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HIGH-AFFINITY Ca^{2+}–Mg^{2+}-ATPase IN KIDNEY OF EURYHALINE *GILLICHTHYS MIRABILIS*: KINETICS, SUBCELLULAR DISTRIBUTION AND EFFECTS OF SALINITY

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Abstract—1. Two components of Ca^{2+}–Mg^{2+}-ATPase are observed in kidneys of *G. mirabilis*. The high-affinity component has a $K_{0.5Ca}$ of 0.23 μM; the low-affinity activity $K_{0.5Ca}$ is 90–110 μM. The high-affinity activity requires Mg^{2+}, displays Michaelis–Menten kinetics, has peak activity at 1.2 μM Ca^{2+}, and is insensitive to ouabain and Na^+ azide.

2. In subcellular fractions, the high-affinity component segregates with Na^+–K^+-ATPase and is localized predominantly in BLM. The low-affinity component is broadly distributed among membranous organelles, including brush border, and may be equivalent to alkaline phosphatase.

3. Specific activity of the high-affinity Ca^{2+}–Mg^{2+}-ATPase is modestly increased following adaptation of fish to FW, but total renal high-affinity activity is greatest in the hypertrophied kidneys of FW-adapted fish and is least in kidneys of fish adapted to 200% SW.

4. High-affinity Ca^{2+}–Mg^{2+}-ATPase may be associated with active Ca^{2+} transport or with regulation of intracellular Ca^{2+} concentration of tubular cells.

INTRODUCTION

The kidney functions in maintenance of Ca^{2+} homeostasis in vertebrates (Dacke, 1979). In euryhaline fish the rate of Ca^{2+} movement across renal tubules and possibly its directionality (secretion or absorption) is altered to promote (in marine environments) or to minimize (in FW) urinary losses. The kidney of marine teleosts can apparently secrete Ca^{2+}, although Mg^{2+} is normally the major secreted divalent cation (Hickman, 1968; Renfro, 1978). The kidney of FW-adapted euryhaline teleosts is specialized for Ca^{2+} absorption (Lahlou, 1967; Nishimura and Imai, 1978). In the American eel, 80% of plasma Ca^{2+} is ultrafilterable; fractional Ca^{2+} tubular reabsorption in FW-adapted animals is 75% greater than in marine animals (Schmidt-Nielsen and Renfro, 1974; Foster, 1976).

There are few studies of mechanisms of Ca^{2+} absorption in kidney of FW-adapted teleosts. In the mammalian kidney, Ca^{2+} absorption occurs in all segments (Suki, 1979). Some components of Ca^{2+} transport are linked to electrochemical gradients established by co-transport of monovalent ions, such as Cl^- in the Loop of Henlé (Dennis *et al.*, 1979; Suki, 1979). In the proximal, distal and collecting tubules Ca^{2+} transport appears to be coupled to the movement of Na^+, but can be dissociated from Na^+ co-transport in some segments by parathyroid hormone, thiazide or Ca^{2+} infusion (Agus, 1977). Na^+-independent Ca^{2+} transport was first attributed to a low affinity Ca^{2+}-ATPase in the rat kidney (Perez-Gonzalez de la Manna *et al.*, 1980), but this seems an unlikely role for this activity when its Ca^{2+} requirement (in the mM range) is compared to those of proven Ca^{2+} transporting enzymes identified in mammalian red blood cells (Sarkardi, 1980), in sarcoplasmic reticulum (Berman, 1982; Haynes, 1983) and in some transporting epithelia (Nandi *et al.*, 1981; Proverbio *et al.*, 1982). These ATPases are activated by less than 1 μM Ca^{2+}. A more plausible renal Ca^{2+} transporter is the Na^+-independent ouabain-insensitive, high-affinity Ca^{2+}-ATPase ($K_{0.5Ca} = 0.3$–0.4 μM) of the rabbit nephron which is most enriched in basolateral membranes of the distal and collecting segments, the principle sites of Ca^{2+} absorption (Doucet and Katz, 1982).

In the teleost kidney, the only mechanistic studies of Ca^{2+} transport have focussed on the marine eel (Renfro, 1978; Renfro *et al.*, 1982) and the hypothesized tubular secretion of Ca^{2+}. The low concentration of urinary Ca^{2+} of FW teleosts (Hickman, 1968; Foster, 1976) implicates an efficient renal Ca^{2+} reabsorption system, but no studies comparable to

Abbreviations—FW, freshwater; SW, seawater; AP, alkaline phosphatase; SDH, succinate dehydrogenase; BLM, basolateral membrane; DMSO, dimethylsulfoxide; EDTA, (ethylenedinitrilo)-tetraacetic acid; EGTA, ethylene-bis(oxyethylenenitrile) tetraacetic acid; ELON; *p*-methylaminophenol sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MS-222, tricaine methanosulphonate; Tris, tris (hydroxymethyl) aminomethane; TCA, trichloroacetic acid.

those in marine fish exist. In particular, the possibility that one component of Ca^{2+} reabsorption is mediated by a membrane ATPase has not been investigated. In the major site of Ca^{2+} regulation in teleosts, the branchial epithelium, a high-affinity Ca^{2+}-ATPase has been implicated in Ca^{2+} absorption. A Ca^{2+}-ATPase potentially involved in ion uptake was first identified in the gills of *G. mirabilis*, but was not accurately analyzed for Ca^{2+} affinity (Doneen, 1981). A high-affinity Ca^{2+}-ATPase has been extensively characterized in branchial epithelium of two species of eel (Flik *et al.*, 1984; Naon and Mayer-Gostan, 1989). These high-affinity activities are more likely agents of Ca^{2+} translocation in gill chloride cells than the low-affinity Ca^{2+}-ATPases first proposed (Fenwick, 1979; Ho and Chan, 1980). Chloride cells in the gill and skin of FW teleosts have been shown to be specific sites of Ca^{2+} absorption (Ishihara and Mugiya, 1987; McCormick *et al.*, 1992).

This paper is concerned with identification and characterization of a high-affinity Ca^{2+}-ATPase in the kidney of the euryhaline teleost, *Gillichthys mirabilis*. Certain kinetic features diagnostic of Ca^{2+} transporting enzymes are described; the subcellular distribution of, and effects of environmental salinity on, activity are also presented.

MATERIALS AND METHODS

Animals

Gillichthys mirabilis collected in salt ponds of San Francisco Bay, CA, were shipped by air to Ann Arbor, MI. Animals were maintained in an artificial seawater system at 18°C and fed frozen brine shrimp.

In salinity-adaptation experiments, fish were transferred from 100% SW to 200% SW (made by addition of Instant Ocean salts; Aquarium Systems Inc., Woods Hole, MA) for 30 or more days. In FW-adaptation, fish were first transferred to 15% SW for 3–4 days, and then placed in pond water (Ca^{2+} = 0.9–1.2 mM) for at least 30 days.

To obtain tissues, fish were anaesthetized with MS-222 and perfused with heparinized ice-cold *Gillichthys* Ringer through the bulbus arteriosus using a peristaltic pump (Doneen, 1981). The small amount of blood remaining in the kidney was removed by insertion of the perfusion needle next into the dorsal aorta, and then into several sites along the kidney. Verification of the complete elimination of contaminating blood cells was confirmed by microscopic examination of tissue smears. Kidneys were dissected to the level of the heart, blotted and weighed. The animal was also weighed. Tissues were homogenized (200 mg per ml) in 0.21 M mannitol, 0.07 M sucrose, 10% DMSO, pH 7.5. In salinity-adaptation experiments, high- and low-affinity Ca^{2+}-ATPase activities were assayed before freezing the homogenate. Other aliquots were taken for measurement of protein and DNA. The homogenate was quickly frozen in a polyethylene bag immersed in liquid N_2, and stored at −70°C.

Subcellular fractionation

Subcellular fractions were prepared in two different ways using perfused kidneys. Fractions were first obtained using conventional differential centrifugation techniques (Fleisher and Kervina, 1974a). Kidneys from 2 to 4 animals were pooled and homogenized in 0.25 M sucrose, 10 mM HEPES, pH 7.5, filtered through sterile cheesecloth, frozen in liquid N_2 and stored at −70°C for 1–2 days. Homogenates were rapidly thawed by addition of warmed homogenization buffer (Fleisher and Kervina, 1974b). The nuclear pellet (960 ***g***; 10 min) was homogenized a second time and this supernatant added to the first. The combined post-nuclear supernatants formed the starting material for sequential isolation of the following fractions: mitochondrial (25 K g_{av}: 16 min; Beckman SW 27.1 rotor); heavy microsomal (34 K g_{av}; 110 min; SW 27.1 rotor). Pelleted fractions were resuspended in sucrose–HEPES buffer, frozen and stored at −70°C for less than 5 days. To assay enzymatic activities, fractions were rapidly thawed as before and resuspended with a Dounce homogenizer.

In one experiment kidneys from FW-adapted fish were subjected to the differential calcium precipitation method of Booth and Kenny (1974) to separate basolateral from brush border membranes. The modifications of this technique described by Eveloff *et al.* (1979) for the flounder kidney, including removal of renal hemapoietic cells, were followed with one further modification. This was removal of the Ca^{2+} necessarily added in preparation of membrane fractions. Membrane pellets were rinsed in EGTA buffer (200 μM EGTA, 12.5 mM HEPES, pH 7.5) twice and repelleted (120 K g_{av}; 30 min, Beckman SW 50.1 rotor) in Ca^{2+}- and EGTA-free buffer (0.25 M sucrose; 12.5 mM HEPES, pH 7.5).

Ca^{2+}-ATPase

Ca^{2+}-dependent ATPase activity was assayed at concentrations ranging from less than 1 μM to 10 mM. Assay solutions were made with double-distilled water which was distilled a third time from 0.1 mM EDTA and stored in EDTA-rinsed plastic containers. To obtain Ca^{2+} concentrations below 1 μM, a series of Ca^{2+}–EGTA buffered solutions were constructed using 200 μM EGTA, 20 mM Na^+ azide, 0.5 mM ouabain, 12.5 mM HEPES, 1 mM Tris–ATP, pH 7.5 (Perez-Gonzalez de la Manna *et al.*, 1980). Increasing amounts of $CaCl_2$ were added and the quadratic equations of Katz *et al.* (1970) solved for free Ca^{2+} concentration using the calculator program of Fabiato and Fabiato (1979). These calculations assume a negligible binding of 1 μM Ca^{2+} by 1 mM Tris–ATP, and used 7.83 M^{-1} as the apparent association constant (log k') for the binding of the predominant chelator ($EGTA^{4-}$) to Ca^{2+} as described by Pershadsingh and McDonald (1980).

Total $CaCl_2$ was added incrementally from 100 to 197 μM to yield calculated free Ca^{2+} concentrations ranging from 0.015 to 0.97 μM. For characterization of the low-affinity component, assay buffers contained Ca^{2+} but no EGTA. Total Ca^{2+} concentration was measured by atomic absorption spectrophotometry. ATPase activities were measured from the enzymatic release of P_i as described below. Blank values were determined in EGTA buffer without added Ca^{2+}.

Mg^{2+}-dependence of Ca^{2+}-ATPase

Mg^{2+} was chelated by substitution of 200 μM CDTA for EGTA in the HEPES, Na azide, ouabain, Tris–ATP buffer described above. Total Ca^{2+} was varied between 125 and 160 μM to yield solutions containing free Ca^{2+} of 0.28–0.58 μM (log $k' = 6.98\ M^{-1}$; Pershadsingh and McDonald, 1980), and ATPase activity assayed.

Salinity adaptation

Following salinity adaptation, high- and low-affinity Ca^{2+}–Mg^{2+}-ATPases were assayed using 1.0 μM and 0.8 mM $CaCl_2$, respectively, in 1 ml HEPES, ouabain, Na azide, Tris–ATP buffer without added ligand for 20 min at 21°C. Exogenous Mg^{2+} was not added as residual tissue contact was apparently adequate to satisfy the Mg^{2+} requirement of high-affinity Ca^{2+}-ATPase (see Results). Twelve to 27 μg protein were assayed in triplicate. Na^+–K^+-ATPase was measured in the absence of detergent from differences in the rate of Na_2ATP (vanadate-free; Sigma, St Louis, MO) hydrolysis in matched tubes with and without 0.5 mM ouabain (G-strophanthin, Sigma). The assay buffer was 0.1 M imidazole–HCl, 5 mM ATP, 7.5 mM $MgCl_2$, 180 mM NaCl, 60 mM KCl, pH 7.2 (21°C × 40 min; Doneen, 1981). ATPase reactions were terminated by addition of 1 vol cold 10% TCA. Liberated P_i was measured in this, and other ATPase reactions, according to Peterson (1978), with the substitution of 2% ELON (Eastman Kodak, Rochester, NY) in 5% sodium sulfite as reducing agent (LeBel *et al.*, 1978).

SDH activity was assayed from the reduction of 2,3,5-triphenyltetrazolium Cl^- (Sigma) with succinate as electron donor (Clark and Porteus, 1964). AP was measured from the hydrolysis of *p*-nitrophenyl phosphate (Sigma) in 42 mM glycine, 4.2 mM $MgCl_2$, 0.83 mM $ZnCl_2$ and 15 mM substrate at pH 9.2 (McComb *et al.*, 1979).

Protein was estimated using a commercial version of the Coomassie Brilliant Blue reaction (Bio-Rad, Richmond, CA) with gamma-globulin as standard. DNA was determined using the diphenylamine reaction (Burton, 1956).

Statistics

Kinetic values for enzymes were determined from a Lineweaver–Burk plot of the Michaelis–Menten equation. Lines were drawn using a least-squares method. In salinity adaptation experiments, the *t*-test (two-sided) was used with 100% SW-adapted fish as controls. Significance level was set at $P < 0.05$.

RESULTS

Figure 1 illustrates the effect of free Ca^{2+} concentration on Ca^{2+}-dependent ATPase activities in the heavy microsomal fraction derived from pooled kidneys of FW-adapted *Gillichthys*. This fraction was selected for kinetic analysis when adaptational studies (presented below) showed it to be highly enriched in Ca^{2+}-ATPase. Ca^{2+}-ATPase activity can be resolved into two separate kinetic components. Peak ATPase activity occurs at 1 mM Ca^{2+}, but a higher affinity component is detected at Ca^{2+} concentrations of less than 1 μM. The low-affinity activity displays an apparent $K_{0.5Ca}$ of 0.09–0.11 mM, and is progressively inhibited by Ca^{2+} concentrations in excess of 1 mM. However, as noted by Pershadsingh and McDonald (1980), the decline in activity can probably be attributed to the reduction in substrate from interaction of ATP^{4-} and cation at higher Ca^{2+} concentrations. The high-affinity Ca^{2+}-ATPase can only be resolved with ligands capable of buffering Ca^{2+} concentrations in the μM range. Peak high-affinity activity is measured at the plateau marking the onset of low-affinity activity and occurs at 1.20 μM Ca^{2+} (Fig. 1).

Figure 2 illustrates the effect on the high-affinity activity of varying the Ca^{2+} concentration from 0.15 to 0.97 μM using EGTA buffer. The Ca^{2+}-activation curve can be fitted to the Lineweaver–Burk plot of the Michaelis–Menten equation with acceptable linearity (not shown). The $K_{0.5Ca}$ is 0.23 μM Ca^{2+}. Figure 2 also illustrates the requirement of high-affinity Ca^{2+}-ATPase for Mg^{2+}. Ca^{2+}-dependent activity is abolished by chelation of Mg^{2+} with 200 μM CDTA. The actual Mg^{2+} concentration required to permit Ca^{2+}-stimulation was not determined. However, the low concentration of Mg^{2+} in the microsomal fraction is apparently adequate to maintain Ca^{2+}–Mg^{2+}-ATPase activity as inferred from the loss of activity in CDTA, a ligand having a Mg^{2+}-affinity more than four orders of magnitude greater than that of EGTA (Pershadsingh and McDonald, 1980). Subsequent assays of the high- and low-affinity activities were also done without addition of exogenous Mg^{2+}. Whether or not Mg^{2+} is essential for the low-affinity activity was not studied.

The next study is concerned with the subcellular distribution of the high- and low-affinity components of Ca^{2+}-ATPase, and also with the possibility that differences in activities and subcellular location might occur upon adaptation to different salinities. Table 1 illustrates the effects of adaptation to three salinities on Na^+–K^+-ATPase, high- and low-affinity Ca^{2+}-ATPases, SDH and AP in kidney homogenates. Renal Na^+–K^+-ATPase activity is the same in FW- and SW-adapted animals, but is significantly reduced

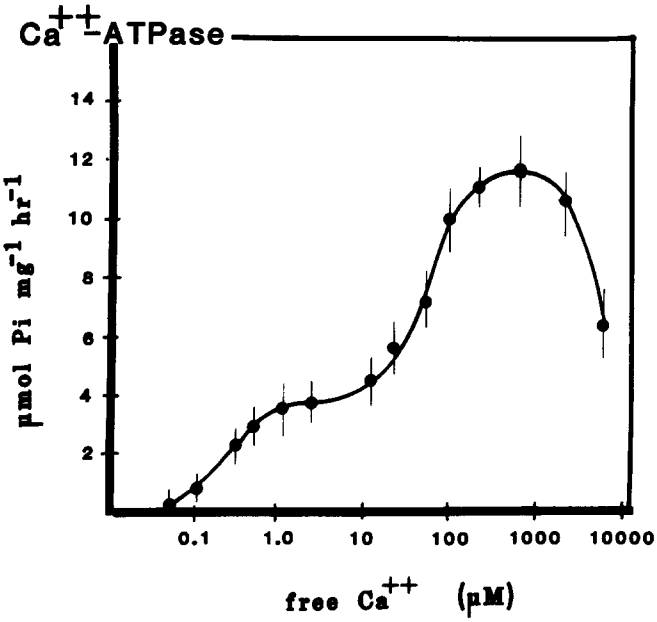

Fig. 1. Effects of free Ca^{2+} concentration on Ca^{2+}-ATPase activity of microsomal fraction of FW-adapted *Gillichthys* kidney. The high-affinity component is the minor peak on the left ($K_{0.5Ca} = 0.23\ \mu M$); the low-affinity activity is the major peak on the right ($K_{0.5Ca} = 90$–$110\ \mu M$). EGTA buffer was used to obtain Ca^{2+} concentrations of 0.97 μM and less. Values are means ± SEM of quadruplicate determinations. Line fitted by least-squares method.

($P < 0.05$) in fish adapted to 200% SW. High-affinity Ca^{2+}-ATPase activity is moderately elevated in kidneys of FW-adapted fish, but is depressed in the 200% SW group compared with 100% SW fish ($P < 0.05$). The low-affinity Ca^{2+}-ATPase is also least active in 200% SW-adapted animals ($P < 0.05$). SDH

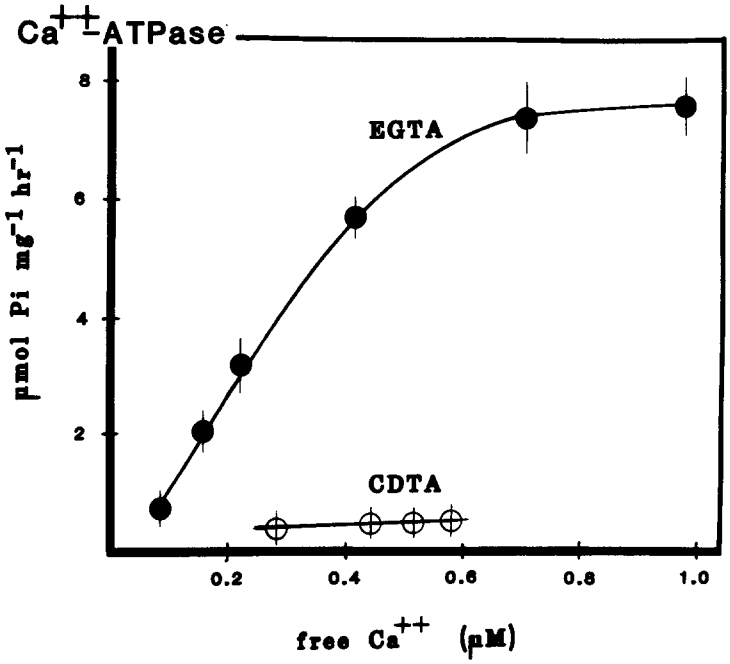

Fig. 2. Dependence of Ca^{2+}-ATPase activity on Mg^{2+}. Upper curve: activity in the presence of an unknown (see text) concentration of Mg^{2+} with Ca^{2+} concentrations buffered by EGTA. Lower curve: activity in the absence of Mg^{2+} (obtained with the chelator, CDTA). Values are the mean ± SEM of quadruplicate determinations using heavy microsomal fraction of kidneys of FW-adapted fish.

Table 1. Effects of salinity on enzyme activities in kidney homogenates

Salinity	N	Na^+–K^+-ATPase	High-Aff. Ca^{2+}-ATPase (μmol P_i/mg protein/hr)	Low-Aff. Ca^{2+}-ATPase
200% SW	6	1.44 ± 0.17*	1.08 ± 0.14*	3.10 ± 0.12*
100% SW	7	2.36 ± 0.17	1.56 ± 0.09	4.37 ± 0.40
FW	8	2.98 ± 0.30	1.92 ± 0.11*	4.96 ± 0.61
		Succinate dehydrogenase (nmol formazan/mg/hr)	Alkaline phosphatase (nmol *p*-nitrophenol/mg/hr)	
200% SW	6	48.4 ± 1.8	513.8 ± 16.3	
100% SW	5	46.4 ± 6.1	516.6 ± 27.5	
FW	5	82.8 ± 0.6**	536.7 ± 20.9	

Values are means ± SEM; N = number of animals. *$P < 0.05$; **$P < 0.01$; t-test (100% SW = control). High- and low-affinity Ca^{2+}-ATPases assayed at 1.0 μM and 0.8 mM Ca^{2+}, respectively, without use of EGTA buffers.

is highly stimulated in FW-adapted fish ($P < 0.01$), but AP displays equal activities in the three salinities.

In Table 2 the activities of the two Ca^{2+}-ATPases and of marker enzymes for BLM (Na^+–K^+-ATPase) and the mitochondrial fraction (SDH) are presented. These data show that high-affinity Ca^{2+}-ATPase is most active in the heavy microsomal fractions derived from each adaptational group. The greatest activity is in the microsomal fraction of FW-adapted fish, but this value is not significantly greater than in SW-adapted fish. In contrast, high-affinity Ca^{2+}-ATPase is significantly reduced in microsomes of 200% SW-adapted fish kidney ($P < 0.05$). The high-affinity activity appears to segregate with Na^+–K^+-ATPase. However, the relative enrichment of Na^+–K^+-ATPase in the heavy microsomal fraction (generally 8–10-fold) exceeds that of the high-affinity Ca^{2+}-ATPase (4–6-fold). The low-affinity component is approximately equally active in the various fractions among adaptational groups, with the microsomal preparations having the greatest activity. The low-affinity activity is again somewhat higher in the microsomal fraction of kidneys of FW fish, but this difference is not statistically significant. The mitochondrial fraction of the 200% SW group is relatively more enriched in Na^+–K^+-ATPase (and also in high-affinity Ca^{2+}-ATPase) than the same fractions prepared from SW- and FW-adapted fish. As expected, the microsomal fractions contained the greatest enrichment of Na^+–K^+-ATPase; microsomal Na^+–K^+-ATPase activity is greatest in the FW group, and least in the 200% SW, but neither is significantly different from the 100% SW group.

The next experiment is concerned with a more precise definition of the membrane location of Ca^{2+}-ATPase activities. Figure 3 illustrates the distribution of Ca^{2+}-ATPases and of marker enzymes between BLM and brush border fractions of kidneys of FW-adapted *Gillichthys*. The plasma membranes of FW-adapted fish were subfractionated (by the Ca^{2+} precipitation method) because this group displayed the greatest activities of high- and low-affinity Ca^{2+}-ATPases (Table 2). In Fig. 3, the activities in BLM and brush border of the five enzymes assayed in this study are displayed relative to activities in homogenate. Thus, values in excess of one indicate enrichment compared to homogenate. BLM is most enriched in Na^+–K^+-ATPase (5.2-fold) and in high-affinity Ca^{2+}-ATPase (3.3-fold). The brush border fraction is approximately equally enriched in low-affinity Ca^{2+}-ATPase (6.3-fold) and in AP (6.6-fold). However, both of these activities are also found to a lesser extent in the BLM. The brush border is deficient (compared to homogenate) in both Na^+–K^+-ATPase and high-affinity Ca^{2+}-ATPase.

The final study sought to determine the total amounts of the high- and low-affinity components of Ca^{2+}-ATPase and other enzymes in renal tissues of fish in each adaptational group. This approach recognizes the differences in kidney mass (or protein) and

Table 2. Subcellular distribution of Ca^{2+}-ATPases and marker enzymes

Fraction	Salinity	Na^+–K^+-ATPase	Ca^{2+}-ATPase High-Aff.	Ca^{2+}-ATPase Low-Aff.	SDH
Nuclear	200% SW	1.41 ± 0.05	1.18 ± 0.13	3.71 ± 0.29	74.9 ± 8.7
	100% SW	1.78 ± 0.09	1.31 ± 0.24	4.91 ± 0.22	67.3 ± 10.1
	FW	1.82 ± 0.11	1.49 ± 0.14	4.82 ± 0.39	137.2 ± 12.2*
Mitochon.	200% SW	2.32 ± 0.25*	1.81 ± 0.09*	2.56 ± 0.33	145.3 ± 5.6
	100% SW	1.03 ± 0.06	0.97 ± 0.07	2.21 ± 0.16	119.0 ± 14.1
	FW	0.81 ± 0.07	0.93 ± 0.11	2.66 ± 0.21	275.5 ± 24.4*
Microsom.	200% SW	12.97 ± 1.31	4.98 ± 0.57*	4.14 ± 0.48	26.4 ± 2.3
	100% SW	15.73 ± 2.31	7.12 ± 0.83	5.10 ± 0.65	27.6 ± 1.6
	FW	18.27 ± 2.58	8.74 ± 0.65	7.21 ± 0.72	19.3 ± 2.0

Values are means ± SEM. $N = 5$ for each assay. Activities expressed as μmol P_i/mg protein/hr (Na^+–K^+ and Ca^{2+}-ATPases), and nmol formazan/mg protein/hr (SDH). Nuclear fraction: 960 g × 10′. Mitochondrial fraction: 25 kg × 16′. Microsomal fraction: 34 kg × 107′. *$P < 0.05$ (100% SW = control).

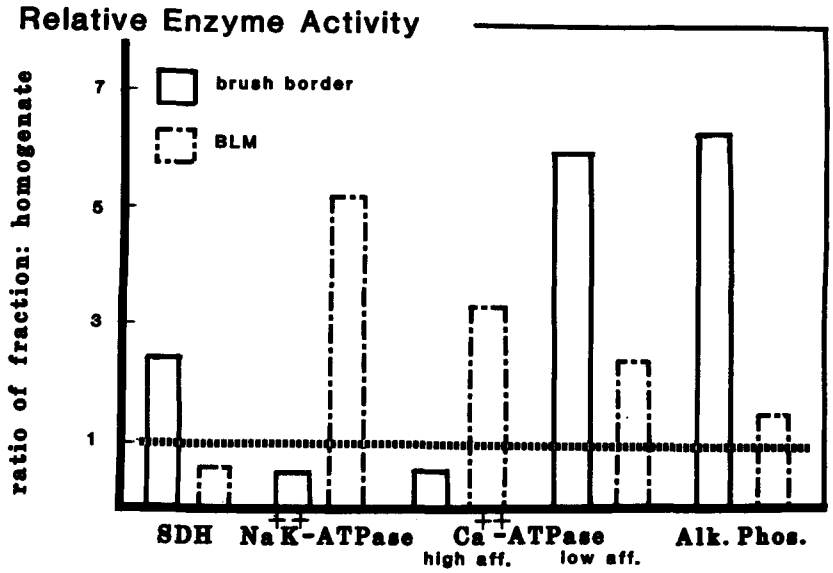

Fig. 3. Enzyme activities in brush border and basolateral membrane (BLM) fractions relative to activities in homogenate. Activities in excess of 1.0 indicate enrichment compared to the homogenate, and values of less than 1.0 indicate a relative depletion. Assays were done in triplicate on five pooled kidneys of FW-adapted *Gillichthys*; SEM was less than 8% of the mean value in each assay. Brush border prepared by the Ca^{2+}-precipitation method (see Methods).

number of tubular cells (DNA) in fish acclimated to different salinities.

Differences in wet weight, protein, and DNA content (each normalized to body wet weight) are summarized in Table 3. Wet weight ($P < 0.05$), protein and DNA contents ($P < 0.01$) are significantly greater in perfused kidney of FW-adapted fish compared with the SW-adapted group. Size and number of cells of 200% SW-adapted fish kidney do not differ from the SW group.

Figure 4 compares total activity (assayed in homogenate) of the renal enzymes in the three adaptational groups (normalized to body weight which is not different among groups). Total kidney Na^+–K^+-ATPase in FW fish is significantly greater ($P < 0.05$) than in 100% SW-adapted fish, and this activity is further depressed in the 200% SW-adapted group in relation to 100% SW fish ($P < 0.05$). The total amount of high-affinity Ca^{2+}-ATPase activity is also elevated following adaptation to FW ($P < 0.05$). As with Na^+–K^+-ATPase, high-affinity Ca^{2+}-ATPase is somewhat reduced ($P < 0.05$) in 200% SW-adapted animals compared to 100% SW-acclimated *Gillichthys*. Total SDH, total low-affinity Ca^{2+}-ATPase and total AP activities are also greatest in the FW-adapted fish kidney ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Activities of these three enzymes do not differ in the two groups adapted to the marine environments.

Table 3. Wet weight, protein and DNA content of *Gillichthys* kidney following salinity adaptation

Salinity	N	Wet weight (mg)	Protein (mg)	DNA (μg)
		(per g body weight)		
200% SW	13	3.56 ± 0.22	0.55 ± 0.05	31.3 ± 5.9
100% SW	7	3.42 ± 0.16	0.52 ± 0.03	35.5 ± 3.3
FW	8	4.59 ± 0.27*	0.69 ± 0.02**	50.2 ± 1.1**

Values are means ± SEM. Adaptation time: 200% SW (6 weeks); 100% SW (>4 months); FW (6 weeks). *$P < 0.05$; **$P < 0.01$; *t*-test (100% SW = control).

DISCUSSION

This study identifies a high-affinity Ca^{2+}–Mg^{2+}-ATPase in the kidney of a euryhaline teleost, and has provided partial characterization of the enzyme with respect to sensitivity to Ca^{2+} and Mg^{2+}, lack of inhibition by ouabain and azide, and subcellular location. Certain properties suggest a role of the enzyme in transepithelial Ca^{2+} transport, in regulation of intracellular Ca^{2+} concentration of tubular cells, or both. Identification of Ca^{2+}-dependent ATPase sensitive to Ca^{2+} concentrations between 10^{-7}–10^{-6} M requires EGTA buffers. These solutions contain calculated free Ca^{2+} concentrations which duplicate low activities of the divalent cation actually observed in cytoplasm, typically less than 1 μM (Godfraind deBecker and Godfraind, 1980). The high-affinity ATPase is maximally stimulated by *ca* 1.0 μM Ca^{2+} and its $K_{0.5Ca}$ is 0.23 μM. The high-affinity activity also displays a requirement for Mg^{2+} as indicated by complete inhibition of Ca^{2+}-dependent ATP hydrolysis in buffers containing CDTA (Fig. 2). The concentration of Mg^{2+} required for activity was not determined; the addition of Mg^{2+} was intentionally omitted in standard assay buffers because modest amounts of this cation (< 125 μM)

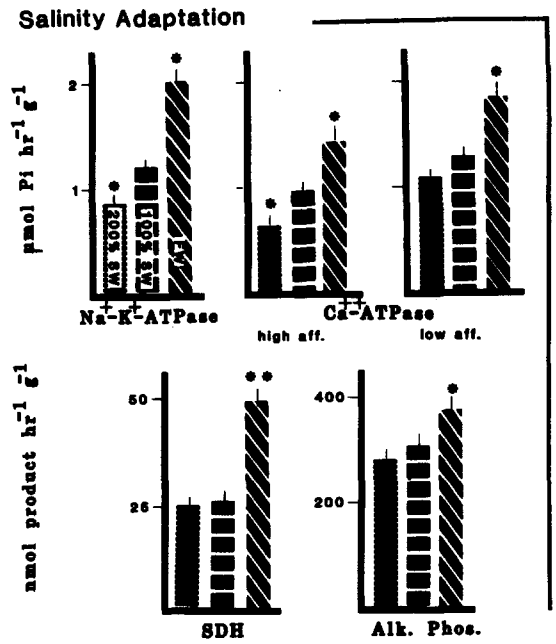

Fig. 4. Effects of adaptation to 200 and 100% SW and to FW on renal enzyme activities measured in perfused kidney homogenates. Activities are expressed as total kidney activity per g body weight; values are means ± SEM ($N = 6$–9).

can activate Mg^{2+}-ATPases of endoplasmic reticulum and mitochondria (Pershadsingh and McDonald, 1980). Apparently, tissue homogenates and subcellular fractions contain adequate Mg^{2+} to support Ca^{2+}-dependent stimulation of the high-affinity ATPase.

The evidence presented in Table 2 and in Fig. 3 favors a predominantly BLM localization of the high-affinity Ca^{2+}-ATPase. Whereas this activity is also distributed in nuclear and mitochondrial fractions prepared by conventional ultracentrifugation, these fractions also contain BLM as indicated by Na^+–K^+-ATPase. The high-affinity Ca^{2+}-ATPase is most enriched in BLM, and is less abundant in brush border. Whether BLM is the exclusive site of high-affinity Ca^{2+}-ATPase cannot be decided from the data presented, since the BLM and apical membrane fractions are, to a limited extent, mutually contaminated as shown by distribution of marker enzymes. Both membrane fractions also contain other organelles, such as mitochondria (Table 2, Fig. 3). These impurities obviously complicate characterization of subcellular location. Mitochondria, for example, possess a high-affinity Ca^{2+}-ATPase (Pershadsingh and McDonald, 1980), as well as Mg^{2+}-ATPase; endoplasmic reticulum also contains both activities (Pershadsingh and McDonald, 1980; Black *et al.*, 1981). However, mitochondrial ATPases can be inactivated by Na azide, and the low-affinity Mg^{2+}-ATPases (typically with $K_{0.5\,Mg} > 5\,\mu M$) are inactive in the absence of added Mg^{2+} (Pershadsingh and McDonald, 1980), conditions employed in this study. Overall, initial fractionation results indicate that BLM is the major site of tubular high-affinity Ca^{2+}–Mg^{2+}-ATPase but also suggest that this enzyme occurs in other organelles.

High-affinity Ca^{2+}-ATPases have previously been identified in plasma membranes of a number of cell types, including some Ca^{2+}-transporting epithelia, such as mammalian kidney (Doucet and Katz, 1982), duodenum (Ghijsen *et al.*, 1980, 1982) and gill of teleost fishes (Doneen, 1981; Flik and Wendelaar Bonga, 1984; Flik *et al.*, 1984; Naon and Mayer-Gostan, 1989).

In most epithelia, high-affinity Ca^{2+}–Mg^{2+}-ATPase has not been directly linked with active Ca^{2+} transport as in sarcoplasmic reticulum (Berman, 1982) and some plasma membrane vesicle preparations (Lichtman *et al.*, 1981). The linkage is more often indirect, and depends on association of peak enzyme activities with conditions of maximum Ca^{2+} transport. In the rabbit kidney, for example, the enzyme is most enriched in the distal tubule, though it is observed with varying activity in all tubular regions. And it is in the distal tubule in which ATP-dependent Ca^{2+} transport can be dissociated from Na^+ reabsorption (Doucet and Katz, 1982).

High-affinity Ca^{2+}-ATPase of rabbit kidney displays a $K_{0.5Ca}$ of 0.3–0.4 μM, and is maximally stimulated by 1.1–2.3 μM Ca^{2+} (Doucet and Katz, 1982). The latter property is similar to that of the *Gillichthys* renal enzyme, whereas the $K_{0.5Ca}$ of the fish enzyme is somewhat smaller (0.23 μM; Fig. 2).

In *A. rostrata* and *Oreochromis mossambicus*, Flik *et al.* (1985a,b) have provided evidence that branchial Ca^{2+} absorption might be specifically associated with high-affinity Ca^{2+}-ATPase. The earliest

characterization of a teleost high-affinity Ca^{2+}-ATPase (in the gill of *Gillichthys*; Doneen, 1981) cannot be considered accurate with respect to Ca^{2+} affinity since the appropriate ligands were not used to control Ca^{2+} concentration. The only reliable $K_{0.5Ca}$ values for teleost high-affinity Ca^{2+}-ATPases are those of Naon and Mayer-Gostan (1989) and Flik *et al.* (1984). The former authors described an activity in the gill of the FW-adapted eel (*A. anguilla*) having a $K_{0.5Ca}$ of 0.36 μM and the latter authors (*A. rostrata*), 0.22 μM. The second value is essentially identical to that described herein. The difference in Ca^{2+} affinity between the branchial enzyme characterized by Naon and Mayer-Gostan (1989) and the renal enzyme in *Gillichthys* is relatively small and could be attributed to use of different species or osmoregulatory tissues. Alternatively, different temperatures were used in assay of Ca^{2+}-ATPase activities (37 vs the 21°C used in this work).

The V_{max} of the *Gillichthys* kidney microsomal high-affinity activity is also lower (Table 2; *ca* 5–8 μmole Pi/mg/hr) than reported for the branchial enzyme of *A. anguilla* by Naon and Mayer-Gostan (1989; >20 μmol P_i/mg/hr). However, the V_{max} observed in this study is similar to that obtained in the North American eel gill by Flik *et al.* (1984; *ca* 5 μmol P_i/mg/hr). This variation may reflect differences in species, adaptation regimes, tissues, assay temperatures, conditions of storage or amount of Mg^{2+}. In particular Mg^{2+} concentration in excess of the minimum required can inhibit Ca^{2+}-ATPase activity (Naon and Mayer-Gostan, 1989). EGTA can also increase the velocity of Ca^{2+}–Mg^{2+}-ATPase independently of its role in regulation of free Ca^{2+} concentration (Kotagal *et al.*, 1983); all investigations in teleosts have used different EGTA concentrations, the lowest being the 200 μM used in this study. Finally, as suggested above, there is no reason to expect enzyme velocity to be similar in transporting epithelia as disparate as gill and kidney.

The high-affinity Ca^{2+}-ATPase in the kidney of *Gillichthys* can be implicated in tubular Ca^{2+} reabsorption for three reasons. First, the enzyme is activated by submicromolar concentrations of Ca^{2+} as required to translocate cytoplasmic ions to extracellular compartments. Second, its enrichment in the BLM is consistent with the direction of Ca^{2+} reabsorption, at least in FW fish which translocate Ca^{2+} from tubular cells to blood (Foster, 1975; Nishimura and Imai, 1982). Third, maximum activity is observed in FW-adapted fish in which the rate of Ca^{2+} reabsorption is accelerated (Schmidt-Nielsen and Renfro, 1974; Foster, 1976). However, in fish adapted to marine environments, kidney high-affinity Ca^{2+}-ATPase is also observed. In these salinities, the enzyme may contribute to the reduced rate of Ca^{2+} absorption that occurs (Renfro, 1978), or possibly to a component of Ca^{2+} secretion activated by low filtration rate (Renfro *et al.*, 1982).

It is notable that, in the *Gillichthys* kidney, high affinity Ca^{2+}-ATPase is markedly elevated in the FW-adapted group only when activity is calculated on the basis of total tissue content (compare Table 1 and Fig. 4). This suggests that the activity of Ca^{2+}-ATPase is nearly the same in SW and in FW fish per unit membrane surface, but that FW kidneys display heightened activity as a consequence of elaboration of BLM and an increased number of tubular cells (Table 3). Renal hyperplasia, particularly increased cell number and growth of BLM, has been observed in euryhaline fish transferred to FW (Hickman and Trump, 1969) or treated with prolactin (Wendelaar Bonga, 1976; Gona, 1981).

In contrast to the fish gill (Flik *et al.*, 1985a,b), there is no direct evidence linking Ca^{2+}-ATPase to Ca^{2+} transport in *Gillichthys* kidney. The high-affinity Ca^{2+} pump might exist simply to maintain low intracellular concentrations; one possibility is that low cellular Ca^{2+} concentrations produced in all environments become specifically coupled to transepithelial Ca^{2+} transport only upon exposure to FW.

The final issue raised by this work is the possible role of low-affinity Ca^{2+}-ATPase. As noted previously (Doneen, 1981; Naon and Mayer-Gostan, 1989), considerable misunderstanding exists regarding the possible Ca^{2+} transporting role of this activity. In fish gill, most earlier studies were not concerned with the high-affinity activity (Fenwick, 1979; Ho and Chan, 1980), and the low-affinity activity stimulated by Ca^{2+} concentrations in excess of 0.1 mM cannot act in transport of the divalent ion from cytosol. Moreover, high concentrations of Ca^{2+} can non-specifically activate various Mg^{2+}-ATPases (Flik *et al.*, 1983). This study supports the view that some portion of the low-affinity Ca^{2+}-ATPase is probably equivalent to AP, an enzyme which has somewhat non-specific requirements for both divalent cation and phosphorylated substrate (McComb *et al.*, 1979) and is located predominantly in the apical membrane of transporting cells (Flik *et al.*, 1983, 1984; Yusufi *et al.*, 1983). There is no convincing evidence linking AP with transport of ions or other molecules (Yusufi *et al.*, 1983; Flik *et al.*, 1984). Therefore, the increased total AP activity of FW-adapted *Gillichthys* kidney probably merely reflects its greater apical membrane surface area (Hickman and Trump, 1969).

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