

Regulation of Fish Gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ by Selective Sulfatide-enriched Raft Partitioning during Seawater Adaptation*

Received for publication, June 20, 2005, and in revised form, July 13, 2005. Published, JBC Papers in Press, August 11, 2005, DOI 10.1074/jbc.M506670200

Daniel Lingwood^{†1}, George Harauz[§], and James S. Ballantyne[‡]

From the [‡]Department of Integrative Biology and the [§]Department of Molecular and Cellular Biology, University of Guelph, Ontario N1G 2W1, Canada

$\text{Na}^+\text{-K}^+\text{-ATPase}$ is arguably the most important enzyme in the animal cell plasma membrane, but the role of the membrane in its regulation is poorly understood. We investigated the relationship between $\text{Na}^+\text{-K}^+\text{-ATPase}$ and membrane microdomains or “lipid rafts” enriched in sulfatide (sulfogalactosylceramide/SGC), a glycosphingolipid implicated as a cofactor for this enzyme, in the basolateral membrane of rainbow trout gill epithelium. Our studies demonstrated that when trout adapt to seawater (33 ppt), $\text{Na}^+\text{-K}^+\text{-ATPase}$ relocates to these structures. Arylsulfatase-induced desulfatation of basolateral membrane SGC prevented this relocation and significantly reduced $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in seawater but not freshwater trout. We contend that $\text{Na}^+\text{-K}^+\text{-ATPase}$ partitions into SGC-enriched rafts to help facilitate the up-regulation of its activity during seawater adaptation. We also suggest that differential partitioning of $\text{Na}^+\text{-K}^+\text{-ATPase}$ between these novel SGC-enriched regulatory platforms results in two distinct, physiological Na^+ transport modes. In addition, we extend the working definition of cholesterol-dependent raft integrity to structural dependence on the sulfate moiety of SGC in this membrane.

In all animal cells, the major role of the $\text{Na}^+\text{-K}^+\text{-ATPase}$ is to maintain ion electrochemical gradients across the plasma membrane. It has been estimated that this action accounts for ~25% of standard metabolic rate (1, 2), making the pump energetically among the most important proteins in the cell. In vertebrate osmoregulatory epithelia, this role is modulated to meet the demands of ionic homeostasis (3). We investigated the possible connection between such modulation and membrane rafts.

Plasma membrane lipid rafts are temporary, non-random aggregates of primarily cholesterol and sphingolipids that exist as relatively rigid units in an otherwise more fluid heterogeneous lipid milieu (4). These structures serve as dynamic foci for transmembrane signaling (5) and membrane trafficking (6). Many ion transport processes have been localized in these domains (7), possibly because of regulation by phosphatidylinositol-4,5-bisphosphate (8). The fact that $\text{Na}^+\text{-K}^+\text{-ATPase}$ is not similarly regulated (8) might explain the few functional microdomain studies on this enzyme. Raft-localized $\text{Na}^+\text{-K}^+\text{-ATPase}$ has been linked to signal transduction events (9–11), caveolar-related endocytosis (12), and cytoskeletal associated anchoring (13); however, none of these roles address how raft insertion relates to the primary function of

the enzyme of pumping ions. This role is, however, modulated by association with the glycosphingolipid, sulfogalactosylceramide (SGC)² (14–17), which can be enriched in rafts (18, 19). Therefore, the lack of functional studies concerning the partitioning of $\text{Na}^+\text{-K}^+\text{-ATPase}$ between membrane subdomains represents a significant gap in understanding ion transport.

Rafts are operationally defined as detergent-insoluble at 4 °C (20). Although this facilitates their separation from soluble/non-raft material via density gradient centrifugation, it complicates an investigation of how raft insertion may relate to the activity of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (or any enzyme for that matter). Comparisons between the kinetics of raft- versus non-raft-associated enzymes are questionable because they would be derived from two thermodynamically different environments (detergent-insoluble versus detergent-soluble). Given these limitations, we chose to implement a physiological approach to the conundrum: an investigation of the membrane-based regulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity as a function of saltwater (SW) adaptation in the basolateral membrane (BLM) of rainbow trout gill epithelium. SW acclimation in trout involves an increase in gill BLM $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity associated with the transformation of a salt-absorbing epithelium (freshwater) to a salt-secreting epithelium (saltwater) (21). Thus, an examination of SW and freshwater (FW) acclimated systems facilitates an evaluation of the role of rafts in $\text{Na}^+\text{-K}^+\text{-ATPase}$ dynamics in two physiologically relevant situations of differing enzyme activity.

Furthermore, we have recently obtained evidence supporting a regulatory role for SGC in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in this membrane (22). SGC has been implicated as a cofactor in $\text{Na}^+\text{-K}^+\text{-ATPase}$ -associated K^+ transport (23, 24). This cofactor model stated that SGC functions to donate a K^+ (SGC exhibits a $\text{K}^+ > \text{Na}^+$ selective charge interaction via its sulfate group (25)) to the enzyme gate site (*i.e.* SGC is responsible for K^+ import) and has since been supported by extensive biochemical evidence (14–17). Karlsson (23) proposed that such cofactor functioning was achieved via the formation of an annulus of SGC around $\text{Na}^+\text{-K}^+\text{-ATPase}$. We hypothesized that the formation of such an annulus might occur in SGC-enriched membrane rafts.

Our studies were designed to determine whether changes in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity during SW acclimation were related to the dynamics of raft partitioning during this process. We demonstrate that raft-based SGC interaction provides a novel regulatory platform for this enzyme.

MATERIALS AND METHODS

Animals—Rainbow trout obtained from Alma Research Centre (Alma, ON, Canada) were acclimated to freshwater ($n = 60$) and 33 ppt saltwater ($n = 60$) for 60 days in the Hagen Aqualab, University of

* This work was supported by a National Sciences and Engineering Research Council of Canada Postgraduate Scholarship (to D. L.), a NSERC Discovery grant (to J. S. B.), and an operating grant from the Multiple Sclerosis Society of Canada (to G. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom correspondence should be addressed: Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany. E-mail: lingwood@mpi-cbg.de

² The abbreviations used are: SGC, sulfogalactosylceramide; SW, salt water; BLM, basolateral membrane; FW, fresh water; CHAPS, 3-[[3-cholamidopropyl]dimethylammonia]-1-propane-sulfonate; GM1, monoganglioside 1.

Regulation of Fish Gill $\text{Na}^+ - \text{K}^+ - \text{ATPase}$

Guelph. Water temperature was 10 °C, and the lighting was set to a 12 light:12 dark photoperiod. Fish were fed a commercial salmon feed (Biodiet Grower food pellets, Bio Oregon Inc., Warrenton, OR) once daily to satiety.

Gill Basolateral Membrane Isolation—Following the acclimation period, trout were sacrificed, transferred to an ice bucket, and cannulated through the bulbus arteriosus (23-gauge needle, P50 polyethylene tubing, BD Biosciences). Gills were perfused with saline solution (0.9% NaCl, 0.5 mM Na_2EDTA , 10 U/ml heparin, pH 7.8) at a rate of 4 ml/min for 5 min (Gilson peristaltic pump, model minipuls 3, Villers le Bel, France). The gill tissue was then obtained, and the BLM was purified as described (26). Membrane samples were frozen at -80°C for subsequent analysis.

Lipid Raft Isolation—Lipid rafts were isolated according to a modification of Radeva and Sharom (27). Gill BLM (1000 μg of BLM protein) was incubated on ice with 1 ml of lysis buffer (9 mM Tris, 51 mM NaCl, 0.7 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 , and 2% protease inhibitor mixture (Roche Pharmaceuticals Nutley, NJ), pH 7.5), which contained 1.5% CHAPS, 0.5% Brij 96V, or 0.5% Triton X (1% disruptive as determined from the profile of the raft marker GM1). During the 30-min extraction period, samples were vortexed periodically. Samples were then centrifuged at $10,000 \times g$ for 10 min at 4 °C. The postnuclear supernatant (400 μl) was mixed with 800 μl of 60% sucrose solution (25 mM Tris-HCl and 140 mM NaCl, pH 7.5) and then subjected to density gradient centrifugation on a discontinuous sucrose gradient. The 40% sucrose sample mixture (800 μl) was transferred to a 5-ml Quick Seal tube (Beckman Coulter Canada, Mississauga, ON, Canada) and carefully overlaid with 30% and then 5% sucrose solutions (25 mM Tris-HCl and 140 mM NaCl, pH 7.5). Samples were centrifuged at $402,000 \times g$ in a VTi 65.2 rotor (Beckman Coulter Canada) for 180 min at 4 °C (L7–65 Beckman ultracentrifuge). After ultracentrifugation, sucrose gradients were separated from top to bottom into 13 fractions (400 μl) by displacement with a density gradient fractionator (model 640, ISCO, Lincoln, NE).

Effect of Arylsulfatase and Cholesterol Depletion on Lipid Rafts—Rafts in the presence of arylsulfatase (Sigma-Aldrich) were isolated by CHAPS detergent extraction as stated above for gill BLM of SW exposed trout (1000 μg of BLM protein), except that BLM was preincubated with 0.25 units arylsulfatase/ μg of BLM protein for 30 min at 25 °C. We defined this as mild arylsulfatase treatment. Enhanced arylsulfatase treatment involved the addition of another 250 units of arylsulfatase to the postnuclear supernatant, which was then incubated on ice for 30 min prior to sucrose gradient overlay. SW gill BLM (1000 μg of BLM protein) was also extracted with 75 mM methyl- β -cyclodextrin as described (19).

Lipid Raft Analysis—Sucrose gradient fractions (2 μl) were spotted on nitrocellulose and probed for GM1 by cholera toxin horseradish peroxidase-conjugate (27) and for SGC by Sulf 1 monoclonal antibody (28). Sulf 1 was a generous gift from Dr. Pam Fredman (Sahlgrenska Academy at Goteborg University, Sweden). Rainbow trout gill BLM contains no other cholera toxin-binding gangliosides³ or Sulf 1-binding lipids (22). Gradient fractions were prepared for Western blotting using a modification of Wessel and Flugge (30). Briefly, 4 μl of 5% deoxycholic acid was added to 200 μl of each fraction, and the samples were incubated on ice for 10 min. Two-hundred eighty microliters of chloroform and 160 μl of methanol were then added to the 204 μl of solubilized samples and mixed well, and samples were spun at $10,000 \times g$ for 10 min at 4 °C to concentrate the proteins at the aqueous/organic interface. The

aqueous (top) layer was decanted without disturbing the interface, and 100 μl of 100% ethanol was then added to the organic (lower) phase to promote its evaporation under a gentle stream of nitrogen. Precipitated proteins were separated by PAGE as described (31); α -5 monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa) was used to probe (1/100 dilution) for the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ α subunit during Western blotting.

$\text{Na}^+ - \text{K}^+ - \text{ATPase}$ Activity in the Presence and Absence of Arylsulfatase—Gill BLM $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity was measured at 25 °C as described previously (32). Samples were preincubated for 30 min with 10 mM ouabain, 0.1 unit of arylsulfatase/ μg of BLM protein, 0.5 units of arylsulfatase/ μg of BLM protein, or 10 mM ouabain + 0.5 units of arylsulfatase/ μg of BLM protein for 30 min, and NADH oxidation-dependent ATP hydrolysis was measured spectrophotometrically at 340 nm (Cary 50 Varian spectrophotometer, Varian, Palo Alto, CA). $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity was defined as ouabain-sensitive ATPase and expressed as μmol of ADP/mg of BLM protein/h.

RESULTS AND DISCUSSION

We discovered that SGC microdomains do exist in rainbow trout BLM and that $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ partitions into these structures during trout salinity adaptation. Raft isolation by Triton X, Brij 96V, or CHAPS detergents showed that SGC was solely localized to glycosphingolipid-enriched microdomains from both FW and SW BLM (co-fractionated with ganglioside raft marker GM1), whereas $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ only localized to these domains after SW acclimation and up-regulation of enzyme activity (Figs. 1 and 2); $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity (ouabain-sensitive ATPase) was reduced following SGC desulfation by exogenous arylsulfatase only in SW-acclimated fish (Fig. 2). Moreover, mild treatment of BLM with arylsulfatase disrupted $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ raft localization as monitored by CHAPS detergent extraction (Fig. 3). Further additions of arylsulfatase disrupted GM1 profiles of CHAPS rafts in a similar manner to that seen by treatment with methyl- β -cyclodextrin (Fig. 4). Cumulatively, our results suggest a new ionically adaptive connection between a membrane-bound transport enzyme and a raft environment.

These results initially appear counterintuitive; $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ partitioned into a raft environment of increased rigidity during the up-regulation of enzyme activity associated with SW exposure. This issue may be rationalized. We hypothesize that the functioning of an enzyme in a raft environment might be accomplished by energetic compensation. Enthalpic-entropic compensation is a feature of cold-adapted enzymes whereby thermodynamic parameters are adjusted to increase flexibility and allow for higher catalytic efficiency at low temperatures (33); additionally enzymes adapted to higher pressure can exhibit pressure-insensitive kinetic properties related to reductions in the volume change (ΔV^\ddagger) associated with the formation of the activated complex of the enzyme (33). The problems associated with catalysis within the rigid structure of a raft may be analogous to those faced by enzymes operating at high pressures and/or low temperatures; therefore, raft-associated $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ (or for that matter any raft enzyme) may also exhibit reductions in ΔV^\ddagger and/or increases in structural flexibility that are more conducive to functioning in a more restrictive environment.

The $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ sensitivity to arylsulfatase-induced SGC desulfation we observed is expected, given the precedents in the literature (14–17). Furthermore, differential sensitivity of SW- and FW-adapted fish is rational based on the differential raft partitioning we observed. Arylsulfatase sensitivity is attributable to $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity within SGC-enriched rafts. In both FW and SW acclimated trout, SGC was restricted to GM1-enriched rafts and so desulfation by arylsulfatase

³ D. Lingwood, unpublished data.

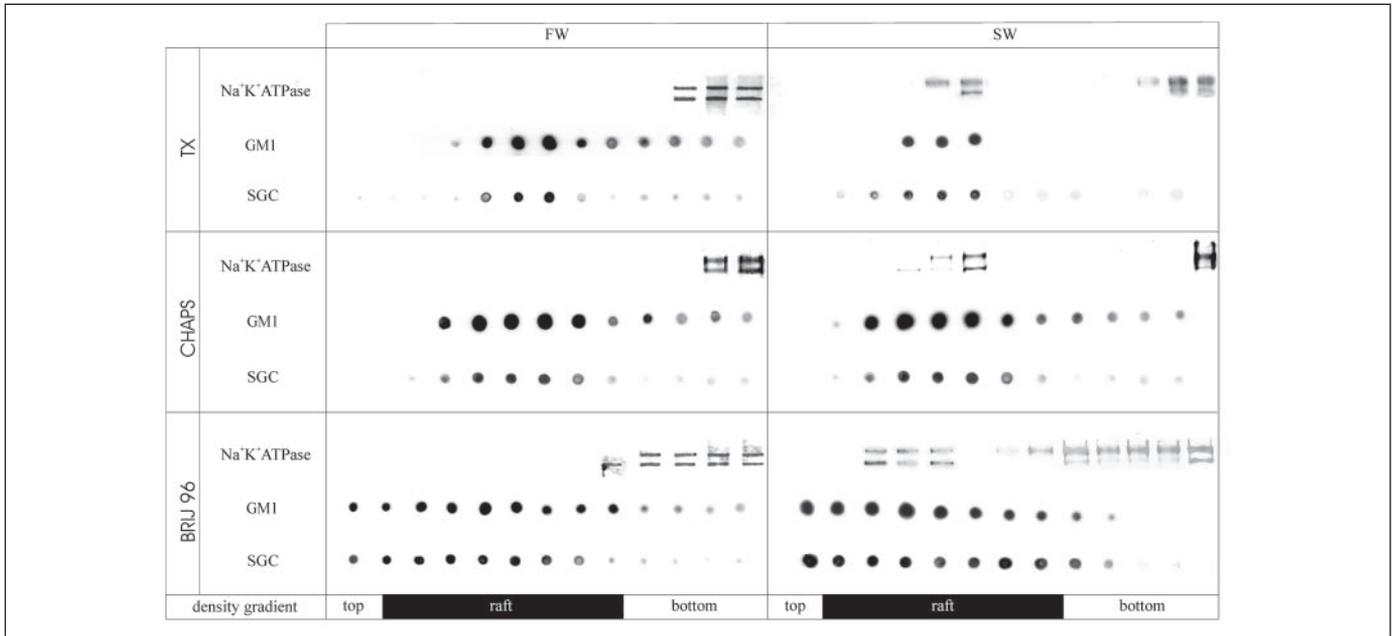


FIGURE 1. Lipid rafts from the BLM of gill epithelium from rainbow trout acclimated to freshwater (left column) and saltwater (right column) as visualized by the density gradient profiles of GM1 (raft marker), SGC, and $\text{Na}^+\text{-K}^+\text{-ATPase}$ (immunoreactive bands correspond to the accepted masses for the teleost gill $\text{Na}^+\text{-K}^+\text{-ATPase}$ α subunit (52) (~110 kDa) and $\alpha\beta$ complex (29) (~150 kDa) extracted with Triton (TX), CHAPS, and Brij 96V detergents.

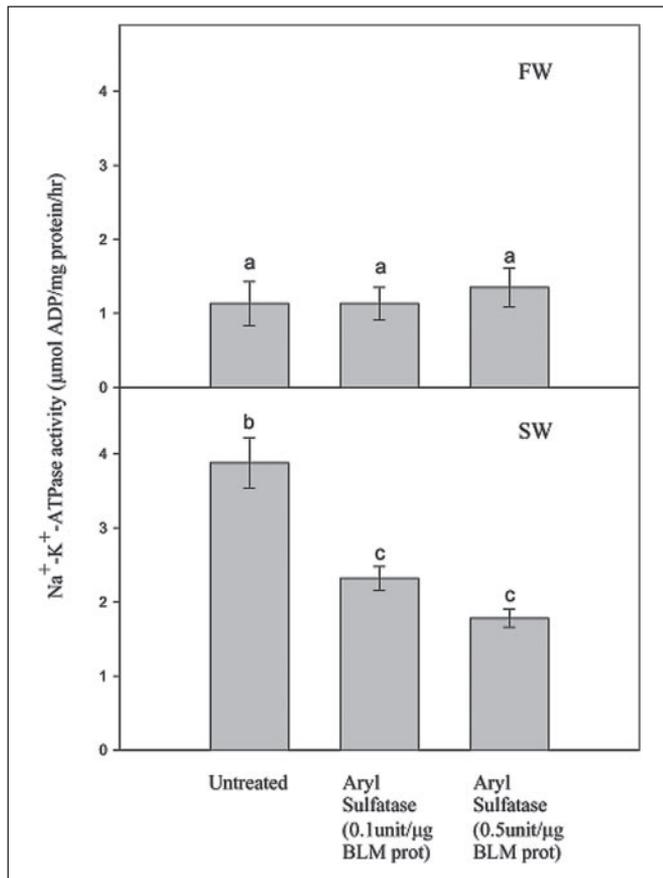


FIGURE 2. Effect of arylsulfatase on gill BLM $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity (mean \pm S.E.) in freshwater- (upper panel) and saltwater- (lower panel) exposed rainbow trout. Different letters indicate significant differences within each salinity treatment group (analysis of variance with Tukey's test, $n = 6$, $p < 0.05$); 0.5 units of arylsulfatase/ μg of BLM protein had no effect on ouabain-insensitive ATPase activity (not shown).

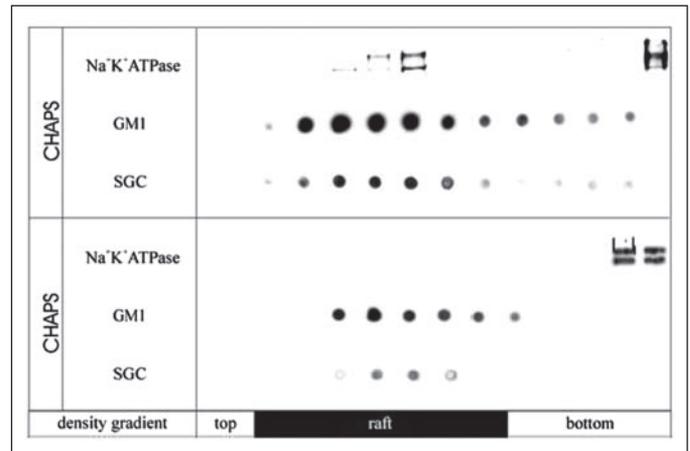


FIGURE 3. Effect of arylsulfatase on $\text{Na}^+\text{-K}^+\text{-ATPase}$ partitioning into CHAPS rafts of gill epithelial BLM of rainbow trout acclimated to saltwater as visualized by the density gradient profiles of GM1 (raft marker), SGC, and $\text{Na}^+\text{-K}^+\text{-ATPase}$. Upper panel denotes control rafts, and the lower panel refers to rafts isolated from BLM pretreated with 0.25 units of arylsulfatase/ μg of BLM protein.

must have occurred in rafts; SGC was only available to the raft-partitioned $\text{Na}^+\text{-K}^+\text{-ATPase}$. Therefore, we conclude that the arylsulfatase-sensitive fraction of $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in the SW situation is a measure of catalysis generated from the raft-associated enzyme (~50% of total original activity). Furthermore, mild treatment of SW BLM with arylsulfatase destabilized $\text{Na}^+\text{-K}^+\text{-ATPase}$ raft partitioning, indicating that an enzyme interaction involving the sulfate of SGC is required for raft insertion. A polar interaction between the enzyme and SGC has been suggested (23), because an additional salt extraction step is required to purify $\text{Na}^+\text{-K}^+\text{-ATPase}$ from preparations in which SGC is also extracted. Such arylsulfatase sensitivity and SGC interactions were not apparent in FW-adapted fish because $\text{Na}^+\text{-K}^+\text{-ATPase}$ and SGC were located in different membrane subdomains. Furthermore, rainbow trout gill α -1a and α -1b $\text{Na}^+\text{-K}^+\text{-ATPase}$ subunit isoforms are

Regulation of Fish Gill $\text{Na}^+ - \text{K}^+ - \text{ATPase}$

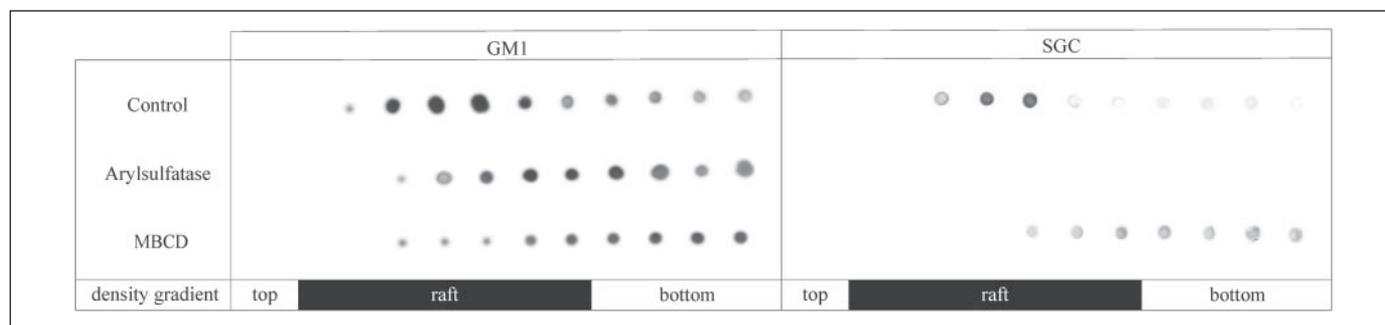


FIGURE 4. Effect of methyl- β -cyclodextrin (MBCD) and enhanced arylsulfatase treatment on GM1 and SGC BLM raft profiles from gill BLM of saltwater-exposed rainbow trout. BLM was preincubated with 0.25 units of arylsulfatase/ μg of BLM protein and then treated with another 250 units of arylsulfatase following CHAPS detergent extraction.

differentially expressed following SW transfer (34), which we previously suggested might be differentially sensitive to SGC (22). Perhaps only the isoform up-regulated in SW has the ability to partition into SGC-enriched microdomains.

We consider partitioning of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ into SGC-enriched rafts adaptive with respect to both enzyme activity and saltwater adaptation. The insertion of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ into rafts facilitates an arylsulfatase-sensitive co-localization of enzyme and cofactor, which would allow SGC to form an annulus around the enzyme, thus satisfying the conditions for SGC-assisted K^+ co-transport according to Karlsson (23, 24) and increase the efficiency of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity. Saltwater adaptation/salt secretion is accomplished by an up-regulation of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity to drive Cl^- extrusion against a concentration gradient, which is matched to the passive paracellular exit of Na^+ (21). The fraction of arylsulfatase-sensitive ATPase accounted for more than two-thirds of the increase in $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity from FW- to SW-acclimated fish (Fig. 2). Therefore, we propose that $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ localization to SGC-enriched rafts represents a posttranslational, membrane-based, up-regulation of the activity required for salt secretion. Because SGC is also a cofactor for other K^+ transporters (35), partitioning into SGC subdomains may be a regulatory mechanism for plasma membrane K^+ transport systems in general.

Raft partitioning because of signal transduction (9–12) and cytoskeletal (13)-related roles cannot be ruled for $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ of SW-acclimated trout. Increases in gill Cl^- secretion upon SW exposure may be linked to activation of the α_2 -adrenergic receptor pathway in killifish (36, 37). This pathway involves phospholipase C-associated increases in inositol triphosphate and intracellular Ca^{2+} , a system that is thought to also be regulated by the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ raft-dependent activation of Src kinase (11). Additionally, expression of cytoskeletal elements (e.g. actin-binding protein and a member of the Rho family known to control actin) is elevated in the gill of SW-acclimated killifish (38–41). Furthermore actin can directly regulate $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ in some cells (42). These observations correlate with the finding that raft-localized $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ can associate with ankyrin, an actin-binding protein (13). The extent to which these non-transport-related roles occur for raft-associated $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ in the gill BLM of SW-exposed trout is not clear; however we would suggest that these mechanisms (transport function included) are not mutually exclusive of one another.

Given the adaptive significance of a raft-mediated co-localization of enzyme and cofactor, it is interesting that $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is not raft-associated in FW trout. Given the experimental impossibility of directly measuring raft-related enzyme catalysis (see Introduction), an assessment of the functional consequences of segregating $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ into different membrane subdomains cannot be performed at this time. Nevertheless the literature does generate viable predictions concerning the result of such a segregation. In $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ preparations in

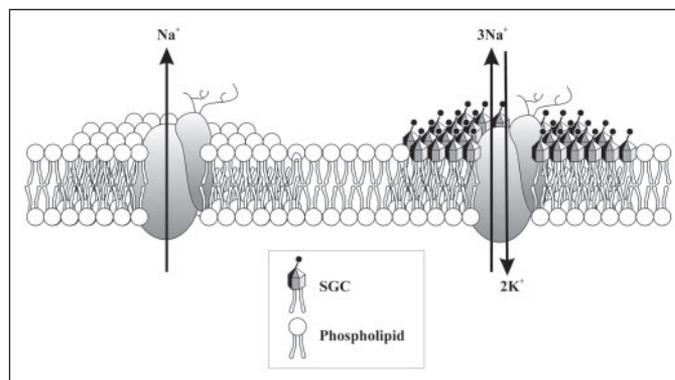


FIGURE 5. Hypothesis for SGC-enriched raft-based modulation of $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ transport stoichiometry.

which SGC is removed, there is Na^+ efflux with no K^+ influx (43). Consequently, Karlsson (24) proposed that when SGC and $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ are localized to different cellular compartments, the pump performs Na^+ uniport without reciprocal K^+ transport. In the absence of external K^+ , such transformations have been observed (44). Interestingly, an ouabain-sensitive Na^+ efflux rate equivalent to that obtained in a medium lacking K^+ is produced from erythrocytes treated with arylsulfatase (15); native ouabain-sensitive Na^+ efflux is only restored by SGC repletion and not K^+ addition. Also, arylsulfatase treatment of microsomes from pig kidney medulla inhibits only the ouabain-sensitive, K^+ -sensitive activity (16) and K^+ -dependent dephosphorylation of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ phosphointermediate (17); restoration in both cases only occurs following SGC repletion and not K^+ addition. Cumulatively, the literature suggests that when $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ and SGC are separated *in vitro*, the pump transports Na^+ only. Our data shows that $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ and SGC can be selectively segregated, *in vivo*, within the membrane itself; the fact that $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is sensitive to exogenous arylsulfatase treatment in SW trout but not FW trout implies that the segregation is a functional separation. Given the above precedents in the literature we hypothesize that the FW non-SGC-enriched raft $\text{Na}^+ - \text{K}^+ - \text{ATPase}$, having limited access to SGC, also transports Na^+ only, whereas the $3 \text{Na}_{\text{cyt}}^+ : 2 \text{K}_{\text{ext}}^+$ transport stoichiometry is predominately conserved by the SW SGC/raft-localized enzyme (Fig. 5).

This decoupling scheme may explain why no $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is raft-associated in the FW trout, since rainbow trout gill $\text{Na}^+ - \text{ATPase}$ activity is higher in fish acclimated to FW than those adapted to SW (45). Indeed, it is not known whether $\text{Na}^+ - \text{ATPase}$ and $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ are distinct or represent two different “formulations” of the same enzyme (46). In this model, the enormous energetic loss due to passive K^+ leak (24) would be minimized; in situations where $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ is segregated from SGC subdomains, K^+ leak at a lower level

that matches housekeeping levels of Na⁺-K⁺-ATPase catalyzed K⁺ influx could be assumed. This does not necessarily predict that intracellular K⁺ is lower in FW branchial epithelium, but rather, is recycled at a lower rate. In any event, Na⁺-ATPase is generally high in all electrolyte-adsorbing epithelia (47); therefore the extent to which such decoupling relates to kidney and intestinal systems warrants investigation.

In addition to its relationship with Na⁺-K⁺-ATPase, SGC appears responsible for maintaining raft structure in general. Increased desulfation (as monitored by loss of Sulf 1 reactivity) resulted in a disruption of GM1 profiles in a manner similar to that seen when cholesterol is removed by methyl- β -cyclodextrin (Fig. 4). The majority of lipid rafts are defined by their cholesterol dependence (4); these results indicated that rafts of rainbow trout gill BLM are also dependent on the SGC sulfate moiety. How SGC and its sulfate moiety maintain raft integrity is not clear. SGC can actually interfere with raft-mediated receptor-ligand binding *in vitro* (48). Furthermore, artificial SGC micelles exhibit lower transition temperatures than those of galactosyl ceramide indicating that the negatively charged sulfate contributes a repulsive force which weakens the head group interactions (49). Additionally, SGC and galactosyl ceramide transition temperatures and enthalpies do not depend significantly on *N*-acyl chain length suggesting that hydrogen bonding between the head groups may be stronger than the Van der Waals interactions between the acyl chains (49). However, OH-SGC fatty acids show higher transition temperatures than do those of galactosyl ceramide (49). Furthermore, changes in head group angle because of hydrogen bonding between fatty acid hydroxyls and head group components has been modeled for SGC (50). It is, therefore, possible that such intramolecular hydrogen bonding occurs within the OH-SGC fatty acid isoforms present in rainbow trout gill BLM (22), perhaps offsetting the repulsion due to a condensed anionic head group. Head group repulsion could also be offset by a counter-ion. Sphingomyelin, a common raft component (20), is a suitable lipid-based candidate, but cations should also be considered. In the presence of 2 M K⁺, the transition temperature of SGC is significantly increased, indicating that this cation can shield the repulsive charge and allow for stronger hydrogen bonding (49). The relative affinity of the SGC sulfate group for cations is Ca²⁺ > Mg²⁺ > K⁺ > Na⁺ > Li⁺ (25); perhaps cations in addition to K⁺ could act to stabilize SGC in rafts.

We have presented evidence that raft-based SGC interactions provide a regulatory platform for Na⁺-K⁺-ATPase catalysis in the BLM of rainbow trout gill epithelium. Upon SW acclimation, a population of Na⁺-K⁺-ATPase becomes raft-associated, facilitating a direct arylsulfatase-sensitive interaction between enzyme and cofactor. This is, therefore, partly responsible for the increase in Na⁺-K⁺-ATPase associated with SW adaptation. We also suggest a physiologically based mechanism of Na⁺-K⁺-ATPase decoupling: the traditional 3 Na_{cyt}⁺:2 K_{ext}⁺ transport stoichiometry is predominately conserved by the SGC/raft-localized enzyme, whereas non-SGC/raft-associated Na⁺-K⁺-ATPase may act as a Na⁺-ATPase. This is a novel concept whereby a single enzyme could carry out two distinct functions according to its molecular environment. Although the effects of differential partitioning to SGC domains are not yet clear for other K⁺ transporters, it suggests a new role for Na⁺-K⁺-ATPase in gill epithelium of freshwater fishes and perhaps other absorptive tissues. Future studies concerning Na⁺-K⁺-ATPase regulation must account for the relationship of the enzyme to SGC-enriched membrane rafts; additionally SGC knock-out systems (51) would prove useful systems to study the effects of SGC-related manipulations of Na⁺-K⁺-ATPase transport stoichiometry. Moreover, we illustrate the importance of the SGC sulfate moiety in maintaining

raft integrity in this membrane. The extent to which this applies to raft structure in general warrants further investigation.

Acknowledgments—We thank Dr. P. Fredman for kindly donating the Sulph 1 antibody, Dr. L. S. DeBruin, J. D. Haines, Dr. P. T. K. Woo, Dr. F. J. Sharom, C.-W. Tan, and S. Costigan for technical help and Dr. C. A. Lingwood, J. Bystriansky, and B. Speers-Roesch for helpful discussion.

REFERENCES

- Land, S. C., and Hochachka, P. W. (1994) *Am. J. Physiol.* **266**, C1028–C1036
- Rolfe, D. F., and Brown, G. C. (1997) *Physiol. Rev.* **77**, 731–758
- Blanco, G., and Mercer, R. W. (1998) *Am. J. Physiol.* **275**, F633–F650
- Simons, K., and Ikonen, E. (1997) *Nature* **387**, 569–572
- Simons, K., and Toomre, D. (2000) *Nat. Rev. Mol. Cell. Biol.* **1**, 31–39
- Schuck, S., and Simons, K. (2004) *J. Cell Sci.* **117**, 5955–5964
- Tillman, T., and Cascio, M. (2003) *Cell. Biochem. Biophys.* **38**, 161–190
- Hilgemann, D. W., Feng, S., and Nasuhoglu, C. (2001) *Sci. STKE* **111**, RE19
- Xie, Z. (2003) *Ann. N. Y. Acad. Sci.* **986**, 497–503
- Wang, H., Haas, M., Liang, M., Cai, T., Tian, J., Li, S., and Xie, Z. (2004) *J. Biol. Chem.* **279**, 17250–17259
- Yuan, Z., Cai, T., Tian, J., Ivanov, A. V., Giovannucci, D. R., and Xie, Z. (2005) *Mol. Biol. Cell* **16**, 4034–4045
- Liu, J., Liang, M., Liu, L., Malhotra, D., Xie, Z., and Shapiro, J. I. (2005) *Kidney Int.* **67**, 1844–1854
- Dalskov, S.-T., Immerdal, L., Niels-Christiansen, L.-L., Hansen, G. H., Schousboe, A., and Danielsen, E. M. (2005) *Neurochem. Int.* **46**, 489–499
- Gonzalez, M., Morales, M., and Zambrano, F. (1979) *J. Membr. Biol.* **51**, 347–359
- Zambrano, F., Morales, M., Fuentes, N., and Rojas, M. (1981) *J. Membr. Biol.* **63**, 71–75
- Gonzalez, E., and Zambrano, F. (1983) *Biochim. Biophys. Acta* **728**, 66–72
- Jedlicki, A., and Zambrano, F. (1985) *Arch. Biochem. Biophys.* **238**, 558–564
- Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, K. (1999) *Biochem. Biophys. Res. Comm.* **258**, 616–623
- DeBruin, L. S., Haines, J. D., Wellhauser, L. A., Radeva, G., Schonmann, V., Bienzle, D., and Harauz, G. (2005) *J. Neurosci. Res.* **80**, 211–225
- Brown, D. A., and Rose, J. K. (1992) *Cell* **68**, 533–544
- Evans, D. H., Piermarini, P. M., and Choe, K. (2005) *Physiol. Rev.* **85**, 97–177
- Lingwood, D., Fisher, L. J., Callahan, J. W., and Ballantyne, J. S. (2004) (2004) *J. Membr. Biol.* **201**, 77–84
- Karlsson, K.-A. (1977) in *Structure of Biological Membranes* (Abrahamsson, S., and Pascher, I., eds) pp. 245–274, Plenum Press, New York
- Karlsson, K.-A. (1982) in *Biological Membranes* (Chapman, D., ed) Vol. 4, pp. 1–74, Academic Press, London
- Abramson, M. B., Katzman, R., Curci, R., and Wilson, C. E. (1967) *Biochemistry* **6**, 295–304
- Perry, S. F., and Flik, G. (1988) *Am. J. Physiol.* **254**, R491–R498
- Radeva, G., and Sharom F. J. (2004) *Biochem. J.* **380**, 219–230
- Kushi, Y., Arita, M., Ishizuka, I., Kasama, T., Friedman, P., and Handa, S. (1996) *Biochim. Biophys. Acta* **1304**, 254–262
- Ura, K., Soyano, K., Omoto, N., Adachi, S., and Yamauchi, K. (1996) *Zool. Sci.* **13**, 219–227
- Wessel, D., and Flugge, U. I. (1984) *Anal. Biochem.* **138**, 141–143
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Gibbs, A., and Somero, G. N. (1989) *J. Exp. Biol.* **143**, 475–492
- Hochachka, P. W., and Somero, G. N. (1984) *Biochemical Adaptation*, Princeton University Press, New Jersey
- Richards, J. G., Semple, J. W., Bystriansky, J. S., and Schulte, P. M. (2003) *J. Exp. Biol.* **206**, 4475–4486
- Zambrano, F., and Rojas, M. (1987) *Arch. Biochem. Biophys.* **253**, 87–93
- Marshall, W. S., Bryson, S. E., and Garg, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5504–5508
- Marshall, W. S., Duquesney, R. W., Gillis, J. M., Bryson, S. E., and Liedtke, C. M. (1998) *J. Exp. Biol.* **201**, 1959–1965
- Singer, T. D., Tucker, S. J., Marshall, W. S., and Higgins, C. F. (1998) *Am. J. Physiol.* **274**, C715–C723
- Suzuki, M., Itakura, M., Kashiwagi, M., Nakamura, N., Matsuki, T., Sakuta, H., Naito, N., Takano, K., Fujita, T., and Hirose, S. (1999) *J. Biol. Chem.* **274**, 11376–11382
- Pelis, R. M., Zydlewski, J., and McCormick, S. D. (2001) *Am. J. Physiol.* **280**, R1844–R1852
- Yasunaga, H., Sakamoto, T., Yokota, S., and Ando, M. (2001) *J. Comp. Physiol.* **172B**, 447–453
- Nelson, W. J., and Hammerton, R. W. (1989) *J. Cell Biol.* **108**, 893–902

Regulation of Fish Gill $\text{Na}^+ - \text{K}^+ - \text{ATPase}$

43. Goldin, S. M., and Tong, S. W. (1974) *J. Biol. Chem.* **249**, 5907–5915
44. Cornelius, F. (1991) *Biochim. Biophys. Acta* **1071**, 19–66
45. Ventrella, V., Trombetti, F., Pagliarani, A., Trigari, G., Pirini, M., and Borgatti, A. R. (1992) *Comp. Biochem. Physiol.* **101B**, 1–7
46. Ventrella, V., Pagliarani, A., Trombetti, F., Pirini, M., Trigari, G., and Borgatti, A. R. (2001) *Physiol. Biochem. Zool.* **74**, 694–702
47. Proverbio, F., Marin, R., and Proverbio, T. (1991) *Comp. Biochem. Physiol.* **99A**, 279–283
48. Nutikka, A., and Lingwood, C. (2004) *Glycoconj. J.* **20**, 33–38
49. Boggs, J. M. (1987) *Biochim Biophys Acta* **906**, 353–404
50. Karlsson, K.-A., Angström, J., Bergström, J., and Lanne, B. (1992) *APMIS Suppl.* **27**, 71–83
51. Ishibashi, T., Dupree, J. L., Ikenaka, K., Hirahara, Y., Honke, K., Peles, E., Popko, B., Suzuki, K., Nishino, H., and Baba, H. (2002) *J. Neurosci.* **22**, 6507–6514
52. Lee, T.-H., Tsai, J.-C., Fang, M.-J., Yu, M.-J., and Hwang, P.-P. (1998) *Am. J. Physiol.* **275**, R926–R932