 mM, respectively (see Table 2 in Ref. 824). These species all live in waters with a pH between 9.4 and 10.0 that inhibit passive NH_3 excretion via diffusion trapping (see sect. VII*B2*) and appear to elevate urea synthesis to partially (Lahontan cutthroat and cyprinid), or completely (Lake Magadi tilapia), compensate for reduced ammonia excretion (636). A few other fishes have elevated plasma urea concentrations, either during stress or confinement (gulf toadfish; Ref. 828) or air exposure (e.g., lungfishes; Ref. 824). By far, the highest blood urea concentrations are found in fishes that are ureosmotic, which include coelacanths and almost all elasmobranchs (Table 1). The only species of the latter group known to be ammonotelic belong to a single family of stenohaline freshwater stingrays in South America, the Potamotrygonidae (745).

As with ammonia, the majority of urea appears to be synthesized in the liver of fishes, regardless of the enzymatic pathways used to synthesize it (reviewed by Refs. 785, 824). Arginase and uricolytic enzymes synthesize the majority of urea in ammonotelic fishes and appear to be present in most species. A fully functional ornithine-urea cycle (OUC) appears only in chondrichthyes, coelacanths, and a limited number of teleosts (503). Because the majority of teleosts are ammonotelic, few studies have investigated mechanisms of urea transport in the gills of teleosts until the past decade. These have largely focused on two species that can be ureotelic and express a fully functional OUC, the gulf toadfish and the Lake Magadi tilapia (reviewed recently in Ref. 802).

1. Pulsatile urea excretion

Gulf toadfish are normally ammonotelic when they are first acquired from the wild, but soon become facultatively ureotelic (>70% of nitrogen excreted as urea) when crowded or confined in aquaria. Under these conditions, gulf toadfish excrete urea from the gills in discrete pulses that last under 3 h each and occur about one time per day (828). Remarkably, urea excretion rates rise from near zero ($\sim$10 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}) during steady-state, nonpulse times to over 300 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} during pulses, and blood concentrations decrease by $\sim$1 mM (827, 829). This excretion pulse is not due to rapidly increased blood urea concentrations or urea synthesis rates, because neither increases sharply before or during a pulse event (829). A series of in vivo experiments ruled out two of three hypothetical excretion mechanisms that could explain the observed pulses: *1*) involvement of a normally active back-transport system similar to elasmobranchs (see sect. VII*C4*) and *2*) changes in the general permeability of the gills (827). For example, neither Na^+ removal from nor urea analog addition to the external water increased urea excretion from toadfish during nonpulse steady-state periods, suggesting the absence of a back-transport system as occurs in elasmobranchs (see sect. VII*C4*). Such treatments would be expected to inhibit a back-transport system and increase urea excretion. A general increase in gill perfusion or permeability was also ruled out by experiments that demonstrated a lack of increased urea excretion after injection of a strong branchial dilator (L-isoprenaline) and by experiments that showed no change in the permeabilities of polyethylene glycol-4000 or water during urea pulses (827). Further experiments suggested that the urea permeability could be regulated by periodic translation, insertion, and/or activation of specific urea transporters. For instance, elevating urea concentrations in the external water to three times the blood concentration (30 mM) caused absorption of urea into the blood at each pulse event that was proportional to the relative concentrations in the water and blood, demonstrating that the transport system is reversible. This is a characteristic of rat inner medullary collecting ducts, which express the facilitated urea carrier UT-2 (696). Walsh et al. now have cloned, sequenced, and functionally expressed a urea transporter from toadfish gills (tUT), which was the first urea transporter sequenced from fish gills (787). The 1,814-bp cDNA (GenBank accession no. 165893) codes for a protein that is over 55% homologous to mammalian, amphibian, and elasmobranch kidney urea transporters and exhibits phloretin-sensitive urea transport when expressed in oocytes. Interestingly, of six tissues assayed with Northern blots including the kidneys, tUT expression was only detected in the gills of toadfish (787), confirming earlier divided chamber, and pharmacological studies that suggested that gills are the primary site of urea excretion. Therefore, it was concluded that tUT is the protein responsible for transient urea permeability in toadfish gills. However, no statistical change in tUT expression levels were detected with Northern blots during urea pulses, suggesting that regulation of urea excretion is posttranscriptional (787). In support of this hypothesis, Laurent et al. (394) used transmission electron micrographs to demonstrate an increase in vesicle numbers and proximity of vesicles to the apical membranes of PVCs in urea-pulsing toadfish. The authors suggested that tUT might be expressed in these vesicles, which are delivered to the apical membrane during a urea pulse.

2. Urea excretion at high external pH

The Lake Magadi tilapia is another unique teleost with respect to nitrogen metabolism. As mentioned earlier, it lives in the highly basic (pH 10.0) and buffered (CO_2 content = 180 mM) waters of Lake Magadi in Kenya that originate from volcanic hot springs (826). The Lake Magadi tilapia thrives in these conditions by having a fully functional OUC and excreting essentially all of its waste nitrogen as urea through the gill, which has an exceptionally high urea permeability (4.74×10^{-5} cm^{-1} $\cdot$ s^{-1}) (636). Furthermore, the nitrogen excretion rates ($\sim$3,302 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}) are high for teleosts, presumably because of high temperatures in the lake and the high nitrogen content of their cyanobacteria diet (826).

The results of in vivo, molecular, and microscopy experiments suggest that the gills of Lake Magadi tilapia excrete urea by a mechanism similar to the gulf toadfish gills during a pulsing event (786). For instance, the ratio of thiourea to urea permeability was 0.18, and urea transport was shown to be reversible and proportional to the relative concentrations in the external water and blood plasma, demonstrating a bidirectional transport system. These are both characteristics of toadfish gills during urea pulses, and of the rat inner medullary collecting duct (786). In addition, a 1,699-bp cDNA (GenBank accession

no. AF278537) coding for a 475-amino acid protein (mtUT) was cloned, sequenced, and functionally expressed from Lake Magadi tilapia (786). It is 75% identical to the tUT protein and is thought to be responsible for the exceptionally high urea permeability of the gills in this species. Finally, vesicles structurally similar to those proposed to contain tUT in toadfish were observed near the apical membranes of PVCs in Lake Magadi tilapia gill and were likewise proposed to contain mtUT. Clearly, immunolocalization and in situ hybridizations are needed to further test this hypothesis, especially in light of the more recent immunolocalization of a homologous UT in the MRC of eels (see below).

One important difference between urea excretion from Lake Magadi tilapia and toadfish is the lack of pulses in the former. Interestingly, although the amino acid sequences of the mtUT and tUT proteins are similar, they do differ with respect to potential PKC phosphorylation sites at their carboxy termini. Walsh et al. (786) have speculated that this could potentially explain the differences in how urea excretion is regulated between the two species.

3. Urea transporters in ammonotelic fishes

It was assumed that the relatively small amounts of urea excreted by ammonotelic teleosts crossed the gills through nonspecific pathways, such as via diffusion through lipid membranes (reviewed in Ref. 788). However, when a radiolabeled probe for tUT was hybridized with RNA from 14 species of marine teleosts under low-stringency conditions, homologous transcripts were detected in almost all of them (789). This suggests that specific facilitated urea transporters are expressed in the gills of most, if not all, teleosts regardless if they are ureotelic or not. In support of this hypothesis, Mistry et al. (498) cloned and sequenced a urea transporter from the ammonotelic Japanese eel (eUT) that is 486 amino acids and used a homologous antibody to localize it to MRCs. The function of this branchial urea transporter in an ammonotelic teleost is uncertain but could be a way of ensuring secretion of urea that is synthesized via non-OUC pathways under control conditions (e.g., cleavage of dietary arginine, and from uricolytic pathway of purine catabolism; Ref. 824). Interestingly, the expression of this branchial urea transporter was higher in seawater than in fresh water (498). The reason for higher expression in seawater is unclear but may be related to reduced urinary flow rates (and therefore reduced urea excretion by the kidneys) in high salinities requiring increased secretion by the gills (498).

Therefore, the expression of branchial facilitated urea transporters may be common to all teleosts but is probably most important in species that elevate urea synthesis to detoxify ammonia. The immunolocalization of eUT in MRCs of eels is not consistent with the hypothesis that vesicles near the apical membranes of PVCs contain urea transports (tUT and mtUT) in gulf toadfish and Lake Magadi tilapia (see sect. VII*C*, *1* and *2*). Clearly, immunohistochemical localization of tUT and mtUT are needed to evaluate this discrepancy.

4. Retention mechanisms in elasmobranchs

Despite being ureotelic and excreting over 80% of their nitrogenous waste as urea, elasmobranchs require branchial mechanisms to minimize loss of urea that is required to maintain osmotic balance in seawater. For instance, Pärt et al. (573) pointed out that the urea excretion rates of elasmobranchs would be ~10,000 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1} if their gills had urea permeabilities similar to teleosts, which is 40-fold greater than their actual excretion rates (250 μmol $\cdot$ kg^{-1} $\cdot$ h^{-1}). A low urea permeability by elasmobranch gills was first reported for the spiny dogfish by Boylan (55) and later verified by Fines et al. (213) to be 3.2×10^{-8} cm/s, which is over 60-fold less than the permeability of rainbow trout and eel gills (213). Unfortunately, detailed investigations of the mechanisms responsible for this low permeability have only begun in the past 10 years, and large gaps still exist in the current models (reviewed recently in Ref. 802). Although the low urea permeability of elasmobranch gills was originally assumed to be a purely structural adaptation (55), recent data suggest a combination of structural and active transport mechanisms (Fig. 36).

The structural adaptations may be due to an unusual composition of the plasma membranes in the gill epithelium. For instance, basolateral membrane vesicles (BLMV) from spiny dogfish shark gill tissue were shown to have the highest cholesterol-to-phospholipid ratios ever recorded for a natural biological membrane (3.68; Ref. 213). This was proposed as a mechanism to minimize urea entry into the epithelial cells across the basolateral membrane, because cholesterol is known to reduce urea permeability by allowing a tighter fit between adjacent phospholipid molecules (509).

Data from in vivo and isolated perfused head experiments suggested the presence of an active, back-transport system in the gill epithelium spiny dogfish sharks. For instance, urea excretion rates from this species were unaltered by up to 50% changes in urea concentrations of plasma or isolated-head perfusion fluids, suggesting a saturated transport mechanism (573, 820). In addition, urea excretion rates from spiny dogfish were increased by the competitive urea analogs acetamide and thiourea (820) and by the noncompetitive inhibitor phloretin (573), suggesting the presence of a carrier-mediated transport system. Finally, a washout experiment with isolated perfused heads of spiny dogfish sharks demonstrated a 14-fold greater urea transport rate across the basolateral membrane than across the apical membrane (573). These data

support a model of the gill epithelium as an "intermediary compartment" where the urea gradient across the apical membrane is lowered by active transport of intracellular urea across the basolateral membrane. More recently, a detailed study of BLMV from spiny dogfish shark gills further characterized the putative carrier-mediated transporter (213). Urea uptake by BLMV was inhibited by the urea analogs *N*-methylurea and nitrophenolthiourea and was inhibited in a dose-dependent manner by phloretin, confirming the presence of carrier-mediated transport. Furthermore, urea uptake was stimulated by ATP, and this stimulation was sensitive to ouabain, suggesting that the urea transporter was secondarily active, dependent on Na^+ and/or K^+ gradients created by Na^+-K^+-ATPase. Other experiments showed that Na^+ gradients, and not K^+ gradients, stimulated uptake, suggesting that the urea transporter is Na^+ dependent and probably uses the Na^+ gradients created by Na^+-K^+-ATPase. Finally, K_m for urea was 10.1 mM, which is much lower than the blood plasma urea concentration. The authors suggested that this may be indicative of an intracellular urea scavenging role for the transporter that actively returns urea to the blood (213).

Therefore, the current model for urea retention by elasmobranch gills suggests that the role of basolateral membrane is to minimize entry of urea with a low permeability via a high cholesterol-to-phospholipid ratio. Most of the urea that does enter the cells is then transported back out through a yet to be identified Na^+-stimulated urea transporter in the basolateral membrane, which maintains a low intracellular urea concentration to minimize urea diffusion across the apical membrane (Fig. 36) (213, 802). Obviously, further experiments are needed to identify and localize the putative basolateral, Na^+-stimulated urea transporter(s). Transcripts that are homologous to those cloned from the kidneys of elasmobranchs are expressed at low levels in the gills of spiny dogfish and Atlantic stingrays (326, 697), but they are passive and not thought to be a part of the basolateral membrane back transport mechanism (802). Further experiments on other species are also needed to determine if the proposed basolateral membrane adaptations of spiny dogfish sharks are shared by other elasmobranchs.

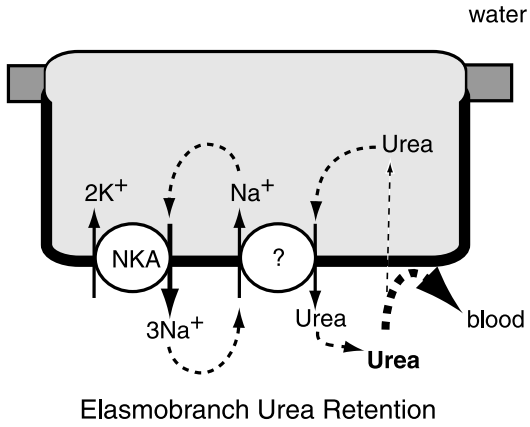

FIG. 36. Model of urea retention mechanisms proposed for marine elasmobranchs. The basolateral membranes of spiny dogfish shark gills were shown to have a high cholesterol content, which reduces the membrane's permeability to urea (indicated by a thick line for the basolateral membrane). Although this is thought to minimize urea entry into the cells, some still leaks in and is thought to be transported back across the basolateral membrane by an unidentified, Na^+-dependent urea transporter. The Na^+ gradient required for this urea transporter is thought to be maintained by Na^+-K^+-ATPase. See text for abbreviations and details.

VIII. NEURAL, HORMONAL, AND PARACRINE CONTROL

A. Introduction

Given the complexity of the perfusion pathways in the fish gills (see sects. III and IV) and the location of specialized transporting cells and paracellular pathways (see sects. V–VII), one might hypothesize that complex control systems have evolved to provide for homeostasis and the potential for rapid and long-term responses to environmental or internal changes. Recent reviews supporting this hypothesis include References 155, 188, 284, 443, 473, 531, 544, 546, 728. Interestingly, many of these signaling agents have both hemodynamic and ionic transport effects.

B. Intrinsic Control

1. Cholinergic neurons

Stimulation of the "vagosympathetic" nerve trunk to the isolated third gill arch of the Atlantic cod produced an increase in branchial vascular resistance, which was reversed by the addition of atropine (599), corroborating studies that demonstrated that acetylcholine produced an increase in branchial resistance in the rainbow trout (e.g., Refs. 54, 301, 698, 821). The early proposition that constriction of postlamellar, efferent vessels underlies this response (e.g., Ref. 698) has been confirmed by in vivo videomicroscopy that visualized constriction of the efferent filamental vessels in the rainbow trout, which was blocked by atropine (723). The site of constriction is generally assumed to be the prominent sphincter in the proximal portion of the efferent filamental artery that is innervated by vagal cholinergic fibers in a variety of teleost species (18, 163). Constriction in the postlamellar, efferent filamental artery is accompanied by increased blood flow into the ILV and increased flow through the lamellae, both of which are blocked by atropine (723). Similar, atropine-sensitive, changes in the perfusion pat-

tern and gill resistance can be elicited by hypoxia, which would increase the functional surface area of the gills, appropriate to compensate for hypoxic conditions (723). Moreover, diversion of blood into the ILV may provide greater perfusion of MRCs (although no evidence exists suggesting perfusion limitation to transport) and/or plasma skimming to enrich the hematocrit of the efferent filamental blood (e.g., Refs. 323, 542). Atropine sensitivity suggests a muscarinic receptor in the efferent gill vasculature, but no biochemical or molecular characterization of the putative receptor has been published. However, an M_3 type muscarinic receptor has been characterized in the spiny dogfish ventral aorta (193), so it is possible that this receptor mediates the cholinergic effects in the gills also.

To our knowledge only a single study has demonstrated that cholinergic innervation may have a direct effect on ionic transport across the gill epithelium. May and Degnan (469) found that acetylcholine inhibited the I_{sc} across the isolated killifish opercular epithelium.

2. Adrenergic neurons and chromaffin tissue

Epinephrine and norepinephrine reach gill tissues via both neurotransmission from sympathetic innervation (apparently only in teleosts; see sect. III*B*), and from stimulation of chromaffin cells in the teleost and elasmobranch interrenal gland, which is associated with the head kidney (e.g., Ref. 284). Intravenous infusion of catecholamines into a variety of teleost species is associated with a biphasic response in gill resistance: an initial, α-adrenergic-mediated increase, followed by a longer-lasting, β-adrenergic-mediated fall in resistance (38, 41, 572, 577, 588, 600, 784, 821). Stimulation of the sympathetic chain anterior to the celiac ganglion in the cod produced the same α-mediated vs. β-mediated responses (599). The β-adrenergic-mediated fall in gill resistance was associated with an increased oxygen uptake (e.g., Refs. 572, 588) and was presumed to be the result of lamellar recruitment (54). Accordingly, hypoxia elicits an increase in plasma catecholamine levels (e.g., Ref. 240), with the attending changes in gill perfusion (e.g., Ref. 529), as part of a complex response to maximize oxygen delivery to the tissues (254).[4] Measurement of branchial venous outflow in the cod in vivo has demonstrated that the α-mediated increase in resistance is secondary to constriction of the arteriovenous anastomoses between the efferent filamental artery and the ILV (730), where nerve terminals have been observed (778). The β-mediated fall in branchial resistance is generally considered to be the result of dilation of afferent lamellar arterioles, which produces lamellar recruitment (728). It is noteworthy that catecholamines also produce preferential arterio-arterial flow in the hagfish gill pouch (720), suggesting an ancient origin for at least the sympathetic, branchial control mechanisms.

In addition to potential effects of catecholamines on gill permeability and transport produced by changes in perfusion patterns (e.g., Ref. 537), it is clear that these vasoactive mediators can have direct effects on branchial ionic transport. The initial characterization of the killifish operculum as a model for teleost gill salt extrusion demonstrated that epinephrine inhibited the I_{sc} produced by the tissue (147). Subsequent studies demonstrated that Cl^- fluxes across the tissue could be inhibited by α-adrenergic activation and stimulated by β-adrenergic activation (148) and that inhibition versus stimulation of the I_{sc} was via α_2-adrenergic and β_1-adrenergic receptors, respectively (467). Moreover, cAMP is the second messenger for the β-stimulation (468). A more recent study has demonstrated that stimulation of the trigeminal nerve associated with the opercular epithelium produced a α-mediated inhibition of the I_{sc} with an associated increase in intracellular inositol trisphosphate (IP_3) levels (451). Adrenergic neurons may also play a role in modulating Ca^{2+} balance, because one study demonstrated that epinephrine injection or stimulation of adrenergic branchial nerves inhibited $^{45}Ca^{2+}$ uptake into the gill tissue of the rainbow trout (154).

3. Serotonergic neurons and neuroepithelial cells

The fact that the constrictory response to nerve stimulation in the cod could not be totally abolished in some preparations by pretreatment with either 10 μM atropine or phentolamine (α-adrenergic antagonist) suggested that some nonadrenergic, noncholinergic (NANC) fibers may exist in the gills (599). Serotonergic fibers now have been described in the gills of a variety of teleost species (19, 163, 721, 853), and serotonin increased branchial resistance in a number of species in vivo and in vitro (e.g., Refs. 242, 329, 583, 727). Video observation of blood flow through the rainbow trout gills has shown that serotonin redistributes blood flow to the more proximal parts of the filament, by constriction of distal portions of the efferent filamental artery and possibly the afferent filamental artery (725, 726). The resulting reduction of lamellar perfusion presumably accounts for the reduction in gas exchange when serotonin is infused into two species of teleosts (242, 721). In the cod, serotonin also dilates the arteriovenous anastomoses, shunting more blood into the ILV (718). Interestingly, serotonin apparently stimulates the release of catecholamines in at least the rainbow trout (241). Sensitivity to methysergide suggests that the serotonin effect is mediated by 5-HT_2 receptors in at least three species of teleosts (242, 583, 721, 725, 726). Seroto-

[4] In addition, gill vessels may respond to hypoxia directly as has been described for the efferent branchial artery and other vessels in the trout (704) as well as various systemic vessels from agnathan species (562).

nin is also produced in gill neuroepithelial cells (17, 162, 463, 853), which are on efferent side of gill filaments and assumed to be O_2 sensors (see sect. IV*D1*), but the relative roles of NEC release versus neuronal release are unclear.

To our knowledge the effects of serotonin on fish gill transport are unstudied, but the nonmetabolized, serotonin agonist 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT) stimulates Na^+-K^+-ATPase expression and enzymatic activity in the opossum proximal tubule (61). Therefore, similar studies in fish gills would be interesting.

Figure 37 presents a working model for the autonomic control of the teleost gill vasculature.

4. Nitrergic neurons and neuroepithelial cells

Branchial NANC innervation may also include nitrergic fibers, because NADPH-diaphorase reactivity was seen in 55–85% of branchial nerves in the cod (252), as well as in the putative sympathetic trunk (which supplies the gill arches) in stage 13 (4.5 days of development) of the spotted tilapia (*Tilapia mariae*; Ref. 776). NADPH-diaphorase-reactive nerves also have been described in the vasculature in the gills of the Indian catfish (851). It has been suggested that NADPH-diaphorase histochemistry may not be as specific for nitric oxide synthase (NOS) as formerly assumed (399), but immunoreactive nitrergic fibers have now been described associated with the efferent filamental arteries in the Indian catfish (463, 851). In addition, neuronal NOS (nNOS) immunoreactivity is seen in neuroepithelial cells adjacent to the efferent filamental artery along the length of the filament in the Indian catfish (463), in the interlamellar region in both the killifish and longhorn sculpin (Hyndman and Evans, unpublished data), and in cells distinct from but adjacent to Na^+-K^+-ATPase-containing MRCs in the operculum of the killifish (188). Partial clones for nNOS from the gills of the spiny dogfish (GenBank accession no. 232227) and killifish (GenBank accession no. AY533030) now have been reported.

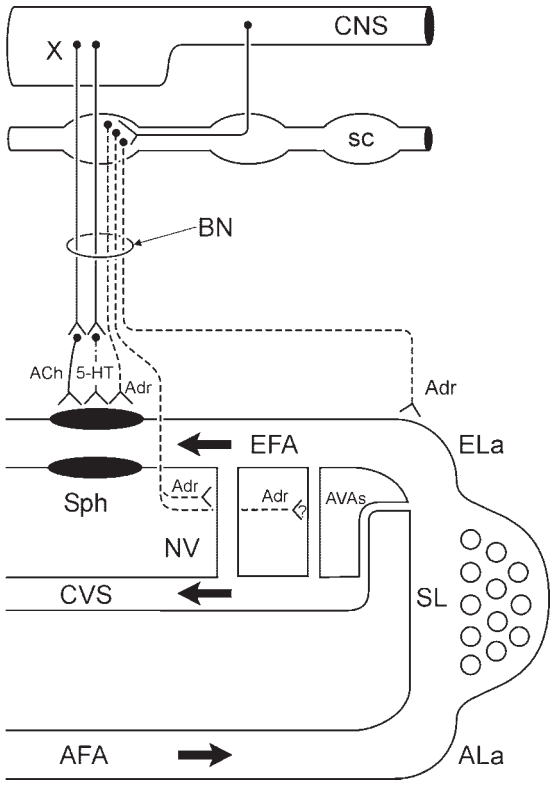

FIG. 37. Working model for the autonomic control of teleost gill vasculature. The sphincter (Sph) at the base of the efferent filamental arteries (EFA) is the site of cholinergic (ACh), adrenergic (Adr), and serotonergic (5-HT) innervation. ACh and 5-HT innervation provide constriction, whereas Adr innervation may cause dilation, via β-adrenoceptors. Other potential sites for perfusion control are the nutritive vasculature (NV) and arteriovenous anastomoses (AVAs). Adr innervation of NV, and possibly AVAs, produces vasoconstriction via α-adrenoceptors. SLA, secondary lamella (=lamella); BN, branchial nerve; CNS, central nervous system; sc, sympathetic chain, X, vagus nerve. See text for other abbreviations, details, and supporting evidence. [From Sundin and Nilsson (723).]

There is clear evidence that either a NO donor (sodium nitroprusside, SNP) or NO itself is vasodilatory in systemic vessels (and the ventral aorta) in the rainbow trout and eel (195, 563, 695), and infusion of NOS inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-nitro-L-arginine (L-NNA) constricted the rainbow trout coronary system (512). On the other hand, SNP constricted the ventral aorta, anterior mesenteric artery, and posterior intestinal vein of the dogfish shark (187, 194) and the ventral aorta of the hagfish (195) and produced constriction followed by dilation in the ventral aorta of the sea lamprey (195). In contrast to the finding that SNP dilated the eel ventral aorta (195), another study has found that SNP and SIN-1 (another NO donor) contracted the perfused gill preparation of the same species (584). The effect could be mimicked by the stable cGMP analog 8-bromo-cGMP and inhibited by the addition of the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), demonstrating that intracellular cGMP was involved (584). To our knowledge, there are no published data suggesting that NO affects gill vasculature directly, but it likely does, given the extensive expression of nNOS in the gills. A more specific description of NO effects on gill vessels awaits the kind of video-microscopic studies that have been described for other effectors (see sect. VII*B*, *2* and *3*). Despite obvious vasoactivity of NO in fish vessels, it appears that a prostanoid, not NO, is the endothelium-derived relaxing factor in at least the rainbow trout and spiny dogfish (194, 563), so it appears that branchial NO is likely produced by the NOS-containing neurons and neuroepithelial cells that have been described above.

Recent evidence suggests that NO may have a role in modulating salt extrusion by the gill epithelium. For ex-

ample, if L-NAME was applied to the killifish opercular epithelium, the I_{sc} increased by 15%, suggesting tonic, inhibitory control by NO. In addition, inhibition of NOS by L-NAME reduced the inhibitory effect of subsequent addition of sarofotoxin S6c [endothelin (ET) agonist], suggesting that a component of the ET-mediated inhibition (see sect. VII*B7*) is secondary to stimulation of NO production (204).

5. Neuropeptides

Gastrin-like immunoreactivity has been described in neurons in the gills of the cod (unpublished results presented in Ref. 728), and cholecystokinin (CCK)-8 and caerulein constricted both arterio-arterial and arteriovenous pathways in perfused gills in the cod (727). Moreover, sulfated-CCK-8 constricted arteries in the gills of the New Zealand hagfish (*Eptatretus cirrhatus*) (720), suggesting an ancient origin of this putative modulatory system.

Neuropeptide Y (NPY)-like immunoreactivity has been demonstrated in the gill vessels of three elasmobranchs (45, 46), and it produces concentration-dependent contraction of the afferent branchial artery of the smallspotted dogfish shark, mediated by a Y1-type receptor (46). To our knowledge, no data have been published suggesting a role for NPY in branchial ionic transport, but NPY does inhibit Cl^- extrusion by the spiny dogfish rectal gland, a tissue that expresses the same NaCl extrusion proteins as does the teleost gill (687).

Vasoactive intestinal polypeptide (VIP) immunoreactive fibers have been described in the gills of the cod (530), and VIP produced a concentration-dependent dilation in perfused gills of the brown trout (52). Interestingly, the dilation was inhibited by indomethacin, suggesting a role for prostanoids in the response (52). VIP (10 μM) stimulated the I_{sc} across the opercular epithelium of tilapia, but a concentration dependence was not examined (231). However, this finding is corroborated by the fact that VIP plays a major role in stimulation of NaCl secretion by the rectal gland of the spiny dogfish (e.g., Ref. 689).

6. Adenosine

The paracrine adenosine also may affect gill blood flow. Adenosine increased branchial vascular resistance in rainbow trout (e.g., Ref. 113), and this was associated with a vasoconstriction of the efferent filamental artery and shunting of blood to the ILV and more proximal parts of the filament, which was mediated by an A_1-type receptor (722). Activation of A_1 receptors also increased gill resistance (and decreased oxygen uptake) in the Antarctic borch (*Pagothenia borchgrevinki*; Ref. 719), but adenosine did not affect the resistance in perfused hagfish gills, although it did reduce systemic resistance (16). Adenosine agonist assays demonstrated the presence of both A_1 (constrictory) and A_2 (dilatory) receptors in the ventral aorta of the dogfish shark (184), so it is possible that both receptors may be in the gill vasculature. We are unaware of any published studies on the putative effects of adenosine agonists on gill epithelial transport, as has been described for ionic transport in the mammalian renal tubules (e.g., Ref. 12).

7. ET

ET is as potent in constricting fish vasculature as it is in mammals, and the effects are both systemic and branchial. An early study demonstrated that rat ET-1 increased gill resistance in the rainbow trout (554), and rainbow trout ET (isolated from rainbow trout kidney extracts) potently (and concentration dependently) constricted a variety of rainbow trout vascular rings, including the efferent branchial artery (790). Interestingly, the trout vascular preparations were more sensitive to rat ET-1 than the homologous peptide, but the maximum tensions produced by both peptides were equivalent. Heterologous ET-1 also produced an increase in branchial resistance in the cod (711) and constricted systemic vessels in two agnathans, the spiny dogfish and the eel (187, 191, 195). Agonist sensitivity suggests that the gill ET receptor in the spiny dogfish is an ET_B type (191, 192). Lodhi et al. (411) suggested that the ET receptor in the gills of the rainbow trout was not a classical ET_A type, because of its insensitivity to BQ-123, but they did not test for an ET_B-like receptor (411). They localized the ET receptor to the lamellae by ^{125}I-labeled ET-1 autoradiography, which is consistent with the subsequent, video-microscopic demonstration that ET-1 constricted lamellar pillar cells in both the rainbow trout and cod, thereby shunting blood to the outer margins of the lamellae (711, 724). Our recent video-microscopic studies with the longhorn sculpin have demonstrated a nearly complete shutdown of flow to the distal filament and lamellae, consistent with pillar cell contraction and possibly constriction of the afferent filamental artery or prelamellar arterioles (Fig. 38). Moreover, we have immunolocalized ET_B-like receptors to the afferent and efferent filamental arteries in the gills of the killifish and longhorn sculpin (as well as the postlamellar, arteriovenous anastamoses), but not to the pillar cells (K. A. Hyndman and D. H. Evans, unpublished data). Thus species differences may exist in the specific distribution of ET receptors in the fish gills. The site of ET synthesis appears to be vascular endothelial and neuroepithelial cells in the filamental epithelium in a variety of fish species including teleosts and elasmobranchs, (852, 854). In Zaccone's studies, big ET (ET precursor) immunoreactivity colocalized with serotonin and neuropoeptide immunoreactivity in some neuroepithelial cells. Big ET has been localized to Na^+-K^+-ATPase-

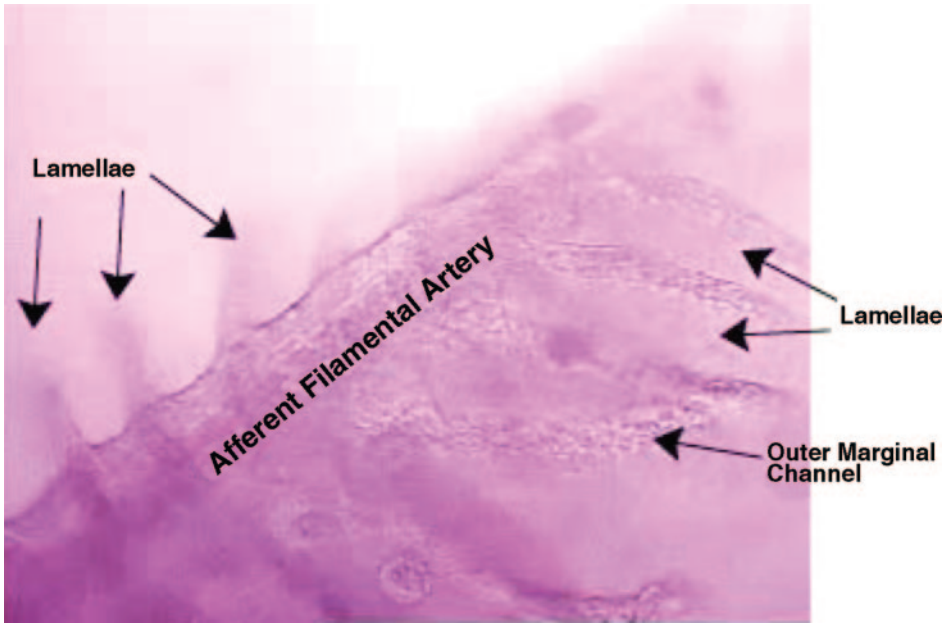

FIG. 38. Video of the effect of endothelin on the flow of blood through the afferent filamental artery and a series of afferent lamellar arterioles leading into lamellae of a single filament in the gill of the longhorn sculpin *Myoxocephalus octodecimspinosus*. Go to http://physrev.physiology.org/cgi/content/full/00050.2003/DC1.

containing MRC in the gill epithelium of the Atlantic stingray (188).

Endothelin has direct effects on mammalian renal transport (e.g., Refs. 251, 856), and recent data suggest that epithelial effects of ET can be demonstrated in fish also. Specifically, ET-1 inhibited the I_{sc} produced by the killifish opercular epithelium in a concentration-dependent manner, and the effect is mimicked by the ET_B-specific agonist sarafotoxin S6c, suggesting a role for ET_B-like receptors (204).

8. Superoxide

It is now well established that cellular production of superoxide ions (O_2^-) has direct metabolic effects and indirect effects because of the instantaneous reaction of O_2^- with NO to produce the very toxic peroxynitrite ($ONOO^-$) and also reducing the NO concentration (e.g., Ref. 37). In addition, $ONOO^-$ itself has been found to be dilatory in the rat pulmonary artery (85). This is a relatively unstudied area in fishes, but a recent study demonstrated that acclimation of the Adriatic sturgeon (*Acipenser naccarii*) to seawater was accompanied by a significant increase in superoxide dismutase (SOD) activity, suggesting an increased production of O_2^- during an osmotic stress (460). Such a response also may have vascular effects, because it has been shown recently that pretreatment with SOD potentiated the constrictory effect of the NO donor SIN-1 on the perfused gill preparation of the rainbow trout, suggesting that either O_2^- or $ONOO^-$ may be dilatory or $ONOO^-$ production reduces NO concentrations in this system (584). Effects of this complex chemical interaction on gill ionic transport are also possible, because we have recently demonstrated that pretreatment of the killifish opercular epithelium with the SOD mimetic TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) reduced the ET-induced inhibition of the I_{sc} across the tissue by 34% (204). Further studies on a putative role for this system on gill perfusion and transport are warranted.

9. Prostanoids

Branchial production of prostanoids may play a role in gill perfusion, because a cyclooxygenase (COX) inhibitor, indomethacin, attenuated the VIP-induced dilation of perfused rainbow trout gills (52) (see sect. VIII*B5*). However, the data on the vasoactivity of prostanoids in fish vessels are conflicting. For example, Piomelli et al. (609) demonstrated a prostacyclin (PGI_2)-induced decline in gill vascular resistance in perfused heads of two elasmobranchs, but an increase in resistance in the same preparation in four teleosts, as well as perfused arches of two other teleosts and an elasmobranch. In all cases, adrenergic receptor inhibitors had no effect, suggesting that the effects were direct and not mediated by secondary release of epinephrine. A subsequent study showed that PGE_2 increased gill resistance in eels in vivo (328), and this has been corroborated by a video study that showed that infusion of $PGF_{2\alpha}$, but not PGE_2, decreased the diameter of the afferent filamental arteries in the cod (712). In all, it appears that the prostanoids are constrictory in the gills and may have prelamellar effects on the afferent filamental artery. Gill tissue from both the rainbow trout and European eel produces prostanoids, including PGE_1, PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, and PGD_2, and in the eel (but not rainbow trout) the levels of each fell when the fish were acclimated to seawater (59). Using a heterologous anti-

body, COX immunoreactivity has been localized to the ILV of the Atlantic stingray gills (188) and in MRCs in the killifish gills (Piermarini and Evans, unpublished data), and we have recently cloned a COX-2-like fragment from the gill of the killifish (Rose, Evans, and Choe; GenBank accession no. AY532639).

Prostanoids (specifically PGE_2) also may play an important role in modulating NaCl extrusion by the marine teleost gills. Two early studies demonstrated that PGE_2 inhibited the I_{sc} across the killifish opercular epithelium (171, 770), and a recent study found that PGE_2 inhibited the I_{sc} produced by a cultured epithelium of PVCs from the sea bass (*Dicentrarchus labrax*; Ref. 15). However, it should be noted that this cultured epithelium is presumably lacking MRCs and produces a very small I_{sc} compared with the killifish opercular preparation. As mentioned above, the ET-1 (actually the ET_B agonist sarafotoxin S6c) mediated reduction of the I_{sc} across the killifish opercular epithelium can be inhibited partially by preincubation of the tissue with either L-NAME (17%) or TEMPOL (34%), suggesting that both NO and O_2^- may be secondary mediators of the ET response (204). Preincubation of the tissue with indomethacin inhibited the sarafotoxin S6c effect by 90%, suggesting that COX-produced prostanoids are the major effectors of this axis (204), just as they appear to be the dominant endothelium-derived relaxing factor (EDRF) in fishes (see sect. VIII*B4*). Moreover, PGE_2 produced a concentration-dependent inhibition of the I_{sc}. Other prostanoids [e.g., thromboxane A_2, $PGF_{2\alpha}$, PGI_2 (actually carbaprostacyclin), and PGD_2] were not inhibitory. The COX-2 specific inhibitor NS-398 was much more effective than the COX-1 inhibitor SC560, and an $EP_{1/3}$-receptor specific agonist, sulprostone, produced a concentration-dependent inhibition of the I_{sc}, but butaprost (an EP_2-specific agonist) produced a concentration-dependent stimulation (204). A working model for this new signaling axis in the fish gill is diagrammed in Figure 39.

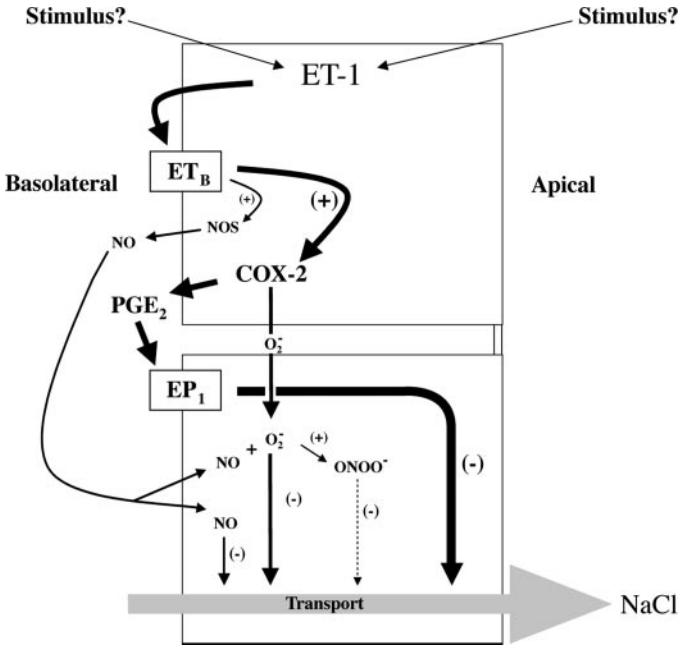

FIG. 39. Working hypothesis for the putative pathways of endothelin (ET)-inhibited NaCl transport across the fish gill. Two cells are diagrammed, but the system may be expressed within a single cell. The width of the arrows is proportional to the presumed importance of the specific pathway in the axis. See text for details. ET-1, endothelin; ET_B, endothelin B receptor; NOS, nitric oxide synthase; COX-2, cyclooxygenase-2; NO, nitric oxide; PGE_2, prostaglandin E; EP_1, PGE_2 receptor; O_2^-, superoxide ion; $ONOO^-$, peroxynitrite ion. [From Evans et al. (204).]

C. Extrinsic Control

1. Prolactin

We are unaware of any studies on the hemodynamic effects of prolactin in fishes, but it has been clear for 45 years that the adenohypophysial peptide plays a major role in gill function in freshwater osmoregulation (e.g., Ref. 437). In a classic paper, Pickford and Phillips (603) demonstrated that hypophysectomized killifish could survive in fresh water only if they were treated with prolactin, and subsequent isotopic studies showed that this survival was associated with a reduction in ionic loss, rather than stimulation of ionic uptake (429, 624). More recently, it has been shown that prolactin can also restore osmoregulatory ability to the hypophysectomized channel catfish in freshwater (165). Prolactin injections reduced gill Na^+-K^+-activated ATPase activity in the killifish, the thick-lipped grey mullet (*Chelon labrosus*), gilthead sea bream, and rainbow trout (247, 419, 488, 602). However, prolactin had no effect on either expression of Na^+-K^+-ATPase (by Northern blot) or enzymatic activity in the gill epithelia of the brown trout (421) and tilapia (293), as well as the opercular epithelium in tilapia (293). In the latter study, prolactin did not affect MRC number, but it reduced the size of individual cells, and this was corroborated by a subsequent study in a related species (*O. niloticus*) that found that prolactin injection stimulated the formation of small MRCs, similar to the "beta cells" of freshwater acclimated teleosts (611) (see sect. II*B3*B). A similar cellular remodeling was produced by prolactin injection into the silver sea bream (*Sparus sarba*; Ref. 355). These studies corroborate earlier work that found that prolactin injections into tilapia were associated with a reduction of both I_{sc} and conductance across the in vitro opercular epithelium, suggesting epithelial remodeling in addition to direct effects on ionic transport (234). As one might expect, plasma levels of prolactin are inversely related to salinity (506, 843). A recent study has

demonstrated the importance of prolactin in freshwater ion regulation directly, by showing that a cultured gill epithelium (that contains both PVCs and MRCs) can only transport Na^+ and Cl^- inward when prolactin and cortisol are in the culture medium (857). Interestingly, Na^+-K^+-activated ATPase activity was unaffected by these treatments.

Prolactin receptors have been cloned in several fish species, including two species of tilapia and rainbow trout (627, 662), goldfish (759), and gilthead sea bream (663), and expression was high in the gill in all cases. Using either in situ hybridization or immunohistochemistry, the receptors could be localized to the MRCs in most of these species (627, 662, 663, 800). Interestingly, in the tilapias and rainbow trout, transfer to hyperosmotic salinities was associated with equivalent or increased expression of the receptor, suggesting that prolactin might have some role in acclimation to salinities other than fresh water (13, 627, 661).

2. Cortisol

Cortisol is the major corticosteroid produced by the interrenal gland in fishes and functions as both a glucocorticoid and mineralocorticoid (272, 284). Until recently, cortisol has been considered to be involved primarily in seawater osmoregulation because it reversed the reduction in Na^+-K^+-activated ATPase activity produced by hypophysectomy of the killifish (602), stimulated the enzyme's activity in a variety of fish species, and increased tolerance to high salinities (reviewed in Ref. 474). Moreover, there was a correlation between Na^+-K^+-activated ATPase activity, Na^+ effluxes, and plasma cortisol concentration as the eel was acclimated to seawater (227, 470), and there was a rapid increase in plasma cortisol concentrations (325, 452) preceding the increase in CFTR expression in the killifish gills after transfer to seawater (452), as well as associated with the changes in expression of Na^+-K^+-ATPase α_{1a}- versus α_{1b}-mRNA in the gills of the rainbow trout after transfer to seawater (645). Injection of cortisol stimulated Na^+-K^+-activated ATPase activity in the gills of the gilthead bream (383, 488) and tilapia (731). Cortisol also increased the expression of NKCC (as well Na^+-K^+-activated ATPase activity) in the gills of freshwater Atlantic salmon (581), and the increase was associated with proliferation of MRCs in the epithelium, as had been demonstrated in other salmonids (418). At least some of this cytogenic effect is due to direct stimulation (rather than stimulation of other hormones in vivo), because it can be produced in the tilapia opercular epithelium cultured in vitro (471). These studies suggest that the effect of cortisol on the fish gills is primarily cytogenic, and this is corroborated by the finding that cortisol does not stimulate the I_{sc} produced by the killifish operculum (230).

Radioligand binding protocols have identified cortisol receptors in the gills of the eel, two salmonids, and tilapia (57, 88, 464, 660), and in tilapia, acclimation to seawater is accompanied by an increase in the number of receptors (57). The receptors were localized to two types of MRC (defined by Na^+-K^+-ATPase immunoreactivity) in chum salmon fry, using both immunological and molecular protocols (763). In this study, cortisol receptors were also localized to undifferentiated cells in the interlamellar region, suggesting a role in MRC development, which corroborated an earlier study that found that a mineralocorticoid receptor antagonist, spironolactone (but not the glucocorticoid antagonist RU486), inhibited the proliferation of MRCs in the gills of the rainbow trout that had been exposed to ion-deficient tap water (694). In addition to supporting a cytogenic rather than physiological role for cortisol, this study also supports the idea of a role for cortisol in freshwater fishes, which will be discussed further below. A cortisol receptor for the rainbow trout has been cloned and shares more homology with the mammalian glucocorticoid receptor (GR) than mineralocorticoid receptor (MR) (160). However, an MR-like receptor has now been cloned from the same species (114), and recent studies have reported two GRs in the trout gills (68) and four, distinct corticosteroid receptors in the cichlid *Haplochromis burtoni*, one MR and two GRs, one of which has two splice variants (272).

Early plasma assays found that cortisol levels increased when fish were acclimated to seawater (11, 20), but more recent studies have shown that cortisol concentrations also increase after transfer to fresh water in eel, tilapia, killifish, mullet, flounder, and sea bass (reviewed in Ref. 473), suggesting a role for cortisol in freshwater osmoregulation. Early studies demonstrated that cortisol injection increased Na^+ uptake in intact and interrenalectomized European eels and intact goldfish in fresh water (e.g., Refs. 89, 427), and cortisol treatment increased the plasma osmolarity of hypophysectomized European eels and goldfish in fresh water (90, 381). Importantly, the hypophysectomized black bullhead and Indian catfish need both prolactin and cortisol to osmoregulate in freshwater (229, 574). More recent studies found that the cortisol-enhanced Na^+ uptake was associated with an increased apical MRC surface area in the rainbow trout, European eel, tilapia, and catfish (392, 590), and Dang et al. (136) demonstrated that cortisol treatment increased the intracellular tubular system in the MRCs, as well as the expression of immunoreactive Na^+-K^+-ATPase. In addition, cortisol may stimulate ionic transport steps directly, because Lin and Randall (509) found that H^+-ATPase activity in gills homogenates was stimulated by cortisol (409), and Kelly and Wood (356) found that cor-

tisol increased Na^+ and Cl^- uptake by cultured PVCs from the rainbow trout. As noted in section VIII*C1*, this group has recently demonstrated that both cortisol and prolactin are necessary to stimulate net Na^+ and Cl^- uptake by a cultured epithelium that contains both PVCs and MRCs from trout gills (857).

In summary, current evidence suggests that the primary mode of action of cortisol is cytogenic, mediating the growth and differentiation of transport cells in the gill epithelium in both seawater and fresh water. In fresh water, cortisol appears to work in concert with prolactin, but in seawater another synergistic system is involved: growth hormone and insulin-like growth factor I (IGF-I) (see Ref. 473).

3. Growth hormone and IGF

As might be expected, fish growth hormone (GH) has morphogenic effects (independent of GH-enhanced size), and the major effect appears to be stimulation of osmoregulation in seawater. Injection of GH promotes salinity tolerance, associated with increased Na^+-K^+-activated ATPase activity and MRC size and density, in salmonids as well as two species of tilapia, the killifish, and silver sea bream (355, 421, 433, 435, 626, 657, 658, 674, 840). GH injections were associated with increased expression of Na^+-K^+-ATPase in MRC of the brown trout (674) and expression of NKCC1 in the MRCs in the gills of the Atlantic salmon (581). GH receptors have been localized to gills in the coho salmon by radioreceptor assay (271), and two splice variants of a recent clone from the black sea bream (*Acanthopagrus schlegeli*) can be localized to a variety of tissues, including the gills (760). In the killifish, Atlantic salmon, and brown trout, some of the effects of GH can be mimicked by injection of IGF (433, 472, 674). For example, IGF-I injection stimulated MRC development and Na^+-K^+-ATPase expression in the MRC in the gills of the brown trout (674). Transfer to seawater is associated with an increase in plasma concentrations of GH in salmonids (657), as well as increased message for IGF-I in the gills of coho salmon (656). The gene for IGF-I and its product can be localized to MRC in the tilapia gills (641), and an IGF-I receptor has been cloned and localized to the gills in two sculpins (317, 412). It is generally considered that IGF-I is the direct mediator of increased Na^+-K^+-ATPase activity in the MRCs but that GH is needed for the response (e.g., Ref. 420).

The fact that cortisol and GH together stimulate salinity tolerance and Na^+-K^+-activated ATPase activity better than either one alone in various salmonids and the killifish (and NKCC1 expression in the Atlantic salmon) suggests that cortisol and GH interact to promote seawater osmoregulation (418, 423, 434, 472, 581). There also appears to be some synergism between cortisol and IGF-I injections (472, 674). One site of GH/cortisol interaction may be cortisol receptors because GH treatment was associated with an increase in the number of cortisol receptors in the gills of the coho and Atlantic salmon (684, 685). The synergism may be cytogenic, however, because GH increased general mitotic activity in the gills of the rainbow trout, but cortisol specifically stimulated the number of MRC (388). A working model for the interplay between cortisol, GH, and IGF-I is diagrammed in Figure 40.

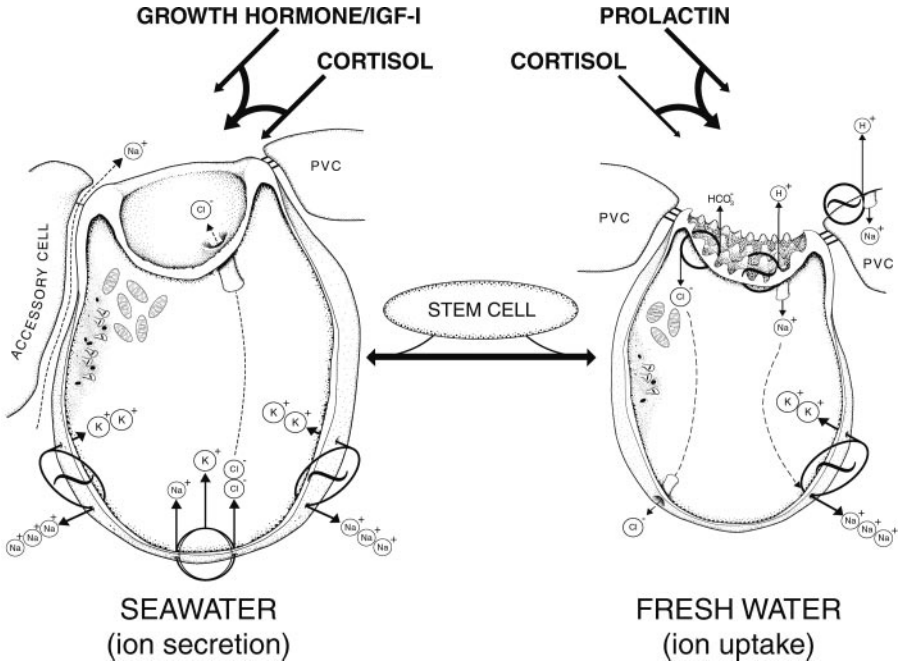

FIG. 40. Working model for the effects of prolactin, cortisol, growth hormone, and insulin-like growth factor I on the morphology and transport mechanisms of gill mitochondrion-rich cells in the gill epithelium of teleost fish. In seawater, MRCs are generally larger and contain a deep apical crypt; in fresh water, the apical surface is broad and contains numerous microvilli. Growth hormone and cortisol can individually promote the differentiation of the seawater MRC, and also interact to control epithelial transport capacity. Prolactin inhibits the formation of seawater MRCs and promotes the development of freshwater MRCs. Cortisol also promotes acclimation to fresh water by maintaining ion transporters and MRCs, and by interacting to some degree with prolactin. See text for details and supporting evidence. [From McCormick (473).]

4. Angiotensin

Despite earlier uncertainty (532), it is now clear that a renin-angiotensin system (RAS) is expressed in fishes (533, 655), including elasmombranchs (7, 286) and agnathans (112), although fish produce little or no aldosterone (e.g., Refs. 70, 642). ANG II constricted perfused gills in both the trout (548) and eel (211), but local, tonic production of ANG II appears to be relatively unimportant in gill hemodynamics, because inhibition of ANG II production in the trout in vivo by the angiotensin converting enzyme (ACE) inhibitor lisinopril did not affect gill resistance, although systemic resistance was reduced by nearly 50% (549).

Fish ANG II has three amino acid substitutions in the basic octapeptide, compared with mammals (284). There generally is a direct correlation between plasma ANG II (and renin) levels and salinity in fishes, including the river lamprey (112, 637), two eel species (534, 747), and two salmonids (705). Enzymatic analyses have found ACE activity in the gills of the trout (246) and river lamprey (112) as well as in the corpuscles of Stannius in the rainbow trout and American eel (71, 246). Fish corpuscles of Stannius are located within the renal tissue of teleosts and are the site of the production of a unique hormone, stanniocalcin, important in Ca^2 regulation by the gills (e.g., Ref. 794) (see sect. VIII*C12*). Within the rainbow trout gill, ACE can be localized to pillar cells nearest the inner lamellar margin and, to a lesser extent, the lumen of the afferent filamental artery and afferent lamellar arterioles (558).

ANG II receptors have been localized to gill tissue in the banded houdshark (*Triakis scyllia*) by radioligand binding (746), and antibodies raised against the mammalian AT_1 receptor localized the receptor to MRCs and PVCs in the gill epithelium in the eel (457) and icefish (*Chionodraco hamatus*; Ref. 456). A partial sequence for the eel ANG II receptor has been reported (Tran Van Chuoi et al., GenBank accession no. AJ005132), but no expression data are available. In the eel, acclimation to seawater was associated with a threefold increase in immunoreactive AT_1 in the gills (458). Infusion of ANG II into the European eel was associated with a concentration-dependent increase in Na^+-K^+-activated ATPase activity in the gills of freshwater acclimated fish, but a biphasic (stimulation inhibition) response with the same concentrations in the seawater acclimated fish (458). This may appear to be counter to the finding that ANG II inhibited the transepithelial potential across perfused flounder gills (415), but it is generally accepted that the transepithelial potential in vivo is the result of differential gill ionic permeabilities, not active transport (e.g., Ref. 202). Unfortunately, no data have been published on the effect of ANG II on the I_{sc} across the killifish opercular epithelium.

5. Bradykinin

At least some components of the kallikrein-kinin cascade are present in the plasma of teleosts, lungfish, and lower actinopterygians such as gar, bowfin, and sturgeon, but apparently not in the plasma of either agnathans or elasmobranchs (21, 118). The sequences of trout, cod, and lungfish bradykinin (BK) are now published, and it appears that trout may express two kinogen genes, which produce distinctive BK precursors (120, 121, 330, 401, 618). Infusion of homologous BK into the trout produced a triphasic response (pressor-depressor-pressor) in dorsal aortic pressure. The second phase was characterized by a slight increase in gill resistance, which was abolished by pretreatment with indomethacin, suggesting a role for prostanoids in the response (551). Isolated, efferent branchial arteries from the trout were unresponsive to homologous BK (120), and we found that aortic rings from the spiny dogfish were unresponsive to mammalian BK (0.1 nM to 0.1 μM; D. H. Evans and J. Nowacki, unpublished data). Thus a role for BK in modulating gill perfusion remains unclear, despite the fact that there is clear activity in other vascular beds (e.g., Refs. 330, 679). Structure-activity protocols suggest that the fish BK receptor is different from either B_1 or B_2 receptors (e.g., Refs. 21, 118), but a B_2 ortholog has been sequenced (and functionally expressed) recently from the zebrafish (164). There are no published data suggesting that BK may modulate salt transport across the fish gills, but it has been shown to activate a Ca^{2+}-dependent Cl^- conductance in the mouse inner collecting duct epithelium (371), so further investigation is warranted.

6. Arginine vasotocin

Like all nonmammalian vertebrates, fishes (including the agnatha) produce the neurohypophysial arginine vasotocin (AVT), with a single amino acid substitution compared with vasopressin (284, 384). AVT is one of the most potent vasoconstrictors in the teleost gills, decreasing flow through the arterio-arterial pathway in the eel gills in vivo (564) and decreasing flow through the arteriovenous pathway in the rainbow trout in vivo at low doses and through the arterio-arterial pathway at higher concentrations (115). In the trout, a bolus injection of AVT doubled the total gill resistance, while only increasing systemic resistance by 15% (115). AVT also contracted ventral aortic and efferent branchial arterial rings from the trout (116). The pharmacological profile of the effect of AVT on the trout efferent branchial artery suggested a receptor similar to the mammalian arginine vasopressin (AVP) V1a and oxytocin receptors (116), but an earlier study of the effects of putative AVP receptor agonists and antagonists on adenylate cyclase activity in rainbow trout gill plasma membranes suggested that the receptor was distinct from either V1 or V2 receptors (275). However, a V1-type re-

ceptor has now been cloned from the European flounder, and its message could be localized to the gills (791).

The relationship between fish plasma AVT levels and external salinity is unclear. Early radioimmunoassay studies, using a fish-specific antibody, found no difference in plasma AVT concentrations in seawater versus freshwater acclimated European flounder, rainbow trout, and European eel (22, 792), and an ELISA protocol confirmed this finding for the rainbow trout (608). In contrast, other studies determined that plasma AVT concentrations increased when the European flounder or rainbow trout was acclimated to fresh water (22, 374), and a recent study showed a direct correlation between plasma AVT levels and salinity in the European flounder (53). Some of these discrepancies may be due to diurnal changes in plasma levels (375).

Recent evidence suggests that AVT also may play a role in modulating salt transport across the fish gill epithelium, although the current model system consists of a cultured epithelium of PVCs from the sea bass, which maintains an extremely low I_{sc} compared with the killifish operculum (3–5 vs. >50 $\mu A/cm^2$). In the sea bass preparation, AVT stimulates the I_{sc} (15), and the agonist profile suggests mediation by a V1-type receptor (274). Effective doses, however, were much higher than those measured in plasma (e.g., Refs. 22, 53, 374, 375).

7. Natriuretic peptides

Various components of the natriuretic peptide (NP) system are expressed in all fish groups that have been examined to date (including the elasmobranchs and agnatha), and NPs play important roles in cardiovascular, renal, and gill physiology (182, 186, 205, 413, 740, 741, 752). Amino acid sequences have been published for atrial NP (ANP) in the Japanese eel (*Anguilla japonica*) and rainbow trout, and for C-type NP (CNP) in four species of elasmobranchs and three species of teleosts (319, 413). Four distinct CNPs have been cloned recently from the medaka (*Oryzias latipes*) and fugu (318). Brain NP (BNP) has not been isolated from fish, but a unique NP, termed VNP, has been isolated from ventricular tissue from the Japanese eel and two species of salmonids (413). Teleosts produce both ANP and CNP, but elasmobranchs apparently express only CNP (318), and a recent study suggests that the agnathan NP is unique (354). Four NP receptors have been cloned from the Japanese eel (termed NPR-A through D), with mammalian-like structures and ligand specificities in the first three (reviewed in Ref. 297). NPR-D has only been described in fishes and is closely related to NPR-C, sharing a short cytoplasmic carboxy-terminal tail that does not include the guanylyl cyclase catalytic domain (297). NPR-B has been cloned from the rectal gland of the spiny dogfish shark (1) and from the medaka (738), and partial sequences for an NPR-A/B and NPR-C/D have been reported from the New Zealand hagfish (*Eptatretus cirrhatus*) and pouched lamprey, respectively (72, 754). A partial sequence for a putative NPR-C/D also has been reported for the spiny dogfish (157). Radioligand binding protocols have localized NPR-A/B and NPR-C /D receptors in the gills of gulf toadfish (156), Atlantic hagfish (753), pouched lamprey (754), and New Zealand hagfish (72). It is generally assumed that the CNP/NPR-B couple is the ancestral vertebrate condition (297).

It has been pointed out that the use of heterologous antibodies for radioimmunoassay determination of plasma NP concentrations often has produced conflicting results (739), and recent results, using homologous antibodies for Japanese eel peptides, have shown that salinity changes in this species are associated with differential secretion of specific NPs. For instance, the hypernatremia associated with acclimation to seawater is associated with an increase in ANP secretion (and a secondary stimulation of cortisol secretion); acclimation of eels to freshwater is associated with an upregulation of both CNP expression and its specific receptor, NPR-B (reviewed in Ref. 741).

NPs are potent dilators of vascular smooth muscle in fishes, including teleosts, elasmobranchs, and agnathans (e.g., Refs. 183, 206, 553, 559), and ANP decreased gill resistance in the rainbow trout in vivo (550), corroborating an earlier study that demonstrated that ANP decreased arterio-arterial resistance in the epinephrine-contracted, perfused gills from that species (559). Unfortunately, no in vivo videomicroscopy studies have been done to visualize the effects of NPs on gill blood flow directly.

Since an early study found that a truncated ANP stimulated the I_{sc} across the killifish opercular epithelium (668), it has been accepted generally that one of the prime roles of NPs in seawater teleosts is the stimulation of gill Na^+ excretion by the branchial epithelium (e.g., Refs. 297, 413, 741). However, a recent study was unable to elicit any effect on the I_{sc} across this epithelium by either eel ANP or porcine CNP (0.1 nM to 0.2 μM) (203), so it is not clear that NPs stimulate salt extrusion by the marine teleost gills, despite the fact that ANP apparently may increase the Na^+-K^+-activated ATPase activity in isolated gill cells from the eel (Flik and Takei, unpublished data reported in Ref. 413).

8. Thyroid hormones

No data have been published suggesting that thyroid hormones affect gill perfusion, but they may play an indirect, stimulatory role in gill salt extrusion (reviewed in Ref. 188). For instance, thyroxine (T_4) or triiodothyro-

nine (T_3) treatment stimulated Na^+-K^+-activated ATPase activity and/or MRC number in the Atlantic salmon (422), rainbow trout (757, 773), climbing perch (397), and tilapia (598), as well as immunoreactive Na^+-K^+-ATPase in MRC in the gills of the summer flounder (*Paralichthys dentatus*; Ref. 671). However, it appears that these effects may be mediated via the cortisol and/or GH-IGF axes (see Ref. 473), although at least one study found that the effects on Na^+-K^+-activated ATPase activity of GH and T_3 were additive (397). For instance, T_4 treatment alone did not stimulate Na^+-K^+-activated ATPase activity in the gills of tilapia, but it potentiated the stimulation produced by cortisol (140), as it did the effect of GH on enzyme activity in the amago salmon (*Oncorhynchus rhodurus*; Ref. 500). Moreover, T_3 treatment increased the number of cortisol receptors in the gills of the Atlantic salmon, which was further potentiated with GH addition (685), and it increased the number of cortisol receptors (and the cortisol stimulation of Na^+-K^+-activated ATPase activity) in rainbow trout gills (686).

9. Glucagon

As is true for thyroid hormones, pancreatic glucagon has not been shown to affect gill perfusion, but this peptide may play a role in gill transport. In an early study of the tilapia opercular epithelium, Foskett et al. (231) found that glucagon could stimulate the I_{sc} across the epithelium in a concentration-dependent manner, with a minimum effective dose of 1 nM and a maximum stimulation of 72% at 10 μM. Moreover, glucagon stimulated adenylate cyclase in the rainbow trout gills (276), as it does in the process of stimulating NHE3 in the mammalian kidney (3), a transporter of relevance to fish gills (see sect. VII*B*).

10. Urotensins

Since the initial characterization of urotensins (UT) from the fish urophysis (reviewed in Ref. 42), it has become apparent that these neuropeptides may be major cardiovascular effectors in fishes and amphibians (122, 414), as well as mammals (e.g., Refs. 158, 431, 461). In fishes, urotensins (UI and UII) are secreted by neurosecretory cells (Dahlgren cells) in the distal spinal cord, which secrete into the neurohemal urophysis in most groups (e.g., Ref. 510). In amphibians and mammals, U1 and related peptides (CRF, sauvagine, urocortin) are expressed in the central nervous system (e.g., Refs. 119, 372, 772), and this apparently is also true in both teleosts and elasmobranchs (484, 766, 849). UT-secreting neurons have not been described in fish gills to date. UI and/or UII have now been sequenced from many fish species, including teleosts, elasmobranchs, and lampreys (284), and UI-related receptors (CRF-like receptors) have been cloned from the chum salmon (620) and brown bullhead catfish (8).

Infused UII (a somatostatin-like peptide) produced an increase in gill vascular resistance in the rainbow trout in vivo and also a concentration-dependent contraction of isolated, efferent branchial artery rings (399a), and UII contracted rings from the afferent branchial artery from the smallspotted dogfish shark (285). These data are consistent with the recent demonstration that UII is a very potent vasoconstrictor in mammals (5, 431). However, some of the vascular effects of UII may be secondary to stimulation of the parasympathetic nervous system, because α-adrenergic blockade completely abolished the UII generated hypertension in the smallspotted dogfish shark in vivo (285). UI may be a vasodilator, because infusion into the trout was associated with vascular hypertension, which became hypotension when an α-adrenergic blocker was applied (496). This study corroborated earlier data from the smallspotted dogfish shark, which demonstrated a transient hypotension, followed by an α-adrenergic-mediated hypertension, subsequent to infusion of UI (619). None of these experiments examined gill responses specifically, but they suggest that the two UTs may have opposing vascular effects.

The fact that immunoreactive UII appeared to be greater in the urophysis of seawater species versus freshwater species suggested an osmoregulatory role for at least UII (566). This was corroborated by a subsequent study that demonstrated increased immunoreactivity for both UI and UII in the urophysis of freshwater acclimated longjaw mudsucker, but this could be secondary to increased synthesis and storage, decreased release of stored peptide, or decreased peptide degradation (385). Similar results were published for the euryhaline bogue (*Boops boops*; Ref. 497). Subsequent determinations of plasma UT concentrations by homologous radioimmunoassay in the euryhaline, European flounder have found a direct correlation between plasma osmolarity and UII levels (53, 815). UII inhibits the I_{sc} across the seawater longjaw mudsucker jaw skin epithelium, and UI stimulates the I_{sc} (444, 445), but both UI and UII stimulate cortisol secretion by the rainbow trout or European flounder interrenal gland (10, 357), suggesting that UTs may have both direct and indirect effects on fish osmoregulation. Because the urophyses from brook trout (*Salvelinus fontinalis*) from acidified lakes in Canada displayed lower UII content (93), it may be that UTs also play a role in acid-base balance. Further studies clearly are warranted.

11. Calcitonin and calcitonin gene-related peptide

Calcitonin (CT) is produced in the ultimobranchial glands of fishes and tetrapods, except for mammals,

where it is synthesized in the parafollicular "C" cells in the thyroid gland (e.g., Refs. 284, 795). Agnathans lack the gland, and they were thought to lack CT until it was recently immunolocated in the plasma of freshwater (but not seawater)-acclimated, Japanese lamprey (*Lampetra japonica*) and also in the inshore hagfish (*Eptatretus burgeri*), where concentration was correlated with plasma Ca^{2+} levels (732). Calcitonin immunoreactivity has been measured in a number of species of teleosts and elasmobranchs (e.g., Refs. 47, 48, 236, 237, 525, 715, 733), and the peptide sequence has been determined for a number of species (e.g., Refs. 126, 524, 535, 665, 667), including fugu (110). The CT gene also has been sequenced from many species (e.g., Ref. 735), and the gene and its transcript can be localized to the gills of the Atlantic salmon, so the ultimobranchial gland may not be the only site of CT production (459). Moreover, a CT-receptor like gene has been cloned from the gill cDNA library of the Japanese flounder (*Paralichthys olivaceus*) (734).

Calcitonin is hypocalcemic in tetrapods, and current evidence suggests that this also is the case in fishes (e.g., Refs. 86, 665, 667), although hypercalcemic effects are sometimes described (e.g., Ref. 565). Infusion of Ca^{2+} into the Japanese eel was associated with an increase in plasma CT immunoreactivity (666), but transfer of this species from freshwater to seawater did not elicit a change in plasma CT levels, despite increased plasma Ca^{2+} concentrations (733). However, transfer of rainbow trout to seawater was associated with significant increases in plasma CT concentrations (514). Several studies have demonstrated that CT inhibits Ca^{2+} uptake by the gills (e.g., Refs. 489, 490, 783), consistent with a hypocalcemic role.

Calcitonin gene-related peptide (CGRP), a splice variant of the CT mRNA, is also found in fishes (e.g., Ref. 327) and may play a role in vascular dynamics, osmoregulation, Ca^{2+} balance, and gas exchange. CGRP-containing fibers have been described in the brain and/or gut of all the major fish taxa (35, 58, 73, 306, 501, 848). The peptide also has been identified in the plasma of the pink salmon (*Oncorhynchus gorbuscha*; Ref. 462) and in gills of the rainbow trout by radioimmunoassay (238), where it binds specifically (9) and stimulates adenyl cyclase by a GTP-binding process (235, 236). The gene for a CGRP receptor has been cloned from the gills of the Japanese flounder (734). Acclimation of the rainbow trout to seawater is associated with an increase in plasma levels of CGRP as well as specific binding to gill membrane receptors (514). Interestingly, this study also found increased gill CA levels in seawater, and this group previously had demonstrated that CGRP can stimulate production of this enzyme in trout gill extracts (513). Finally, it is possible that CGRP could be vasodilatory in the fish gills, as it is in small arteries from the rainbow trout gut (335).

12. Stanniocalcin

Stanniocalcin (STC, formerly termed hypocalcin or teleocalcin) is a glycoprotein that is produced by the corpuscles of Stannius, which are located in renal tissue in teleost and holostean (gar, bowfin) fishes only (340, 568, 780, 794). However, it is now clear that STC and its receptors also are expressed in a variety of mammalian (and human) tissues, including kidney and gut (e.g., Ref. 477), and play roles in mineral metabolism, neural differentiation, reproduction, and even cancer (e.g., Refs. 321, 417, 538).[5] It has been demonstrated that CS extracts inhibit Ca^{2+} uptake by three salmonids and the American eel (380), and stanniectomy of the eel was associated with increased plasma Ca^{2+} levels, which were corrected when STC was injected (280), so STC is generally considered to be the major hypocalcemic factor in fishes (767). The cellular site of STC production in the CS has been localized by immunocytochemistry (798), and hypercalcemia in the rainbow trout is associated with increased release of immunoreactive STC from the CS (219). Moreover, intraperitoneal injection of $CaCl_2$ into the rainbow trout and European eel stimulated STC release from the CS (279), as did acclimation of the eel to seawater versus fresh water (278). It appears that the Ca^{2+} stimulation of CS secretion of STC may be direct and/or via cholinergic innervation (82, 210), but it also may involve direct stimulation of transcription (781). A calcium-sensitive receptor (CaR) has been sequenced from rainbow trout CS, and transcripts are also expressed in the gills (628). A CaR was concurrently cloned from dogfish shark, winter flounder, and Atlantic salmon and expression shown in the kidney and gills (see sect. v*H*), which was suggested to play a role in salinity perception (521). Corpuscles of Stannius extracts also produce hypocalcemia in the Japanese stingray (*Dasyatis akajei*; Ref. 707). The gene for coho and chum salmon STC has been sequenced (779, 842), and initial studies on the distribution of the STC mRNA and product suggested that they are expressed only in cells in the CS in fishes (439, 713). However, more recent in situ hybridization and Northern blot studies have delineated STC expression in a variety of tissues in the rainbow trout and arawana (*Osteoglossum bicirrhosum*), including the kidney and gills (4, 478), suggesting that STC may act as a paracrine as well as circulating hormone. Interestingly, two distinct STCs are expressed in the CS of the Atlantic salmon (782).

[5] It has been suggested that the emerging roles for both STC and UII in mammalian physiology and pathophysiology are good examples of the "singular contributions of fish neuroendocrinology to mammalian regulatory peptide research" (119).

STC is thought to inhibit Ca^{2+} uptake across the apical membrane of the MRC (222), because stanniectomy in the European eel is not associated with any measurable changes in either Ca^{2+}-activated ATPase or Na^{+}/Ca^{2+}exchanger, both of which are thought to be basolateral (see sect. v*B6* and Refs. 767, 775). These authors conclude that the increased Ca^{2+} influx subsequent to stanniectomy is secondary to an increase in the number and/or size of the MRCs.

13. Parathyroid hormone-related protein

Fishes lack an encapsulated parathyroid gland (e.g., Ref. 797), but parathyroid hormone (PTH)-like immunoreactivity has been reported in the pituitaries and plasma of several teleosts (239, 282, 342). However, the recently described PTH-related protein (PTHrP) is also a hypercalcemic factor in humans, shares some common regions at the amino terminus, and binds to a common PTH/PTHrP receptor (e.g., Ref. 315). PTHrP-like immunoreactivity has now been localized in the pituitary, plasma, or other tissues in a variety of fishes, including teleosts, elasmobranchs, and agnathans (141, 142, 239, 316, 756), and immunoreactivity was localized to the gill epithelium in some of these studies, with expression confirmed by molecular techniques (e.g., Refs. 143, 756). The gene for PTHrP was initially cloned from fugu (625), but a sequence now has been reported from the gilthead sea bream, and in situ hybridization has localized expression to, among other sites, MRCs in the gill epithelium (214). A radioimmunoassay also has been developed and validated for the peptide transcribed from this sequence, and immunoreactivity was localized to the gills, as well as the pituitary, esophagus, kidney, and intestine (650). Two relevant receptors have now been cloned from the zebrafish, one more sensitive to fugu PTHrP than human PTH (654) and another sensitive to human PTH and not human PTHrP (653). Expression of these receptors in relevant fish tissues has not been published.

In an unpublished study (reported in Ref. 143), it was shown that PTHrP-like immunoreactivity in the plasma of the European flounder was inversely correlated with salinity (highest in gill tissue from freshwater acclimated individuals), suggesting that the peptide may play some role in Ca^{2+} regulation, which differs with salinity (see sect. v, *B6* and *E*). Exposure of larval gilthead sea bream to an amino-terminal peptide from the fugu sequence increased $^{45}Ca^{2+}$ influx in both 100 and 33% seawater-acclimated fish and reduced Ca^{2+} efflux from the seawater-acclimated fish, both in a concentration-dependent manner (273). To our knowledge, these are the only published data demonstrating that PTHrP is a functional hypercalcemic factor in fishes.

14. Other hypercalcemic factors: prolactin, somatolactin, and cortisol

In artificial, calcium-deficient seawater, hypophysectomized killifish displayed reduced plasma Ca^{2+} levels, without any discernable changes in other plasma electrolytes, and the hypocalcemia was not seen in seawater with normal Ca^{2+} levels (569) or after replacement therapy with either pituitary homogenates or heterologous prolactin (570). Ovine prolactin injections were associated with hypercalcemia in the intact European eel, stimulation of Ca^{2+} uptake from the fresh water, and increased branchial Ca^{2+}-activated ATPase activity (216, 224). Similar results have been published for tilapia (215, 221) and carp (87). As one might expect for a putative hypercalcemic factor, pituitary prolactin content is often inversely related to external Ca^{2+} concentrations (reviewed in Ref. 341).

Somatolactin (SL) is a novel pituitary protein that was initially isolated from the pars intermedia of the cod (630) and has been described in the pituitary of a variety of fish species (e.g., Refs. 92, 249, 382, 466, 631, 742, 777, 858). It is structurally related to both prolactin and growth hormone and was initially described only in teleosts (339), but has more recently been found in the African lungfish pituitary (465), suggesting that it may be described in tetrapods in the future. The fact that low environmental Ca^{2+} levels are associated with activation of SL cells, and increased mRNA levels, in the pituitary of the rainbow trout (as well as plasma SL levels) suggests that SLs is a hypercalcemic factor (337). Somatolactin also may function in reproduction, acid-base regulation, and stress responses in fishes (e.g., Refs. 338, 339).

Cortisol also may be a hypercalcemic factor in fishes, because plasma concentrations of cortisol increased when the rainbow trout was acclimated to freshwater with a reduced Ca^{2+} concentration, and 8 days of this treatment resulted in a stimulation of branchial Ca^{2+} transport and Ca^{2+}-activated ATPase activity (220). In freshwater with normal Ca^{2+} concentrations, treatment with cortisol was associated with a stimulation of Ca^{2+} uptake, branchial transport capacity, and hypercalcemia (220). Although these effects may be related to the cytogenic effects of cortisol, it has been shown recently that cortisol treatment is rapidly (30 min) followed by a stimulation of gill Ca^{2+}-activated ATPase activity, suggesting that the cortisol effect is not via changes in transcription (731).

D. Metabolism of Intrinsic Signaling Agents and Xenobiotics

Largely due to studies from a single research group, it has become clear that the fish gill vasculature also is the

site of substantial metabolism and/or inactivation of circulating and paracrine signaling agents (reviewed in Refs. 545, 546). As we have stated previously, the gill vasculature receives 100% of the cardiac output, and it can be calculated that the vascular surface area of a 300 g rainbow trout may be 900 cm^2, nearly twice the respiratory surface area (546). Using perfused gill arches or cannulated free-swimming rainbow trout, the Olson group (545) has measured fractional extractions of a variety of molecules of physiological importance. For instance, 35% of infused ANG I is converted to ANG II in the arterioarterial pathway during a single pass through the perfused rainbow trout gills (557), and 60% of infused ANP, 55% of infused ET-1, and 80% of infused serotonin is extracted (539, 546). The extraction of other peptides, amines, purines, and COX products is somewhat less (Table 2). The ANG conversion is via ACE, which can be localized to the lamellar pillar cells (see sect. VIII*C4*) and NPR-C/D type receptors in the gill vasculature (see sect. VIII*C7*) presumably function as clearance receptors as they do in mammalian vasculature. It is interesting to note that branchial clearance of plasma ANP and VNP can be demonstrated in freshwater Japanese eels but not in eels acclimated to seawater, suggesting that there may be physiological control of gill clearance of at least NPs (336). The ET presumably is extracted by the very abundant ET_B receptors that can be localized to both afferent and efferent filamental arteries (see sect. VIII*B7*).

The fish gill is also a major site of the damage produced by environmental pollutants (e.g., Refs. 181, 295), and recent evidence suggests that it may be a site for metabolism and/or excretion of toxins (e.g., Ref. 546). For instance, cytochrome *P*-450 enzymes (e.g., CYP1A) have been localized in the pillar cells and vascular endothelium of the rainbow trout and scup (*Stenotomus chrysops*), and β-naphthoflavone induced a 10-fold increase in the enzyme (492, 710). In a subsequent study of the scup, gill CYP1A produced epoxyeicosatrienoic acids (EETs), which have been shown to affect renal transport and hemodynamics (483). Other environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or polychlorinated biphenyl (PVCB) can induce CYP1A expression in the zebrafish, tilapia, and rainbow trout gills (60, 398, 765). Sadly, CYP1A has been found in gill tissue from oceanic, mesopelagic fishes of the western North Atlantic, suggesting a possible global occurrence of the contamination problem (709). Metallothioinein (MT), the heavy metal binding protein, can be localized to MRCs in the Atlantic salmon, and treatment with cadmium was associated with increased MT expression (138). Treatment of tilapia with copper induced MRC MT expression (139), as well as increased turnover of MRCs (137). Members of the multidrug resistant protein family of toxin export carriers (e.g., P-glycoprotein and Mrp2) have not been identified in the fish gills to date (D. Miller, personal communication), despite their expression in the fish brain (491) and liver (638). It is clear that more investigations of the roles of the fish gill vasculature and epithelium in the metabolism of intrinsic signaling agents and xenobiotics are needed.

TABLE 2. *Percent recovery of hormones and paracrines after a single pass through the gill vasculature of the rainbow trout*

Signaling Agent	Recovery, %
Peptides	
ANG II	65
BK	75–80
ANP	40
AVT	65–80
ET-1	45
Amines	
EPI	95
NorEPI	84
Serotonin	20
Arachidonic acid	<1
Prostanoids	
PGE_2	96
PGI_2	87
Purines	
Adenosine	60

Values were measured either in dorsal aorta of intact fish after a single pass or in effluent from perfused gill. BK, bradykinin; ANP, atrial natriuretic peptide; AVT, arginine vasotocin; ET-1, endothelin-1; EPI, epinephrine. [Redrawn from Olson (545).]

IX. SUMMARY AND CONCLUSIONS

The fish gill is a morphologically and functionally complex tissue that is the site of numerous, interconnected physiological processes, which are vital to maintaining systemic homeostasis in the face of changing internal (e.g., acidosis) and environmental (e.g., salinity) conditions. Early studies established a basic understanding of these processes, and during the past half-century, with the development of cellular, biochemical, and molecular techniques, our understanding of the specific pathways and control mechanisms involved has been greatly enhanced. Many of these mechanisms are homologous to those found in mammals (and other tetrapods), but important differences do exist, largely due to an aquatic versus terrestrial evolution and existence. Thus the general morphometric and irrigation/perfusion constraints of gas exchange are mediated by respiratory structures and processes similar to those in mammals, but the structures and processes in fish are adapted to the specifics of aquatic versus aerial gas exchange and mediated by gills rather than by lungs.

The features of the gills that enhance gas exchange also make the gills susceptible to osmotic and ionic movements between the environment and extracellular fluids;

thus osmoregulation is necessary. The branchial epithelium expresses an array of transporters and channels that are homologous to those in the mammalian kidney and balance the passive movements of water and solutes across the tissue (in coordination with intestinal and renal processes to maintain blood tonicity). The gill epithelium is also the primary site of plasma pH regulation and excretion of excess nitrogen, using transporters and channels that probably arose in marine ancestors, but have been secondarily adapted for osmoregulation in fresh water species.

These numerous functions by the gills require control and coordination by a complex web of neural, endocrine, and paracrine signaling pathways that are expressed in functionally analogous systems in mammals. However, some control systems (e.g., urotensins and stanniocalcin) were first described in fishes and are now known to play pivotal roles in mammalian physiology. Thus physiological research on "lower" vertebrates, such as fishes, is sure to lead to important discoveries in the realm of mammalian physiology.

In short, much has been learned, but even more remains to be discovered. We are hopeful that with the application of new and powerful molecular techniques, such as RT-PCR, microarrays, siRNA, etc., a more complete understanding of the physiology of the fish gill is soon to arrive.

This review honors Profs. W. T. W. Potts and L. B. Kirschner and the memory of Dr. Jean Maetz, true pioneers in the physiology of the fish gill.

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