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Na^+,K^+-ATPase Na^+ Affinity in Rat Skeletal Muscle Fiber Types

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Abstract Previous studies in expression systems have found different ion activation of the Na^+/K^+-ATPase isozymes, which suggest that different muscles have different ion affinities. The rate of ATP hydrolysis was used to quantify Na^+,K^+-ATPase activity, and the Na^+ affinity of Na^+,K^+-ATPase was studied in total membranes from rat muscle and purified membranes from muscle with different fiber types. The Na^+ affinity was higher (K_m lower) in oxidative muscle compared with glycolytic muscle and in purified membranes from oxidative muscle compared with glycolytic muscle. Na^+,K^+-ATPase isoform analysis implied that heterodimers containing the β_1 isoform have a higher Na^+ affinity than heterodimers containing the β_2 isoform. Immunoprecipitation experiments demonstrated that dimers with α_1 are responsible for approximately 36% of the total Na,K-ATPase activity. Selective inhibition of the α_2 isoform with ouabain suggested that heterodimers containing the α_1 isoform have a higher Na^+ affinity than heterodimers containing the α_2 isoform. The estimated K_m values for Na^+ are 4.0, 5.5, 7.5 and 13 mM for $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_2$ and $\alpha_2\beta_2$, respectively. The affinity differences and isoform distributions imply that the degree of activation of Na^+,K^+-ATPase at physiological Na^+ concentrations differs between muscles (oxidative and glycolytic) and between subcellular membrane domains with different isoform compositions. These differences may have consequences for ion balance across the muscle membrane.

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Introduction

Ion gradients across muscle membranes are important for muscle function. Activity-induced changes in ion distribution affect muscle excitability and may lead to impairment of force development.

Na^+,K^+-ATPase (Na,K pump) maintains normal transmembrane gradients for Na^+ and K^+ and counteracts changes in ion gradients due to muscle activity. Therefore, regulation of the pump is important for muscle function. The minimal functional pump is a heterodimer comprising one α- and one β-subunit. Each subunit is expressed in a number of isoforms, of which α_1, α_2, β_1 and β_2 are important in rat skeletal muscle (Clausen 2003). The α_1 and α_2 isoforms are found in all muscle fiber types; however, they have different cellular localizations (Kristensen and Juel 2009). The α_1 isoform is localized to the outer membrane, whereas the α_2 isoform is found in the outer membrane and the t-tubuli (Williams et al. 2001). The β_1 isoform is mainly found in oxidative fibers, whereas the β_2 isoform is found in glycolytic fibers (Fowles et al. 2004; Thompson and McDonough 1996). The different localizations of these isoforms suggest that the heterodimers have different roles (He et al. 2001) and are regulated differently.

The properties of the different heterodimers (isozymes) have only been intensely studied in expression systems such as *Xenopus* oocytes, HeLa cells, *Saccharomyces*, Sf-9 insect cells and *Pichia pastoris* (Blanco and Mercer 1998; Crambert et al. 2000; Lifshitz et al. 2006, 2007; Müller-Ehmsen et al. 2001; Zahler et al. 1997), i.e., away

from the native environment. In some of these model systems the affinities for Na^+ and K^+ are higher in heterodimers including the α_1 isoform than in heterodimers containing the α_2 isoform (Bibert et al. 2008; Crambert et al. 2000, 2002; Müller-Ehmsen et al. 2001; Zheng et al. 2008), although similar affinities have also been reported (Lifshitz et al. 2006). For the β-subunits, Na^+ and K^+ affinities seem to be higher if β_1 is present rather than β_2 (Crambert et al. 2000); however, conflicting data have also been reported for this (Blanco and Mercer 1998). In addition, the K_m values differ between these studies. The Na^+ affinity of Na^+,K^+-ATPase has not been studied directly in membrane fractions of skeletal muscle because of membrane separation difficulties and the extremely high background ATPase activity not related to Na^+,K^+-ATPase.

In recent years, it has become evident that in different tissues the Na^+,K^+-ATPase complex can include members of the FXYD protein family. In skeletal muscle the FXYD1 (PLM) protein is associated with Na^+,K^+-ATPase (Crambert et al. 2002; Reis et al. 2005), and in *Xenopus* oocytes PLM associated with the $\alpha\beta$ complex significantly increased the K_m for Na^+ (Crambert et al. 2002).

Based on the different ion affinities of the Na^+,K^+-ATPase subunits in expression systems and the different distribution of pump isoforms in muscle fiber types, it is hypothesized that the affinities for Na^+ and K^+ differ between different muscles. It has previously been shown that the Na^+ affinity of Na,K-ATPase differs between muscles and that the affinity can change with muscle activity (Juel 2009). In the present study the different fiber type-dependent expression of subunits is used to investigate the enzyme properties of the individual isozymes. Therefore, one aim of the present study was to investigate the Na^+ affinities for the various Na^+,K^+-ATPase isoforms present in rat muscle, to characterize muscle fiber type-specific variations in Na,K-pump function. Another aim was to evaluate the relative importance of the dimers containing α_1 and α_2. For that purpose, we used total membranes from muscle with specific Na^+,K^+-ATPase isoform content and purified membranes from different muscles. Muscle ATPase activity in a number of previous studies has been determined with an assay that calculates the maximal K^+-dependent phosphatase activity using 3-*O*-methylfluorescein as the substrate (Fraser and McKenna 1998). The advantage of this method is that the problem of high background ATPase activity is avoided; however, the calculated K_m values for K^+ are not reliable, and Na^+ affinities cannot be measured. We have therefore used an Na^+,K^+-ATPase assay based on quantification of ATP hydrolysis (Rasmussen et al. 2008).

Materials and Methods

Animals and Muscle Sampling

Animals were handled in accordance with Danish animal welfare regulations. Male Wistar rats (body weight 130–150 g, age 6 weeks) were provided with unlimited food and water and kept under a 12 h/12 h dark/light cycle. Rats were killed with a blow to the neck followed by cervical dislocation, and muscle tissue was immediately removed. All sample preparations were carried out at a maximum temperature of 4°C unless otherwise stated. The protein content of samples was determined using a bovine serum albumin (BSA) standard (DC protein assay; Bio-Rad, Hercules, CA, USA).

Muscle Preparations and Membrane Fractionating: Total Membranes

After mincing, muscle fragments were homogenized for 30 s (Kinematica, Lyzern, Switzerland) in 250 mM mannitol, 30 mM L-histidine, 5 mM EGTA and 0.1% deoxycholate (pH 6.8). The sample (crude homogenate) was subjected to $3{,}000\times g$ spinning for 30 min, and the supernatant was subjected to $190{,}000\times g$ spinning for 90 min (4°C). The final pellet, called "total membranes," was resuspended in assay buffer (see below) and used for the activity measurements. For immunoprecipitation 0.125 mg/ml $C_{12}E_8$ was added (see below). Sample protein recovery was about 5% of the total protein content (including soluble proteins) in the crude homogenate, and the Na,K-ATPase α_2 isoform protein content in the final total membrane fraction (quantified with Western blotting) was more than half of the content in the crude homogenates. The production of total membranes removed some of the background ATPase activity, thereby increasing the maximal Na^+-stimulated Na,K-ATPase activity to about 11% of the total ATPase activity (including Ca^{2+} ATPase).

Sarcolemma Giant Vesicles: Purified Membranes

Because the homogenate contained a large background from other ATPases, we also used sarcolemmal giant vesicles as a membrane purification method. Sarcolemma was isolated as giant vesicles in accordance with the technique of Juel (1991). This method is well characterized (Juel et al. 1994; Kristensen et al. 2006; Nielsen et al. 2003; Pilegaard et al. 1993) and often used in our laboratory. Vesicles contain soluble proteins from the cell; however, previous characterizations have demonstrated that the vesicles contain no t-tubule membranes and very low contamination with sarcoplasmatic reticulum. Briefly, muscle tissue was incubated for 45 min at 34°C in KCl-MOPS buffer

(140 mM KCl, 5 mM MOPS [pH 7.4]) containing 150 U/ml collagenase. Samples were washed and subjected to 50×*g* three-layer gradient centrifugation for 45 min at room temperature. After harvesting, giant vesicles were washed in KCl-MOPS buffer and recovered by centrifugation at 830×*g* for 30 min at room temperature.

Fiber type-specific samples containing glycolytic (white) and oxidative (red) muscle fibers were made from the following muscles: white gastrocnemius (WG), white vastus lateralis (VLw), white tibialis anterior (glycolytic), soleus (SOL), red gastrocnemius (RG) and vastus intermedius (oxidative). The fiber-type distribution I:IIA:IID/X:IIB in these preparations has been calculated, in accordance with Delph and Duan (1996), to be about 0:0:4:96 (glycolytic) and 69:23:7:1 (oxidative).

Immunoprecipitation

Immunoprecipitation experiments were made with total membranes (see above) from VLw muscle. After ultracentrifugation, pellet was resuspended in assay buffer (see below) containing 0.125 mg/ml $C_{12}E_8$ and the protein concentration measured. Each sample was split in two, and α_1 antibody-conjugated agarose beads (C464.6; Santa Cruz Biotechnology, Santa Cruz, CA) washed in $C_{12}E_8$-containing assay buffer were added to half of the split samples. $C_{12}E_8$-containing assay buffer was added to the remaining samples (control samples) to equal sample volume. Samples were slowly stirred overnight. After incubation, samples were subjected to 1,000×*g* spinning, the supernatants were removed, Na^+-stimulated Na^+,K^+-ATPase activity was determined by measuring the hydrolysis of ATP (see below) and the relative amounts of α_1- and α_2-subunits were measured using Western blotting. Preliminary experiments demonstrated that the ATPase measurements were not affected by the presence of the diluted detergent.

Measurement of Na^+-Stimulated Na^+,K^+-ATPase Activity in Total Membranes and Purified Membranes

Na^+-stimulated Na^+,K^+-ATPase activity was determined by measuring the hydrolysis of ^{33}P-ATP (Rasmussen et al. 2008; Sandiford et al. 2005). Purified membranes or total membranes were suspended in assay buffer (10 mM KCl or 80 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris base, 5 mM EGTA [pH 7.4]). The purified membranes (vesicles) were subjected to five freeze–thaw cycles to permeabilize them (Sandiford et al. 2005).

In studies with purified membranes, each sample contained 9–95 μg of protein, including soluble protein. In total membrane studies, each sample contained 8–100 μg of protein. Na^+ was added to the samples to a final concentration of 0–80 (0, 2, 4, 6, 10, 20, 40, 80, 0) mM (ionic strength was kept constant by substituting NaCl with choline chloride) or K^+ was added to the samples to a final concentration of 0–10 (0, 0.5, 1, 2, 3, 5, 7.5, 10, 0) mM with Na^+ kept constant at 80 mM. After 15 min of preincubation at 37°C, the reaction was started by adding ^{33}P-ATP (Perkin-Elmer, Oak Brook, IL) plus Mg-ATP (Sigma, St. Louis, MO) to a final concentration of 5 mM. After 30 min, samples were placed on ice and the reaction was stopped by adding 1 ml of a solution containing 1 M H_2SO_4 and 0.5% $(NH_4)Mo_7O_{24}$. Inorganic phosphate ions were isolated by addition of 2 ml isobutanol, extracted by 15 s vortexing and centrifuged at 1,000×*g* for 5 min. A 1-ml aliquot of the isobutanol phase was counted in Ultima Gold (Perkin-Elmer) using a *β*-scintillation counter. All samples were run in triplicate (0 mM Na^+ or K^+ six times), and the activity at 0 mM Na^+ or 0 mM K^+ was subtracted from all activity values. Preliminary experiments demonstrated that the Na^+-stimulated activity was completely inhibited by preincubation with 2 mM ouabain (data not shown).

In an alternative method for measurements of ATPase activity, Pi was detected with the malachite-based Biomol Green reagent (AK-111; Biomol, Plymouth Meeting, PA). The incubation solution was as above, but protein per sample was reduced to 8–20 μg. The reaction was terminated by adding 1 ml Biomol reagent. After 30 min the absorbance was read at 620 nm and Pi was calculated from a standard curve.

Western Blotting

Samples were mixed with sample buffer (2 mM Tris–HCl, 0.2 mM EDTA, 20 mM DTT, 4% SDS, 10% glycerol, 0.04% bromophenol blue [pH 8.0]). Equal amounts of protein were loaded into each lane and separated by 8–18% SDS–PAGE (Excel 8–18% gradient gel, GE Healthcare, Uppsala, Sweden) or 12.5% SDS–PAGE (ExcellGel, GE Healthcare, Uppsala, Sweden) for PLM measurements. The proteins were then electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Billerica, MA, USA). The membrane was blocked for 60 min at room temperature in TS buffer (10 mM Tris base, 0.9% NaCl [pH 7.4]) containing 2% BSA, 1% skim-milk powder and 0.1% Tween-20 before incubation with primary antibody diluted in a similar buffer overnight (4°C). After treatment with a horseradish peroxidase-coupled secondary antibody (Dako, Copenhagen, Denmark) for 90 min at room temperature, the membrane was repeatedly washed in TS buffer with or without 0.05% Tween-20. The membrane was incubated with enhanced chemiluminescence reagent (Amersham) and visualized on Hyperfilm (Amersham). Samples to be compared were loaded on the same gel. Relative protein concentrations were quantified by scanning the film and analyzing band intensities with UN-SCAN-IT version 5.1

software (UN-SCAN-IT, Silk Scientific, Orem, Utah, USA). For each isoform, the values are calculated relative to the mean of the highest value for that isoform. This method does not allow comparison of isoform protein levels.

Antibodies

The α_1 isoform was detected with the monoclonal α6F antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). A monoclonal antibody (McB2) used to detect the α_2 isoform and a polyclonal antibody to detect the β_1 isoform were generously provided by Dr. P. A. Pedersen (University of Copenhagen, Denmark). The β_2 isoform was detected with a polyclonal antibody (06-171; Upstate Biotechnology, Lake Placid, NY). For detection of PLM (FXYD1), we used polyclonal C2 antibody, generously provided by Dr. J. Cheung (Thomas Jefferson Univ. Philadelphia, PA, USA).

Statistics

Apparent affinity constant $K_{0.5}$ (K_m) and V_{max} for Na^+ were determined for each individual experiment by nonlinear regression (SigmaPlot software; Systat Software, Richmond, CA) with a Hill equation in the following form: rate of ATP hydrolysis $= (V_{max}\ [Na]^b)/([Na]^b + K_m^b)$, with a variable Hill coefficient (b). The calculated mean ($n = 6$–16) Hill parameters for each series of experiments were used for construction of the curves in Fig. 2.

A two-way ANOVA for repeated measurements was used to detect differences in Na^+-stimulated Na^+,K^+-ATPase activity (SOL vs. VLw muscle or oxidative vs. glycolytic). Post hoc analysis was performed using Tukey's test. The mean K_m values were compared using unpaired t-test (oxidative vs. glycolytic samples or for comparison between muscles). $P < 0.05$ was considered significant.

Results

Relative Distribution of Pump Subunits in Total Membranes and Purified Membranes

The relative distribution of isoforms in total membranes from various muscles is depicted in Fig. 1a. The α isoforms were present in all muscles without a clear fiber type dependence, whereas the distribution of β isoforms was clearly dependent on fiber type: β_1 mainly in oxidative fibers and β_2 mainly in glycolytic fibers. The relative distributions of subunits in the purified membranes (sarcolemmal giant vesicles) used below are depicted in Fig. 1b. The α_1 and α_2 isoforms were present in both fiber types. The β_1 isoform was present mainly in oxidative fibers, whereas the β_2 isoform was present mainly in glycolytic fibers. PLM protein was also present in both fiber types; however, it had a higher density in oxidative fibers compared with glycolytic fibers.

Na^+-Stimulated Na^+,K^+-ATPase Activity in Total Membranes

ATPase activity in total membranes from SOL, RG, VLw and WG muscles was measured at Na^+ concentrations of 0–80 mM with the K^+ concentration constant at 10 mM. For oxidative muscles (SOL and RG), Na^+,K^+-ATPase activity reached a maximal value within the Na^+ interval used for the measurements. Na^+,K^+-ATPase in the most glycolytic muscles (VLw and WG) did not fully reach an apparent maximal value within the Na^+ concentration interval used, and only partial saturation was obtained. Examples of Na^+ dependence of the Na,K-ATPase in oxidative (SOL) and glycolytic (VLw) muscle are shown in Fig. 2. The K_m for Na^+ in individual muscle was quantified from a Hill plot. The mean K_m obtained in SOL was significantly lower ($P < 0.01$, $n = 16$) than that obtained in VLw (5.0 ± 0.8 vs. 11.3 ± 1.8 mM). Similarly, the K_m was lower in RG compared to WG.

The calculated Na^+ affinity (mean K_m) in total membranes from different muscles is included in Fig. 3. It can be concluded that the Na^+ affinity is significantly higher (K_m lower) in more oxidative muscle compared with glycolytic muscle; the Na^+ affinity in 100% oxidative muscle is probably double compared to 100% glycolytic fiber.

Na^+-Stimulated Na^+,K^+-ATPase Activity in Purified Membranes from Oxidative and Glycolytic Fibers

ATPase activity in purified membranes from oxidative and glycolytic fibers was measured at Na^+ concentrations of 0–80 mM. The stimulatory effect of Na^+ was saturated at high concentrations in both oxidative and glycolytic fibers. Purified membranes from oxidative fibers had a significantly lower mean K_m (higher affinity) compared with glycolytic fibers (6.1 ± 1.6 vs. 14.0 ± 1.7 mM), which supports the findings with total membranes. Mean K_m values are included in Fig. 3.

Removal of α_1-Subunits by Immunoprecipitation

This experiment was performed to determine the ratio of ATPase activities in dimers with α_1- or α_2-subunits. An α_1-specific antibody was used in immunoprecipitation experiments to remove α_1 from the lysate, and the α and β contents in the lysate before and after were compared by Western blotting. Immunoprecipitation removed 74 ± 9%

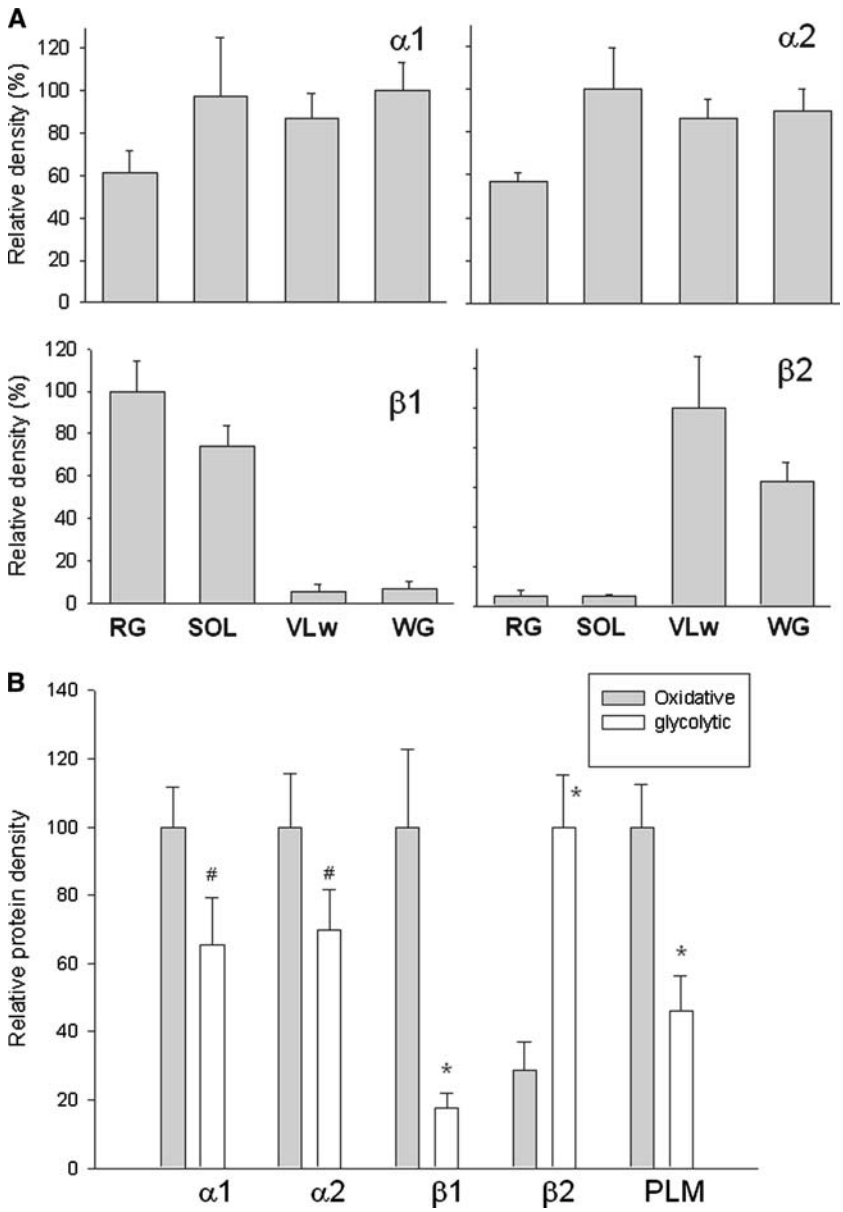

Fig. 1 **a** Relative distribution of Na^+,K^+-ATPase subunits in total membranes from four selected muscles (*SOL* soleus, *RG* red gastrocnemius, *WG* white gastrocnemius, *VLw* white vastus lateralis). **b** Distribution of subunits in purified membranes produced from oxidative (*gray columns*) and glycolytic (*white columns*) fibers determined with Western blotting. Each lane in **a** and **b** was loaded with the same amount of protein, and samples to be compared were loaded on the same gel. Note that the values are calculated relative to the mean of the highest value ($n = 7$), mean ± SE.
* Different from membranes produced from oxidative fibers ($P < 0.05$), # tended to be different ($P < 0.1$)

of α_1 (determined by Western blotting, $n = 6$, $P < 0.05$), whereas the α_2 content remained unchanged (97.7 ± 10% left in the lysate after immunoprecipitation) (Fig. 4a, b). The β_1 content after immunoprecipitation was 76 ± 13% and the β_2 content was 82 ± 20% compared to the respective control values. The Na^+-sensitive ATPase activity of the lysate was measured in control samples and in samples where α_1 was removed by immunoprecipitation (Fig. 4c). K_m values were 13.5 and 16.3 mM in control lysate and in lysate after immunoprecipitation, respectively (not significantly different). The 74% removal of α_1 reduced ($P < 0.05$) the ATPase activity by 27% at 20 mM Na^+. It is therefore expected that 100% removal of α_1 reduces the ATPase activity by approximately 36%; i.e., the ATPase activity mediated by dimers with α_1 makes up one-third of the total ATPase activity.

Separation of α Isoforms with Ouabain

Ouabain is expected to primarily inhibit heterodimers containing the α_2 isoform. One experiment used total membranes from VLw muscle, which predominantly contains β_2. The active dimers after ouabain treatment are therefore mainly $\alpha_1\beta_2$. Ouabain reduced the maximal activity by 64%. High Na^+ did not inhibit the activity either in the control experiments or in the ouabain experiments (Fig. 5c). Ouabain reduced K_m in VLw from 10.4 to 7.7 mM ($n = 6$, $P < 0.05$).

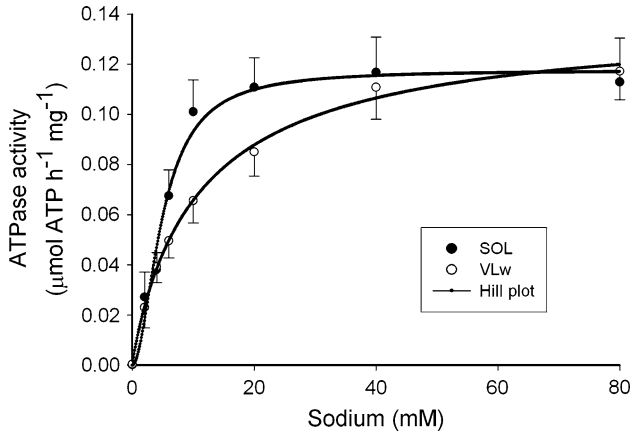

Fig. 2 Na^+-stimulated Na^+,K^+-ATPase activity at various Na^+ concentrations and 10 mM K^+ in total membranes of muscle with different fiber-type composition. *SOL* soleus (representing oxidative muscle), *VLw* white vastus lateralis (representing glycolytic muscle). All measurements were subtracted from the activity at 0 mM Na^+. Each value represents mean ± SE ($n = 16$). *Curves* represent a Hill plot constructed from the mean values of the Hill parameters obtained from the individual experiments. Mean K_m was 5.0 ± 0.8 and 11.3 ± 1.8 mM and mean Hill coefficient was 1.9 and 0.9, for SOL and VLw, respectively

A similar experiment used total membranes from SOL muscle, which predominantly contains β_1. The active dimers after ouabain treatment are therefore mainly $\alpha_1\beta_1$. Ouabain reduced the maximal activity by 61%. In the control experiments high Na^+ (80 mM) slightly reduced the activity (10%) compared to the highest value, whereas high Na^+ reduced the activity by approximately 81% in the ouabain-treated samples (Fig. 5b). This inhibition at high Na^+ makes the calculation of K_m difficult, but inspection of the data at low Na^+ concentrations suggests that K_m for Na^+ was reduced (to ~4 mM) in the ouabain-treated samples compared to control.

Purified membranes from mixed muscle (both β_1 and β_2) were treated with 10^{-5} M ouabain for 1 h before activity was measured. Ouabain treatment reduced the maximal activity by 60% in the Na^+ concentration interval used. The control values showed a small (~23%) reduction in activity with high Na^+ (80 mM) compared to the highest value obtained, whereas this inhibition was 76% in the ouabain-treated samples (Fig. 5a).

Discussion

This study confirmed the hypothesis that Na^+ affinity is fiber type-specific, due to the different distribution of the ATPase isoforms.

Affinity Differences: Role of Subunit Distribution

The affinities (apparent EC_{50} or K_m) for Na^+ reported in the literature have been obtained from studies in expression systems and show large variations. For example, the K_m for Na^+ was 9.6 and 13.8 mM for the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ dimers, respectively, expressed in yeast (Müller-Ehmsen et al. 2001); 8.3–9.2 and 12.8–13.6 mM for the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ dimers, respectively, expressed in *Xenopus* oocytes (Crambert et al. 2000, 2002); and 16.4 and 8.8 mM for the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers expressed in an insect cell line (Blanco and Mercer 1998). In transfected HeLa cells, the K_m for Na^+ was 12 mM for the $\alpha_1\beta_1$ isoform and 22 mM for the $\alpha_2\beta_1$ isoform (Zahler et al. 1997). These values were obtained without PLM expression. Systematic measurements of ion affinities

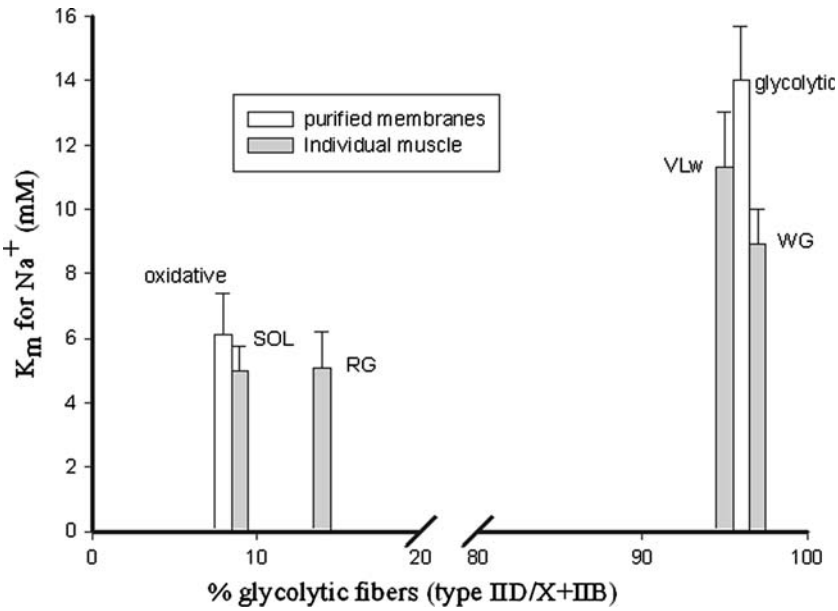

Fig. 3 K_m for Na^+ and fiber type. Mean K_m values (±SE) in total membranes from individual muscle (SOL, RG, VLw and WG; $n = 8$ or 16) and in purified membranes from glycolytic or oxidative fibers ($n = 8$) are depicted as a function of the percentage glycolytic fibers (% type IID/X + IIB)

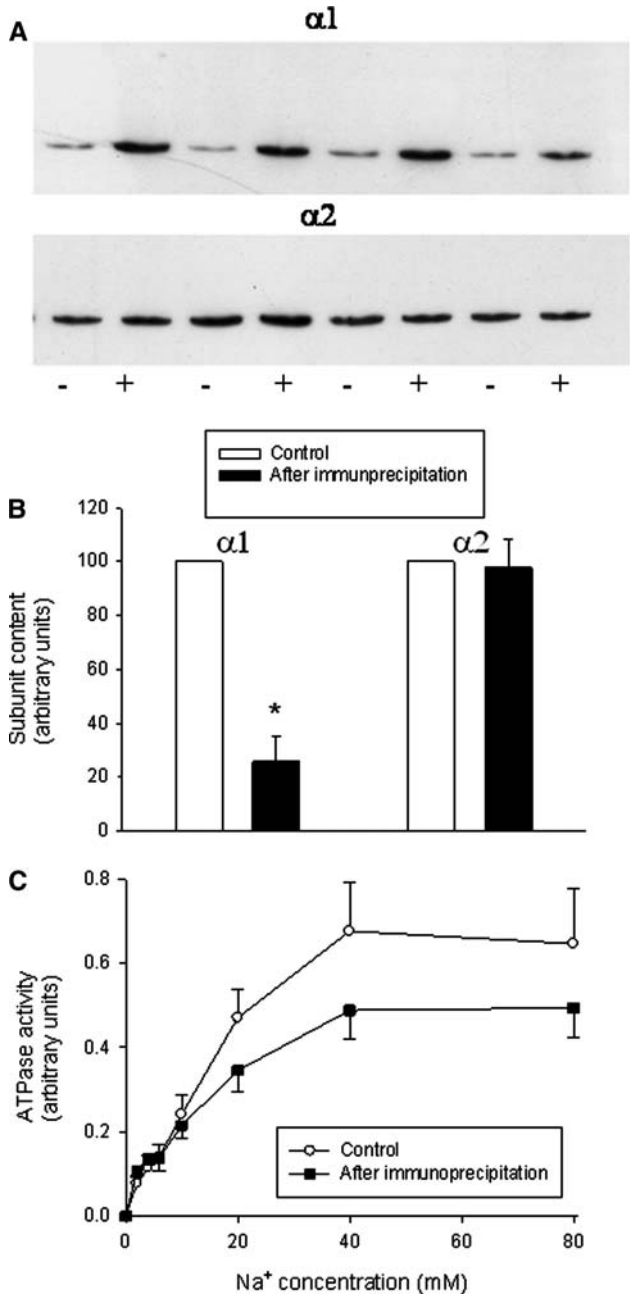

Fig. 4 Separation of α isoforms with immunoprecipitation. **a** Western blot of lysates of four different VLw muscles before and after immunoprecipitation of α_1. Symbols "*plus*" indicates total lysate, "*minus*" indicates lysate after immunoprecipitation. *Upper panel* shows labeling with α_1 antibodies, *lower lane* labeling with α_2 antibodies in the same samples. The same amount of protein was loaded on each lane. **b** α_1 and α_2 content in lysate before and after immunoprecipitation. * Significantly different from before immunoprecipitation ($n = 6$ VLw muscles). **c** ATPase activity in lysate before and after α_1 immunoprecipitation

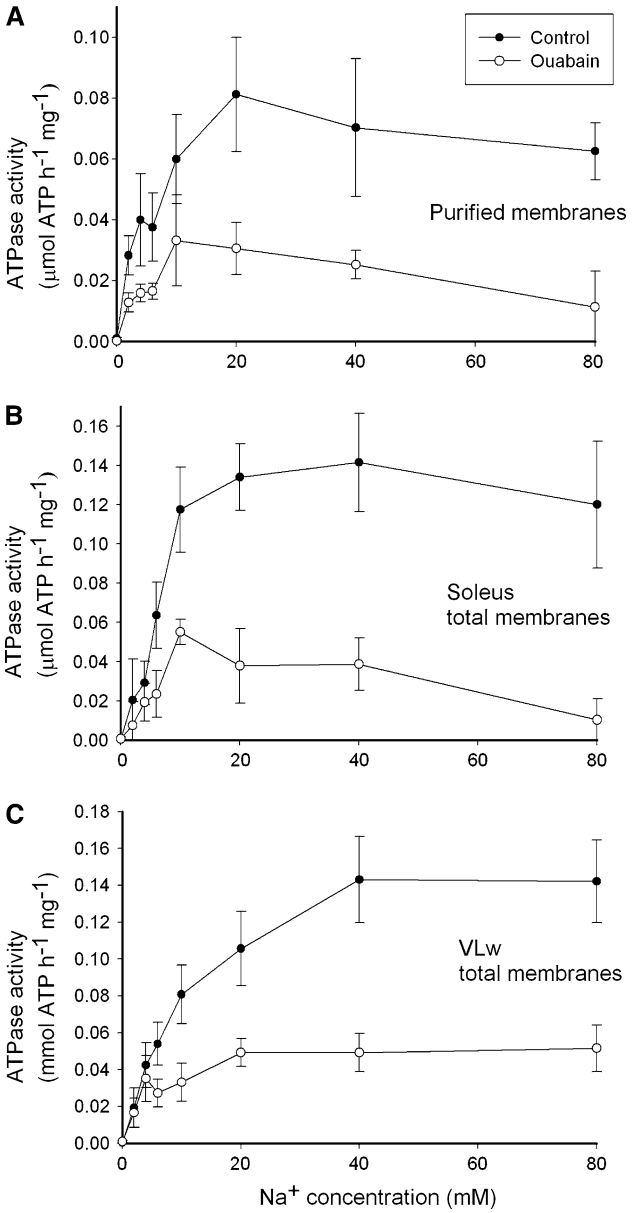

Fig. 5 Effect of 10^{-5} M ouabain. ATPase activity in the presence of 0–80 mM Na^+ was measured in purified membranes from mixed muscle (**a**), total membranes from soleus (**b**) and total membranes from VLw (**c**) with and without ouabain treatment ($n = 6$)

in skeletal muscle have not been available until now; however, a K_m of >35 mM for Na^+ can be deduced from one study that used isolated muscle and quantification of ^{86}Rb uptake (Buchanan et al. 2002).

In the present study with total membranes and purified muscle membranes, the Na^+ affinity of Na^+,K^+-ATPase was higher in oxidative fibers compared with glycolytic fibers (Fig. 3). This difference can probably be explained by the isoform distribution.

The distribution of Na^+,K^+-ATPase isoforms in rat muscle was studied both in total membranes and in purified membranes (Fig. 1). In short, α_1 and α_2 are present both in oxidative and in glycolytic muscle. In contrast, the distribution of the β isoforms is clearly fiber type-dependent: β_1 is present mainly in oxidative and $\beta 2$ mainly in glycolytic

fibers. A similar distribution has been obtained previously (Fowles et al. 2004; Thompson and McDonough 1996). Consequently, membranes from oxidative fibers contain mainly $\alpha_1\beta_1$ and $\alpha_2\beta_1$ heterodimers, whereas membranes from glycolytic muscle contain mainly $\alpha_1\beta_2$ and $\alpha_2\beta_2$ heterodimers.

Based on the isoform distribution and the activity measurements in single muscle, it can be concluded that heterodimers containing β_1 isoforms have a higher Na^+ affinity (lower K_m) than heterodimers containing β_2 isoforms (Figs. 2, 3). It is tempting to conclude that the different Na^+ affinities exclusively are due to the variation in β isoforms. The two β isoforms can be separated by using different muscles or dissecting different fibers, whereas this is not possible for the α isoforms, which are more uniformly distributed.

Separation of α_1- and α_2-Containing Dimers with Immunoprecipitation

To separate the effect on ATPase activity of the two α isoforms, we used immunoprecipitation to selectively remove dimers with α_1 (Fig. 4). Immunoprecipitation removed 74% of α_1, whereas α_2 content remained unchanged; thus, the removal was selective. The K_m value after removal of α_1 was not different from control (16.3 vs. 13.5 mM), which could be expected as α_1 makes up a minor fraction of the total isoform amount. The 74% removal of α_1 reduced ATPase activity by 27%; it is therefore expected that total removal could reduce the activity by approximately 36%, indicating that the dimers with α_1 are responsible for 36% of the total activity. The fraction of α_1 of the total α content has been measured in only one study using kidney ATPase as standard and Western blotting (Hansen 2001). This study found that α_1 represents 15–25% of the total content in rat SOL and extensor digitorum longus (EDL) muscle. The present study represents a new method to quantify the functional importance of the α_1 isoform. However, the estimated 36% reduction in activity with all α_1 removed does not necessarily mean that this subunit makes up 36% of the total α protein content in rat as the turnover number for dimers with α_1 could be higher than that for α_2, which has been reported from studies in oocytes (Crambert et al. 2000).

Ouabain Experiments

In an attempt to separate the affinities of the α isoforms we used the different ouabain sensitivities of the two isoforms in rat muscle (Fig. 5). Treatment with 10^{-5} M ouabain is expected to mainly inhibit dimers containing the α_2 isoform (Zheng et al. 2008). In the present study with purified membranes from mixed muscle and total membranes from SOL and VLw muscles, ouabain reduced maximal Na^+,K^+-ATPase activity by 61, 60 and 64%, respectively. These values are in line with the immunoprecipitation data (above), which suggest that α_1 dimers are responsible for one-third of the activity. Although treatment with ouabain is not expected to be totally isoform-specific, it is expected that the activity after ouabain treatment of the purified membranes mainly represents dimers with the α_1 isoform.

Ouabain treatment of total membranes from VLw muscle reduced K_m from 10.4 to 7.7 mM ($P < 0.05$) (Fig. 5c). The ATPase activity was not inhibited by high Na^+, indicating that the dimers with β_2 isoform are not inhibited in the Na^+ concentration interval up to 80 mM (a slight inhibition could be present but is not detectable with the method used here). The selectivity of ouabain could have been only partial and not completely specific; the K_m for a pure $\alpha_1\beta_2$ dimer is therefore expected to be slightly lover than that obtained in the ouabain experiments ($\sim$7.5–7.6 mM). Based on the isoform composition, the K_m for a pure $\alpha_2\beta_2$ dimer is expected to be higher than that for the total membranes ($\sim$13 mM).

In SOL, which has low β_2 isoform content, the activity after ouabain treatment mainly represents the $\alpha_1\beta_1$ dimers. It is difficult from the data in Fig. 5b to calculate the K_m for the ouabain-treated samples, but inspection of the data suggests that K_m may be lower than for the control samples ($K_m = 5.0$ mM), indicating that the $\alpha_1\beta_1$ dimers possess the highest Na^+ sensitivity of the four possible combinations of isoforms (K_m probably $\sim$ 4.0 mM). The Na^+ dependence in SOL muscle seems to consist of two processes: an activation mainly seen at low Na^+ and an inhibition at high Na^+. The high Na^+ sensitivity of the $\alpha_1\beta_1$ dimer seems to be combined with an inhibition of activity at high Na^+ concentrations. In Fig. 5b the activity values from 40 to 80 mM Na^+ are parallel, which seems to indicate that the dimers with $\alpha_1\beta_1$ are solely responsible for the reduction in activity with high Na^+. Based on the finding above that the α_2 dimers possess approximately two-thirds of the total activity, it is expected that the K_m for the pure $\alpha_2\beta_1$ dimer is slightly higher than that for the total SOL membranes ($\sim$5.5 mM).

A graphic overview of the measured and expected K_m values is provided in Fig. 6.

Role of PLM

Another factor of importance in the affinity of Na^+,K^+-ATPase could be the presence of the PLM subunit, which is mainly phosphorylated in the preparations used here (Rasmussen et al. 2008). The association of the PLM protein with Na^+,K^+-ATPase influences the transport properties of Na^+,K^+-ATPase (Crambert et al. 2002). The possibility that PLM is involved in the differences in

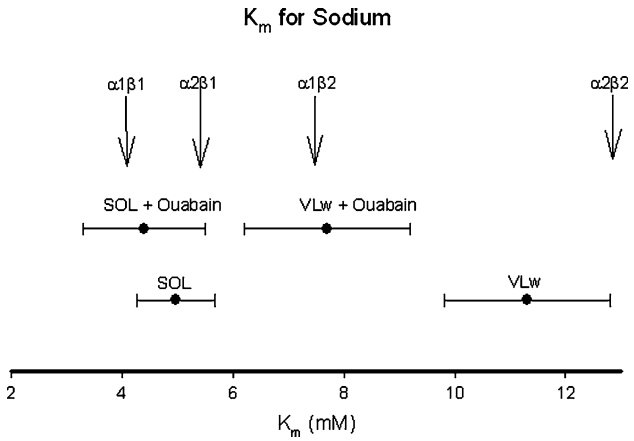

Fig. 6 Estimates of K_m for Na^+ in the four dimers. *Below* K_m values for Na^+ (±SD) obtained in total membranes from SOL and VLw muscle (data from Fig. 2). *Middle* estimated K_m values in ouabain-treated total membranes from SOL and VLw muscles (data from Fig. 5). *Arrows* indicate estimated K_m values for the four pure dimers

affinity of the pump in different muscle fiber types must therefore be considered. However, PLM expression is very similar in homogenates of oxidative and glycolytic muscle (Rasmussen et al. 2008; Reis et al. 2005), which seems to exclude a role for PLM. PLM is probably found in free and bound forms. An immunoprecipitation study found that at least 30% of α_1 and α_2 isoforms are associated with PLM (Rasmussen et al. 2008). In another study, PLM was predominantly associated with the α_1 isoform (Crambert et al. 2002). Based on these findings, it is possible that PLM contributes to the fiber-type differences in pump affinity, but this is not the most important difference.

In the purified membranes used in the present study, PLM was about twice as abundant in membranes prepared from oxidative fibers compared with glycolytic fibers (Fig. 1). This difference between total membranes and purified membranes is probably due to the localization of PLM. This protein is present in the outer membrane and the t-tubuli; however, the t-tubuli membrane fraction is not included in the purified membranes. The different distribution of PLM in the two fractions of purified membranes is therefore based on the preparation technique.

Maximal Pump Activity: V_{max}

Maximal pump activity is often used to compare the efficiency of ion regulation in different muscles. However, it can be seen from Fig. 2 that the maximal pump activity is obtained at Na^+ concentrations far higher than the reported intracellular Na^+ concentration in resting muscle (10–20 mM). In the literature, "maximal pump activity" has been measured at 120 mM Na^+ and 10–15 mM K^+ (Fowles et al. 2004; Zheng et al. 2008). The use of maximal pump activity may be misleading for two reasons: firstly, because such intracellular Na^+ concentrations are never obtained in muscle and, secondly, because ATPase activity in oxidative muscle (SOL in Fig. 2) tends to be reduced by high Na^+. A comparison of pump capacity at physiological intracellular Na^+ concentrations (~15–20 mM Na^+) is therefore more relevant (see below).

Physiological Consequences of Fiber-Type Differences in Na^+ Affinity

Figure 2 shows that the degree of Na^+ activation of Na^+,K^+-ATPase at 15 mM differs between fiber types. For oxidative fibers, the activity at this concentration is close to the maximal ion-dependent activity; however, activity is only approximately 60% for VLw. This pattern is confirmed in the purified membranes. Thus, in resting muscle the pump seems to be activated to a higher degree in oxidative fibers compared with glycolytic fibers. Furthermore, the activity of $\alpha_1\beta_1$ even seems to be inhibited by high Na^+ concentrations. In contrast, the activation curves in the physiological range of Na^+ are steeper for glycolytic fibers, suggesting that these fibers possess a spare capacity that can be activated if intracellular Na^+ is increased during muscle activity.

It could be expected that the higher Na^+ affinity of the pump in oxidative fibers would result in increased pump activity and a lower intracellular Na^+ concentration than in glycolytic fibers. In contrast, the basal rate of pump activity in isolated EDL muscle is higher than that in isolated SOL muscle (Everts and Clausen 1992), and the intracellular Na^+ at rest is slightly higher in SOL than in EDL muscle (16.5 vs. 12.9 mM) (Clausen et al. 2004) or (13.7 vs. 9.8 mM) (Fong et al. 1986). However, an understanding of the degree of pump activation must take into account the greater amount of ion leakage in glycolytic fibers (Clausen et al. 2004) and that a higher fraction of the pumps in glycolytic fibers are located in t-tubuli.

Different Subcellular Pools of Pumps

One consequence of the isoform-dependent ion affinities could be differences in pump activation in t-tubuli compared with the outer sarcolemmal membrane. This is because of the distribution of α isoforms: The α_1 isoform is exclusively located in the outer membrane, whereas the α_2 isoform is located in both membrane areas. It would therefore be expected that the Na^+ affinity and degree of activation are higher in the outer sarcolemmal membrane compared with t-tubuli. The physiological consequences of this difference remain unclear. The ouabain experiments which isolated the activity of the $\alpha_1\beta_1$ dimers indicated that this fraction of pumps may even be inhibited by Na^+ in the range 15–20 mM.

This opens the possibility that the fractions of pumps could be regulated differently. Interestingly, Sandiford et al. (2005) found very different K_m values for K^+ between whole homogenates, sarcolemma-enriched fractions and endosomal fractions obtained by centrifugation and the 3-*O*-MFPase technique. Although the 3-*O*-MFPase technique is not directly comparable to the ATPase technique used in the present study, the two methods suggest that pump affinities may vary in different compartments of the cell. One factor could be that the pumps are located inside and outside caveolae, which can be free and membrane-associated. The location of Na^+,K^+-ATPase in caveolae is supported by immunoprecipitation studies reporting that at least 17% of α_1 and α_2 isoforms are located with caveolin-3 (Kristensen et al. 2008). Furthermore, fractionation studies suggest that the distribution of α subunits is not uniform in the cell membranes (Lavoie et al. 1997). Collectively, these findings suggest that the pumps are located in pools that may have different roles and regulatory mechanisms.

Limitations of Study

The properties of the different heterodimers (isozymes) have previously been studied in expression systems, whereas the present study used membrane preparations from skeletal muscle. Although the proteins in the present experiments are situated in the natural membrane, the study did not use intact cells with Na^+ and K^+ gradients across the sarcolemma, which may affect the K_m values for Na^+. The most likely effect is a shift of ATPase activity along the concentration axis.

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