Decoupling the Na\(^+\)–K\(^+\)–ATPase in vivo: A possible new role in the gills of freshwater fishes

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Abstract

The literature suggests that when Na\(^+\)–K\(^+\)–ATPase has reduced access to its glycosphingolipid cofactor sulfogalactosyl ceramide (SGC), it is converted to a Na\(^+\) uniporter. We recently showed that such segregation can occur within a single membrane when Na\(^+\)–K\(^+\)–ATPase is excluded from membrane microdomains or ‘lipid rafts’ enriched in SGC (D. Lingwood, G. Harauz, J.S. Ballantyne, J. Biol. Chem. 280, 36545–36550). Specifically we demonstrated that Na\(^+\)–K\(^+\)–ATPase localizes to SGC-enriched rafts in the gill basolateral membrane (BLM) of rainbow trout exposed to seawater (SW) but not freshwater (FW). We therefore proposed that since the freshwater gill Na\(^+\)–K\(^+\)–ATPase was separated from BLM SGC it should also transport Na\(^+\) only, suggesting a new role for the pump in this epithelium. In this paper we discuss the biochemical evidence for SGC-based modulation of transport stoichiometry and highlight how a unique asparagine–lysine substitution in the FW pump isoform and FW gill transport energetics gear the Na\(^+\)–K\(^+\)–ATPase to perform Na\(^+\) uniport.

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1. Introduction

Na\(^+\)–K\(^+\)–ATPase (EC 3.6.1.37) is a membrane bound enzyme that uses the energy from the hydrolysis of one molecule of ATP to transport two K\(^+\) into and three Na\(^+\) out of most animal cells. As with all lipoprotein enzymes, a membrane environment is necessary for this activity (Roelofsen and Deenen, 1973). Na\(^+\)–K\(^+\)–ATPase can, however, exhibit specificity in its lipid requirement, in particular for the glycosphingolipid sulfogalactosyl ceramide (SGC). Karlsson (1977, 1982) proposed that SGC is a cofactor for Na\(^+\)–K\(^+\)–ATPase-catalyzed K\(^+\) translocation. His cofactor model, which has subsequently been supported by extensive biochemical evidence (Gonzalez et al., 1979; Zambrano et al., 1981; Gonzalez and Zambrano, 1983; Jedlicki and Zambrano, 1985), stated that SGC functions to donate a K\(^+\) (SGC exhibits a K\(^+\)Na\(^+\) selective charge interaction via its sulfate group (Abramson et al., 1967)) to the enzyme gate site (i.e., SGC is responsible for K\(^+\) import). Furthermore, Karlsson (1982) proposed that when SGC and Na\(^+\)–K\(^+\)–ATPase are localized to different cellular compartments, the pump performs Na\(^+\) uniport without reciprocal K\(^+\) transport. This was based on the observation that in Na\(^+\)–K\(^+\)–ATPase preparations where SGC is absent there is Na\(^+\) efflux with no K\(^+\) influx (Goldin and Tong, 1974).

In a recent study we showed that this enzyme and cofactor can be functionally segregated within a single membrane: Na\(^+\)–K\(^+\)–ATPase localizes to SGC-enriched microdomains or ‘lipid rafts’ (see Lai (2003) for a recent lipid raft review) in the gill basolateral membrane (BLM) of rainbow trout exposed to seawater (SW) (electrolyte secreting gill epithelia) but not freshwater (FW) (electrolyte absorbing epithelia) (Lingwood et al., 2005). Additionally, we found that arylsulfatase-induced desulfation of BLM SGC reduced Na\(^+\)–K\(^+\)–ATPase activity in SW but not FW trout; suggesting that partitioning between SGC-enriched rafts results in a functional difference with respect to enzyme catalysis. We proposed that the raft-mediated, co-localization of enzyme and cofactor of the SW gill was...
2. Decoupling the Na$^+$–K$^+$–ATPase

2.1. Modulation of transport stoichiometry by SGC

Through the manipulation of ionic medium Na$^+$–K$^+$–ATPase can be induced to perform Na$^+$ uniport without reciprocal K$^+$ transport:

1. Uncoupled Na$^+$ efflux: in the absence of extracellular Na$^+$ and K$^+$ the Na$^+$–K$^+$–ATPase catalyzes an ouabain-sensitive ATP supported Na$^+$ extrusion (Garrahan and Glynn, 1967a,b; Glynn et al., 1974; Karlish and Glynn, 1974; Glynn and Karlish, 1975; Lew et al., 1973; Glynn, 1988); the widely accepted Albers–Post scheme (Fahn et al., 1966; Post et al., 1969) predicts a transport stoichiometry of 3Na$^+_{cyt}$/1ATP hydrolyzed (no compensating ions are returned) which has been measured in shark rectal gland (Cornelius, 1989).

2. Na$^+$/K$^+$-congener exchange: in the absence of extracellular K$^+$ the Na$^+$–K$^+$–ATPase catalyzes an ouabain-sensitive ATP supported Na$^+$/Na$^+$ exchange in which Na$^+$ substitutes for extracellular K$^+$ (Lee and Blostein, 1980; Blostein, 1983); transport stoichiometry is 3Na$^+_{cyt}$/2Na$^+_{ext}$/1 ATP hydrolyzed (Cornelius and Skou, 1987; Yoda and Yoda, 1987).

Although such decoupling events have not been thought to occur in vivo (De Weer et al., 1988), we propose that differential partitioning of Na$^+$–K$^+$–ATPase to SGC membrane subdomains or lipid rafts represents a novel physiological mechanism for similar departures from 3Na$^+_{cyt}$/2K$^+_{ext}$ transport stoichiometry. Several earlier studies are consistent with this concept. An ouabain-sensitive Na$^+$ efflux rate equivalent to that obtained in a medium lacking K$^+$ is produced from erythrocytes treated with arylsulfatase (Zambrano et al., 1981); native ouabain-sensitive Na$^+$ efflux is only restored by SGC repletion and not K$^+$ addition. Additionally, arylsulfatase treatment of microsomes from pig kidney medulla inhibits both ouabain-sensitive, K$^+$-sensitive activity (Gonzalez and Zambrano, 1983) and K$^+$-inducible dephosphorylation of the Na$^+$–K$^+$–ATPase phospho-intermediate (Jedlicki and Zambrano, 1985); restoration in both cases only occurs following SGC repletion and not K$^+$ addition. These data indicate that when Na$^+$–K$^+$–ATPase has limited access to SGC, it transports Na$^+$ only. Since arylsulfatase treatment mimics the transport dynamics of systems lacking extracellular K$^+$, we contend that non-SGC-enriched raft Na$^+$–K$^+$–ATPase also pumps Na$^+$ only. The resultant transport stoichiometry is not clear and ADP stimulated Na$^+$/Na$^+$ exchange (Garrahan and Glynn, 1967a,b; De Weer, 1970; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979; Kennedy et al., 1986) (in absence of K$^+$ the enzyme can catalyze a one for one Na$^+$ exchange; it is electroneutral and net ATP hydrolysis is zero) cannot be ruled out. Nevertheless, we predict that the 3Na$^+_{cyt}$/2K$^+_{ext}$ coupling ratio is predominantly conserved by SGC/raft associated enzyme.

2.2. Na$^+$–K$^+$–ATPase vs Na$^+$–ATPase

K$^+$ independent, ouabain insensitive Na$^+$–ATPase activity has been detected in a number of different tissues of different organisms (Proverbio et al., 1991). These systems have been generally distinguished from Na$^+$–K$^+$–ATPase by preferential inhibition by ethacrynic acid and furosemide (Proverbio et al., 1989). However, ethacrynic acid is not an ideal inhibitor because it penetrates the cell and progressively inhibits other cell functions (Epstein, 1972a,b; Gaudemer and Foucher, 1967; Gordon, 1968). Furthermore, specificity according to furosemide sensitivity should be assessed with caution as furosemide is only a general inhibitor of Cl$^-$/cation co-transport (Engström et al., 1991). Na$^+$–ATPase and Na$^+$–K$^+$–ATPase do exhibit different responses to adenosine (Caruso-Neves et al., 1997), angiotensin II (Rangel et al., 1999) and bradykinin (Caruso-Neves et al., 1999). However, Na$^+$–K$^+$–ATPase isoforms also possess considerably different kinetic properties and modes of regulation (Mobasheri et al., 2000). Moreover, differentiation according to glycoside sensitivity is questionable since Na$^+$–K$^+$–ATPase isoforms differ in ouabain sensitivity (Blanco and Mercer, 1998). Finally, K$^+$ independence as a criterion for defining Na$^+$–ATPase is suspect since in the absence of K$^+$, the Na$^+$–K$^+$–ATPase will perform ATP-consuming Na$^+$ transport (see Na$^+$/K$^+$-congener exchange described above). The question of whether the functionally different Na$^+$–ATPase and Na$^+$–K$^+$–ATPase possess distinct structures or whether they represent two different isoform expressions of the same enzyme remains unanswered (Ventrella et al., 2001).

2.3. The solvent capacity of water

Compared with Na$^+$, K$^+$ is relatively large and therefore has a lower charge density. This means that Na$^+$ packs more densely with water than does K$^+$ (Hochachka and Somero, 1984). Several workers (Hazelwood, 1979; Ling, 1979; Wiggins, 1971, 1979) have proposed that as intracellular water is highly structured due to the presence of proteins, membranes, nucleic acids and thousands of organic metabolites, the different demands of K$^+$ and Na$^+$ on the solvent capacity of water can govern the ease with which these two ions enter the cell. For an inorganic cation to be accommodated in such a structured solvent, it requires a relatively low demand on solvent capacity. Selective accumulation of K$^+$ instead of Na$^+$ may, therefore, be a physical–chemical consequence of incorporating ions into already densely packed solutions. Ling (1979) suggested that specific ATPase ion pumps may not even be needed to maintain high intracellular K$^+$ and low Na$^+$. We are not advocating this extreme view of ion transport, nevertheless the different
influences of K⁺ and Na⁺ on water structure strengthen the notion of Na⁺–K⁺–ATPase-catalyzed Na⁺ uniport without reciprocal transport in vivo.

3. Decoupling the pump in the freshwater gill

3.1. SW and FW Na⁺–K⁺–ATPase isoforms have different transport stoichiometric capacities

Richards et al. (2003) discovered that two gill Na⁺–K⁺–ATPase catalytic (α) subunit isoforms are differentially expressed during rainbow trout salinity transfer. We suggested that in contrast to the FW isoform (α1a), the isoform upregulated in SW (α1b) had an ability to partition into SGC-enriched membrane microdomains (Lingwood et al., 2005). There are also differences in the sequences of these isoforms that contribute to a resultant decoupled Na⁺–K⁺–ATPase in FW.

The efficacy of ion-binding is a function of the amino acids residues that make up a transporter’s binding site. With respect to Na⁺–K⁺–ATPase, the residues Asn324 and Asn776 on the α-subunit have been found to act as cation coordination centres (Jorgensen and Pedersen, 2001). Specifically, ion occlusion studies on modified α-subunit from the kidney of Sus scrofa show that single substitution of Asn776 to Lys completely eliminates high affinity binding of Tl⁺ (a K⁺ analogue); replacement of Asn324 to Lys also causes a large depression in high affinity Tl⁺ occlusion. Substitution of both residues for Lys, however, increases Tl⁺ binding to one Tl⁺ ion occluded per ouabain binding site. Energy analysis shows that the single substitution of Asn324 or Asn776 to Lys reduces the free energy of binding with 5.7 or 15.8 kJ/mol, respectively, while the interaction between the two Lys residues after double Lys substitution favours Tl⁺ binding by ~18.3 kJ/mol (Jorgensen and Pedersen, 2001).

Such a double Lys substitution has been found to occur in an α-subunit isoform expressed in the chloride cells of the salt gland of the brine shrimp Artemia salina (Baxter-Lowe et al., 1989; Escalante et al., 1995; Fig. 1); additionally, this isoform is up-regulated upon increasing salt exposure (Jorgensen and Domenech, 2005). Jorgensen and Pedersen (2001) suggested that since the electrochemical work required to transport Na⁺ from hemolymph to the brine shrimp’s high salt environment (200 to 300 ppt) is greater than the free energy if ATP hydrolysis, the double Lys substitutions facilitated a compensatory alteration in Na⁺–K⁺–ATPase transport stoichiometry. Specifically the pump (activity is 10–20% with double Lys substitution) would transport one K⁺ and two Na⁺ per ATP hydrolyzed. Upon examination of the rainbow trout FW (α1a) and SW (α1b) isoforms it becomes apparent that in FW there is a similar Asn to Lys substitution, but only at Asn776 (Fig. 1); the SW form exhibits no Lys substitutions. The replacement therefore differs from that of the shrimp in that it is in the direction of FW adaptation and is only a single substitution. Given the fact that Lys replacement at Asn776 eliminates K⁺ binding (interestingly much more than a single substitution at Asn324, see above), and that in this case there is no recovery of K⁺ occlusion by an additional Lys substitution at Asn324, we can propose that the FW isoform should transport Na⁺ only. The nature of that stoichiometry, given the costs of Na⁺ across gill epithelium will be discussed later.

3.2. Physiological considerations

In order to maintain ionic homeostasis, the fish gill absorbs salt in freshwater. Although the mechanism is controversial, gill chloride cells (also termed mitochondria rich cells) house much of the machinery thought to perform this function (Perry, 1997). In the current model of salt uptake (Fig. 2A; reviewed by Marshall, 2002) an apical vacuolar type H⁺–ATPase generates an electrochemical gradient favourable for the passive uptake of Na⁺ through an epithelial Na⁺ channel and acidifies the boundary layer. In the apical membrane, a Cl⁻–HCO₃⁻ antiporter, possibly driven by low HCO₃⁻ in the boundary layer, drives Cl⁻ into the cell. Intracellular H⁺ and HCO₃⁻ are thought to be derived from the hydration of CO₂. Cl⁻ probably exits the cell via basolateral Cl⁻ channels, while Na⁺ uptake is completed by the Na⁺–K⁺–ATPase.

Although freshwater chloride cells exhibit lower Na⁺–K⁺–ATPase levels than their seawater counterparts (Sakamoto et al., 2001), these levels are far greater than those possessed by their surrounding non-chloride gill cell types (Perry, 1997). Current models of salt uptake do not require Na⁺–K⁺–ATPase activity beyond a generic housekeeping level. The reason for heightened Na⁺–K⁺–ATPase in FW chloride cells therefore remains unclear (Perry, 1997; Evans et al., 2005).

Na⁺–ATPase is generally found in higher levels in the basal membranes of electrolyte-adsorbing epithelia (Proverbio et al., 1991). It is suggested to produce a vectorial flux of Na⁺ into the interstitium that is thought to facilitate salt absorption in kidney, small intestine and gill epithelium (Proverbio et al., 1991). If non-SGC/raft associated Na⁺–K⁺–ATPase acting as a Na⁺–ATPase performs a similar function, it explains why no Na⁺–K⁺–ATPase was raft associated in the FW trout (Lingwood et al., 2005); rainbow trout gill Na⁺–ATPase activity is higher in fish acclimated to FW than those adapted to SW (Ventrella et al., 1992). This addresses the unclear role of Na⁺–K⁺–ATPase in salt uptake by the gill epithelium of freshwater fishes. Based on their existence in SW adapted gill epithelia (Evans et al., 2005), current models of salt uptake in freshwater fish gill assume putative, unidentified basolateral K⁺ channels to account for K⁺ recycling (Fig. 2A). Passive K⁺
leak constitutes an enormous energetic loss for absorptive epithelia (Karlsson, 1982). Under our scheme, the majority of Na\(^+\)-K\(^+\)-ATPase pumps Na\(^+\) only (Fig. 2B). Therefore, 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.

Transforming Na\(^+\)-K\(^+\)-ATPase to a Na\(^+\)-ATPase may also help facilitate Cl-uptake in the FW gill. Experimental evidence indicates that both uncoupled Na\(^+\) efflux and Na\(^+\)/K\(^+\)-congener exchange are electrogenic (Cornelius and Skou, 1987; Yoda and Yoda, 1987; Cornelius, 1989). However, uncoupled Na\(^+\) efflux from red blood cell ghosts is accompanied by an ouabain-sensitive efflux of the anions present in solution (SO\(_4\)^{-2}) (Cornelius, 1991). Additionally, an electroneutral uncoupled Na\(^+\) efflux has been observed in proteoliposomes at pH 7.0 (Goldshleger et al., 1990); the authors suggest co-transport of Cl\(^-\) or counter-transport of H\(^+\) in order to explain this. Finally, in Na\(^+\)-K\(^+\)-ATPase preparations where SGC is absent

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Fig. 2. Current model for freshwater gill chloride cell salt uptake (A) (Redrawn from Marshall, 2002) and proposed model with decoupled Na\(^+\)-K\(^+\)-ATPase (B).
Goldman Equation (P. Goldman and Tong, 1974). Current models of salt uptake by the FW gill predict that Cl$^{-}$ absorption into the blood is completed by a Cl$^{-}$ channel in the gill basolateral membrane (Fig. 2A); a translocation that would therefore be favourable in the presence of decoupled Na$^{+}$ transport (Fig. 2B).

3.3. Decoupled Na$^{+}$ transport energetics

Decoupled Na$^{+}$ transport itself appears more thermodynamically plausible in the FW gill. Using available literature values (Wright, 1991; Morgan et al., 1994) we calculated the energy barriers for Na$^{+}$ transport across whole epithelium, apical membrane and BLM of FW acclimated (Na$^{+}$ absorbing) and SW adapted (Na$^{+}$ secreting) gill epithelia (Table 1). Assuming no junctional potentials these values are reliable because energy is conserved in FW (EPITHFW Na$^{+}$ ~ BLMFW Na$^{+}$ AMFW Na$^{+}$) and EPITHNa$^{+}$ is less than zero in SW (a negative value is indicative of the passive paracellular Na$^{+}$ extrusion driven by the transepithelial potential associated with apically concentrated Cl$^{-}$ (Evans et al., 2005)). BLMNa$^{+}$ represents the cost of Na$^{+}$ transport by the basolaterally restricted Na$^{+}$-K$^{+}$-ATPase in SW and FW, whereas the cost associated with AMFW Na$^{+}$ is consistent with the energy consumption by the apical H$^{+}$-ATPase modeled for salt uptake in FW (Evans et al., 2005). Although total energetic costs to Na$^{+}$ movement appear similar in FW and SW (EPITHFW Na$^{+}$ ~ BLMFW Na$^{+}$ AMFW Na$^{+}$ ~ BLMSW Na$^{+}$ EPITHSW Na$^{+}$ ~ 11 kJ/mol), the FW gill divides this energy into two active steps (Table 1) making it energetically cheaper to transport Na$^{+}$ across its BLM (BLMFW Na$^{+}$<BLMFW Na$^{+}$). Conservatively, if we assume Na$^{+}$-K$^{+}$-ATPase operates at <60% efficiency (Kirschner, 2004), the energy released by ATP hydrolysis in a cycle (∼ 63 kJ (Civan et al., 1983)) could support a novel 6–7Na$^{+}$cyt/1ATP stoichiometry (5× BLMFW Na$^{+}$) in FW but only the traditional 3Na$^{+}$cyt/1ATP stoichiometry in SW (3× BLMFW Na$^{+}$). We would therefore contend that decoupling Na$^{+}$-K$^{+}$-ATPase to a Na$^{+}$-ATPase is more favourable in the Na$^{+}$ absorbing system.

To summarize, we have proposed a thermodynamically and physiologically viable mechanism whereby Na$^{+}$-K$^{+}$-ATPase may be transformed to a Na$^{+}$-ATPase via its separation from SGC due to partitioning into different membrane subdomains. This concept is of wide interest since it relates to the functioning of the main ion transporter in most cells with applications in understanding the regulation of all epithelial electrolyte absorbing/transport processes; particularly those of the FW gill where it is complemented by adaptations in amino acid residues and gill transport energetics. The challenge now is to consistently account for altered Na$^{+}$-K$^{+}$-ATPase transport stoichiometry both as a function of cation binding residues and pump position relative to SGC microdomains within the membrane.
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