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AN INVESTIGATION INTO ACID-BASE BALANCE AT THE GILLS OF RAINBOW TROUT (*Oncorhynchus mykiss*) USING *in situ* HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY FOR H^+-ATPase AND BAND 3

By

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A thesis submitted to the School of Graduates Studies and Research
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Title: An Investigation Into Acid-Base Balance at the Gills of Rainbow Trout (*Oncorhynchus Mykiss*) Using *in situ* Hybridization and Immunocytochemistry for H^+-ATPase and Band 3

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AN INVESTIGATION INTO ACID-BASE BALANCE AT THE GILLS OF RAINBOW TROUT (*Oncorhynchus mykiss*) USING *in situ* HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY FOR H^+-ATPase AND BAND 3

SUMMARY

This thesis examines changes that occur in the epithelial cells of rainbow trout (*Oncorhynchus mykiss*) gills with respect to the expression of Na^+ and Cl^- ion-transporting proteins during periods of acid-base disturbance.

In the first part of the study, the expression of the V-type proton ATP-ase (H^+-ATPase) was examined in the gill of trout to provide direct evidence for its existence, determine its cellular distribution, and assess its response to acid-base disturbance. To this end, immunocytochemistry was used in concert with laser scanning confocal or electron microscopy and Western blotting. A synthetic peptide consisting of the carboxy-terminal region of the 31 kDa sub-unit of the bovine renal H^+-ATPase was used to generate an antiserum in rabbits suitable for immunodetection of H^+-ATPase in gill tissue.

Gill epithelial cells demonstrated specific immunoreactivity, the intensity of which was increased markedly after 18 h of exposure to hypercapnia (1% CO_2 in air). The increased intensity of H^+-ATPase immunoreactivity was associated with elevated branchial net acid excretion. In the hypercapnic fish, the specific immunoreactivity was associated with apical membrane and sub-apical cytoplasm. Electron microscopy revealed specific immunoreactivity localized to the pavement cells, particularly associated with the apical membrane and sub-apical cytoplasmic vesicles. The specificity of the antiserum for the 31- kDa subunit of the H^+-ATPase was supported by Western blotting with the presence of an immunoreactive band at 31-kDa.

The increased H^+-ATPase immunoreactivity in the epithelial cells of hypercapnic fish demonstrates an "up-regulation" of this protein in response to respiratory acidosis. The results are discussed with reference to current models of acid-base and ion regulation in the freshwater fish gill.

Oligonucleotide probes, complementary to the mRNA of the 31- and 56-kDa subunits of the bovine renal H^+-ATPase were constructed. Similarly, an oligonucleotide probe for Band 3, an AE1 anion exchange protein (Cl^-/HCO_3^- exchanger) from bovine erythrocytes was synthesized and these probes were used in conjunction with *in situ* hybridization techniques to determine the cellular distribution of the transcripts for both proteins, and once again, the effects of acid-base disturbance on their degrees of expression and regulation. Specific hybridization signals were demonstrated in gill epithelial cells by all probes with particular patterns of expression. The H^+-ATPase mRNA expression was increased markedly in the gills of acidotic fish and was accompanied by a pronounced elevation in branchial H^+ excretion. The probe for the Band 3 mRNA also demonstrated a specific hybridization signal in gill epithelial cells which was greatly increased in the gills of alkalotic fish, which were excreting large amounts of HCO_3^-.

The results of this thesis provide direct evidence for the presence of an electrogenic H^+ pump located on the apical surface of the pavement cell which is actively up-regulated during periods of acidosis through increased transcription and translation of the protein as well as increased membrane association. Further evidence is provided for

the presence of Band 3 or a Band 3-like protein which is responsible for Cl^-/HCO_3^- exchange across the gill.

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CHAPTER 1

GENERAL INTRODUCTION

Freshwater (FW) fishes occupy diverse and often unstable habitats with respect to water chemistry. Thus many species have developed adaptive physiological mechanisms to regulate ionic, osmotic, and acid-base disturbances arising from environmental fluctuations. As a result, particular organs have evolved in connection with these mechanisms, such as the fish gill. Along with their role in respiratory gas exchange, the gills of teleosts are also the major sites of ion exchange with the external medium and are vital in acid-base regulation. Osmotic balance is achieved by the excretion of large volumes of dilute urine while a major contribution to ionic balance is attained by the absorption of Na^+ and Cl^- ions across the gill (Evans, 1993) in exchange for acidic and basic equivalents, respectively, as originally proposed by Krogh in 1938. Thus, acid-base status is simultaneously regulated via extrusion of protons and bicarbonate.

The multifunctional nature of the fish gill stems from its unique cellular structure coupled with its dynamic morphology. The actual surface area of the gills is extremely large in comparison to the body surface, a prerequisite for efficient gas transfer . During periods of increased oxygen demand, such as exercise, the functional surface area of the gills is actually augmented by increased blood pressure causing lamellar recruitment. Another similar, important dynamic feature of the gills, which shall be discussed in detail shortly, is the ability of the fish to manipulate anatomical and morphological characteristics of the gill epithelial cells as well as regulating the expression of particular cell types under various conditions. Following an acid-base perturbation, the requirement for internal homeostasis necessitates the implementation of compensatory mechanisms in response to changing physiological parameters. During a respiratory acidosis, for instance, the fish

experiences an acute lowering of blood pH outside the optimal range. In order to compensate for this, there is a net retention of base in the form of HCO_3^- owing to an increase in H^+ excretion to the external medium. It has been shown by Goss *et al.* (1992a) that during such an acidosis, there is a reduction in the number and exposed surface area of chloride cells (cc), apparently as a result of covering over by adjacent pavement cells (pc), thus reducing their exposed surface area and functional exchange surfaces while increasing that of pavement cells. Upon removal of the cause of respiratory acidosis, there is a transient but acute metabolic alkalosis accompanied by a reappearance of the chloride cells, presumably to clear the excess of base (HCO_3^-) which has accumulated to buffer the decreased pH (Goss *et al.*, 1992b). A similar response is seen when normal fish are exposed to an acute metabolic alkalosis. Presumably, these observed morphological changes in response to acid-base disturbances act to regulate ion translocation mechanisms at the cellular level, and it is the nature of these mechanisms that is addressed in this thesis.

EPITHELIAL CELL FUNCTION

Cl^-/HCO_3^- exchange

The gill epithelium consists of three predominant cell types: the mitochondria-dense chloride cells (cc) which have been implicated in hyperosmoregulation in freshwater fish (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989), pavement cells (pc) which are known to be responsible for transepithelial gas exchange, and mucus cells.

Much attention has been given to the chloride cell in past years (see reviews by: Karnaky *et al.*, 1977; Evans, 1979; Foskett et al., 1983; Degnan, 1984) and it has

generally been considered to be the major site of Na^+ and Cl^- uptake in FW teleosts, due to its morphological features. The chloride cell is a fairly large, round cell with a dense concentration of mitochondria, presumably to provide ATP for active ion transport, and an extensive tubular network associated with the basolateral membrane (Laurent and Dunel, 1980; Philpott, 1980). Ion specific translocating mechanisms have also been identified in the chloride cell, including the Na^+/K^+-ATPase (Sardet *et al.*, 1979; Philpott, 1980) and the Ca^{2+}-ATPase (reviewed by Fenwick, 1989). A study by Laurent and Perry (1990), suggested that the chloride cells are the site of Na^+ and Cl^- uptake. This was also based on observations that a correlation existed between gill ionic uptake rates and chloride cell fractional area (Perry and Laurent, 1989). Work by Perry and Goss (1992) also proposed a relationship between cc fractional area and ion uptake based on morphological data and measurement of ion fluxes. In light of evidence presented by Goss *et al.* (1992a,b) the role of cc's in transepithelial Na^+ transport has been disputed. During respiratory acidosis in brown bullheads (*Ictalurus nebulosus*), pc fractional area is increased, covering over the cc's and thereby functionally uncoupling them from the medium. There is also an associated decrease in Cl^- uptake and an increase in Na^+ uptake which would not be predicted if cc's were the site of H^+-Na^+ movement.

Na^+/H^+ movement

As was mentioned previously, the nature of ionic uptake across the FW fish gill has not been firmly established. In particular, the molecular mechanism(s) of Na^+ uptake are unresolved, the participating cell types are unknown, and the manner by which ionic

uptake is adjusted to regulate acid-base disturbances is unclear. The classical model for Na^+ uptake across the FW fish gill (Krogh, 1938) involves electroneutral Na^+/H^+ exchange on the apical plasma membranes of, as yet, unidentified gill epithelial cells. This theory was reinforced by findings reported by Wright and Wood (1985). Work by Lin and Randall (1991), however, showed no effect of amiloride, a blocker of Na^+/H^+ exchange, on net proton or ammonia excretion, indicating that there is no direct coupling of Na^+ influx and proton or ammonia excretion. On the basis of theory and pharmacological data, (Lin and Randall, 1991; Lin and Randall, 1993; Laurent *et al.*, 1994) there is considerable debate surrounding the classical model and an alternate model proposes that Na^+ uptake occurs through apical membrane Na^+ channels that are coupled to an electrogenic H^+ pump (Avella and Bornancin, 1989). These findings proposed the existence of an electrogenic proton pump or proton ATPase on the fish gill, similar to that found in mammalian kidney (Steinmetz, 1985), turtle urinary bladder (Al-Awqati, 1978), and frog skin (Ehrenfeld *et al.*, 1985). This proposed H^+ pump (Gluck, 1992; Gluck and Nelson, 1992) would be coupled to a Na^+ channel and generate an appropriate electrochemical gradient to allow passive movement of Na^+ through this channel into the cell. Recently, Lin *et al.* (1994) demonstrated H^+-ATPase immunoreactivity in the trout gill but did not clearly demonstrate involvement of a specific cell type. High magnification, transmission electron microscopy performed by Laurent *et al.* (1994) demonstrated the presence of vesicles and associated "studs" in the apical region of the pavement cell, reminiscent of those reported by Brown *et al.* (1987) in toad urinary bladder and in the mammalian kidney. In the same study by Laurent *et al.*, these vesicles were observed actively fusing

with the apical membrane of the pc. It would appear that these vesicles are responsible for the transport of the proton pump enzyme from the Golgi to the apical membrane for subsequent incorporation into the membrane during periods of acid-base disturbance. These morphological findings, along with correlative data presented by Goss *et al.* (1992), which is discussed further in chapter 2, as well as x-ray microanalysis studies (Morgan *et al.*, 1994), provides considerable indirect evidence that the pavement cell is involved in Na^+ and H^+ translocation

H^+-ATPases

In a variety of acid secreting epithelia including turtle urinary bladder (Gluck *et al.*, 1982a) mammalian collecting duct (Brown *et al.*, 1992), amphibian skin (Ehrenfeld *et al.*, 1985), and fish gill (Avella and Bornancin, 1989; Lin and Randall, 1991), a vacuolar type H^+-ATPase (V-ATPase or proton pump) has been implicated in both acid-base and ionic regulation. Typically, these epithelia are composed of two predominant cell types, one that contains abundant mitochondria (e.g. mitochondria-rich cells, intercalated cells, chloride cells) and one that contains few mitochondria (e.g. granular cells, principal cells, pavement cells). Although the cellular location of the apical membrane proton pump is generally believed to be the mitochondria-rich cell in non-piscine model systems, the situation is unclear in the fish gill model. Indeed, both the chloride cell (Avella and Bornancin, 1989; Lin and Randall, 1991) and pavement cell (Goss *et al.*, 1992a; Perry and Laurent, 1993; Laurent *et al.*, 1994) have been proposed as sites of apical membrane proton pump activity in the fish gill. The existence of an apical membrane proton pump in

the mitochondria-poor pavement cell of fish is in stark contrast to other acid secreting epithelia, yet is consistent with the profound morphological transformation of the pavement cell observed during acidosis in which mitochondrial density increases markedly.

Proton ATPases are generally divided into three classes of which the V-type or vacuolar ATPase (V-ATPase) is the most likely candidate to be considered in the fish gill. These proton ATPases are extremely common in eukaryotic cells and are located on the vacuolar membranes of all such cells as well as providing the major mechanism of maintenance of organelle pH. These enzymes also have the specific role of proton translocation in specialized acid-transporting cells such as in the case of renal intercalated cells (Brown *et al.*, 1987), which is why they have been implicated for a possible role in acid excretion at the fish gill. This H^+-ATPase is a hetero-oligomer (300 - 700 kDa) composed of the V_1 peripheral domain and the V_0 integral domain. V-ATPases have been well characterized in a variety of tissues, including bovine brain and kidney (Gluck *et al.*, 1987), and cDNA clones for three major subunits of the V-ATPase (31-, 56-, and 70-kDa) have been isolated and sequenced from bovine kidney. Each of the subunits demonstrates remarkable sequence homology in primary structure for several diverse species including *Neurospora crassa* (Bowman *et al.*, 1988), carrot (Zimniak *et al.*, 1988), and *Saccharomyces cerevisiae* (Hirata *et al.*, 1990), suggesting that specific reactivity of antibodies to these proteins in fish gill is quite likely. It is the sequence of the carboxy terminal peptide of the 31-kDa subunit of bovine H^+-ATPase which we have used to generate the antibody used for immunological detection of proton pump in this study.

ANION EXCHANGE PROTEINS

The Anion Exchanger (AE) gene family is a group of genes coding for several anion translocating proteins, all of which are highly homologous to erythrocytic Band 3 (AE1). The cDNA for Band 3 was originally cloned by Kopito and Lodish (1985) from the erythropoietic spleen cells of mouse which led to the discovery of the other related members of the AE family. Although Band 3 was once believed to exist exclusively in erythroid cells, it has been immunolocalized in several other tissues, most notably, kidney (Jennings *et al.*, 1985; Kopito *et al.*, 1988; Alper *et al.*, 1989), stomach (Kellokumpu *et al.*, 1988; Thomas *et al.*, 1989), and turtle bladder (Drenckhahn *et al.*, 1987). In each of these cases, the AE proteins are found associated with functionally analogous, acid-secreting cells although they are not necessarily coded for by the AE1 gene as is the case with the intercalated cells of mammalian kidney collecting duct, but rather the AE2 or AE3. The A-type intercalated cells of the mammalian kidney are a site of basolateral bicarbonate exchange through a Band 3 anion exchanger and while it has not been specifically identified (specific immunoreactivity as in the A-type cells has not been demonstrated) in the B-type intercalated cells, Band 3 is believed to be present as an apical anion exchanger. The kidney Band 3 is actually a truncated version of the mouse erythroid Band 3 with part of the N-terminus cytoplasmic domain at the 5' end missing. The presence of Band 3 in several diverse yet functionally similar tissues, along with current indirect evidence (Laurent *et al.*, 1985; Avella *et al.*, 1987; Perry and Laurent, 1987; Perry *et al.*, 1992), strongly supports the contention that Band 3 is involved in Cl^-

/HCO_3^- exchange at the fish gill, most likely associated with the apical membrane of the chloride cell.

OBJECTIVES OF THE STUDY

The purpose of this study involved several main objectives: i) to determine whether it is actually a H^+-ATPase involved in H^+ translocation in the gill; ii) to provide further evidence for a Band 3 or Band 3-like protein associated with Cl^-/HCO_3^- exchange; iii) to localize these ion transport proteins with respect to the particular cell(s) involved; iv) to determine whether these transport proteins are regulated during acid-base disturbances. In the subsequent chapters of this thesis, an array of immunocytochemical and in situ hybridization techniques have been utilized to provide unequivocal evidence for i) the existence of an apical membrane vacuolar proton pump in trout gill pavement cells and its modulation by internal acid-base status by an augmentation of gene expression; and ii) the presence of a functional transcript coding for the Band 3 protein in trout gill epithelial cells and its regulation during an acid-base disturbance.

CHAPTER 2

IMMUNOLOCALIZATION OF PROTON PUMPS (H^+-ATPASE) IN PAVEMENT CELLS OF RAINBOW TROUT GILL

INTRODUCTION

As explained in Chapter 1, in freshwater teleosts, Na^+ and Cl^- ions are absorbed across the gill epithelium from the dilute external environment. This serves to counterbalance the continual loss of these ions by diffusion or by renal excretion and thereby help to establish ionic balance (see review by Evans, 1993). There is a general consensus, arising from the results of numerous indirect studies, that Cl^- uptake is achieved by a Cl^-/HCO_3^- exchange mechanism located on the apical membranes of branchial chloride cells (for reviews see Goss *et al.* 1992b; Avella and Bornancin 1990; Perry and Laurent 1993; Shuttleworth 1989; McDonald *et al.* 1989). The nature of Na^+ uptake, however, is less certain and currently there is considerable debate surrounding its mechanism and cellular location. In recent years, the classical model incorporating an electroneutral Na^+/H^+ exchanger (Krogh 1938) has been challenged on the basis of i) thermodynamic criteria (Avella & Bornancin 1989), ii) analogy with other acid excreting "tight" epithelia (Goss *et al.* 1995), and iii) the results of several studies using an array of pharmacological (Lin & Randall 1991), biochemical (Lin & Randall 1993) and morphological (Lin & Randall 1994; Laurent *et al.* 1994) techniques. The new model of Na^+ uptake across the gill epithelium of freshwater fish incorporates an electrogenic apical membrane proton pump that establishes a favorable electrochemical gradient for the inward movement of Na^+ through apical membrane Na^+ channels. The most compelling evidence for this model emanates from the recent work of Lin *et al.* (1994) in which

proton pump (vacuolar type H^+-ATPase) immunoreactivity was clearly demonstrated in the gill of rainbow trout. However, the branchial cell type in which the proton pump/Na^+ channel is localized is less obvious and is currently debated. Lin and Randall (1991) proposed the mitochondria-rich chloride cell as the site of the proton pump/Na^+ channel mechanism yet recent immunocytochemical results (Lin & Randall 1994) suggest distribution of proton pumps in both chloride cells and pavement cells. The problematic resolution of the light microscopy techniques employed by Lin & Randall (1994), however, did not permit precise identification of the immunoreactive cells. Laurent *et al.* (1994), employing transmission electron microscopy, identified presumptive proton pump vesicles that were located almost exclusively in pavement cells. Furthermore, Morgan *et al.* (1994) concluded on the basis of x-ray microanalysis that the pavement cell is the site of the sodium uptake mechanism in brown trout (*Salmo trutta*). These results concur with the findings of Goss *et al.* (1992a) who observed physical covering of gill chloride cells by pavement cells in hypercapnic brown bullhead (*Ictalurus nebulosus*), at times of stimulated acid excretion and Na^+ uptake. Owing to the absence of any correlation between Na^+ uptake and chloride cell surface area in hypercapnic fish, Goss *et al.* (1992a) postulated that the pavement cell was involved in Na^+ absorption and H^+ excretion.

With this general background, the primary goals of the present study were i) to provide direct evidence for the localization of the proton pump in the pavement cell of the rainbow trout gill and ii) to establish further its role in acid-base balance. These goals were accomplished by evaluating the gills of normocapnic and hypercapnic fish using

immunological techniques in conjunction with laser scanning confocal, and electron microscopy.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout, *Oncorhynchus mykiss*, weighing 245-310 g were obtained from Linwood Acres trout farm, (Cambellcroft, Ontario) and held in large (300 L) fibreglass tanks supplied with flowing, dechlorinated City of Ottawa tap water (PO_2 = 20.0-21.3 kPa, PCO_2 = 0.033-0.047 kPa, $[Na^+]$ = 0.15mmol/l, $[Cl^-]$ = 0.18 mmol/l, $[Ca^{2+}]$ = 0.42 mmol/1, Temp. = 10 °C). All fish were maintained in the aquarium for a minimum of four weeks prior to experimentation and fed to satiation on alternate days with a commercial salmonid diet. They were not fed 24 h prior to experimentation. The photoperiod was held at 12 h dark/12 h light.

Surgical Procedures

Fish were anesthetized in a 1:10000 (w/v) solution of MS 222 (Sigma) buffered to pH 7.0 with $NaHCO_3$. To allow blood sampling, indwelling dorsal aortic (DA) cannulae were surgically implanted according to the procedure of Soivio *et al.* (1975). Fish were placed into individual, opaque acrylic boxes (approx. vol. = 3 L) and allowed to recover for at least 24 h prior to experimentation.

Hypercapnia

Five periods were examined in the present study: Control (pre-hypercapnia), 12, 18, 24, 36 and 48 h of hypercapnia (1% CO_2 in air; P_wCO_2 = 1.01 kPa). In order to induce respiratory acidosis, the inflowing water was rendered hypercapnic by bubbling a mixture of CO_2 in air (Wösthoff gas mixing pump) through a gas equilibration column (Perry *et al.* 1987a,b) to obtain a final $PwCO_2$ of 1.01 kPa. $PwCO_2$ was monitored continuously by circulating inflowing water through a PCO_2 electrode (E5037; Radiometer) connected to a meter (PHM 72; Radiometer) and chart recorder. The final 3 h of each exposure period was used as an ion flux period during which water flow was stopped and hypercapnia was maintained by bubbling 1% CO_2 in air (commercial gas cylinders) directly into individual boxes.

Acid-Base Parameters

Upon concluding each of the flux periods, a 1 mL blood sample was taken from the DA cannula of each fish for immediate analysis of acid-base variables. A 10 mL water sample also was taken from each box at the beginning and end of each flux period for measurements of titratable alkalinity (TA) and total [ammonia] ([Amm]). Whole blood pH was determined with a microcapillary electrode (Radiometer G299A) in conjunction with a Radiometer PHM 71 acid-base analyzer and a BMS3 Mk2 Blood Microsystem thermostated to 10° C. Blood was centrifuged (12000 g) for 30 s and plasma total CO_2 was measured on 50 uL samples using a total CO_2 analyzer (Corning Model 965). Plasma

bicarbonate (HCO_3^-) concentrations were calculated from the total CO_2 and pH measurements according to the Henderson-Hasselbalch equation and appropriate constants (Boutilier *et al.* 1984). Water [Amm] was determined colorometrically by the salicylate-hypochlorite method (Verdouw *et al.* 1978) and TA and net acid fluxes were determined as described by McDonald and Wood (1981).

Tissue Preparation

At the end of each experimental period, the second and third gill arches from the right and left gills were removed from control and hypercapnic fish and immediately emersion-fixed for immunocytochemistry (ICC) using light or electron microscopy. In total, the gills of 12-16 fish were examined for each period of hypercapnia or normocapnia. Upon close examination, it was determined that gills from the 18 h hypercapnia exhibited the most intense immunoreactive signal, therefore four additional groups (36 fish) were exposed to 18 h of hypercapnia. For light microscopy, the tissue was rinsed briefly in cold phosphate buffered saline (PBS) and then placed in Bouin's fixative for 2 h, followed by three X 10 min washes in cold PBS and finally 24 h of fixation in Bouin's without acetic acid. For electron microscopy, the tissue was rinsed briefly in cold PBS and immersed for 24 h in a fixative designed for immunoelectron microscopy (4% paraformaldehyde, 0.1% gluteraldehyde, 0.1% picric acid in PBS). All tissues then were dehydrated in a series of ethanols (12 h in 70% EtOH, 2 h in 90% EtOH, 2 h in 95% EtOH, 2 X 2 h in 99% EtOH). Tissue for light microscopy was trimmed to

remove any bone and a portion of arch with 7-12 lamellae was obtained. These were placed in xylene (2 X 1 h) and then in paraffin (Ameraffin, Baxter Diagnostics Inc.) at 60° C for 12 h to allow infiltration before embedding in paraffin blocks. For immunoelectron microscopy, individual lamellae were removed from gill arches and trimmed to a length of approximately 2 mm. This tissue was placed in a solution of 50% EtOH/ 50 % LR White acrylic resin (J.B.EM Services Inc.) for 2 h and then transferred to LR White (a water soluble embedding medium) for 2 h. Finally, the pieces were embedded in LR White in gelatin capsules (No.00, T.U.B. Enterprises) and allowed to cure overnight at 60° C.

Immunological Techniques

An antiserum was raised in rabbits against the carboxy-terminal peptide of the 31-kDa subunit of the bovine renal V-type ATPase (**Cys - Gly - Ala - Asn - Ala - Asn - Arg - Lys - Phe - Leu - Asp**; Hirsch *et al.* 1988). Pre-immune blood samples were drawn from 2 rabbits and the serum was collected, frozen, and stored at -80° C. Rabbits were inoculated with the peptide antigen and blood samples were drawn after 6 weeks. Serum was frozen and stored at -80° C. Rabbits were then boosted with another inoculation and a second bleed was performed after 6 weeks. When tested on rat kidney, the serum obtained from the second bleed resulted in the strongest immunoreactive signal (Y. Okawara, personal communication). Sections (7 μm) were cut from paraffin blocks using a microtome, mounted on glass slides, and allowed to dry on a slide warmer for at least 6 h. Sections were incubated overnight at room temperature with primary antiserum diluted

1:500 in Tris buffer (0.1 M Tris and 0.14 M NaCl) containing 0.6% carrageenan and 0.3% Triton X-100 (TCT). After washes in Tris buffer (2 x 7 min), sections were incubated with biotinylated anti-rabbit IgG (secondary antibody) diluted 1:50 with TCT for 30 min. Following another series of washes, final labeling was performed with a 30 min incubation in Streptavadin-CY3 (Sigma) diluted 1:50 in TCT. Positive immunoreactivity was visualized using fluorescence microscopy and confocal microscopy. Confocal images were generated by an upright, confocal, laser scanning microscope (Leica) using an air-cooled, krypton/argon laser. Specific immunoreactivity (Steptavidin-CY3 conjugate, Sigma) was visualized following excitation at a green wavelength (545 ± 20 nm). In order to visualize the entire gill tissue, images were generated from excitation at a red wavelength (580 ± 34 nm) which caused autofluorescence of the tissue. To ensure valid comparisons between fish and treatment groups, all settings for the confocal microscope and laser were kept constant.

Thin sections (50-60 nm) for immunoelectron microscopy were cut using an ultramicrotome and collected on nickel grids. Sections were incubated overnight in antiserum diluted 1:500 in TBS-Tween (Tris buffered saline and Tween). Sections were then washed in TBS (3 x 5 min) and incubated with biotinylated anti-rabbit secondary antibody as above. Following another series of TBS washes, sections were incubated for 30 min in either Streptavadin-5 nm or-20 nm colloidal gold conjugate (Sigma). Sections were then briefly incubated with uranyl acetate (15 min) and lead citrate (7 min) in order to stain cellular structures. Visualization was achieved using transmission electron

microscopy. Control sections for immunocytochemistry using light or electron microscopy were processed using secondary antibody only (primary antiserum was replaced with buffer), pre-immune serum in place of antiserum or primary antiserum which had been pre-incubated overnight with antigen (50 X excess antigen).

Image Processing

All color images were exported from the confocal microscope as TIFF (Tagged Image File Format) files and imported into a commercial software package (Adobe Photoshop). All images were treated in an identical manner, using minimal processing to enhance overall brightness and contrast. Finished images were pasted into Powerpoint (Microsoft) for final cropping, arrangement, and labeling before printing.

Western Blotting

Fish were killed by a blow to the head. A cannula was inserted into the bulbus arteriosus and the gills were perfused *in situ* for approximately 3 min with ice-cold PBS. After the gills were cleared of blood, the epithelial tissue was scraped from gill arches with a single edged razor blade and collected in 0.75 - 1.0 ml of lysis buffer containing protease inhibitors. Samples were boiled in 1.5 ml micro-centrifuge tubes for 5 min and the tissue was homogenized with a 23 gauge needle and 1 ml syringe. Samples were centrifuged for 10 min at 9000 g and the supernatant was collected and stored in 100 µl aliquots at -80°C. Total protein in the samples was determined by the bicinchonic acid

(BCA) method (Pierce). Electrophoresis was carried out on a vertical mini-gel apparatus (Bio-Rad) according to the procedure of Laemmli (1970) with a 12% running gel. Tissue homogenates were diluted 1:1 with sample buffer and boiled for 5 min. Appropriate volumes of sample were loaded into each well to yield protein concentrations of 25 µg/well. Gels were run for 90 min at constant voltage (90 v) and gels were equilibrated in transfer buffer for 30 min. Proteins were transferred to nitrocellulose using a mini Trans-Blot Cell (Bio-Rad) at a constant voltage (100 v) for 1 h. Membranes were blocked overnight with PBS containing 5% skimmed milk. Membranes were washed three time in PBS and incubated for 90 min with a 1:2000 dilution of primary H^+-ATPase antiserum at room temperature. Membranes were then washed three times in PBS and incubated for 1 h in a 1:2000 dilution of biotinylated sheep anti-rabbit IgG (Sigma) at room temperature. Next, membranes were washed three times in PBS and incubated for 1 h in a 1:5000 dilution of streptavadin-HRP conjugate. Finally, membranes were washed three times in PBS and immunoreactive bands were visualized using ECL Western blotting protocol on Hyperfilm-ECL (Amersham).

Statistical analysis

Data shown in figure 2.1 are means ± 1 standard error of the mean. Differences between the control and experimental fish were established using a two-tailed Student's t-test; the limit of significance was set at 5%. Differences between experimental and pre-exposure (pre) values were established using a 2 way analysis of variance (ANOVA).

RESULTS

Acid-Base Parameters

Exposure to hypercapnia caused a significant, but transitory, reduction in arterial blood pH (figure 2.1a). Blood pH was reduced by approximately 0.2 units after 12 - 18 h but had returned to control values within 24 h. Plasma bicarbonate concentration displayed an immediate and continuous increase during the initial 36 h of hypercapnia and then stabilized (figure 2.1b). There was a pronounced increase in net H^+ excretion after 18 h of hypercapnia as shown in figure 2.1c; net H^+ excretion remained elevated throughout the 48 h period of hypercapnia. The control fish displayed no changes in any measured variable (figure 2.1a,b,c).

Immunocytochemistry

Positive immunoreactive labeling for H^+-ATPase was clearly evident in the gills of both normocapnic and hypercapnic fish and in both situations the labeling was localized to cells of the lamellar epithelium (figure 2.2). The intensity of labeling and the number of H^+-ATPase immunoreactive cells was markedly increased during hypercapnia (e.g. compare figure 2.2a and c). Maximal labeling was achieved after 18 h of hypercapnia and this corresponded to the period of maximal net branchial H^+ excretion (figure 2.1c). Thus, while gill tissues from all time periods were examined, this study focuses on comparisons between 18 h of normocapnia and 18 h of hypercapnia.

Figure 2.3 illustrates at higher magnification the pattern of proton pump immunolabeling in hypercapnic fish. While specific labeling was apparent on the majority of lamellar epithelial cells, a sub-population of these cells displayed intense H^+-ATPase immunoreactivity. Specific labeling was abolished by incubating the gills of hypercapnic fish with pre-immune serum rather than antiserum (figure 2.3c, d) or by incubating with secondary antibody, alone (figure 2.3e, f).

Figures 2.4 and 2.5 demonstrate that the H^+-ATPase immunoreactivity was localized to apical (water-facing) regions of lamellar epithelial cells. The intense thin band of immunolabeling apparent in figure 2.4b is reminiscent of the appearance of the pavement cell apical membrane and cytoplasm with respect to the flat, elongated morphology often observed in these cells. Serial optical sections through a single immunoreactive lamellar epithelial cell are shown in figure 2.5. These micrographs provide a partial three-dimensional representation of the distribution of H^+-ATPase labeling within a cell. An optical section from the edge of the cell shows consistent labeling throughout the cell which represents a cut through and on a tangent to the exposed membrane where the labeling is most concentrated. Further sections exhibit a ring of intense labeling associated with the apical membrane with a distinct punctate distribution suggestive of the presence of sub-apical cytoplasmic vesicles. No immunoreactivity was evident in the basolateral regions of the cell.

Immunoelectron microscopy

Transmission electron micrographs clearly showed positive H^+-ATPase immunoreactivity localized within cells in the gills of acidotic fish (figures 2.6 - 2.8). Immunogold labeling of localized sites was present in pavement cells, particularly associated with the apical membrane and also the apical region of the cytoplasm (figures 2.6 and 2.8). The pavement cells of normocapnic fish expressed greatly reduced or no immunogold labeling of H^+-ATPase in any region of the cell (figure 2.6c). It is likely that the total absence of labeling in the gills of normocapnic fish, in part, reflected the very thin EM sections (0.1% of the thickness of the paraffin sections used for light microscopy).

Close examination of the apical region of pavement cells from hypercapnic fish (figures 2.6a and 2.8) not only revealed labeling on the apical membrane, but also distinct concentrations of gold particles in the cytoplasm and in association with the presumptive Golgi apparatus. Further, there was marked immunoreactivity in the proximity of vesicles which appear as electron lucent "voids" in the micrographs (figures 2.6a, 2.8). In order to optimize immunolabeling, fixation with osmium tetroxide could not be performed and thus the visualization of cell membranes was impaired. Immunolabeling was essentially abolished when sections were incubated with secondary antibody only (figure 2.7a), pre-immune serum (figure 2.7b), or primary antiserum, pre-incubated with excess antigen (figure 2.7c).

Figure 2.6a is a high magnification TEM showing a pavement cell overlying a chloride cell. In this particular section, positive immunoreactivity was restricted to the

pavement cell with no apparent labeling within the chloride cell. This pattern of preferential immunoreactivity in pavement cells was observed on numerous occasions.

Western blotting

Western blots of gill tissue from hypercapnic and normocapnic fish resulted in major immunoreactive bands at 31 kDa (figure 2.9) when incubated with primary antiserum. The immunoreactive band for the hypercapnic fish appeared more intense than that of the normocapnic fish, but both bands for hypercapnic and normocapnic displayed the same molecular weight. No immunoreactive bands were present on the blots incubated with pre-immune serum in place of antiserum.

Figure 2.1. The temporal effects of hypercapnia on blood pH (a); plasma bicarbonate concentration ($[HCO_3^-]$) (b); and branchial net acid excretion ($J_{net}\ H^+$) (c) in control (filled circles) and hypercapnic (filled squares) fish. In (c) positive values indicate acid equivalent uptake (base excretion) and negative values indicate acid equivalent excretion. * denotes a significant difference from control values. † indicates a significant difference from pre-values within the same group. Values shown are means ± 1 standard error of the mean.

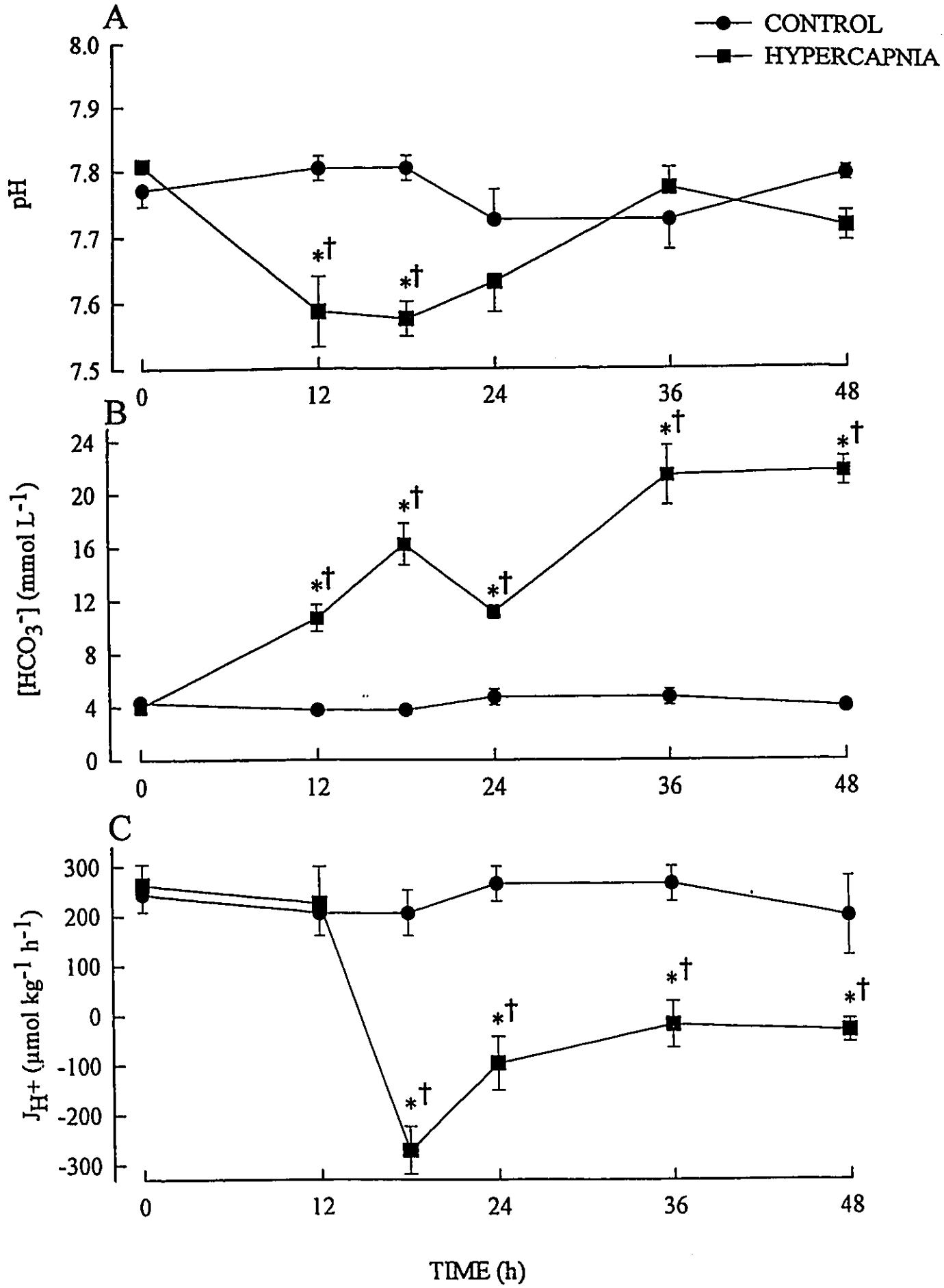

A
CONTROL
HYPERCAPNIA
pH
8.0
7.9
7.8
7.7
7.6
7.5
*†
*†
0
12
24
36
48
B
[HCO3-] (mmol L-1)
24
20
16
12
8
4
0
*†
*†
*†
*†
*†
0
12
24
36
48
C
JH+ (µmol kg-1 h-1)
300
200
100
0
-100
-200
-300
*†
*†
*†
*†
0
12
24
36
48
TIME (h)

Figure 2.2. Confocal micrographs of gills from normocapnic (a,b) and 18 h hypercapnic (c,d) trout showing specific immunoreactivity for the V-ATPase antibody (arrows). Note the increase in positive immunoreactivity both in intensity and lamellar distribution in the gills of the hypercapnic fish. Figures a and c were generated by scanning with an excitation wavelength of 545 ± 20nm. Figures b and d make use of the autofluorescence properties of the tissue at an excitation wavelength of 580 ± 34nm (shown as green). The immunoreactive signal in figures b and d (arrows) is created by superimposing the first images onto this one. Bar = 50 μm.

fig 2

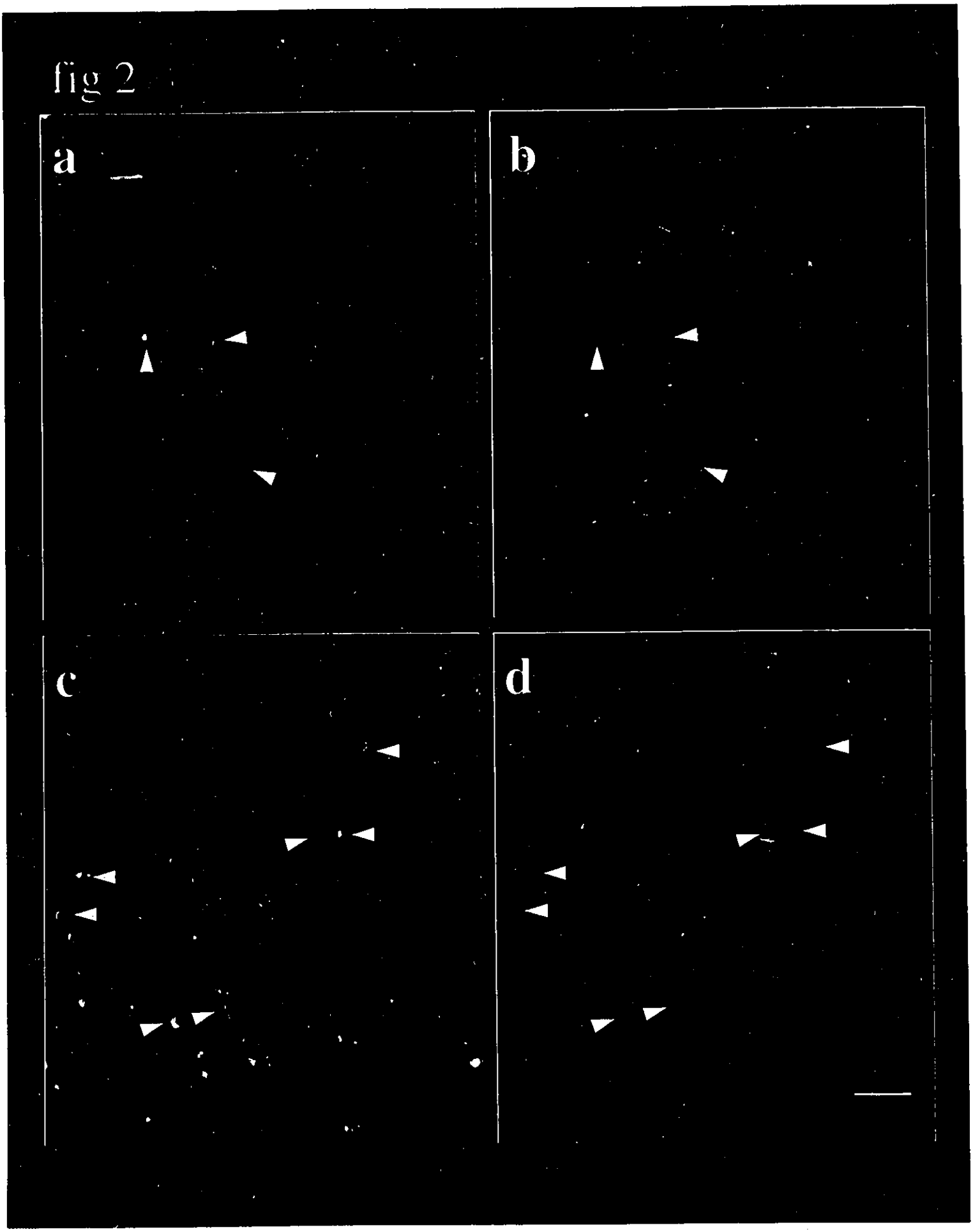


Figure 2.3. Confocal micrographs of gills from hypercapnic fish. Figures a and b show a gill section which was incubated with antiserum and immunopositive cells can be seen distributed over the lamellar surface (arrows). Figures c and d show a gill section which was treated with pre-immune serum in place of antiserum. Specific immunoreactivity has been abolished. Figures e and f show a gill section in which primary antiserum was replaced with buffer and incubation was with secondary antibody only. Again, no positive immunoreactivity is detectable. Images were obtained as described in figure 2.

Bar = 15 μm.

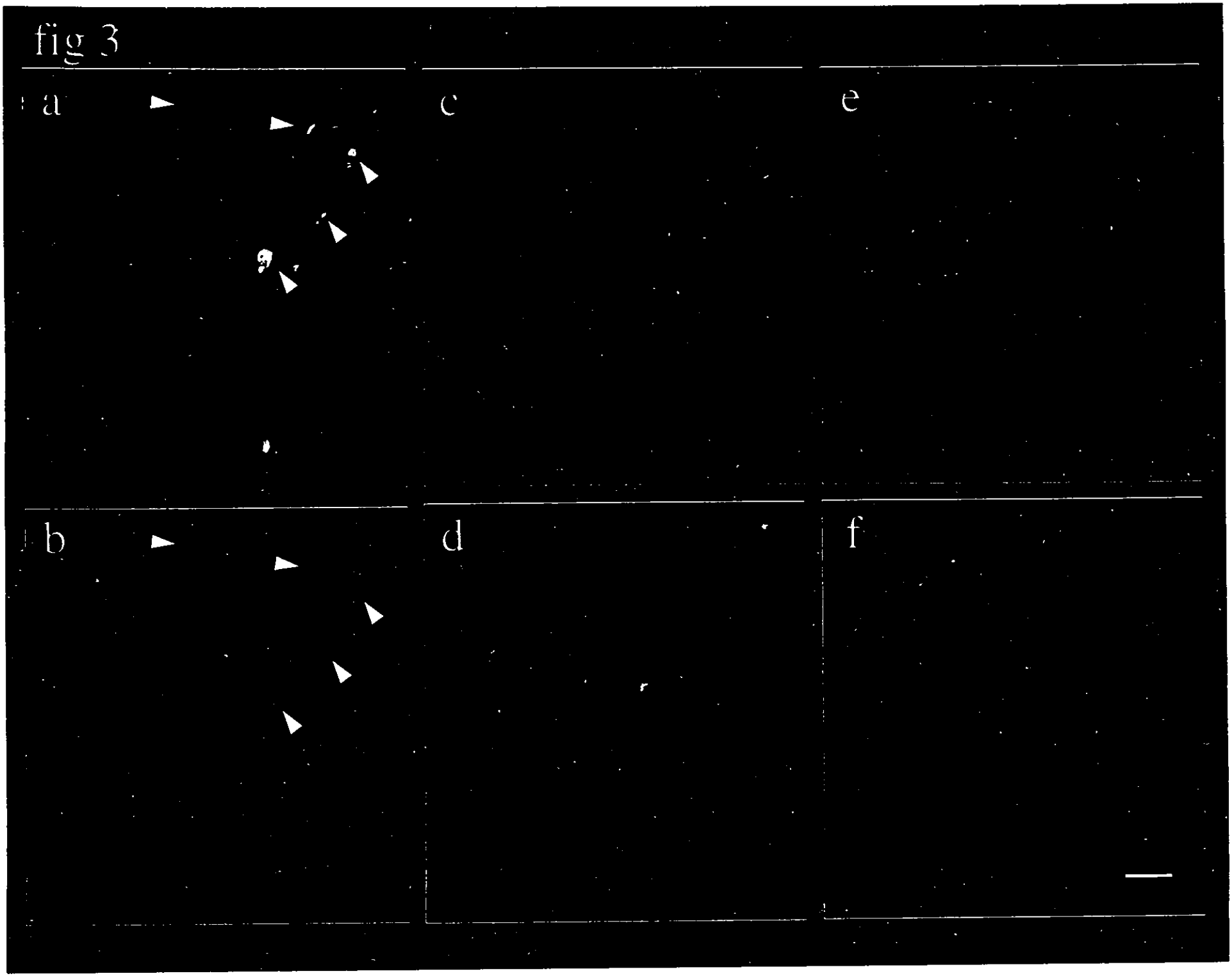


fig 3
a
c
e
b
d
f

Figure 2.4. High magnification confocal micrographs showing positive immunoreactivity associated with epithelial cells. The positive signal is apparently localized to the apical regions of the cells. Bar = 15 μm.

fig 4

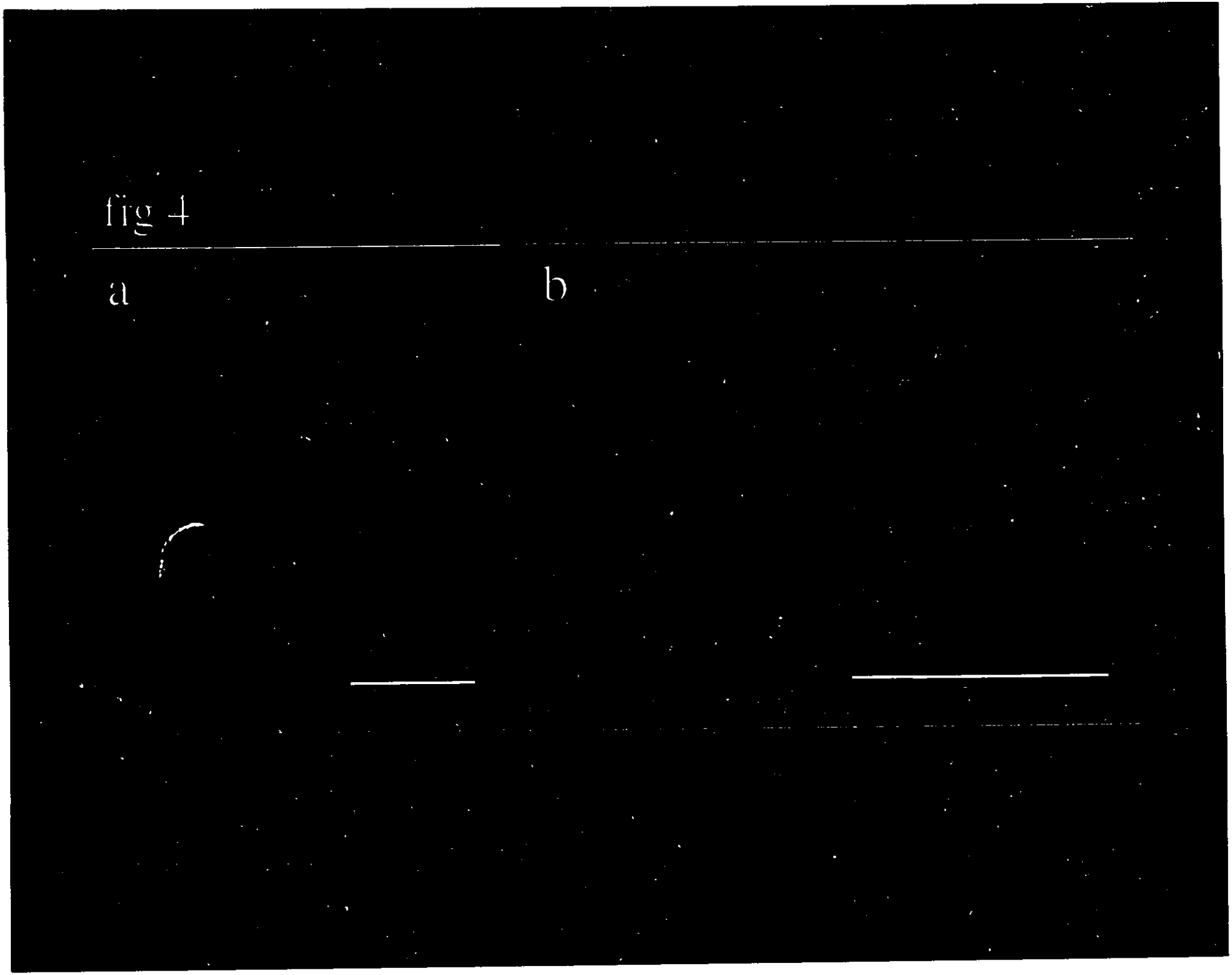


Figure 2.5. A series of confocal, optical sections made through one cell from the lamella of a fish exposed to 18 h of hypercapnia. Optical sections were made by scanning the section at increasing depths from the apex of the cell to a plane at approximately the middle of the cell. Yellow arrows indicate immunoreactivity associated with the outer apical membrane. White arrows indicate immunoreactivity associated with cytoplasmic vesicles. The attached diagram (a) represents the orientation of the planes of section. Bar = 8μm.

fig 5

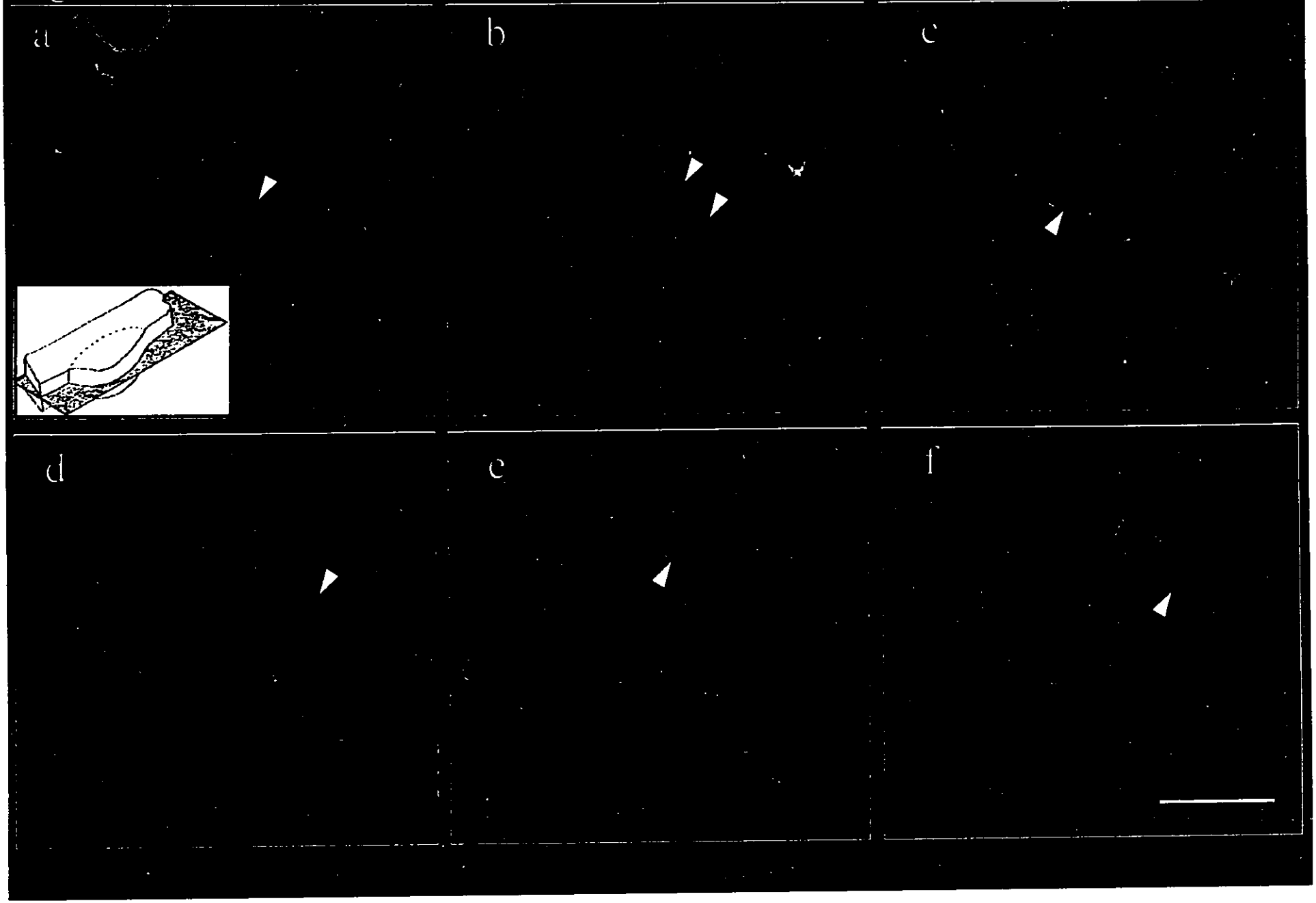


Figure 2.6. Transmission electron micrographs of gill sections from fish incubated with proton pump antiserum. (a) A pavement cell (pc) and a chloride cell (cc) from a fish exposed to 18 h of hypercapnia. Note the positive immunoreactivity (5 nm gold depicted by arrows) associated with the pc in the vicinity of the apical membrane as well as the Golgi complex(G) and cytoplasmic vesicles (v). n = nucleus. (b) An adjacent cc and pc from a normocapnic fish. Note the absence of any immunoreactivity. Bar = 500nm.

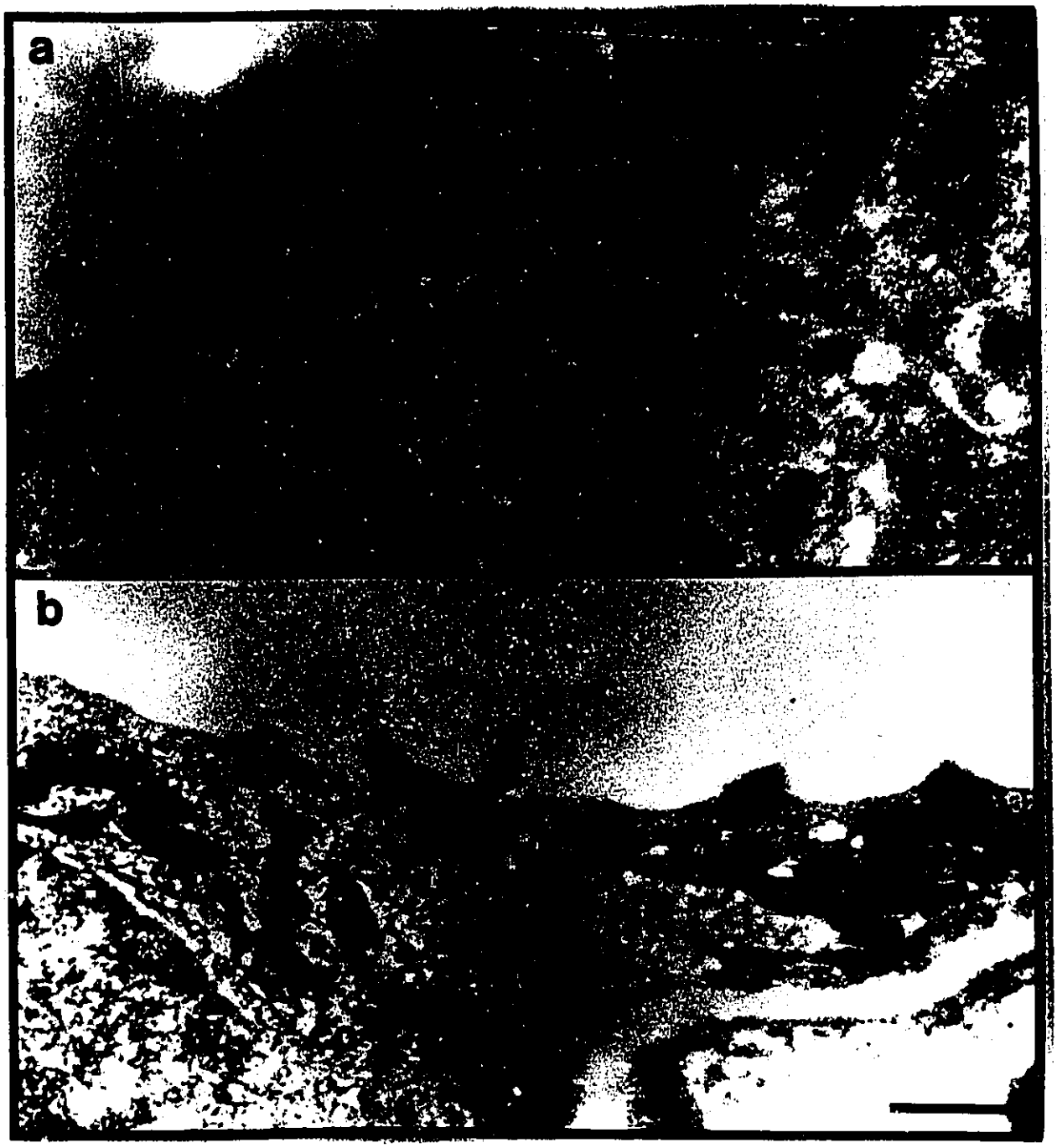

a
b

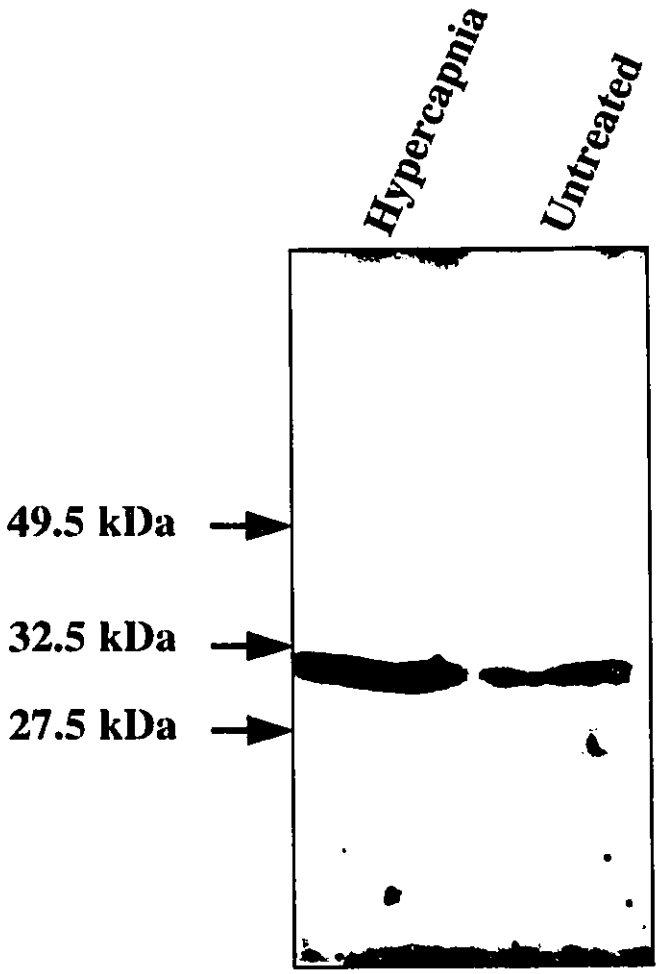
Figure 2.7. Electron micrographs of control gills from 18 h hypercapnic fish. (a) The primary antiserum was pre-incubated with excess antigen. (b) The primary antiserum was replaced with pre-immune serum. (c) The primary antiserum was replaced with buffer. (a) Bar = 1 μm. (b) and (c) Bar = 500nm.

a
b
c

Figure 2.8. (a) A pc displaying immunoreactivity (5 nm gold depicted by arrows) associated with the apical membrane as well as cytoplasmic vesicles (v) and Golgi complex (G). Vesicles appear to be in the process of migrating from the Golgi to the apical membrane. (b) Apical region of a single pc after 18 h of hypercapnia. Note immunoreactivity associated with the apical membrane and apical regions of the cytoplasm (20 nm gold depicted by arrows).


b
a
v
v

Figure 2.9. A Western blot of tissue from the gills of hypercapnic and normocapnic fish. The blot was incubated with primary antiserum and immunoreactive bands are evident just below 32.5 kDa. A greater degree of immunoreactivity is associated with the band for the hypercapnic fish, suggesting a greater expression of the protein in this tissue.


Hypercapnia
Untreated
49.5 kDa
32.5 kDa
27.5 kDa

DISCUSSION

This is only the second study to demonstrate proton pump (H^+-ATPase) immunoreactivity in fish gill (see also Lin *et al.*,1994) and is the first study to provide direct evidence that the proton pump is located in lamellar pavement cells. Furthermore, the simultaneous assessment of branchial net acid excretion and proton pump immunoreactivity during hypercapnia that was employed in the present study has clearly identified "up regulation" of the proton pump as an important mechanism of acid-base regulation during respiratory acidosis.

Currently, homologous antibodies to fish gill proton pump sub-units are unavailable. Thus, as in the study of Lin *et al.* (1994), non-homologous antibodies were used for immunolocalization. The polyclonal antibody used in the present study was raised in rabbit against a synthetic peptide (CGANANRKFLD) which is the carboxy-terminal 11 residues of the predicted 226 amino acid sequence of the 31 kDa sub-unit of bovine H^+-ATPase (Hirsch *et al.*, 1988). Preliminary experiments established that this antibody recognizes the mammalian proton pump antigen based on positive specific immunoreactivity in the intercalated cells of rat kidney (Y. Okawara, personal communication) and thus it was deemed suitable for testing in the fish gill. The specificity in the fish gill was established by comparing labeling patterns in a number of control situations including pre-absorption of the antibody with excess antigen, application of

secondary antibody alone, and use of pre-immune serum instead of antiserum. Additionally, Western blotting revealed a single immunoreactive band corresponding to a molecular weight of 31 kDa (see figure 8). These experiments clearly established the specificity of the antibody and together with the apical polarization of the labeling and the obvious correlation between the distribution and intensity of immunoreactivity with branchial net acid excretion, provide convincing evidence that the antibody is recognizing trout proton pump antigenicity. Finally, the amino acid sequence of the same 31 kDa bovine sub-unit was used to generate an oligonucleotide mRNA probe that also specifically labeled rat kidney intercalated cells (Fryer *et al.* 1994) and trout lamellar epithelial cells . Thus, we agree with Lin *et al.* (1994) that the use of a non-homologous antibody may be used to localize fish H^+-ATPase when appropriate control experiments have been performed for appropriate validation.

The presence of proton pump immunoreactivity in the trout gill supports the alternate model of Na^+ uptake across the gills of freshwater fish (Avella and Bornancin 1989) which incorporates inward Na^+ movement through apical membrane Na^+ channels. In this scheme, the electrochemical gradient for inward Na^+ movement is, in part, provided by electrogenic outward H^+ pumping. Although the existence of proton pump in trout gill supports the notion of coupled H^+ pumping and diffusive Na^+ influx, it does not preclude the existence of electroneutral Na^+/H^+ exchange that has been advocated in the classical model of Na^+ uptake in freshwater fishes (Krogh 1938). However, given the low concentration of Na^+ in freshwater (e.g. 0.15 mmol l^{-1} in City of Ottawa tapwater) and the

relatively high concentration of Na^+ in pavement cells (e.g. 60 mmol l^{-1} in pavement cells of brown trout, *Salmo trutta*; Morgan *et al.* 1994), an electroneutral Na^+/H^+ exchanger seems unlikely. Such a scheme is more likely in the gills of seawater adapted fishes in which the concentration of external Na^+ is high (approximately 450 mmol l^{-1}). Indeed, Lin *et al.* (1994) reported a significant reduction in proton pump immunoreactivity in the gills of seawater adapted rainbow trout.

On the basis of the measured Na^+ ion concentration in the pavement cells of brown trout, Morgan *et al.* (1994) estimated that an apical membrane potential of about 130 mV (inside negative) would be required to allow net uptake of Na^+ from dilute freshwater (0.1 mmol l^{-1}) through apical membrane Na^+ channels. It is unknown whether such large potentials could be generated across the apical membrane. On the other hand, the technique utilized by Morgan *et al.* (1994) could not distinguish between free and bound intracellular Na^+ and thus the actual intracellular activities of Na^+ conceivably could have been markedly lower. If so, the required apical membrane potential would also be lowered.

In teleost fish, respiratory acidosis is regulated metabolically by the gradual accumulation of bicarbonate within the extracellular fluid compartments (for reviews see Cameron 1978; Randall *et al.* 1982; Heisler 1984). Such a compensatory mechanism was clearly evident in the present study. The accumulation of bicarbonate is achieved predominantly by an increase in branchial acid excretion and to a lesser extent, increased renal acid excretion (e.g. Perry *et al.* 1987a, b). Previous research has identified two

mechanisms that contribute to the increased branchial acid excretion during respiratory acidosis, a reduced rate of Cl^-/HCO_3^- exchange and an accelerated rate of Na^+/H^+ exchange (for reviews see Cameron 1989; Cameron & Iwama 1989; McDonald *et al.* 1989; Wood & Goss 1990; Goss *et al.* 1992b). Recently, a series of studies employing morphological techniques have provided evidence that the mechanism of reduced Cl^-/HCO_3^- exchange during respiratory acidosis is the physical covering of chloride cells by neighboring pavement cells (Goss *et al.* 1992; Goss & Perry 1993; Goss *et al.* 1994a, b). This is believed to uncouple Cl^-/HCO_3^- exchange sites (on chloride cell apical membranes) from the water and thus constrain Cl^-/HCO_3^- exchange (see reviews by Goss *et al.* 1992b; Perry & Laurent 1993). Conversely, during alkalosis, the surface area of exposed chloride cells increases and this is thought to contribute to the compensatory increase in Cl^-/HCO_3^- exchange that is observed at such times (Goss & Perry 1994; Perry & Goss 1994).

In the light of the recent empirical evidence and theoretical considerations (see above), stimulation of Na^+/H^+ exchange is unlikely to contribute significantly, if at all, to enhanced branchial acid excretion during acidosis. On the other hand, the obvious correlation between proton pump immunoreactivity and net acid excretion observed in this study suggests that electrogenic proton pumping is an important mechanism of branchial net acid excretion. Thus, the increase in Na^+ uptake that often accompanies respiratory acidosis may reflect indirect coupling via the proton pump/Na^+ channel mechanism rather than direct coupling via Na^+/H^+ exchange (see also Potts 1994).

The distinct apical polarization of proton pump immunoreactivity in trout gill and its apparent localization in sub-apical cytoplasmic vesicles is reminiscent of the situation in other acid-secreting epithelia in which vesicles containing proton pumps fuse with the apical plasma membrane during acidosis to form active domains of intense proton pump activity (Gluck *et al.* 1982b; Brown *et al.* 1992; Gluck & Nelson 1992). In addition to the possible cellular redistribution of proton pumps, the increased immunoreactivity during hypercapnic acidosis was, at least in part, a result of increased proton pump synthesis as revealed by the Western blots. Recently, using *in situ* hybridization, we showed an increase in the expression of the mRNA for the 31 kDa subunit of the proton pump in hypercapnic trout (G. Sullivan, J. Fryer, S. Perry, unpublished results) to further support the notion of increased proton pump synthesis during respiratory acidosis.

The presence of a proton pump in the trout gill pavement cell is in stark contrast to other acid excreting epithelia such as frog skin (Ehrenfeld *et al.* 1985), turtle urinary bladder (Gluck *et al.* 1982a) and mammalian renal collecting duct (Brown *et al.* 1992). In these epithelia, the proton pump is located exclusively in mitochondria-rich cells that are structurally analogous to the fish gill chloride cell. However, the finding of proton pump immunoreactivity in the trout pavement cell was expected given the results of several previous indirect studies. In particular, Goss *et al.* (1992a, 1994a) observed that the gill pavement cell of brown bullhead (*Ictalurus nebulosus*) undergoes a pronounced morphological transformation during hypercapnic acidosis in which the numbers of mitochondria and surface area of apical membrane microvilli increase markedly. These

changes occur concomitantly with increased branchial acid excretion and Na^+ uptake. More recently, Laurent *et al.* (1994) reported the occurrence of vesicles in brown bullhead pavement cells that were similar in structure to the proton pump vesicles reported in other acid secreting epithelia (e.g. Brown *et al.* 1987). Moreover, the numbers of cytoplasmic vesicles and the occurrence of pear-shaped pits (indicative of vesicle fusion with the plasma membrane) were increased during hypercapnia (Laurent *et al.* 1994). Proton pump vesicles and pear-shaped pits were not observed in chloride cells. Thus, the increased surface area of pavement cell apical membrane microvilli during hypercapnia noted by Goss *et al.* (1992a) may represent insertion of proton pump vesicles into the apical plasma membrane while the increased numbers of mitochondria presumably correspond to the increased energetic requirements of pavement cell proton pump activity. Acid excretion by proton pump activity requires the presence of carbonic anhydrase and indeed the fish gill pavement cell is known to contain abundant carbonic anhydrase activity (Rahim *et al.* 1988). Clearly, in light of the current study and other recent evidence (Goss *et al.* 1992a; Laurent *et al.* 1994), the traditional view that the fish gill pavement cell is involved only in respiratory gas transfer is probably incorrect. Indeed, Potts (1994), in a recent editorial review hypothesized that Na^+ uptake is confined to the pavement cells while Cl^- uptake occurs in the chloride (mitochondria-rich) cells.

Although the results of this study demonstrate proton pump immunoreactivity in pavement cells, we cannot totally exclude its presence in chloride cells because insufficient numbers of chloride cells were examined. The focus of this study was the pavement cell

and there was no attempt to quantify the distribution of proton pump immunoreactivity in the various cell types of the gill. However, the results of several other studies indicate that the chloride cell may not contain proton pump activity. First, during hypercapnic acidosis in brown bullhead (Goss *et al.* 1992a) and rainbow trout (Goss & Perry 1993) gill chloride cells are covered by pavement cells during periods of intense branchial acid excretion. Second, Laurent *et al.* (1994) failed to observe proton pump vesicles in chloride cells during an extensive analysis of brown bullhead gills. Clearly, further work will be required to clarify the roles of the pavement cell and chloride cell in acid-base regulation in freshwater fishes.

CHAPTER 3

LOCALIZATION OF mRNA FOR PROTON PUMP (H^+-ATPASE) AND BAND 3 (CL^-/HCO_3^- EXCHANGER) IN EPITHELIAL CELLS OF RAINBOW TROUT GILL

INTRODUCTION

With the presence of proton pump protein identified in the pavement cells of trout gill, a major question has been answered regarding the nature of H^+ excretion. With this in mind, further studies were performed to clarify the actual method of regulation of this mechanism during an acid-base challenge. All proteins are translated from a mRNA template produced in the nucleus of cell, the sequence of which is based on the nucleotide sequence of the gene responsible. The technique of in situ hybridization permits the identification of a particular mRNA, which is complementary to a probe consisting of all or part of the complementary sequence. At such times as there is a requirement for increased protein production, there is an increase in the rate of mRNA transcription, followed by subsequent protein translation. When both antigen and transcript for a protein are identified, the nature of increased protein expression can be more clearly understood. In particular, an increase in protein expression which is not accompanied by a similar increase in mRNA would suggest utilization of stored proteins or post translational modifications brought upon by the particular stimulus. If both immunoreactivity and hybridization signal are increasing in response to a stimulus, the suggestion is that transcription is the determining factor in regulation.

The possible mechanisms for H^+ excretion have been outline in Chapters 1 and 2, but in this chapter, the question of Cl^-/HCO_3^- is raised and in the absence of an appropriate antibody, *in situ* hybridization is the technique used to elucidate the nature of this mechanism. Considerable indirect evidence has suggested that Cl^- uptake is achieved by a

Band 3 or Band 3-like Cl^-/HCO_3^- exchanger located on the apical membrane of chloride cells on the branchial epithelium (Avella & Bornancin 1990; Perry & Laurent 1993; Shuttleworth 1989; McDonald *et al.* 1989). In particular, it has been shown that during periods of alkalosis, as well as during recovery from respiratory acidosis, when large quantities of HCO_3^- are excreted, there is a marked increase in chloride cell fractional area (Goss *et al.* 1992 a); however, direct evidence is still lacking.

The goals of this study were to isolate mRNA for Band 3 and a major subunit of the H^+-ATPase in gill epithelial cells and to determine whether there is regulation of transcription during acid-base disturbances. This was achieved using *in situ* hybridization techniques with oligonucleotide probes complementary to the particular mRNA in conjunction with light microscopy.

MATERIALS AND METHODS

Experimental Animals

Holding conditions and handling of experimental animals are described in detail in chapter 2.

Surgical Procedures

Surgical procedures used in this chapter are described in detail in chapter 2.

Hypercapnia

Procedures for exposing animals to hypercapnia are described in detail in chapter 2.

Base Infusion

In order to induce metabolic alkalosis, fish were exposed to four different periods of base ($NaHCO_3$) infusion: 0 (pre-infusion), 6, 12, and 24 h. Fish were infused via the dorsal aortic cannulae (Manostat Cassette Pump) with a $NaHCO_3$ solution (140 mmol/l) at an approximate infusion rate of 800 μmol/kg/h. Control fish were infused at the same rate with 140 mmol/l NaCl. The flow rate of each pump channel was adjusted proportionally to the mass of the individual fish so as to maintain infusion rates approximately equal to the nominal rate. As with the hypercapnia experiments, during the final 3 h of each

exposure period, water flow was stopped to allow a flux period during which time infusion was maintained.

Acid-Base Parameters

Procedures for measuring, recording, and calculating physiological acid-base parameters are described in detail in chapter 2.

Tissue Preparation

At the end of each experimental period, complete gill arches were removed from control, base infused, and hypercapnic fish and immediately emersion fixed for *in situ* hybridization (ISH). The gills of 12 - 14 fish were examined for each period of hypercapnia and normocapnia. For hypercapnia, the most intense hybridization signal was obtained from the gills of fish exposed for 12 or 18 h, so an additional three groups (24 fish) were exposed to 18 h. For base infusion, the gills of 12-14 fish were examined for each period of $NaHCO_3$ infusion and NaCl infusion and the most intense hybridization signal was obtained from gills of fish exposed to 12 h of $NaHCO_3$ infusion. 3 additional groups (22 fish) were exposed to this condition. Tissue was rinsed briefly in cold (4°C) phosphate buffered saline (PBS) and then emersed in 4% paraformaldehyde in PBS and fixed for 12 - 18 h at 4°C. All tissues were dehydrated in a series of ethanols (12 h in 70 % EtOH, 2 h in 90% EtOH, 2 h in 95% EtOH, 2 X 2 h in 99% EtOH). Tissue was trimmed to remove any bone and a portion of arch with 7 - 12 lamellae was obtained. The tissue pieces were then placed in xylene (2 X 1 h) and then in paraffin (Ameraffin, Baxter

Diagnostics Inc.) at 60° C for 12 - 16 h to allow infiltration before embedding in paraffin blocks.

In situ Hybridization

A 28 mer oligonucleotide probe, complimentary to the mRNA for the 31-kDa subunit of the bovine renal H^+-ATPase was sythesized along with a 24 mer oligonucleotide probe complimentary to nucleotides 1381 - 1404 of rat kidney Band 3 cDNA. Probes were 3'-end labeled with digoxigenin using a terminal transferase end-labeling kit (Boehringer). Sections (7μm) were cut from paraffin blocks using a microtome, mounted on glass slides, and allowed to dry on a slide warmer for at least 6 h. Sections were deparaffinized, rehydrated and digested for 10 min in protease K (5 μg/ml in 0.1 M Tris-HCl) at 37° C followed by washing and air drying. Slides were then incubated in the dark overnight with a probe concentration of 500pg/μl in hybridization buffer (50% formamide, 6X SSC, 50 mM Tris-HCl, 2X Denhardt't solution, 0.2% SDS) at room temperature in a humidified box containing 50% formaldehyde. Following incubation, slides were given a series of SSC washes and incubated for 2 h at room temperature with anti-digoxigenin conjugated with alkaline phosphatase (Sigma). The final step in the reaction was to develop a colour reaction using a solution of 4-nitro blue tetrasolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate-4-toluisin salt (BCIP). Positive hybridization signals were visualized using bright field light microscopy. Control sections for *in situ* hybridization were processed using excess unlabeled probe (the labeled probes were added with 50X excess unlabeled probe), hybridization buffer

only (no probe), and a corticotropin releasing factor (CRF) probe (used as a negative control).

Statistical analysis

Data shown in figure 1 are means ± 1 standard error of the mean. Differences between the control and experimental fish were established using a two-tailed Student's t-test; the limit of significance was set at 5%.

RESULTS

Acid-Base Parameters

As described in Chapter 2, exposure to hypercapnia caused a significant, but transitory, reduction in arterial blood pH (figure 2.1a). Base infused fish displayed an initial increase in arterial blood pH at 6 h which had already returned to control values by 12 h (figure 3.1a). During both hypercapnia and base infusion (figure 3.1b), plasma bicarbonate concentration displayed a continuous increase, however the increase was less pronounced in the base infused fish. A maximum level was reached during hypercapnia at approximately 36 h (figure 2.1b) and a maximum level at 24 h of base infusion (figure 3.1b). During hypercapnia there was a pronounced increase in net H^+ excretion, reaching a maximum at 18 h and remaining elevated above control levels during the 48 h exposure period (figure 2.1c). During base infusion, there was a net base excretion (acid retention) which was continuing to increase at the last sample time of 24 h (figure 3.1c). The control fish displayed no changes in any measured variable (figure 3.1a,b,c).

In situ Hybridization

Positive hybridization signals were observed for both oligonucleotide probes in all tissues that were tested. Obvious differences were present, however, with respect to intensity and distribution of signal. The H^+-ATPase probe signal was present in the normocapnic fish, but was generally associated with the epithelial cells of the lamellae rather than on the filament (figure 3.2b). This signal was greatly enhanced in the tissue of

hypercapnic fish, particularly after 18 h of hypercapnia, and although there were obvious areas of hybridization on the filamental epithelium, the majority was concentrated on the lamellae (figure 3.2c). When the tissue was incubated with an excess of unlabeled probe, the hybridization signal was no longer present on any area of the gill (figure 3.2a). Similarly, when hybridization buffer alone was substituted for the probe, or a probe for the neuropeptide coticotropin releasing factor (CRF) was used, a signal was no longer detectable.

The oligonucleotide probe for Band 3 demonstrated a similar positive hybridization signal in untreated tissue as did the H^+-ATPase probe with a similar intensity; however, the distribution of signal differed in that it was generally seen associated with the interlamellar regions and lower portions of the lamellae (figure 3.3b). Base infusion resulted in a marked increase in hybridization signal (figure 3.3c) accompanied by a maximum degree of observed base excretion (figure 3.1c). The increase in signal was obvious not only in association with the interlamellar filament, but was also observed in a greater number of cells on the lamellae. As with the H^+-ATPase probe, the signal was abolished when an excess of unlabeled probe was used (figure 3.3a) as well as when tissues were incubated with hybridization buffer alone or the CRF probe.

Figure 3.1. The temporal effects of base infusion on blood pH (a); plasma bicarbonate concentration ($[HCO_3^-]$) (b); and branchial net acid excretion ($J_{net}\ H^+$) (c) in control (filled circles) and base infused (filled squares) fish. In (c) positive values indicate acid uptake (base excretion) and negative values indicate acid excretion. * indicates a significant difference from control values. † indicates a significant difference from pre-values within the same group. Values shown are means ± 1 standard error of the mean.

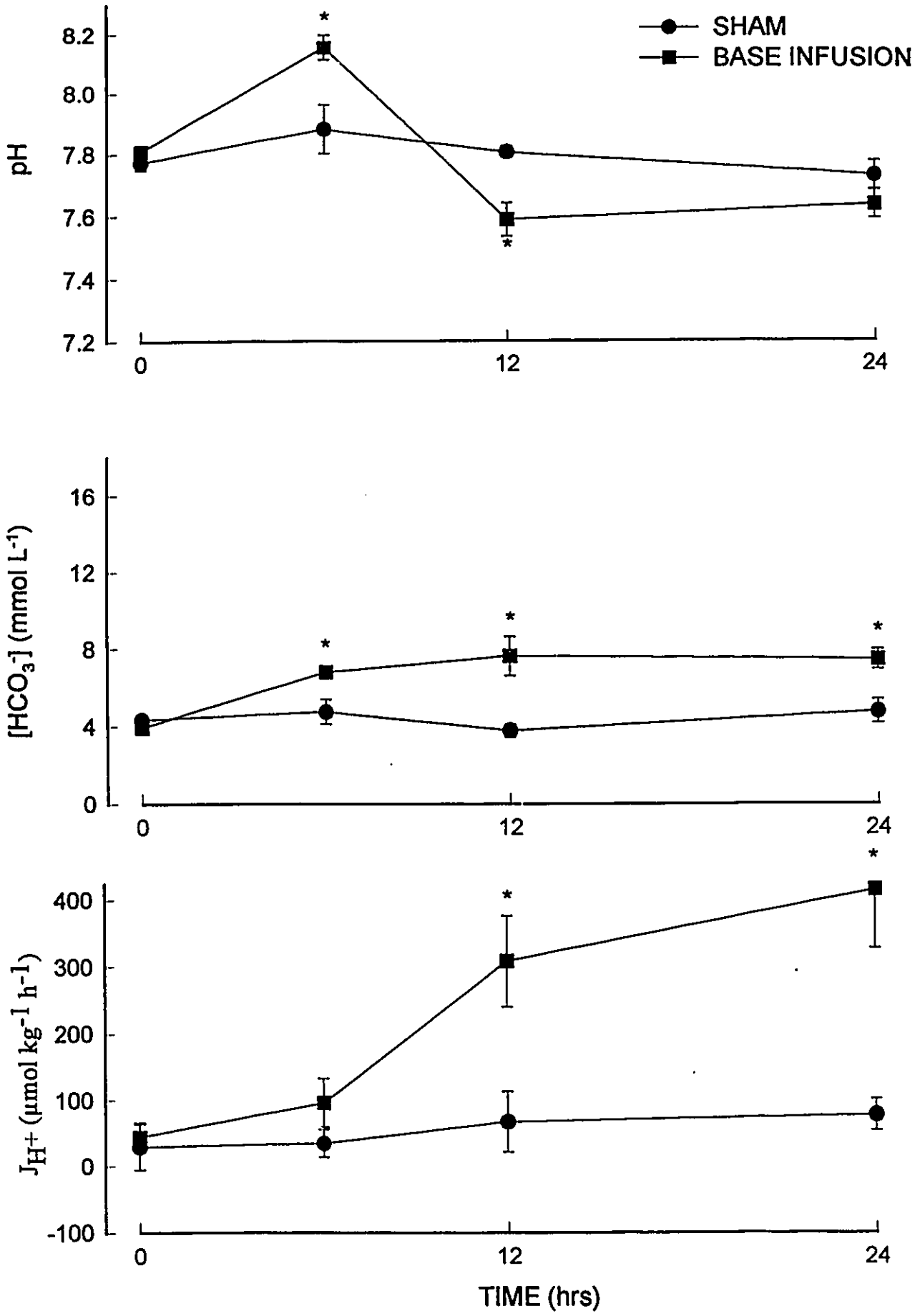
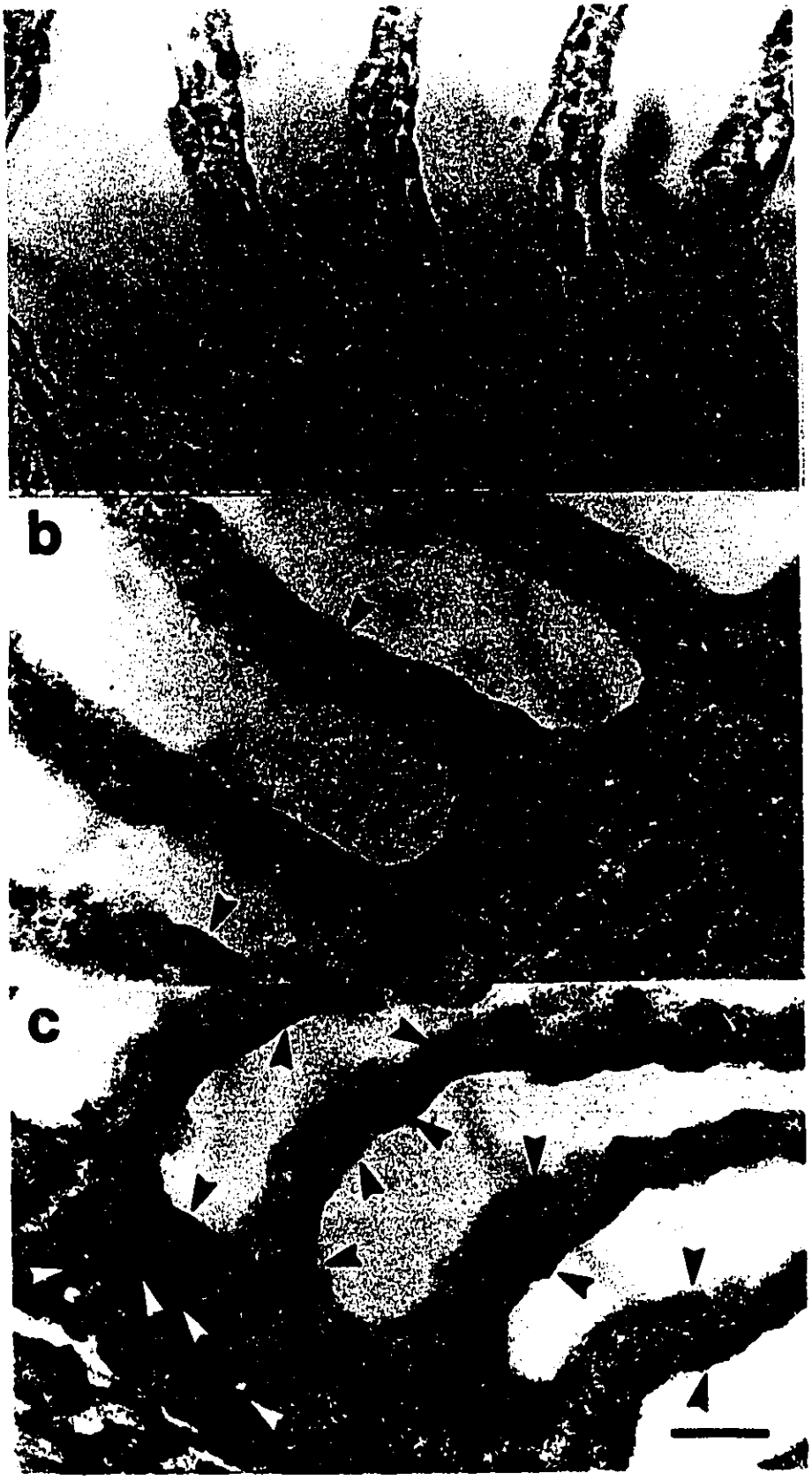

SHAM
BASE INFUSION
pH
8.2
8.0
7.8
7.6
7.4
7.2
0
12
24
[HCO3-] (mmol L-1)
16
12
8
4
0
JH+ (µmol kg-1 h-1)
400
300
200
100
0
-100
TIME (hrs)

Figure 3.2. Light micrographs of gills comparing the intensity and distribution of hybridization signal for the H^+-ATPase probe. (a) Tissue from fish exposed to 18 h of hypercapnia when incubated with excess unlabeled probe , (b) tissue from normocapnic fish incubated with labeled probe, (c) tissue from fish exposed to 18 h of hypercapnia, incubated with labeled probe. Bar = 20 μm.

Figure 3.3. Light micrographs of gills comparing the intensity and distribution of hybridization signal for the Band 3 probe. (a) Tissue from fish exposed to 24 h of base infusion when incubated with excess unlabeled probe , (b) tissue from untreated fish incubated with labeled probe, (c) tissue from fish exposed to 24 h of base infusion, incubated with labeled probe. Bar = 20 μm.


b
c

DISCUSSION

This is the first study to employ *in situ* hybridization as a method to provide direct evidence for the presence of either Band 3 or H^+-ATPase in the gills of fish by demonstrating the existence of transcripts for these respective proteins. The correlation between positive hybridization signals and associated branchial net acid fluxes provides strong evidence for distinct yet opposing roles for these ion transporting proteins during acid base disturbances as well as their obvious regulation in response to these disturbances.

The *in situ* hybridization protocols used in this study were performed with oligonucleotide probes with lengths of 28 mer and 24 mer for proton pump and Band 3 respectively. The sequences were chosen from the area of the cDNA clones which would theoretically provide a probe sequence most likely to hybridize specifically with the desired mRNA, based on known oligonucleotide design strategies. The disadvantage of these oligonucleotide probes as opposed to RNA or cDNA probes is the small number of nucleotides, which increases the chances of non-specific binding as well as weaker binding to the target RNA. The advantages, however, include the ease with which they can be obtained, unlike cDNA or RNA which require a degree of molecular biological expertise; the relative ease with which they may penetrate a target tissue; since they are single stranded, there is no reannealing of sense and antisense strands during hybridization; and there is little chance of hybridization with conserved sequence homologies found in many mRNA species.

The probes used were constructed based on sequences from bovine tissue, but in the case of Band 3, as mentioned in Chapter 1, considerable sequence homology exists between species within the AE gene family (see review by Kopito, 1990). The Band 3 probe used here was shown to exhibit a positive hybridization signal in cells of the cortical collecting ducts of mammalian (rat) kidney (Fryer *et al.*, unpublished results) and was further verified in the same study through various control experiments. The control experiments performed in the present study were also vital in establishing the specificity of the probe for Band 3 in gill tissue. As mentioned in the methods section of this chapter, tissue sections were hybridized with excess unlabeled probe, hybridization buffer only, and other oligonucleotide probes of similar length. Like the Band 3 probe, the H^+-ATPase probe is also derived from a bovine cDNA sequence. Vacuolar ATPase purified from various species including *Neurospora* (Bowman *et al.*, 1988), carrot (Zimniak *et al.*, 1988), and *Saccharomyces cerevisiae* (Hirata *et al.*, 1990) as well as the H^+-ATPase of kidney, all posess a cytoplasmic domain similar to that of the F_0F_1 H^+-ATPases and which all show specific immunoreactivity to antibodies for the 70-, 56-, or 31-kDa kidney H^+-ATPase subunits. Such sequence conservation between species provides strong evidence for the ability of this non-homologous probe to recognize the desired mRNA in fish tissue. Positive hybridiztion signals were observed once again in studies by Fryer *et al.* (unpublished data) using rat kidney. As with the Band 3 probe, appropriate control experiments were performed in the context of this study and similar positive results were obtained with respect to the specificity of this probe.

Performing in situ hybridization at the electron microscopy level is problematic and therefor it was not possible to demonstrate the ultrastuctural detail present in chapter 2. The comparatively thin sections required for TEM reduces the possibility of obtaining an appreciable amount of the required sequence, complementary to the probe, within the section. Also, the degree to which tissue must be fixed to provide sufficient structural integrity for TEM is not conducive to optimal hybridization conditions. Despite this, intuitively, several possibilities may be suggested with respect to the cellular localization of both mRNA species, based on distribution of signal as well as physiological and morphological evidence from previos studies.

It is apparent in figure 3.2b that mRNA for the proton pump protein is generally present in a small number of cells on the lamellar epithelium, and although chloride cells may be found here (Avella and Bornancin, 1990; Laurent and Perry, 1991; Perry and Laurent, 1992), they are normally more concentrated in the interlamellar regions. During hypercapnia, when the signal is considerably more intense, more interlamellar cells are involved, but the lamellar epithelium is unquestionably the site of the greatest density of positive cells. This along with the observations by Goss *et al.* (1992a) which reported a 95% reduction in exposed area of chloride cells in brown bullheads during hypercapnia, implicate the pavement cell as the site of the positive hybridization signal for the proton pump. Perhaps the most compelling evidence comes from the immunocytochemical evidence presented in chapter 2 which immunolocalizes the H^+-ATPase in the apical membrane and cytoplasmic vesicles of pavement cells. Since the probe and the antigen against which the antiserum was raised were based on the same sequence, it is reasonable

to assume, in light of the other evidence, that both are being localized in the same cells. The similarity between the distribution of immunoreactive signal and hybridization signal would also tend to agree with this contention (compare figures 3.2b and c with figures 2.2a-d).

Figure 3.3b shows the distribution of mRNA for Band 3 which, in contrast to figure 3.2b, shows more signal associated with the interlamellar regions as compared to the lamellae which is characteristic of the distribution of chloride cells. This becomes more apparent during alkalosis (figure 3.3c) at which time there is a dramatic increase in signal in the interlamellar regions as well as an increase on the lamellae. This occurs concurrently with an uptake of acid (figure 3.1c) which is seen in this case as a substantial increase in branchial HCO_3^- excretion. This also correlates with the maintenance of plasma HCO_3^- concentrations (figure 3.1b) during the metabolic alkalosis, as it is being actively cleared. Morphological studies by Goss and Perry (1993) demonstrated an increase in gill chloride cell surface area as a result of metabolic acidosis and in a subsequent study (Perry and Goss, 1994) they demonstrated that fish with an increased area of chloride cells displayed a greater ability to regulate acid-base status (e.g. base clearance) during base infusion. These morphological and physiological observations (i.e. increased chloride cell surface area and increased requirement for HCO_3^- excretion) and the presence of mRNA for a known Cl^-/ HCO_3^- exchanger, implicate the chloride cell as an inportant site of acid-base regulation with respect to Cl^-/ HCO_3^- exchange. Presumably, based on these observations, it is most likely that during recovery from a respiratory acidosis, when there is a rapid reappearance of chloride cells (Goss *et al.*, 1992a), there

would be an increase in positive hybridization signal as accumulated base from the acidosis compensation has to be excreted.

Results of this study provide further credibility to evidence presented in chapter 2 supporting the Na^+ uptake model proposed by Avella and Bornancin (1989), based on positive immunoreactivity for the proton pump antiserum. The presence of a functional transcript for the H^+-ATPase, especially one that is upregulated concurrently with an increased requirement for acid excretion, is indicative of a regulatory mechaninism designed to respond to an acid load by increasing transcription of mRNA and subsequently increased translation of protein (see chapter 2). Although there is no immunological evidence to support the proposal that Band 3 is present in the gills, based on the presence of mRNA and indirect evidence from past studies reviewed here, a role for Band 3 is likely real.

CHAPTER 4

GENERAL DISCUSSION

In light of indirect evidence from the numerous studies investigating ionic and acid-base regulation by the gills of freshwater fish, the purpose of this study was to provide direct evidence for the existence and cellular localization of ion translocating mechanisms, specifically Cl^-/HCO_3^- exchanger and H^+-ATPase, and whether or not they are actively regulated in response to acid-base disturbances. In particular, it was suggested that a proton pump in the form of a V-ATPase is present on the gills, specifically the pavement cell, and provides one possible method of H^+ excretion. Such a protein may be coupled to the passive entry of Na^+ through a Na^+ channel although investigation of this mechanism was not a component of this study. The possibility of the presence of a Cl^-/HCO_3^- exchanger from the AE1 family of anion exchangers, on the chloride cell, was also addressed. This protein would presumably be similar to the Band 3 protein found in other tissues such as kidney and red blood cells. To this end, the techniques of *in situ* hybridization and immunocytochemistry were employed, thereby allowing for observations to be made at the cellular level.

As mentioned in Chapter 2, this is only the second study to attempt to immunolocalize proton pump in the gills of freshwater fish. Indeed, successful immunocytochemistry depends on many factors related to the tissue being investigated as well as the characteristics of the particular antibody. In this study, as with any, the choice of fixative was made to insure optimal immunoreactivity while also preserving tissue integrity. In the case of paraffin sections for light microscopy, a combination of Bouin's fixative and modified Bouin's fixative (see Chapter 2) was used and resulted in satisfactory signal being produced and sufficient tissue preservation for the resolution required. Slight

complications were encountered in the case of immunoelectron microscopy as sections are much thinner and fixation must be slightly more intense without excessive loss of immunoreactivity. Although there may be more appropriate methods of fixation, the one used was adequate within the scope of this study. The antibody itself may also display fixation-dependent characteristics, and indeed there are many examples of cases where an antibody will work well on one type of fixed tissue, but not another. Again, since positive results were obtained with the procedures used, it was unnecessary to experiment with such parameters. Finally, the specificity of an antibody is of primary concern, especially when a polyclonal antibody is being used as in this case. The extensive control experiments explained in Chapter 2 as well as the localization of the proton pump to intercalated cells of the rat and human kidney, along with the positive Western blot results should dispel any concerns regarding the validity of results obtained.

To the best of my knowledge, *in situ* hybridization has never been used to provide evidence of either proton pumps or Cl^-/HCO_3^- exchangers in fish gill. Such a step is a natural progression following successful immunolocalization in order to elucidate the nature of increased protein concentration in a tissue with respect to whether or not there is also an increase in transcription. In the case of Cl^-/HCO_3^- exchanger, *in situ* hybridization provides an alternate method for identification of a potential cellular component in the absence of an antibody for immunocytochemistry. To reiterate what was explained in Chapter 3, *in situ* hybridization is a technique by which a particular mRNA species is localized by using a probe which is complementary to the mRNA molecule or a portion thereof. Ribonucleotide probes were used in this study, which have the advantage of

being readily obtained as well as having (due to their relatively small size) easy access to the target tissue mRNA. Once again, the choice of fixative is important for successful *in situ* hybridization and paraformaldehyde is commonly used for paraffin embedded sections. Paraffin embedding of fixed tissue does result in the loss of some mRNA (up to 30% in some cases) but it has the advantage of being easy to work with and store, and is quite stable for indefinite periods of time after embedding and sectioning. Since a large amount of tissue was being dealt with and accurate quantification was not required, the methods chosen proved quite effective for the application, and due to the relative simplicity of the procedure, results were readily reproducible.

REGULATION OF H^+ AND Na^+ MOVEMENT ACROSS THE GILL

The observations reported here support the existence of an H^+-ATPase on the gills of fish. Microscopic evidence has localized both the protein and the mRNA for the proton pump in the epithelial cells with the protein being identified particularly in pavement cells by immunoelectronmicroscopy. Furthermore, during such times as there is a requirement for increased excretion of H^+, such as during acidosis, there is a definite upregulation of both. The fact that increased concentrations of both mRNA and protein have been found in close association under similar conditions, namely acidosis, strongly suggest that they are changing in response to the same stimulus and are most likely localized within the same cells. It is noteworthy that levels of both protein and mRNA are low throughout the cell during normal conditions, and both increase similarly during periods of acidosis. This suggests that very little, if any, protein is stored in vesicles for subsequent incorporation

into the membrane when required, but rather increased transcription, followed by increased translation occurs in response to the acid base insult, producing as much protein as is required. When the evidence from previous studies is considered (Chapter 2), the data is most convincing supporting the contention that H^+ excretion, in the form of an electrogenic proton pump, and a mechanism such as a Na^+ channel, are associated with the pavement cell. Although it cannot be stated that a Na^+/H^+ antiporter does not exist in the fish gill epithelium, arguments presented with regard to internal Na^+ concentrations and electrochemical gradients (Chapter 2) make this very unlikely. For the most part, the conclusions drawn here regarding a proton pump agree with the findings of Lin *et al.* (1994) except that the present study provides direct evidence that these structures are located in the pavement cells as in contrast to the suggestion by Lin *et al.* (1994) that chloride cells are the cells involved.

Cl^-/HCO_3^- ACROSS THE GILL

The direct evidence in this study supporting the existence of a Band 3-like Cl^-/HCO_3^- exchanger is based on *in situ* hybridization evidence, since an antibody against this particular protein was not available. A positive hybridization signal was observed in epithelial cells as described in Chapter 3 and an obvious increase in intensity was seen in tissue from fish exposed to base infusion. Presumably, the alkalosis which accompanies such an exposure is the stimulus for the increased transcription and/or reduced degradation of mRNA. The absence of immunocytochemistry precludes making any conclusions regarding the amount and distribution of protein, but when results are

compared to those found for proton pump, increased protein translation seems quite likely. The indirect evidence suggesting the presence of a Cl^-/HCO_3^- exchanger in the chloride cells was presented in Chapter 3, and when this is taken into consideration with the observations in this study, the chloride cell seems to be the most likely site for this mechanism. Although the distribution of hybridization signal for the Cl^-/HCO_3^- exchanger probe could not be localized at the level of the cell using EM techniques, the relative distribution of the signal is suggestive of its origin. As was proposed in Chapter 3, the distribution of signal in untreated tissue and during alkalosis is reminiscent of the pattern of chloride cell distribution under the same conditions.

FUTURE DIRECTION

It was the intention of this study to provide direct evidence for the presence of a proton pump and a Cl^-/HCO_3^- exchanger on the gills of fish, localize such structures to particular cell types, and determine whether or not they are actively regulated during acid-base disturbances. All of these issues were addressed with different degrees of success. The case of the proton pump provides the most definite evidence in that both the protein and the mRNA have been detected and the protein has been localized in pavement cells specifically. In order to complete the story, however, it would be beneficial to perform successful *in situ* hybridization at the EM level to determine the cell types with which the mRNA is associated. There has also been considerable success with techniques to perform colocalization of both antigen and mRNA within the same tissue and this would provide definitive evidence for the relationship between regulation of transcription and

translation. As I have mentioned, the possibility of proton pump being present on chloride cells has not been ruled out, rather it was not specifically addressed. A similar immunoelectron microscopic investigation should be performed with emphasis on observation of chloride cells.

In relation to H^+ excretion in the manner proposed here, there is a need for a mechanism by which Na^+ is taken into the cell. The most reasonable possibility is the presence of a Na^+ channel as outlined in Chapter 2. Currently, assays for Na^+ channels are performed and the identification of such a channel would fit perfectly into the model of the fish gill proposed in this thesis.

It is obvious that if the Band 3 Cl^-/HCO_3^- exchanger is to be specifically identified as an acid-base regulation mechanism, immunocytochemistry must be performed and at the present time, we are having an antibody raised based on the sequence from which the oligonucleotide probe was taken. Once again, isolation of both the antigen and the mRNA at the EM level would be required. Other valuable colocalization techniques which might be employed would allow simultaneous viewing of hybridization signal for both proton pump and Cl^-/HCO_3^- exchanger within the same tissue so as to determine their respective locations (ie. cell types).

Many questions still remain unanswered with respect to the mechanisms of acid-base regulation in freshwater fish, but the information provided by this study represents several new approaches which may be taken to resolve them.

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