The Molar Ratios of α and β Subunits of the Na^+-K^+-ATPase Differ in Distinct Subcellular Membranes from Rat Skeletal Muscle†

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ABSTRACT: The Na^+-K^+-ATPase consists of α and β subunits proposed to function as an α−β heterodimer. Skeletal muscle is characterized by expression of α1, α2, β1, and β2 subunit isoforms. The relative molar proportions of each subunit or each protein isoform are not known, yet their subcellular distribution and expression in muscles of different fiber types are markedly different. In this study, the molar ratio of each pump subunit isoform was measured in purified membranes from skeletal muscle and compared with those in kidney and brain microsomes. Recombinant proteins were used as standards to quantitate each isoform by immunoblotting in combination with measurements of [^{3}H]ouabain binding. The results indicate that in kidney microsomes, which express predominantly α1 and β1 isoforms, the α:β subunit molar ratio is approximately 1:1. In brain microsomes, the sum of all α (α1, α2, and α3) and all β (β1 and β2) subunits also yielded a molar ratio of aproximately 1:1. In contrast, in red (oxidative) skeletal muscles, the all α:β subunit ratio was 0.2 in plasma membranes and 0.4 in intracellular membranes. The ratio of α2 subunits to α1 subunits ranged from 1.6 in surface membranes to up to 7 in internal membranes, while the β1 subunits exceded the β2 subunits by ≈4-fold in all membrane fractions. Thus, intracellular membranes of red skeletal muscles contain primarily α2 and β1 subunits. When these intracellular membranes were further subfractionated by velocity gradient centrifugation, the α2:β1 subunit ratio was 0.5 in the faster migrating (larger) membranes and 1.0 in the slower migrating (smaller) ones. This was due to a progressive decrease in abundance of the β1 subunits without a change in the concentration of α2 subunits per unit protein. The Na^+-K^+-ATPase hydrolytic activity was higher in the larger vesicles than in the smaller ones along the sucrose gradient. These results suggest that the ratio of β to α subunits may serve to regulate the catalytic activity of the Na^+-K^+-ATPase in skeletal muscle.

The sodium- and potassium-dependent adenosinetriphosphatase (Na^+-K^+-ATPase; EC 3.6.1.37) is an integral membrane protein which catalyzes the extrusion of three Na^+ in exchange for the uptake of two K^+ across the plasma membrane of all mammalian cells. This enzyme consists of two integral membrane proteins, a catalytic α subunit of 110 kDa and a glycosylated β subunit of ≈50 kDa (Sweadner, 1989). The α subunit contains the binding sites for ATP, Na^+, and K^+, and for the specific inhibitor ouabain. The precise role of the β subunit is not known but is thought to be important for the stability of the complex and for the maintenance of ATP hydrolytic activity and ion currents (Geering, 1991; Blanco et al., 1994a,b; Jaisser et al., 1992; Jaunin et al., 1993; Eakle et al., 1992, 1994). In mammalian cells, at least three isoforms of each subunit have been described (α1−3 and β1−3) of distinct but not exclusive tissue distribution (Sweadner, 1989; Martin-Vassallo et al., 1989; Malik et al., 1996).

To date, most studies on the biosynthetic pathways of the pump polypeptides have relied on heterologous expression of the α1 and β1 isoforms. Thus, in cRNA-injected *Xenopus* oocytes, α1 and β1 are cotranslated and associate in the endoplasmic reticulum. Unassociated α subunits are degraded, and unassociated β subunits appear to be prevented from progressing through the biosynthetic pathway (Geering, 1991; Jaunin et al., 1992; Geering et al., 1996; Ackermann & Geering, 1990). In contrast, in mammalian MDCK cells, β1 subunits can mature in excess of α1 subunit complements (Mircheff et al., 1992). In yeast, β1 subunit cDNA must be cotransfected with α1 for the latter protein to display catalytic activity (Horowitz et al., 1990). Finally, in insect cells, α1 and β1 subunits appear capable of reaching the plasma membrane independently, and assemble at this location to produce bona fide ion pump activity (DeTomaso et al., 1993, 1994). Collectively, these results suggest that α1 and β1 subunits must interact to display catalytic activity, and that in certain cellular backgrounds, α and β subunits may reach the plasma membrane independently of the other. However, the molar ratio of the natural complement of these polypeptides has not been examined in tissues where more than one isoform of the α and β subunits is expressed. Rat skeletal muscles are particularly interesting in this regard because the α1 and α2 isoforms are expressed in all fiber types, the

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β1 subunit is preferentially expressed in slow-twitch, oxidative fibers, the β2 subunit is specifically expressed in fast-twitch, glycolytic fibers, and fast-twitch oxidative fibers express both β isoforms (Hundal et al., 1993). Most individual muscles contain a combination of fiber types, and red muscles are composed of slow- and fast-twitch oxidative fibers which express β1 and β2 subunits. Whereas α1 is almost exclusively present in surface membranes, α2, β1, and β2 subunits populate both surface and intracellular membranes (Hundal et al., 1992; Lavoie et al., 1996). Moreover, insulin mobilizes pump subunits from intracellular membranes to surface membranes in red muscle, specifically the α2 and β1 isoforms (Hundal et al., 1992; Lavoie et al., 1996). However, the relative proportion of all these subunits is not known.

The aim of the present work was to evaluate the molar proportions of all Na^+-K^+-ATPase isoforms in purified membranes from red skeletal muscles composed of fast- and slow-twitch, oxidative fibers. To this effect, recombinant proteins comprising α1, β1, or β2 subunits were used as standards on immunoblots, and the α2 subunit was quantitated by [^{3}H]ouabain binding. The results indicate that there are different α:β subunit ratios among the purified muscle membranes; α2 always exceded α1 protein in a proportion ranging from 1.6 in surface membranes to 3−7 in internal membranes. The β subunits exceded the molar content of the sum of α1 and α2 subunits in surface membranes and in intracellular membranes, but a stoichiometry of 1:1 (predominantly α2 and β1) was observed in a subgroup of intracellular membranes separated by sucrose gradient velocity centrifugation. The membranes characterized by an α:β subunit ratio of 1 showed lower Na^+-K^+-ATPase activity than other membranes, suggesting that an excess of β subunits over α subunits is required for optimal enzymatic activity of the pump in skeletal muscle.

EXPERIMENTAL PROCEDURES

Purified Proteins and Antibodies. A fusion protein comprising amino acid residues 338−513 (18 kDa) of the rat α_1 Na^+-K^+-ATPase linked to TrpE (37 kDa) (Shyjan & Levenson, 1989) was used as reference standard for quantitative immunoblotting of α1 in isolated membrane samples. Truncated Na^+-K^+-ATPase β1 and β2 proteins (27 kDa) were generated as reported earlier (Gonzalez-Martinez et al., 1994): A His-tagged β1 polypeptide encompassed all the extracellular domain (amino acids 70−303 coded by human β1 cDNA), and a His-tagged β2 polypeptide encompassed all the extracellular domain and a portion of the transmembrane domain (amino acids 55−290 coded by the human β2 cDNA) (Gonzalez-Martinez et al., 1994). A polyclonal antiserum against the α1 subunit of the Na^+-K^+-ATPase was purchased from Upstate Biotechnology (Lake Placid, NY). The specificity of this antiserum was previously established (Shyjan & Levenson, 1989) and assessed in isolated rat skeletal muscle membranes (Hundal et al., 1992). The monoclonal antibody McB2 specific for the α2 subunit of the Na^+-K^+-ATPase was kindly provided by Dr. K. J. Sweadner (Harvard University). Polyclonal antisera SpETb1 and SpETb2 against the β1 and β2 subunits of the Na^+-K^+-ATPase, respectively, were raised against the fusion proteins described above (Gonzalez-Martinez et al., 1994). Polyclonal antiserum against the GLUT4 glucose transporter was from East Acres Biologicals (Southbridge, MA).

Subcellular Fractionation and Immunoblotting. Rat kidney and brain microsomes were prepared as described previously (Jorgensen, 1974). Membranes of rat skeletal muscle composed of slow- and fast-twitch oxidative fibers (soleus, red gastrocnemius, red rectus femoris, red vastus lateralis) were isolated by differential centrifugation and discontinuous sucrose grandients, and characterized as previously described (Klip et al., 1987; Douen et al., 1989). Fraction F25 is enriched in plasma membrane markers (5′-nucleotidase, GLUT1 glucose transporter, Na^+-K^+-ATPase α1 isoform); fraction F30 also contains plasma membrane markers but to a lesser extent than F25; fraction F35 contains the internal insulin-sensitive pools of Na^+-K^+-ATPase α2 and β1 subunits (Lavoie et al., 1996) and the GLUT4 glucose transporters (Marette et al., 1992); the pellet (PEL) of the gradient is enriched in sarcoplasmic reticulum but also contains Na^+-K^+-pumps including insulin-sensitive β1 subunits. Considering the α1 Na^+-K^+-ATPase as a marker of plasma membranes, it is estimated that less than 20% of the mass of the intracellular fraction F35 could be of plasma membrane origin.

The intracellular F35 fraction was further fractionated by velocity centrifugation (120000*g* for 50 min) in continuous sucrose gradients. Approximately 3 mg of F35 protein was applied to a 10 mL 15−35% linear sucrose gradient. Fractions were collected from the bottom of the gradients to measure refractive index and protein concentration by the bicinchoninic acid procedure. Samples were subjected to SDS-PAGE and immunoblotting analysis as previously described (Marette et al., 1992). Quantitation of immunoreactive bands was performed with a Molecular Dynamics PhosphorImager system (Sunnyvale, CA). All signals acquired were within the linear range of the detection scale.

Quantification of Na^+-K^+-ATPase α and β Subunits. The TrpE−α1 Na^+-K^+-ATPase fusion protein migrated as a single band in SDS−PAGE, assessed by Coomassie blue staining. No other polypeptides were detected in the sample, which was then used as reference standard for α1 Na^+-K^+-ATPase.

The concentration of the Na^+-K^+-ATPase α2 protein was measured in a sample of the F25 fraction of skeletal muscle plasma membranes, by binding of [^{3}H]ouabain (43 nM) in the presence of vanadate (Omatsu-Kanbe & Kitasato, 1990). Vanadate renders the α subunits fully accessible to ouabain (Hansen, 1979). At the concentration used, the binding of ouabain to the α1 subunit is negligible. Skeletal muscle does not express the α3 isoform which also binds ouabain with high affinity. The maximum binding of ouabain to α2 subunits was then calculated by the equation: $B_{max} = B + K_d(B/O_f)$, where B_{max} is the maximum binding, B is the measured binding, $K_d = 0.34\ \mu$M is the dissociation constant for ouabain binding to the α2 subunit (mean value measured in homogenates of rat skeletal muscle; Norgaard et al., 1983), and O_f is the concentration of free ouabain at which binding is measured (Kjeldsen, 1986). The B_{max} value of the reference F25 membrane fraction yields the content of α2 subunit per milligram of protein. This F25 membrane fraction was used as a reference standard in immunoblots using anti-α2 antibody, to calculate the amount of α2 subunits in other skeletal muscle fractions and brain microsomes. In addition, [^{3}H]ouabain binding to brain microsomes was used to calculate the amount of α2 + α3 subunits. From this value, the concentration of α3 subunits

was calculated after subtracting the concentration of α2 subunits calculated from the immunoblots.

The recombinant His-tagged Na^+-K^+-ATPase β1 subunit was solubilized from inclusion bodies with Triton-X100 and urea by the procedure described by Sambrook et al. (1989). The solubilized material was analyzed by SDS−PAGE whereby the 30 kDa band corresponding to the β1 subunit represented 48% of the total protein applied. Known amounts of the purified β1 subunit were then used as reference standard to calculate the molar content of the β1 subunit in membrane samples analyzed by immunoblotting.

The recombinant β2 His-tagged protein was analyzed by SDS−PAGE, and the amount of protein loaded on each gel was calculated from the absorbance of the Coomassie blue staining of the sample compared with the absorbance of a standard curve of BSA (0.25, 0.5, and 1.0 μg) loaded in the same gel (e.g., the densitometric values of the BSA band generated by 0.5 and 1.0 μg of BSA were 0.325 and 0.636, and the scanned value of the 27 kDa band generated by loading 10 μL of the β2 sample was 0.138). Known amounts of β2 polypeptide were then used as a reference standard to calculate the molar content of β2 subunits in the membrane samples analyzed on the same immunoblots.

In these calculations, it is assumed that the reactivity of the anti-β1 (or anti-β2) antibody for its cognate His-tagged protein is similar to that for the native enzyme in the rat tissue samples. Deviations from this assumption could lead to minor underestimates in the net amount of β subunits in the tissues, but would not affect the comparisons among different tissues or subcellular fractions.

Na^+-K^+-ATPase Activity Determination. Na^+-K^+-ATPase activity was measured as the K^+-dependent 3-*O*-methylfluorescein phosphatase activity (Nørgaard et al., 1984) using 25 μg of protein of freshly prepared, intracellular membranes. Briefly, 3-*O*-methylfluorescein phosphate (19.5 mM) was added to the reaction mixture containing the membrane sample in a buffer containing 4 mM $MgCl_2$, 1 mM EDTA, and 80 mM Tris, pH 7.6, to initiate the reaction. The hydrolysis of 3-*O*-methylfluorescein phosphate was followed for 2 min as the fluorescence of hydrolyzed methylfluorescein, measured at 465 nm excitation and 515 nm emission wavelengths, to measure the basal rate of hydrolysis (spontaneous and by nonspecific phosphatases). KCl (10 mM) was then added, and the increased rate of hydrolysis was recorded for another 2 min. Activity is expressed in micromoles of activity per hour per milligram of protein. This assay is preferentially used for skeletal muscle because it shows lower background due to lesser reactivity of the substrate with other ATPases such as that of actomyosin. This activity is ouabain-sensitive in addition to K^+-dependent. Norgaard et al. (1984) have shown that the number of ouabain binding sites correlates with the 3-*O*-methylfluorescein activity in skeletal muscle homogenates. Intracellular membranes do not form tight seals to K^+ and are oriented with the cytoplasmic ATP binding side facing the solution. In some experiments, the membranes were treated with SDS (0.3 mg/mL) prior to the assay, but no differences in the ATPase activity were noted, supporting the notion that the membranes are leaky.

Statistical Analysis. Fischer ANOVA test was performed for the comparison of different fractions. A value of $p < 0.05$ was considered statistically significant.

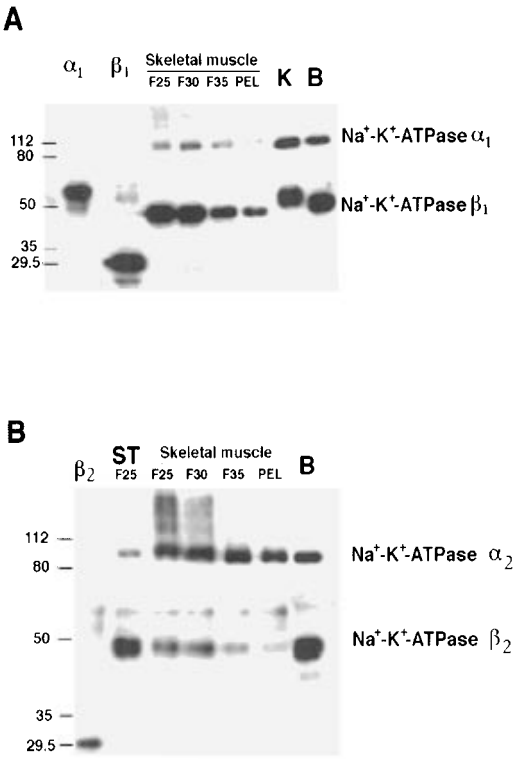

FIGURE 1: Quantification of α1, α2, β1, and β2 subunits of the Na^+-K^+-ATPase in purified membranes from skeletal muscle, and in kidney and brain microsomes. The figure illustrates one representative experiment. The means ± SE of four experiments are presented in Table 1. (**A**) The reference standards Na^+-K^+-ATPase α1 fusion protein (α_1, 60 ng applied = 1.1 pmol in the immunodetected band) and β1 protein (β_1, 250 ng applied = 4.2 pmol in the immunodetected band), and test samples consisting of purified membranes from red skeletal muscles (F25, F30, F35, PEL, each 15 μg), kidney (K, 10 μg), and brain (B, 10 μg) microsomes, were subjected to SDS−PAGE and immunoblotted with antibodies specific for the α1 and β1 isoforms of the Na^+-K^+-ATPase. (**B**) The reference standards Na^+-K^+-ATPase β2 subunit (β_2, 3.7 pmol in the immunoreactive band) and F25 fraction from skeletal muscle (ST, 10 μg, in which $[^3H]$ouabain was 16.3 pmol/mg of protein) are compared with test samples from red skeletal muscles (F25, F30, F35, PEL, each 15 μg) and brain microsomes (B, 10 μg) that were subjected to SDS−PAGE and immunoblotted with antibodies specific for the α2 and β2 isoforms of the Na^+-K^+-ATPase. Numbers on the left side denote the molecular mass of reference standard proteins in kilodaltons.

RESULTS

We have previously reported that rat skeletal muscle expresses the α1, α2, β1, and β2 isoforms of the Na^+-K^+-ATPase (Hundal et al., 1992). The kidney expresses the α1 and β1 isoforms of the enzyme but not α2 or β2, and brain expresses α1, α2, and α3, as well as β1 and β2 isoforms (Shyjan & Levenson, 1989; Shyjan et al., 1990). In addition, both latter tissues express mRNA of the newly described mammalian β3 isoform, though at very low levels compared to its expression in testis (Malik et al., 1996). We used kidney, brain, and muscle tissues to quantitate the molar proportions of each of their major isoforms. Figure 1A shows a representative immunoblot of α1 and β1 subunits in the reference standards and in isolated membranes prepared from red skeletal muscle (F25, F30, F35, and PEL), kidney (K), and brain (B). The α_1 fusion protein standard migrated in SDS−PAGE with an apparent molecular mass of 55 kDa. Densitometric scanning of the standard samples was used to calculate the picomoles per milligram of protein

Table 1: Abundance of Na^+-K^+-ATPase α and β Subunit Isoforms in Kidney Microsomes, Brain Microsomes, and Purified Membranes from Red Skeletal Muscles[a]

fraction	abundance (pmol/mg of protein)					all α:β ratio
	α1	α2	α3	β1	β2	
kidney microsomes	60 ± 14			88 ± 10		0.70
brain microsomes	31 ± 2	43 ± 6	295[b]	227 ± 23	151 ± 32	0.97
muscle membranes:						
F25[c]	18 ± 5	30 ± 6		164 ± 38	44 ± 11	0.23
F30[c]	16 ± 3	26 ± 4		121 ± 29	40 ± 10	0.26
F35[d]	6 ± 1	18 ± 4		46 ± 7	12 ± 1	0.41
PEL[e]	2 ± 0.4	14 ± 3		30 ± 6	7 ± 5	0.44

[a] Results are the mean ± SE of four independent experiments. For the α:β ratio, the sums of all α subunits and of all β subunits were calculated, in picomoles per milligram of protein. [b] Calculated by subtracting from the maximum [^{3}H]ouabain binding (in picomoles per milligram of protein) the α2 content obtained from quantitative immunoblotting (in picomoles per milligram of protein). [c] Plasma membranes. [d] Intracellular membranes. [e] Sarcoplasmic reticulum.

of α1 and β1 subunits in the muscle, brain, and kidney membrane samples. The data in Figure 1A and Table 1 indicate the following pattern for the concentration of the α1 subunit per milligram of protein: kidney microsomes > brain microsomes > purified plasma membranes from red skeletal muscle. In contrast, the order of concentration of the β1 subunit was brain microsomes > plasma membranes from red skeletal muscle > kidney microsomes.

Figure 1B shows a representative immunoblot of α2 and β2 subunits in the samples used as reference standards, as well as in purified skeletal muscle membranes and in brain microsomes. The moles per milligram of protein of the Na^+-K^+-ATPase α2 isoform were calculated from measurements of [^{3}H]ouabain binding in the reference standard F25 sample, as described under Experimental Procedures. The data obtained from several experiments indicate that the molar content of α2 subunit was roughly comparable to that of the α1 subunit in brain microsomes. The concentration of the α3 isoform in brain microsomes was estimated by subtracting the α2 molar value from the B_{max} of [^{3}H]ouabain bound to microsomes (under the conditions used, B_{max} is a measure of the total number of α2 and α3 subunits but not α1 subunits). By this calculation, rat brain was shown to contain almost 10 times more α3 than α2 or α1 subunits (Table 1). In skeletal muscle, the α2 isoform was more abundant than the α1 isoform, especially in the internal membrane fractions (F35 and pellet) (Table 1). This tissue does not express the α3 isoform (Jewell et al., 1992).

The skeletal muscle fractions F25 and F30, enriched in plasma membrane markers, contained more β2 subunits than the intracellular membranes F35 and PEL (Table 1). Brain microsomes contained more β2 subunits than any of the membrane fractions isolated from red skeletal muscles (Table 1). This latter result is in keeping with the obsevation that the β2 isoform is less abundant in red muscles (composed of oxidative fibers) than in white muscles (composed of glycolytic fibers) (Hundal et al., 1993). Indeed, in F25 from white (fast-glycolytic) muscle, the molar concentration of β2 isoform was comparable to the amount present in brain microsomes (results not shown).

The above results also allow us to calculate the molar ratios of α to β subunits (Table 1). Thus, in kidney microsomes the α:β subunit (α1:β1) ratio was 0.70. In brain microsomes, the ratio of all α subunits to all β subunits was 0.97 (α:β = 1:1), with α3 being by far the most abundant α isoform. In contrast, in purified skeletal muscle plasma membranes, the α:β subunit ratio ranged from 0.23 to 0.26 (α:β = 1:4), and in intracellular membranes, α:β ranged from 0.41 to 0.44 (α:β = 2:5).

The intracellular fraction F35 of red skeletal muscle contains primarily α2 and β1 subunits (Lavoie et al., 1996; Figure 1), both of which respond to insulin by translocating to the plasma membrane. To analyze whether this fraction contains a homogeneous mixture of membranes, F35 from red muscles was further fractionated by velocity centrifugation in continuous sucrose gradients. A total of 30 fractions of 0.35 mL each were collected from the bottom of the tube and were analyzed for subunit composition. During velocity gradient centrifugation, the vesicles separate according to their size, with larger sized vesicles migrating toward the bottom of the tube. The first five fractions collected preceded the elution of protein and were discarded; the rest of the samples were pooled into four distinct groups (FP1 through FP4) as shown in Figure 2A. Fraction FP1 contained the bulk of the protein. Although of low protein content, FP2, FP3, and FP4 also contained significant amounts of pump subunits (see below).

The amount of the Na^+-K^+-ATPase α2 and β1 subunits in the sucrose velocity gradient fractions FP1−FP4 was quantitated as described in Experimental Procedures. Figure 2B illustrates a representative immunoblot of these fractions, using antibodies to the α2 and β1 subunits of the Na^+-K^+-ATPase as well as to the GLUT4 glucose transporter. Figure 2C shows the results of three to four independent experiments. Whereas the amount of α2 and β1 subunits was not significantly different in FP1 and FP2, there was a marked decrease in the amount of β1 in FP3, and in FP4 the amount of both subunits decreased to approximately 5% of the total recovered from the gradient. The proportion of α2:β1 subunits varied from less than 0.5 in FP1 and FP2 to 1.0 in FP3 and FP4 (Table 2). The change in α2:β1 subunit ratio from FP2 to FP3 was due to a marked decrease in the number of β1 subunits (Table 2) without a change in the corresponding amount of α2 subunit per unit protein.

The contents of α1 and β2 in fractions FP1 through FP4 were also determined, and found to be lower than those of α2 and β1 (Table 2). Isoform α1 was low because these fractions are derived from intracellular membranes (F35), and β2 was low because the fractions were derived from red muscles which express only low levels of this isoform. The sum of all α subunits was compared to the sum of all β subunits. Fractions FP1, FP2, and FP3 had a similar amount of α subunits (12, 16, and 13 pmol/mg of protein), but FP3 had a significantly lower amount of β subunits (17

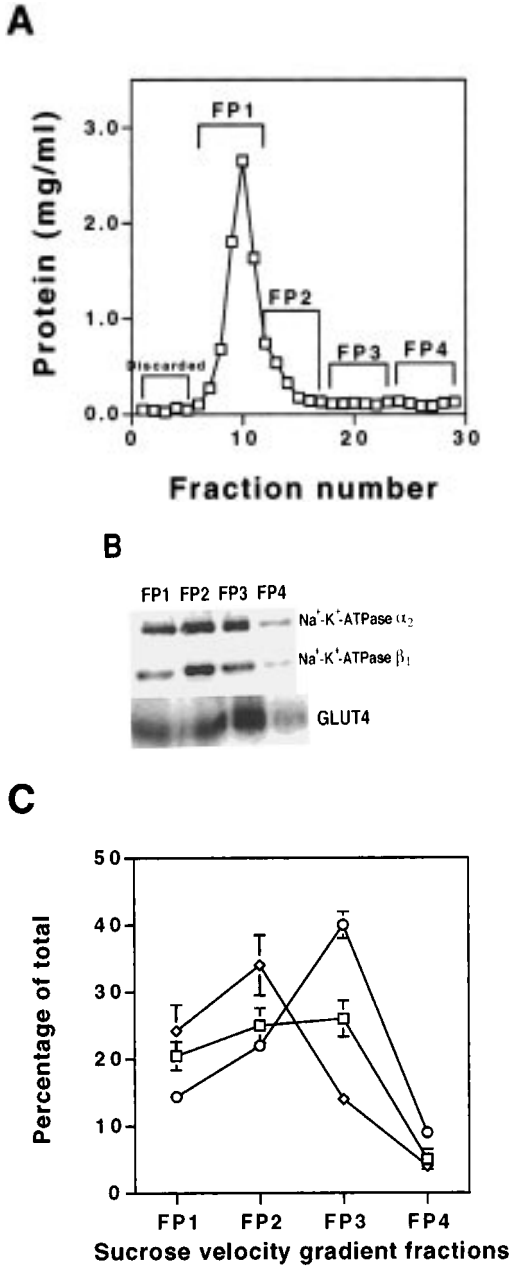

FIGURE 2: Na^+-K^+-ATPase α2 and β1 subunits and GLUT4 glucose transporters in intracellular membranes of red skeletal muscles fractionated by continuous sucrose velocity gradient centrifugation. (**A**) Internal membranes of F35 (≈3 mg of protein) were further separated by centrifugation in continuous sucrose velocity gradients (15 to 35%) as described under Experimental Procedures. A typical profile of the protein distribution in the gradient is shown. Grouping of fractions into FP1−FP4 is indicated. (**B**) Fractions FP1−FP4 (25 μg of protein each) were analyzed by SDS−PAGE and immunoblotted for α2 and β1 subunits or GLUT4 glucose transporters. Parallel lanes were run containing reference standards consisting of β1 purified protein and an F25 fraction of known α2 content (see legend to Figure 1 for details). (**C**) Content of α2 (squares) and β1 (diamonds) subunits of the Na^+-K^+-ATPase, and of GLUT4 glucose transporters (circles) in fractions FP1−FP4, expressed as a percentage of the total content recovered in all fractions. Results are means ± SE of three to four independent fractionation experiments. $p < 0.05$ for the α2 content in FP4 vs FP1, FP2, or FP3. $p < 0.05$ for the β1 content in FP3 vs FP1, FP2. $p < 0.05$ for the β1 content in FP4 vs FP1, FP2. $p < 0.05$ for the GLUT4 content in any of the fractions.

pmol/mg of protein) than FP1 or FP2 (27 and 37 pmol/mg of protein). These results support the notion that the main diverging factor between fractions FP1 and FP3 is the content of β subunits.

Table 2: Abundance of the Na^+-K^+-ATPase α and β Subunits in Intracellular Membranes from Red Skeletal Muscle, Subfractionated by Continuous Sucrose Velocity Gradients[a]

fraction	pmol/mg of protein α2	pmol/mg of protein β1	α2:β1 ratio	pmol/mg of protein α1	pmol/mg of protein β2	all α:β ratio
FP1	8 ± 1	17 ± 3	0.5	4 ± 1	10 ± 3	0.4
FP2	10 ± 1	24 ± 3	0.4	6 ± 2	13 ± 2	0.4
FP3	10 ± 2	9 ± 2	1.1	3 ± 1	8 ± 2	0.8
FP4	2.5 ± 1	2.3 ± 1	1.1	1 ± 0.5	2 ± 2	0.8

[a] Results are the mean ± SE of three to four independent experiments, expressed in picomoles per milligram of protein: $p < 0.05$ for α2 in FP4 vs any other fraction; $p < 0.05$ for β1 in FP3 vs any other fraction, and for FP4 vs any other fraction; $p < 0.05$ for α1 or β2 in FP4 vs any other fraction.

Table 3: Na^+-K^+-ATPase Activity Associated with Intracellular Membranes of Rat Skeletal Muscle[a]

fraction	Na^+-K^+-ATPase activity [μmol h^{-1} (mg of protein)$^{-1}$]
FP1	0.55 ± 0.07
FP2	0.44 ± 0.06
FP3	0.28 ± 0.06
FP4	0.19 ± 0.02

[a] Na^+-K^+-ATPase activity was measured from the K^+-dependent hydrolysis of methylfluorescein phosphate as indicated under Experimental Procedures. The samples examined for α:β ratios and content in Table 2 were analyzed. Results are expressed in micromoles per hour per milligram of protein and are the means ± SE of four independent determinations. $p < 0.05$ for the activity of FP1 vs FP3 or FP4, and for FP2 vs FP4.

The subfractionation of F35 by velocity gradient centrifugation was also used to compare whether the GLUT4 glucose transporter comigrated with any of the Na^+-K^+-ATPase subunits. As seen in Figure 2B,C, the bulk of the GLUT4 protein was found in FP3, a fraction that was not particularly enriched in either the α2 or the β1 subunits of the Na^+-K^+-ATPase. These results agree with previous studies (Lavoie et al., 1995; Aledo & Hundal, 1995) demonstrating that the majority of the GLUT4 glucose transporter and α2 subunit reside in different intracellular vesicles in rat skeletal muscle. Our results also suggest that the glucose transporter and the Na^+-K^+-ATPase β subunit do not colocalize in intracellular membranes. Thus, the three different polypeptides translocated to the plasma membrane, in response to insulin from intracellular membrane storage sites, may originate from distinct intracellular loci.

The distinct pattern of segregation of α and β subunits raises the question of whether the differential expression of β subunits was correlated with distinct levels of catalytic activity of the pump. Table 3 shows that the activity of the Na^+-K^+-ATPase was lower in FP3 and FP4 than in FP1 and FP2. A statistically significant difference in Na^+-K^+-ATPase activity was observed between fractions FP1 and FP3; fraction FP2, with the same amount of α2, also had a numerically higher Na^+-K^+-ATPase activity than FP3, which, however, did not reach statistical significance. These values were not altered by the inclusion of SDS (0.3 mg/mL) in the samples, confirming that the difference in activity between fractions FP1, FP2 and FP3, FP4 was not due to differences in the accessibility of the pump to its substrates (results not shown). The fractions with higher catalytic

activity (FP1 and FP2) had a higher β:α subunit ratio than those with low activity (FP3 and FP4). Moreover, FP2 and FP3 have the same amount of α subunits per milligram of protein but different amount of β subunits (Table 2).

DISCUSSION

Biosynthetic Assembly of Pump Subunits and Stoichiometry of Complexes. It is generally accepted that the stoichiometry of the α and β subunits of the Na^+-K^+-ATPase in mammalian cell is 1:1. This premise is based primarily on observations that unassociated subunits of multimeric proteins are retained in the endoplasmic reticulum until they are properly assembled for packaging toward their final destination (Helenius et al., 1992). Molecular chaperones are endoplasmic reticulum proteins that bind transiently to intermediates during protein folding and assembly, preventing export of misfolded proteins and allowing time for assembly to occur. It was recently shown that the chaperone protein BiP/GRP78 coprecipitates with both the α1 and β1 subunits of the Na^+-K^+-ATPase expressed in *Xenopus laevis* oocytes (Beggah et al., 1996), and full processing of the carbohydrate of the Na^+-K^+-ATPase β1 subunit of *Xenopus* requires coexpression of α1 subunits (Geering, 1991; Ackermann & Geering, 1990), suggesting that the two subunits associate early on in the endoplasmic reticulum of the oocytes. However, the existence of unaccompanied β subunits has also gained experimental support. Thus, expression of the *Torpedo californica* β subunit proceeds without α subunit expression in the same *Xenopus* oocytes (Ueno et al., 1995). Moreover, brush border membranes from rat distal colon have immunodetectable β1 subunits in the absence of immunodetectable α subunits (Marxer et al., 1989), and MDCK cells have a pool of unassociated β subunits (Mircheff et al., 1992). This indicates that β subunits can exist independent of α subunits.

Our results indicate that in red skeletal muscle there is an overall excess of β over α subunits.[1] The all α:β ratio was 0.23 in plasma membranes and 0.41 in intracellular membranes. We hypothesize that, in this tissue, Na^+-K^+-ATPase β subunits in excess of α subunits might escape interaction with BiP in the endoplasmic reticulum and subsequent degradation. Alternatively, interaction with BiP might protect the β subunit from degradation and allow it to reach its final cellular destinations unaccompanied by the α subunit. It is of interest that the proportion of α to β subunits in skeletal muscle varies depending on the origin of the membranes. Thus, we found intracellular membranes characterized by small size in which the proportion of these subunits is 1:1, intracellular membranes characterized by large size vesicles in which the proportion is 2β per each α, and plasma membranes in which the proportion is 4β (sum of β1 and β2) per each α (sum of α1 and α2) subunit. We cannot distinguish whether complexes exist of stoichiometries such as 1α−2β, 1α−3β or 1α−4β, or if the only complexed form is 1α−1β and the rest of the β subunits are unassembled, because there are no antibodies available that can immunoprecipitate the α2 subunit. It is also plausible that α subunits of other P-type ATPases, so far undescribed in skeletal muscle, might be complexed to the extra β subunits. Based on the observations made in insect cells (DeTomaso et al., 1993, 1994), it is conceivable that association of higher order α and β subunits could potentially occur at the plasma membrane of skeletal muscle. In contrast, the stoichiometric proportion of α to β subunits in purified Na^+-K^+-ATPase preparations from the outer renal medulla of mammalian kidney was shown to be 1:1 (Jorgensen, 1980). The quantitative approach used in the present study, applied to kidney microsomes, approximates this value. Similarly, the immunoprecipitated α1 and α3 subunits of brain axolemmal membranes show stoichiometries of the complex of α1−β1 and α3−β1 which are each close to 1:1 (Therien et al., 1996). Although the molar content of each subunit was not measured in that study, the proportions of associated α−β were calculated by comparison with kidney microsomes in which the stoichiometry of α−β complexes was considered to be 1. Using a similar approach, the molar ratio of α−β was calculated to be 1 in lamb liver microsomes (Sun & Ball, 1992). In our hands, brain microsomes showed almost comparable molar levels of α and β subunits, in close agreement with the above studies. These observations are consistent with the view that in skeletal muscle, but not in brain, there are spare or excess β subunits.

Na^+-K^+-ATPase Subunit Proportions in Skeletal Muscle: Implications for Functional Activity. The skeletal muscle Na^+-K^+-ATPase α2−β1 complex is particularly interesting in view of the fact that there is an insulin-induced specific translocation of these two isoforms from intracellular to plasma membranes as assessed by immunodetection in purified membranes and immunogold labeling on ultrathin cryosections (Hundal et al., 1992; Lavoie et al., 1996; Marette et al., 1993). Both subunits are detected in subsarcolemmal vesicles and in intermyofibrillar membranes close to the triads where the transverse tubules appose the sarcoplasmic reticulum (Marette et al., 1993; Lavoie et al., 1995). However, it is not known if the diverse locations represent biochemically different organelles. In the present study, we began to approach this question by separating intracellular membranes first by density and then by size. The distinct membrane fractions isolated displayed different proportions of pump subunits and pump activity. Two groups of membranes could be identified: those with an α:β subunit ratio of around 1:2, which had the higher catalytic activity, and those with an α:β subunit ratio of about 1:1, which had lower catalytic activity. These results suggest the possibility that a molar excess of β subunits may confer higher enzymatic activity in skeletal muscle. Thus, we hypothesize that dynamic changes in the amount of β subunits could be an effective mechanism to stimulate pump activity. This possibility has important implications for the regulation of the rate of ion fluxes by a variety of hormones as well as exercise (Ewart & Klip, 1995; Clausen, 1996). Indeed, experiments carried out by Geering et al. (1996) indicate that the β subunit may serve to modulate the K^+-activation of the α subunit catalytic activity and of ion transport. It is hypothetically possible that higher order oligomers of β subunits could render the α2 subunit accessible to the ion, thereby creating functionally exposed and cryptic forms of the enzyme. Whereas there is no experimental report of β oligomers, evidence is emerging that α subunits can form oligomers (Blanco et al., 1994b), and that the carbohydrate

[1] Due to the absence of antibodies to the β3 subunit, it is not possible at present to assess the contribution of this isoform to the total pool of β subunits. However, if present in a significant degree, this would only strengthen the conclusion that there is an excess of β over α subunits in skeletal muscle.

moiety of the β subunit can physically interact with the α subunits (Schmalzing et al., 1997). We speculate that acute or sustained changes in pump activity could be brought about by recruiting to the suface or synthesizing, respectively, more β subunits without necessarily changing the abundance of α subunits.

In conclusion, whereas the α:β subunit molar ratio in kidney and brain microsomes is close to 1:1, in purified membranes from red skeletal muscles α:β molar ratios of 1:4 were observed in plasma membranes, 1:2 in large internal membranes, and 1:1 in the lighter membranes of the internal pool. We speculate that α2-β1 complexes of 1:1 stoichiometry might form at the endoplasmic reticulum, and that additional unassociated β1 subunits may be synthesized and exported along the secretory pathway to the plasma membrane. There, excess β1 subunits might support a higher enzymatic activity of the Na^+-K^+-ATPase.

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