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Review

Cellular composition and ultrastructure of the gill epithelium of larval and adult lampreys

Implications for osmoregulation in fresh and seawater

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Summary

Lampreys, one of the only two surviving groups of agnathan (jawless) vertebrates, contain several anadromous species that, during their life cycle, thus migrate from fresh to seawater and back to freshwater. Lampreys have independently evolved the same overall osmoregulatory mechanisms as the gnathostomatous (jawed) and distantly related teleost fishes. Lamprey gills thus likewise play a central role in taking up and secreting monovalent ions. However, the ultrastructural characteristics and distribution of their epithelial cell types [ammocoete mitochondria-rich (MR) cell, intercalated MR cell, chloride cell and pavement cell] differ in several respects from those of teleosts. The ultrastructural characteristics of these cells are distinctive and closely resemble those of certain ion-transporting epithelia in other vertebrates, for which the function has been determined. The data on each cell type, together with the stage in the life cycle at which it is found, i.e. whether in fresh or seawater, enable the following proposals to be made regarding the ways in which lampreys use their gill epithelial cells for osmoregulating in hypo- and hypertonic environments. In freshwater, the intercalated MR cell takes up Cl^- and secretes H^+, thereby facilitating the uptake of Na^+ through pavement cells. In seawater, the chloride cell uses a secondarily active transcellular transport of Cl^- to provide the driving force for the passive movement of Na^+ through leaky paracellular pathways between these cells.

Key words: lamprey, Agnatha, osmoregulation, gill epithelium, mitochondria-rich cell, chloride cell, pavement cell.

Introduction

The agnathan (jawless) stage in vertebrate evolution is represented in the contemporary fauna only by the hagfishes (Myxiniformes) and lampreys (Petromyzontiformes) (Hardisty, 1982). Although analyses of morphological and physiological data strongly indicate that lampreys are more closely related to the gnathostomatous (jawed) vertebrates than to the hagfishes (Hardisty, 1982; Forey and Janvier, 1993), the results of certain molecular studies support the view that the hagfishes and lampreys constitute a monophyletic group (Stock and Whitt, 1992; Mallatt and Sullivan, 1998; Kuraku et al., 1999; Delarbre et al., 2002). Yet, there can be no doubt that these two agnathan groups have been separated for a very long period and probably from as long ago as the lower Cambrian (Janvier, 1999).

Hagfishes, which are found only in marine habitats, are osmoconformers (McFarland and Munz, 1965; Cholette et al., 1970). The concentrations of Na^+ and Cl^- in their sera approximate those in full-strength seawater, a situation unique amongst vertebrates and which results in their internal milieu being essentially iso-osmotic with that of their marine environment (Robertson, 1974). These characteristics imply that hagfishes have always lived in marine habitats (Robertson, 1957, 1974; Lutz, 1975).

In contrast to hagfishes, the anadromous species of lampreys spend a substantial part of their life cycle in freshwater (Hardisty and Potter, 1971; Hardisty et al., 1989). The efficient osmoregulatory mechanisms evolved by lampreys enable the concentrations of Na^+ and Cl^- in their internal milieu to be maintained at levels well above that of freshwater, when the animal is living in rivers, and well below that of full-strength seawater, when the animal is residing in marine environments (Morris, 1972; Beamish et al., 1978). Since the osmolality of their serum is far lower than that of seawater, it has been concluded that lampreys have an ancient freshwater history and that the marine parasitic phase of their life cycle was developed relatively late in their evolution (Hardisty et al., 1989).

However, the recent discovery of a lamprey-like fossil in lower Cambrian marine deposits (Shu et al., 1999) strongly suggests that the initial evolution of lampreys occurred in a marine environment at a very early date, i.e. over 545 million years ago, and thus prior to the time when this group invaded freshwater.

The marked differences between both the ionic composition and osmolality of the body fluids of hagfishes and lampreys are consistent with the long period that these two agnathan groups are believed to have been separated. Indeed, as long ago as 1932, Homer Smith stated that "*these two groups lead back to a parting of the ways in the evolution of body fluids*".

The teleost fishes are found in a wide range of fresh, brackish and marine habitats and thus, as a group, are faced with the same variety of osmotic problems as those experienced by the anadromous species of lampreys during the course of their life cycle. The diversity and abundance of teleosts account for this group having been the subject of the majority of the studies aimed at elucidating the mechanisms by which fish regulate the ionic composition and osmolality of their serum (Smith, 1930, 1932; Krogh, 1939; Karnaky, 1980, 1986; Zadunaisky, 1984; Perry, 1997; Wilson et al., 2000a,b). Although lampreys are not closely related to teleosts, their gills and kidneys likewise constitute the main organs responsible for osmoregulation, and their overall mechanisms for regulating the concentrations of Na^+ and Cl^- in their body fluids are similar (Morris, 1972; Beamish, 1980; Hardisty et al., 1989). However, ultrastructural studies have demonstrated that, particularly when lampreys are in freshwater, the cellular composition of their gill epithelium differs from that of teleosts (Bartels et al., 1996, 1998). These studies have also revealed that certain epithelial cells in the lamprey gill possess highly distinctive ultrastructural features, which they share with particular cell types in specific ion-transporting epithelia in other vertebrates and for which a function has been determined.

In the present review, the ultrastructural characteristics of each of the cell types present in the gill epithelium at the different stages in the life cycle of lampreys are described. The characteristics of each cell type are then considered in the context of both the type of environment in which the animal containing that cell is found, i.e. freshwater or seawater, and the roles played by analogous cells in other vertebrates. This then enables each of these various cell types to be assigned a presumptive role in lamprey osmoregulation.

Osmoregulatory mechanisms in freshwater

When in freshwater, fish are subjected to an osmotic influx of water and efflux of ions across their skin and gills. This problem is overcome by an active uptake of monovalent ions across the gills and the excretion of a copious and dilute urine by the kidney (Fig. 1A; Smith, 1932; Krogh, 1939; Parry, 1966; Perry, 1997). The uptake of Na^+ and Cl^- by various vertebrates in freshwater, i.e. under *in vivo* conditions, is stoichiometrically balanced and occurs independently by exchanging H^+ for Na^+ and HCO_3^- for Cl^- (Krogh, 1939; Kirschner, 1983). The models developed for these ion exchange mechanisms were derived mostly from the results of studies carried out in Ussing-like chambers. Since such studies require the use of flat sheets of tissues, they have been performed mainly on amphibian epidermis, which, in contrast to the fish gill, has a flat surface (Garcia-Romeu and Ehrenfeld, 1975; Kirschner, 1983; Larsen, 1988). The results of these studies have led to the general acceptance that the exchange of Na^+ for H^+ involves an active secretion of H^+ by an electrogenic H^+-ATPase and the uptake of Na^+ through an epithelial Na^+ channel (ENac), rather than occurring simply through an Na^+/H^+-antiport. This mechanism is dependent on the activities of both cytosolic carbonic anhydrase in the H^+ secreting cell and Na^+/K^+-ATPase in the basolateral membrane of the cell whose apical membrane contains the ENac (Harvey and Ehrenfeld, 1986; Harvey et al., 1988; Nagel and Dörge, 1996; Ehrenfeld and Klein, 1997). Furthermore, the toad and turtle urinary bladders, in which the epithelium has a cellular composition comparable with that of amphibian skin and consists also of granular cells and mitochondria-rich (MR) cells (Wade et al., 1975; Wade, 1976; Rick et al., 1978; Durham and Nagel, 1986; Brown and Breton, 1996), employ essentially the same mechanisms for reabsorbing Na^+ from the urine and for secreting H^+ into that urine (Stetson and Steinmetz, 1985; Durham and Nagel, 1986; Lang, 1988). In addition, the immunocytochemical demonstration that H^+-ATPase and ENac are present in the gill epithelium of teleosts (Sullivan et al., 1995; Wilson et al., 2000a; Marshall, 2002) is consistent with the hypothesis that this mechanism for taking up Na^+ from a hypotonic environment is universal amongst vertebrates and invertebrates such as crustaceans, annelids and molluscs (Kirschner, 1983).

In contrast to the indirect coupling of Na^+ uptake to H^+ secretion, as described above, the uptake of Cl^- from an hypotonic environment by all epithelia studied thus far occurs directly by means of an HCO_3^-/Cl^- antiport (Garcia-Romeu and Ehrenfeld, 1975; Larsen, 1991; Marshall et al., 1997). The cells engaged in Cl^- uptake are further characterised by the presence of cytosolic carbonic anhydrase and a Cl^- channel in their basolateral membrane (Larsen, 1991).

Osmoregulatory mechanisms in seawater

When in seawater, teleosts and lampreys are confronted with the reverse situation to that in freshwater, i.e. they experience an osmotic loss of water to the environment. The further loss of water through the kidneys is minimised by reducing excretion from this source to just a small amount of concentrated urine. The osmotic loss of water is overcome by swallowing seawater, resorbing monovalent ions and water from the gut and retaining the 'free' water that remains following the secretion of an hypertonic solution of Na^+ and Cl^- across the gills (Fig. 1B; Smith, 1930; Karnaky, 1980, 1986; Loretz, 1995). As long ago as 1932, Keys and Willmer considered that Cl^- is secreted by the gill epithelium through

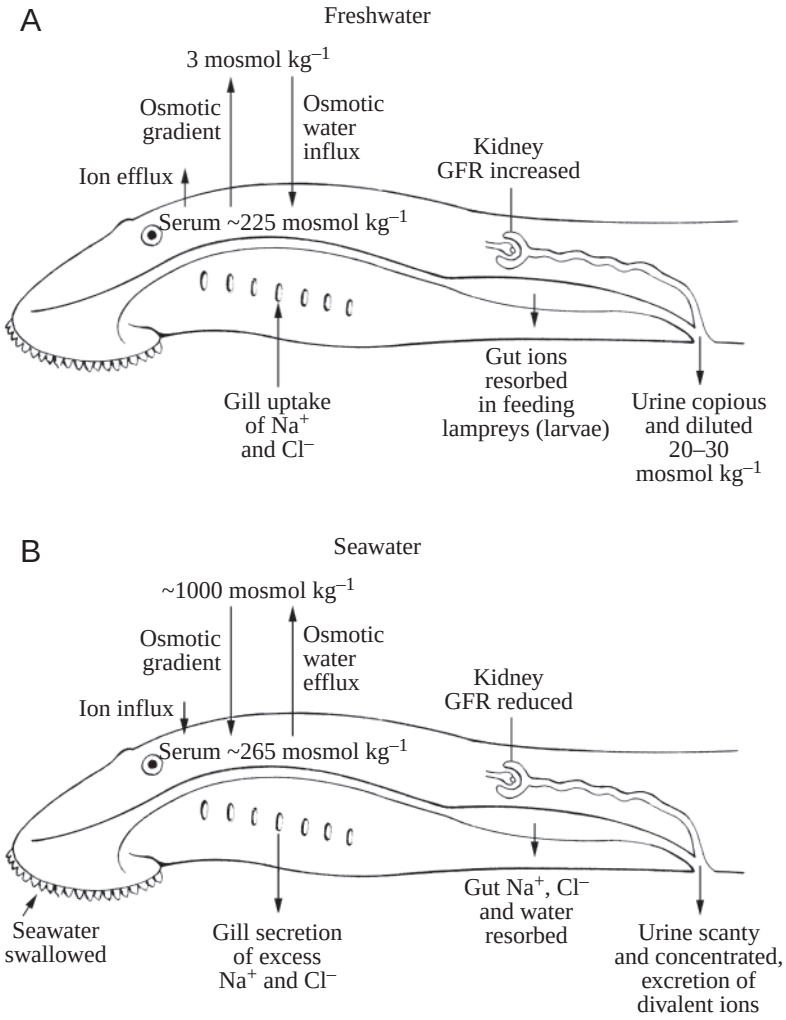

Fig. 1. Diagrams showing osmoregulatory mechanisms employed by anadromous lampreys during the freshwater (A) and seawater (B) phases in their life cycles. Modified from Hardisty et al. (1989).

a large eosinophilic cell that resembles the parietal cell in the gastric mucosa of mammals and which was consequently termed the 'chloride secreting cell' (Keys and Willmer, 1932). The cytoplasm of this cell contains numerous mitochondria and a system of membranous tubules (Philpott and Copeland, 1963; Karnaky et al., 1976a; Pisam and Rambourg, 1991). This membranous tubular system, which represents a vast intracellular amplification of the basolateral cell membrane, is the site of Na^+/K^+-ATPase activity and an $Na^+/K^+/2Cl^-$ cotransport system (Karnaky et al., 1976b; Karnaky, 1980, 1986; Eriksson et al., 1985). The mechanism by which Na^+ and Cl^- are transported by the gill epithelium of marine teleosts has been resolved through work on the opercular epithelium, which, in some teleost species, contains numerous chloride cells and, in contrast to the gill surface, is flat and thus suitable for studies in an Ussing-like chamber (Karnaky and Kinter, 1977).

Concomitant morphological and electrophysiological studies using the opercular epithelium have confirmed that, when teleosts are in seawater, Cl^- is secreted by this cell type through a secondary active transport that provides the driving force for the passive transport of Na^+ through leaky pathways between adjacent chloride cells (Karnaky et al., 1977; Ernst et al., 1980; Foskett and Scheffey, 1982; Foskett and Machen, 1985). The channel through which Cl^- is secreted by the teleost chloride cell has now been identified as a homologue of the mammalian cystic fibrosis transmembrane conductance regulator (CFTR; Singer et al., 1998; Wilson et al., 2000b; Marshall et al., 2002). Identical mechanisms for hypertonic salt secretion are also present in the secretory cells of both the rectal gland of elasmobranch fishes and the nasal salt gland of some species of marine birds (Kirschner, 1977, 1980; Riddle and Ernst, 1979; Ernst et al., 1981). It is thus assumed that this mechanism is universally employed by vertebrates for osmoregulation in hypertonic environments.

Osmoregulation during the life cycle of lampreys

The life cycle of all species of lampreys contains a microphagous larval phase, which is spent in freshwater (Potter, 1980). After a number of years, the larvae (ammocoetes) undergo a radical metamorphosis into young adults that, in the case of anadromous species, migrate downstream to the sea where they feed on predominantly teleost fishes (Potter and Hilliard, 1987). Following the completion of their marine trophic phase, adult lampreys re-enter freshwater and migrate to upstream areas, where spawning and then death occur (Hardisty and Potter, 1971).

Ammocoetes of the sea lamprey *Petromyzon marinus* are unable to osmoregulate in environments in which the osmolality exceeds that of their own serum, i.e. ~225 mosmol kg^{-1} (Beamish et al., 1978; Morris, 1980). Although ~50% of larval *P. marinus* died within 24 h of being placed in water of 350 mosmol kg^{-1}, i.e. about one-third of full-strength seawater, the recently metamorphosed young adults of anadromous species can readily be acclimated to full-strength seawater and are then able to maintain their serum osmolality at ~260 mosmol kg^{-1} (Beamish et al., 1978). This ability to change from hyper-osmotic regulation in freshwater to hypo-osmotic regulation in seawater is so effective that more than 80% of young adult *P. marinus* can even survive direct transfer from fresh to full-strength seawater (Potter and Beamish, 1977). Similar results have been obtained with young adults of the anadromous lampreys *Lampetra fluviatilis* and *Geotria australis* (Potter and Huggins, 1973; Potter et al., 1980). The ability to osmoregulate in hypertonic environments is lost by adult lampreys soon after they have completed their marine trophic phase and embarked on their upstream

spawning migration (Morris, 1956, 1958; Pickering and Morris, 1970).

The gill epithelium during the life cycle of lampreys

The gill epithelium undergoes marked changes during the life cycle of anadromous lampreys and, in particular, as the animal migrates from fresh to seawater and *vice versa* (Table 1). These changes include alterations to the composition and spatial relationships of the cells and to the structure of their tight junctions. The outer cellular layer of the gill filaments and lamellae of ammocoetes comprises two types of MR cell (i.e. the ammocoete MR cell and the intercalated MR cell) and pavement cells (Bartels et al., 1998). During metamorphosis, the ammocoete MR cells disappear and the chloride cells develop (Peek and Youson, 1979b). Thus, following the completion of metamorphosis, the gill epithelium of the downstream migrating young adult possesses chloride cells, pavement cells and intercalated MR cells. The last of these cells disappears after the young adult has entered seawater and does not reappear until the adult has finished growing and has re-entered freshwater on its upstream spawning migration (Bartels et al., 1998). By contrast, the chloride cells gradually disappear during the spawning run (Morris, 1957; Morris and Pickering, 1976).

The presence or absence of the different cell types at the surface of the gills at different phases in the life cycle of anadromous lampreys can now be used to propose which cells are involved in osmoregulation in hypo- and hyper-osmotic environments. For example, since the intercalated MR cell is the only cell type present throughout all freshwater phases, and is absent during the marine phase, it presumably plays a crucial role in osmoregulation when lampreys are in hypo-osmotic media. Likewise, since the chloride cell develops just prior to the marine phase and disappears soon after the completion of this phase, it can only be involved in osmoregulation when lampreys are in an hyper-osmotic medium. Although the pavement cell is the only cell type that is present on the gill surface throughout the entire life cycle of lampreys and is thus a potential candidate as an osmoregulatory cell in both fresh and seawater, there is currently no evidence that this cell type is required for osmoregulation in seawater (see below). The fact that the ammocoete MR cell is never found after the completion of the larval phase demonstrates that any role that it plays in osmoregulation in ammocoetes must be undertaken by other cell type(s) during the freshwater phases of post-larval life.

The freshwater phases in the life cycle

The intercalated MR cell

The cell in the lamprey gill epithelium that we have termed the intercalated MR cell (Bartels et al., 1998) corresponds to that designated by Youson and Freeman (1976) and Mallatt and Ridgway (1984) as a chloride cell in the ammocoete gill. Our choice of the term intercalated MR cell is based on the fact that, while these cells differ from the chloride cells in the gills of adult lampreys in that they lack a membranous tubulous system (see above), they do have the same ultrastructural characteristics as the MR cells that, for example, are intercalated in the epithelium of both the skin and urinary bladder of amphibians, of the urinary bladder of reptiles and of the collecting duct of the amphibian and mammalian kidney (Figs 2A, 3; Brown and Breton, 1996). Thus, the branchial intercalated MR cell of lampreys is characterized by the presence of numerous membranous vesicles and tubules between the mitochondria and also often immediately beneath the apical membrane (Fig. 2A). Moreover, the apical surface of the intercalated MR cell is enlarged by slender, branching microfolds that, from scanning electron microscopy, can be seen to produce a honeycomb appearance (Figs 2A, 3C). As primarily shown in the epithelium of the turtle urinary bladder, the extent of such enlargements, which varies markedly amongst intercalated MR cells, is inversely related to the number of membranous tubules and vesicles in the apical cytoplasm, which are incorporated into and removed from the apical membrane by exo- and endocytosis, respectively (Stetson and Steinmetz, 1983; Brown, 1989; Brown and Breton, 2000). A coat of studs projects about 12 nm outwards from the cytoplasmic side of the apical membrane (Fig. 2B). In freeze-fracture replicas, the cell membrane and the membranes of the cytoplasmic vesicles and tubules of the intercalated MR cell are characterized by the presence of rod-shaped particles on the protoplasmic (P) face and by complementary pits on the exoplasmic (E) face (Fig. 3A,B). These particles, which are either 16–18 nm or 24–27 nm in length and 8–9 nm in width, consist of two or three globular subunits. They are located in the apical membrane of almost all of the intercalated MR cells and in the basolateral membrane of a few of these cells (Bartels and Welsch, 1986; Bartels et al., 1998).

In the lamprey gill epithelium, these cuboidal intercalated MR cells are generally confined to the filaments, where they typically occur singly at their base and between the lamellae (Bartels et al., 1998). They are intercalated between ammocoete MR cells in larval lampreys (Fig. 3C) and either between chloride and pavement cells or between pavement

Table 1. *Cell types in the gill epithelia at different stages in the lamprey life cycle*

	Cell types			
Life cycle stage	IMRC	AMRC	PC	CC
Larva (FW)	+	+	+	–
Downstream migrant (FW)	+	–	+	+
Adult (SW)	–	–	+	+
Upstream migrant (FW)	+	–	+	d

FW, freshwater; SW, seawater; AMRC, ammocoete mitochondria-rich cell; CC, chloride cell; IMRC, intercalated mitochondria-rich cell; PC, pavement cell; +, present; –, absent; d, disappear during upstream migration.

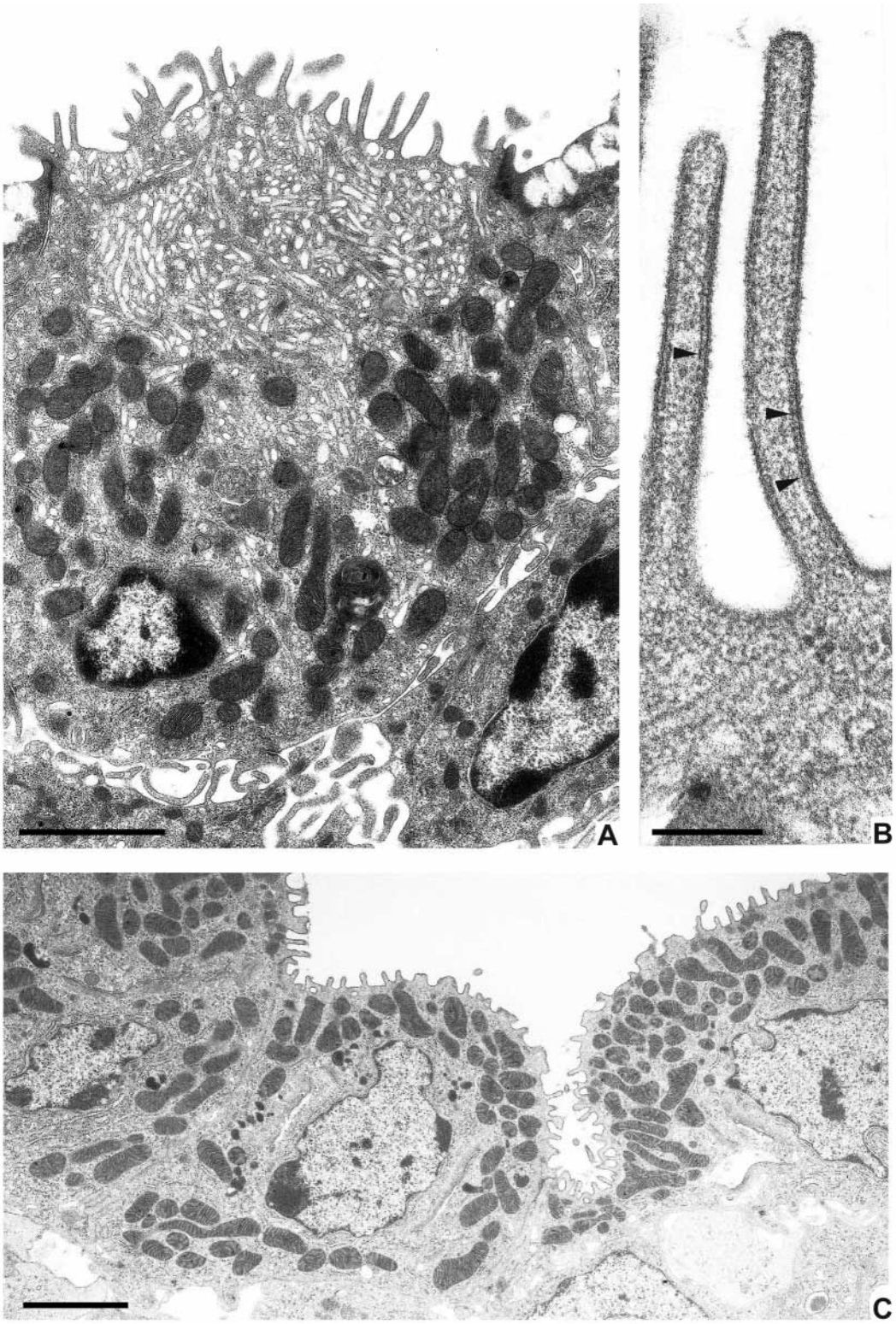

Fig. 2. Mitochondria-rich (MR) cells in the lamprey gill epithelium. (A) Intercalated MR cell between two pavement cells in the gill epithelium of a downstream migrant (young adult) of *Geotria australis*. The apical surface is enlarged by slender microplicae and the apical cytoplasm contains large numbers of membranous tubules and vesicles. (B) Higher magnification of the membrane of the microplicae, showing a coat of studs on the cytoplasmic side (arrows). (C) Ammocoete MR cells in the gill epithelium of a *Petromyzon marinus* larva, which, in contrast to the intercalated MR cells, lack cytoplasmic vesicles and tubules and exhibit only a moderate enlargement of their apical surface. Scale bars, 2 μm (A), 0.2 μm (B) and 2 μm (C).

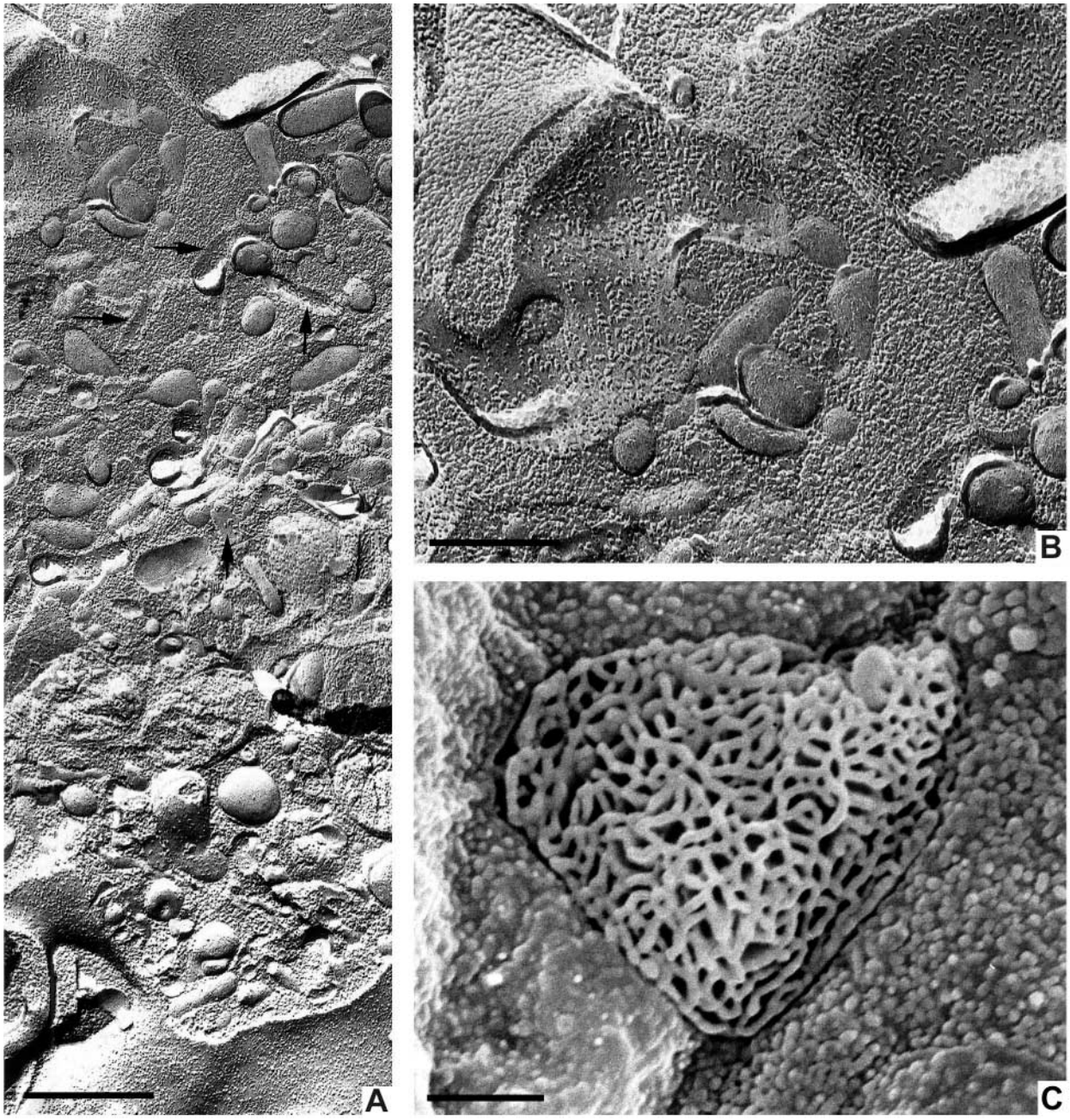

Fig. 3. (A) Freeze fracture of an intercalated mitochondria-rich (MR) cell of a *Petromyzon marinus* larva. Rod-shaped particles are present in the apical membrane (top of the micrograph) and in the membranes of cytoplasmic tubules and vesicles (arrows), while the basolateral membrane (bottom of micrograph) contains only globular particles. (B) Higher magnification of the apical part of the intercalated MR cell shown in A. The apical and cytoplasmic membranes contain a few globular particles in addition to rod-shaped particles. (C) Scanning electron micrograph of an intercalated MR cell surrounded by ammocoete MR cells in the gill epithelium of a larval *Geotria australis*. Scale bars, 0.5 μm (A), 0.25 μm (B) and 2 μm (C).

cells in downstream migrating lampreys and between pavement cells in upstream migrating lampreys.

The intercalated MR cells in urinary epithelia and the amphibian epidermis are rich in cytosolic carbonic anhydrase (CA II) and contain, within their plasma membrane and the membranes of cytoplasmic vesicles, a vesicular type of proton pump (Brown and Breton, 1996, 2000). The peripheral cytoplasmic subunit V1 of this pump has been immunolocalized to the studs on the cytoplasmic side of the MR cell membrane (Brown et al., 1987). Furthermore, a combination of physiological and morphological studies in various urinary epithelia indicates that the rod-shaped particles are either the transmembrane portion or an intimate associate of this pump (Stetson and Steinmetz, 1986; Brown et al., 1987;

Kohn et al., 1987). This view is supported by the presence of H^+ V-ATPase activity in all of those membranes that have been shown by freeze-fracture replicas to contain rod-shaped particles (Brown and Breton, 1996).

On the basis of differences in the locations of the H^+ V-ATPase, rod-shaped particles and a bicarbonate exchanger, two subtypes of intercalated MR cells (A and B) were initially distinguished in the collecting duct of the mammalian kidney and turtle urinary bladder (Stetson and Steinmetz, 1985; Brown et al., 1988; Brown and Breton, 1996). The subtype A contains the H^+ V-ATPase and rod-shaped particles in its apical and cytoplasmic vesicular membranes and possesses, in its basolateral membrane, the anion exchanger, identified as an alternatively spliced kidney form of the band 3 protein AE-1 (Fig. 4A). This subtype is responsible for electrogenic H^+ secretion. The subtype B of the intercalated MR cell is characterized by an apical membrane that possesses a bicarbonate exchanger, which, although functionally detectable, does not immunoreact with antisera against AE-1 or any other anion exchanger (Fig. 4B). This subtype is responsible for HCO_3^- secretion. Although the AE-1-negative subtype B was originally distinguished from subtype A by the presence of H^+ V-ATPase and rod-shaped particles in its basolateral membrane, immunocytochemical studies have now shown that the H^+ V-ATPase can occur in various locations in these AE-1-negative MR cells (Brown and Breton, 1996). This has led to the identification of a third subtype (C) of the intercalated MR cell in the amphibian epidermis (Fig. 4C), which is characterised by the presence of an anion exchanger and the H^+ V-ATPase in its apical membrane and thus provides a mechanism for the uptake of Cl^- from a dilute solution (Larsen et al., 1992).

The ultrastructural and functional characteristics of intercalated MR cells have been conserved in certain ion-transporting epithelia of vertebrates as diverse as amphibians, reptiles and mammals (Brown and Breton, 1996). Since the intercalated MR cells in the lamprey gill epithelium have the same unique ultrastructural characteristics as the other members of this group of cells, they presumably perform the same basic functions as those cells. The vast majority of the intercalated MR cells in the lamprey gill epithelium contains rod-shaped particles in their apical membrane and thus belong to the subtypes A or C of these cells. It is assumed that both subtypes, which cannot be distinguished on the basis of the location of the rod-shaped particles (and H^+ V-ATPase) in their apical membranes alone, are present in the lamprey gill epithelium. Subtype A is envisaged as actively secreting H^+, whereas subtype C is responsible for taking up Cl^-. The presence of an anion exchanger in parallel with the H^+ V-ATPase in the apical membrane, as is the case in subtype C cells, would enable the actively secreted hydrogen ions to bind the HCO_3^- as it leaves the cell and thereby establish an HCO_3^- gradient across this membrane, which would drive the uptake of Cl^-. The low external pH generated by nearby subtype A intercalated MR cells would further enhance this effect and help overcome the unfavourable Cl^- gradient, with the intracellular Cl^- concentration being approximately 10–20-fold greater than the extracellular concentration. Thus, the characteristics of subtype C make it much more efficient for taking up Cl^- from the environment than would those of subtype B, whose apical membrane contains the anion exchanger but not the H^+ V-ATPase. It is assumed that the primary role of the subtype B of the intercalated MR cell is HCO_3^- secretion during alkalosis, e.g. in urinary epithelia where these cells frequently occur (Alper et al., 1989), rather than the uptake of Cl^- from a dilute solution. This conclusion would explain why the subtype B is very rare in the lamprey gill epithelium.

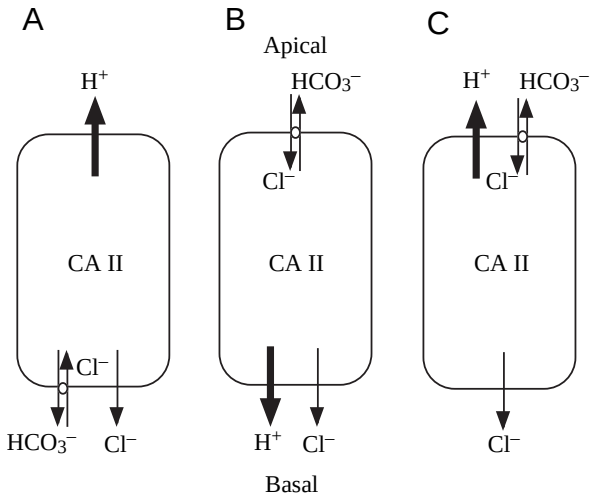

Fig. 4. Diagram showing the three subtypes (A–C) of the intercalated mitochondria-rich (MR) cell, based on the different distribution of the H^+ V-ATPase, anion exchanger and Cl^- channel. Thicker and narrower arrows denote active and passive transport, respectively. CA II, cytosolic carbonic anhydrase.

Finally, there is the question of whether epithelial Na^+ channels are present in the apical membrane of intercalated MR cells and provide a route for the uptake of Na^+ through these cells. Ehrenfeld et al. (1989) proposed that, in the frog skin under 'natural' conditions, i.e. low external Na^+ concentrations and open circuit, a significant amount of Na^+ is taken up through the MR cells and that only under Ussing-like conditions, i.e. high mucosal Na^+ concentration and short circuit, is Na^+ taken up through the granular cells. By contrast, Nagel and Dörge (1996) concluded that, even under natural conditions, the uptake of Na^+ *via* MR cells is negligible and occurs almost exclusively through the granular cells.

The pavement cell

The cells that form the surface of the lamellae in adult lampreys were called pavement cells by Bartels (1989) to be consistent with the terminology used for comparable cell types in the gills of teleosts and other fishes (Laurent, 1984; Wilson and Laurent, 2002). These cells correspond to those which, in ammocoetes, were designated as mitochondria-poor cells by Youson and Freeman (1976), mucous-platelet cells by Mallatt

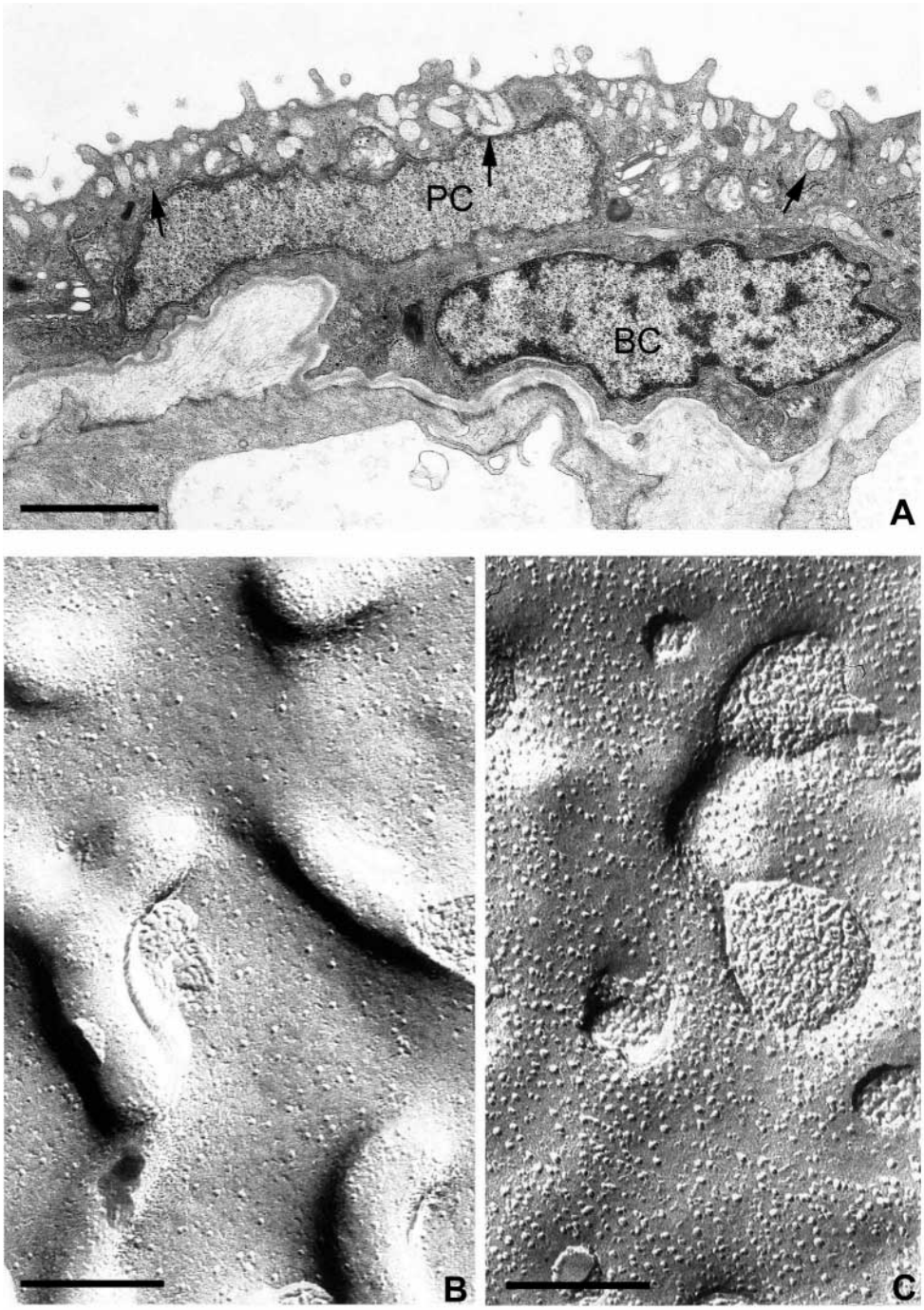

Fig. 5. Pavement cells in the gill epithelium of an upstream migrant of Lampetra fluviatilis. (A) The two-layered lamellar epithelium contains an outer layer of pavement cells (PC) and an inner layer of basal cells (BC). The pavement cells contain numerous ovoid mucous granules (arrows) and a few mitochondria. Freeze fracture of the apical membrane of the pavement cell showing few particles on the P face (B) and numerous particles on the E face (C). Scale bars, 2 μm (A) and 0.25 μm (B,C).

and Ridgway (1984) and mucous pavement cells by Mallatt et al. (1995). In ammocoetes, the pavement cells are restricted to the apex of the lamellae and thus, as will be described later, beyond the region occupied by the ammocoete MR cells (Youson and Freeman, 1976), whereas in adults they cover the entire lamellar surface and, at least in those in freshwater, also part of the interlamellar region of the filament. They are squamous on the lamellae and columnar in the interlamellar region, particularly in upstream migrants.

The pavement cell is ultrastructurally very similar to the granular cell in the amphibian urinary bladder (Wade et al., 1975; Bartels, 1989). It is thus characterized by the presence of numerous ovoid mucous granules, which are located in the apical cytoplasm, a rough endoplasmic reticulum with few cisternae, a well-developed Golgi apparatus and less mitochondria than are present in the two types of MR cells (Fig. 5A). The apical surface bears microplicae or short microvilli. Freeze-fracture replicas have shown that the majority of the intramembranous particles of the apical membrane are located on its E face and that few particles are present on its P face (Fig. 5B,C). This pattern of distribution of intramembrane particles differs from that found in the plasma membrane of most other vertebrate cells, including the basolateral membrane of both the lamprey pavement cell and the granular cell of the amphibian urinary bladder. Most of the particles on the E face of the apical membrane are relatively large (diameter 10–13 nm) and of greater size than those on the corresponding P face (diameter 6–8 nm).

The granular cell in the toad urinary bladder epithelium facilitates ionic and osmotic regulation by acting as the effector cell through which water and Na^+ are taken up independently. These two functions are hormonally controlled by the antidiuretic hormone (ADH) and the mineralocorticosteroid aldosterone. The permeability of the apical membrane of the granular cell to water is very low, unless this cell has been stimulated by ADH (DiBona et al., 1969; Harris et al., 1991). This low permeability has been related to the presence of only a few particles on the P face of the apical membrane (Bourguet et al., 1976), which in turn may reflect an unusual composition

or arrangement of lipids in the exoplasmic half of this membrane (Harris et al., 1991). Since this unusual and highly specialised membrane structure is shared by the pavement cell in the lamprey gill epithelium, the apical membrane of the latter cell is likewise assumed to be relatively impermeable to water. This conclusion is supported by the observation by Bentley (1962) that the permeability of the body surface of adult *Lampetra fluviatilis* to water is far lower than that of either the isolated toad urinary bladder or even the frog skin. It thus appears relevant that, particularly in adult lampreys, the contribution made by pavement cells to the area of the body surface that is exposed to the environment is far greater than that of any other cell type. Thus, when adult lampreys are in freshwater, the possession by these cells of a relatively impermeable apical membrane helps protect these animals against an osmotic influx of water across the gills.

During periods of water shortage in toads, ADH increases the permeability of the apical membrane of the granular cells to water by recruiting water channels (aquaporin 2) into this membrane from a cytoplasmic pool (Wade et al., 1981; Brown et al., 1989). Thus, since lampreys are not threatened by dehydration when in freshwater and thus do not require an ADH-mediated mechanism for either conserving or taking up water, it is hardly surprising that this hormone does not elicit an hydro-osmotic response in these animals (Bentley and Follett, 1962).

The uptake of Na^+ from the urine by the granular cell in the toad bladder, which is regulated by aldosterone and ADH, occurs through ENac in the apical membrane and is driven by Na^+/K^+-ATPase activity in the basolateral membrane (Macknight et al., 1980; Garty, 1986; Garty and Palmer, 1997). Although lampreys do not produce aldosterone (Bentley and Follett, 1962), the administration of this hormone (but not of neurohypophyseal hormones) to these agnathans likewise increases the extrarenal uptake of Na^+ (Bentley and Follett, 1962, 1963). This finding raises the possibility that, as with the granular cells of Na^+-resorbing epithelia such as the toad urinary bladder and epidermis, ENac are present in the lamprey pavement cell. The pavement cell thus becomes the main candidate for the uptake of Na^+ and the target of unidentified mineralocorticoid hormone(s) in lampreys.

The ammocoete MR cell

The cell that we term the ammocoete MR cell (Bartels et al., 1998) corresponds to the mitochondria-rich cell of Morris and Pickering (1975), the mitochondria-rich platelet cell of Youson and Freeman (1976) and the ion-uptake cell of Mallatt and Ridgway (1984). The latter three groups of authors considered that this cell is responsible for taking up ions from the environment.

The ammocoete MR cells represent up to 60% of the cells at the surface of the gill lamellae of larval lampreys. They are arranged in large groups at the base of the lamellae and in the region between the lamellae. Their mitochondria, which occupy about one-third of the cell volume (Mallatt et al., 1995), have an unusually electron-dense matrix (Fig. 2C). Elongated mucous granules, which are smaller than those of pavement cells, lie directly beneath the apical membrane of some of these cells. In freeze-fracture replicas, the apical membrane of the ammocoete MR cell exhibits the typical characteristics of vertebrate cell membranes, i.e. the P face of the cleaved membrane contains most of the globular particles (diameter 8–9 nm) whereas the E face contains few particles but numerous pits (Bartels et al., 1998).

The presence of numerous mitochondria and a positive histochemical reaction for carbonic anhydrase by the ammocoete MR cell (Conley and Mallatt, 1988) are consistent with the view that, in larval lampreys, this cell is responsible for exchanging Na^+ for H^+ and/or Cl^- for HCO_3^- (Morris and Pickering, 1975; Youson and Freeman, 1976; Mallatt and Ridgway, 1984). However, our preliminary studies show that, when ammocoetes of *G. australis* are held in distilled water, in which the uptake of Na^+ and Cl^- is maximally stimulated, the characteristics of the ammocoete MR cells (and pavement cells) are unaffected whereas the density of the intercalated MR cells increases significantly beyond that found with these cells in ammocoetes held in 10% seawater (H. Bartels, J. Rosenbruch and I. C. Potter, unpublished observations). Furthermore, adult lampreys in freshwater still possess the osmoregulatory capacity to overcome the same osmotic challenges as ammocoetes even though they do not possess ammocoete MR cells. The ammocoete MR cell may thus have a function additional to or other than osmoregulation. Since lampreys feed in freshwater only during their larval phase, it is possible that the ammocoete MR cell, which apparently has no morphological counterpart in other vertebrate epithelia, may be involved in excreting ions and/or waste products that have been derived from the digestion of their algal and detrital food (Bartels et al., 1998). The ability to take up ions *via* the branchial epithelium would be particularly valuable during the upstream spawning run since, during that migration, the lamprey ceases feeding and its gut degenerates (Youson, 1981) and thus no longer has the potential to acquire ions from food.

Models for Na^+ uptake by the branchial epithelium of lampreys in freshwater

Since the intercalated MR cell and pavement cell are the only two cell types that are present on the gill surface of both larval and adult lampreys in freshwater, it has been assumed that Na^+ and Cl^- must be taken up through either one or both of these cell types (Bartels, 1989; Bartels et al., 1998). Since the putative role of the subtype C of the intercalated MR cell in the uptake of Cl^- has been discussed above, we will now focus on the uptake of Na^+.

The arrangement and ultrastructure of the intercalated MR cell and pavement cell in the gill epithelium of adult lampreys in freshwater bear a striking resemblance to those of the intercalated MR cells and granular cells in the amphibian skin and urinary bladder, respectively. This led to the proposal that, in the lamprey gill, the intercalated MR cells (subtypes A and C) likewise facilitate the uptake of Na^+ by active H^+ secretion and that the pavement cell is the primary candidate

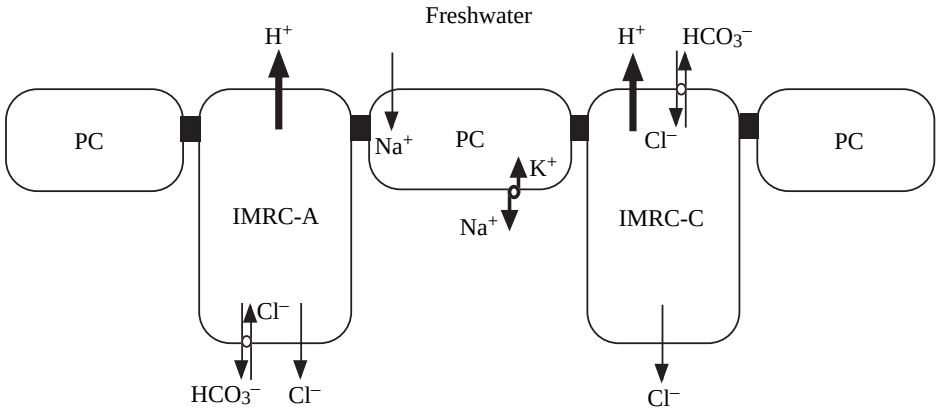

Fig. 6. A proposed model for Na^+ and Cl^- uptake by adult lampreys in freshwater. Thicker and narrower arrows denote active and passive transport, respectively. IMRC-A and IRMC-C, subtype A and C of intercalated MR cells, respectively; PC, pavement cell.

for the uptake of Na^+ (Fig. 6; Bartels, 1989; Bartels et al., 1998). Except in 'very tight' epithelia, such an indirect coupling of H^+ secretion and Na^+ uptake requires a close spatial relationship between the cell types involved. This is the case in both of the freshwater stages of adult lampreys, i.e. during their downstream and upstream migration, when the MR cells are intercalated between (or directly neighbouring) the granular cells. However, in larval lampreys, the pavement cells are always separated from the intercalated MR cells by large groups of ammocoete MR cells. The distance between these two cell types would thus presumably be too large to produce an effect on the electrical potential generated by the active H^+ secretion through intercalated MR cells on the pavement cells. Although the electrical resistance of the gill epithelium of larval lampreys has not yet been determined, the tight junctions, as revealed in freeze-fracture replicas, do not possess the structural characteristics considered responsible for epithelial tightness, e.g. a large number of superimposed junctional strands, exhibiting a high degree of branching and anastomosing and consisting of solid fibrils rather than particles (see fig. 3A in Bartels et al., 1998; Claude and Goodenough, 1973; Claude, 1978; Cerejido et al., 1989).

Provided that the gill epithelium of larval lampreys is not very tight and the ammocoete MR cells do not contribute to the uptake of Na^+, there are two possible alternative mechanisms for this transport in ammocoetes. The first hypothesis is based on the assumption that, in ammocoetes, Na^+ uptake is coupled to H^+ secretion. Under this condition, only the intercalated MR cell can be responsible for the uptake of Na^+. This model, which is consistent with that proposed for the MR cells in the frog skin under natural conditions (see above; Ehrenfeld et al., 1989; Ehrenfeld and Klein, 1997), has the advantage of being equally applicable to larval and adult lampreys. It requires the presence both of the H^+-ATPase and the ENac in the apical membrane and of significant amounts of Na^+/K^+-ATPase in the basolateral membrane of the intercalated MR cell. However, there is as yet no direct evidence from patch–clamp or immunocytochemical studies that the ENac is present in intercalated MR cells of the amphibian epidermis or in any other location in which these cells occur. Furthermore, studies on the cellular distributions of Na^+/K^+-ATPase in the toad and turtle urinary bladders and frog skin have shown that the vast majority of Na^+ pumps are localised in the granular cells and that the intercalated MR cell possesses only a few of these pumps. This distribution pattern is consistent with the model that the granular cell compartment is responsible for Na^+ uptake (Rick et al., 1978; Durham and Nagel, 1986; Nagel and Dörge, 1996) and suggests that the few Na^+ pumps present in the intercalated MR cells are sufficient to maintain the balance of the Na^+ and K^+ gradients across the basolateral membrane but do not significantly contribute to Na^+ uptake.

The alternative model assumes that Na^+ is taken up by pavement cells and is energized only by the activity of the Na^+ pump, located in the basolateral membrane of this cell, and is not facilitated by H^+ secretion by intercalated MR cells. The question then arises as to whether the rate of this branchial Na^+ uptake, possibly in concert with intestinally resorbed dietary Na^+, is sufficient to substitute the passive loss of Na^+. Under such a condition, H^+ secretion might provide the energy for Cl^- uptake through the subtype C of the intercalated MR cells (see above). By contrast, since the requirements for a coupling of Na^+ uptake and H^+ secretion are met in adults, H^+ secretion through the subtypes A and C could also facilitate Na^+ uptake in the adult stages of the life cycle in addition to driving the uptake of Cl^-. This model implies that the mechanisms for regulating the Na^+ concentration are not the same in larval and adult lampreys. It also takes into account the fact that the composition and arrangement of the branchial epithelial cells in larval and adult lampreys differ and that the larval lamprey feeds whereas the adult lamprey in freshwater does not.

The marine phase in the life cycle

The chloride cell

Lamprey chloride cells are disc-like and form long continuous rows that extend throughout the interlamellar region of the filament and into the basal region of the filament where lamellae are absent (Figs 7A, 8; Bartels et al., 1996). The cytoplasm of these cells contains numerous membranous tubules, between which large mitochondria are intercalated (Fig. 7A), thereby paralleling the situation with the chloride cells of teleost fishes. Since these tubular membranes represent a vast intracellular amplification of the basolateral cell

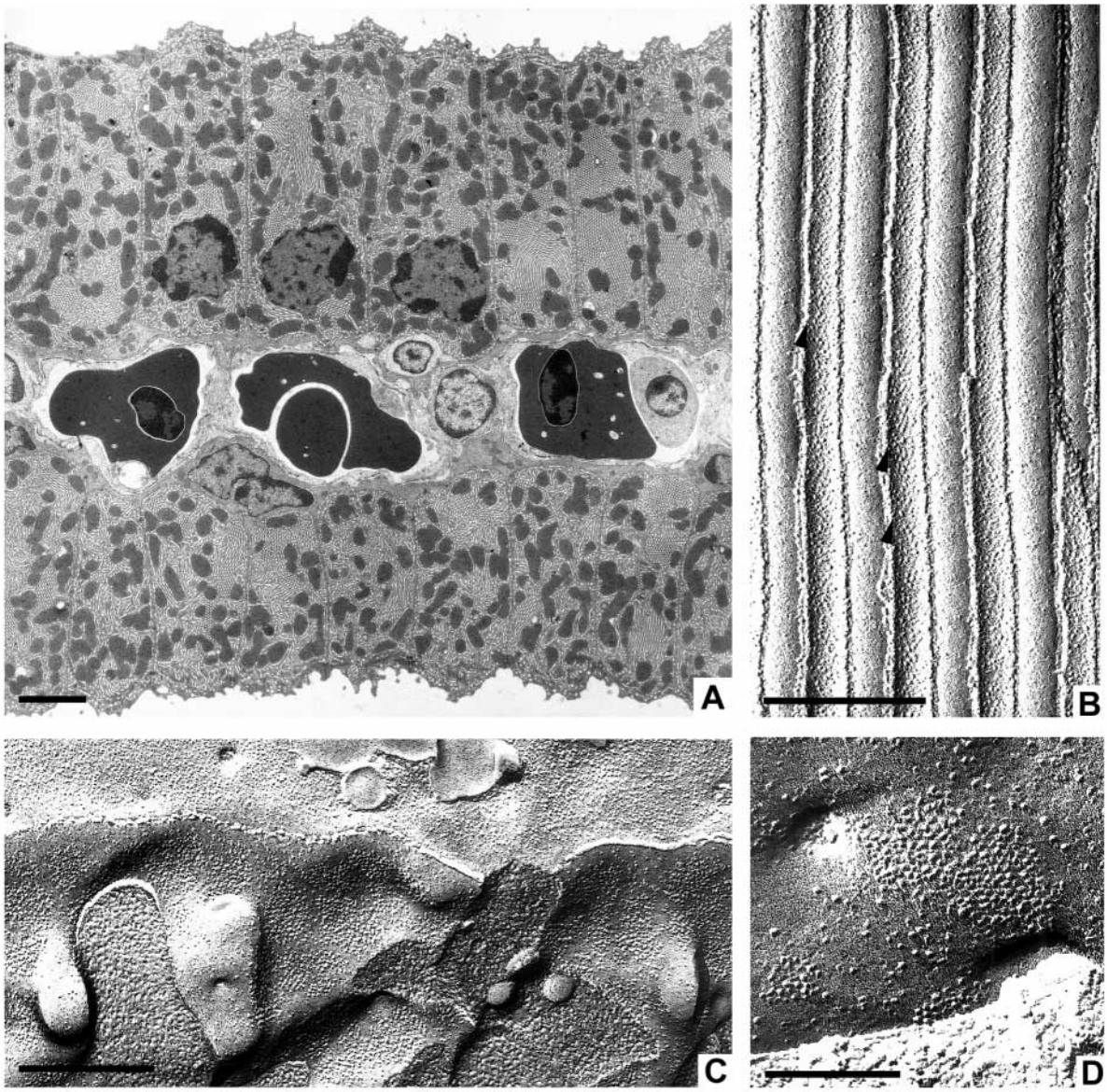

Fig. 7. Chloride cells in the gill epithelium of an adult *Geotria australis* in seawater. (A) Cross section of the filament, showing a row of chloride cells on each side of the central blood space. (B) Freeze fracture of a bundle of parallel membranous tubules, showing the helicoidally arranged particles (arrowheads). (C) Zonula occludens between two chloride cells consisting of a single strand. The particles in the apical membrane of the chloride cell are clustered on the P face. (D) Clusters of particles on the E face. Scale bars, 3 μm (A), 0.5 μm (B,C) and 0.25 μm (D).

membrane, the lumen of the tubules constitutes part of the extracellular space (Philpott and Copeland, 1963; Philpott, 1966; Nakao, 1974; Karnaky et al., 1976a; Peek and Youson, 1979a). The particles in the membranes of these tubules have been shown by freeze fractures to be located mainly on their P face and to be arranged in linear and helicoidally twisted arrays (Fig. 7B; Hatae and Benedetti, 1982; Bartels and Welsch, 1986).

When young adult lampreys are still in freshwater, the surface of all but a small circular central region of their chloride cells is covered by the flanges of adjacent pavement cells (Fig. 8A). After young adults have entered the marine environment, their pavement cell flanges retract, with the result that the surface of each chloride cell that is exposed to the environment then occupies a rectangle, the length of which corresponds to the entire width of the interlamellar region (Fig. 8B). In addition, the apical surface loses its short microvilli and thus becomes relatively smooth (Fig. 8B; Bartels et al., 1996). Freeze-fracture replicas demonstrate that, when these lampreys are still in freshwater, the particles in the apical membrane of the chloride cells are randomly distributed on the P face. However, after the lamprey has entered seawater, most of the particles present on both fracture faces are hexagonally arranged into clusters with a periodic spacing of approximately 19 nm (Fig. 7C,D; Bartels et al., 1993).

As a consequence of the changes at the gill epithelium

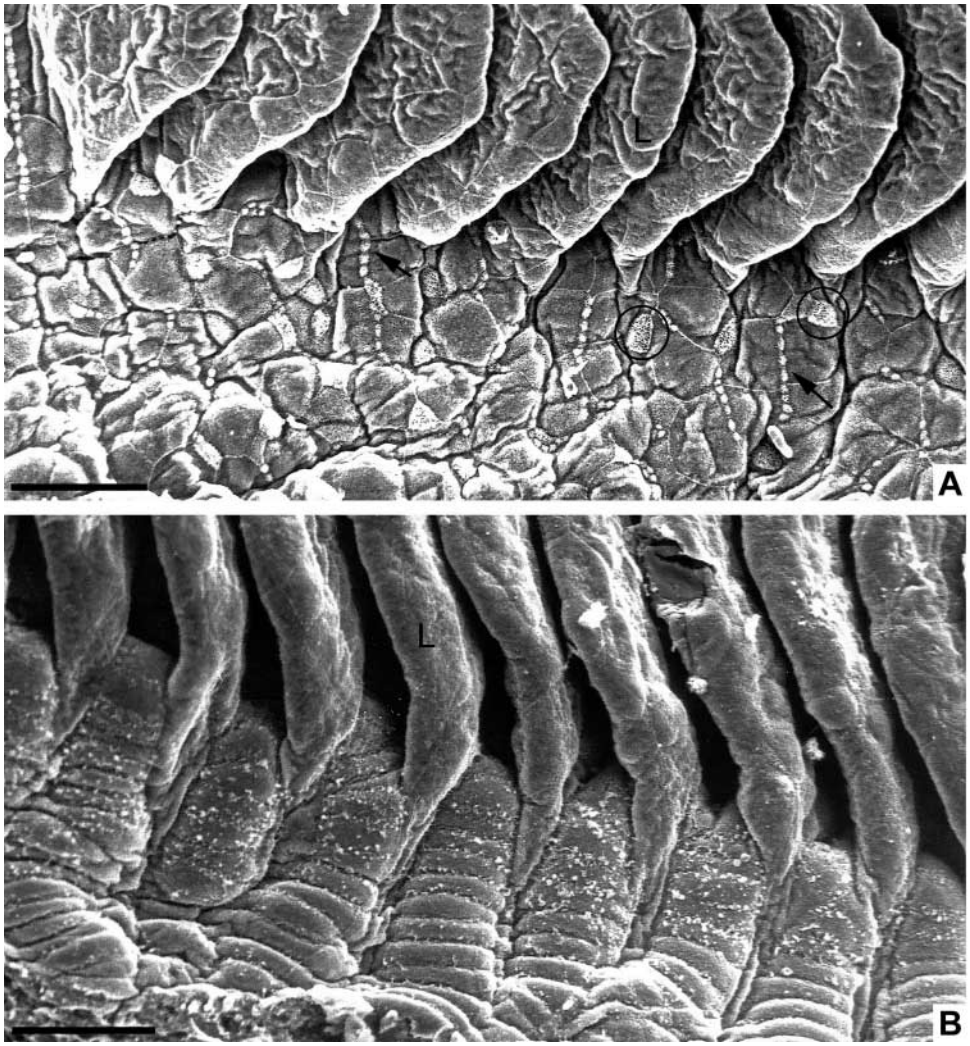

Fig. 8. Apical surfaces of the chloride cells of young adult *Geotria australis* in freshwater (A) and after migration to seawater (B). Lateral views of the filament, showing at the bottom its base, where lamellae are absent. The chloride cells form rows (arrows) at the base of the filament, which continue into the interlamellar region. Note the changes of the apical surface of the chloride cell from a small circular region enlarged by microvilli in freshwater (A) to a comparably large rectangular region lacking microvilli in seawater (B). Intercalated MR cells are encircled in A and absent in B. L, lamellae. Scale bars, 30 μm (A,B). Reprinted from Bartels et al. (1996) with kind permission from the publishers.

surface, the length of the paracellular pathway between adjacent chloride cells is increased by four to five times. At the same time, the tight junctions between chloride cells become greatly reduced in depth and their strands decline in number from four to either one or two (Fig. 7C), whereas those between chloride and pavement cells remain deep and the number of their strands declines only from four to three (Bartels and Potter, 1991). Finally, the membranous tubules in the cytoplasm, which are convoluted when the lamprey is still in freshwater, become aligned and organised into tight bundles when the animal enters seawater. After the fully grown lamprey has left the sea and embarked on its spawning run, the chloride cells become covered by the flanges of adjacent pavement cells and undergo apoptosis.

In the gills of marine teleosts, the site of Na^+/K^+-ATPase activity lies predominantly in the membranous tubules in the cytoplasm of their chloride cells (Karnaky et al., 1976b), which contains intramembranous particles that form a tight and regular (‘cobblestone’) arrangement. Since these particles are considered to correspond to the Na^+ pumps (Sardet et al., 1979; Sardet, 1980), it has been proposed that the particles of the helicoidally twisted linear arrays in the corresponding membrane of lamprey chloride cells are also the sites of Na^+/K^+-ATPase activity (Hatae and Benedetti, 1982; Bartels and Welsch, 1986). Furthermore, in teleosts, this membrane contains a furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (Eriksson et al., 1985), through which Cl^- enters the cell on its basolateral side, a process driven by the steep Na^+ gradient maintained across this membrane by the activity of the Na^+ pump (Karnaky, 1986).

The type of clusters of intramembranous particles, which are present in the apical membrane of the chloride cells of lampreys in seawater, has not been observed in any of the Cl^- secretory cell types engaged in osmoregulation in marine environments. However, ‘crystalline-like plaques’, whose particles are of a similar size and spacing as those in the lamprey chloride cell membrane, are present in the apical membrane of the principal cells in the renal collecting tubule of the salamander *Amphiuma means* (Biemesderfer et al., 1989), i.e. in a cell engaged in taking up rather than secreting Na^+ and Cl^- (Hunter et al., 1987). Since the changes in the apical membrane of the lamprey chloride cell described earlier take place at the time when the animal becomes faced with the need to excrete monovalent ions in a hypertonic environment (and when an extended leaky paracellular pathway is developed between the chloride cells), the clusters of particles in the apical membrane of lamprey chloride cells are likely to be associated with the transport of Cl^- across this membrane. The occurrence of these clusters of particles in cell membranes, across which Cl^- is transported in opposite directions, implies that the direction of Cl^- movement depends on the electrochemical gradient.

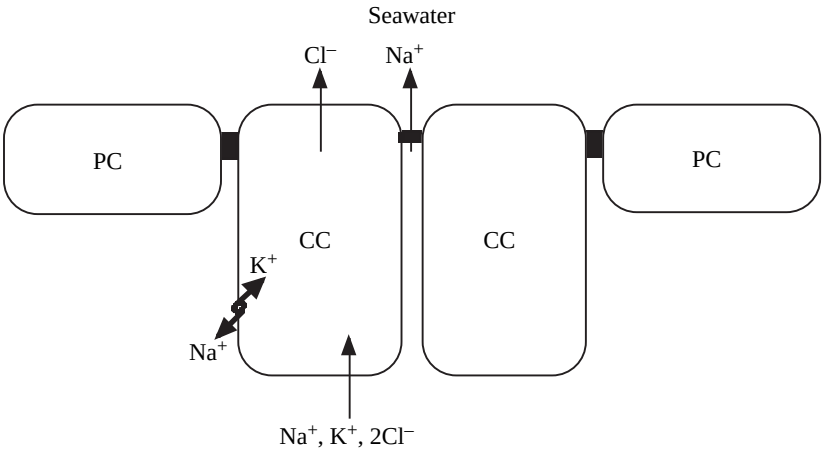

Fig. 9. A proposed model for Na^+ and Cl^- secretion by lampreys in seawater. Thicker and narrower arrows denote active and passive transport, respectively. CC, chloride cell; PC, pavement cell.

The above description of the lamprey chloride cell demonstrates that, when lampreys are in seawater, this cell possesses the morphological characteristics that, in ion-secretory cells in epithelia such as those of the teleost gill and operculum, the elasmobranch rectal gland and the avian nasal gland, have been shown to be involved in secreting excess monovalent ions when the animal is in hypertonic environments (Kirschner, 1980). In this regard, the three most salient features are: (1) the secretory cells are organised into multicellular units, (2) there is an extended leaky paracellular pathway (shunt) between the secretory cells and (3) the (baso)lateral membrane is greatly amplified, which thereby provides space for a large number of Na^+ pumps. It is thus concluded that, as in the gills of marine teleosts, the chloride cell of lampreys in hypertonic environments is responsible for secreting the excess component of the Na^+ and Cl^- that has been absorbed through the alimentary canal (Fig. 9). The mechanisms used for such secretion by lamprey chloride cells are assumed to be essentially the same as those employed by the chloride cells of teleosts in seawater. They thus involve a secondary active transport of Cl^- through the chloride cells, which drives the passive movement of Na^+ through the leaky pathways between these cells (Bartels and Potter, 1991; Bartels et al., 1996).

The pavement cell

The only morphological change observed in the pavement cells of young adult lampreys as these animals migrate from fresh to seawater was a reduction from five to four in the number of strands in their tight junctions (Bartels and Potter, 1993). Since the number of strands of the tight junctions between pavement and chloride cells also declined, in this case from four to three (Bartels and Potter, 1991), the paracellular pathway in the lamprey gill epithelium becomes more leaky during the transition from fresh to seawater. Although such a conclusion is consistent with the results of studies on teleosts, which have shown that the permeability of their gills to Na^+ is greater in sea than freshwater (Potts, 1984), any such increase in the permeability to Na^+, when young adult lampreys enter seawater, would be due mainly to the pronounced changes that occur to the structure and length of the occluding junctions between the chloride cells. Since, as in marine teleosts, the chloride cell of lampreys apparently possesses all of the structural characteristics required to fulfil the osmoregulatory function of gills in seawater, there are at present no indications that the osmoregulation of lampreys in seawater requires an involvement of the pavement cell.

Comparisons between epithelial cell types in the gills of lampreys and teleosts

Although the gills of lampreys and teleosts are assumed to perform similar, if not identical, osmoregulatory functions, the cellular composition of their epithelia differs considerably in some respects (Laurent, 1984; Wilson and Laurent, 2002). Thus, in contrast to the situation in lampreys, the gill epithelia of teleosts always only contain pavement and chloride cells, irrespective of whether the fish is living in fresh or seawater. Furthermore, teleosts possess two types of chloride cell. There is thus a freshwater chloride cell, which is typically singly intercalated between the pavement cells, as is the case with the intercalated MR cells in lampreys, and a seawater chloride cell, which is similar to the chloride cells of adult lampreys in seawater (see below).

The freshwater chloride cell of teleosts differs from the intercalated MR cell of lampreys through its possession of a tubular system, which is less elaborate than that of the chloride cell in seawater, and by the absence of rod-shaped particles in its plasma and cytoplasmic membranes. Although the term ‘MR cell’ has been proposed for the freshwater chloride cell of teleosts (Pisam and Rambourg, 1991), the gills of teleosts in freshwater do not contain cells with the cytological characteristics of either the intercalated MR cells of lampreys and higher vertebrates or the ammocoete MR cell. Experimental studies indicate that, in teleosts, the freshwater chloride cell exchanges Cl^- for HCO_3^- while the pavement cell exchanges Na^+ for H^+ (Goss et al., 1992; Perry, 1997). The latter conclusion is supported by the results of immunocytochemical studies, which showed that the pavement cell is the only cell in the teleost gill epithelium that contains both H^+-ATPase and ENac (Sullivan et al., 1995; Wilson et al., 2000a). The freshwater chloride cell of teleosts thus performs the role that we consider is carried out in lampreys by the subtype C of the intercalated MR cell. The function performed by the pavement cell of teleosts in freshwater, in turn, is ascribed in lampreys to the activities of the subtypes A and C of the intercalated MR cell and the

pavement cell, coupled in adults and possibly uncoupled in ammocoetes.

The differences between the gill epithelium of teleosts and lampreys in marine environments are less pronounced than those described above for freshwater and are mainly associated with chloride cells, which in both groups are responsible for excreting excess Cl^- and Na^+. They thereby constitute the main effector cells for osmoregulation in seawater. Although the chloride cells of teleosts and lampreys are both located close to the afferent filament artery and form multicellular complexes, the arrangement and size of these complexes in the two groups differ. Thus, the chloride cells in the gills of teleosts form small groups of 2–4 cells whereas those of lampreys form long rows. Furthermore, while accessory cells, which lack an extensive tubular system and associated Na^+/K^+-ATPase, also frequently contribute to the groups of chloride cells in teleosts (Hootman and Philpott, 1980), such cells are not found in lampreys (Bartels and Potter, 1991). Moreover, in teleosts, each group of chloride cells and of chloride and accessory cells share an apical crypt (Karnaky, 1986), a structure not found in association with these cells in lampreys (Nakao, 1974; Peek and Youson, 1979a; Bartels and Potter, 1991; Bartels et al., 1993, 1996). The paracellular pathways between the chloride cells in both lampreys and teleosts and between chloride and accessory cells in teleosts contain leaky occluding junctions through which Na^+ enters the environment passively (Sardet et al., 1979; Ernst et al., 1980; Bartels and Potter, 1991). However, the increase that occurs in the length of this shunt, following the migration of individuals from fresh to seawater, is achieved in teleosts through the development of interdigitations amongst the cells that form multicellular complexes (Sardet et al., 1979; King et al., 1989) and in lampreys by the retraction of pavement cells (Bartels et al., 1996). Finally, the apical membrane of the chloride cells of teleosts in seawater does not contain the prominent clusters of particles that are found in this membrane in lampreys (Sardet et al., 1979; Ernst et al., 1980; Bartels et al., 1993).

As mentioned earlier, the fact that the osmolality of the sera of all stages in the life cycle of lampreys is far less than that of seawater argues that this agnathan group spent a considerable period in freshwater (Hardisty et al., 1989). However, the discovery of a lamprey-like fossil in Cambrian marine deposits from 545 million years ago strongly indicates that the Petromyzontiformes evolved in marine environments (Janvier, 1999; Shu et al., 1999). If this is the case, the specialised parasitic phase in the life cycle of contemporary anadromous lampreys represents a secondary return to a marine environment. By contrast, the teleosts are believed to have evolved in freshwater and then undergone extensive adaptive radiation in the sea during the Mesozoic, with some groups subsequently reinvading freshwater (Lutz, 1975). The possession of the same basic mechanisms for osmoregulation by lampreys and teleosts thus presumably represents the result of convergent evolution. Such independent evolution of the same mechanisms would account for differences between the characteristics of the cell types used for osmoregulation by these two divergent groups.

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