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Hypokalemia downregulates cardiac α1 and skeletal muscle α2 isoforms of Na^+,K^+-ATPase in ferrets

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K^+ deficiency decreases amount of Na^+,K^+-ATPase in cardiac and skeletal muscle (SM) and differentially regulates α- and β-subunit isoforms. Expression of the α3 isoform during K^+ deficiency is unknown, however. In the myocardium of normal adult ferrets, α1 and α3 isoforms are expressed. Therefore, we examined in ferrets the effects of K^+ deficiency in levels of α- and β-subunit isoforms. A K^+ deficient diet greatly reduced plasma K^+ concentrations. Tissue K^+ content in myocardium remained unchanged, whereas that of SM decreased markedly. Corresponding to these changes, Na^+,K^+-ATPase enzyme activity decreased in SM but not in heart. Levels of α1 in heart and α2 in SM decreased, whereas those of α3 in heart and α1 in SM remained unchanged; β1 remained unchanged in both tissues. Thus, in ferrets, K^+ deficiency differentially regulated the levels of Na^+,K^+-ATPase isoforms in an isoform- and tissue-dependent manner.

Na^+,K^+-ATPase is an integral membrane protein that maintains the electrochemical gradient across the plasma membrane by regulating the influx of K^+ and efflux of Na^+. The enzyme consists of an α- (MW=110,000) and a β-subunit (MW=35,000). Three isoforms of the α-subunit (α1, α2, and α3), as well as the β-subunit (β1, β2, and β3), have been cloned and sequenced (1-3). Although precise functions of these isoforms remain unclear, expression of the subunit isoforms are species- and tissue-dependent (4,5).

Expression of the isoforms is differentially regulated under various physiological and pathological conditions (6-8). Changes in extracellular K^+ concentration regulate the number of functional Na pumps. K^+ deficiency decreased ^{3}H-ouabain binding sites in rat heart (9) and skeletal muscle (10), and the decrease corresponded to reduced Na^+,K^+-ATPase activity (9,11). Recent reports (8) further demonstrated that the effect of hypokalemia is isoform specific;

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expression of α2 protein in rat heart, skeletal muscle, and brain decreased, whereas that of α1 was unchanged.

In human heart, all three α-subunit mRNA are expressed (12,13). In rat heart, by contrast, only α1 and α2 isoforms are expressed in adult cardiac muscle cells (4), although a small amount of α3 has been detected in the conduction system (14). Due to lack of an appropriate animal model, expression of α3 isoform during K^+ deficiency is unknown. In ferrets, we recently demonstrated that α1 and α3 isoforms are expressed in myocardium (15). Therefore, ferrets provide a unique opportunity to examine regulation of the α3 isoform in the myocardium. The present study was undertaken to investigate effects of K^+ deficiency on expression of the α- and β-subunit isoforms in ferret heart and skeletal muscle.

MATERIALS AND METHODS

Hypokalemic ferrets- Male ferrets (about 1 Kg) were fed either a low K^+ diet (ICN Biomedicals Inc., Costa Mesa, CA., Cat. No. 960228) or regular cat chow (Purina Mills Inc., St. Louis, MO) for 20 days before being sacrificed. Plasma were collected by cardiac puncture at the time of death. Heart, mixed hindlimb and temporalis skeletal muscles were quickly removed, a small specimen from each tissue (about 200 mg) was dissected and weighed. The rest of the tissues was quickly frozen in liquid nitrogen. To determine tissue K^+ content, a modified method of Kjeldsen et al. (10) was used. Dissected samples were homogenized immediately in a 5% trichloroacetic acid (TCA) solution (4 ml) using a Polytron (Brinkmann, Westbury, NY) at a setting of 6.5 for 1 min. Precipitated protein was removed by centrifugation at 2,000 x g for 10 min, and the resultant supernatant was used for K^+ measurement. Potassium levels were assayed using a Instrumentation Laboratory Model 443 flame photometer (Lexington, MA). Briefly, K^+ standards were prepared in a 0.025% TCA/15 mM LiCl mixture. TCA extracts of tissues were diluted 200 fold in 15 mM LiCl just prior to flame photometry. For plasma K^+ determination, plasma was diluted in 15 mM LiCl.

During the course of study, a ferret in the control group died of unknown causes. A ferret treated under identical conditions as the other controls was later added to the control group. K^+ measurement was not performed in this ferret.

SDS-Gel electrophoresis and Western blot- In order to minimize potential selective enrichment of the isoforms during membrane purification, crude tissue homogenates were used in most experiments. Left ventricular cardiac muscle, and temporalis skeletal muscle were minced and homogenized in a 50 mM Tris-HCl buffer (pH 7.4 at 4°C) in the presence of 0.5 mM phenylmethyl-sulfonyl fluoride (PMSF) using a Polytron (Brinkmann, Westbury, NY), filtered through a 250 μm nylon mesh screen, and frozen at -40°C until use. For determination of β-subunit isoform in skeletal muscle, because of presence of high levels of non-specific bands in crude homogenates, partially purified crude membrane preparations were used (16). Briefly, tissues were minced and homogenized in a 50 mM Tris-HCl buffer (pH 7.4 at 4°C) in the presence of 0.5 mM PMSF using a Polytron. Homogenates were centrifuged at $3,000xg_{max}$ for 20 min, and the resultant supernatant fractions were centrifuged at $65,000xg_{max}$ for 35 min. The pellets were resuspended in 10 mM Tris-HCl buffer with 0.5 mM EDTA, and frozen at -40°C until use.

Slab gel electrophoresis was performed according to the method of Laemmli (17) with slight modification as previously described (18). Alpha- and β-subunits were separated on 5% and 7.5% SDS-gels, respectively. Gels were transferred onto Immobilon PVDF membrane (Millipore, Bedford, MA), blocked with Blotto-Tween (when α1, α2, and β1 specific antibodies

were used) or with Tween-20 (0.2%) in Tris (10 mM, pH7.4) (when αN was used), and incubated with various primary antibodies for 1.5 hr. For detection of β1, crude homogenates (cardiac tissue) or crude membrane preparations (skeletal muscle) were first treated with N-glycosidase F (Boehringer Mannheim, Indianapolis, IN) as previously described (16). α1, α2, and β1 isoform-specific antisera were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). αN, which was produced in our laboratory, is an antipeptide antibody which reacts equally well with ferret α1 and α3 isoforms (15). Bound antibodies were detected by ^{125}I-labeled goat anti-rabbit IgG. The blots were exposed to multiple X-ray films to ensure that signals were within the linear range of the film. In several experiments, duplicate blots were analyzed.

Enzyme activity- K^+-dependent nitrophenylphosphatase activity (K^+-pNPPase) was measured according to the method described by Bers (19) with slight modifications (16). Left ventricular tissues (about 100 mg) were minced and homogenized in 2.5 ml of a 10 mM Tris/1 mM EDTA buffer using a Potter type homogenizer with a motor driven Teflon pestle. The homogenate was filtered through a 250 μm nylon screen mesh, and incubated with 0.03% SDS for 25 min at room temperature. The mixture was diluted 8-fold with cold 10 mM Tris/1 mM EDTA buffer. Skeletal muscle homogenates were not treated with SDS because repeated attempts showed that SDS could only decrease, rather than increase, enzyme activity (data not shown). The reaction mixture (0.9 ml) contained 20 mM KCl or NaCl, 5 mM $MgCl_2$, 1.0 mM EDTA, 50 mM Tris-HCl buffer (pH 7.4), and appropriate amounts of tissue homogenates (0.05 to 0.5 mg). After a 5-min incubation at 37°C for temperature equilibration, the reaction was started by the addition of 0.1 ml p-nitrophenyl phosphate (final concentration 5 mM). The reaction was terminated 10-25 min later by adding 0.1 ml 50% trichloroacetic acid and cooling on ice. After the samples were centrifuged to remove precipitated protein, two ml of 0.5 M Tris were added to 0.9 ml of the supernatant. Absorbance was recorded at 410 nm, and K^+-pNPPase activity was calculated as the difference in values observed in the presence and absence of K^+.

RESULTS

Hypokalemic ferrets- Ferrets fed a K^+ deficient diet lost on average 143 g of their body weight during the 20 day treatment period, whereas control ferrets gained on average 184 g (Table 1). Ferrets in the experimental group became hypokalemic, as evident by a significant reduction in plasma K^+ concentrations compared to control ferrets (Table 1). Potassium content of temporalis and hindlimb skeletal muscles decreased significantly to 78.6% and 71.2% of controls, respectively. By contrast, K^+ content in cardiac muscle remained unchanged.

Effects of hypokalemia on expression of subunit isoforms and enzyme activity in heart- We previously showed that ferret heart expresses α1 and α3 isoforms (15). Immunoblotting of cardiac muscle homogenate using isoform-specific antibodies showed that hypokalemia significantly decreased levels of α1 to 73.7% of controls (Fig.1; C=10.14±0.54; K=7.47±0.43; $p<0.01$). By contrast, the level of α3 (C=6.25±0.68; K=5.67±0.32) and β1 (C=7.77±1.57; K=8.69±1.56) remained unchanged.

Despite the decreased level of α1 in hypokalemic ferret heart, K^+-stimulated pNPPase, an estimation of Na^+,K^+-ATPase activity, was unchanged (C=310.3±14.1; K=304.5±5.5 nmol/mg prot/25 min).

TABLE 1

	Body Weight (g)		Plasma K (mM)	Tissue Potassium Content (μmol/g wet wt)		
	Initial	Final		Heart	TS	HLS
CONTROL	1068±47(4)	1252±70(4)	6.19±0.33(3)	23.53±0.50(3)	26.43±0.72(3)	26.63±0.12(3)
TREATED	1043±29(4)	900±21(4)[a]	2.70±0.13(4)[b]	23.95±0.50(4)	20.78±0.21(4)[a]	18.95±0.56(4)[a]

TS: Temporalis skeletal muscle; HLS: hindlimb skeletal muscle.

a Significantly different from controls, $p<0.005$.
b Significantly different from controls, $p<0.0005$.

Numbers in parentheses indicate number of subjects.

Effects of hypokalemia on expression of subunit isoforms and enzyme activity in skeletal muscle- Ferret skeletal muscle expresses predominately α1 and α2 isoforms; α3 is undetectable (15). Hypokalemia markedly reduced the level of α2 to 49.2% of controls (Fig.2; C=8.19±0.24; K=4.03±0.53; $p<0.005$). By contrast, the level of α1 isoform (C=4.55±1.12; K=4.77±0.34) and β1 (C=3.33±0.58; K=2.79±0.66) remained unchanged.

In skeletal muscle, corresponding to the decrease in the level of α2 protein, K^+-stimulated pNPPase activity decreased to 73.4% of controls (C=21.8±1.8 nmol/mg prot/15 min; K=16.0±1.6 nmol/mg prot/15 min; $p<0.05$).

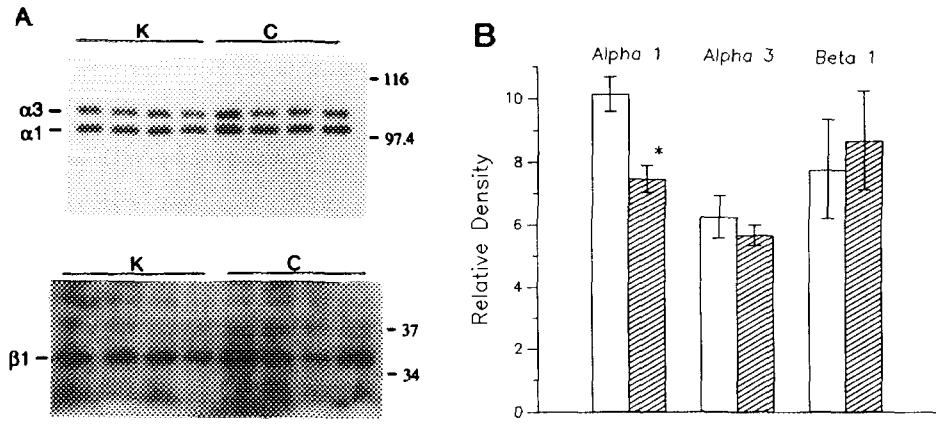

Fig. 1. Relative abundances of Na^+,K^+-ATPase subunit isoforms in cardiac muscle of hypokalemic ferrets. Crude homogenate preparations (50 μg/lane) of cardiac muscle from control (C; n=4) and hypokalemic (K; n=4) ferrets were subjected to SDS-gel electrophoresis in a 5% gel. Relative abundances of α1 and α3 were quantitated by immunoblotting using αN antibody and ^{125}I-anti-IgG as primary and secondary antibodies, respectively. Some samples were analyzed twice by two independent Western blots. For detection of β1, crude homogenates were first treated with N-glycosidase F as described in Methods, subjected to SDS-gel electrophoresis in a 7.5% gel (40 μg/lane), and immunoblotted with β1 specific antiserum. A: Typical autoradiograms; B: Relative densities of the bands (open bar: control; hatched bar: hypokalemic ferrets). Molecular weight markers are shown on the right. (*$p<0.01$ vs. control ferrets).

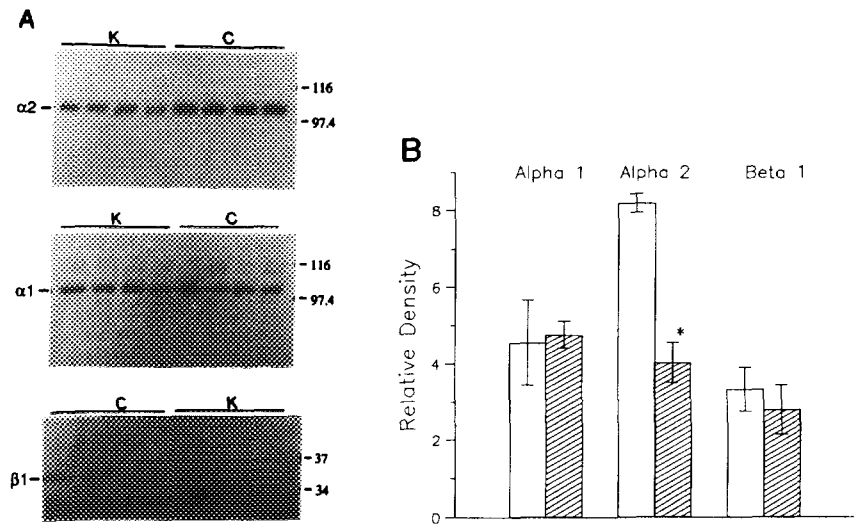

Fig. 2. Relative abundances of Na^+,K^+-ATPase subunit isoforms in skeletal muscle of hypokalemic ferrets. For detection of α-isoforms, crude homogenate preparations (50μg/lane) of skeletal muscle from control (C; n=4), hypokalemic (K; n=4) ferrets were subjected to SDS-gel electrophoresis in a 5% gel and immunoblotted with α1- and α2-specific antibodies. For detection of β1 subunit, crude membrane preparations were first treated with N-glycosidase F as described in Methods, subjected to SDS-gel electrophoresis in a 7.5% gel (40 μg/lane) and immunoblotted with β1 specific antiserum. For quantitation of α1 and α2, samples were analyzed twice in two independent immunoblots. A: Typical autoradiograms; B: Relative densities of the bands (open bar: control; hatched bar: hypokalemic ferrets). Molecular weight markers are shown on the right. (*$p<0.005$ vs. control ferrets).

DISCUSSION

The significant observation in the present study is that in ferrets K^+ deficiency elicited differential regulation of the Na^+,K^+-ATPase isoforms in a tissue-dependent manner: the level of α1 isoform in myocardium was downregulated, whereas that of α1 in skeletal muscle was unaltered. By contrast, the level of α3 in myocardium was unaltered, whereas that of α2 in skeletal muscle markedly decreased. Thus, in both tissues expression of only one of the two constituitive α-isoforms was altered.

In myocardium of hypokalemic ferret, enzyme activity was unaltered, despite decreased α1 level. The apparent disparity may be attributable to the unchanged level of β1, which could be a rate limiting factor in assembly of functional enzyme units (20). Although overall enzyme activity did not change, a decrease in the α1 level without changes in the α3 level suggests an increased ratio of α3/β1 to α1/β1. Because α3 exhibits a different affinity for Na^+ than the α1- and α2-isoforms (21,22), such a change may influence Na^+ and/or K^+ handling by the myocardial cell. In addition, because α3 and α2 isoforms have higher affinity for cardiac glycosides than the α1 isoform, changes in ratios of the isoforms may also influence sensitivity of the Na^+ pump for the cardiac glycosides (6) or endogenous digitalis-like factors (23). By contrast, in skeletal

muscle, decreased α2 was accompanied by a reduction in enzyme activity. We postulate that in skeletal muscle during hypokalemia, levels of α-subunit may be overriding in the assembly of functional enzyme units. Alternatively, the marked decrease in α2 may cause a more easily detectable decrease in enzyme activity.

In hypokalemic ferrets, paralleling the changes in Na^+,K^+-ATPase enzyme activity, tissue K^+ content decreased in skeletal muscle but remained unchanged in myocardium. Thus, decreased plasma K^+ concentration as a result of reduced K^+ intake may be minimized by a reduction in K^+ uptake into skeletal muscle, the largest K^+ pool in the body. By contrast, a constant level of K^+ in cardiac muscle is probably crucial, considering the importance of K^+ ion in the electrophysiological activities of the heart. In rats, however, K^+ contents of both cardiac and skeletal muscle have been shown to be reduced, although with a much larger decrease in skeletal muscle than in cardiac muscle (8). These decreases coincide with decreased enzyme activities both in rat cardiac and skeletal muscle. It remains to be determined whether in human heart, in which all three α-isoforms are expressed, K^+ content in myocardial cells is also protected from changes associated with variations in extracellular K^+ concentrations.

We previously demonstrated that expression of α3 isoform in myocardium of adult ferret was insensitive to thyroid hormone regulation (6) and to pressure-overload hypertrophy (24). Thus, our results appear to indicate that expression of α3 in ferret heart is much less sensitive to physiological and pathological stimuli. By contrast, the α2 isoform in rat heart appears to be very sensitive to such conditions (7,8,25). It may be speculated that in human heart differential expression of the isoforms may serve to maintain intracellular Na^+/K^+ homeostasis under various physiological and pathological conditions.

Molecular mechanisms underlying isoform- and tissue-specific regulation of the Na^+,K^+-ATPase isoforms remain to be investigated. Azuma et al. (8) reported that downregulation of the isoforms in cardiac and skeletal muscle at the protein level was accompanied by a lesser reduction in mRNA levels. By contrast, Hsu and Guidotti (26) showed that hypokalemia decreased the level of α2 protein in rat skeletal muscle, despite increased α2 mRNA level. Although reasons for this apparent discrepancy remain unclear, these results suggest involvement of transcriptional as well as post-transcriptional modification of the isoforms.

In conclusion, hypokalemia differentially regulates levels of Na^+,K^+-ATPase isoforms in cardiac and skeletal muscle. Potassium content in skeletal muscle was decreased, whereas that of cardiac muscle remained unchanged. Cellular signals directly responsible for the isoform-specific regulation remain to be investigated.

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