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Effects of Adaptation to Sea Water, 170% Sea Water and to Fresh Water on Activities and Subcellular Distribution of Branchial Na^+–K^+-ATPase, Low- and High Affinity Ca^{++}-ATPase, and Ouabain-Insensitive ATPase in *Gillichthys mirabilis**

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Summary. 1. Branchial activities of Na^+–K^+-ATPase, ouabain-insensitive ATPase, (Mg^{++}-ATPase) and Ca^{++}-ATPase were measured in *Gillichthys mirabilis* after adaptation to salinities ranging from 170% SW to FW. Stabilities of these activities against freezing and deoxycholate solubilization and the temperature-dependence of activity rates were also investigated. Subcellular distribution and some kinetic properties of these activities, and of SDH were compared in branchial tissues of fish adapted to 170% SW and to FW.

2. Na^+–K^+-ATPase was maximally active at a Na^+ concentration of 180 mM and K^+ concentration of 60 mM. This enzyme was least active in 100% SW-adapted animals, but showed elevated activity after adaptation to 170% SW and to FW. *Gillichthys* is unusual (but not unique) among euryhaline teleosts by displaying higher Na^+–K^+-ATPase in FW- than in SW-adapted animals. Greatest activity, however, was observed in the heavy microsomal fraction ($34{,}000 \times g$) of the 170% SW-adapted group. Maximum SDH activity was also observed in the mitochondrial fraction ($25{,}000 \times g$) of 170% SW-adapted fish.

3. Activity of Ca^{++}-ATPase displayed a complex Ca^{++}-dependence. Two kinetic forms of this activity could be resolved, one with a high Ca^{++}-affinity ($K_m = 2.9$ μM), the other having low Ca^{++}-affinity ($K_m = 0.88$ mM). The low-affinity activity was reduced in branchial homogenates of 170% SW-compared with FW-adapted fish; the heavy microsomal fraction ($34{,}000 \times g$) derived from gills was most enriched in this Ca^{++}-dependent ATPase in FW-adapted *Gillichthys*. In contrast, activities of the high-affinity form were equally enriched in heavy microsomal fractions of 170% SW- and FW-adapted animals. The high-affinity Ca^{++}-ATPase was far more sensitive to inhibition by deoxycholate treatment than the low-affinity component. The possible roles of heterogeneous Ca^{++}-dependent activities in branchial Ca^{++} transport are discussed.

4. Gill Mg^{++}-ATPase activity was significantly elevated in FW and in 5% SW-adapted animals compared with the marine (170% SW- and 100% SW-adapted fish). The Mg^{++}-dependent activity was most concentrated in the heavy microsomal fraction and to a lesser extent in the light microsomal and mitochondrial fractions in both 170% SW- and FW-adapted *Gillichthys*.

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Abbreviations: Ca^{++}-ATPase: calcium-activated adenosinetriphosphatase(EC 3.6.1.3.);DMSO:dimethylsulfoxide;EDTA:ethylenediamine tetracetic acid; EGTA: ethyleneglycol-bis- (-aminoethyl ether) N,N′-tetracetic acid; ELON: p-methylaminophenol sulfate; FW: freshwater; HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Mg^{++}-ATPase: magnesium-activated adenosinetriphosphatase (EC 3.6.1.3.); $Na^+ - K^+$-ATPase: sodium, potassium-activated adenosinetriphosphatase (EC 3.6.1.3.); SDH: succinate dehydrogenase (EC 1.3.99.1); SW: seawater

Introduction

The branchial epithelium of euryhaline teleosts adapted to SW contains chloride cells specialized for elimination of Na^+ and Cl^-. The gills of FW-adapted fish become modified to absorb these ions, but little direct evidence supports the hypothetical role of chloride cells in salt absorption (Philpott 1980). Indeed, other cell types in the gill, such as respiratory epithelial cells, may also participate in monovalent ion absorption in FW-adapted fish (Girard and Payan 1980). Fluxes of Na^+ and Cl^- across the gill are driven by multiple mechanisms and can be partitioned into components mediated by diffusion, exchange diffusion and by active transport (Maetz and Bornancin 1975; Kirschner and Howe 1981). Net transport of

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Na^+ and Cl^- in both directions depends, albeit quite indirectly for some flux components, on Na^+–K^+-ATPase (Epstein et al. 1980; Kirschner 1980) which is localized predominantly on the extensive basolateral membranes of chloride cells (Ernst and Philpott 1970; Hootman and Philpott 1980). This enzyme maintains appropriate intracellular concentration of Na^+ and K^+ (and indirectly, Cl^-) which, along with the particular ionic permeabilities of apical and basolateral membranes, allows the establishment of potentials and ionic gradients actually driving a major fraction of the fluxes. The importance of Na^+–K^+-ATPase in ion transport has been demonstrated following its selective inhibition using ouabain. This poison quickly eliminates some components of monovalent ion transport in gills of SW- and FW-adapted fish (Maetz and Bornancin 1975) and is most effective when applied to the serosal side of the gill (Kamiya and Utida 1968; Silva et al. 1977).

In most commonly studied euryhaline species, the activity of Na^+–K^+-ATPase is higher in gills or in chloride cells of SW-adapted fish than in those adapted to FW (Jampol and Epstein 1970; Sargent et al. 1975; Folmar and Dickhoff 1979; Kirschner 1980). In fish adapted to SW or to hypersaline conditions, Na^+–K^+-ATPase activities vary directly with net Na^+ efflux (Karnaky et al. 1976; Epstein et al. 1980). Reduced Na^+–K^+-ATPase activity in FW may be correlated with the lower rate of net branchial Na^+ and Cl^- absorption often observed in this environment compared with elevated NaCl secretion in SW (Kamiya and Utida 1968; Epstein et al. 1980), but it is important that activity of Na^+–$^{K+}$-ATPase in branchial homogenates cannot give direct information on the rate or direction of net transport for either Na^+ or Cl^-. Only two exceptions to the common euryhaline pattern of highest Na^+–K^+-ATPase in SW-fish have been reported. *Chelon labrosus* and *Dicentrarchus labrax* display gill Na^+–K^+-ATPase activities which are greater in FW- than in SW-adapted animals (Lassere 1971; Gallis et al. 1979). One possibility is that in these species efficient branchial salt absorption in FW is associated with elevated Na^+–K^+-ATPase. This conclusion, however, requires extrapolation of results from other euryhaline species in which branchial Na^+–K^+-ATPase activities have been directly correlated with rates of net Na^+ transport, measured isotopically, such as the eel (Maetz and Bornancin 1975).

Some evidence shows that branchial epithelium may also function in concert with kidney to maintain uniform serum Ca^{++} concentrations in diverse salinities or Ca^{++} environments (Potts 1977; Pang et al. 1980). An unproven assumption is that the Ca^{++}-dependent ATPase characterized in gills of some euryhaline species (Ma et al. 1974; Fenwick 1979; Ho and Chan 1980) may mediate transepithelial Ca^{++}-exchanges. The functional importance of the Ca^{++}-ATPases described previously must be questioned, however, since elevated Ca^{++} concentrations (mM) were used in assays, and the possibility of activating non-specific ATPases exists under these conditions. Moreover, the well-characterized Ca^{++}-transporting ATPases of red blood cells (Sarkadi 1980) and sarcoplasmic reticulum (Bennett et al. 1980) operate at physiological intracellular Ca^{++} concentrations, i.e., less than 1 μM (Godfraind-de Becker and Godfraind 1980), as would be required to move intracellular Ca^{++} to the extracellular compartment. The allegation that low affinity Ca^{++}-ATPase could be irrelevant for Ca^{++}-exchange by the gill is in opposition to the conclusions drawn by several authors (cited above), and is treated further in the Discussion. An unexplored possibility is that intracellular Ca^{++} may be set at differing concentrations by plasma-membrane-bound Ca^{++}-ATPase and that the divalent ion is used as an intracellular regulator of transporting enzymes or structures. For example, external Ca^{++} is required for the normal passive branchial permeability to Na^+ and Cl^- in SW-adapted *Mugil capito* (Pic and Maetz 1981), and the reduction of intracellular Ca^{++} disrupts the structure of membranes, microfilaments and microtubules in chloride cells (Pic and Lahitette 1981). In other transporting epithelia, e.g., amphibian urinary bladder and renal tubules, Ca^{++} fluxes across basolateral membranes are modulated to regulate intracellular Ca^{++} concentrations which appear to be directly coupled to water and Na^+ permeability and to Na^+ transport rate (Hardy 1978; Taylor and Windhager 1979). In the frog skin, a transient increase in Na^+ influx was obtained by increasing intracellular Ca^{++} using a Ca^{++} ionophore (Balaban and Mandel 1979).

This paper reports results of investigations in *Gillichthys mirabilis* aimed at characterization of biochemical differences in the gill of SW- and FW-adapted fish which may be associated with salinity-specific patterns of ion metabolism. This euryhaline goby has a wide range of salinity tolerance, from FW to 200% SW (Owens et al. 1977), and has been studied before chiefly with respect to salinity adaptations and responses to hormones in urinary bladder (Doneen 1976; Loretz and Bern 1980). Ion-transporting properties of *Gillichthys* skin and opercular epithelium have also been described (Marshall and Bern 1979; Marshall and Nishioka 1980). This study examines transport enzymes in whole gill and in subcellular fractions. Effects of adaptation to salinities ranging from 170% SW to FW on activities and subcellular distribution of branchial Na^+–K^+-ATPase, ouabain-

insensitive ATPase (Mg^{++}-ATPase) and Ca^{++}-ATPase are reported. Special attention has been given to identification of Ca^{++}-dependent activities which operate at normal intracellular Ca^{++} concentrations, in the μM range. Biochemical information of this type can contribute, along with force-flux and electrophysiological studies, to the construction of a generalized model of vectorial ion transport by the fish gill.

Materials and Methods

Animals

Gillichthys mirabilis were collected in San Francisco Bay, California by a commercial supplier (Laine Co., Alviso, California) and air-shipped to Ann Arbor. Animals were maintained in an aerated, filtered artificial seawater ($Na^+ = 464$ mM) system (Instant Ocean) at 18 °C, and were fed frozen brine shrimp on alternate days.

In salinity-adaptation experiments, fish were transferred directly to aquaria containing 170% SW (made by adding Instant Ocean salts) or to 15% SW (diluted with tap water). After 3–4 days in 15% SW, fish were then transferred either to 5% SW or to FW (dechlorinated tap water). SW-adapted fish were handled in a manner similar to transferred-fish, but were returned to SW-aquaria. Branchial enzyme activities in homogenates were measured on 4 separate occasions in fish adapted to SW for periods ranging from 12 to 60 days. As mean enzyme activities among these groups varied by less than 12%, results were pooled into single SW-adapted values (Table 2). Likewise, adaptation experiments were performed twice and the similar results obtained in each salinity treated together (Table 2).

Homogenization and Assay of Enzymes in Whole Homogenates

Gill bars were dissected from fish immediately after transection of the spinal cord. Gills were rinsed in ice-cold homogenization buffer (0.25 M sucrose, 17 mM NaCl, 5.5 mM Na_2EDTA, 10 mM imidazole-HCl, pH 7.4; Fenwick 1979). 1.5 ml of this buffer was used to homogenize 4 gill bars (190–220 mg wet weight) using 10 strokes (500 RPM) in a Potter-Elvehjem grinder with teflon pestle. Homogenates were filtered through two layers of cheesecloth. Grinders and filters were rinsed with 1.5 ml cold distilled water, and this was added to the homogenate. One-half of the homogenate was treated with 1/10 volume 1% Na deoxycholate and mixed with gentle vortexing. The 0.1% deoxycholate-treated homogenate was used for assay of Na^+–K^+-ATPase activity. The untreated portion was used for assay of ouabain-insensitive and Ca^{++}-ATPase (see Results).

Methods used in experiments which characterized optimal assay conditions (with respect to effects of ionic concentration and effects of freezing or 0.1% Na deoxycholate-treatment) are described in the Results. The standard assay conditions ultimately developed for salinity-adaptation studies are summarized here.

Na^+–K^+-ATPase. This activity was measured in the usual manner (Johnson et al. 1977) from differences in the rate of ATP (disodium salt, vanadate-free, Sigma) hydrolysis in assay tubes containing or lacking 0.5 mM ouabain (G-strophanthin, Sigma). Freshly-prepared 0.1% deoxycholate-treated homogenate (50–150 μg protein) was assayed in 1 ml assay buffer with and without ouabain and also containing 0.1 M imidazole-HCl, 10 mM Na_2ATP, 1.5 mM $MgCl_2$, 180 mM NaCl, 60 mM KCl, pH 7.2 (21 °C; 40 min with constant shaking). Reactions were stopped with 1 ml cold 10% trichloroacetic acid. Gill preparations were assayed in duplicates which varied by 12% or less.

Mg^{++}-ATPase. Ouabain-insensitive ATPase was assayed (50–150 μg protein) using 1 ml of the buffer described above containing 0.5 mM ouabain. Triplicate determinations varied by less than 5%.

Low-Affinity Ca^{++}-ATPase. This activity was assayed with only minor modifications of the technique of Fenwick (1976). Briefly, 0.05 ml of freshly-prepared homogenate (25–75 μg protein) was added to 1.85 ml 20 mM Tris-HCl, 70 mM NaCl, 4 mM $CaCl_2$, pH 7.4. The reaction was started by addition (to 5 mM) of vanadate-free Na_2ATP (21 °C; 40 min). Reactions were stopped with 2 ml cold 10% trichloroacetic acid. Quadruplicate values differed by less than 6%.

High-Affinity CA^{++}-ATPase. Ca^{++}-free water was prepared by the triple-distillation of twice-deionized water. Assays were performed and solutions prepared and stored in plastic containers which had been rinsed with 10 mM EDTA. Low Ca^{++} concentration assay buffers were made by the serial dilution of 100 μM $CaCl_2$ using the standard (nominally Ca^{++}-free) assay buffer. Ca^{++}-free blanks contained 5 mM EDTA. ATPase activity was measured from the Ca^{++}-dependent hydrolysis of vanadate-free Na_2ATP (5 mM) as described above. In all ATPase assays, phosphate was measured in 0.5 ml aliquots as described by Peterson (1978), except that 2% ELON (Eastman) in 5% sodium sulfite was used as reducing agent (LeBel et al. 1978). This method was selected instead of the original method of Fiske and Subbarrow (1925) and another modification (LeBel et al. 1978) because of superior reproducibility and color stability.

SDH. Activity of the mitochondrial enzyme was assayed at 21 °C using 50–150 μg protein according to Clark and Porteus (1964). This spectrophotometric method measures the production of formazan by the enzymic reduction of 2,3,5-triphenyltetrazolium chloride (Sigma) with succinate as electron donor. Protein was measured according to Lowry et al. (1951) after heating 50 μl homogenate in 0.25 N NaOH to 80 °C. Bovine serum albumin was used as standard.

Subcellular Fractionation

In this study, perfused gills from 170% SW and FW-adapted fish were used. Perfusion was via the bulbus arteriosus using heparinized *Gillichthys* Ringer solution. Care was taken to preserve integrity of membranes during homogenization, freezing and thawing by using DMSO in the homogenization buffer (Fleisher and Kervina 1974b). Briefly, pooled filaments of 5 perfused animals were cut from gill bars and homogenized in 1 volume (1 ml/g) of 0.21 M mannitol, 0.07 M sucrose, 10% DMSO, pH 7.5, using a loose-fitting teflon pestle. Homogenates were placed in polyethylene bags and rapidly frozen in liquid N_2. Homogenates were stored at −70 °C and rapidly thawed later by addition of 5 volumes of warmed (50 °C) buffer (0.25 M sucrose, 10 mM HEPES, pH 7.5). Thawed homogenates were kept at 4 °C and homogenized in a Potter-Elvehjem device using first a loosely-fitting, then a tightly-fitting pestle (7 strokes each). Subcellular fractionation of thawed-filtered (4 layers cheesecloth) homogenates was achieved by differential centrifugation (Beckman L5–50 Ultracentrifuge) as described by Fleisher and Kervina (1974a). In centrifugation steps which required rotor substitutions from the published scheme, velocities were altered to provide the specified g_{av} and centrifugation times were adjusted using the K′ value for 0.25 M sucrose provided by the manufacturer (Beckman). The differential centrifugation protocol is summarized in Table 3. Pelleted fractions were rinsed in 0.25 M sucrose, 10 mM HEPES, pH 7.5 and frozen (dry ice-ethanol bath) in the same buffer. Thawed fractions were resuspended in a Dounce homogenizer (5 strokes with pestle A) and

assayed for enzyme activities and for protein content as described above.

Statistics

In adaptational studies, mean enzyme activities in various salinities were compared with SW-adapted values (controls) using Students' *t*-test (one-sided).

Results

Figure 1 shows the effects of Na^+ and K^+ concentration in assay media on Na^+–K^+-ATPase activity in 0.1% Na^+ deoxycholate-treated homogenates of SW-adapted gills. Maximum enzyme activity was obtained using several Na:K ratios provided that the Na^+ concentration was at least 180 mM; 180 mM NaCl and 60 mM KCl were adopted for the standard assay.

Figure 2 displays Ca^{++}-ATPase activities in SW- and FW-adapted gill homogenates in response to increasing Ca^{++} concentrations from 0.25 mM to 6 mM. In both salinities maximum activity was obtained with 2.0 mM $CaCl_2$. The standard low-affinity Ca^{++}-ATPase assay used 4 mM Ca^{++}.

Figure 3 illustrates the effects of temperature on Na^+–K^+, ouabain-insensitive, and Ca^{++}-ATPase activities. Q_{10}-values for each activity measured between 15 and 25 °C (extrapolated) were 1.95 for Na^+–K^+-ATPase, 1.82 for Ca^{++}-ATPase, and 1.73 for Mg^{++}-ATPase. For convenience, assays were performed at 21 °C, a temperature at which the relative rates of the three activities were similar to those measured at the adaptational temperature (18 °C).

Table 1 presents the effects of freezing (dry ice-ethanol bath, storage for 3 days at −70 °C) and rapid thawing (50 °C bath × 10 min) and of treatment of homogenates with 0.1% deoxycholate on branchial enzyme activities in SW- and FW-adapted fish. Na^+–K^+-ATPase activity was quite stable to one cycle of freezing and thawing, losing less than 10% activity. The activity of this enzyme was increased in both salinities by 50–60% when 0.1% deoxycholate was added to the homogenate. In contrast, Mg^{++}-ATPase and Ca^{++}-ATPase showed highly significant losses in activity after freezing and also after treatment with 0.1% deoxycholate. Therefore, enzyme assays performed in the salinity adaptation experiments reported below used freshly prepared homogenates. These were divided and Na^+–K^+-ATPase assayed 1–2 h after addition of 0.1% deoxycholate. The remaining homogenate was not treated with deoxycholate and was used for assay of ouabain-insensitive ATPase and Ca^{++}-ATPase.

Table 2 summarizes the effects of adaptation to concentrated and to dilute salinities on branchial Na^+–K^+-ATPase, Mg^{++}-ATPase and Ca^{++}-ATPase activities. Na^+–K^+-ATPase activities were significantly higher ($P<0.01$) in FW- and in 170% SW-than in SW-adapted animals. Na^+–K^+-ATPase activities following transfer to 170% SW (from SW) and to FW (from 15% SW) increased in a time-depen-

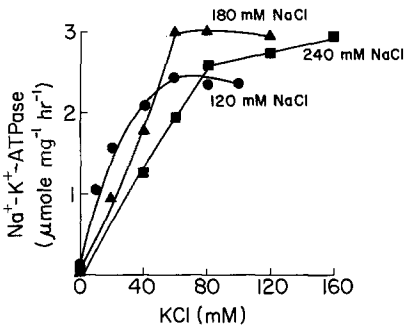

Fig. 1. Effects of NaCl and KCl concentrations on SW-adapted branchial Na^+–K^+-ATPase activity. Enzyme activity expressed as µmoles P_i/mg protein·h. Values are means of triplicate determinations which differed by 12% or less. *Circles* 120 mM NaCl; *triangles* 180 mM NaCl; *squares* 240 mM NaCl

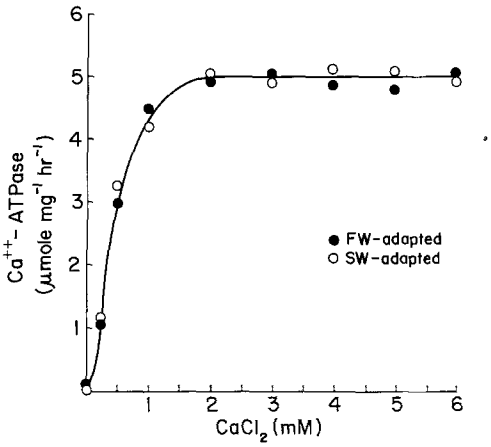

Fig. 2. Effects of $CaCl_2$ concentration on branchial Ca^{++}-ATPase activities in SW- (*open circles*) and FW- (*filled circles*) adapted animals. Enzyme activity expressed as µmoles P_i/mg protein·h. Values are means of triplicate determinations which differed by 7% or less

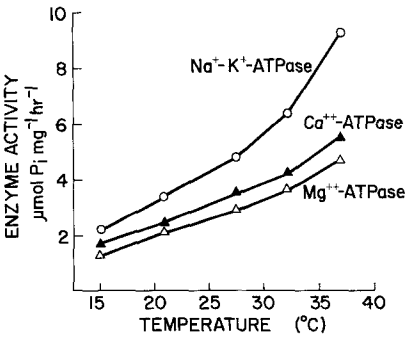

Fig. 3. Effects of temperature on activity rates of Na^+–K^+-ATPase, Ca^{++}-ATPase and Mg^{++}-ATPase. Values are means of three determinations made using pooled whole branchial homogenates of four 170% SW-adapted fish. Standard errors were ±11% of the mean value or less

Table 1. Effect of freezing and 0.1% deoxycholate treatment on Na^+–K^+, Mg^{++}-, and Ca^{++}-ATPase in homogenates of SW- and FW-adapted gills. Enzyme activities expressed as μmoles P_i $mg^{-1} \cdot h^{-1}$ (±S.E.). $n=4$ in each group. Assay temperature=21 °C. Statistical significance (*t*-test): $*P<0.01$; $**P<0.001$. (Control=freshly-prepared homogenate)

Enzyme	Salinity	Fresh	Freeze-thaw	% Change	Fresh	0.1% deoxycholate	% Change
Na^+–K^+-ATPase	SW	3.72±0.22	3.45±0.37	− 6±2	1.87±0.20	3.15±0.29	+66±7**
	FW	5.72±0.34	5.26±0.41	− 7±2	4.12±0.39	6.53±0.49	+54±6**
Mg^{++}-ATPase	SW	2.76±0.21	2.29±0.27	−15±3*	3.76±0.45	2.21±0.25	−37±6**
	FW	3.12±0.34	2.79±0.31	−11±4*	2.80±0.20	2.16±0.29	−20±8**
Ca^{++}-ATPase	SW	5.92±0.48	4.12±0.32	−27±5**	5.27±0.54	2.51±0.32	−45±7**
	FW	5.63±0.36	4.51±0.42	−19±7**	4.95±0.39	2.97±0.37	−42±4**

Table 2. Effects of adaptational salinity and duration on Na^+–K^+-ATPase, Mg^{++}-ATPase and Ca^{++}-ATPase. Assay temperature=21 °C. Statistical significance (*t*-test): $*P<0.01$; $**P<0.001$; (100% SW=control)

Adaptational salinity	Time (days)	*n*	Na^+–K^+-ATPase	Mg^{++}-ATPase	Ca^{++}-ATPase
			(μmoles P_i $mg^{-1} \cdot h^{-1}$)		
100% SW	12–60	15	2.78±0.21	2.91±0.22	5.50±0.30
170% SW	4	11	3.99±0.42**	2.09±0.18*	4.13±0.23**
	8	12	5.17±0.29**	3.02±0.27	2.91±0.37**
15% SW	7	7	3.21±0.36	2.98±0.36	6.00±0.51
	15–20	9	2.76±0.17	2.45±0.26	6.12±0.37
5% SW	60–80	11	6.33±0.41**	3.39±0.21*	4.93±0.24
FW	4	9	4.88±0.31**	2.67±0.26	4.79±0.55
	9	6	5.27±0.32**	3.82±0.19*	5.58±0.21

dent fashion. Adaptation to 5% SW for a period exceeding 60 days also elicited elevated Na^+–K^+-ATPase. However, transfer of SW-adapted fish to 15% SW for 20 days did not stimulate Na^+–K^+-ATPase.

Branchial Ca^{++}-ATPase activities measured in SW- and in FW-adapted fish did not differ significantly (Table 2). Ca^{++}-ATPase in the intermediate salinities of 15% and 5% SW were also similar to the SW- and FW-adapted values. Only in 170% SW-adapted fish did Ca^{++}-ATPase activities deviate significantly from a rate of ATP hydrolysis of 5–6 μmoles $mg^{-1} \cdot h^{-1}$. After 9 days in 170% SW, Ca^{++}-ATPase activity was one-half the SW-adapted level; the decline in Ca^{++}-ATPase was correlated with time of exposure to hypersaline conditions.

Mg^{++}-ATPase activities were rather similar in all groups. A significant decline ($P<0.01$) from the SW-adapted activity was observed 4 days after transfer to 170% SW, but branchial Mg^{++}-ATPase activity similar to SW-adapted animals was reestablished after 9 days in the hypersaline environment. A modest but significant ($P<0.01$) elevation in branchial Mg^{++}-ATPase was observed in FW- and in long term 5% SW-adapted *Gillichthys*.

Table 3 presents the subcellular distribution of the Na^+–K^+-, Mg^{++}- and Ca^{++}-ATPase activities and also of the mitochondrial marker, SDH in fractions derived from perfused 170% SW- and FW-adapted *Gillichthys*. SW-adapted fish were not subjected to a complete subcellular analysis since 170% SW-adapted fish appeared to represent a hyperstimulated version of adaptation to the marine environment. Na^+–K^+-ATPase activities were somewhat lower in whole homogenates in this experiment than shown in Table 2 since deoxycholate treatment was eliminated prior to subcellular fractionation. Na^+–K^+-ATPase, Mg^{++}-ATPase and Ca^{++} activities were most enriched in the heavy microsomal fractions. As expected, SDH was most prevalent in the conventional mitochondrial fraction. In the mitochondrial fractions, as well as in the homogenates, branchial SDH activity was somewhat higher in the 170% SW-adapted group than in the FW-adapted fish. Salinity-specific differences in the distribution of the various activities were observed. Na^+–K^+-ATPase activity was higher in the mitochondrial, and in heavy and light microsomal fractions of 170% SW-derived tissue, whereas Ca^{++}- and Mg^{++}-ATPases were approximately twice as enriched in the heavy microsomal fraction of FW-adapted fish compared with the hypersaline group. This low-affinity Ca^{++}-ATPase was also more highly concentrated in the light microsomal fraction of the FW-adapted group. With few exceptions, enzyme activities recovered totalled those in the starting material (postnuclear supernatant). The major exception was Ca^{++}-ATPase, in which summed activity in fractions was somewhat greater than in starting material (+28% in 170% SW; +40% in the FW-adapted group; Table 3).

Whereas complete characterization of the subcellular distribution of enzymes in SW-adapted fish was not done, Na^+–K^+-ATPase was measured in the

Table 3. Subcellular distribution of branchial Na^+–K^+-, Mg^{++}- and Ca^{++}-ATPase and SDH in 170% SW-and FW-adapted *Gillichthys*. ATPase activities expressed as μmoles P_i $mg^{-1} \cdot h^{-1}$. SDH activity expressed as nmoles formazan $mg^{-1} \cdot h^{-1}$. Assay temperature = 21 °C

Fraction	Na^+–K^+-ATPase		Mg^{++}-ATPase		Ca^{++}-ATPase		SDH	
	170% SW	FW	170% SW	FW	170% SW	FW	170% SW	FW
Homogenate	2.56	1.76	2.81	2.91	2.05	3.26	56.5	44.9
Nuclear pellet (960 × *g*)	2.73	1.79	1.37	1.79	1.21	2.38	58.8	46.7
Post-nuclear sup. (960 × *g*)	2.01	1.24	2.58	2.67	3.92	2.15	31.4	58.8
Mitochondrial (25,000 × *g*)	7.97	4.80	3.57	3.76	7.65	6.51	214.4	151.3
Heavy microsomal (34,000 × *g*)	11.65	8.39	6.26	13.22	10.05	21.30	45.0	42.3
Light microsomal (120,000 × *g*)	2.16	1.04	4.83	5.70	6.73	15.92	12.1	7.2
Soluble (120,000 × *g*)	< 0.2	< 0.2	< 0.2	0.61	0.82	0.58	< 0.5	< 0.5

Fractionation protocol: mitochondrial: 25,000 × *g*; r_{av} = 11.30 cm, time (*t*) = 16 min; heavy microsomal: 34,000 × *g*; r_{av} = 11.30 cm; *t* = 110 min; light microsomal and soluble: 120,000 × *g*; r_{av} = 8.35 cm; *t* = 90 min. Conditions for enzyme assays summarized in Methods. Percent protein recovered from starting material (post-nuclear supernatant) was 170% SW: 85%; FW: 91%. Recovery of enzyme activities as a percentage of protein yield was: Na^+–K^+-ATPase – 170% SW: +14%; FW: −5%. Mg^{++}-ATPase – 170% SW: +12%; FW: +22%. Ca^{++}-ATPase – 170% SW: +28%; FW +40%. SDH – 170% SW: −12%; FW: −9%

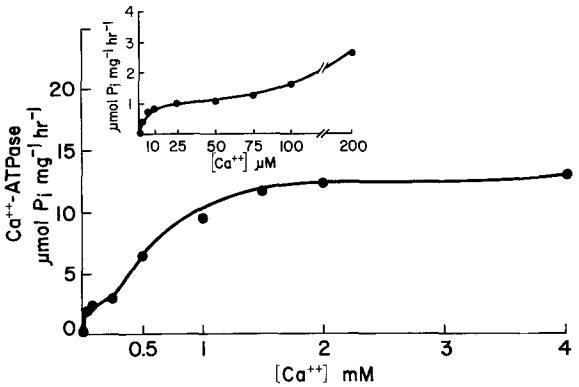

Fig. 4. Effects of $CaCl_2$ concentration on Ca^{++}-ATPase activities in heavy microsomal fraction (34,000 × *g*) of pooled FW-adapted fish. Values are means of quadruplicate values which differed by less than 6%. *Inset*: Ca^{++}-ATPase activity at Ca^{++} concentrations ranging from 0 (5 mM EGTA) to 200 μM

heavy microsomal fraction (34,000 × *g*) derived from 3 perfused SW-adapted animals. As in homogenates (Table 2), the SW-adapted activity in this fraction (6.09 μmoles · $mg^{-1} \cdot h^{-1}$) was less than either the 170% SW- or FW-adapted activities (11.65 and 8.39 μmoles · $mg^{-1} \cdot h^{-1}$, respectively; Table 3).

The remaining results (Figs. 4–6) summarize experiments designed to identify separate kinetic components of the Ca^{++}-dependent ATPase activity. These experiments utilized the heavy microsomal fraction of perfused gills taken from FW-adapted fish, because this group showed the greatest Ca^{++}-dependent activity. The overall form of the activity curve at Ca^{++} concentrations in excess of 0.25 mM was similar to that observed in homogenates (Fig. 4). However, Ca^{++}-ATPase activity could be resolved into two kinetic components when assay conditions included Ca^{++} concentration of 50 and 100 μM. The biphasic response to Ca^{++} suggested the presence of a microsomal high-affinity form of Ca^{++}-ATPase. The insert in Fig. 4 presents Ca^{++}-ATPase activity in buffers nominally containing reduced Ca^{++} concentrations ranging from 2 to 200 μM. The Ca^{++}-free value was obtained in buffer containing 5 mM EGTA. The high-affinity form appeared to show maximum activity at 10 μM Ca^{++}. The low-affinity activity (or activities) displayed V_{max} at 2 mM $CaCl_2$. Additional kinetic features of the low- and high-affinity activities can be derived from the Lineweaver-Burk curves plotted in Fig. 5. From the intercept of the ordinate, V_{max} for the high affinity Ca^{++}-ATPase was 1.20 μmole P_i mg protein$^{-1} \cdot h^{-1}$ (21 °C) in this heavy microsomal preparation which had been frozen (−70 °C) and rapidly thawed twice. The dissociation constant, K_m, for Ca^{++} was 2.9 μM. The low affinity form of Ca^{++}-ATPase displayed a V_{max} of 17.54 μmoles P_i mg protein$^{-1} \cdot h^{-1}$ (21 °C) after freezing and thawing (Fig. 5, insert). Obviously, a maximum rate of 1.20 μmoles P_i $mg^{-1} \cdot h^{-1}$ could have been contributed by high Ca^{++}-affinity ATPase to the low affinity activity. The

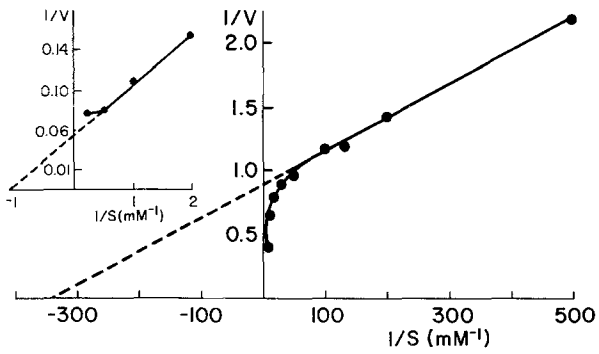

Fig. 5. Lineweaver-Burk plot of high-affinity Ca^{++}-ATPase activity measured in heavy microsomal fraction (34,000 × *g*) of pooled FW-adapted fish. Values are means of quadruplicate determinations which differed by less than 6%. *Inset*: Lineweaver-Burk plot of low-affinity Ca^{++}-ATPase in heavy microsomal fraction of FW-adapted fish gills

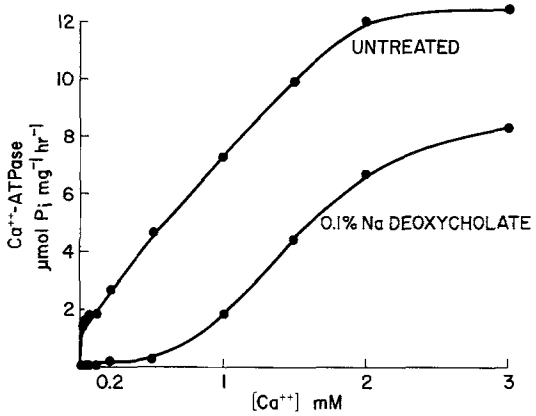

Fig. 6. Effects of 0.1% Na deoxycholate treatment on Ca^{++}-ATPase in heavy microsomal fraction (34,000 × *g*) of FW-adapted *Gillichthys* gill. Values are means of quadruplicate determinations which differed by less than 6%

Table 4. Activities of high-affinity and low-affinity Ca^{++}-ATPase in heavy microsomal fractions prepared from perfused gills of 170% SW- and FW-adapted *Gillichthys*. Assay temperature = 21 °C. Statistical significant (*t*-test): $^{**}P < 0.001$; *NS* not significant

	Ca^{++}-ATPase (µmoles P_i $mg^{-1} \cdot h^{-1}$)		
	N	170% SW	FW
High affinity $[Ca^{++}] = 100$ µM	5	3.56 ± 0.31	3.84 ± 0.29^{NS}
Low affinity $[Ca^{++}] = 4$ mM	5	12.26 ± 1.05	$20.67 \pm 1.85^{**}$

$K_{mCa^{++}}$ measured for the low affinity activity was 0.88 mM.

Figure 6 illustrates the effect of 0.1% Na deoxycholate treatment of the heavy microsomal fraction on the kinetics of Ca^{++}-ATPase. Activity in the full range of Ca^{++} concentrations used (25 µM–3 mM $CaCl_2$) was depressed by treatment with detergent. Magnitude of losses observed in low Ca^{++}-affinity activity in the microsomal fraction was similar to that observed previously in whole homogenates (Table 2). Deoxycholate inhibition was relatively greater for the high-affinity component than for low-affinity Ca^{++}-ATPase. At 100 µM $CaCl_2$, 97% of the Ca^{++}-ATPase activity was destroyed by deoxycholate treatment. At 1 mM Ca^{++}, 45% of Ca^{++}-ATPase activity was destroyed by deoxycholate and one-third of the decline could be attributed to the reduced contribution of high-affinity Ca^{++}-ATPase to the total Ca^{++}-dependent activity.

Table 4 compares high- and low-affinity Ca^{++}-dependent ATPase activities in heavy microsomal fractions (34,000 × *g*) prepared from perfused gills of 170% SW- and FW-adapted fish. High-affinity Ca^{++}-ATPase activities (assayed using 100 µM Ca^{++}) were not significantly different in the two salinities. In contrast, low-affinity Ca^{++}-ATPase (assayed using 4 mM Ca^{++}) were significantly ($P < 0.01$) elevated in the fraction derived from the FW-adapted group.

Discussion

There are two principle findings in this paper. First, two peaks of branchial Na^+–K^+-ATPase activity were observed following adaptation to hypersaline and to FW environments. Activities in both concentrated and dilute salinities exceeded that of SW-adapted fish. Second, two kinetic components of branchial Ca^{++}-ATPase were detected, one having a high, the other a low Ca^{++}-affinity.

Maximum Na^+–K^+-ATPase activity was obtained in the presence of 180 mM NaCl and 60 mM KCl (Fig. 1). Na^+–K^+-ATPase activity was enhanced in all salinities by pretreatment with 0.1% deoxycholate (Table 1). Deoxycholate was apparently required to disrupt membranes which allowed access of substrate to enzyme. Na^+–K^+-ATPase was enriched in both mitochondrial (25,000 × *g*) and, to the greatest extent, in heavy microsomal (34,000 × *g*) fractions in 170% SW- and FW-adapted fish (Table 3). But, in contrast with whole branchial homogenates (Table 2), Na^+–K^+-ATPase activities were higher in fractions from the hypersaline group than in FW-adapted fish (Table 3). It can be concluded from these results that branchial Na^+–K^+-ATPase activities can be modulated in *Gillichthys* to produce *graded* responses to the Na^+ requirements experienced in different salinities. An elevated metabolic requirement for adaptation to 170% SW was supported by enhanced mitochondrial SDH activity in the marine animals (Table 3). Elevated Na^+–K^+-ATPase activity in

170% SW seems consistent with ultrastructural evidence for the proliferation of branchial chloride cells in this concentrated environment (Doneen and Kenny, unpublished). In the only tracer experiments reported for this species, SW-adapted *Gillichthys* showed branchial sodium efflux having kinetic features typical of most marine teleosts (Thompson 1972). The SW-pattern of Na^+ and Cl^- secretion can reasonably be expected to be magnified after adaptation to a more concentrated environment as has been observed in *Gillichthys* opercular membrane (Marshall and Nishioka 1980). Though no observations have been made on branchial Na^+ transport in FW-adapted *Gillichthys*, the second peak of Na^+–K^+-ATPase in FW- and 5% SW-adapted fish might be linked with Na^+ absorption. However, it is not possible to extrapolate transport rate or direction on the basis of this biochemical measurement alone. Increased specific activity of Na^+–K^+-ATPase in homogenates and in microsomal fractions of 170% SW- and FW-adapted animals, compared with SW-adapted fish, might also correlate with proliferation of membranes of the tubular labyrinth of chloride cells or with enhanced turnover of the transport enzyme. Since enzyme activity was normalized to protein content, increased activity could also reflect a selective decrement in other kinds of membrane protein. Stimulation of gill Na^+–K^+-ATPase in concentrated-SW has been observed in other euryhaline species. In *Cyprinodon variegatus* adapted to 200% SW, Na^+–K^+-ATPase activity exceeded the SW-adapted level (Karnaky et al. 1976). An endocrine mechanism for producing elevated branchial Na^+–K^+-ATPase in marine environments has been identified in SW-adapted *Chelon labrosus*; in these fish cortisol treatment further stimulated Na^+–K^+-ATPase activity above the SW-adapted level (Gallis et al. 1979). *C. labrosus*, like *Gillichthys*, showed a greater Na^+–K^+-ATPase activity in FW than in SW (Gallis et al. 1979). Two maxima in branchial Na^+–K^+-ATPase activity have also been reported in *Fundulus heteroclitus*, one in SW-adapted, the second in FW-adapted fish (Towle et al. 1977). Thus, these species are exceptions to the usual pattern in euryhaline fish, many of which show maximum enzyme activity in SW.

The teleost gill functions in maintenance of Ca^{++} balance (reviewed by Potts 1977), but much evidence is indirect. Sixty per cent of ingested Ca^{++} in SW-adapted *Paralichthys lethostigma* was eliminated by extral-renal mechanisms, with the gill as one likely site (Hickman 1967). The branchial epithelia of three species of salmon absorb Ca^{++} in FW (Milhaud et al. 1977). The major site of Ca^{++} absorption in fresh water is the primary lamellae of the rainbow trout gill which contains most chloride cells (Payan et al. 1981). Ca^{++} also plays a regulatory role in branchial transport by interaction with cellular structures regulating permeability or with intercellular junctions (Pic and Lahitette 1981). Considerable evidence shows that environmental Ca^{++} concentration is intimately linked with rates of branchial Na^+ exchange (Maetz and Bornancin 1975; Potts 1977; Pic and Maetz 1981). Ultrastructural evidence for Ca^{++} deposits in the mitochondrial matrix of SW-adapted chloride cells and their relative paucity in FW-cells (Shirai and Utida 1970) suggests that intracellular Ca^{++} concentration or its subcellular distribution show salinity-specific differences. In contrast with Na^+ transport, however, the enzymes directly or indirectly responsible for branchial Ca^{++} fluxes or for regulation of intracellular Ca^{++} have not been identified. Ca^{++}-ATPases have been described in gills of *Salmo gairdneri* (Ma et al. 1974), in SW- and FW-adapted Japanese (Ho and Chan 1980) and in North American (Fenwick 1979) eels and in the FW-adapted roach exposed to differing environmental Ca^{++}-concentrations (Shephard 1981). With regard to effects of salinity, notable discrepancies exist among species. *Anguilla japonia* showed elevated Ca^{++}-ATPase in SW-adapted animals (assayed using 5 mM Ca^{++}; Ho and Chan 1980), whereas Fenwick (1979) reported that *A. rostrata* displayed peak activity (assayed using 2 mM Ca^{++}) following adaptation to FW. Shephard (1981) showed that in the FW-adapted roach, branchial Ca^{++}-ATPase activity (assayed at 2 mM Ca^{++}) varied in proportion to external Ca^{++} concentration. Each of the cited studies, however, was concerned with low-affinity Ca^{++}-dependent ATPase activities, and there are at least two reasons for skepticism about the physiological significance of this activity for branchial Ca^{++} transport. First, high concentrations of Ca^{++} (mM) can substitute for Mg^{++} in activation of Mg^{++}-ATPase (Ma et al. 1974), a ubiquitous activity of cells and mitochondria (Pullman et al. 1960). Thus some low-affinity Ca^{++}-ATPase in homogenates or subcellular fractions may not interact directly with Ca^{++} in vivo. In *Gillichthys* gill, distribution of Mg^{++}-ATPase paralleled that of the low-affinity Ca^{++}-ATPase, both in subcellular fractions (Table 3), and in effects of salinity adaptation (Table 2). These results indicate that Mg^{++} may have simply substituted for Ca^{++} in activation of divalent cation-sensitive ATPases. Another enzyme which will hydrolyze ATP in the presence of Ca^{++} is alkaline phosphatase (Russell et al. 1972; Ghijsen et al. 1980), a widely distributed enzyme having uncertain function (Tenenhouse et al. 1980). Though this enzyme has been implicated in phosphate transport and, indirectly, in calcification

processes, it does not transport Ca^{++} (McComb et al. 1979). A second difficulty with the described branchial Ca^{++}-ATPases is that proven Ca^{++}-transporting ATPases, such as those in red blood cells (Sarkadi 1980), sarcoplasmic reticulum (Bennett et al. 1980) and adipose cells (Pershadsingh and McDonald 1980) are maximally activated by Ca^{++} concentrations of less than 1 μM, the approximate upper limit of Ca^{++} concentration in cells (Godfraind-DeBecker and Godfraind 1980). At least one author (Shepard 1981) described the Ca^{++}-ATPase assayed in the fish gill at 2 mM Ca^{++} as identical with the high-affinity activity studied in blood cells. These activities cannot be comparable since they are activated by Ca^{++} concentrations which differ a thousand-fold. The low-affinity form of Ca^{++}-ATPase (K_m=0.88 mM) was also observed in *Gillichthys* gill (Tables 1–4). Activity of this enzyme was enriched in the microsomal fraction and was elevated in FW-adapted fish compared with those adapted to 170% SW (Table 4), though the low-affinity activity was not different in the SW- and FW-adapted groups (Table 2). The biochemical identification of a low-affinity Ca^{++}-dependent ATP-hydrolyzing activity cannot be directly linked with the transcellular movement of Ca^{++} until the possibility of activation of non-specific ATPases by Ca^{++} is eliminated, and additional kinetic features of and energy requirements for Ca^{++} transport across the branchial epithelium are learned. The same reservation applies, of course, to the high-affinity activity described in this paper. However, this activity does share important features with well-characterized Ca^{++}-transporters by having a high Ca^{++} affinity (K_m=2.9 μM), a predominant localization in the microsomal fraction, and also sensitivity to deoxycholate (Figs. 4, 5 and 6). Inhibition of muscle Ca^{++}-ATPase was produced by extraction of microsomal lipids using deoxycholate, a treatment which also caused denaturation of this proven Ca^{++}-transporting enzyme (Bennett et al. 1980). However, activity of high affinity Ca^{++}-ATPase did not differ in the microsomal fractions of gills adapted to 170% SW and to FW (Table 4). Therefore, this form of Ca^{++}-ATPase may only be involved in maintenance of intracellular Ca^{++} concentration, a general cellular requirement (Godfraind-DeBecker and Godfraind 1980) irrespective of adaptational salinity or level of ambient Ca^{++}. Or the enzyme may actually be involved in transepithelial Ca^{++} transport in both marine- and FW-animals. Both high- and low-affinity forms of Ca^{++}-ATPase could possibly be involved in Ca^{++} transport by acting at different sites or forming parts of separate mechanisms. These possibilities can only be suggested in the absence of necessary data linking force-flux measurements with enzyme biochemistry. The Ca^{++} concentration appropriate for a Ca^{++}-transporter requires knowing the electrochemical gradients at apical and basolateral surface of branchial cells, the precise cellular location of the transport enzyme or enzymes, and the direction of net transport. Finally, in excitable cells and in some epithelia, one component of Ca^{++} transport is actually independent of Ca^{++}-ATPase. Intracellular Ca^{++} can be exchanged for Na^{+} across the plasma membrane (or its basolateral segment), the net movement of Ca^{++} thus being coupled to the Na^{+} gradient, and therefore, indirectly, to the activity of Na^{+}–K^{+}-ATPase (reviewed by Godfraind-DeBecker and Godfraind 1980). The discovery of a high-affinity Ca^{++}-ATPase in the teleost gill expands the number of mechanisms potentially involved in branchial Ca^{++} transport or metabolism.

Conclusions

Branchial Na^{+}–K^{+}-ATPase activities in *Gillichthys mirabilis* showed two peaks of activity after adaptation to concentrated SW (170%) and to FW or to 5% SW. Na^{+}–K^{+}-ATPase activities in the concentrated and the dilute environments exceeded that in SW-adapted fish. However, Na^{+}–K^{+}-ATPase activity in heavy microsomal fractions was greater in 170% SW-adapted than in FW-adapted fish. Mitochondrial SDH activity was also greater in gills of 170% SW-adapted than in FW-adapted fish.

Two kinetic components of branchial Ca^{++}-ATPase were detected, one with high affinity for Ca^{++} (2.9 μM), the other having low affinity (0.88 mM). The high-affinity form was more sensitive to membrane solubilization by deoxycholate than the low-affinity form. Both Ca^{++}-dependent components were localized predominantly in the heavy microsomal fraction of 170% SW- and FW-adapted fish. The low-affinity activity was elevated following FW-adaptation, but the high affinity Ca^{++}-ATPase displayed equivalent activities in marine and FW-adapted animals. By analogy with known Ca^{++}-transporting enzymes, high-affinity Ca^{++}-ATPase may be concerned with Ca^{++} transport or with regulation of intracellular Ca^{++} concentration.

Mg^{++}-ATPase (ouabain-insensitive) activity was somewhat elevated in FW-adapted fish, especially in the heavy microsomal fraction, but this activity may include some low-affinity Ca^{++}-ATPase.

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