THE EFFECTS OF ACID-BASE DISTURBANCES ON BRANCIAL AND RENAL CALCIUM FLUXES IN THE FRESHWATER RAINBOW TROUT

(*Oncorhynchus mykiss*)

by

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SUMMARY

Whole body calcium influx, branchial calcium efflux, and renal Ca^{2+} excretion were measured in rainbow trout (*Oncorhynchus mykiss*) either exposed to environmental hypercapnia or infused intra-arterially with $NaHCO_3$. These experiments were performed to assess the potential impact on Ca^{2+} balance of the changes in gill morphology that are known to accompany acid-base disturbances in this species.

After 48 hours of environmental hypercapnia, gill filamental chloride cell fractional area was significantly reduced. Despite this reduction, and the presumed involvement of the chloride cell in calcium influx, whole body calcium influx was increased after 12 hours of hypercapnia and remained elevated for 48 hours. Branchial calcium efflux was unaltered during hypercapnia exposure, whereas renal Ca^{2+} excretion was elevated over preflux values only at 6 hours of hypercapnia. Measurement of the kinetics of whole body calcium influx after 48 hours of hypercapnia revealed a significant increase in the maximal uptake rate of Ca^{2+} yet the affinity constant of Ca^{2+} uptake was unaffected. Measurements of high-affinity Ca^{2+}-ATPase activities and ATP-dependent Ca^{2+} transport of gill basolateral membrane vesicles revealed that the ATP-dependent Ca^{2+} extrusion mechanism of the gills was not affected by hypercapnia. The results of this study clearly show that the reduced chloride cell surface area that accompanies hypercapnia in trout does not impair calcium homeostasis. Although adjustments to the basolateral membrane high affinity Ca^{2+} transporter do not appear to play a role, the mechanism(s) underlying the maintenance of calcium homeostasis under hypercapnic conditions are unresolved.

Whole body Ca^{2+} influx was significantly increased after 6 hours of $NaHCO_3$ infusion and remained elevated throughout the duration of the experiment. Branchial and renal Ca^{2+} effluxes were largely unaffected by $NaHCO_3$ infusion. Plasma total Ca^{2+} concentrations were significantly decreased after 6 hours of $NaHCO_3$ infusion and remained so until 48 hours. Such results suggest relocation of Ca^{2+} from the plasma to other body compartments, such as bone. Analysis of the kinetics of whole body Ca^{2+} uptake revealed that infusion of $NaHCO_3$ for 48 hours caused a significant increase in the maximal uptake rate of Ca^{2+}; the affinity constant of Ca^{2+} uptake was unchanged. Measurement of various enzymatic activities from gill basolateral membranes revealed that although Na^+,K^+-ATPase activity was significantly increased in $NaHCO_3$-infused fish, neither Ca^{2+}-ATPase activity nor ATP-dependent Ca^{2+} transport were affected. These findings suggest that the basolateral membrane Ca^{2+} transporter does not alter its capacity to move Ca^{2+} under alkalotic conditions. We suggest that the chloride cell apical membrane is the principle regulator of branchial Ca^{2+} uptake in rainbow trout under alkalotic conditions. Such a suggestion concurs with the original hypothesis that an increase in apical surface area would lead to increased Ca^{2+} uptake.

The results of this thesis research indicate that disturbances in acid-base balance do affect Ca^{2+} fluxes. Ca^{2+} uptake was enhanced when the trout experienced either a respiratory acidosis or a metabolic alkalosis. This enhancement was expected in the alkalotic fish but was not expected in the acidotic fish. Such enhancement of Ca^{2+} uptake must require some apparent change in the mechanisms allowing Ca^{2+} across the chloride cell. Since it was found in this study that this enhancement does not occur at the

basolateral membrane in either case, the most likely other location is the apical membrane. Thus, it appears that the apical membrane of the chloride cell may exhibit significant control over Ca^{2+} uptake in rainbow trout, at least during acid-base disturbances.

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LIST OF ABBREVIATIONS

ANOVA analysis of variance

BSA bovine serum albumin

°C degrees Celsius

Ca_t total calcium

Ca_{uf} ultrafiltrable calcium

CC chloride cell

CCFA chloride cell fractional area

DPM disintegrations per minute

DTT dithiothreitol

Ef final external water radioactivity

Ei initial external water radioactivity

EGTA ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid

ESA external specific activity

g gram

h hour

HEDTA N-hydroxyethylethylene diaminetriacetic acid

Ho homogenate remaining after first centrifugation

$J_{in}Ca^{2+}$ calcium influx

$J_{out}Ca^{2+}$ calcium efflux

kg kilogram

K_m affinity constant

kPa kilopascal

L litre

m mitochondria

mg milligram

min minute

ml millilitre

mm millimeter

mmol millimole

MS 222 ethyl m-aminobenzoate

N nucleus

nm nanometer

NMWL nominal molecular weight limit

NTA nitrilotriacetic acid

P3 final pellet, Pi inorganic phosphate

PC pavement cell

PMSF phenylmethylsulfonyl fluoride

PSA plasma specific activity

P_wCO_2 water partial pressure of carbon dioxide

SEM standard error of the mean

T time

TCA trichloroacetic acid

UFR urine flow rate

µCi microCurie

µg microgram

µl microlitre

µm micron

µmol micromol

V external water volume

V_{max} maximal uptake rate

V_{spec} specific activity

W body weight

WBA whole body activity

CHAPTER 1

GENERAL INTRODUCTION

The fish gill is a large, complex organ with a surface area 10 to 60 times greater than that of the skin covering the body (Parry, 1966). The gill is involved in a multitude of functions enabling the fish to inhabit various aquatic environments. Although its primary function is respiratory gas exchange, the gill is also involved in ionoregulation (reviewed by Laurent and Perry, 1995), acid-base balance (Evans, 1975; Cameron 1976 and 1978; reviewed by Heisler, 1984 and 1993), and removal of metabolic wastes (Smith, 1929; Maetz, 1971 and 1973; Cameron and Heisler, 1983). Such processes are possible due to the complex, yet strategic, organization of the gill.

FRESHWATER GILL STRUCTURE AND FUNCTION

The gills of fishes are considered external organs, although they are covered by the protective operculum (Withers, 1992). The gill basket is composed of 8 gill arches. Each gill arch is subdivided into filaments; each filament is composed of lamellae which tend to be the main sites of gas transfer (Laurent, 1982). These subcomponents increase the surface area of the gill, thus increasing its capacity for gas transfer, ionoregulation, acid-base regulation, and nitrogen excretion. These physiological processes are made possible by the functioning of specialized gill cells which form a barrier between the external environment (water) and the internal environment (blood).

The most abundant type of cell found on the gill is the pavement cell. These cells, which make up about 90% of the gill epithelium (Avella and Bornancin, 1990; Laurent and Perry, 1995), are responsible for respiratory gas exchange, with CO_2 moving out of the fish and O_2 moving into the fish. Because of their role in gas transfer, pavement cells tend to be more

flattened than other gill cells, being approximately 3 to 5 μm thick (Straus, 1963; Maetz, 1971). The apical (water-facing) surface is characterized by numerous microridges (Olson and Fromm, 1973; Kendall and Dale, 1979) which may be responsible for more effective attachment of mucus to the epithelium and/or increasing the surface area of the gill, thereby increasing gas exchange potential (Perry and Laurent, 1993). Ultrastructurally, these cells are populated densely with small vesicles and lysosomes and sparsely with mitochondria. Extensive Golgi apparati and rough endoplasmic reticula and a typically elongated nucleus are also present (Straus, 1963; Maetz, 1971; Laurent and Dunel, 1980; Laurent, 1982). These cells were once believed to be responsible for respiratory gas exchange only; their lack of energy-producing mitochondria seemed to exclude the possibility of any role in acid-base or ion regulation. But evidence has been accumulating in the past 25 years suggesting that these cells may be involved in maintenance of ionic balance. Maetz (1971) suggested that pavement cells were involved in the passive movement of ions. Girard and Payan (1980) proposed that a Na^+/NH_4 exchanger was located on pavement cells in freshwater fish. Goss *et al.* (1992a) proposed that the pavement cell, under certain circumstances, may be the site of Na^+ uptake and acid excretion mechanisms. Morgan *et al.* (1994) suggested that pavement cells may be responsible for Na^+ uptake in freshwater fishes. And, Sullivan *et al.* (1995) were the first to demonstrate, using immunocytochemistry, laser scanning confocal and electron microscopy, that a proton pump is located on the lamellar pavement cell (Figure 1.1).

Chloride cells, also known as mitochondria-rich cells or ionocytes, generally comprise less than 10% of the total surface area of the freshwater fish gill (Avella and Bornancin, 1990; Perry and Laurent, 1993). These cells are usually cuboidal or columnar in shape with a

smooth, slightly concave or invaginated apical plasma membrane (Threadgold and Houston, 1964; Kendall and Dale, 1979; Peek and Youson, 1979; Franklin, 1990). Numerous mitochondria and extensively branching smooth endoplasmic reticula distinguish these cells. The apical region is characterized by vesicles derived from the endoplasmic reticulum membranes while the basal region is characterized by a large spherical or oval nucleus and a few Golgi apparati (Philpott and Copeland, 1963; Straus, 1963; Threadgold and Houston, 1964; Morgan and Tovell, 1973; Peek and Youson, 1979). Chloride cells were first identified by Keys and Willmer (1932) as salt-secreting cells in marine fishes. Their presence and function in freshwater fishes was not determined until later. In freshwater fish, chloride cells are believed to be responsible for active uptake of ions from the surrounding environment (Krogh, 1938; Girard and Payan, 1980; Laurent and Dunel, 1980; Evans, 1982; Laurent *et al.*, 1985; Laurent and Perry, 1990; Perry *et al.*, 1992b). Numerous Na^+,K^+-ATPases on the extensive endoplasmic reticula (Karnaky *et al.*, 1976) and numerous ATP-producing mitochondria (Threadgold and Houston, 1964; Morgan and Tovell, 1973) support the idea that active ion transport occurs at the chloride cell.

The 3 remaining cell types found on the fish gill epithelium are the stem cell, the neuroepithelial cell and the mucous cell. Stem cells are undifferentiated cells of unknown function while neuroepithelial cells are involved in epithelial serotonergic innervation of the gill (Bailly *et al.*, 1989; Goss *et al.*, 1995). Mucous cells are usually oval with a centrally or basally located nucleus. The remainder of the cell appears to be composed of secretory granules, vacuoles, and, depending on the state of activity, various amounts and forms of mucus (Bevelander, 1935; Ishihara and Mugiya, 1987). Mucous cells are not believed to have any

fundamental role in ion regulation. They may, however, have an indirect role in reducing ion loss from freshwater fish. Handy (1989) suggested that gill mucus may provide an ion-rich environment separating the relatively ion-poor water from the blood plasma. Such an environment could, potentially, limit ion escape and decrease the amount of energy required to maintain ion levels in the blood.

ACID-BASE BALANCE

Maintaining acid-base balance and blood pH are of the utmost importance to all vertebrates; teleost fish are no exception. However, the mechanisms employed by fish for maintaining these parameters are quite different from those employed by terrestrial organisms. In terrestrial animals, the kidney and lung are the main organs of acid-base balance (Pitts, 1974). In freshwater fish, however, the gills are the primary organs of acid-base balance (Cameron, 1976 and 1980); the kidney plays an important, but secondary, role in acid-base homeostasis in fish (Wheatly *et al.*, 1984; Heisler, 1993).

Acid-base regulation in freshwater fish involves regulation of the following ions: Na^+, Cl^-, HCO_3^-, H^+, and NH_4^+. Transfer of these ions between the fish and the surrounding milieu, mainly via the gills, allows compensation for acid-base disturbances (Evans, 1975 and 1982; Cameron, 1976 and 1980; Heisler, 1982). Movement of Na^+ into the fish appears to be coupled with movement of acid equivalents (H^+ and/or NH_4^+ ions) out of the fish; Cl^- movement into the fish appears to be coupled to movement of base equivalents (HCO_3^- ions) out of the fish (reviewed by Heisler, 1984 and 1993; see Figure 1.1). Removal of H^+, HCO_3^-, NH_4^+ and uptake of Na^+ and Cl^- are important not only in acid-base regulation, but also in

ionoregulation, removal of metabolic wastes, and respiratory gas exchange. Removal of HCO_3^- facilitates loss of respiratory CO_2 and loss of base equivalent; removal of H^+ facilitates loss of acid equivalent; and, removal of NH_4^+ facilitates removal of acid equivalents and nitrogenous metabolic wastes (Evans, 1975).

Although the branchial location of the mechanism responsible for removal of base equivalents from the fish has not been recently challenged, the form of this mechanism has. All evidence suggests that this mechanism is located on the apical membrane of the gill chloride cell. There is abundant evidence to suggest that this mechanism exists in the form of an electroneutral Cl^-/HCO_3^- exchanger. Such an exchanger was first proposed by Krogh (1939) on the basis that the amount of CO_2 excreted via the gills was sufficient to allow functioning of the Cl^-/HCO_3^- exchanger, considering the presence of carbonic anhydrase (Maetz, 1971). Since then, numerous studies have reaffirmed the existence of the exchanger and its placement on the chloride cell (reviewed by Evans, 1984, Heisler, 1984 and 1993, Goss *et al.*, 1995; see Figure 1.1). However, questions have been raised recently as to the ability of such an exchanger to function on the apical surface of the chloride cell, given the unfavorable Cl^- gradient at this membrane. Lin and Randall (1991) suggested that the branchial Cl^-/HCO_3^- exchanger alone plays little role in acid-base regulation. It could, however, play a greater role if there was a favorable HCO_3^- gradient across the apical membrane, as could be attained via the functioning of a HCO_3^--ATPase, stimulated by both Cl^- and HCO_3^-. Although such a mechanism has been suggested (Bornancin *et al.*, 1980; DeRenzis and Bornancin, 1984), a direct relationship between it and the Cl^-/HCO_3^- exchanger remains to be established.

The exact branchial mechanism responsible for removal of acid equivalents from the fish is also in question. It was traditionally believed that this mechanism existed in the form of a Na^+/H^+ (NH_4^+) exchanger (Krogh, 1938; Kerstetter *et al.*, 1970). Avella and Bornancin (1989) questioned the existence of a Na^+/H^+ exchanger, proposing instead, that Na^+ uptake occurs via an apically located channel while H^+ extrusion occurs via an electrogenic proton pump. Since then, more evidence has come to light supporting the existence of such a mechanism. Lin and Randall (1991 and 1993) provided *in vivo* and *in vitro* evidence, respectively, that a functional H^+-ATPase existed on the trout gill; morphological evidence (Lin *et al.*, 1994; Sullivan *et al.*, 1995) supported these findings. Thus, it now appears that although Na^+ uptake and H^+ extrusion are coupled, the mechanism(s) responsible exists in two possible forms: Na^+ channel/ H^+-ATPase and/or a Na^+/H^+ (NH_4^+) exchanger.

The location of the mechanism(s) responsible for Na^+ uptake and acid excretion has also recently been modified. For many years, evidence suggested that Na^+ uptake and/or H^+ extrusion occurred via the branchial chloride cells (Laurent *et al.*, 1985; Avella *et al.*, 1987; Avella and Bornancin, 1990; Laurent and Perry, 1990; Lin and Randall, 1991 and 1993). But evidence has begun to surface suggesting that the mechanism(s) is located on the gill pavement cells. Goss *et al.* (1992a) first suggested a relationship between Na^+ uptake/H^+ excretion and pavement cells. Additional evidence since then has lent support to the proposition that pavement cells are the principle site of Na^+ uptake and H^+ extrusion in freshwater fish (see Goss *et al.*, 1992b; Morgan *et al.*, 1994; Sullivan *et al.*, 1995; reviewed by Goss *et al.*, 1995; see Figure 1.1).

One of the major findings that suggested the mechanism(s) responsible for Na^+ uptake and H^+ extrusion was located on the gill pavement cell was the discovery that gill cells alter their morphology in response to acid and base disturbances (Figure 1.1). Cameron and Iwama (1987) originally suggested a relationship between acid-base disturbances and gill morphology. This relationship was reexamined in 1992 by Goss *et al.* who looked at the effects of hypercapnia on brown bullhead (*Ictalurus nebulosus*). Goss *et al.* (1992a) found that hypercapnia caused a severe reduction in exposed chloride cell apical membrane, such that after 48 h of exposure, chloride cell fractional area was decreased by 90%. Examination of rainbow trout (*Oncorhynchus mykiss*) under similar conditions revealed the same results, although the reduction was not as great (Goss and Perry, 1993). Infusion of rainbow trout (*Oncorhynchus mykiss*) for 19 hours with $NaHCO_3$ caused a 70% increase in chloride cell fractional area (Goss *et al.*, 1994b). The explanation for such changes appears to lie in the mechanisms responsible for acid-base balance and ionoregulation.

When a fish experiences an internal acidosis, there is a need to remove acid equivalents (H^+ ions) from the blood but retain base equivalents (HCO_3^- ions). This can be achieved by physically covering the apical surface of gill chloride cells, thus facilitating an uncoupling of the Cl^-/HCO_3^- exchanger and preventing removal of HCO_3^- ions. Retention of base equivalents is analogous to extrusion of acid equivalents. At the same time, the apical surface area of the pavement cells increases, leading to greater functioning of the mechanism(s) responsible for removal of acid equivalents. The opposite phenomenon occurs when a fish experiences an internal alkalosis. In this case, the fish needs to remove base equivalents; increased functioning of the Cl^-/HCO_3^- exchanger is required. During such a disturbance, there is also a change in gill

morphology such that the exposed apical surface area of chloride cells increases. This increase permits greater functioning of the Cl^-/HCO_3^- exchangers, thereby allowing increased removal of base equivalents. The resultant decrease in pavement cell apical surface area also means decreased functioning of mechanisms responsible for removal of acid equivalents. Thus, the fish is able to compensate for acid-base disturbances by altering the proportion of pavement and chloride cells exposed to the environment. These changes in CCFA in response to acid-base disturbances are due to changes in the morphology of surrounding pavement cells. Marshall and Nishioka (1980) observed few desmosomal junctions between chloride and adjacent pavement cells, suggesting that movement of gill cells is quite plausible. Pavement cells appear to alter their cellular organization and gradually "crawl" over the apical surface of adjacent chloride cells from all directions during acidosis. During alkalosis, they retract and allow greater exposure of the chloride cells (Figure 1.1). The chloride cell does not appear to play an active role in either process (Laurent and Perry, 1995).

Examination of ion fluxes during these acid-base disturbances reveals that movements of Cl^- and Na^+ are also affected. Respiratory acidosis in the brown bullhead catfish (*Ictalurus nebulosus*) caused decreased Cl^- uptake and increased Na^+ uptake (Goss *et al.*, 1992a). The same disturbance in rainbow trout (*Oncorhynchus mykiss*) led to decreased Cl^- uptake and a slightly decreased Na^+ uptake (Goss *et al.*, 1992b). These changes occurred simultaneously with decreased chloride cell fractional area. Internal alkalosis, caused by infusion of $NaHCO_3$, in rainbow trout (*Oncorhynchus mykiss*) led to increased chloride cell fractional area, decreased Na^+ uptake and increased Cl^- uptake (Perry and Goss, 1994). Such findings lend

further support to the contention that Cl^- uptake/HCO_3^- extrusion occurs via the branchial chloride cell while Na^+ uptake/H^+ extrusion occurs via the pavement cell.

CALCIUM HOMEOSTASIS

Calcium homeostasis in fishes has generated much interest in the past 50 years or more. $[Ca^{2+}]$ in the aquatic environment can range in from 0.1 mM (freshwater) to 10 mM (seawater). Yet, fishes are able to maintain relatively constant plasma calcium levels despite living in these diverse environments. Such an ability is the result of effective calcium transporting systems consisting of branchial and renal components.

Because of the dilute environment in which they live, freshwater fish are always in danger of losing Ca^{2+} to the environment. There is an on-going need to prevent such a loss and, simultaneously, maintain storage and uptake. Freshwater fish, unlike other vertebrates, which rely on ingested calcium as their main source, are able to absorb calcium from the external milieu (Simmons, 1971; Pang *et al.*, 1980; reviewed by Fenwick, 1989). Although they do take up some Ca^{2+} via the intestine from ingested food and across the skin, the main site of Ca^{2+} uptake in freshwater fish is the gill, accounting for approximately 50% of total whole body uptake of Ca^{2+} (Berg, 1968 and 1970; Milhaud *et al.*, 1977; Mugiya and Ichii, 1981; Ichii and Mugiya, 1983; Flik *et al.*, 1985b; Marshall *et al.*, 1992 and 1995; Perry and Wood, 1985; reviewed by Fenwick, 1989; reviewed by Flik and Verbost, 1993 and 1995).

Although it was established that the gill was the main site of Ca^{2+} uptake in freshwater fish, the question remained as which cell(s) was responsible for this action. Payan *et al.* (1981) compared the appearance of ^{47}Ca in arterial and venous fluids and determined that the cell type

responsible for Ca^{2+} uptake was the chloride cell. Since then, the fact that the chloride cells are the main sites of Ca^{2+} uptake has been further verified, for example by Flik *et al.* (1984, 1985a and b), Perry and Flik (1988), Marshall *et al.* (1992) and Perry *et al.* (1992a). It is believed that this uptake begins with passive diffusion of Ca^{2+} ions across the chloride cell apical membrane via presumptive Ca^{2+} channels. This movement is passive since the ions move down an electrochemical gradient into the negatively charged chloride cell cytoplasm, and down a concentration gradient, from the water where $[Ca^{2+}]$ is normally in the mmol l^{-1} range to the cytoplasm where $[Ca^{2+}]$ is normally in the μmol l^{-1} range. Once across, the ions are transported to the basolateral membrane via Ca^{2+}-binding proteins. Here, they must be actively transported into the extracellular space, up concentration and electrochemical gradients, before being picked up by the circulatory system (reviewed by Fenwick, 1989; see Figure 1.1). It was once believed that the transporter responsible for this movement was a low-affinity Ca^{2+}-ATPase. But conflicting data lead to further assessment of this enzyme's functioning. This lead to the discovery that the enzyme in question was actually an alkaline phosphatase and speculation that another enzyme, a high-affinity Ca^{2+}-ATPase, was the true transporter of Ca^{2+} across the basolateral membrane (Doneen, 1981). Studies since then have shown that there is, in fact, a calmodulin-dependent high-affinity Ca^{2+}-ATPase present on the gill basolateral membrane and that this enzyme is responsible for active Ca^{2+} movement (Flik *et al.*, 1984, 1985b and c; Perry and Flik, 1988). This is not, however, the only transporter of Ca^{2+} ions across the gill basolateral membrane. A Na^{+}/Ca^{2+} exchanger has recently been found on the gill basolateral membrane (Flik *et al.*, 1993; Flik and Verbost, 1993; Verbost *et al.*, 1994). In fish, this mechanism was first identified in the intestine, transporting Ca^{2+} from the mucosa to the serosa.

It is driven by an inward Na^+ gradient maintained by the Na^+/K^+-ATPase. It now appears to be the main transporter of Ca^{2+} in the fish intestine, with the high-affinity Ca^{2+}-ATPase playing only a minor role (Flik *et al.*, 1990; Schoenmakers *et al.*, 1993). This is not the case, however, on the gills. Functioning of the Ca^{2+}-ATPase is much higher than that of the Na^+/Ca^{2+} exchanger on the gill basolateral membrane. The exchanger appears to function only when the intracellular $[Ca^{2+}]$ reaches 1 μmol l^{-1} or greater (Flik *et al.*, 1993). Since the $[Ca^{2+}]$ within the cytoplasm of the chloride cell rarely rises this high, activity of the Na^+/Ca^{2+} exchanger is low. Thus, this exchanger may only function in the gills on those rare occasions when the intracellular $[Ca^{2+}]$ exceeds 1 μmol l^{-1} or when there are isolated pockets of excess Ca^{2+} within the cytoplasm, such as near the apical Ca^{2+} channels.

Although the Ca^{2+} that enters the fish via the gills enters in the form of ionic Ca^{2+}, once in the blood, it can exist in a few different forms. Approximately half exists in the form of free ionic Ca^{2+}; the other half exists as bound Ca^{2+}, complexed to proteins and large molecules (Andreasen, 1985). The blood serves as a pool of Ca^{2+} which can be quickly mobilized when necessary. Stored Ca^{2+} is located in various locations throughout the body. The liver, fins, scales, and skeletal muscle serve as minor reservoirs for body Ca^{2+}; bone is the major reservoir (Ruben and Bennett, 1981). It is well known that changes in the acid-base status of vertebrates with osseous skeletons can potentially impact the displacement of Ca^{2+} in the body. Acidosis is characterized by increased $[H^+]$ in the blood. Such an increase can lead to the displacement of Ca^{2+} ions from their position bound to proteins and molecules. Simultaneously, there can also be a dissolution of the hydroxyapatite skeleton of the bone. Calcium phosphate in bone is relatively insoluble but becomes soluble when the blood pH decreases. Thus, during an internal

acidosis, both Ca^{2+} and PO_4^{3-} are released into the blood. The plasma PO_4^{3-} is then capable of buffering the excess H^+ ions in the blood. Both actions can lead to increased ionic [Ca^{2+}] in the plasma. An internal alkalosis can have the opposite effect, leading to increased binding of plasma free ionic Ca^{2+} by proteins and molecules and increased uptake by the bone. This consequently, causes decreased free ionic Ca^{2+} in the plasma (Gardner, 1978).

Removal of excess Ca^{2+} from the freshwater teleost fish occurs via 2 principle routes: the gills and the kidney. Movement across the gills is paracellular and passive. Ca^{2+} ions move from the blood, into the extracellular space, between the chloride cell and adjacent pavement cell, and then into the water (Fenwick, 1989; see Figure 1.1). Measurements of Ca^{2+} concentrations in the urine of freshwater teleosts has revealed that the amount of Ca^{2+} excreted this way is quite low. Ca^{2+} appears to be reabsorbed in the kidney, against a strong concentration gradient (Hickman and Trump, 1969).

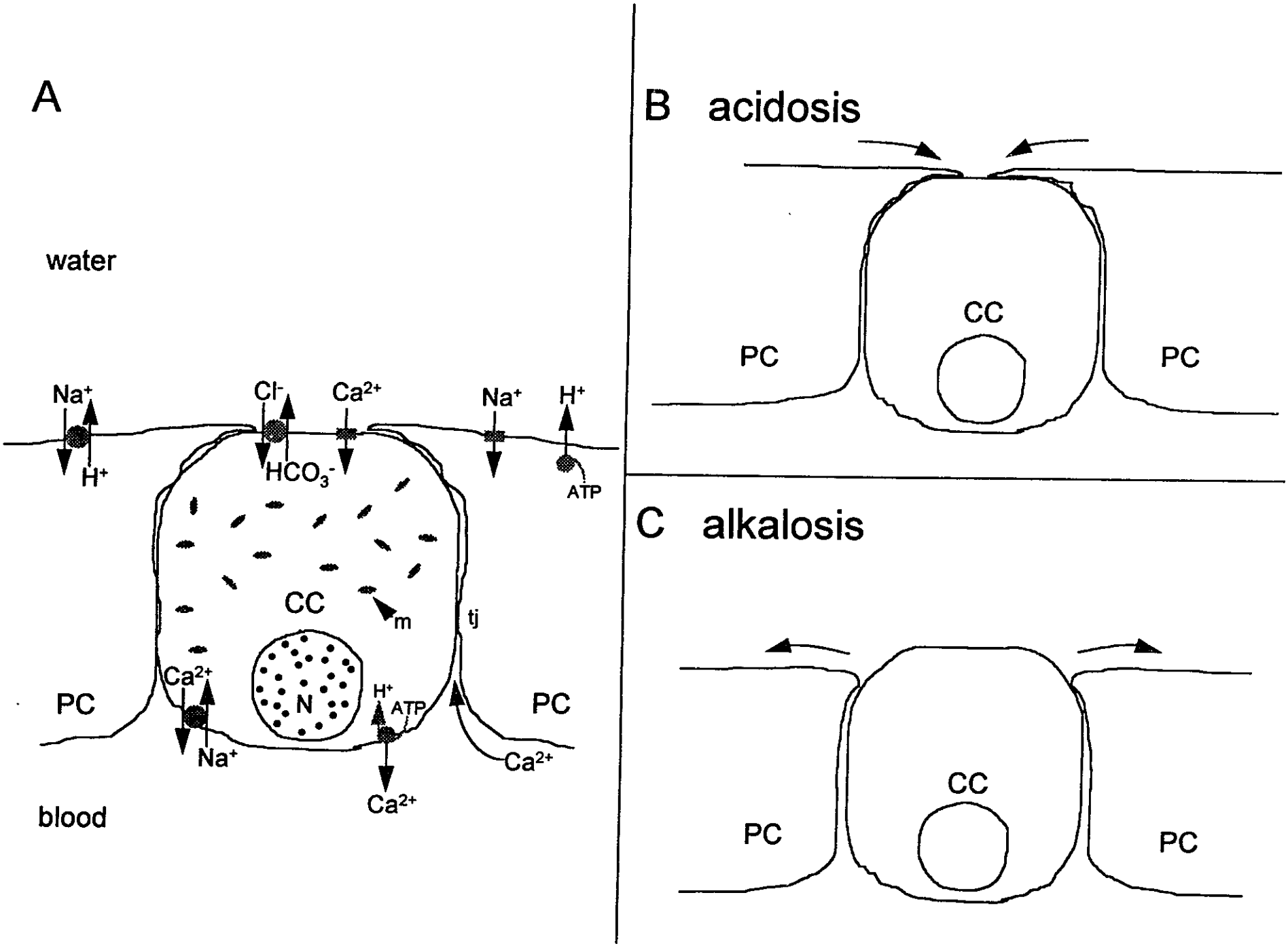
SUMMARY AND HYPOTHESIS

The gill is composed of no less than 5 major cell types, 3 of which have been implicated in some way in ionoregulation and/or acid-base regulation. Uptake of environmental ions in freshwater fish and removal of acid and base equivalents are believed to occur predominently via the gill pavement and chloride cells. Changes in the morphology of these cell types have been seen during acid-base disturbances. These changes appear to affect uptake of ions such as Na^+ and Cl^-. However, the effect of acid-base disturbances on Ca^{2+} movement has not been previously investigated. The majority of Ca^{2+} uptake is believed to occur transcellularly across the branchial chloride cells. Thus, the goal of this thesis was to determine if acid-base

disturbances affect Ca^{2+} flux. The hypothesis tested was **acid-base disturbances will alter transbranchial Ca^{2+} flux owing to changes in the surface area of chloride cells exposed to the environment.** This hypothesis was further subdivided into 2 more specific hypotheses: i) that acidosis will cause decreased Ca^{2+} uptake owing to decreased exposed chloride cell apical surface area, and ii) that alkalosis will cause increased Ca^{2+} uptake owing to increased exposed chloride cell apical surface area.

To test this hypothesis, acid-base disturbances were induced in rainbow trout (*Oncorhynchus mykiss*) and whole body Ca^{2+} fluxes were monitored. Trout were exposed to environmental hypercapnia, inducing a respiratory acidosis. The effects of this treatment on renal and branchial Ca^{2+} fluxes are presented in Chapters 2. The effects of a metabolic alkalosis, caused by infusion of $NaHCO_3$, on renal and branchial Ca^{2+} fluxes in trout are presented in Chapter 3.

Figure 1.1 Schematic diagram of branchial chloride cell **(A)** under normal conditions, **(B)** during an internal acidosis, and **(C)** during an internal alkalosis. In **(B)**, pavement cells have covered the apical membrane of the chloride cell, thus reducing its fractional area. In **(C)**, pavement cells have retracted from the chloride cell and increased the chloride cell fractional area. Apical and basolateral exchangers have been indicated where they are believed to be located in **(A)**. Pictured on the chloride cell apical membrane are a Cl^-/HCO_3^- exchanger and a Ca^{2+} channel and, on the basolateral membrane, are a Ca^{2+}-ATPase and a Na^+/Ca^{2+} exchanger. Pictured on the pavement cells are a Na^+/H^+ exchanger (left cell) and a Na^+ channel/H^+-ATPase (right cell). CC = chloride cell; m = mitochondria; N = nucleus; PC = pavement cell; tj = tight junction;  = ATPase; = channel; = exchanger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H+
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blood
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PC
C alkalosis
CC
PC
PC

CHAPTER 2

THE EFFECTS OF HYPERCAPNIA ON BRANCHIAL AND RENAL CALCIUM FLUXES IN THE RAINBOW TROUT (*Oncorhynchus mykiss*)

INTRODUCTION

The uptake of calcium from the external environment, rather than dietary entry, is the principle route of calcium absorption in freshwater teleosts (Mayer-Gostan *et al.*, 1983; Flik et *al.*, 1985a). The major organ of calcium uptake is the gill (Fenwick and So, 1974; Ma *et al.*, 1974; Flik *et al.*, 1985a; Perry and Flik, 1988) with the skin and intestine serving only as supplementary routes (Pang *et al.*, 1980). Chloride cells, also known as mitochondria-rich cells or ionocytes, are responsible for active calcium uptake at the gill (Payan *et al.*, 1981; Flik *et al.*, 1985b and c; Perry and Wood, 1985; Perry and Flik, 1988; Marshall *et al.*, 1992; McCormick *et al.*, 1992; Perry *et al.*, 1992a). Transport across the gill begins with passive diffusion of Ca^{2+} ions across the chloride cell apical membrane via presumptive calcium channels. Calcium-binding proteins then bind the ionic Ca^{2+} and transport it to the basolateral membrane. Here, a high-affinity Ca^{2+}-ATPase and a Na^{+}/Ca^{2+} exchanger actively move Ca^{2+} against an electrochemical gradient between the chloride cell cytoplasm and the blood (Flik *et al.*, 1985b; Perry and Flik, 1988; reviewed by Fenwick, 1989; Flik and Verbost, 1993; Flik *et al.*, 1993; Verbost *et al.*, 1994; Flik *et al.*, 1995; see Figure 1.1).

Upon exposure of freshwater teleosts to certain environmental conditions, apical chloride cell surface area may be altered markedly. Recent studies using brown bullheads (*Ictalurus nebulosus*; Goss *et al.*, 1992a and 1994a) and rainbow trout (*Oncorhynchus mykiss*; Goss and Perry, 1993 and 1994; Goss *et al.*, 1994b; Perry and Goss, 1994) have shown that acid-base disturbances elicit alterations in both chloride cell and surrounding

pavement cell structure (reviewed by Goss *et al.*, 1992b and 1995; see Figure 1.1). In particular, during an internal acidosis, pavement cells physically change shape and cover adjacent chloride cells, such that 30 - 90% of the exposed chloride cell surface area may be covered, depending upon the species (Goss *et al.*, 1992a and 1995). Because Ca^{2+} uptake is directly proportional to exposed chloride cell surface area (McCormick *et al.*, 1992; Perry *et al.*, 1992a), acid-base disturbances could potentially affect calcium balance in fish owing to alteration in Ca^{2+} influx. Ca^{2+} efflux is less likely to be affected because it is thought to occur via paracellular pathways (Perry and Flik, 1988). Thus, we hypothesized that a respiratory acidosis in rainbow trout would elicit a decrease in branchial calcium influx owing to the reduction of exposed chloride cell surface area that is known to accompany respiratory acidosis in this species (e.g. Goss and Perry, 1993; Goss *et al.*, 1994b). Alternatively, compensatory mechanisms might allow Ca^{2+} uptake to be maintained despite the loss of exposed chloride cell surface area. Thus, in the present study we have assessed gill chloride cell morphometry, branchial and renal Ca^{2+} fluxes *in vivo*, and basolateral membrane Ca^{2+} transport *in vitro* in trout maintained under normocapnic or hypercapnic conditions.

MATERIALS AND METHODS

Experimental animals

Two size ranges of rainbow trout (*Oncorhynchus mykiss*) were used in this study. One group, used in the determination of calcium influx ($J_{in}Ca^{2+}$), calcium efflux ($J_{out}Ca^{2+}$), exposed chloride cell fractional area (CCFA), and the isolation of gill basolateral membranes, weighed between 158 and 435 g (mean mass = 260.0 ± 4.8 g SEM, N = 144). A second group, used in the study of Ca^{2+} uptake kinetics, weighed between 17 and 39 g (mean mass = 24.8 ± 0.4 g SEM, N = 60). All trout were obtained from Linwood Acres Trout Farm, Campbellcroft, Ontario, and were transported to the University of Ottawa in oxygenated water. Fish were held indoors in large fiberglass tanks supplied with flowing, aerated, dechlorinated, City of Ottawa tap water ($[Na^+]$ = 0.15 mmol l^{-1}; $[Cl^-]$ = 0.15 mmol l^{-1}; $[K^+]$ = 0.02 mmol l^{-1}; $[Ca^{2+}]$ = 0.40 mmol l^{-1}; pH = 7.5 to 8.0; temperature = 10° C) under a constant photoperiod of 12 hours light : 12 hours dark. Fish were fed daily with commercial trout pellets.

Experimental protocol

Individual rainbow trout were placed in opaque acrylic flux boxes (approximate volume = 3.6 L for large fish and 0.6 L for small fish) and were allowed to adjust to these conditions for at least 19 h prior to commencing experiments. Fish were separated into two groups; experimentals and controls. Experimental fish used to determine the rates of $J_{in}Ca^{2+}$ and $J_{out}Ca^{2+}$ were exposed to either 6, 12, 18, 24, 36, or 48 hours (h) of

environmental hypercapnia (1% CO_2 in air; P_wCO_2 = 7.6 torr). Experimental fish used to isolate gill basolateral membranes, to study Ca^{2+} uptake kinetics and to determine exposed chloride cell surface area were exposed to 48 h of environmental hypercapnia. Environmental hypercapnia was attained by bubbling CO_2/air mixtures into a large surface area gas exchange column as described by Perry *et al.* (1987a). The CO_2/air mixtures were supplied by either a Wösthoff gas mixing pump (model M301 A-F) or a gas mixing flowmeter (Cameron Instrument Company model GF-3/MP). P_wCO_2 was monitored constantly by passing water from the gas exchange column over a PCO_2 electrode (E5036-0; Radiometer) maintained at 10° C and attached to a meter (PHM 71 Acid-Base Analyzer; Radiometer) and chart recorder (SE120; BBC). Water flow was maintained at 750 ml min^{-1} box^{-1} for large fish and 500 ml min^{-1} box^{-1} for small fish. Seven large fish or 12 small fish could be supplied with inflowing hypercapnic water simultaneously. When water flow was stopped (i.e. during flux periods), 1% CO_2 in air was bubbled into each box. Each experimental group had its own corresponding control group. Control fish were maintained under normocapnic conditions for the same duration as their experimental counterparts. During flux periods, air alone was bubbled into individual boxes.

Gill morphometry

The central portion of the second left gill arch was excised from the fish immediately after death and fixed overnight at 4°C in 5% glutaraldehyde buffered with 0.05 mol l^{-1} Na-cacodylate. Tissue was cut into pairs of filaments still attached to the

septum of the arch and placed in 0.15 mol l^{-1} Na-cacodylate at 4°C until ready to use. Filaments were then post-fixed in 1% OsO_4 for 1 h, rinsed in 0.15 mol l^{-1} Na-cacodylate for 15 min., and then dehydrated in an ethanol series followed by 2 successive baths (2 min. each) of 1,1,1,3,3,3-hexamethyldisilizan. Filaments were air-dried overnight before being glued with silver paint onto specimen stubs used with a scanning electron microscope (Phillips model 505) and sputter-coated with gold. Filaments were oriented such that the lateral side of the filament was maintained parallel with the stub plate.

Epithelial surfaces on the trailing edges of the filaments at the point of separation from the septum and close to the base of the lamellae were located and photographed at a magnification of 1050 x. At least 4, and normally 6, non-contiguous fields were randomly photographed from each fish for subsequent morphometric analysis.

The apical surface area of individual chloride cells (CC) and their density on the filament epithelium were determined by tracing CC perimeters using a digitizing tablet and morphometry software (SigmaScan, Jandel Scientific Inc.). From these measurements, the mean CC fractional area per unit epithelium was calculated. CC's were distinguished from other epithelial cells using criteria established in previous studies (Laurent and Hebibi, 1989; Laurent and Perry, 1990; Goss *et al.*, 1992a and b; Perry *et al.*, 1992a and b; Goss and Perry 1993 and 1994; Goss *et al.*, 1994a and b; Goss *et al.*, 1995).

Determination of $J_{in}Ca^{2+}$

Whole body calcium influx ($J_{in}Ca^{2+}$) was determined using a modification of the method of Perry and Wood (1985). At time = -15 min., water flow to individual boxes was stopped and 10 μCi l^{-1} of ^{45}Ca ($CaCl_2$; Amersham) was added to each box. At t = 0 min., an initial water sample was taken. At t = 240 min., a final water sample was taken and fish were killed by an overdose of anaesthetic (0.8 g l^{-1} ethyl m-aminobenzoate (MS-222; Sigma), 1.6 g l^{-1} $NaHCO_3$, 1.0 g l^{-1} $CaCl_2$) and lethal blow to the head. Carcasses were softened in a microwave oven for 3 to 5 minutes before being homogenized (Proctor-Silex Blend Master Blender) with 65% of body weight in water. Four aliquots of tissue homogenate, weighing approximately 0.5 g each, were taken from each fish and dissolved in 10 ml of aqueous fluor (ACS II; Amersham). Water samples (1 ml) were also dissolved in 10 ml of aqueous fluor. ^{45}Ca activity was measured by liquid scintillation counting (Tri-Carb 2500TR; Canberra-Packard). Water total [calcium] ($[Ca_t]$) was determined using flame emission spectroscopy (Spectra AA 250 Plus; Varian). A pre-hypercapnia flux was also performed.

Determination of $J_{out}Ca^{2+}$

Surgical procedures - Rainbow trout were anaesthetized in a solution of ethyl m-aminobenzoate (0.1 g l^{-1} MS-222) adjusted to a pH of approximately 7.5 with $NaHCO_3$. Fish were placed onto an operating table which allowed continuous retrograde irrigation of the gills with the same anaesthetic solution maintained at 10° C. To allow blood

sampling, each fish was fitted with a chronic indwelling cannula (PE 50 tubing; Clay Adams) inserted into the dorsal aorta as described by Soivio *et al.* (1975). To allow urine sampling, each fish was fitted with an internal bladder catheter (PE 60 tubing; Clay Adams) as described by Wood and Patrick (1994).

Protocol - Branchial calcium efflux (branchial $J_{out}Ca^{2+}$) was determined using a modification of the method of Perry and Flik (1988). Each fish was injected, via the dorsal aorta cannula, with 90 μCi of ^{45}Ca in 1 ml of Cortland saline (Wolf, 1963), followed by 2 ml of Cortland saline, 24 h prior to commencing experiments (established in preliminary experiments). At t = -30 min., water flow to individual boxes was stopped and at t = 0 min., an initial water sample was taken. At t = 240 min., a final water sample and a dorsal aortic blood sample were taken. The same fish were used to calculate $J_{out}Ca^{2+}$ at each hypercapnia exposure period. A pre-hypercapnia flux was also performed.

Blood, plasma, water, and urine analysis - At the end of each 4 h flux period, approximately 0.4 ml of blood was removed from the dorsal aorta. Red blood cells were separated from plasma, resuspended in an equal volume of Cortland saline, and re-injected into the fish. Plasma was analyzed for total [calcium] (Ca_t; ionic and bound forms of calcium) and ultrafiltrable [calcium] (Ca_{uf}; an index of ionic Ca^{2+}). Plasma Ca_{uf} was obtained by filtering plasma through a 10 000 NMWL filter (Ultrafree-MC Filter Unit; Millipore) and measuring the $[Ca^{2+}]$ of the filtrate. Plasma $[Ca_t]$ and $[Ca_{uf}]$ were determined on diluted samples (150x) using flame emission spectroscopy. Water $[Ca_t]$ also was determined using flame emission spectroscopy. Water (5 ml) and plasma (20 μl)

samples were dissolved in aqueous fluor and ^{45}Ca activity was measured by liquid scintillation counting.

Urine samples were collected during each flux period in pre-weighed plastic vials anchored outside each box below water level. Urine [Ca_t] was determined on diluted (20x) samples using flame emission spectroscopy.

Calcium uptake kinetics

At time = -20 min (47 hours, 40 minutes of hypercapnia exposure), water flow to boxes was stopped and the water level in each box was reduced by half and replaced with water containing normal levels of Na^+, Cl^- and K^+ but altered [Ca_t]. Replacement water [Ca_t]was either 0.025, 0.4, 0.8, 1.6, or 3.2 mmol l^{-1}. Water with a [Ca_t] < 0.4 mmol l^{-1} was prepared from distilled water by adding NaCl, KOH and $Ca(NO_3)_2$. Water with [Ca_t] ≥ 0.4 mmol l^{-1} was prepared using City of Ottawa dechlorinated water by adding $Ca(NO_3)_2$. The pH of the replacement water was tested before flushing the boxes to ensure it was comparable to inflowing water pH. Each box was flushed 7 times with replacement water; preliminary experiments showed that this protocol was sufficient to change the water [Ca_t] of box water to that of the replacement water.

At t = -10 min, sufficient ^{45}Ca was added to each box to provide an average water specific activity of approximately 500 000 DPM/μmol Ca. At t = 0 and t = 30 min, water samples were removed from each box. Duplicate 1 ml samples were measured for radioactivity and water [Ca_t] was determined as described above. At t = 30 min, fish were

killed and $J_{in}Ca^{2+}$ was determined as described above except that the volume of water added to carcasses to aid in blending was increased to 100% of body weight. Less water than this caused the homogenate to become lodged beneath the homogenizer blades.

Gill plasma membrane transport

Gill basolateral membrane isolation - Gill basolateral plasma membrane vesicles were prepared using a modification of the method of Flik *et al.* (1985c). Rainbow trout were killed by a blow to the head. Material from 2 rainbow trout were pooled for each preparation (i.e. N = 1). The heart of each fish was exposed and the branchial apparatus was perfused, via cannulation of the ventral aorta, with 30 ml of the following solution: 20 U ml^{-1} ammonium heparin, 5 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} dithiothreitol (DTT), 0.5 mmol l^{-1} EDTA, 0.2 mmol l^{-1} phenylmethylsulfonyl flouride (PMSF), pH 8.0, in order to clear red blood cells. Gill arches were removed, rinsed in ice-cold 0.9 % (W/V) saline, blotted dry, and scraped to remove the epithelium. Approximately 30 ml of hypotonic buffer (25 mmol l^{-1} NaCl, 1 mmol l^{-1} Hepes/Tris, 1 mmol l^{-1} DTT, 0.2 mmol l^{-1} PMSF, pH 8.0) was added to individual scrapings for homogenization. Scrapings were homogenized (Polytron tissue homogenizer; 2 min., setting 2) on ice and then centrifuged at 550 g (10 min.; 4 to 12°C) to remove nuclei and cellular debris. A 500 µl aliquot of the resulting supernatant (designated as Ho) was retained and immediately sonicated for later analysis. The remaining Ho was centrifuged at 30 000 g (30 min.; 4 to 12°C). The resulting pellets were pooled in groups of two and dounced 200x by hand in a glass dounce homogenizer

in 30 ml of an isotonic sucrose buffer (250 mmol l^{-1} sucrose, 5 mmol l^{-1} Hepes/Tris, 5 mmol l^{-1} $MgCl_2$, pH 7.4). The preparation was then centrifuged 3 times: 1000 **g** (10 min.), 10 000 **g** (10 min.), and 30 000 **g** (30 min.). The final pellet (designated as P3) was resuspended in 1 ml of isotonic sucrose buffer by passing it 10x through a 23 gauge needle. Ho and P3 samples were prepared and analyzed on the same day.

Enzyme assays - Protein concentrations of Ho and P3 were determined using a commercial reagent kit (BioRad) with bovine serum albumin (BSA) as a reference.

Na^+,K^+-ATPase activities of Ho (the supernatant which remained after the first centrifugation) and P3 (the final pellet) were assayed using a modification of the method of Flik *et al.* (1983). Two solutions (solutions A and E) were prepared, each containing 100 mmol l^{-1} NaCl, 30 mmol l^{-1} imidazole/Hepes/Tris, 5 mmol l^{-1} $MgCl_2$, 0.1 mmol l^{-1} EDTA, 3 mmol l^{-1} Na_2ATP, 2 mg ml^{-1} NaN_3, pH 7.4. Solution A also contained 12.5 mmol l^{-1} KCl while solution E contained 2 mg ml^{-1} ouabain. A 500 µl sample of either solution A or E was added to 20 µg of Ho or P3 protein and samples were incubated at 37°C for 15 min. The reaction was stopped by adding 500 µl of ice-cold 30% trichoroacetic acid (TCA). The amount of inorganic phosphate (Pi) produced was determined by adding 500 µl of a color reagent (3.66% H_2SO_4, 9.2 mmol l^{-1} ammonium molybdate tetrahydrate, and 331 mmol l^{-1} $FeSO_4$), leaving samples on ice for 5 min and then at room temperature for 20 min. Samples were measured by spectrophotometry (Spectronic 1001 Plus; Milton Roy) at a wavelength of 700 nm.

Ca^{2+}-ATPase activities of Ho and P3 were assayed using a modification of the method of Flik *et al.* (1983). Two solutions were prepared (solutions 1 and 2), each containing 150 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, 0.5 mmol l^{-1} N-hydroxyethylethylenediaminetriacetic acid (HEDTA), 0.5 mmol l^{-1} nitrilotriacetic acid (NTA), 0.5 mmol l^{-1} ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 3 mmol l^{-1} Na_2ATP, 1 mmol l^{-1} free Mg^{2+}, 2 mg ml^{-1} ouabain, 2 mg ml^{-1} NaN_3, pH 7.4. Solution 1 also contained 1 µmol l^{-1} free Ca^{2+}; solution 2 contained no Ca^{2+}. A 500 µl sample of either solution 1 or 2 was added to 20 µg of Ho or P3 protein and samples were incubated at 37°C for 30 min. The reaction was stopped and Pi production was assayed as described above.

ATP-dependent Ca^{2+} transport - ATP-dependent Ca^{2+} transport of P3 was measured using a modification of the method of Flik *et al.* (1985c). Two assay media were prepared, each containing 150 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes/Tris, 0.5 mmol l^{-1} EGTA, 0.5 mmol l^{-1} HEDTA, 0.5 mmol l^{-1} NTA, 1 µmol l^{-1} free Ca^{2+}, 0.8 mmol l^{-1} free Mg^{2+}, and 3 µCi ml^{-1} ^{45}Ca, pH 7.4. In addition, one of the solutions also contained 3 mmol l^{-1} Tris-ATP. A 500 µl sample of either solution was added to 100 µg of P3 protein. Samples were incubated at 37° C and at time = 1, 3, and 5 min., 150 µl aliquots were removed and placed in 1 ml of stopping solution (150 mmol l^{-1} KCl, 20 mmol l^{-1} Tris-HCl, 0.1 mmol l^{-1} $LaCl_3$, pH 7.4) on ice. Stopping solution, containing vesicles, was filtered through 0.45 µm filters (Millipore Corp.). Filters were dissolved in 10 ml of aqueous fluor (ACS II; Amersham)

and ^{45}Ca activity was counted by liquid scintillation counting. Free Ca^{2+} and Mg^{2+} concentrations were calculated as described by Schoenmakers *et al.* (1992).

Calculations

Gill filament chloride cell fractional area (CCFA) was calculated as the product of the apical membrane surface area of exposed chloride cells in μm^2 and the density of exposed chloride cells in # of cells/mm^2.

Whole body $J_{in}Ca^{2+}$ was calculated as follows:

$$J_{in}Ca^{2+} = \frac{WBA}{ESA \cdot T \cdot W}$$

where WBA (whole body activity) is the total activity accumulated in the fish in DPM, ESA (external specific activity) is the average specific activity of the external water in DPM μmol^{-1} Ca^{2+}, T is the flux duration in hours, and W is the mass of the fish in kg (Perry and Wood, 1985).

Branchial $J_{out}Ca^{2+}$ was calculated as follows:

$$J_{out}Ca^{2+} = \frac{(Ei - Ef) \cdot V}{PSA \cdot T \cdot W}$$

where Ei and Ef are the initial and final external water radioactivities in DPM ml^{-1}, V is the external water volume in ml, PSA is the final specific activity of the plasma in DPM μmol^{-1} Ca^{2+}, T is the flux duration in hours, and W is the mass of the fish in kg (Perry and Flik, 1988).

Renal Ca^{2+} efflux was calculated as follows:

$$\text{renal } Ca^{2+} \text{ efflux} = \text{urine } [Ca_t] \bullet \text{UFR}$$

where urine $[Ca^{2+}]$ is given in $\mu mol\ ml^{-1}$ and UFR (urine flow rate) is given in $ml\ kg^{-1}\ h^{-1}$ (Perry *et al.*, 1987b).

Statistical analysis

Values shown in figures and tables are sample means ± 1 SEM. Unpaired Student's t-tests were used to compare sample means of experimental and corresponding control groups. One-way ANOVA ($J_{in}Ca^{2+}$ data) /one-way repeated measures ANOVA (plasma, urine and $J_{out}Ca^{2+}$ data) and Dunnett's tests were used to compare sample means within experimental and control groups; 5% was taken as the fiducial limit of significance.

RESUI TS

Gill morphometry

Exposure of trout to 48 h of hypercapnia reduced the fractional area of exposed chloride cells on the gill filamental surface to 68% of the control value (Figure 2.1C). The percentage of exposed chloride cells on the gill filamental epithelium decreased from 11.1 ± 2.0 % in normocapnic trout to 3.4 ± 0.6 % in hypercapnic trout. This reduction was due to a significant reduction in chloride cell density (Figure 2.1B); the surface area of individual chloride cells was unaffected after 48 h of hypercapnia (Figure 2.1A).

Plasma calcium levels

Plasma [Ca_t] declined initially during hypercapnia and was significantly reduced after 18 h of exposure (Figure 2.2A). Following this, plasma [Ca_t] was not significantly different from the pre-hypercapnia values. Although significant differences were observed between experimental and control plasma Ca_t concentrations at nearly all sampling times during hypercapnia, this difference existed before the commencement of hypercapnia. No significant change in plasma [Ca_t] was seen in the control trout.

Plasma [Ca_{uf}] was not significantly different from the pre-hypercapnia value among experimental trout. However, among control trout, values at 36 and 48 h of exposure were significantly higher than the pre value (Figure 2.2B). This upward trend began after 18 h of exposure and could account for the significant difference between control and experimental values at 24, 36 and 48 h.

Calcium fluxes

Whole body $J_{in}Ca^{2+}$ increased during hypercapnia (Figure 2.3A); at 36 and 48 h the rate of $J_{in}Ca^{2+}$ was significantly higher than corresponding control values. Moreover, within the hypercapnic fish, the rate of $J_{in}Ca^{2+}$ had significantly increased at all time periods, except 6 h, when compared to the preflux period.

Branchial $J_{out}Ca^{2+}$ was not affected by environmental hypercapnia (Figure 2.3B). No significant differences were detected between experimental fish and their corresponding controls. Nor were there any significant differences within the control or experimental groups.

After 6 and 36 h of exposure to environmental hypercapnia, renal Ca^{2+} efflux was significantly higher than in corresponding normocapnic fish (Figure 2.4C). However, only after 6 h was renal Ca^{2+} efflux significantly higher than the preflux value. This was a consequence of significantly increased urine flow rates (UFR; Figure 2.4B) at these exposure times rather than to changes in urine $[Ca_t]$, which remained unaltered with hypercapnia exposure (Figure 2.4A). Renal Ca^{2+} efflux among control trout decreased significantly from the pre value after 12 h and remained lower throughout the rest of the experiment. This was due to significantly lowered urine $[Ca_t]$ at these times (Figure 2.4A). UFR remained unchanged among control trout.

Calcium uptake kinetics

Both hypercapnic and normocapnic trout displayed typical Michaelis-Menten kinetics for $J_{in}Ca^{2+}$ (Figure 2.5A). Exposure to 48 h of hypercapnia resulted in significant increases in $J_{in}Ca^{2+}$ at water Ca_t concentrations of 0.025, 0.8, 1.6, and 3.2 mmol l^{-1}. Unexpectedly, uptake was not significantly greater in experimental trout at a water [Ca_t] of 0.4 mmol l^{-1}, which is where the significant difference had been observed previously (Figure 2.3A). This may have been caused by a high degree of variation in this particular data set.

The maximum transport velocity of Ca^{2+} uptake (V_{max}) and affinity constant of Ca^{2+} uptake (K_m) of trout exposed to 48 h of hypercapnia were 140.2 ± 9.8 μmol h^{-1} kg^{-1} and 0.38 ± 0.08 mmol l^{-1}, respectively. V_{max} and K_m values of normocapnic trout were 103.1 ± 6.2 μmol h^{-1} kg^{-1} and 0.39 ± 0.07 mmol l^{-1}, respectively (Figure 2.5B). Although there was no significant difference between K_m values (P = 0.9193), the V_{max} values were significantly different from each other (P = 0.0061).

Gill basolateral membrane enzyme activities and ATP-dependent Ca^{2+} transport

The P3 fraction of gill basolateral membranes from rainbow trout exposed to 48 h of environmental hypercapnia contained 1.05 ± 0.06% of the protein found in the Ho fraction. This was not significantly different from the recovery value of 1.20 ± 0.09% obtained from control trout. Na^+,K^+-ATPase and Ca^{2+}-ATPase specific activities of the P3 membrane fraction of the hypercapnic trout (33.4 ± 5.3 and 9.3 ± 1.5 μmol Pi h^{-1} mg^{-1}

protein, respectively) also did not differ from control values (25.1 ± 1.9 and 10.7 ± 1.6 μmol Pi h^{-1} mg^{-1} protein, respectively). Hypercapnia did not affect ATP-dependent Ca^{2+} transport; the experimental transport value of 4.08 ± 0.54 nmol Ca^{2+} min^{-1} mg^{-1} protein was not significantly different from the control value of 3.76 ± 0.49 nmol Ca^{2+} min^{-1} mg^{-1} protein (Table 2.1). Enrichment of the basolateral membranes was confirmed via a 5' cyclic AMP assay. Enrichment was found to be the same as values obtained from the Na^+,K^+-ATPase assay.

Figure 2.1 The effects of 48 h of normocapnia (unfilled bars) or environmental hypercapnia (1% CO_2 in air, P_wCO_2 = 7.6 torr; filled bars) on gill filament chloride cell (CC) morphometry, including (A) exposed apical surface area of individual CCs, (B) exposed CC density and (C) gill chloride cell fractional area. Values shown are means ± 1 SEM; N = 6 for each group. * indicates a significant difference from a corresponding value in normocapnic fish (t-test; $P \leq 0.05$).

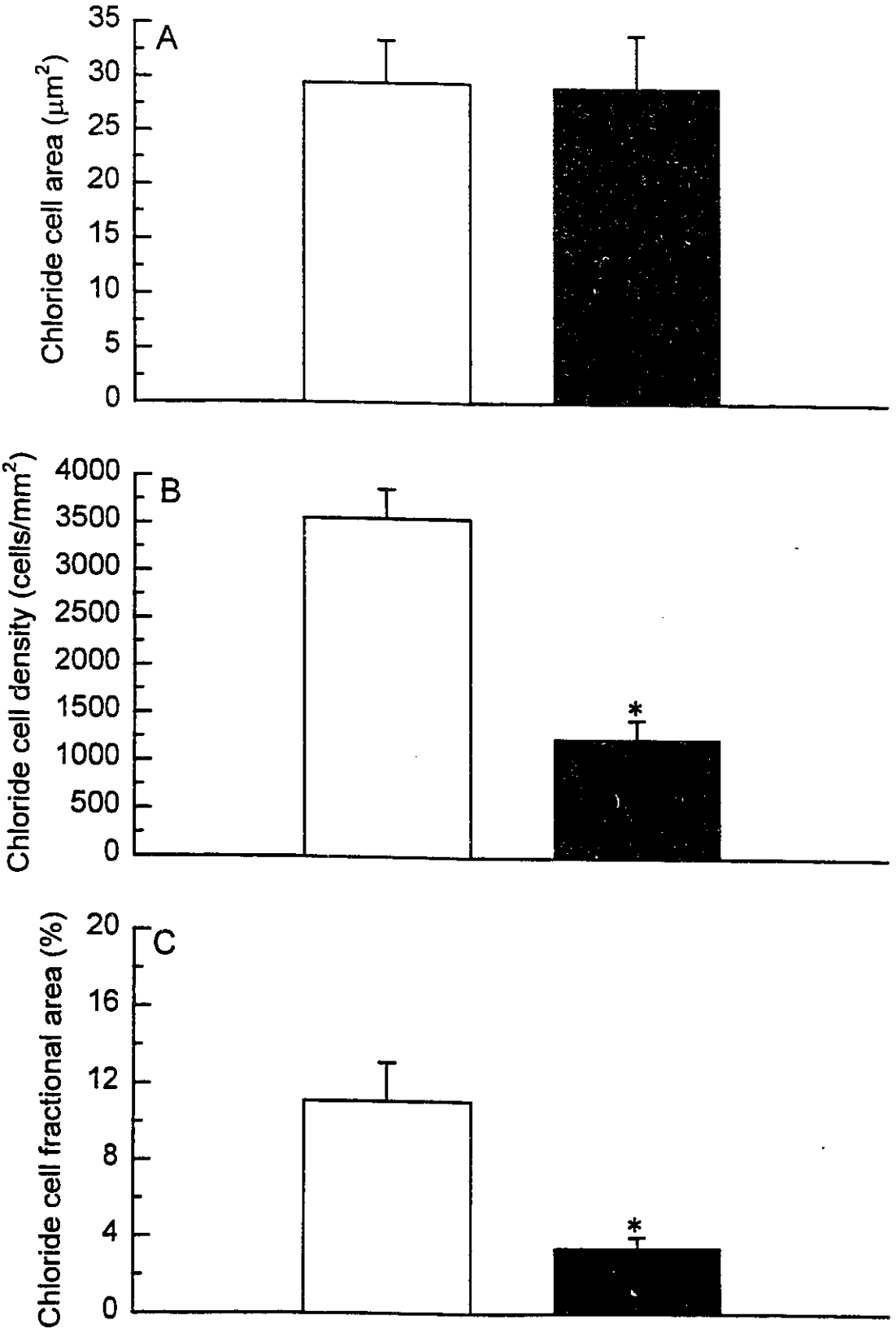

A
Chloride cell area (μm²)
35
30
25
20
15
10
5
0
B
Chloride cell density (cells/mm²)
4000
3500
3000
2500
2000
1500
1000
500
0
*
C
Chloride cell fractional area (%)
20
16
12
8
4
0
*

Figure 2.2 The effects of 48 h of environmental hypercapnia (1% CO_2 in air, P_wCO_2 = 7.6 torr; circles) or normocapnia (triangles) on (A) plasma total [calcium] and (B) plasma ultrafiltrable [calcium]. Values shown are means ± 1 SEM; N numbers are indicated above or below the data points. * indicates a significant difference from a corresponding value in normocapnic fish (t-test; $P \leq 0.05$). † indicates a significant difference from preflux value (one-way repeated measures ANOVA; $P \leq 0.05$).

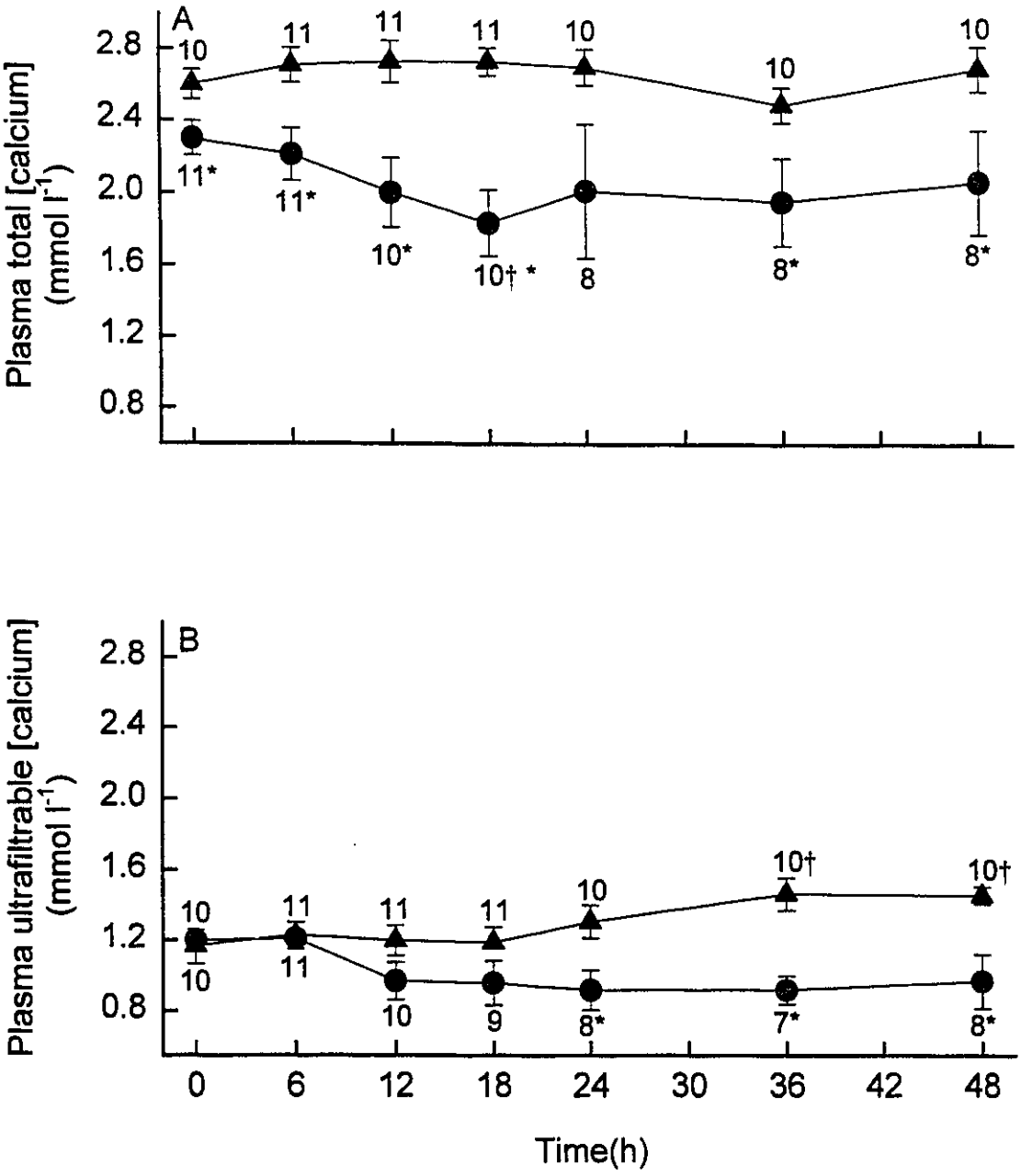

A
Plasma total [calcium] (mmol l-1)
2.8
2.4
2.0
1.6
1.2
0.8
10
11
11
11
10
10
10
11*
11*
10*
10† *
8
8*
8*
B
Plasma ultrafiltrable [calcium] (mmol l-1)
2.8
2.4
2.0
1.6
1.2
0.8
10
11
11
11
10
10†
10†
10
11
10
9
8*
7*
8*
0
6
12
18
24
30
36
42
48
Time(h)

Figure 2.3 The effects of normocapnia (unfilled bars) or environmental hypercapnia (1% CO_2 in air, P_wCO_2 = 7.6 torr; filled bars) on (A) whole body calcium influx ($J_{in}Ca^{2+}$) and (B) branchial calcium efflux ($J_{out}Ca^{2+}$). Values shown are means ± 1 SEM; N values are indicated above the error bars. * indicates a significant difference from a corresponding value in normocapnic fish (t-test; $P \leq 0.05$). † indicates a significant difference from preflux value (one-way ANOVA; $P \leq 0.05$).

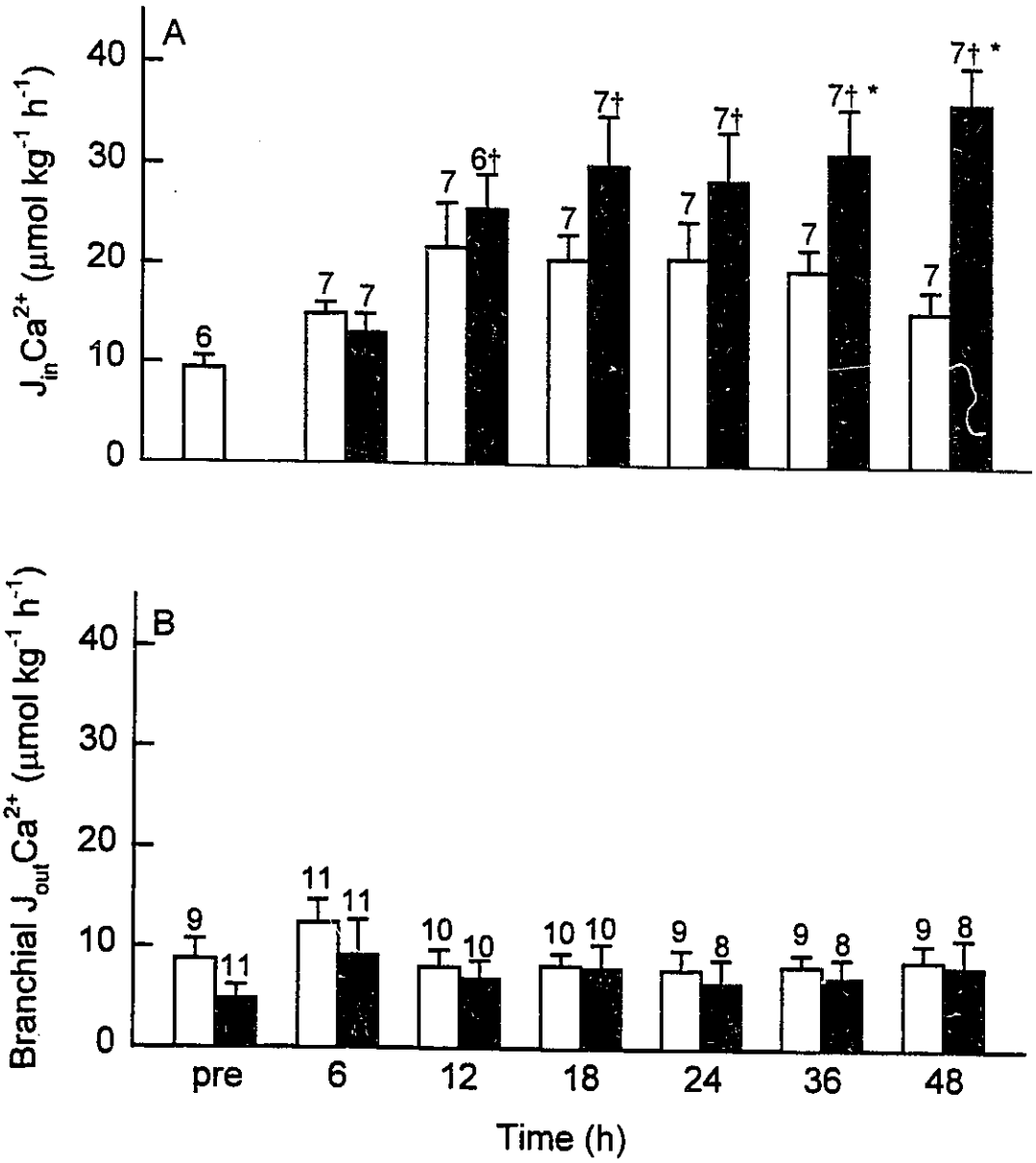

A
$J_{in}Ca^{2+}$ (µmol kg^{-1} h^{-1})
40
30
20
10
0
6
7
7
7
6†
7
7†
7
7†
7
7† *
7
7† *
B
Branchial $J_{out}Ca^{2+}$ (µmol kg^{-1} h^{-1})
40
30
20
10
0
9
11
11
11
10
10
10
10
9
8
9
8
9
8
pre
6
12
18
24
36
48
Time (h)

Figure 2.4 The effect of environmental hypercapnia (1% CO_2 in air, P_wCO_2 = 7.6 torr; circles) or normocapnia (triangles) on (A) urine total [calcium], (B) urine flow rate (UFR), and (C) renal calcium efflux ($J_{out}Ca^{2+}$). Values shown are means ± 1 SEM; N numbers are indicated above or below the data points. * indicates a significant difference from a corresponding value in normocapnic fish. † indicates a significant difference from preflux value (one-way repeated measures ANOVA; $P \leq 0.05$).

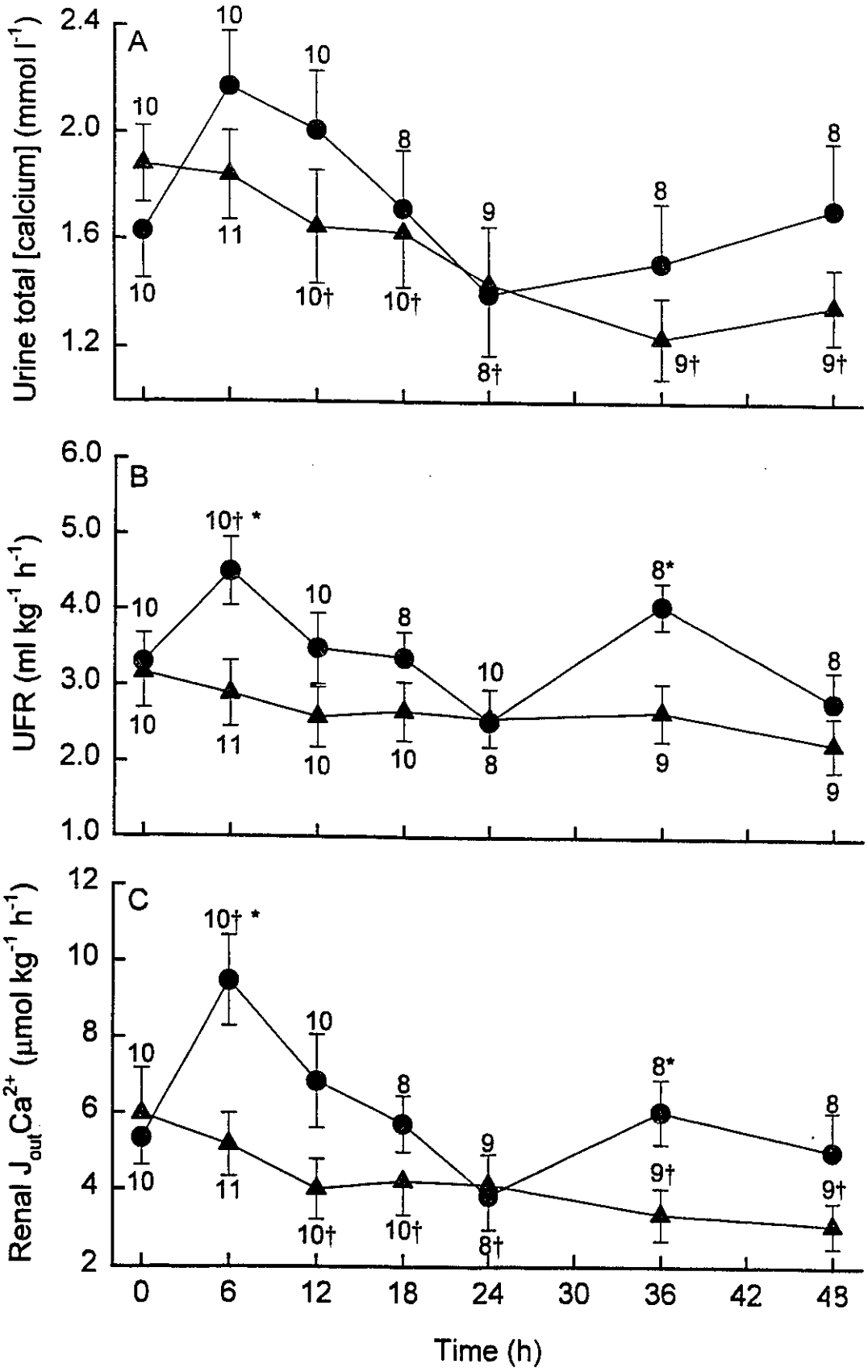
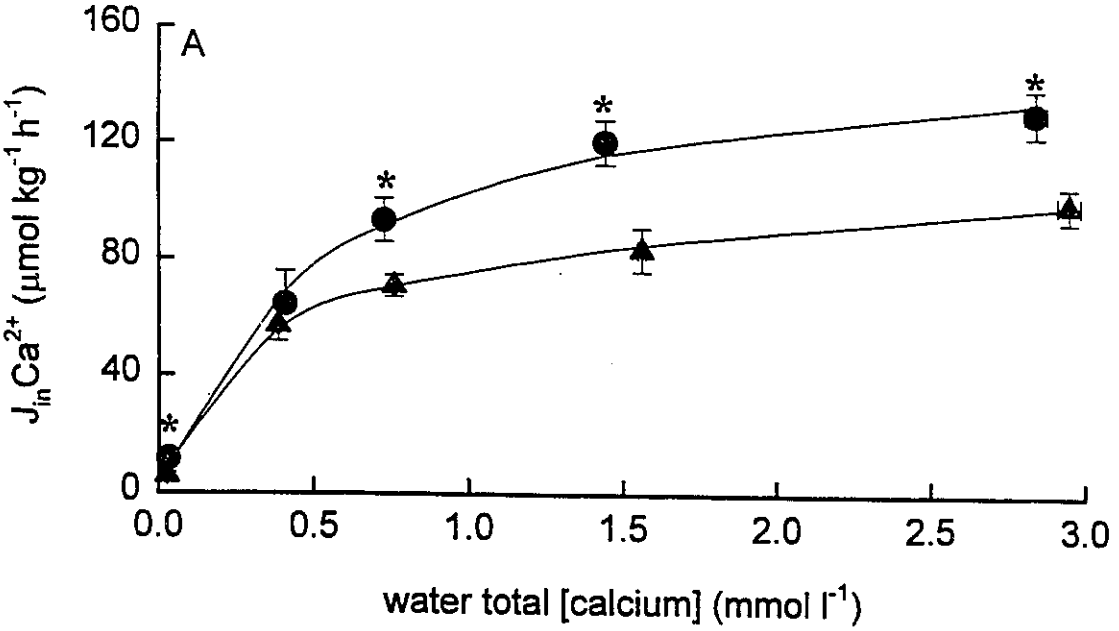

A
Urine total [calcium] (mmol l-1)
2.4
2.0
1.6
1.2
B
UFR (ml kg-1 h-1)
6.0
5.0
4.0
3.0
2.0
1.0
C
Renal Jout Ca2+ (µmol kg-1 h-1)
12
10
8
6
4
2
0
6
12
18
24
30
36
42
48
Time (h)

Figure 2.5 (A) Whole body calcium influx ($J_{in}Ca^{2+}$) in rainbow trout (*Oncorhynchus mykiss*) at various water [Ca^{2+}] following exposure to 48 h of environmental hypercapnia (1% CO_2 in air, P_wCO_2 = 7.6 torr; circles) or normocapnia (triangles). (B) Kinetic analysis of $J_{in}Ca^{2+}$ in rainbow trout following exposure to 48 h of environmental hypercapnia. Results are presented as an Eadie-Hofstee plot. Exposure to 48 h of environmental hypercapnia caused a significant (t-test, $P \leq 0.05$) increase in V_{max} (control V_{max} = 103.1 ± 6.2 μmol kg^{-1} h^{-1}; hypercapnia V_{max} = 140.2 ± 9.8 μmol kg^{-1} h^{-1}; P = 0.006) but not in K_m values (control K_m = 0.39 ± 0.07 mmol l^{-1}; hypercapnia K_m = 0.38 ± 0.08 mmol l^{-1}; P = 0.92). * indicates a significant difference from a corresponding value in normocapnic fish. N = 6 for all groups.


A
160
120
80
40
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
$J_{in}Ca^{2+}$ (µmol kg^{-1} h^{-1})
water total [calcium] (mmol l^{-1})

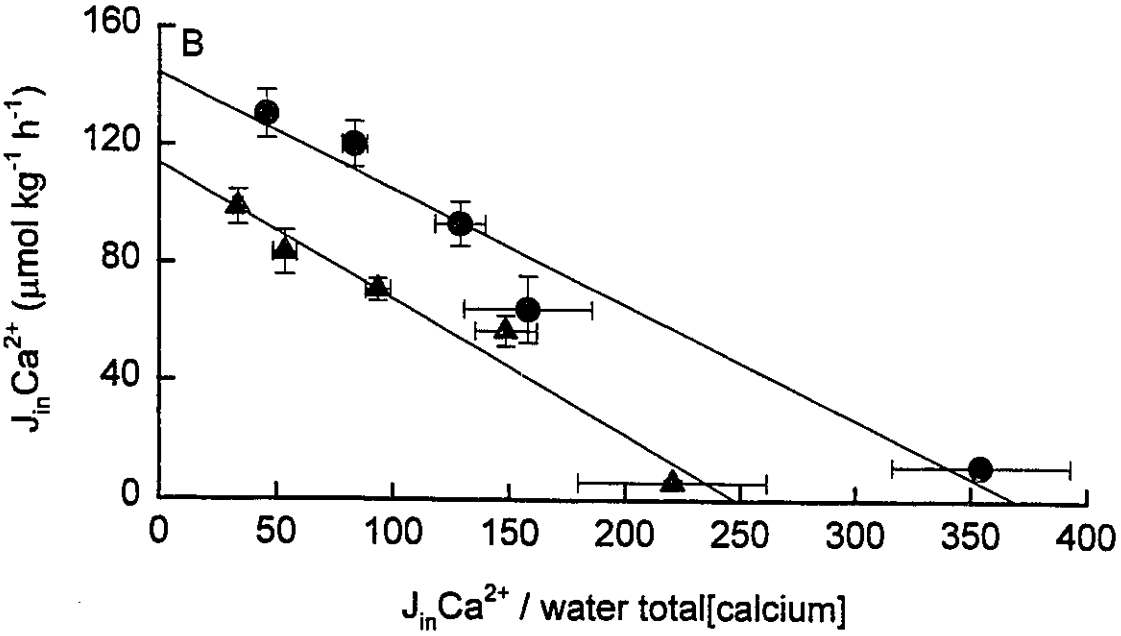

B
160
120
80
40
0
0
50
100
150
200
250
300
350
400
$J_{in}Ca^{2+}$ (µmol kg^{-1} h^{-1})
$J_{in}Ca^{2+}$ / water total[calcium]

Table 2.1 Protein percentage recoveries, Na^+,K^+-ATPase and Ca^{2+}-ATPase total and specific activities, and ATP-dependent Ca^{2+} transport values from gill basolateral plasma membranes prepared from rainbow trout (*Oncorhynchus mykiss*) exposed to 48 h of normocapnia (N = 9) or hypercapnia (N = 9). Values shown are means ± 1 SEM.

	Normocapnic		Hypercapnic	
	[1]Ho	[2]P3	[1]Ho	[2]P3
Body mass (g)	332.4 ± 14.0		307.4 ± 12.4	
[3]Protein percentage recovery (%)	1.20 ± 0.09		1.05 ± 0.06	
[4]Total Na^+,K^+-ATPase	1028 ± 337	184.9 ± 13.4	1379 ± 478	226.9 ± 36.9
[5]V_{spec} Na^+,K^+-ATPase	1.71 ± 0.58	25.11 ± 1.89	2.14 ± 0.76	33.40 ± 5.33
[4]Total Ca^{2+}ATPase	204.8 ± 71.5	81.5 ± 13.4	355.1 ± 81.1	60.2 ± 8.5
[5]V_{spec} Ca^{2+}-ATPase	0.33 ± 0.11	10.73 ± 1.58	0.55 ± 0.13	9.26 ± 1.53
[6]ATP-dependent Ca^{2+} transport	3.75 ± 0.48		4.08 ± 0.54	

[1]Ho is designated as the supernatant that remains after the first centrifugation.

[2]P3 is designated as the final pellet which contains the basolateral membrane vesicles.

[3]Protein percentage recoveries are expressed as percentages of total protein in Ho.

[4]Total activity is equal to the product of V_{spec} and total protein.

[5]Na^+,K^+-ATPase and Ca^{2+}-ATPase specific activities (V_{spec}) are expressed as μmol Pi h^{-1} mg^{-1} protein at 37° C.

[6]ATP-dependent Ca^{2+} transport values are expressed as nmol Ca^{2+} min^{-1} mg^{-1} protein at 37° C.

DISCUSSION

The results of the present investigation indicated that the reduction in exposed chloride cell surface area caused by exposure of rainbow trout to environmental hypercapnia (Goss *et al.*, 1992b and 1995; Goss and Perry, 1993; present investigation) did not impair whole body calcium uptake ($J_{in}Ca^{2+}$) and thus our initial hypothesis predicting a reduced rate of $J_{in}Ca^{2+}$ during hypercapnia was not supported. Although chloride cell fractional area (CCFA) decreased by 68% after 48 h of hypercapnia exposure, whole body $J_{in}Ca^{2+}$ was significantly increased. Previous studies have demonstrated *in vivo* (Perry *et al.*, 1992a) or *in vitro* (Marshall *et al.*, 1992; McCormick *et al.*, 1992) that $J_{in}Ca^{2+}$ is directly proportional to chloride cell numbers or surface area when measured under steady state conditions. In hypercapnic trout, there was a reduction in chloride cell surface area yet $J_{in}Ca^{2+}$ was stimulated; this implies that mechanisms were activated to compensate for the loss of transport cell surface area.

In an attempt to elucidate the mechanism(s) of increased $J_{in}Ca^{2+}$ during hypercapnia, the kinetics of whole body $J_{in}Ca^{2+}$ were investigated. The results demonstrated that the stimulation of $J_{in}Ca^{2+}$ was a consequence of an elevated maximum transport velocity (V_{max}) and that the affinity (K_m) of the Ca^{2+} uptake process (all steps considered) was unaffected. The elevated rate of Ca^{2+} uptake in these fish compared to the rates seen in the fish used only in the determination of whole body $J_{in}Ca^{2+}$ was due to the smaller size of the fish (Flik *et al.*, 1986).

To uncover the mechanism(s) underlying the increased V_{max}, Ca^{2+}-ATPases activity and ATP-dependent Ca^{2+} transport were measured *in vitro* using isolated gill basolateral membrane vesicles. These experiments showed that neither Ca^{2+}-ATPase activity nor ATP-dependent Ca^{2+} transport were significantly altered by 48 h of hypercapnia. This suggests that the enhancement of $J_{in}Ca^{2+}$ during hypercapnia was related to modulation of other mechanisms involved in Ca^{2+} uptake.

Possible mechanisms of increased $J_{in}Ca^{2+}$ during hypercapnia

The results of the present study appear to exclude a specific modification of basolateral membrane ATP-dependent Ca^{2+} transport as a mechanism of increased $J_{in}Ca^{2+}$ during hypercapnia. Based on current models of trans-epithelial Ca^{2+} uptake in fish (Flik *et al.*, 1993 and 1995), other potential sites of control include the apical membrane Ca^{2+} channels or the basolateral Na^{+}/Ca^{2+} exchanger.

It is possible that a change in the number of apical membrane Ca^{2+} channels or in their permeability characteristics may have contributed to the increased whole body $J_{in}Ca^{2+}$ during hypercapnia. Indeed, it has been suggested that changes in apical membrane permeability play an important role in the control of calcium uptake in freshwater teleosts (Pang *et al.*, 1980; Mayer-Gostan *et al.*, 1983; Verbost *et al.*, 1993; Flik *et al.*, 1995). A major regulator of the chloride cell apical membrane presumptive Ca^{2+} channel is the calciotropic hormone stanniocalcin (e.g. Flik *et al.*, 1993). Stanniocalcin is known to affect branchial calcium influx; removal or destruction of the teleost corpuscles of Stannius

results in increased $J_{in}Ca^{2+}$ (Fenwick and So, 1974; Pang *et al.*, 1980; Perry *et al.*, 1989; Verbost *et al.*, 1993) while treatment with stanniocalcin results in decreased $J_{in}Ca^{2+}$ (Lafeber *et al.*, 1988; Perry *et al.*, 1989; Verbost *et al.*, 1989). Lafeber *et al.* (1988) and Verbost *et al.* (1989) hypothesized that stanniocalcin affects the apical Ca^{2+} channel of gill chloride cells. In the present study, the apical entry step of Ca^{2+} may have been accelerated during hypercapnia by reduced stanniocalcin secretion or blocking of receptors responsible for stannniocalcin's effect on the gill.

Neither Na^+/Ca^{2+} exchanger activity nor Na^+-dependent Ca^{2+} transport were assayed in the present study. In recent years, however, substantial evidence has accumulated to implicate a Na^+/Ca^{2+} exchanger in the movement of Ca^{2+} across the basolateral membrane of gill chloride cells (Flik and Verbost, 1993; Flik *et al.*, 1993; Verbost *et al.*, 1994; Flik *et al.*, 1995). At common physiological intracellular Ca^{2+} concentrations (< 1 μmol l^{-1}), the Na^+/Ca^{2+} exchanger, owing to its lower affinity for Ca^{2+}, is likely to be less important than the high affinity Ca^{2+}-ATPase. However, at intracellular Ca^{2+} concentrations of 1 μmol l^{-1} or greater, the Na^+/Ca^{2+} exchanger becomes more important in moving Ca^{2+} across the basolateral membrane (Flik *et al.*, 1993; Verbost *et al.*, 1994). It is possible, as suggested by Flik and Verbost (1993), that Na^+/Ca^{2+} exchangers function in areas of locally elevated Ca^{2+} concentrations, such as near the apical membrane Ca^{2+} channels. Thus, modulation of the Na^+/Ca^{2+} exchanger, also must be considered as a possible mechanism contributing to the increase in $J_{in}Ca^{2+}$ during hypercapnia.

Physiological significance of increased $J_{in}Ca^{2+}$ during hypercapnia

Branchial $J_{out}Ca^{2+}$ did not change during hypercapnia while renal Ca^{2+} excretion was increased transiently and only slightly (<4 μmol kg^{-1} h^{-1}). Thus, the increase in whole body $J_{in}Ca^{2+}$ during hypercapnia (~20 μmol kg^{-1} h^{-1} after 48 h) clearly was not a compensatory response to counteract increased Ca^{2+} losses. We suggest that the physiological significance of the increased $J_{in}Ca^{2+}$ is related to acid-base regulation. Fish regulate respiratory acidosis by accumulating plasma HCO_3^- (see review by Heisler, 1993). Traditionally, the compensatory rise in plasma [HCO_3^-] has been attributed to dynamic modulation of gill epithelial apical membrane ionic exchangers/transporters (reviewed by Goss *et al.*, 1995). Specifically, fish increase branchial net acid excretion during hypercapnia by a reduction of Cl^-/HCO_3^- exchange and an increase in H^+ excretion via a vacuolar type H^+-ATPase (Lin *et al.*, 1994; Sullivan *et al.*, 1995). Current models of trans-epithelial Ca^{2+} uptake in fish indicate that the pumping of Ca^{2+} across the basolateral membrane via the Ca^{2+}-ATPase is coupled to inward movement of H^+ ions (Flik *et al.*, 1993; Flik and Verbost, 1994; see Figure 4.1). Thus, the increased rate of $J_{in}Ca^{2+}$ during hypercapnia may be a supplementary mechanism to remove H^+ ions from the blood compartment and thereby raise plasma [HCO_3^-]. Because plasma [Ca_t] and [Ca_{uf}] (a measure of ionic Ca^{2+}) were virtually constant during hypercapnia (see also Andreasen, 1985), the additional Ca^{2+} entering the blood must have been transferred to other internal compartments such as bone. Owing to the constraints of electroneutrality, such transfer of Ca^{2+} would be accompanied by equimolar influx of cations or efflux of anions into or from

the blood compartment, respectively; this would serve to maintain the changes in plasma [HCO_3^-] accompanying the increased branchial influx of Ca^{2+}.

CHAPTER 3

BRANCHIAL AND RENAL CALCIUM FLUXES IN RAINBOW TROUT (*Oncorhynchus mykiss*) DURING METABOLIC ALKALOSIS

INTRODUCTION

Although it is well established that acid-base disturbances in freshwater fish are compensated for by adjustments of branchial ion fluxes, the underlying mechanisms remain uncertain (Cameron, 1976; McDonald and Wood, 1981; Wood *et al.*, 1984; Claiborne and Heisler, 1986; Perry *et al.*, 1987a and b; Goss and Wood, 1990a and b; Goss *et al.*, 1992a and b; Goss and Perry, 1993 and 1994). A recent model (see Goss *et al.*, 1992b; see Figure 1.1) proposes that alteration in the structure of the gill epithelium is used as a mechanism of regulation. Specifically, it was shown that acid-base disturbances cause branchial pavement and chloride cells to change their shape, thus altering the proportion of each cell type exposed to the environment. Internal alkalosis elicits an increase in the exposed chloride cell surface area, while internal acidosis elicits a decrease (Goss *et al.*, 1992a and b; Goss and Perry, 1993 and 1994; Goss *et al.*, 1994a and b; Perry and Goss, 1994). Such changes in the conformation of the gill epithelium are thought to influence acid-base regulation by affecting the branchial net fluxes of Na^+, Cl^-, H^+ and HCO_3^- (Goss and Perry, 1994; Perry and Goss, 1994). Such structural changes, however, could potentially affect Ca^{2+} movement across the gills. This is because the gill chloride cell is believed to be the principle site of Ca^{2+} uptake in freshwater teleosts (Payan *et al.*, 1981; Flik *et al.*, 1985c; Perry and Wood, 1985; Perry and Flik, 1988; Marshall *et al.*, 1992; McCormick *et al.*, 1992; Perry *et al.*, 1992a; reviewed by Fenwick, 1989). Since Ca^{2+} uptake from the environment was found to be directly proportional to exposed chloride cell numbers or surface area (McCormick *et al.*, 1992; Perry *et al.*, 1992a), conformational alterations in chloride cell surface area during acid-base disturbances could potentially influence Ca^{2+} fluxes. In the present study, we hypothesized that a metabolic

alkalosis would cause an increase in whole body Ca^{2+} uptake in rainbow trout (*Oncorhynchus mykiss*) due to the increased branchial chloride cell surface area that is known to occur in this species under such conditions (see Goss *et al.*, 1994b; Goss and Perry, 1994; Perry and Goss, 1994). To test this hypothesis, branchial and renal Ca^{2+} fluxes *in vivo* and basolateral Ca^{2+} transport *in vitro* were assessed in rainbow trout infused with either NaCl or $NaHCO_3$.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) weighing between 123 and 659 g (mean mass = 281.7 ± 8.6 g SEM, N = 206) were obtained from Linwood Acres Trout Farm, Campbellcroft, Ontario, and transported to the University of Ottawa in oxygenated water. Fish were held indoors in large fiberglass tanks supplied with flowing, aerated, dechlorinated, City of Ottawa tap water ($[Na^+]$ = 0.15 mmol l^{-1}; $[Cl^-]$ = 0.15 mmol l^{-1}; $[K^+]$ = 0.02 mmol l^{-1}; $[Ca^{2+}]$ = 0.40 mmol l^{-1}; pH = 7.5 to 8.0; temperature = 15° C) under a constant photoperiod of 12 hours light : 12 hours dark. Fish were fed daily with commercial trout pellets.

Surgical procedures

Cannulation of the dorsal aorta of rainbow trout was conducted as described in Chapter 2.

Experimental protocol

Cannulated rainbow trout were placed into individual opaque acrylic flux boxes (approximate volume = 3.6 L) and were allowed to adjust to these conditions for at least 19 hours (h) prior to commencing experiments. Water flow was maintained at 750 ml min^{-1} box^{-1}. Fish were separated into two groups, experimentals (alkalotic fish) and controls. Experimental fish used to determine the rates of calcium influx ($J_{in}Ca^{2+}$) and calcium efflux ($J_{out}Ca^{2+}$) were infused via the dorsal aorta cannula with 140 mmol l^{-1} $NaHCO_3$ for 6, 12, 18, 24, 36, or 48 h at an infusion rate of 800 μmol kg^{-1} h^{-1}. Experimental fish used to isolate gill basolateral

membranes and to study Ca^{2+} uptake kinetics were infused for 48 h only. Each experimental group had its own corresponding control group. Control fish were infused with 140 mmol l^{-1} NaCl for the same duration and at the same rate as their experimental counterparts. Solutions were infused using a Manostat cassette pump. During flux periods, water flow was stopped, and air was bubbled into individual boxes. Plasma [Na^+] and [Cl^-] have been found not to change during such a regime (Goss *et al.*, 1994b).

Determination of $J_{in}Ca^{2+}$

Whole body calcium influx ($J_{in}Ca^{2+}$) was determined as described in Chapter 2. The only modification made was that the amount of water added to carcasses before homogenization was decreased to 50% of body weight.

Determination of $J_{out}Ca^{2+}$

Branchial calcium efflux (branchial $J_{out}Ca^{2+}$) was determined as described in Chapter 2.

Whole blood pH was determined using a microcapillary pH electrode (G299 A; Radiometer) housed within a BMS 3 MK2 blood micro system (Radiometer) at 15° C and attached to a meter (PHM 71 Acid-Base Analyzer; Radiometer). Red blood cells were separated from plasma, resuspended in an equal volume of Cortland saline, and re-injected into the fish. Plasma was analyzed further for total CO_2 (50 µl samples; Corning 965 Carbon Dioxide Analyzer) and total [calcium] (Ca_t; ionic and bound forms of calcium). Plasma [Ca_t], water [Ca_t], and water and plasma radioactivities were determined as described in Chapter 2.

To allow urine sampling, each fish was fitted with an internal bladder catheter as described in Chapter 2.

Although 10 experimental and 10 control fish were originally set up for either $NaHCO_3$ or NaCl infusion, over the duration of the infusion regime, some fish were able to remove dorsal aortic cannulae and/or bladder catheters. These fish were excluded from the study and only data from fish that participated in all sampling periods were used.

Calcium uptake kinetics

The protocol used in the determination of calcium uptake kinetics was identical to that used in Chapter 2 except that 2 modifications were made. First, the replacement water [Ca_t] was either 0.025, 0.1, 0.4, 0.8, 1.6, or 3.2 mmol l^{-1}. Second, the amount of ^{45}Ca added to each box was sufficient to provide an average water specific activity of approximately 300 000 DPM/μmol Ca.

Gill plasma membrane transport

Gill basolateral membrane isolation - Gill basolateral plasma membrane vesicles were prepared and assays were conducted as described in Chapter 2. The one modification made was that the assay for ATP-dependent Ca^{2+} transport was not conducted on the original homogenate (Ho).

Calculations

Whole body $J_{in}Ca^{2+}$, branchial and renal $J_{out}Ca^{2+}$ were calculated using the formulae presented in the previous chapter. Branchial $J_{net}Ca^{2+}$ was calculated as follows:

$$J_{net}Ca^{2+} = \frac{([Ca_i] - [Ca_f]) \cdot V}{T \cdot W}$$

where $[Ca_i]$ and $[Ca_f]$ are the initial and final water Ca^{2+} concentrations, respectively, in μmol ml^{-1}, V is the external water volume in ml, T is the flux duration in hours, and W is the mass of the fish in kg (Hobe and Laurent, 1984).

Statistical analysis

Values shown in figures and tables are sample means ± 1 SEM. Unpaired Student's t-tests were used to compare sample means of experimental and corresponding control groups. One-way ANOVA ($J_{in}Ca^{2+}$ data) /one-way ANOVA, repeated measures (blood, plasma, urine data and $J_{out}Ca^{2+}$ data) and Dunnett's tests were used to compare sample means among experimental and control groups; 5% was taken as the fiducial limit of significance.

RESULTS

Blood acid-base status and plasma [Ca_t]

Infusion of trout with $NaHCO_3$ caused significant increases in both blood pH and plasma [HCO_3^-] (Figures 3.1A and B). After 6 h of infusion, blood pH had increased significantly from a pre-infusion value of 7.82 ± 0.03 to 8.03 ± 0.04. Blood pH continued to increase until 18 h and then stabilized for the duration of the experiment (Figure 3.1A).

Plasma [HCO_3^-] was significantly increased during $NaHCO_3$ infusion over the pre-infusion value and corresponding control values at all sampling periods following the pre-infusion sampling. Although infusion of NaCl did not affect blood pH, it did, unexpectedly, affect plasma [HCO_3^-], elevating it slightly over the pre-infusion value at 18 h (Figure 3.1B).

Both $NaHCO_3$-infused experimental and NaCl-infused control trout experienced significant decreases in plasma [Ca_t] after 6 h of infusion. Control trout were able to restore and maintain plasma Ca_t concentrations at all subsequent sampling times. In contrast, the experimental fish displayed significantly lower plasma [Ca_t] until 48 h (Table 3.1).

$J_{in}Ca^{2+}$ and $J_{out}Ca^{2+}$

Infusion of either $NaHCO_3$ or NaCl caused significant increases in whole body $J_{in}Ca^{2+}$ over pre-infusion values. Whole body $J_{in}Ca^{2+}$ in NaCl infused control trout was increased after 6 h and remained relatively constant thereafter. Whole body $J_{in}Ca^{2+}$ in $NaHCO_3$ infused experimental trout, however, continued to increase such that at all sampling periods after 6 h, $J_{in}Ca^{2+}$ was significantly higher than control values (Figure 3.2A). Branchial $J_{out}Ca^{2+}$ was largely unaffected by NaCl or $NaHCO_3$ infusion (Figure 3.2B).

A high degree of variability in the $J_{net}Ca^{2+}$ data prevented statistical confirmation, yet there was an obvious trend for elevated $J_{net}Ca^{2+}$ in the $NaHCO_3$-infused fish (Figure 3.2C).

Renal variables

Urine $[Ca_t]$ was significantly decreased in both experimental and control fish during the 48 h infusion regime (Figure 3.3A). There was an obvious trend toward increased urine flow rate (UFR) in both groups of fish although a high degree of variability precluded statistical confirmation (Figure 3.3B). Renal $J_{out}Ca^{2+}$ remained constant during the 48 h infustion regime (Figure 3.3C).

Calcium uptake kinetics

Trout infused for 48 h with $NaHCO_3$ or NaCl displayed typical Michaelis-Menten kinetics for $J_{in}Ca^{2+}$ (Figure 3.4). It should be noted that the fish used in the kinetics experiments exhibited higher rates of $J_{in}Ca^{2+}$ than the fish in previous experiments (Figure 3.2A). This can be attributed to differences in fish stocks and season (Wagner *et al.*, 1986). Importantly, however, the NaCl and $NaHCO_3$ infusion experiements were performed concurrently.

The V_{max} and K_m values of both $NaHCO_3$-infused and NaCl-infused trout were determined from a Lineweaver-Burk transformation of the data shown in Figure 3.4. The V_{max} and K_m values of fish infused with $NaHCO_3$ for 48 h were 96.6 ± 8.2 μmol kg^{-1} h^{-1} and 0.13 ± 0.03 mmol l^{-1}, respectively. The V_{max} of these fish was significantly higher than the V_{max} of

NaCl-infused trout (74.0 ± 4.1 μmol kg^{-1} h^{-1}) whereas the K_m was not significantly different (control K_m = 0.12 ± 0.04 mmol l^{-1}).

Gill plasma membrane transport

Infusion of $NaHCO_3$ lead to significant increases in Na^+,K^+-ATPase total activity and Na^+,K^+-ATPase specific activity (V_{spec}) in both Ho (homogenate) and P3 (final pellet) samples. Values for protein percentage recovery, ATP-dependent Ca^{2+} transport, total Ca^{2+}-ATPase activity, and Ca^{2+}-ATPase specific activity in experimental trout were not significantly different from control values (Table 3.2).

Figure 3.1 The effects of 48 h of NaCl infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; triangles) or $NaHCO_3$ infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; circles) on (A) blood pH and (B) plasma [HCO_3^-]. Values shown are means ± 1 SEM; N numbers are indicated above or below data points. * indicates a significant difference from a corresponding value in NaCl-infused trout (t-test; $P \leq 0.05$). † indicates a significant difference from pre-infusion values (repeated measures one-way ANOVA; $P \leq 0.05$).

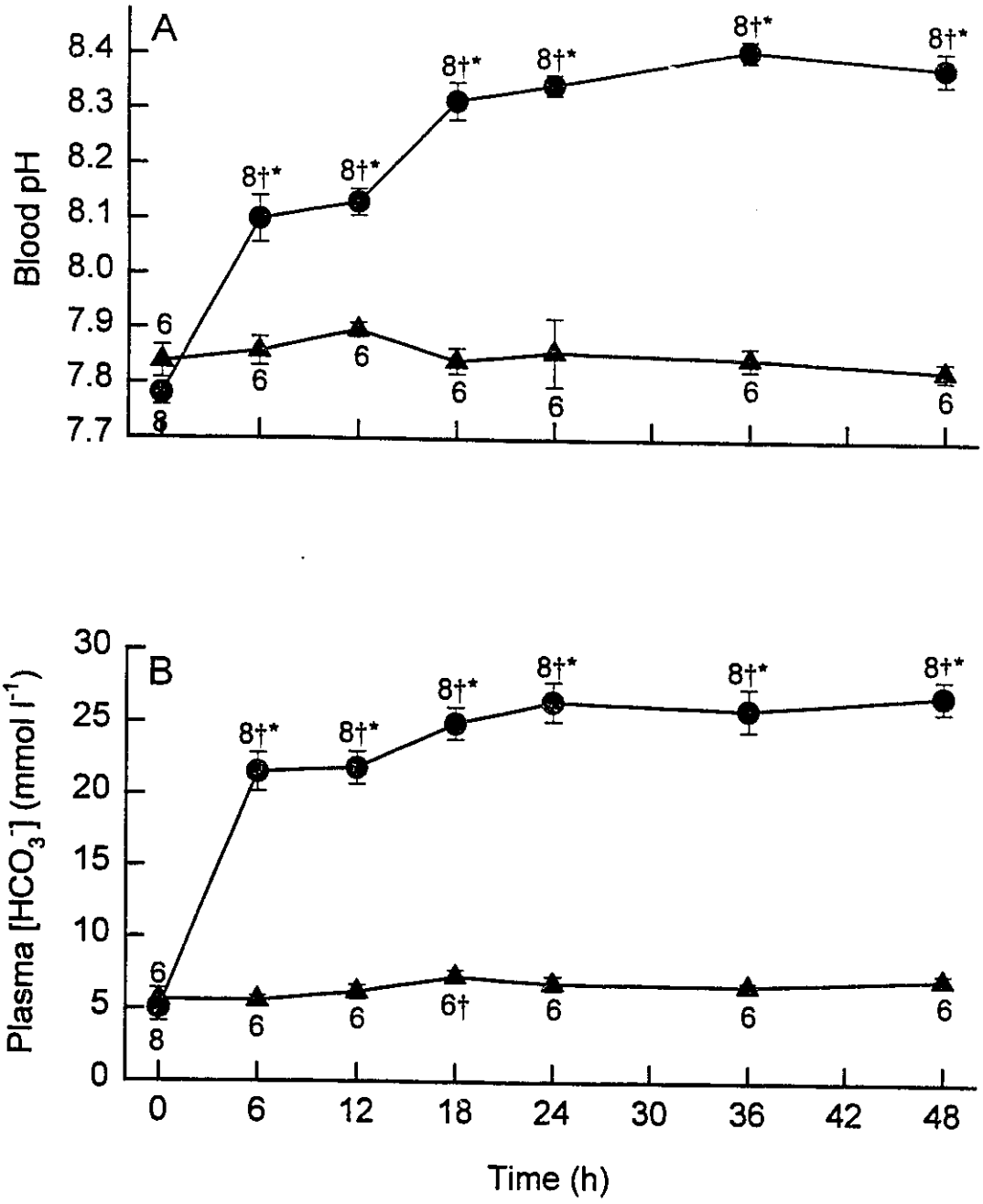

A
Blood pH
8.4
8.3
8.2
8.1
8.0
7.9
7.8
7.7
8†*
6
8
B
Plasma [HCO3-] (mmol l-1)
30
25
20
15
10
5
0
6†
0
6
12
18
24
30
36
42
48
Time (h)

Figure 3.2 The effects of 48 h of NaCl infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; unfilled bars) or $NaHCO_3$ infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; filled bars) on (A) whole body calcium influx ($J_{in}Ca^{2+}$), (B) branchial calcium efflux (branchial $J_{out}Ca^{2+}$) and (C) net branchial Ca^{2+} flux ($J_{net}Ca^{2+}$). Values shown are means ± 1 SEM; N numbers are indicated above or below data points. * indicates a significant difference from a corresponding value in NaCl-infused trout (t-test; $P \leq 0.05$). † indicates a significant difference from pre-infusion values (one-way ANOVA or repeated measures one-way ANOVA; $P \leq 0.05$).

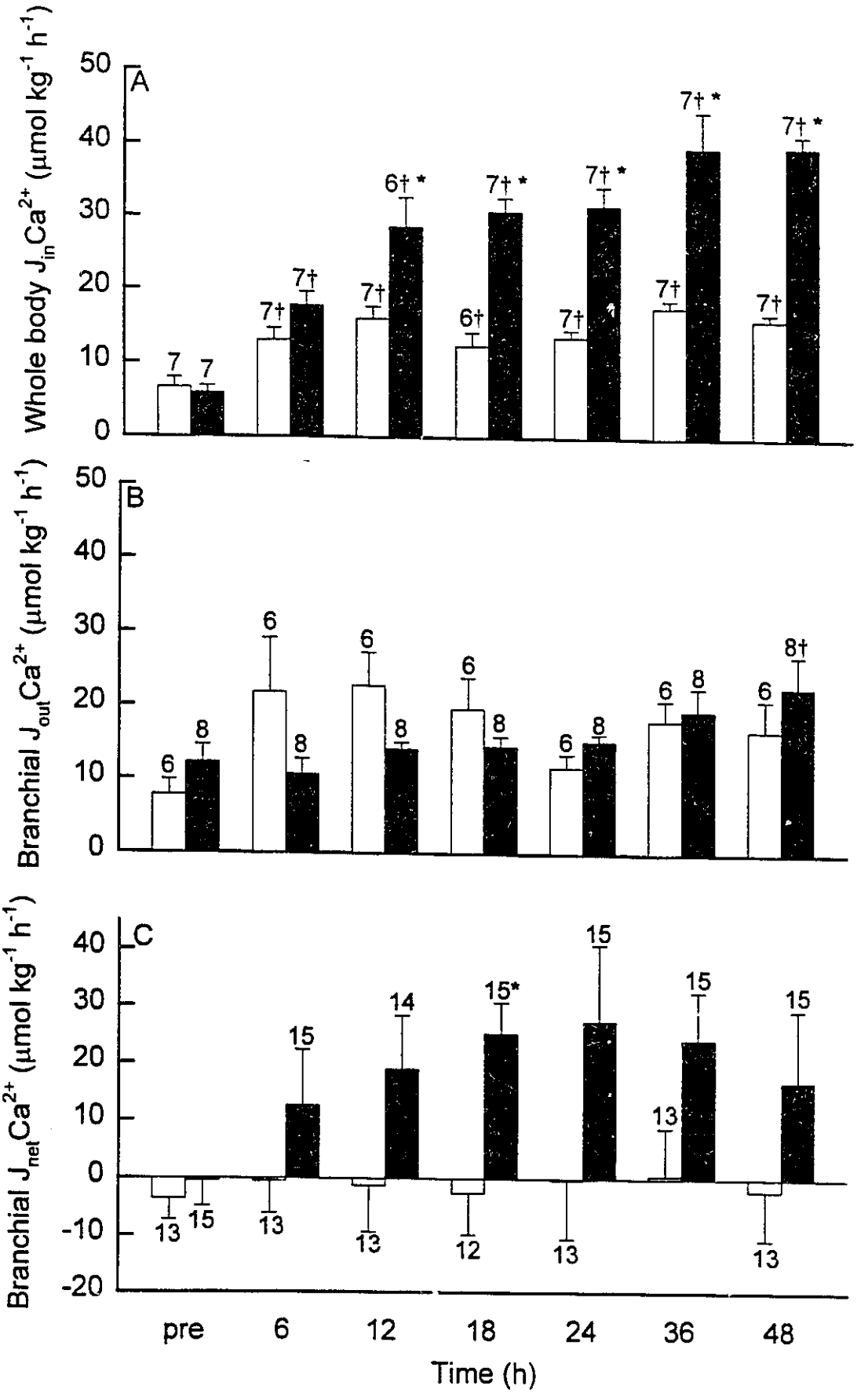

A
Whole body $J_{in}Ca^{2+}$ (μmol kg^{-1} h^{-1})
50
40
30
20
10
0
7 7
7† 7†
7† 7†
6† * 7† *
6† 7† *
7† 7† *
7† 7† *
7† 7† *
B
Branchial $J_{out}Ca^{2+}$ (μmol kg^{-1} h^{-1})
50
40
30
20
10
0
6 8
6 8
6 8
6 8
6 8
6 8
6 8
8†
C
Branchial $J_{net}Ca^{2+}$ (μmol kg^{-1} h^{-1})
40
30
20
10
0
-10
-20
13 15
13 15
13 14
13 15*
12 15
13 13
13 15
13 15
pre
6
12
18
24
36
48
Time (h)

Figure 3.3 The effects of 48 h of NaCl infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; triangles) or $NaHCO_3$ infusion (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; circles) on (A) urine total [calcium], (B) urine flow rate (UFR) and (C) renal calcium efflux (renal $J_{out}Ca^{2+}$). Values shown are means ± 1 SEM; N numbers are indicated above or below data points. * indicates a significant difference from a corresponding value in NaCl-infused trout (t-test; $P \leq 0.05$). † indicates a significant difference from pre-infusion values (repeated measures one-way ANOVA; $P \leq 0.05$).

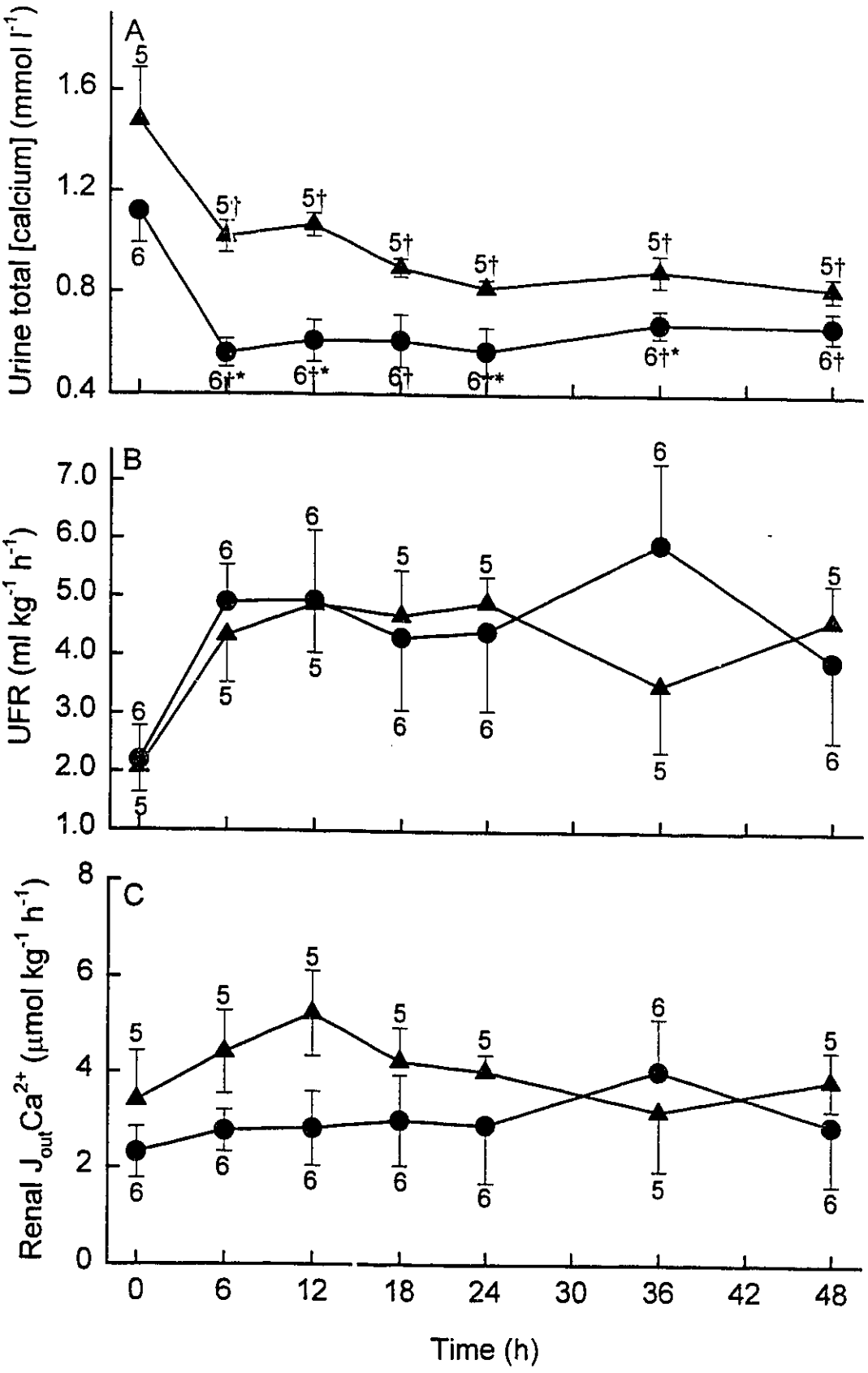

A
Urine total [calcium] (mmol l-1)
1.6
1.2
0.8
0.4
B
UFR (ml kg-1 h-1)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
C
Renal Jout Ca2+ (µmol kg-1 h-1)
8
6
4
2
0
0
6
12
18
24
30
36
42
48
Time (h)

Figure 3.4 Whole body calcium influx ($J_{in}Ca^{2+}$) in rainbow trout (*Oncorhynchus mykiss*) at various water [Ca_t] following ~8 h of infusion of NaCl (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; triangles) or $NaHCO_3$ (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; circles). Values shown are means ± 1 SEM; N = 6 for all groups. Values of fish infused with $NaHCO_3$ were 96.6 ± 8.2 μmol kg^{-1} h^{-1} and 0.13 ± 0.03 mmol l^{-1}, respectively. V_{max} and K_m values of NaCl-infused trout were 74.0 ± 4.1 μmol kg^{-1} h^{-1} and 0.12 ± 0.04 mmol l^{-1}, respectively. The V_{max} and K_m values of both $NaHCO_3$-infused and NaCl-infused trout were determined from a Lineweaver-Burk transformation of the data shown in Figure 3.4.

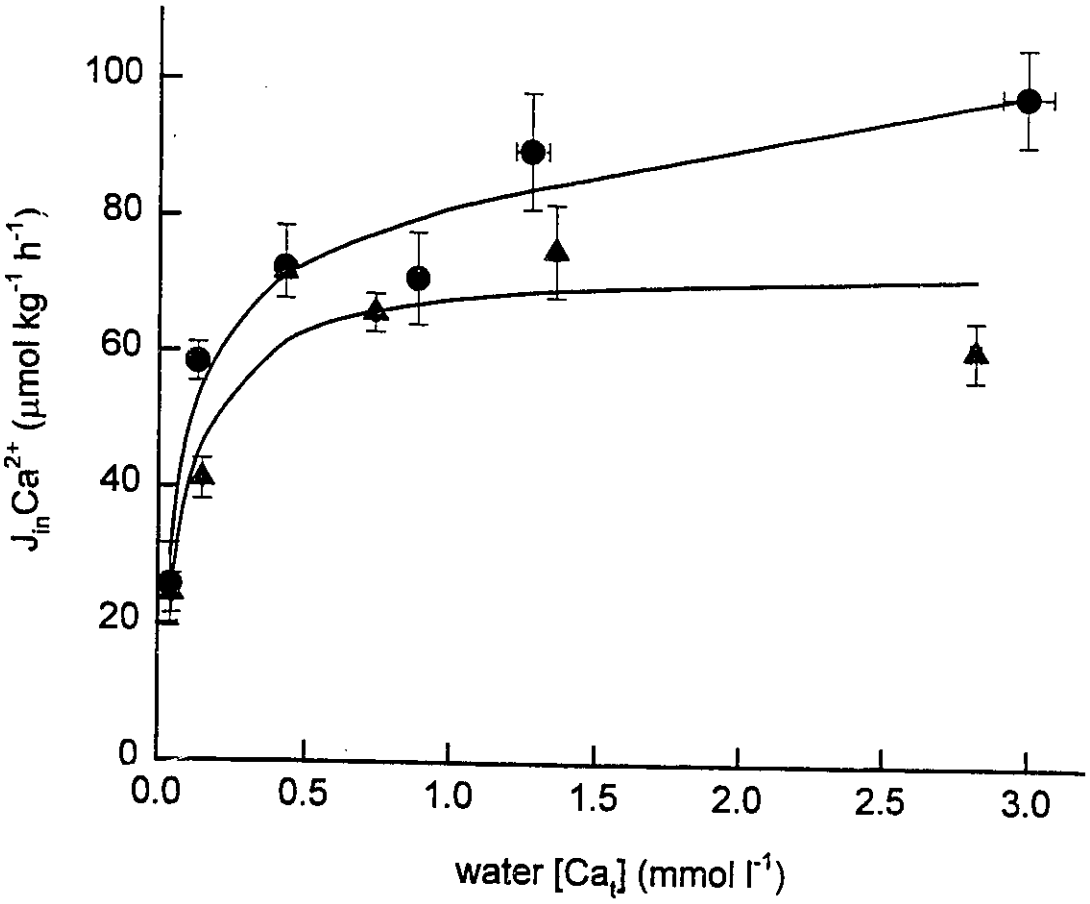

100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
$J_{in}Ca^{2+}$ (µmol kg^{-1} h^{-1})
water [Ca_t] (mmol l^{-1})

Table 3.1 Plasma total calcium concentrations in rainbow trout (*Oncorhynchus mykiss*) infused with NaCl (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}) or $NaHCO_3$ (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}) over a 48 h period. Values shown are means ± 1 SEM; N numbers are indicated in parentheses. All values are given in mmol l^{-1}. * indicates a significant difference from a corresponding value in NaCl-infused trout (t-test; $P \leq 0.05$). † indicates a significant difference from pre-infusion values (repeated measures one-way ANOVA; $P \leq 0.05$).

Sampling period	NaCl-infused	$NaHCO_3$-infused
pre-infusion	2.18 ± 0.12 (6)	2.25 ± 0.11 (8)
6 h	1.58 ± 0.12 (6) †	1.76 ± 0.06 (8) †
12 h	2.18 ± 0.11 (6)	1.82 ± 0.09 (8) †*
18 h	2.35 ± 0.06 (6)	1.93 ± 0.07 (8) †*
24 h	2.05 ± 0.19 (6)	1.93 ± 0.07 (8) †
36 h	2.10 ± 0.08 (6)	1.86 ± 0.08 (8) †*
48 h	2.13 ± 0.09 (6)	2.10 ± 0.06 (8)

Table 3.2 Protein percentage recoveries, Na^+,K^+-ATPase and Ca^{2+}-ATPase total and specific activities, and ATP-dependent Ca^{2+} transport values from gill basolateral plasma membranes prepared from rainbow trout (*Oncorhynchus mykiss*) infused with NaCl (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; N = 9) or $NaHCO_3$ (140 mmol l^{-1}, 800 μmol kg^{-1} h^{-1}; N = 9) for 48 h. Values shown are means ± 1 SEM. * indicates a significant difference from a corresponding value in NaCl-infused trout (t-test; $P \leq 0.05$).

	NaCl-infused		$NaHCO_3$-infused	
	[1]Ho	[2]P3	[1]Ho	[2]P3
Body mass (g)	506.9 ± 38.3		489.8 ± 42.9	
[3]Protein percentage recovery (%)	0.85 ± 0.09		0.89 ± 0.05	
[4]Total Na^+,K^+-ATPase	533 ± 110	114.3 ± 18.9	1538 ± 136 *	208.0 ± 30.2 *
[5]V_{spec} Na^+,K^+-ATPase	1.2 ± 0.3	29.6 ± 2.1	3.6 ± 0.3 *	58.8 ± 10.4 *
[4]Total Ca^{2+}-ATPase	422 ± 91	35.8 ± 8.6	485 ± 103	54.8 ± 13.3
[5]V_{spec} Ca^{2+}-ATPase	1.0 ± 0.2	8.1 ± 1.3	1.3 ± 0.3	15.8 ± 4.3
[6]ATP-dependent Ca^{2+} transport	3.31 ± 0.70		4.62 ± 1.26	

[1]Ho is designated as the supernatant that remains after the first centrifugation.

[2]P3 is designated as the final pellet which contains the basolateral membrane vesicles.

[3]Protein percentage recoveries are expressed as percentages of total protein in Ho.

[4]Total activity is equal to the product of V_{spec} and total protein.

[5]Na^+,K^+-ATPase and Ca^{2+}-ATPase specific activities (V_{spec}) are expressed as μmol Pi h^{-1} mg^{-1} protein at 37° C.

[6]ATP-dependent Ca^{2+} transport values are expressed as nmol Ca^{2+} min^{-1} mg^{-1} protein at 37° C.

DISCUSSION

This is the first study to examine the effects of metabolic alkalosis on whole body and renal calcium fluxes in rainbow trout (*Oncorhynchus mykiss*). The results support our initial hypothesis that alkalosis leads to an increased rate of whole body calcium uptake ($J_{in}Ca^{2+}$) and are readily explained by the increased chloride cell fractional surface area (CCFA) known to occur in this species at such times (Goss *et al.*, 1994b; Goss and Perry, 1994; Perry and Goss, 1994)

Measurement of Ca^{2+} efflux (branchial and renal components) revealed that the increased $J_{in}Ca^{2+}$ in $NaHCO_3$-infused fish was not a response to counteract increased Ca^{2+} losses. Branchial efflux in experimental fish was never significantly higher than in controls and was unable to counter the increased influx, as evident from the Ca^{2+} net fluxes. Nor was renal efflux ever significantly higher in $NaHCO_3$-infused fish than in NaCl-infused fish. Indeed, the urine excreted following infusion of either $NaHCO_3$ or NaCl contained significantly lower concentrations of Ca^{2+} than the pre-infusion urine. The infused fish were simply removing the excess fluid by excreting a more dilute urine. The average amount of urine excreted in an hour was equal to or slightly higher that the amount of fluid infused into the fish on an hourly and per kg basis.

Since the excess Ca^{2+} taken up from the environment during $NaHCO_3$ infusion was not excreted, either branchially or renally, it must have been retained within the fish. Analysis of plasma [Ca^{2+}] showed that the reservoir for this Ca^{2+} was not the blood. Plasma [Ca^{2+}] in $NaHCO_3$-infused trout decreased significantly from pre-infusion values once infusion began and did not return to pre-infusion values until 48 h. Thus, during the initial 36 h of infusion,

Ca^{2+} entering the body, as well as some Ca^{2+} already present in the plasma, appears to have been diverted to other body compartments. It is possible that the receiving compartment was bone since internal alkalosis is known to cause uptake of Ca^{2+} by bone from the plasma (e.g. Gardner, 1978). Infusion, itself, appeared to contribute to the initial reduction in plasma $[Ca^{2+}]$ because the NaCl-infused fish also displayed a decreased plasma $[Ca^{2+}]$ at 6 h. Unlike the $NaHCO_3$-infused fish, however, plasma $[Ca^{2+}]$ returned quickly to pre-infusion levels.

Examination of the kinetics of $J_{in}Ca^{2+}$ in $NaHCO_3$- and NaCl-infused trout revealed that the maximal transport velocity (V_{max}) of the uptake process was significantly higher in $NaHCO_3$-infused trout than in NaCl-infused trout; the affinity constant (K_m) of the process was not affected. The increase in V_{max} is indicative of an increased number of sites available to transport the Ca^{2+}. Chloride cell proliferation is one way in which the number of transporters can be increased. Previous studies have revealed an increased surface area of exposed chloride cells during alkalosis (Goss *et al.*, 1994b; Goss and Perry, 1994; Perry and Goss, 1994) and in this particular study, it also appears that there was proliferation of chloride cells in the $NaHCO_3$-infused trout. This is indicated by the increase in branchial Na^+,K^+-ATPase activity. An increase in Na^+,K^+-ATPase activity signifies a proliferation of a new crop of chloride cells (Karnaky *et al.*, 1976).

Interestingly, there was no accompanying increases in either Ca^{2+}-ATPase activity or ATP-dependent Ca^{2+} transport in the alkalotic trout. Since Ca^{2+}-ATPase is located abundantly in the chloride cell (Flik *et al.*, 1985b and c), one would expect there to be an increase in one or both of these parameters with chloride cell proliferation. A possible explanation is that the newly proliferated chloride cells had not yet fully developed a capacity to transport Ca^{2+}.

Populations of new chloride cells with lower than normal activities of either Na^+,K^+-ATPase or Ca^{2+}-ATPase or both have been reported (G. Flik, personal communication). Thus, it does not appear that chloride cell proliferation, with an accompanying increase in Ca^{2+}-ATPase activity, was the driving force behind the observed increase in V_{max} in $J_{in}Ca^{2+}$ $NaHCO_3$-infused trout.

Because it does not appear that a specific modulation of the basolateral Ca^{2+}-ATPase was responsible for the enhanced uptake in $NaHCO_3$-infused fish, other mechanism(s) must be functioning to allow such a marked increase. The most likely area of regulation is the apical membrane. The increased CCFA that accompanies alkalosis in the rainbow trout (see Goss *et al.*, 1994b; Goss and Perry, 1994; Perry and Goss, 1994) obviously leads to an increase in the number of apical Ca^{2+} channels exposed to the water and directly supports our original hypothesis. Chloride cell proliferation also increases the number of apical Ca^{2+} channels. Such increases allow more entry points for Ca^{2+} ions, thus permitting more Ca^{2+} into the cytoplasm of the chloride cell. This additional Ca^{2+} is then extruded across the basolateral membrane via either the Ca^{2+}-ATPase or a Na^+/Ca^{2+} exchanger. This model assumes that the availability of apical membrane Ca^{2+} channels is the limiting factor in transepithelial Ca^{2+} uptake which seems reasonable considering i) that the inhibitory effects of the calciotropic hormone, stanniocalcin, are believed to be exerted at the level of the apical membrane Ca^{2+} channels (Lafeber *et al.*, 1988; Verbost *et al.*, 1989; Verbost *et al.*, 1993), and ii) the enzymatic activity of the basolateral Ca^{2+}-ATPase is thought to be far in excess of Ca^{2+} transport requirements (Flik and Verbost, 1993; Verbost *et al.*, 1994).

The findings of this study support the findings of Perry *et al.* (1992a), Marshall *et al.* (1992) and McCormick *et al.* (1992) who found, *in vivo* and *in vitro*, that Ca^{2+} uptake is

directly proportional to chloride cell number and/or surface area. Thus, the increase in chloride cell suface area during metabolic alkalosis not only aids acid-base regulation via accelerated Cl^-/HCO_3^- exchange (Wood *et al.*, 1984; Goss and Wood, 1990a and b; Goss and Perry, 1994) but also leads to increased uptake of Ca^{2+} from the water. The apparent absence of any compensatory reduction in the rate of $J_{in}Ca^{2+}$ during prolonged alkalosis (e.g. via stanniocalcin) may reflect the need to counteract the loss of Ca^{2+} from the plasma to bone and/or other body compartments.

CHAPTER 4

GENERAL DISCUSSION

The purpose of this research was to test the hypothesis **that acid-base disturbances would alter transbranchial Ca^{2+} flux owing to changes in the surface area of chloride cells exposed to the environment.** Because the branchial chloride cell is believed to be responsible for the majority of Ca^{2+} uptake, any alteration in chloride cell morphology could potentially affect transbranchial Ca^{2+} flux (e.g. Ca^{2+} uptake). Ca^{2+} efflux was not expected to be affected because it occurs via a paracellular route. It was expected that acidosis would lead to decreased Ca^{2+} uptake, due to decreased exposed chloride cell apical surface area under these conditions. In contrast, it was expected that alkalosis would cause increased Ca^{2+} uptake, due to the increased exposed chloride cell apical surface area under these circumstances.

RESPIRATORY ACIDOSIS

The intent of the acidosis studies (Chapter 2) was to investigate the effects of internal acidosis on Ca^{2+} fluxes in rainbow trout (*Oncorhynchus mykiss*). In this study, the hypothesis tested was *that a respiratory acidosis in rainbow trout would elicit a decrease in branchial Ca^{2+} influx owing to the reduction in exposed chloride cell surface area that is known to accompany respiratory acidosis in this species.* Fish were exposed to environmental hypercapnia, causing a respiratory acidosis, and Ca^{2+} fluxes were monitored. The evidence collected (Chapter 2) did not support this hypothesis. It was found that although chloride cell fractional area decreased 68% after 48 hours of hypercapnia exposure, whole body Ca^{2+} uptake was significantly increased. This increased uptake was found to be due an increased maximum transport velocity (V_{max}) of Ca^{2+}

uptake. This increased V_{max} was not found, however, to be due to any specific modulation of basolateral membrane ATP-dependent Ca^{2+} transport. Thus it appeared that the enhanced Ca^{2+} uptake during hypercapnia was related to modulation of other involved mechanisms, such as the apical membrane Ca^{2+} channels and the basolateral Na^{+}/Ca^{2+} exchanger.

It was postulated that the number of apical membrane Ca^{2+} channels or their permeability characteristics may have contributed to the increased whole body Ca^{2+} uptake. Stanniocalcin (a calciotropic hormone known to decrease branchial Ca^{2+} influx) secretion may have been reduced or receptors responsible for stanniocalcin's effect on the gill may have been blocked, thus allowing increased Ca^{2+} uptake.

Functioning of a basolateral Na^{+}/Ca^{2+} exchanger was also proposed as an explanation for the increased Ca^{2+} uptake during environmental hypercapnia. It was suggested that such an exchanger, which only operates at an intracellular $[Ca^{2+}]$ of 1 μmol l^{-1} or greater, may function in the area of locally elevated Ca^{2+} concentrations within the chloride cell, such as in the vicinity of apical membrane Ca^{2+} channels.

Acid-base regulation was suggested as the physiological explanation for increased Ca^{2+} uptake during hypercapnia. Recent models of Ca^{2+} uptake via a gill basolateral Ca^{2+}-ATPase indicate that Ca^{2+} movement into the blood is coupled to H^{+} movement out of the blood (Figure 4.1). Thus, it was resolved that the increased Ca^{2+} uptake seen in fish experiencing an internal acidosis was probably a supplementary mechanism to remove H^{+} ions from the blood, thereby compensating for the acidosis.

METABOLIC ALKALOSIS

The goal of the alkalosis studies (Chapter 3) was to determine the effects of internal alkalosis on Ca^{2+} fluxes in rainbow trout (*Oncorhynchus mykiss*). In this study, the hypothesis tested was *that a metabolic alkalosis would cause an increase in whole body Ca^{2+} uptake in rainbow trout due to the increased branchial chloride cell surface area that is known to occur in this under such conditions.* To investigate this, rainbow trout were infused with $NaHCO_3$, inducing a metabolic alkalosis, and Ca^{2+} fluxes were monitored. The results obtained (Chapter 3) supported the hypothesis. Whole body Ca^{2+} uptake was significantly increased during $NaHCO_3$ infusion. This increase was found to be due to an increased maximum transport velocity (V_{max}) of Ca^{2+} uptake. Increased V_{max} is indicative of an increase in the number of Ca^{2+} transporting sites, as occurs with chloride cell proliferation. Chloride cell proliferation was found to occur in the present study in the $NaHCO_3$-infused trout, as indicated by increased gill basolateral membrane Na^+,K^+-ATPase activity. But since there was no accompanying increase in either basolateral membrane Ca^{2+}-ATPase activity or ATP-dependent Ca^{2+} transport, it was highly unlikely that this new crop of chloride cells, with an accompanying increase in Ca^{2+}-ATPase activity, was the driving force behind the increased V_{max} in $NaHCO_3$-infused trout.

Since it appeared that alterations in the basolateral Ca^{2+}-ATPase were not responsible for the increased Ca^{2+} uptake, it was suggested that the main area of regulation was the apical membrane. Supporting the original hypothesis, the increased exposed chloride cell apical surface area obviously allowed increased Ca^{2+} uptake. Chloride cell proliferation would have also contributed. The excess Ca^{2+} permitted into the cytoplasm

of the chloride cell would have then been moved across the basolateral membrane via either the Ca^{2+}-ATPase or the Na^{+}/Ca^{2+} exchanger. Thus, the increased exposed chloride cell apical surface area during metabolic alkalosis not only aids acid-base regulation but also allows increased Ca^{2+} uptake from the water. This uptake may compensate loss of Ca^{2+} from plasma to other body compartments such as bone.

FUTURE DIRECTIONS

The purpose of this thesis was to investigate the effects of acid-base disturbances on branchial and renal Ca^{2+} fluxes in freshwater rainbow trout (*Oncorhynchus mykiss).* The results presented provide a basis for further investigation of various components related to this topic. In investigating the effects of hypercapnia and $NaHCO_3$ infusion on Ca^{2+} fluxes, a number of questions arose. A number of questions arose from the results obtained in investigating the effects of hypercapnia and $NaHCO_3$ infusion on Ca^{2+} fluxes. In particular, the following areas warrant further research.

1. What effects do acid-base disturbances have on the accessibility of stanniocalcin to the gill? Upon exposure to both acid and base disturbances, Ca^{2+} uptake in rainbow trout was increased, presumably due to regulation of uptake at the level of the chloride cell apical membrane. A major regulator of the chloride cell apical membrane presumptive Ca^{2+} channel is the calciotropic hormone stanniocalcin. Thus, it would be interesting to investigate what are the effects of acid-base disturbances on stanniocalcin secretion and/or the branchial receptors responsible for stanniocalcin's effect at the gill apical membrane.

Is secretion of stanniocalcin inhibited? Are the branchial receptors blocked? These questions remain to be answered.

2. What is the role of the pavement cell in Ca^{2+} uptake? A previous study (Perry and Flik, 1988) provided indirect evidence that the pavement cell does not participate in uptake of Ca^{2+} from the surrounding water. However, direct evidence is lacking. One way of obtaining direct evidence and determining whether or not the pavement cell participates in Ca^{2+} uptake is to conduct Ca^{2+} fluxes on isolated pavement cells. Such a procedure is possible using the recently developed protocol for growing primary cultures of teleost gill pavement cells *in vitro* (see review by Pärt and Bergström, 1995). Such cells grow in monolayer and maintain their polarity, thus enabling monitoring of Ca^{2+} movement across the apical and basolateral membranes.

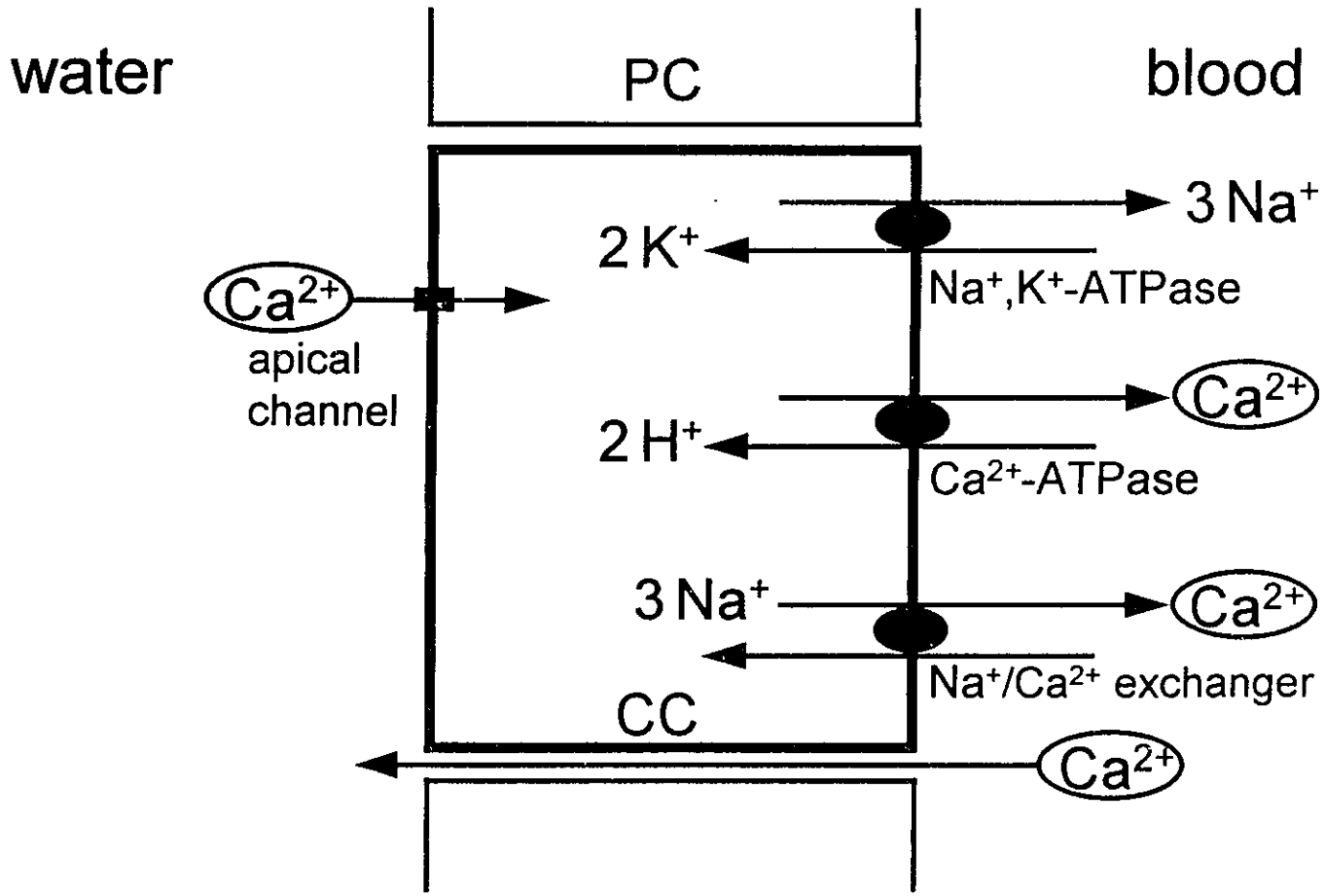
3. Is there any change in the functioning of the Na^{+}/Ca^{2+} exchanger on the basolateral membrane of the branchial chloride cell during acid-base disturbances? Although this exchanger is known to function only at intracellular $[Ca^{2+}]$ of 1 $\mu mol\ l^{-1}$ or greater under normal conditions, does this hold true when the fish experiences an acid-base disturbance? It would be interesting to assay the activity of this exchanger on the branchial epithelium during acid-base disturbances and determine if there is a change in its functioning.

4. How important to acid-base balance is the basolateral Ca^{2+}-ATPase? Current models of transepithelial Ca^{2+} uptake in fish indicate that the pumping of Ca^{2+} across the basolateral membrane via the Ca^{2+}-ATPase is coupled to inward movement of H^{+} ions (Figure 4.1). More research could be conducted into the functioning of this mechanism since there is currently very little known about it.

CONCLUSIONS

The results of this thesis research indicate that disturbances in acid-base balance do affect Ca^{2+} fluxes. Ca^{2+} uptake was enhanced when the trout experienced either a respiratory acidosis or a metabolic alkalosis. This enhancement was expected in the alkalotic fish but was not expected in the acidotic fish. Such enhancement of Ca^{2+} uptake must require some apparent change in the mechanisms allowing Ca^{2+} across the chloride cell. Since it was found in this study that this enhancement does not occur at the basolateral membrane in either case, the most likely other location is the apical membrane. Thus, it appears that the apical membrane of the chloride cell may exhibit significant control over Ca^{2+} uptake in rainbow trout, at least during acid-base disturbances.

Figure 4.1 Schematic diagram of the current model of Ca^{2+} uptake and efflux at the gillvia the branchial chloride cell. Uptake is believed to begin with passive diffusion of Ca^{2+} ions across the apical membrane via presumptive apical Ca^{2+} channels. Ions are then transported to the basolateral membrane via Ca^{2+} binding proteins. Here, ions are believed to be actively moved across the basolateral membrane and into the blood by a high-affinity Ca^{2+}-ATPase and, to a lesser extent, a Na^{+}/Ca^{2+} exchanger. CC = chloride cell; PC = pavement cell. From Flik *et al.* (1993).


water
PC
blood
3 Na+
2 K+
Na+,K+-ATPase
Ca2+
apical
channel
Ca2+
2 H+
Ca2+-ATPase
3 Na+
Ca2+
Na+/Ca2+ exchanger
CC
Ca2+

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