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Na^+,K^+-ATPase trafficking in skeletal muscle: insulin stimulates translocation of both α_1- and α_2-subunit isoforms

Lubna Al-Khalili, Mei Yu, Alexander V. Chibalin*

Section of Clinical Physiology, Department of Surgical Sciences, Integrative Physiology, Karolinska Institutet, von Eulers väg 4, 2 tr, SE-171 77 Stockholm, Sweden

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Abstract We determined insulin-stimulated Na^+,K^+-ATPase isoform-specific translocation to the skeletal muscle plasma membrane. When rat muscle plasma membrane fractions were isolated by discontinuous sucrose gradients, insulin-stimulated translocation of α_2- but not α_1-subunits was detected. However, using cell surface biotinylation techniques, an insulin-induced membrane translocation of both α_1 and α_2-subunits in rat *epitrochlearis* muscle and cultured human skeletal muscle cells was noted. Na^+,K^+-ATPase α-subunit translocation was abolished by the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin, as well as by the protein kinase C inhibitor GF109203X. Thus, insulin mediates Na^+,K^+-ATPase α_1- and α_2-subunit translocation to the skeletal muscle plasma membrane via a PI 3-kinase-dependent mechanism.

Key words: Na-pump; Sodium pump; Insulin; Rat epitrochlearis muscle; Human skeletal muscle cells; Phosphatidylinositol 3-kinase

1. Introduction

Na^+,K^+-ATPase is a plasma membrane (PM) pump essential for maintenance of intracellular and extracellular sodium and potassium concentrations, cell volume and electrochemical gradients [1,2]. Skeletal muscle contains one of the largest pools of Na^+,K^+-ATPase [3] and expresses α- (α_1 and α_2) and β- (β_1 and β_2) subunits [4,5]. Insulin regulates K^+ uptake through stimulation of Na^+,K^+-ATPase, which is not secondary to an increase in $[Na^+]_i$ via Na^+–H^+ antiporter stimulation [6]. Insulin-stimulated Na-pump activity occurs via a protein kinase C (PKC)-dependent pathway [7,8], and increases the number of active Na^+,K^+-ATPase molecules at the cell surface [5,9]. In rat skeletal muscle membrane fractions isolated by differential centrifugation and discontinuous sucrose gradients, insulin promotes α_2- and β_1-subunit translocation to the PM, with no change in α_1- and β_2-distribution [8,9]. A common explanation for this observation is that the ubiquitously expressed Na^+,K^+-ATPase α_1-subunit isoform has a 'housekeeping' function, whereas the α_2-subunit isoform is hormone-sensitive and primarily responsible for effects of insulin on Na^+,K^+-ATPase activity. In skeletal muscle, Na^+,K^+-ATPase α-subunits are phosphorylated on serine/threonine and tyrosine residues in response to insulin [8]. However, the signaling pathways, and nature of the protein kinases involved in insulin-mediated Na^+,K^+-ATPase regulation are unknown.

We determined insulin-stimulated Na^+,K^+-ATPase α-subunit membrane trafficking in rat skeletal muscle by utilizing different cell surface protein isolation techniques. Furthermore we compared species-specific responses for Na-pump trafficking using rat skeletal muscle and differentiated primary human skeletal muscle cells (HSMC). Using cell-surface biotinylation technique and protein kinase inhibitors, we identify a signal transduction pathway involved in insulin-stimulated Na^+,K^+-ATPase α-subunit translocation in skeletal muscle.

2. Materials and methods

2.1. Materials

Cell culture supplies were obtained from Gibco BRL (Life-Technologies, Sweden). Human insulin (Actrapid) was from Novo Nordisk (Copenhagen, Denmark). Wortmannin and GF109203X were from Calbiochem (La Jolla, CA, USA). Streptavidin-agarose beads and EZ-link Sulfo-NHS-SS-biotin were from Pierce, (Rockford, IL, USA). All other reagents were analytical grade (Sigma, St. Louis, MO, USA). Specific anti-α_1-subunit monoclonal [10] and anti-α_2-subunit monoclonal [11] antibodies were obtained from Drs. M. Caplan (Yale University, CT, USA) and K. Sweadner (Massachusetts Central Hospital, MA, USA). Stockholm's North Ethical Committee approved the animal experiments. The Ethical Committee of Huddinge University Hospital approved the human experiments.

2.2. Subcellular fractionation of rat skeletal muscle membranes

Overnight-fasted male Wistar rats (120–130 g) were anesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbital (5 mg/100 g body weight). Animals were injected with either insulin (0.75 U of insulin/100 g body weight, i.p.) or an equal volume of saline (control group) 30 min prior to being sacrificed. Hindlimb muscles were removed and frozen in liquid nitrogen. Cell surface and intracellular membrane (IM) fractions were isolated from ~350 mg of gastrocnemius muscle by differential centrifugation and discontinuous sucrose gradients [8,9]. After centrifugation of the discontinuous sucrose gradients three protein fractions were collected: fraction 25 (on top of the 25% layer), fraction 30 (from the interphase 25–30%), and fraction 35 (from the interphase 30–35%). Protein concentration was determined using the BCA assay (Pierce, Rockford, IL, USA). Fraction 25 corresponds to a cell surface membrane fraction enriched with PM, whereas fraction 35 corresponds to IMs [9,12,13]. Protein recovery yield (calculated as percent of protein content in crude membranes in five membrane preparations) was 3.4 ± 0.3% for PM and 9.8 ± 0.8% for IM fraction, respectively. Protein recovery was not affected by insulin.

*Corresponding author. Fax: +46 (8) 335436.
E-mail address: alexander.chibalin@kirurgi.ki.se (A.V. Chibalin).

Abbreviations: FBS, fetal bovine serum; HSMC, human skeletal muscle cells; PI, phosphatidylinositol; PKC, protein kinase C

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2.3. Epitrochlearis muscle incubation

Overnight-fasted rats were anesthetized with sodium pentobarbital (5 mg/100 g body weight, i.p.). Epitrochlearis muscles were incubated without or with 120 nM insulin in the absence or presence of kinase inhibitors [10]. Muscles were washed three times with ice-cold phosphate-buffered saline (PBS), placed in 12-well cell culture plates on ice and incubated with biotinylated reagent described below.

2.4. Cell culture, differentiation and incubation

Skeletal muscle biopsies were obtained with the informed consent of healthy donors during scheduled abdominal surgery. Satellite cells were isolated and cultures were established as described for human fetal skeletal muscle [14]. At confluence (>80%) cells were trypsinized and subcultured. To initiate differentiation, Hams-F10/20% FBS medium was replaced with DMEM containing 4% FBS for 48 h, and thereafter, with DMEM containing 2% FBS. Fusion and multinucleation of the cells was observed 3 days after initiation of differentiation protocol. Myotubes were used 7 days after differentiation. Studies were performed on the second or third passage.

Cells were serum starved overnight and then incubated in the absence or presence of 120 nM insulin (20 min at 37°C). When pharmacological inhibitors were used, 20 min incubation was introduced to pre-expose cells to the inhibitor. Inhibitors remained present for the duration of the experiment. After incubation, cells were washed three times with ice-cold PBS and incubated with biotinylated reagent described below.

2.5. Cell surface biotinylation

Epitrochlearis muscles or differentiated HSMC were exposed to EZ-link Sulfo-NHS-SS-biotin at final concentration 1.5 mg/ml in PBS at 4°C for 60 min with gentle shaking. Thereafter, medium was aspirated and un-reacted Sulfo-NHS-SS-biotin was quenched with PBS containing 100 mM of glycine. Isolated skeletal muscle or cultured cells were washed three times with ice-cold PBS, and excess PBS was aspirated. Before solubilization, epitrochlearis muscles were frozen and pulverized in liquid nitrogen. Samples were solubilized in 1 ml of ice-cold lysis buffer containing 20 mM Tris, pH 8.0, 135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM Na_3VO_4, 1 μM okadaic acid, 1% Triton X-100, 10% v/v glycerol, 0.2 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, leupeptin and pepstatin. Cell lysates were then transferred to microcentrifuge tubes and rotated for 60 min at 4°C. Insoluble material was removed by centrifugation (12 000×*g* for 10 min at 4°C). Protein was determined using a BCA assay (Pierce, Rockford, IL, USA). Supernatants (1 mg of protein for muscle lysate and 250 μg of protein for cell lysate, respectively) were collected and mixed with 50 μl streptavidin-agarose beads (50% suspension in PBS, Pierce, Rockford, IL, USA) to form a streptavidin–biotin complex. Samples were incubated overnight at 4°C. Beads were washed four times with PBS containing 0.1% Triton X-100, and twice with PBS. Laemmeli buffer (60 μl) was added to each sample, and heated for 56°C for 20 min. Samples were subjected to SDS–PAGE.

2.6. Western blot analysis

Aliquots of plasma or IMs (10 μg of protein) or bead pellets after precipitation of biotinylated proteins were mixed with Laemmli-sample buffer, and proteins were separated on 7.5% SDS–PAGE. Proteins were transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, MA, USA) and subjected to Western blot analysis as described [8]. After incubation with primary antibody, membranes were washed and incubated with secondary antibody linked to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA). Na^+,K^+-ATPase α-subunit isoforms were visualized with enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). Multiple film exposures were used to ensure that Na^+,K^+-ATPase α-subunits detected in linear range of protein bands saturation. Results were quantified by densitometry using Gel Doc 1000 imaging system with Molecular Analyst software, version 1.5 (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Statistics

Comparisons between groups were performed using Student's *t*-test. For multiple comparisons, one-way ANOVA with Sheffe's correction was used. Significance was established at $P<0.05$.

3. Results

3.1. Subcellular fractionation

In rat hindlimb skeletal muscle membranes, insulin increased Na^+,K^+-ATPase α_2-subunit abundance in the PM fraction 41% ($P<0.01$, $n=5$), with a parallel 36% decrease ($P<0.05$, $n=6$) in α_2-subunit abundance in the IM fraction (Fig. 1C,D). α_1-Subunit expression in the PM fraction was not altered, whereas, insulin reduced (22%; $P<0.05$, $n=8$), α_1-subunit content in the IM fraction (Fig. 1B).

3.2. Cell surface biotinylation

Insulin markedly increased cell surface Na^+,K^+-ATPase content in isolated rat epitrochlearis muscle (Fig. 2A,B). In contrast to results obtained using subcellular fractionation methods, insulin induced translocation of α_1- and α_2-subunits to the cell surface (as a percentage of basal: 51% increase for

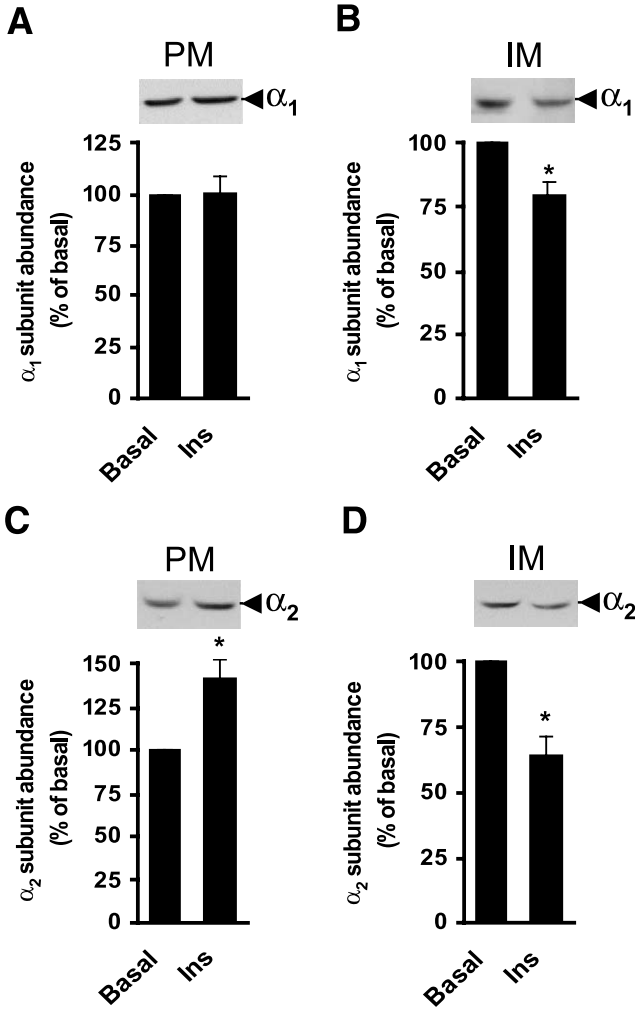

Fig. 1. Subcellular fractionation of rat skeletal muscle membranes. Rats were injected with saline (Basal) or insulin (Ins). Membranes from mixed skeletal muscle were isolated by differential centrifugation and discontinuous sucrose gradient. Protein from cell surface PMs and IMs fractions were resolved by SDS–PAGE and distribution of α_1- (A,B) and α_2-subunits (C,D) of Na^+,K^+-ATPase was determined by Western blot using specific monoclonal antibodies. Representative immunoblots (upper panel) and quantitative data from 5–8 experiments (mean ± S.E.M.) (lower panel) are shown. *$P<0.05$ vs. basal.

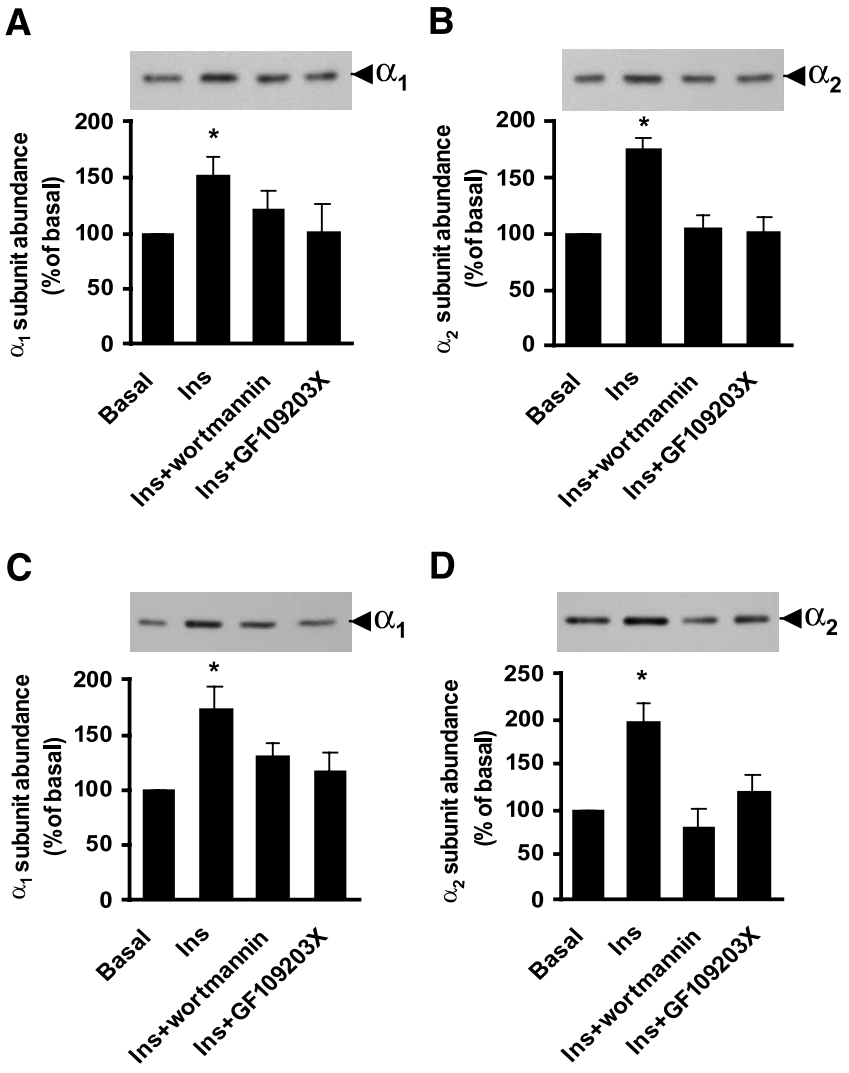

Fig. 2. Translocation of Na^+,K^+-ATPase α-subunit to the cell surface in absence or presence of insulin and PI 3-kinase or PKC inhibitors. Rat epitrochlearis muscle (A,B) and human differentiated myotubules (C,D) were incubated without or with 120 nM insulin and in the absence or presence of either 100 nM wortmannin, or 10 μM GF109203X. Cell surface Na^+,K^+-ATPase abundance was determined by biotinylation with EZ-link Sulfo-NHS-SS-biotin and streptavidin-precipitation as described in Section 2. Na^+,K^+-ATPase α_1- (A,C) and α_2-subunits (B,D) were determined as described in Fig. 1. Representative blots (upper panel) and quantitative data from six experiments (mean ± S.E.M.) (lower panel) are shown. *$P < 0.01$ vs. basal.

α_1 and 74% increase for α_2, respectively, $P < 0.05$, $n = 6$). Translocation of α_1- and α_2-subunits of Na^+,K^+-ATPase to the cell surface was abolished by the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin, as well as the PKC inhibitor GF109203X (Fig. 2A,B).

The primary structure, phosphorylation sites and ouabain sensitivity of Na^+,K^+-ATPase α-subunits differ between rat and human [1,2,15]. Thus, we examined Na^+,K^+-ATPase α-subunit PM content in primary HSMC differentiated to myotubules. Incubation of differentiated HSMC with 120 nM insulin for 20 min increased PM content of the α_1 and α_2-subunit (as a percentage of basal: 73% increase for α_1 and 97% increase for α_2, respectively, $P < 0.01$, $n = 6$, Fig. 2C,D). Insulin-induced Na^+,K^+-ATPase α-subunit translocation to HSMC PM was abolished by PI 3-kinase and PKC inhibitors (Fig. 2C,D). Notably, 1 μM of GF109203X, a concentration that inhibits conventional and novel, but not atypical PKCs [16], did not inhibit insulin action (data not shown).

4. Discussion

Subcellular fractionation experiments provided evidence that the Na^+,K^+-ATPase α_2-subunit, together with glucose transporter GLUT4, are insulin-responsive proteins in skeletal muscle, while α_1-subunit translocation has been thought to be regulated in an insulin-independent manner [2,9,17]. However, using alternative techniques for monitoring protein trafficking, the α_1-subunit has been shown to undergo hormone-sensitive translocation to the PM, at least in cells of kidney origin. Insulin promotes translocation of an exofacially epitope-tagged rat Na^+,K^+-ATPase α_1-subunit to the PM in HEK-293 cells [18]. Cell surface biotinylation has also been used to show translocation of α_1-isoform in rat cortical collecting duct cells in response to an increase in cAMP [19]. Consistent with these findings, using a novel and sensitive biotinylation technique we demonstrate that insulin induces not only Na^+,K^+-ATPase α_2-, but also α_1-subunit translocation to the cell surface in skeletal muscle.

The structural complexity of skeletal muscle tissue, with inherent difficulties in applications of gene transfection and optical techniques has made subcellular fractionation and gradient centrifugation the preferred and almost irreplaceable tool for isolation of membrane compartments to study membrane proteins trafficking [8,9,12,20]. However, skeletal muscle surface membranes include both sarcolemma and T-tubules, and the large content of myofibrils and connective tissue prevents proper separation of membrane fractions. Another difficulty associated with subcellular fractionation is the low yield (0.7–8%) of PM/sarcolemma recovery [21]. Thus, even without a noticeable increase in α_1-subunit abundance in the PM fraction, a reduction of α_1-subunit abundance in the IM fraction can be taken as evidence for an insulin-sensitive α_1-subunit pool. Importantly, a previous comparative study [22] provides evidence that the method utilized in this study, as well as in previously reports [8,9,12,13], concerning isoform-specific translocation of Na^+,K^+-ATPase α_2-subunits in response to insulin, has a better recovery of intracellular compartments as compared to the PM technique. Thus, the subfraction containing the target PM for the insulin-sensitive pool of α_1-subunit may not be recovered on sucrose gradient, whereas an increase in α_1-isoform abundance in response to insulin is significant and measurable by labeling of cell surface Na^+,K^+-ATPase, although the magnitude of the response is greater in case of α_2-subunit. Surface labeling of Na^+,K^+-ATPase molecules in isolated skeletal muscles ex vivo may be an important approach for studying Na^+,K^+-ATPase traffic, as it permits the use of inhibitors of different signaling molecules. The same methodology could be used for visualization of possible Na^+,K^+-ATPase trafficking defects in muscles from experimental animal models. However, despite the caveats mentioned above, subcellular fractionation and gradient centrifugation will remain an important method to study membrane trafficking in skeletal muscle when sufficient quantities of tissue are available; especially as the mild conditions of the method makes it possible to assess enzymatic activity and study protein interactions in membrane fractions.

Rat skeletal muscle and differentiated myotubules of human origin exhibit a similar response of Na^+,K^+-ATPase subunits to insulin and inhibitors of PI 3-kinase and PKC, despite species-specific differences in the primary structure of Na^+,K^+-ATPase α-subunits, sensitivity to ouabain, and specific location of phosphorylation sites recognized by different protein kinases. For example, human α_1-subunit lacks Ser-18, an amino acid residue critically involved in rat α_1-subunit endocytosis [23]. Despite these species-specific differences, our data suggests there are also novel common mechanisms of insulin-regulated Na^+,K^+-ATPase activity. Since cultured HSMC express multiple isoforms of Na^+,K^+-ATPase, they provide a good model for studying differential regulation of multiple human Na^+,K^+-ATPase isoforms, whose functional differences in specific cell types remain poorly understood.

Activation of PI 3-kinase and further activation of atypical PKC ξ and λ by PI 3-kinase lipid products is essential for insulin-stimulated GLUT4 translocation [24]. However, whether the same mechanism(s) are involved in the translocation of Na^+,K^+-ATPase is not clear. Morphological data indicates GLUT4 and Na^+,K^+-ATPase do not co-localize in the same intracellular compartments in skeletal muscle [17]. Nevertheless, pre-incubation with wortmannin or atypical PKC inhibitors abolished insulin-induced activation of Na^+,K^+-ATPase in fibroblasts and epithelial cells [25,26]. Here we demonstrate that α_1- and α_2-subunits of Na^+,K^+-ATPase translocate to the PM in rat and human skeletal muscle via pathways dependent on PI 3-kinase and atypical PKCs, a finding consistent with recent report showing wortmannin and the PKC inhibitor, bisindoleilmalemid, prevents insulin-stimulated translocation of rat α_1-subunit overexpressed in HEK-293 cells [18]. Since atypical, but not conventional and novel PKC isoforms are involved in insulin-stimulated phosphorylation of Na^+,K^+-ATPase α-subunits in intact rat skeletal muscle [8], phosphorylation of the α-subunits may be a triggering signal for translocation to PM.

In summary, insulin induces translocation of α_1- and α_2-subunits of Na^+,K^+-ATPase to the PM in intact skeletal muscle or muscle cells in culture. Our observation highlights the need for verification of results regarding trafficking of membrane proteins obtained by subcellular fractionation techniques by techniques of direct labeling of cell surface or intracellular proteins.

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