Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in rat skeletal muscle: content, isoform, and activity characteristics

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Fowles, J. R., H. J. Green, and J. Ouyang. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in rat skeletal muscle: content, isoform, and activity characteristics. J Appl Physiol 96: 316–326, 2004. First published July 25, 2003; 10.1152/japplphysiol.00745.2002.—The purpose of this study was to investigate the hypothesis that muscle Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity is directly related to Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content and the content of the \(\alpha\)-catalytic isoform in muscles of different fiber-type composition. To investigate this hypothesis, tissue was sampled from soleus (Sol), red gastrocnemius (RG), white gastrocnemius (WG), and extensor digitorum longus (EDL) muscles at rest from 38 male Wistar rats weighing 413 ± 6.0 g (mean ± SE). Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity was determined in homogenates (Hom) and isolated crude membranes (CM) by the regenerating ouabain-inhibitable Na\textsuperscript{+} hydrolitic activity assay (ATPase) and the 3-O-methylfluorescein K\textsuperscript{+}-stimulated phosphatase (3-O-MFase) assay in vitro. In addition, Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content (B\textsubscript{max}) and the distribution of \(\alpha\textsubscript{1}-\), \(\alpha\textsubscript{2}-\), \(\beta\textsubscript{1}-\), and \(\beta\textsubscript{2}\)-isoforms were determined by \(^{[3]}\text{H}\)ouabain binding and Western blot, respectively. For the ATPase assay, differences (P < 0.05) in enzyme activity between muscles were observed in Hom (EDL > WG) and in CM (Sol > EDL = WG). For the 3-O-MFase assay, differences (P < 0.05) were also found for Hom (Sol > RG = EDL > WG) and CM (Sol = WG > RG). For B\textsubscript{max}, differences in the order of RG = EDL > Sol = WG (P < 0.05) were observed. Isoform distribution was similar between Hom and CM and indicated in CM, a greater density (P < 0.05) of \(\alpha\textsubscript{1}\) in Sol than WG and EDL (P < 0.05), but more equal distribution of \(\alpha\textsubscript{2}\) between muscles. The \(\beta\textsubscript{1}\) was greater (P < 0.05) in Sol and RG, and the \(\beta\textsubscript{2}\) was greater in EDL and WG (P < 0.05). Over all muscles, the correlation (r) between Hom 3-O-MFase and B\textsubscript{max} was 0.45 (P < 0.05) and between Hom \(\alpha\textsubscript{2}\) and B\textsubscript{max}, 0.59 (P < 0.05). The \(\alpha\textsubscript{1}\) distribution correlated to Hom 3-O-MFase (r = 0.79, P < 0.05) CM ATPase (r = 0.69, P < 0.005) and CM 3-O-MFase activity (r = 0.32, P < 0.05). The \(\alpha\textsubscript{2}\) distribution was not correlated with any of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity measurements. The results indicate generally poor relationships between activity and total pump content and \(\alpha\textsubscript{1}\) isoform content of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Several factors, including the type of preparation and the type of assay, appear important in this regard.

sodium pump; fiber types; cation cycling; enzyme activity; isoforms

MAMMALIAN RODENT MUSCLE FIBERS are typically classified by histochemical techniques into two major subdivisions (type I vs. type II) and several subdivisions (types Ia, Ix, Iib) (44, 47). These classifications are based on the myosin heavy chain (MHC) isoform composition of the fiber, which is the primary determinant of contraction velocity (45). Of interest is how the MHC composition of a fiber relates to other proteins and processes, such as excitation-contraction coupling. At least for the sarcoplasmic reticulum (SR), it is known that type II fibers, which are fast contracting, possess an extensive SR, which by virtue of its Ca\textsuperscript{2+}-releasing and Ca\textsuperscript{2+}-sequestration functions allows for the rapid regulation of the cytosolic free Ca\textsuperscript{2+}-transient and rapid activation of the myofibrillar proteins (41). Not so clear, however, are the fiber-type differences in the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, the cation pump responsible for active transmembrane Na\textsuperscript{+} and K\textsuperscript{+} transport and for enabling repetitive action potentials to be conducted in the sarcolemma and T tubules. It might be expected that type II fibers in contrast to type I fibers, given the need to conduct a higher frequency of action potentials in the sarcolemma and T tubules to produce similar relative force outputs (21, 22), would express increased Na\textsuperscript{+}-K\textsuperscript{+}-ATPase protein levels and consequently increased Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (21, 22). Moreover, isoform composition of the cation pump may also be different in the different muscle cell types, as a result of the differences between fiber types and the need to respond optimally to its environment (11). It is unclear whether this occurs because few studies have attempted a collective characterization of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase at the protein, isoform, and activity levels in specific fiber types. However, several studies have been published that have examined individual content of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in muscles of different fiber-type composition (9, 19, 34). In general, these studies indicate that the properties of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase reflect not so much the contractile speed of the fiber but more the oxidative potential.

One factor that has mitigated against a coherent description of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase characteristics by fiber type is based on the uncertainty that exists regarding the relationship between different analytical techniques designed to measure the same property and the compatibility of different isolation and purification procedures. The latter consideration is important because the sarcolemma and T tubules represent a small component of the muscle cell (19) and because the Na\textsuperscript{+}-K\textsuperscript{+}-pump appears to be distributed not only on the surface membrane but intracellularly as well (32). The possibility of a selective yield depending on the specifics of the purification protocol remains a continuing concern (19).

A widely accepted procedure for the measurement of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content is the vanadate-facilitated \(^{[3]}\text{H}\)ouabain-binding technique (39). This procedure, which can be performed on small whole muscle tissue samples, is based on the high affinity of cardiac glycosides for binding to the \(\alpha\) subunit of the enzyme (19). It has been reported that the measurement of maximal transmembrane Na\textsuperscript{+} flux in rat muscle is closely related to the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content as measured by the \(^{3}\text{H}\) binding technique (10), indicating that the pumps are all functional.

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Two procedures have been developed to assess the maximal activity and affinity characteristics of the enzyme in skeletal muscle. One procedure is based on a direct measure of hydrolytic activity which allows for the direct assessment of Na\(^{+}\)-K\(^{+}\)-ATPase activity by the measurement of inorganic phosphate (P\(_{i}\)) liberated from the hydrolysis of ATP (19). Direct measurements of ATPase activity in enriched fractions are rare, because, in addition to the large amount of tissue needed, sarcosomal and T tubule yields are notoriously unreliable (19). Direct measurement of Na\(^{+}\)-K\(^{+}\)-ATPase activity in whole muscle homogenates is also complicated because of the relatively low activity relative to other ATPases in the cell and the need to selectively inhibit the other ATPases (38).

These problems have led to an alternate procedure for estimating ATPase activity that is based on the K\(^{+}\)-dependent phosphatase activity of the enzyme (38). Measurement of phosphatase activity is based on the K\(^{+}\)-dependent hydrolysis of the chromogenic substrate, 3-methylfluorescein phosphate (3-O-MFPase), which substitutes for the aspartyl/phosphate intermediate of the ATPase to represent the terminal step in ATP hydrolysis (23). Although the activity measured is specific to the Na\(^{+}\)-K\(^{+}\)-ATPase as indicated by the suppression that occurs with ouabain (38), the possibility exists that the catalytic activity of the phosphatase may not directly relate to the Na\(^{+}\)-K\(^{+}\)-ATPase activity levels per se. In addition, this assay contains a high nonspecific activity, which impairs the sensitivity of the measurement (38). However, recent improvements in the assay have been published (3, 15). As well, direct measurements of Na\(^{+}\)-K\(^{+}\)-ATPase assay in whole muscle homogenates are now possible based on the selective inhibition of other ATPases in the cell (14).

The Na\(^{+}\)-K\(^{+}\)-ATPase content, typically assessed with a vanadate-facilitated \(^{3}\)H\(\)ouabain-binding technique (39), has been shown to be highest in rat muscles possessing a high oxidative potential regardless of the fiber-type composition of the muscle as determined by MHC histochemistry (6). However, no study appears to have reported the relationship between the fiber-type composition and the 3-O-MFPase activity. One study using enriched sarcosomal fractions has found that the ATPase activity is directly related to the oxidative potential of a fiber (28), as has been observed for the pump content (6). Assessment of the Na\(^{+}\)-K\(^{+}\)-ATPase isoform distribution has probably been the least problematic. The Na\(^{+}\)-K\(^{+}\)-ATPase is characterized as having one catalytic (\(\alpha\)) subunit with four known isoforms, one glycoprotein (\(\beta\)) subunit with three isoforms, and an additional regulatory \(\gamma\)-subunit that is expressed in some nonmuscle cell types (4). Antibodies are now available for the measurements of the \(\alpha\)- and \(\beta\)-isoforms using Western blot techniques, and several studies have examined isoform distribution in different muscles (26, 27, 48, 51). In mammalian skeletal muscle, the isoform distribution of the Na\(^{+}\)-K\(^{+}\)-ATPase is such that slow oxidative-type fibers contain \(\alpha_1\beta_1\) and \(\alpha_2\beta_2\)-complexes, fast glycolytic fibers contain \(\alpha_1\beta_2\)- and \(\alpha_2\beta_1\)-heterodimers (26, 27), and fast oxidative-glycolytic have all four combinations (48). In skeletal muscle, only negligible quantities exist for \(\beta_2\) (1) and \(\alpha_3\) (27) isoforms. These results suggest that, in terms of isoform distribution, it is the \(\beta\)-isoform that distinguishes slow from fast fibers. The \(\beta_1\) subunits are believed to confer greater enzymatic activity to the pump than the \(\beta_2\)-subunits (4, 26), so this may have particular importance for characterizing fiber-specific Na\(^{+}\)-K\(^{+}\)-ATPase activity in skeletal muscle. The \(\alpha_1\) is thought to be the major catalytic isoform (27), potentially conferring greater enzyme activity if preferentially expressed in a particular fiber.

In this study, we have hypothesized that the muscle Na\(^{+}\)-K\(^{+}\)-ATPase activity will be directly related to the Na\(^{+}\)-K\(^{+}\)-ATPase content and the content of \(\alpha_1\)-catalytic isoform. Moreover, all of these properties will be higher in muscles of high oxidative potential and no different in muscles of varying fiber-type composition as dictated by the MHC composition. These interrelationships will persist regardless of the preparation used (whole muscle vs. enriched sarcosome) and the technique used to measure ATPase activity (3-O-MFPase vs. direct ATPase assay).

Muscles were selected for study that had previously been characterized by fiber type and oxidative potential (12).

METHODS

Animals. Experiments were carried out with the use of fed male Wistar rats (age 16 wk; mass 413 ± 6 g; means ± SE). Rats were housed in a room where the light-cycle was controlled (12:12-h) and rat chow and water were provided ad libitum. Care and treatment of the animals were in accordance with procedures outlined by the Canadian Council on Animal Care. All procedures were approved by the University of Waterloo Office for Ethics in Research.

Experimental design. A comparative model was used to assess the Na\(^{+}\)-K\(^{+}\)-ATPase characteristics between soleus (Sol), red gastrocnemius (RG), extensor digitorum longus (EDL), and white gastrocnemius (WG) skeletal muscles of the rat. These muscles are representative of primarily type I fibers (Sol), type IIa (RG), fast type IIb (WG) fibers, and muscles having a mixed complement of fast fiber types (EDL) (12). Mitochondrial oxidative potential as measured by the maximal activity of citrate synthase (\(\mu\)mol/min g wet wt\(^{-1}\)) has been reported to be 21.3 ± 2.3, 36.2 ± 1.6, 8.1 ± 0.7, and 21.6 ± 2.1 in Sol, RG, WG, and EDL, respectively (12).

Maximal Na\(^{+}\)-K\(^{+}\)-ATPase activity was measured in whole muscle homogenates (Hom) and isolated crude membranes (CM) for the regenerating ouabain-inhibitable hydrolytic activity assay (ATPase) and the 3-O-methylfluorescein K\(^{+}\)-stimulated phosphatase (3-O-MFPase) assay, in vitro. \(^{3}\)H\(\)ouabain binding was used to quantify pump content [maximum binding (B\(_{\text{max}}\)], as well as ouabain-binding affinity [dissociation constant (K\(_d\))] on Hom only. Isoform distribution of \(\alpha_1\), \(\alpha_2\), \(\alpha_3\), \(\beta_1\), and \(\beta_2\) isoforms was determined in both Hom and CM by Western blot and relative densitometry. Linear and multiple linear regression was used to identify correlations between Na\(^{+}\)-K\(^{+}\)-ATPase activity, content, and isoform distribution.

Muscle preparation and isolation procedures. Sol, RG, EDL, and WG muscle samples were obtained from anesthetized animals. Muscles were rapidly excised, quickly frozen in liquid nitrogen, and stored at −80°C until further preparation and/or analysis. Time between anesthesia and extraction averaged ~5 min. Individual muscles from 10 separate rats were obtained for preparation of Hom in Na\(^{+}\)-K\(^{+}\)-ATPase activity assays and Western blotting. For Hom, tissue from frozen muscle samples was homogenized (5% wt/vol) at 0°C for 2 × 20 s at 25,000 rpm (Polytron) in a buffer containing 250 mM sucrose, 2 mM EDTA, 1.25 mM EGTA, 5 mM Na\(_2\)As, and 10 mM Tris (pH 7.40). The homogenate was aliquoted and quickly frozen in liquid nitrogen until further analyses.

Muscles from both limbs of two rats (depending on muscle size) were pooled to prepare CM for Na\(^{+}\)-K\(^{+}\)-ATPase activity assays and Western blotting. CM were prepared by using a modification of procedures employed by Dombrowski et al. (13), a technique developed to isolate plasma membrane, T-tubular, and internal membrane components of skeletal muscle. This technique uses various centrifugation and sucrose gradient separation steps to purify muscle membrane components. However, because the goal of this study was to use...
small tissue amounts for individual muscles’ Na+-K+-ATPase activity determinations, only the first few steps of this procedure were employed to produce a CM (identical to Dombrowski et al. as “pellet F2”). This fraction contains primarily sarcolemmal membranes and some internal membranes that contain Na+-K+-ATPase subunits. This fraction does have contamination from other internal membranes such as SR (13), and the SR Ca2+-ATPase was identified in this experiment to contribute to background activity in the direct ATPase assay. However, the extent of background relative to Na+-K+-ATPase activity was very stable between repeated analyses of CM samples, as evidenced by a low coefficient of variation (3.3-5.3%). Na+-K+-ATPase activity in the direct assay was calculated as the difference between the presence and absence of the specific inhibitor, ouabain, and this accounts for background activity. It should be emphasized that the primary goal of the isolation procedure was not to obtain a “pure” sarcolemmal preparation but, more so, to obtain a sample adequately purified (~70-10-fold) to assess muscle-specific Na+-K+-ATPase activity by using a direct assay, without extensive tissue requirements. The purification factor was defined as the amplification in Na+-K+-ATPase activity (per mg protein) relative to Hom activity (per mg protein).

Briefly, for preparation of CM, ~1 g of frozen muscle (combined from ~3 rats) was thawed, cleaned of connective tissue, and minced for 5 s, using a homogenizer buffer A (10 mM NaHCO3, 250 mM sucrose, 5 mM NaN3, and 100 μM PMSF; pH 7.0). Tissue was homogenized and centrifuged at 2,000 g for 10 min, the supernatant was saved, the pellet was resuspended in buffer A (500 mg/3.75 ml), and the homogenization and 1,300 g centrifugation were repeated. The supernatants were combined and centrifuged at 9,000 g for 10 min. The supernatant was then centrifuged at 190,000 g for 1 h. The pellet was resuspended in 400 μl of buffer A, aliquoted, and frozen in liquid nitrogen until further analysis. All steps were performed on ice at 4°C.

Protein content of both the Hom and CM was determined by the method of Lowry as modified by Schacterle and Pollock (43). For the CM, the protein yield was calculated as the amount of sarcolemmal protein available at the end of isolation, relative to the known amount of wet weight tissue at the beginning of the isolation (mg protein/g wet wt).

Na+-K+-ATPase activity measurements. Samples were prepared according to the procedures outlined above for Hom and CM and used for analyses in two Na+-K+-ATPase activity assays, namely a direct ATPase assay and a 3-O-MFase assay. Two assays were employed because of the possibility that the pump’s hydrolytic and phosphatase activities can be modified independently of each other. Each assay may distinguish functionally different aspects of Na+-K+-ATPase content. Preparation of both Hom and CM samples, for both the ATPase and 3-O-MFase assays, included four freeze-thaw cycles to permeabilize membranes and expose binding sites for Na+, K+, ATP, and ouabain. Pilot testing indicated that this procedure produced similar results to other permeabilizing agents such as saponin, Triton X-100, and deoxycholate, with less intra-assay variability. Na+-K+-ATPase activity is expressed for all measurements as micromoles per milligram protein per hour.

The procedures for the direct ATPase assay were modified from those published by our laboratory for the Ca2+-ATPase (7, 16). Spectrophotometric (Shimadzu UV 160U) measurement of Na+-K+-ATPase activity was performed by using a regenerating assay. The regenerating assay limits potential buildup of P2−, which in an end-point measure can be inhibitory to maximal activity. After freezing-thawing, the samples were incubated at 37°C for 15 min with or without 2 mM ouabain in the buffer containing (in mM) 120 NaCl, 15 KCl, 50 Tris base, 5 NaN3, 1 EGTA, 5 MgCl2, 10 phospho-eno/pyruvate (pH 7.4), in the presence of pyruvate kinase and lactate dehydrogenase. The assay was started with the addition of 0.3 mM NADH and 5 mM ATP. The linear portion of the slope (~6–8 min) was compared between successive trials for each of the ouabain and no-ouabain conditions to give a calculation of ouabain-inhibitable Na+-K+-ATPase activity. The average of three repeated determinations (no ouabain-ouabain) was used as the Na+-K+-ATPase activity for a given sample. Analyses were identical between Hom and CM with the exception that 15–20 μl of Hom (~150 μg protein) and 10–15 μl of CM (~75 μg protein) were used.

The K+-stimulated 3-O-MFase assay was modified from the procedures of Huang and Askari (24) but using a higher substrate concentration (15, 24). We have confirmed in a separate set of experiments (results not shown) that maximal activity was achieved at ~160 μM 3-O-methylfluorescein phosphate in rat tissue, in both Hom and CM. The use of 1.25 mM EGTA and 5 mM NaN3 was also confirmed to decrease nonspecific activity in the assay and optimize assessment of K+-stimulated activity in rat muscle samples (3). The freeze-thawed samples were diluted one-fifth in cold homogenate buffer before being incubated for 4 min in medium containing (in mM) 5 MgCl2, 1.25 EDTA, 1.25 EGTA, 5 NaN3, and 100 Tris (pH 7.40). The K+-stimulated activity of the Na+-K+-ATPase was determined by the increase in activity after the addition of 10 mM KCl. 3-O-MFase activity was determined by the difference in slope before and after the addition of KCl. This method has greater reliability than interassay activity between ouabain and no-ouabain trials. Incubation with 2 mM ouabain inhibited >90% of K+-stimulated 3-O-MFase activity and confirmed the specificity of the assay. The 3-O-MFase activity was also based on the average of three successive trials. Analysis was identical between Hom and CM samples, with the exception that ~30 μl of Hom (~30 μg protein) and ~15 μl of CM (~15 μg protein) were used.

[3H]ouabain binding and Na+-K+-ATPase content. [3H]ouabain binding was employed to quantify pump content (Bmax), as well as ouabain-binding affinity (Ko) in muscle tissue specimens. Attempts were made to determine Bmax and Ko in CM; however, these data were not reliable. The procedures employed were similar from those used by Kjeldsen (30) and Pickar et al. (42), who also employed muscle tissue specimens. It is generally accepted that the standard ouabain-binding assay (using 1,000 nM ouabain concentration) assesses only the content of α2-isomers in rat skeletal muscle (30) because the α1-isomer in rat skeletal muscle is highly ouabain resistant (4).

Muscle samples are incubated in concentrations of labeled and unlabeled ouabain from 12.5–1,000 nM. Two muscle samples weighing between 2 and 8 mg were prewashed twice for 10-min periods in a Tris-sucrose buffer (10 mM Tris/HCl, 3 mM MgSO4, 1 mM Tris-vanadate, and 250 mM sucrose) containing sodium metavanadate (NaVO3) at 0°C. Samples were incubated in the Tris-sucrose buffer with 12.5–50 nM of [3H]ouabain (0.9 μCi/ml) and unlabeled ouabain (50–950 nM final concentration) for 180 min at 37°C. After the unbound ouabain was removed by washing for three times for 15 min in ice-cold buffer, the samples were blotted, weighed, placed in 1.5-ml Eppendorf tubes, and soaked in 1 ml 5% trichloroacetic acid for 16 h at room temperature, and then 0.5 ml of sample was counted for 3H radioactivity in a scintillation mixture. [3H]ouabain-binding capacity was corrected for loss of specifically bound [3H]ouabain during washout, and unspecific uptake and retention of [3H]ouabain were determined for each individual muscle for each analytical run relative to each ouabain concentration. In our hands, we have determined that the correction factor for washout, given the modifications that we employed, was 1.05 as previously reported by Norgaard et al. (37) for humans. This correction for specific uptake and retention was ~5% at 1,000 pM and ~22% at 5,000 nM total ouabain concentration. The isotopic purity of the [3H]ouabain was 99% as determined by the supplier (New England Nuclear-Du Pont Canada).

The Bmax is calculated by nonlinear regression (i.e., fit) of the saturation binding curve, using the one-component binding model.
The affinity constants ($K_d$) are also obtained by linear regression of a binding curve and represented by linear transform on a Scatchard plot. 

Isomorphism distribution determined by Western blot. Western blot analysis was used to quantify protein subunit contents of $\alpha$- and $\beta$-Na$^+$$-K^+$-ATPase isoforms in the different muscles. Immunoblotting was performed on both Hom and CM, using the primary polyclonal antibodies specific to the $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$-isoforms (Upstate Biotechnologies). In our hands, the polyclonal antibodies identified clear bands and had little background reactivity. Samples of Hom and CM (1.0 mg/ml) were suspended in isolation buffer (described under Muscle preparation and isolation methods) and loaded into gels in equal amounts. We have found, as have others (29), that PMSF is sufficient to protect proteolysis. Exactly 30 $\mu$g of Hom sample and 20 $\mu$g of CM sample from each muscle were electrophoresed in duplicate on separate 7.5% SDS-polyacrylamide gels (Bio-Rad Mini-PROTEAN II) as described by Laemmli (31). After SDS-PAGE and a 15-min equilibration in cold transfer buffer (25 mM Tris, 192 mM glycine, and 20% vol/vol methanol), the proteins were transferred polyvinylidene difluoride membrane (Bio-Rad) by placing the gel in transfer buffer and applying a high voltage (20 V) for 45 min (Trans-Blot Cell, Bio-Rad). The nonspecific binding sites were blocked with 5 or 7.5% BSA in Tris-buffered saline (TBS, pH 7.5) for 2 h at room temperature before incubation. Immunoblotting was performed by using the primary antibodies diluted in 5% BSA ($\alpha_1$, 1:1,000; $\alpha_2$, 1:500; $\beta_1$, 1:1,000; $\beta_2$, 1:500). Incubation with the primary antibodies was performed for 2 h at room temperature. After washing (6 × 5 min) in 0.1% TBS Tween 20 (TBS-T), a secondary antibody (goat anti-rabbit IgG1) was applied for 1 h, diluted at 1:3,000 ($\alpha_1$, $\alpha_2$, $\beta_1$) or 1:4,000 ($\beta_2$) in TBS-T. An enhanced chemiluminescence procedure was used to identify antibody content (Amersham-ECL-RPN2106P1). After exposure to photographic film (Kodak Hyperfilm-ECL), blots were developed for $60$–$90$ s in Kodak GBX developing solution and fixed in Kodak GBX fixer. Protein was determined by using the Bio-Rad assay in which detergent is present (Bio-Rad). Relative isomorphism protein levels were determined by scanning densitometry (Scion Image software). The linearity between the blot signal and the amount of protein applied to the gel was determined in pilot work. 

For any particular isoform, an equal amount of either Hom or CM sample was added to each lane, for each muscle. In total, 13 samples (4 muscles × 3 samples + brain standard) were applied on an individual gel, and each gel was run in duplicate. For each isoform, 8 sets of 2 gels were run in parallel, each gel with a known content (1–2 $\mu$g) of brain standard for relative control. Rat brain has an $\alpha$-to-$\beta$ molar ratio of 1:1 and approximately equal subunit distribution (except $\alpha_3$, which is in high amounts) (32), making it suitable as a relative control between muscle types in this experiment. Data in the results are presented relative to the density of the brain standard for a specific isoform only. The relative density was adjusted relative to 100% for Sol in $\alpha_1$, $\alpha_2$, and $\beta_1$ and against 100% for WG in $\beta_2$.

Data analysis. Statistical analysis was performed on Statistica for Windows R.4.5 software (Statsoft, Tulsa, OK, 1993). Descriptive statistics included means and SE. One-way ANOVA with repeated measures was used to analyze pump characteristics by muscle. Two-way ANOVA with repeated measures was used to analyze differences between assay characteristics and measurements by muscle. Correlational analysis by linear and multiple linear regression was used to relate Na$^+$$-K^+$-ATPase characteristics. Post hoc analysis of mean values was performed using the Tukey’s test. Statistical significance was set at $P < 0.05$.

RESULTS

Na$^+$$-K^+$-ATPase activity in Hom and CM. The Na$^+$$-K^+$-ATPase activity using both the direct (ATPase) and indirect (3-O-MFase) assays was determined in both Hom and CM preparations across four different muscles (Table 1). In Hom, Na$^+$$-K^+$-ATPase activity was higher ($P < 0.05$) in EDL compared with WG. No significant differences were observed between Sol, RG, and EDL. For this assay, the determinations of activity were largely unreliable. For WG, 6 of 10 samples did not exhibit ouabain-inhibitable activity.

In CM, differences were also observed between selected muscles in maximal Na$^+$$-K^+$-ATPase activity. For this preparation, Sol exhibited higher ($P < 0.05$) activities than the EDL and WG muscles. Interestingly, differences ($P < 0.05$) were observed between muscles in protein yield obtained with the CM preparation (RG > Sol; RG > WG). The protein yields (mg protein/g wet wt) were 7.1 ± 0.3, 8.7 ± 0.6, 8.0 ± 0.4, and 5.9 ± 0.3 for the Sol, RG, EDL, and WG, respectively.

Differences between muscles were also examined for the 3-O-MFase assay in both Hom and CM preparations (Table 1). In Hom, WG displayed the lowest activity amongst the four muscles examined, whereas Sol had the highest activity. With the CM preparation, differences were also found in 3-O-MFase activity between muscles (Table 1). For this preparation, both Sol and WG displayed higher ($P < 0.05$) activities than RG.

We have also found differences in recovery of Hom activity (defined as the percentage recovery of activity per gram wet wt of original tissue) between muscles. Percentage recovery was in the order of Sol = RG < EDL < WG ($P < 0.05$).

Correlation coefficients from measurements of ATPase and 3-O-MFase activities were nonsignificant ($r = 0.082; P > 0.05$). For the CM preparation, the correlation coefficient between these two assays was also insignificant ($r = 0.324, P > 0.05$).

$[^3]H$ouabain binding. Differences in $[^3]H$ouabain $B_{max}$ determined on whole muscle samples by using a one-component model, were in the order of RG = EDL > Sol = WG ($P < 0.05$) for the total ouabain-binding range of 12.5–1,000 nM (Table 2). A difference in binding affinity ($K_a$) was only observed between RG and WG ($P < 0.05$). The average saturation curve and Scatchard plot for Sol is presented in Fig. 1. The saturation curve clearly represents a plateau in $B_{max}$, and the Scatchard plot is linear, indicating both a single binding site with low affinity at low ouabain concentration.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Sol</th>
<th>RG</th>
<th>EDL</th>
<th>WG</th>
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<tbody>
<tr>
<td>Hom ATPase</td>
<td>0.97 ± 0.24</td>
<td>0.84 ± 0.29</td>
<td>1.87 ± 0.31</td>
<td>0.57 ± 0.34</td>
</tr>
<tr>
<td>3-O-MFase</td>
<td>0.27 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>CM ATPase</td>
<td>8.12 ± 0.43</td>
<td>6.75 ± 0.66</td>
<td>5.77 ± 0.40</td>
<td>5.10 ± 0.37</td>
</tr>
<tr>
<td>3-O-MFase</td>
<td>1.50 ± 0.10</td>
<td>1.24 ± 0.06</td>
<td>1.39 ± 0.06</td>
<td>1.47 ± 0.07</td>
</tr>
</tbody>
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The $B_{\text{max}}$ determined by using the one-component model was used in subsequent analysis for relationships to other pump characteristics.

**Isoform distribution.** Isoforms were measured in both Hom and CM preparations. The relative distributions of isofrom-specific antibodies against $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ for Hom are presented in Fig. 2. Relative density of each isoform was first calculated relative to a known standard of brain tissue and then relative to the Sol or WG depending on the isoform. Representative blots for each antibody between muscles in Hom are included in WG. The Sol $\alpha_1$ distribution was 1.5- to 2-fold greater ($P < 0.05$) in Sol than in RG, EDL, and WG. The Sol $\beta_1$ distribution was 1.5- to 2-fold greater ($P < 0.05$) in Sol than in RG, EDL, and WG. The Sol $\beta_2$ distribution was opposite to that of the $\beta_1$, being two- to threefold greater in RG, EDL, and WG than in Sol ($P < 0.05$).

Isoform distributions in CM are presented in Fig. 4, and representative blots are presented in Fig. 5. The Sol content of $\alpha_1$ was approximately twofold greater than RG ($P < 0.05$), and RG was 1.5-fold greater than WG and EDL ($P < 0.05$). The distribution for $\alpha_2$ was ~1.3-fold greater in Sol and RG than WG and EDL ($P < 0.05$). Distributions for $\beta_1$-subunits exhibited dramatic differences between muscles. The $\beta_1$ was ~1.5-fold higher in Sol than RG ($P < 0.05$), whereas RG was about twofold higher than EDL ($P < 0.05$) and EDL was about twofold higher than RG ($P < 0.05$). The $\beta_2$ presence was barely detectable in Sol. This isoform was much more pronounced in muscles containing a predominant composition of fast fibers, particularly in WG.

Interrelationships determined from multiple regression correlations between activity and isoform distribution are included in Table 3. No correlation was found between ATPase and 3-O-MFPase activity for either Hom or CM ($P > 0.05$). The correlation between single-site ouabain binding ($B_{\text{max}}$) on whole muscle tissue pieces and Hom 3-O-MFPase was positive and significant ($r = 0.45; P < 0.03$). In addition, $B_{\text{max}}$ was predicted by Hom $\alpha_2$ distribution ($r = 0.59, P < 0.005$). Hom 3-O-MFPase activity was predicted by multiple regression (multiple $r^2 = 0.41, P < 0.05$) with the $\alpha_1$ most prominent ($r = 0.79, P < 0.05$). In CM, ATPase gave the best prediction with $\alpha_1$ most prominent ($r = 0.69, P < 0.5$) compared with the other $\alpha$- and $\beta$-isoforms. CM 3-O-MFPase activity was also predicted (multiple $r^2 = 0.39, P < 0.05$), primarily by $\alpha_1$ distribution ($r = 0.32, P < 0.005$), although $\beta_2$ contribution nearly reached significance ($P = 0.07$). Hom ATPase was not predicted by multiple regression (multiple $r^2 = -0.01, P > 0.5$) and was not correlated to any single isoform or measure of content.

**DISCUSSION**

One major objective of this study was to determine the interrelationships between the content, isoform distribution, and kinetic activities of the Na$^{+}$-K$^{+}$-ATPase. We have hypothesized that muscles with the highest maximal Na$^{+}$-K$^{+}$-ATPase activity would display the highest pump content and the highest $\alpha_2$ isoform content. To investigate this hypothesis, it was first necessary to compare two commonly used assays designed to measure the kinetic properties of the enzyme, namely the direct Na$^{+}$-K$^{+}$-ATPase hydrolytic activity (ATPase) and the phosphatase activity as assessed by 3-O-MFPase in two different preparations. The two different preparations were Hom and CM.

**Enzyme activities.** To assess whether these assays are compatible, we have compared the mean values across muscles and also calculated correlation coefficients from measurements obtained on each tissue. A high concordance between assays and between preparations across muscles would indicate compatibility. For Hom, both ATPase and 3-O-MFPase activities

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### Table 2. Ouabain-binding characteristics in different skeletal muscles

<table>
<thead>
<tr>
<th></th>
<th>Sol</th>
<th>RG</th>
<th>EDL</th>
<th>WG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B_{\text{max}}$ (pmol/g)</td>
<td>261 ± 17†</td>
<td>371 ± 16*</td>
<td>368 ± 35*</td>
<td>244 ± 16††</td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>61 ± 5</td>
<td>84 ± 7</td>
<td>76 ± 16</td>
<td>60 ± 81</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.938</td>
<td>0.958</td>
<td>0.955</td>
<td>0.918</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 6$ for each muscle. $B_{\text{max}}$, maximum binding (pmol/g); $K_d$, dissociation constant (nM); $r^2$, the regression of the prediction equation used to depict the saturation binding plot. These $B_{\text{max}}$, $K_d$, and $r^2$ values were determined on binding isotherms containing 12.5–50 nM [3$^\text{H}$]ouabain to a total concentration of 12.5–1,000 nM ouabain. *Different from Sol ($P < 0.05$). †Different from RG ($P < 0.05$). ††Different from EDL ($P < 0.05$).
were lowest in WG compared with EDL. No further differences were observed in ATPase activities between muscles. In the case of 3-O-MFPase, there appeared to be an increased assay sensitivity as indicated by the additional differences that were observed between muscles. In general, concordance between assays by muscle was low. The fact that the correlation between these two assays for Hom was nonsignificant is further evidence of the dissociation that exists. We attribute the dissociation, in large degree, to the ATPase assay, which was largely unreliable.

For CM, ATPase measurements were much more reliable. For this assay, differences were only found between two muscles (Sol > WG). This difference was not evident for the 3-O-MFPase assay. However, differences in 3-O-MFPase activity were noted between selected muscles (Sol = WG > RG). The correlations between these two assays for tissue samples was not significant ($r = 0.324, P > 0.05$). As with Hom, concordance between assays for CM was low.

There also appeared to be a lack of concordance across tissue preparations for each assay. As an example, for the ATPase assay, WG displayed the lowest activity for both Hom and CM, but no similar association was observed for other muscles. The general lack of association is also evident for the 3-O-ATPase assay.

The results of this experiment indicate that a number of factors dictate the activity of the Na$^+$/K$^+$-ATPase in skeletal muscle. Different activities are observed between techniques, which highlights that the assay, isolation procedure, and muscle used are important factors when interpreting measures of Na$^+$/K$^+$-ATPase activity.

In assessing an ATPase enzyme’s catalytic capacity, the method that most accurately represents enzyme activity or

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**Fig. 2.** Distribution of isoforms in homogenates (Hom) between Sol, red gastrocnemius (RG), extensor digitorum longus (EDL), and white gastrocnemius (WG) muscles. A: $\alpha_1$-subunit. B: $\alpha_2$-subunit. C: $\beta_1$-subunit. D: $\beta_2$-subunit. Values are means ± SE ($n = 6$ for each subunit of each muscle). Relative density is calculated relative to a known standard of brain tissue. The relative density was adjusted relative to 100% for Sol in $\alpha_1$, $\alpha_2$, and $\beta_1$-units and against 100% for WG in $\beta_2$. These plots indicate relative differences between muscles for each isoform only, not between isoforms. *Different from Sol ($P < 0.05$). †Different from RG ($P < 0.05$). ‡Different from EDL ($P < 0.05$).

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**Fig. 3.** Representative Western blots for Hom between soleus (S), red gastrocnemius (R), extensor digitorum longus (E), and white gastrocnemius (W) muscles. Skeletal muscle homogenates (30 μg) were subjected to SDS-PAGE. Isoform protein levels were determined with the polyclonal antibodies specific to $\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$ and enhanced chemiluminescence. Scanning density is calculated relative to a known standard of brain tissue (B) for each plot. Arrow indicates the location of the molecular mass marker, in kDa.
turnover is an assay of ATP hydrolysis. Direct assays of ATP hydrolysis measure either the liberation of Pi or the absorbance change of a light-sensitive product such as NADH, coupled directly to enzyme activity. This can be achieved in either a regenerating assay enzymatically linked to maintain the by-products of reactions in equilibrium or in an end-point assay in which ATP hydrolysis creates a buildup of Pi. For the Na\(^+/\)H\(^-\) pump, hydrolytic activity is composed of Na\(^+/\)H\(^-\)-dependent phosphorylation and K\(^+/\)H\(^-\)-dependent phosphatase steps to complete a pump cycle in the presence of ATP (46).

The literature rarely contains measures of Na\(^+/\)K\(^-/\)H\(^-\) pumps in whole muscle homogenates. This is because of the low proportion of Na\(^+/\)K\(^-/\)H\(^-\) activity relative to the activity of other ATPases in the cell, unreliability due to inhomogeneity of homogenate suspensions, and problems in determining ouabain-inhibitable activity between separate runs of a variable assay (23). Given these difficulties, many researchers use artificial substrates in K\(^+/\)-stimulated phosphatase assays that are more specific to the Na\(^+/\)K\(^-/\)ATPase (23), possibly in combination with an isolation procedure, to improve the measure of Na\(^+/\)K\(^-/\)ATPase relative to the high background activity in skeletal muscle. Although used extensively, the K\(^+/\)-stimulated phosphatase assay can be criticized as not approximating the maximum hydrolytic capacity of the enzyme (23).

In contrast to whole muscle homogenates, an isolation procedure requires greater amounts of tissue than can be supplied by a single rodent muscle, and therefore a mixture of muscles is often used. Isolation procedures can also be criticized as not representing the entire population of pumps in a sample of muscle because of low tissue yields and low recovery of original enzyme activity (19), which may not represent the

Fig. 4. Distribution of isoforms in crude membranes (CM) between Sol, RG, EDL, and WG muscles. Values are means ± SE (n = 6 for each subunit of each muscle). A: \(\alpha_1\)-subunit. B: \(\alpha_2\)-subunit. C: \(\beta_1\)-subunit. D: \(\beta_2\)-subunit. Relative density is calculated relative to a known standard of brain tissue. The relative density was adjusted relative to 100% for Sol in \(\alpha_1\), \(\alpha_2\), and \(\beta_1\) and against 100% for WG in \(\beta_2\). These plots indicate relative differences between muscles for each isoform only, not between isoforms. *Different from Sol (P < 0.05). †Different from RG (P < 0.05). ‡Different from EDL (P < 0.05).

Fig. 5. Representative Western blots for CM between soleus (S), red gastrocnemius (R), extensor digitorum longus (E), and white gastrocnemius (W) muscles. Crude homogenates (20 μg) were subjected to SDS-PAGE. Isoform protein levels were determined with the polyclonal antibodies specific to \(\alpha_1\), \(\alpha_2\), \(\beta_1\), and \(\beta_2\) and enhanced chemiluminescence. Scanning density is calculated relative to a known standard of brain tissue (B) for each plot. Arrow indicates the location of the molecular mass marker, in kDa.
pump capability of the entire muscle pool. For these reasons, the information available on muscle-specific Na\(^+\)-K\(^+\)-ATPase activity is sparse.

For the 3-O-MFPase assay, we found, as have others (23, 24), that the use of the artificial substrate in a phosphatase assay quantifies only a portion of directly assessed Na\(^+\)-K\(^+\)-ATPase activity. For both Hom and CM, the 3-O-MFPase assay exhibited less than one-fifth of the activity of the ATPase assay. In our assay, improved conditions were also employed. As with Horgan and Kuypers (23), we have used the optimal concentration of 3-O-MFPase of 160 \(\mu\)M in our assay. Because a number of researchers have employed a lower 3-O-MFPase concentration in their work, it is possible that this masks identification of tissue specific differences. In this experiment, the differences in Hom 3-O-MFPase activity were highly significant between muscles. Sol was \(\sim\)1.5-fold greater than RG and EDL and threefold greater than that for WG. This is in contrast to a report that K\(^+\)-stimulated phosphatase activity is not different between Sol and WG (49). Thompson et al. (49) used the K\(^+\)-stimulated \(p\)-nitrophenylphosphatase assay for their measurement, which also is limited by the use of a low concentration of the artificial substrate. The activities reported by Thompson et al. were <3% of the theoretical maximum Na\(^+\)-K\(^+\)-ATPase activity for these muscles. In the CM, differences in 3-O-MFPase activity were observed between muscles, with Sol > RG (\(P < 0.05\)) but, surprisingly, RG < WG, as well. This is not the same hierarchical relationship observed from Hom 3-O-MFPase, where Sol was greater than WG, nor is it consistent with the position of Hundal et al. (26) that K\(^+\)-stimulated phosphatase is higher in red than white tissue. The different results obtained in this experiment might be explained, however, by the approximately twofold greater recovery of Hom 3-O-MFPase activity in WG vs. Sol muscle. This greater recovery for WG was specific to the 3-O-MFPase assay, which may explain the discrepant observation.

Interrelationships between Na\(^+\)-K\(^+\)-ATPase activity, ouabain binding, and isoform distribution. To investigate the hypothesis that higher Na\(^+\)-K\(^+\)-pump content would be accompanied by higher ATPase activities and high \(\alpha_2\) isoform content, we compared these properties across muscles using correlation coefficients. As expected, \(B_{\text{max}}\) was positively related to 3-O-MFPase activity in Hom. However, the predictability based on the correlation coefficient was low. Contrary to our original hypothesis that ATPase activity would be predicted by \(\alpha\) subunit distribution and the 3-O-MFPase activity would be predicted by the \(\beta\)-subunit distribution, both the Hom 3-O-MFPase and CM ATPase assays were predicted primarily by the \(\alpha_1\). Neither the ATPase nor the 3-O-MFPase were predicted by the \(\beta\)-subunit distributions. Given these considerations, Sol exhibited the highest Na\(^+\)-K\(^+\)-ATPase activity on these two measures, on the basis of its greater proportion of \(\alpha_1\)-subunit relative to the other muscles. This is also contrary to our hypothesis that \(\alpha_2\) and \(\beta_1\)-subunits would confer higher enzyme activity. Although the standard ouabain-binding technique correlated significantly with the content of \(\alpha_2\) subunits in Hom, \(\alpha_2\) did not significantly correlate to any direct measure of activity, which is also opposite to the classical understanding. To understand the results, it is necessary to examine the nature of the analytical techniques that were employed.

\[^{[1]}\text{H}\text{ouabain binding is the most commonly used assay for characterizing the maximal content of Na\(^+\)-K\(^+\)-ATPase in muscle. Determination of binding affinity (\(K_a\)) in combination with \(B_{\text{max}}\) requires much more extensive measurements than does the standard \(B_{\text{max}}\) assay. Previous reports have also failed to indicate changes in \(K_a\) relative to chronic changes in pump content (5). In these studies and others (30, 39), only a single population of binding sites is quantified in the saturation binding isotherm for whole skeletal muscle pieces, although two distinctly different binding affinities are present for the two \(\alpha\) subunits (4). This has led others (33, 49, 50) to use the binding affinity technique as a sole quantification of \(\alpha_2\)-subunit composition in skeletal muscle.}

Our data suggest that the classic assumption of linearity between ouabain binding and Na\(^+\)-K\(^+\)-ATPase activity is invalid for rat skeletal muscle, where major differences in ouabain affinity exist between some isoforms. It is well established that the \(\alpha_1\)-isoform in rat muscle has a low affinity for ouabain (18). Because the \(\alpha_1\)-isoform represents between 15 and 25% at least in EDL and soleus muscles, the Na\(^+\)-K\(^+\)-ATPase content will be underestimated. Applying this assumption to quantify ion transport capacity (8, 9) and K\(^+\) balance in rat muscle (36), as it relates to muscle fiber specific fatigue, may be inappropriate as a consequence.

An explanation for the discrepancy in \(B_{\text{max}}\) and Na\(^+\)-K\(^+\)-ATPase activities between muscles may also be related to fiber morphology. A study by Harrison et al. (20) on pig muscles found that smaller fibers had significantly greater membrane area per unit volume, which significantly related to ouabain binding. Differences in fiber morphology may also explain difference in specific to non specific enzyme activity assessed on different muscles in this study. Average fiber cross-sectional areas for Sol, RG, EDL, and WG muscles of rats of similar age, gender, and weight used in this study were 4,054, 3,004, 2,922, and 3,923 \(\mu\)m\(^2\), respectively, as calculated from published values (12). The corresponding comparison to pump content in this experiment (261, 371, 368, and 244 pmol/g, respectively) exhibits a striking inverse relationship. The results presented in this study support the theory that the mea-

<table>
<thead>
<tr>
<th>(\text{Na}^+)-K(^+)-ATPase activity</th>
<th>(\alpha_1)</th>
<th>(\alpha_2)</th>
<th>(\beta_1)</th>
<th>(\beta_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(^{[1]}\text{H}\text{ouabain } B_{\text{max}})</td>
<td>-0.12</td>
<td>0.59*</td>
<td>-0.29</td>
<td>-0.09</td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hom ATPase</td>
<td>-0.14</td>
<td>-0.19</td>
<td>0.03</td>
<td>0.37</td>
</tr>
<tr>
<td>3-O-MFPase</td>
<td>0.79*</td>
<td>0.24</td>
<td>0.63</td>
<td>-0.65</td>
</tr>
<tr>
<td>CM ATPase</td>
<td>0.69*</td>
<td>0.53</td>
<td>0.43</td>
<td>-0.64</td>
</tr>
<tr>
<td>3-O-MFPase</td>
<td>0.32*</td>
<td>-0.03</td>
<td>-0.02</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE; \(B_{\text{max}}, n = 6\); Hom, \(n = 10\); CM, \(n = 6\). *Significant correlation in the multiple regression equation for the particular variable (\(P < 0.05\)).
measurement of content of membrane Na,K-pumps is influenced by fiber morphology.

*Isoform distribution quantified by Western blot.* We used Western blot to assess the relative abundance of each of the known Na\(^{+}\)-K\(^{+}\)-ATPase isoforms in skeletal muscle. The relative abundance was calculated in reference to a known standard of brain tissue that expresses all Na\(^{+}\)-K\(^{+}\)-ATPase isoforms and has been used previously for subunit comparisons to skeletal muscle (33, 51). The relative isoform distribution displayed for both Hom and CM clearly demonstrates fiber-specific Na\(^{+}\)-K\(^{+}\)-ATPase isoform complements.

Two features arise from the multiple-regression results between Na\(^{+}\)-K\(^{+}\)-ATPase and isoform distribution in this experiment. First was the fact that the \(\alpha_{2}\)-isoform, believed to be the "major catalytic isoform" in skeletal muscle (27), failed to predict activity in any assay. The rat skeletal muscle \(\alpha_{2}\)-isoform has greater specific activity than the \(\alpha_{1}\)-isoform (53), is present in both surface and internal membranes, and has been reported to exhibit a translocation response to hormones (25, 33) and exercise (29, 52). However, the existence of the translocation phenomenon remains controversial. A recent paper (35) could find no evidence of translocation with either varied contractile protocols or insulin. The \(\alpha_{2}\) also has a more dynamic response to chronic external stress (2, 49-51). The findings from this experiment may mean that the \(\alpha_{2}\)-isoform’s influence on Na\(^{+}\)-K\(^{+}\)-ATPase activity measured in resting muscle is minor, and, therefore, its role may be restricted to acute and chronic modifications of pump activity.

Additionally, our results also suggest that the isolation procedure may preferentially maintain surface membranes and therefore overemphasize the \(\alpha_{1}\) contribution to activity. This might explain results in the CM assays, but not the Hom 3-O-MFPase assay, in which \(\alpha_{1}\) was also the significant predictor. Crambert et al. (11) have recently observed that the \(\alpha_{1}\)-isoform from human muscle has greater activity than \(\alpha_{2}\). This fact introduces the possibility of species-specific molecular activity differences between isoforms from the rats sampled in this experiment, and those used in the previous transfection experiments.

The second feature from the Western blot results is that the \(\beta_{2}\)-isoform contribution nearly reached significance in the CM 3-O-MFPase assay only. This finding may help to explain the peculiar hierarchy established between muscles for the CM 3-O-MFPase, whereby activity in WG exceeded RG and was not different from that in Sol or EDL. It was previously identified that WG muscle exhibited greater purification than the other muscles in the CM 3-O-MFPase assay, likely due to preferential purification of surface membranes. The \(\beta_{2}\)-isoform may be the more structural of the two isoforms, existing primarily in the membrane, because \(\beta_{1}\) exhibits a translocation response to hormones (25) and exercise (29). Therefore, more \(\beta_{2}\) could be maintained in the CM than \(\beta_{1}\) during the isolation. The calculations suggest that, in the CM 3-O-MFPase assay only, \(\beta_{2}\) is likely contributing to activity more than \(\beta_{1}\) because of relative purification differences, not because of a higher specific activity contribution to activity. Given this justification, the \(\beta_{1}\)-isoform likely confers higher kinetic specificity than the \(\beta_{2}\)-isoform in all situations, and the muscle specific activity hierarchy from oxidative muscle (highest) to glycolytic muscle (lowest) is conserved.

A limitation in our Western blot analyses is our inability to express the amount of specific isoforms in absolute quantities. Values are relative, calculated in reference to a brain standard and then relative to a specific muscle such as the Sol. The problem with this approach is that percent differences based on relative comparisons provide little insight into the absolute differences between isoforms. The conversion of Western blot data to absolute terms depends on the preparation of samples that contain specific amounts of each isoform. At present, this is particularly difficult to perform.

*Muscle oxidative potential and Na\(^{+}\)-K\(^{+}\)-ATPase properties.* A second major objective of this research was to examine the hypothesis that the Na\(^{+}\)-K\(^{+}\)-ATPase content, isoform, and kinetic activities in the different muscles would correlate not with the fiber-type distribution based on MHC isoform-based histochemistry but with the oxidative potential of the muscle. It should be emphasized that fiber-type distribution in individual muscles is based on the MHC chain isoform content (45). In adult rodents, muscle fibers are typically classified into type I and type IIa, IIX, and IIb on the basis of the predominance of a single heavy chain isoform (45). This schema is not to be confused with another schema that employs the MHC content to subdivide the fibers into type I and type II and the oxidative and glycolytic potential to further subdivide the type II fibers (40). In general, type I contains a high oxidative and low glycolytic potential. Although all type II fibers contain a high glycolytic potential, large differences exist between fiber types in oxidative potential. On average, type IIa > IIX (D) > IIb in rat adult muscle (17). For the muscles selected in this study, oxidative potential is in the order of RG > Sol = EDL > WG (12).

In comparison to published data, the values of Sol = WG < RG < EDL in Na\(^{+}\)-K\(^{+}\)-ATPase content, as determined by [\(^{3}H\)]ouabain, are consistent to the relationship reported by Bundgaard et al. (5) and Thompson et al. (50) for Sol, EDL, and WG. It is clear that the two muscles (RG and EDL) primarily composed of type II fibers and containing a high oxidative potential also contain high Na\(^{+}\)-K\(^{+}\)-ATPase content. In addition, the WG, which also consists of type II fibers but of low oxidative potential, contains a low pump content. In earlier work from our laboratory, comparison across muscles indicated that Sol values were more similar to the type II, high-oxidative muscles (6). However, in this study the Sol was similar to the WG. The conclusion by Chin and Green (6) that pump content might be related to muscle oxidative capacity may only apply to type II-based muscles. It is clear that Na\(^{+}\)-K\(^{+}\)-ATPase content does not vary with the major MHC composition because RG > WG in pump content and both of these muscles contain primarily type II fibers.

For the ATPase activity assays, WG generally displayed the lowest value across all muscles, consistent with the pump content. Moreover, the type II, high-oxidative muscles (RG, EDL) yielded higher activities across all assay conditions than the WG. In contrast, no clear pattern was found for Sol. Similar to the results of Thompson and McDonough (51), the distribution of \(\alpha\) subunits was such that \(\alpha_{1}\) was greater in two high-oxidative muscles (Sol and RG) than low-oxidative muscle (WG). Interestingly, \(\alpha\) distribution was lower in EDL, a high-oxidative muscle with a predominant type II fiber distribution. The \(\alpha_{1}\) distribution was similar between Hom and CM. Abundance of \(\alpha_{2}\) was greater in RG than in WG, and the
distribution was somewhat modified by the isolation. The latter point confirms the previous suggestion that α₂ may have greater abundance in intracellular compartments (33). The α₁-subunit was thought to have a “housekeeping” function (26) and a more stable position in the surface membrane, whereas α₂-subunit is known to translocate to the membrane in response to insulin and exercise (25, 29). However, both the housekeeping role (11) and the translocation (35) of these isoforms have been challenged. The isolation of CM in this experiment does not fully capture internal membranes (13), so surface membranes may be retained to a greater extent.

For the “structural” β-subunits, similar distributions were observed for Hom and CM. β₁ was greater (P < 0.05) in oxidative Sol than in glycolytic RG, EDL, and WG. β₂ distribution was opposite the β₁, being greater in RG, EDL, and WG than in Sol. This is consistent with previous reports (27, 49, 51). An observation from this experiment was that the CM preparations were “cleaner” than Hom (i.e., there was less of β₂ in Sol and β₁ in WG, in CM vs. Hom). It is possible that this effect may result from cross-reactivity with the polyclonal antibodies used in this experiment. Additionally, some researchers employ deglycosylation to increase antibody binding to β-subunits (50, 51). Our blots were clear and provided unambiguous distinction of relative density between muscles, so it is doubtful that this affected the comparative results for this experiment. Nevertheless, the use of polyclonal antibodies is a limitation that can be addressed with use of monoclonal antibodies in future experiments. An evaluation of the results of Thompson and McDonough (51) might also indicate that the contrast between Hom and CM may be biased by the isolation procedure, as has been identified to affect assessment of other Na⁺-K⁺-ATPase characteristics.

The results for the α₁- and α₂-isomorph distribution in Hom in this experiment are similar to those reported by Thompson and McDonough (51). As well, their results for the β-isomorphs are similar to that obtained for CM in this experiment. Thompson and McDonough (51) used homogenates for their α Western analysis but employed a single isolation step to purify their α² subunit. Although the β-subunits are thought to be more evenly distributed between surface and intracellular compartments, they appear to have intracellular pools (33), susceptible to mobilization (25, 29). These intracellular pools may be preferentially discarded during an isolation and lead to so-called cleaner preparations. This possibly has been documented by Lavoie et al. (33). These researchers identified that each step of their isolation resulted in a loss of β₁-subunits from preparation for “red” skeletal muscle. If this phenomenon is real and can be applied to the results of this experiment, it implies that there may also be a fiber- and isoform-specific distribution of surface to intracellular pools of Na⁺-K⁺-ATPase subunits that are sensitive to the isolation procedure. Further clarification of this issue is required.

In summary, the relationships between activity and isoform distributions provides insight into which assay is the “best” representation of kinetic activity at the molecular level. The activity of Hom 3-O-MFPAse was the best predicted by multiple regression (multiple $r^2 = 0.76, P < 0.001$). The CM ATPase (multiple $r^2 = 0.41, P < 0.05$) and CM 3-O-MFPAse (multiple $r^2 = 0.39, P < 0.05$) were also predicted, and the low $r^2$ values are likely a function of the low sample size ($n = 6$), relative to the number of predictor variables ($n = 5$). Hom ATPase was not predicted at all (multiple $r^2 = -0.01, P > 0.5$) because of the substantial variation in the measure. Therefore, the best assay representing relative molecular measures is likely the Hom 3-O-MFPAse assay, although this requires qualification. The 3-O-MFPAse assay only approximates 13–26% of theoretical maximum capacity of the enzyme. This finding is thought to be related to the lower affinity of the enzyme for the artificial substrate (8), although the low activity may be related to phosphorylation activity being the rate-limiting step in ATP hydrolysis. The fact that β₁ contributed less than α₁ to the multiple regression of Hom 3-O-MFPAse activity may refute this possibility.

The paradox for choosing the best overall measure of Na⁺-K⁺-ATPase activity is that the CM ATPase assay measures hydrolytic activity directly but doesn’t relate as well to molecular findings. Details of the isolation procedure likely influence the relationships established in this experiment and should remain an important consideration for any measure of Na⁺-K⁺-ATPase on purified membrane (19). In summary, it appears that the 3-O-MFPAse is the best measure of Na⁺-K⁺-ATPase activity in homogenates, and the ATPase assay is the best measure of Na⁺-K⁺-ATPase in CM, given that the CM retains a substantial portion of the original Hom activity, as was the case in this experiment.


