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Na,K-ATPase Expression in C_2C_{12} Cells during Myogenesis: Minimal Contribution of $\alpha 2$ Isoform to Na,K Transport

Sandra C. Higham, Jivan Melikian†, Norman J. Karin, Faramarz Ismail-Beigi†, and Thomas A. Pressley
Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas 77225, and †Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Summary. Cells of the murine skeletal muscle line, C_2C_{12}, undergo differentiation from mononuclear myoblasts to multinuclear myotubes that express a number of proteins associated with striated muscle. We examined the relationship between the abundance of the mRNAs encoding the fast-twitch Ca-ATPase and the α isoforms of Na,K-ATPase and the subsequent expression of their respective polypeptides. Both the mRNA and protein levels of the $\alpha 1$ isoform remained constant throughout differentiation. In contrast, the content of mRNAs encoding the $\alpha 2$ isoform and fast-twitch Ca-ATPase increased coordinately with the abundance of their corresponding polypeptides during myotube development. Despite the dramatic increase in $\alpha 2$ expression, estimates of in vitro Na,K-ATPase activity and assessments of in vivo transport activity suggest that $\alpha 2$ contributes little to ionic homeostasis in C_2C_{12} myotubes.

Key Words myogenesis · Na,K-ATPase isoforms · fast-twitch Ca-ATPase · immunoblots · mRNA abundance

Introduction

Since the identification of multiple genes encoding the constituents of the Na,K-pump, attempts have been made to determine the physiological roles played by the various forms. Na^+,K^+-activated adenosine triphosphatase (Na,K-ATPase), the enzymatic equivalent of the pump, is composed of a catalytic α subunit (110 kDa) and a glycosylated β subunit (55 kDa) (Jørgensen & Andersen, 1988; Lingrel et al., 1990). Within mammals, the α subunit exists in at least three isoforms (Shull, Greeb & Lingrel, 1986), and the β subunit exists in at least two (Martin-Vasallo et al., 1989). This subunit diversity is a common motif across a wide phylogenetic range (Pressley, 1992). Indeed, the α isoforms of birds and mammals appear to be highly conserved, with the three isoforms expressed by chickens being very similar to their mammalian counterparts (Takeyasu, Lemas & Fambrough, 1990). The expression of the Na,K-pump isoforms differs with cell type, differentiation, and hormonal state, suggesting that the isoforms may have distinct functional characteristics. Moreover, the presence and abundance of the α isoforms in mammals correlates with differences in substrate affinities and sensitivity to cardiac glycosides (Urayama, Shutt & Sweadner, 1989; McGill & Guidotti, 1991). Nevertheless, it is not clear what selective advantage the expression of different isoforms may confer.

One approach to addressing the role of the Na,K-ATPase isoforms in active Na,K transport would be to study their expression in a model system in which Na,K-pump function can be monitored. Using a murine myogenic cell line, C_2C_{12}, Orlowski and Lingrel (1988) found that the abundance of the mRNA encoding the $\alpha 2$ isoform increased dramatically with differentiation of myoblasts into myotubes. In addition, they reported the emergence of highly ouabain-sensitive ATPase activity and high-affinity ouabain-binding sites. We have extended their work by examining the coordination between the abundances of $mRNA_{\alpha 2}$ and $\alpha 2$ polypeptide and by assessing the consequences of $\alpha 2$ expression on the abundance of the Na,K-ATPase α isoforms and overall Na,K transport. Our results suggest that the $\alpha 2$ isoform contributes minimally to ionic homeostasis in differentiated myotubes under basal conditions.

Preliminary results from this investigation have been reported (Higham, Karin & Pressley, 1991).

Materials and Methods

Cell Culture

The established myogenic cell line, C_2C_{12}, was a generous gift from Dr. Michael T. Crow and was derived from the thigh muscle of a young mouse (Yaffe & Saxel, 1977; Blau, Chiu & Webster,

1983). The undifferentiated myoblasts were grown to near confluence on collagen-coated 100-mm culture plates at 37°C and in a 10% CO_2 atmosphere using Dulbecco's modified Eagle medium (DMEM) containing 20% defined/supplemented bovine calf serum (HyClone Laboratories, Logan, UT). Differentiation into myotubes was induced by substituting DMEM containing 2% horse or calf serum for the normal, high-serum medium; this was designated "Day 0." In addition, 10 μM cytosine arabinoside was included in most experiments to minimize the number of unfused proliferating myoblasts (Blau et al., 1983). Green monkey kidney cells (CV-1) were used as controls in some experiments and were obtained from American Type Culture Collection.

RNA Isolation and Analysis

Total RNA was isolated from C_2C_{12} cells by the method of Chomczynski and Sacchi (1987), using the chaotropic reagent, RNAzol B (Cinna/Biotecx Laboratories, Houston, TX). Equivalent amounts of RNA from each culture plate were separated on a 1% agarose gel containing 2% formaldehyde and transferred to nitrocellulose by capillary action (Thomas, 1980; Pressley et al., 1988). In both the gel and the resulting nitrocellulose blot, ethidium bromide staining of ribosomal RNA was used to confirm the amounts of loaded RNA and to assess the efficiency of transfer. The blot was UV-crosslinked, then pre-hybridized and hybridized with DNA probes as described (Allen et al., 1991). In most experiments, isoform-specific probes were prepared by polymerase chain reaction amplification of full-length cDNA using oligonucleotide primers derived from the α isoform nucleotide sequences; in some experiments, full-length cDNAs were used as probes (Shull et al., 1986). A 173-bp α1-specific probe spanned the α1 sequence from the first nucleotide of the codon for Val_{-1}, within the five-amino-acid sequence cleaved from the amino terminal of the mature polypeptide, to the second nucleotide of the codon for Gly_{57}. A 350-bp α2-specific probe spanned the α2 sequence from the second nucleotide of the codon for Tyr_4 to the third nucleotide of the codon for Asn_{120}. The full-length cDNA templates were kindly provided by Dr. Jerry B. Lingrel. The fast-twitch Ca-ATPase probe was a 1165-bp *Eco*RI-*Nar*I restriction endonuclease fragment from the plasmid p19FCa (Karin, Kaprielian & Fambrough, 1989). Each DNA probe was radiolabeled to high specific activity ($\sim 10^8$ cpm/μg) with deoxycytosine 5'-[α-^{32}P]triphosphate (Amersham, Arlington Heights, IL) by random oligonucleotide priming (Feinberg & Vogelstein, 1983). Washing of the blots after hybridization was as described (Allen et al., 1991), except that a third wash series of five 5-min washes with 0.1 X SSC, 0.1% SDS at 55°C was included to ensure high-stringency (1 X SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Preflashed XAR-5 film (Eastman Kodak, Rochester, NY) and a Cronex Lightning Plus intensifying screen (Du Pont, Wilmington, DE) were used for autoradiography at −70°C (typically a 1- to 2-d exposure). The relative intensities of the bands detected by autoradiography were measured using whole band densitometry software running on a SPARCstation IPC (Sun Microsystems, Mountain View, CA) equipped with an image analysis system (Bio Image, Ann Arbor, MI).

Protein Isolation and Analysis

Culture plates were rinsed twice with ice-cold homogenization medium (250 mM sucrose, 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), and 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, pH 7.4 at 25°C), and then the cells were harvested in a small amount of homogenization medium using a rubber policeman. The resulting cell suspension was centrifuged for 20 sec in a microcentrifuge, and the supernatant was discarded. The cell pellet was disrupted by vigorous mixing with 100 μl of ice-cold lysis buffer (140 mM NaCl, 1.5 mM MgCl, 10 mM Tris-HCl, and 0.5% Nonidet P-40, pH 8.6 at 25°C), and the lysed cells were centrifuged as before to pellet the nuclei. An aliquot of the supernatant was retained for determination of protein concentration (Lowry et al., 1951). The remainder was mixed with an equal volume of gel loading buffer (0.125 M Tris-HCl, 5 mM ethylene diaminetetraacetic acid, 6% sodium dodecyl sulfate (SDS), 20% glycerol, and 5% β-mercaptoethanol, pH 6.8 at 25°C), and then heated at 65°C for 5 min. Subsequent electrophoresis of the protein samples through SDS-polyacrylamide gels (7.5%), electroblotting onto nitrocellulose, and probing of the blots with antibodies were performed as described (Laemmli, 1970; Towbin, Staehelin & Gordon, 1979; Pressley, 1992). The quantity of protein in each lane and the efficiency of transfer were confirmed by staining with Ponceau S. For the detection of the α1 and α2 polypeptides, rabbit polyclonal antibodies directed against specific oligopeptide sequences from each isoform were used. Anti-NASE and anti-HERED antibodies recognize isoform-specific regions within α1 and α2, respectively (Pressley, 1992). The antibodies were purified by binding to their target oligopeptides on affinity columns prior to use. A monoclonal IgG specific for mammalian fast-twitch Ca-ATPase, CaF2, was employed for the detection of this polypeptide (Karin et al., 1989). ^{125}I-labeled *Staphylococcus aureus* protein A or donkey anti-rabbit IgA,IgM,IgG (0.2 μCi/ml) were used to detect the isoform-antibody complex. The secondary antibody for fast-twitch Ca-ATPase was ^{125}I-labeled sheep anti-mouse IgA,IgM,IgG (0.2 μCi/ml). The blots were washed as described previously (Pressley, 1992), and the ^{125}I-labeled complexes were visualized by autoradiography using preflashed XAR-5 film (typically an overnight exposure). Relative intensities of the bands were determined by densitometry as described for RNA analysis.

Na,K-ATPase Activity

C_2C_{12} homogenates and crude membranes were analyzed for ATPase activity by measuring the ouabain-inhibitable release of inorganic phosphate. Culture plates of cells were washed twice with ice-cold homogenization medium and then scraped in 1 ml of medium. The resulting cell suspension was frozen and thawed to promote lysis and then homogenized by hand with a Potter-Elvehjem tissue grinder. For preparation of crude membranes, the cell suspension was homogenized with a motor-driven Potter-Elvehjem grinder and then centrifuged at 300 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 30 min at 4°C. The membrane pellet was resuspended in homogenization medium containing 0.4% sodium deoxycholate. Subsequent assay of ATPase activity was performed as described (Haber et al., 1987), except that whole-cell lysates were preincubated for 30 min at 37°C in the presence of various concentrations of ouabain prior to the addition of ATP. In experiments measuring the concentration dependence of ATPase inhibition by ouabain, crude membranes were preincubated for 2 hr at 37°C. The preincubation steps were included to allow the enzyme-inhibitor binding reaction to approach equilibrium. Inorganic phosphate released during ATP hydrolysis was determined by the method of Baginski and Zak (1960), and the estimates of ATPase activity were standardized against the amount of protein (Lowry et al., 1951). Inhibition of

enzyme activity by ouabain was modeled assuming one- or two-ligand binding sites using Enzfitter software (Biosoft, Miltown, NJ).

Cellular Na^+ and K^+ Content

Estimates of intracellular Na^+ and K^+ content were obtained by flame photometry of cell lysates as described (Pressley et al., 1986), except that the cells were scraped in 15 mM LiCl flame photometry solution rather than $LiNO_3$, and the resulting cell suspension was frozen and thawed once to ensure lysis of the cells. Intracellular Na^+ content was standardized against the total amount of intracellular Na^+ and K^+ (Horn & Zierler, 1975).

Statistical Analysis

All data are expressed as means ± SE. Differences between experimental groups and their controls were examined by analysis of variance and were considered significant for $P < 0.05$.

Results

Cultures of C_2C_{12} cells approaching confluence in normal medium contained predominately thin mononucleated myoblasts. After several days in low-serum differentiation medium, the majority of cells consisted of larger multinucleated myotubes (*data not shown*), presumably the result of myoblasts fusing with their neighbors (Blau et al., 1983). To confirm this apparent myogenesis, we examined the expression of fast-twitch Ca-ATPase, a muscle-specific transport protein usually found in the sarcoplasmic reticulum. At various times following the addition of differentiation medium, cells were harvested for RNA and protein analysis. Northern blots of total RNA revealed a single band corresponding to 26 S when probed with a DNA complementary to the mRNA encoding chicken fast-twitch Ca-ATPase ($mRNA_{FCa}$) (Fig. 1*A*). This band was nearly undetectable in myoblasts, and its intensity increased with differentiation. The increment in the relative abundance of $mRNA_{FCa}$ was accompanied by an increase in Ca-ATPase protein content. Immunoblots of total protein, probed with a monoclonal antibody specific for fast-twitch Ca-ATPase, revealed a single band corresponding to 110 kDa that was nearly undetectable in myoblasts, but whose intensity increased dramatically with differentiation into myotubes (Fig. 1*B*). A compilation of three experiments indicates that the abundance of both $mRNA_{FCa}$ and polypeptide increased coordinately by sixfold during differentation from myoblasts to myotubes (*data not shown*).

With the validity of the differentiation process confirmed, we next examined the expression of $\alpha 1$ and $\alpha 2$, the isoforms of the Na,K-ATPase catalytic subunit expressed in C_2C_{12} cells. Northern blots probed with an $\alpha 1$-specific cDNA revealed a single band corresponding to 27 S in total RNA from both myoblasts and myotubes; the relative abundance of $mRNA_{\alpha 1}$ did not change appreciably during differentiation (Fig. 2*A*). Immunoblots of total protein probed with a site-directed polyclonal antibody specific for $\alpha 1$ displayed a major band in both myoblasts and myotubes that corresponded to 100 kDa and whose abundance remained constant (Fig. 2*B*). In contrast to the constitutive expression of $\alpha 1$, the $\alpha 2$ isoform was only detected in the differentiated myotubes. A single band corresponding to 27 S was observed in Northern blots probed with an $\alpha 2$-specific cDNA, but only in RNA isolated from cells undergoing differentiation (Fig. 3*A*). The larger, 32-S form of $mRNA_{\alpha 2}$, a product of polyadenylation at an alternative, downstream site (Shull et al., 1986), was present as a minor component and was visualized when the autoradiogram was overexposed (*data not shown*). After correction for differences in exposure time, as well as probe size and specific activity, the intensity of the major $\alpha 2$-specific band was approximately one-tenth that of $\alpha 1$ in myotubes after 7 days. A site-directed polyclonal antibody specific for $\alpha 2$ revealed a band corresponding to 100 kDa that was present only in immunoblots of protein from differentiated cells (Fig. 3*B*). Two additional bands were detected as well, corresponding to 120 and 190 kDa, but their abundance did not vary with differentiation, and their relationship with $\alpha 2$, if any, remains unclear. A compilation of several experiments revealed no systematic changes in the abundance of $mRNA_{\alpha 1}$ or $\alpha 1$ polypeptide during myogenesis (Fig. 4*A*). Differentiation into myotubes produced a coordinate tenfold increase in the abundance of both $mRNA_{\alpha 2}$ and $\alpha 2$ polypeptide over their nearly undetectable levels in myoblasts (Fig. 4*B*). The third isoform of the catalytic subunit, $\alpha 3$, was undetectable in myoblasts and myotubes using probes specific for $mRNA_{\alpha 3}$ and $\alpha 3$ polypeptide (*data not shown*).

To assess the functional consequences of Na,K-ATPase α isoform expression in C_2C_{12} cells, we took advantage of the differences in ouabain affinity associated with the $\alpha 1$ and $\alpha 2$ isoforms (Fig. 5). Ouabain inhibition of Na,K-ATPase in crude membranes from myoblasts conformed to a single-site model for glycoside binding with an estimated K_I of 1.1×10^{-4} M. The concentration dependence of inhibition in myotubes fit a two-site model, as might be expected if both $\alpha 1$ and $\alpha 2$ isoforms were functional and contributed to Na,K-ATPase activity. The "low"-affinity site had an estimated K_I of 0.7×10^{-4} M that was similar to that of the myoblasts. The "high"-affinity

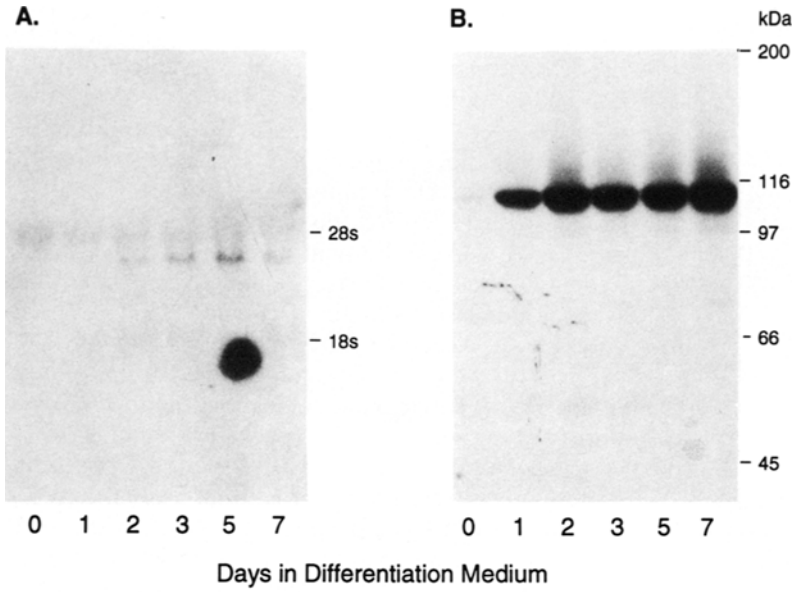

Fig. 1. Expression of fast-twitch Ca-ATPase in differentiating C_2C_{12} muscle cells. RNA and protein were isolated from cultures at various times after addition of differentiation medium. (*A*) Representative Northern blot probed with DNA complementary to $mRNA_{FCa}$; 10 μg of total RNA were loaded per lane. The spot on the lower right part of the autoradiogram was presumably an artifact and was not found in three additional experiments. (*B*) Representative immunoblot probed with a monoclonal antibody specific for fast-twitch Ca-ATPase; 90 μg of total protein were loaded per lane.

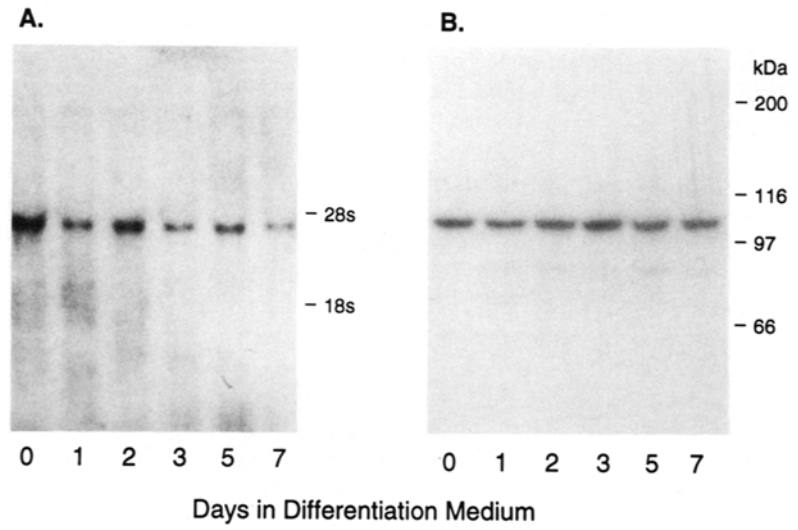

Fig. 2. Expression of the α1 isoform of Na,K-ATPase in differentiating C_2C_{12} muscle cells. Details were as described in Fig. 1, except that probes specific for $mRNA_{\alpha 1}$ and α1 polypeptide were used.

site, presumably resulting from α2 expression, had an estimated K_I of 1.4×10^{-7} M and accounted for only 10% of the total Na,K-ATPase activity.

The contributions of the low- and high-affinity ouabain binding sites to overall enzymatic activity were evaluated in lysates of whole cells (Table 1). From an examination of the concentration dependence data, we selected ouabain concentrations of 1×10^{-6} M and 3×10^{-3} M to partition total Na,K-ATPase activity into high-affinity and low-affinity forms, respectively. Only about 15% of Na,K-ATPase activity in myotube homogenates could be attributed to the high-affinity, ouabain-sensitive α2 form of the enzyme. In contrast, none of the enzymatic activity found in myoblast homogenates could be attributed to the high-affinity form, consistent with the nearly undetectable levels of α2 expression in the undifferentiated state. This agreement between the results obtained in membranes and lysates argues against purification artifacts as an explanation for the small contribution by α2. During myogenesis, α1 expression remained relatively constant and α2 expression was elevated, yet the Na,K-ATPase specific activity did not increase in the myotubes. Indeed, enzyme activity was depressed about 18%. As a control, samples of rat brain microsomes were assayed in parallel and revealed significant amounts of both ouabain-sensitive and -insensitive enzyme activity, demonstrating that the nominal ouabain concentrations were efficacious.

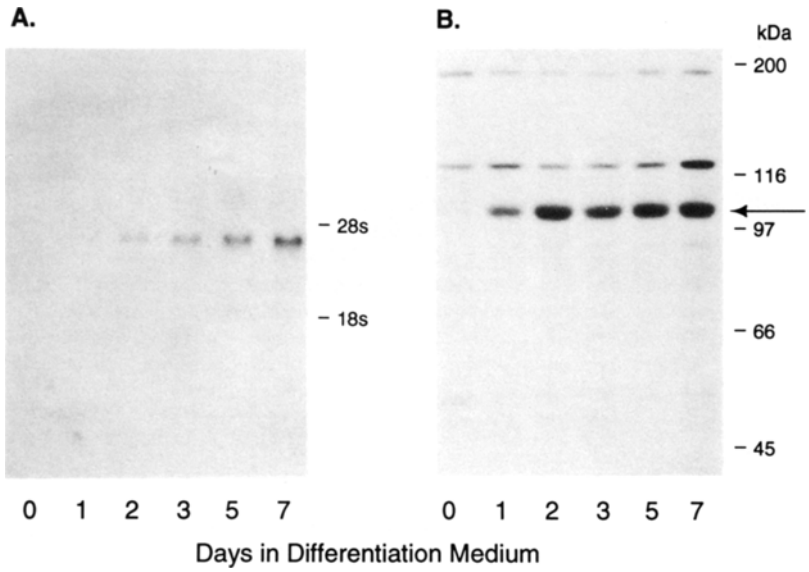

Fig. 3. Expression of the $\alpha 2$ isoform of Na,K-ATPase in differentiating C_2C_{12} muscle cells. Details were as described in Fig. 1, except that probes specific for $mRNA_{\alpha 2}$ and $\alpha 2$ polypeptide were used. The arrow highlights the band with a mobility appropriate for the $\alpha 2$ polypeptide.

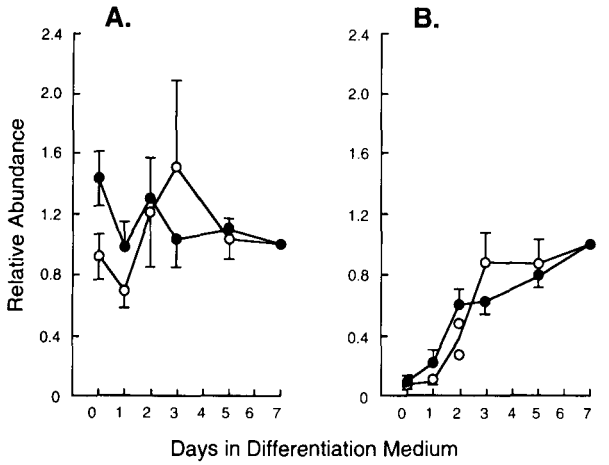

Fig. 4. Time course of Na,K-ATPase α subunit expression in differentiating C_2C_{12} muscle cells. Abundances of mRNA (○) and polypeptide (●) at various times were standardized against their respective abundance at day 7. Data are expressed as mean ± SE, except for $mRNA_{\alpha 2}$ abundance at day 2, where individual values are shown. (*A*) Probes specific for $mRNA_{\alpha 1}$ and $\alpha 1$ polypeptide were used; $n = 5$. (*B*) Probes specific for $mRNA_{\alpha 2}$ ($n = 4$, except for 2 days) and for $\alpha 2$ polypeptide ($n = 5$) were used.

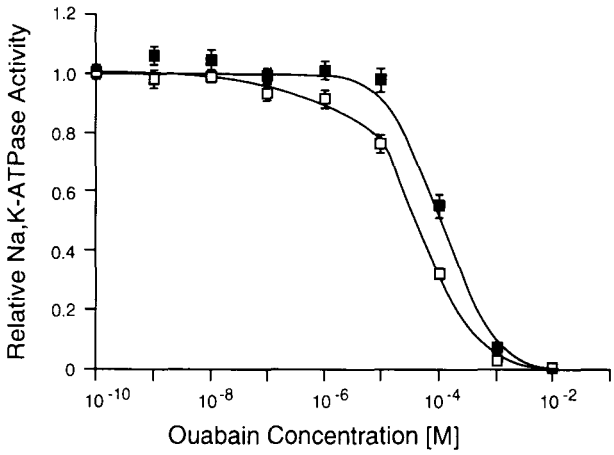

Fig. 5. Ouabain concentration-dependent inhibition of Na,K-ATPase activity in crude membranes of C_2C_{12} myoblasts (■) and myotubes (□). Data are expressed as a fraction of the total Na,K-ATPase activity measured in the presence and absence of 10 mM ouabain (mean ± SE, $n = 4$ and 3 for myoblasts and myotubes, respectively). Curves were generated from a single-site binding model for myoblasts and a two-site model for myotubes.

Despite the dramatic stimulation of $\alpha 2$ expression that accompanied differentiation, the assays of Na,K-ATPase activity suggest that the $\alpha 2$ isoform contributes little to active Na,K transport. An in vitro assay of enzyme activity, however, might misrepresent the situation in the intact cell. For example, a higher fraction of functional $\alpha 2$ isoform could be present in the plasma membrane but remain undetected because of potential problems with the membrane fractionation procedures employed in these assays. If the $\alpha 2$ isoform contributes significantly to the overall transport of Na^+ and K^+, its specific inhibition would be expected to result in a dramatic increase in intracellular Na^+ content. Accordingly, we exposed myotubes to selected concentrations of ouabain for 2 hr and then measured intracellular Na^+ and K^+ content (Table 2). Exposure of myotubes to 1×10^{-6} M ouabain, a concentration calculated from

Table 1. Na,K-ATPase activity in C_2C_{12} muscle cell lysates

	Na,K-ATPase[a]		
	Total	Ouabain-sensitive	Ouabain-insensitive
Myoblasts	00.93 ± 0.03	−0.09 ± 0.04	1.02 ± 0.03
Myotubes	0.76 ± 0.05	0.12 ± 0.03	0.65 ± 0.04[b]
Brain microsomes	22.4 ± 0.9	12.1 ± 0.5	9.6 ± 0.9[b]

Lysates were prepared from cells before (myoblasts) and 7 days after (myotubes) incubation in differentiation medium. Microsomes were prepared from rat brain as a control. Specific activities are expressed as μmol P_i/h/mg protein, mean ± SE, $n = 5$.
[a] Total Na,K-ATPase represents the difference in activities measured in the absence and presence of 3.0×10^{-3} M ouabain and was never less than 60% of total hydrolytic activity. Ouabain-sensitive Na,K-ATPase represents the difference in activities measured in the absence and presence of 1.0×10^{-6} M ouabain. Ouabain-insensitive Na,K-ATPase represents the difference in activities measured in 1.0×10^{-6} M and 3.0×10^{-3} M ouabain.
[b] Denotes $P < 0.05$, as compared to total Na,K-ATPase activity.

Table 2. Effect of ouabain on intracellular Na^+ in C_2C_{12} myotubes

	Control	Ouabain concentration	
		1×10^{-6} M	1×10^{-3} M
Myotubes	0.157 ± 0.008	0.180 ± 0.009[a]	0.696 ± 0.010[b]
CV-1 cells	0.157 ± 0.022	0.686 ± 0.011[b]	0.722 ± 0.009[b]

C_2C_{12} cells after incubation in differentiation medium for 7 days (myotubes) and CV-1 cells were exposed to diluent or ouabain for 2 hr. Intracellular Na^+ content is expressed as a ratio to the sum of intracellular Na^+ and K^+ contents, mean ± SE, $n = 15$ plates for the myotubes and $n = 4$ plates for the CV-1 cells.
[a] Denotes $P < 0.05$, as compared to control.
[b] Denotes $P < 0.001$, as compared to control.

Fig. 5 to inhibit 90% of the α2 form of the enzyme while inhibiting only 1% of the α1 form, produced a barely significant 15% increase in intracellular Na^+ content. Ouabain at 1×10^{-3} M, a concentration calculated to inhibit virtually all of the α2 form and over 90% of the α1, produced a striking, fivefold increase in intracellular Na^+. As a control, green monkey kidney cells (CV-1), which express the ouabain sensitive Na,K-ATPase found in primates, were treated with ouabain in parallel and revealed an elevated intracellular Na^+ content when exposed to 1×10^{-6} M ouabain. Exposure of C_2C_{12} cells to ouabain for periods as long as 48 hr produced results similar to 2-hr exposures (*data not shown*).

Table 3. Effect of ouabain on mRNA abundance in C_2C_{12} myotubes

	Control	Ouabain concentration	
		1×10^{-6} M	1×10^{-4} M
$mRNA_{\alpha 1}$	1.00 ± 0.05	1.02 ± 0.07	1.50 ± 0.10[a]
$mRNA_{\alpha 2}$	1.00 ± 0.10	0.87 ± 0.10	1.48 ± 0.18[b]

C_2C_{12} cells after incubation in differentiation medium for 7 days were exposed to diluent or ouabain for 24 hr. Total RNA was probed as in Figs. 2 and 3. Abundance of $mRNA_{\alpha 1}$ and $mRNA_{\alpha 2}$ is expressed relative to controls, mean ± SE, $n = 12$.
[a] Denotes $P < 0.001$, as compared to control.
[b] Denotes $P < 0.05$, as compared to control.

Prolonged inhibition of the Na-K-pump by ouabain in a variety of systems leads to increased cell Na,K-ATPase content, and the response is associated with an increased abundance of the mRNAs encoding the subunits of the enzyme (Pressley, 1988; Lingrel et al., 1990). As a further measure of the contribution of α2 to ionic homeostasis in C_2C_{12} myotubes, we determined the effect of prolonged exposure to ouabain on the abundance of $mRNA_{\alpha 1}$ and $mRNA_{\alpha 2}$ (Table 3). Consistent with its minimal effect on intracellular Na^+, exposure for 24 hr to 1×10^{-6} M ouabain produced no significant change in the abundance of the mRNAs encoding α1 or α2. That the C_2C_{12} cells were capable of responding to a ouabain challenge was supported by the 50% increase in the abundance of both isoform mRNAs when the concentration of inhibitor was raised to 1×10^{-4} M.

Discussion

The abundance of functional Na,K-pumps in the plasma membrane is controlled by the permeability of the membrane to Na^+ and K^+ and by a variety of hormonal and developmental factors (for a review, *see* Lingrel et al., 1990). Changes in the transcriptional rate of the genes encoding the various isoforms probably accounts for much of this regulation, but discrepancies between observed changes in the subunit mRNAs and Na,K-ATPase abundance suggest that post-transcriptional and post-translational regulatory mechanisms may be important. For example, differentiation of cultured fibroblasts into adipocytes is accompanied by induction of the α2 subunit, but there is no apparent coordination among the changes in mRNA abundance, polypeptide abundance, and enzyme activity (Russo et al., 1990). Thyroid hormone stimulation of cardiac myocytes also results in discordant changes in Na,K-ATPase mRNA and enzyme content (Hensley et al., 1992).

The present study takes advantage of α-isoform-specific antibodies to study regulation of Na,K-ATPase expression during myogenesis of C_2C_{12} cells.

Consistent with the earlier results of Orlowski and Lingrel (1988), we found that differentiation of C_2C_{12} myoblasts into myotubes was accompanied by an increased abundance of $mRNA_{\alpha 2}$. The antibody binding data of the present study extends this earlier work to show that the accumulation of α2 polypeptide followed closely the changes in $mRNA_{\alpha 2}$ content. In addition, the tenfold increase in $mRNA_{\alpha 2}$ was nearly equal to the increment in α2 polypeptide abundance. For these reasons, the biosynthesis and expression of the α2 isoform during C_2C_{12} differentiation appears to be coordinate, and the post-transcriptional or post-translational regulatory mechanisms observed in other systems do not seem to have important roles. Coordinate regulation was also found for the fast-twitch Ca-ATPase, which increased sixfold during myogenesis. In contrast, the α1 polypeptide and its corresponding mRNA remained relatively constant throughout differentiation.

Despite its dramatic increase in myotubes, the α2 isoform did not make a significant contribution to Na^+ and K^+ transport. This conclusion is supported by three experimental results. First, only about 15% of Na,K-ATPase specific activity in myotubes could be attributed to the ouabain-sensitive form associated with α2. This was somewhat less than the 30% estimated by Orlowski and Lingrel (1988) and may reflect differences in technique or cell phenotype. Assuming similar catalytic turnovers for the two forms of the pump (Haber & Loeb, 1988), the small contribution to enzymatic activity in our experiments implies that α2 was a relatively small proportion of the pump population, even after increasing tenfold upon differentiation. Second, incubation of myotubes in the presence of ouabain at a concentration sufficient to inhibit α2-containing Na,K-pumps raised intracellular Na^+ content by no more than 15%. The increase in intracellular Na^+ was likely minimized by the sensitivity of the predominant α1-containing form of the enzyme to subtle changes in Na^+ concentration. On the basis of transport measurements, the binding of intracellular Na^+ by the Na,K-pump appears highly cooperative, such that small elevations in Na^+ concentration produce substantial increases in transport (Haber et al., 1987). Finally, 24-hr exposure of myotubes to an α2-inhibiting concentration of ouabain did not result in upregulation of the mRNAs encoding either α1 or α2. An increase in mRNA abundance would be expected if the ouabain caused significant inhibition of Na,K transport, leading to an elevated intracellular Na^+ concentration (Pressley, 1988). These results place a lower threshold on that regulatory response, because a 15% increase in intracellular Na^+ failed to alter mRNA levels, while a larger increase clearly produced upregulation.

How are we to reconcile the relatively insignificant Na^+ and K^+ transport contribution of α2 with its dramatic increase in abundance during myogenesis? One explanation may be that the α2 isoform does, in fact, contribute significantly to transport, but only under certain physiological conditions. The low-serum medium used to initiate myotube formation may deplete the cells of hormones and factors that could differentially stimulate the α2 form of the enzyme. In the resulting basal state, the functional importance of α2 expression may be minimized. Indeed, the preferential stimulation of α2 produced by insulin in a number of experimental models lends credibility to this explanation (Lytton, 1985; Brodsky, 1990; Hundal et al., 1992). Alternatively, the ion-translocating capability of the α2 form of the pump may be of less importance in myogenic cells. For example, long-term culture of C_2C_{12} cells may not favor the continued expression of α2 at levels comparable to intact skeletal muscle. On the other hand, the α2 isoform may function as a muscle-specific receptor or recognition signal—a role independent of ion transport. Such a dual role has already been postulated for the β2 isoform of the pump, which appears to mediate neuron-astrocyte interactions (Gloor et al., 1990). Additional experiments with C_2C_{12} cells, as well as other culture models of myogenesis, will be necessary to distinguish between these alternatives.

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