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Original Article

Insulin induced translocation of Na^+/K^+-ATPase is decreased in the heart of streptozotocin diabetic rats

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Aim: To investigate the effect of acute insulin administration on the subcellular localization of Na^+/K^+-ATPase isoforms in cardiac muscle of healthy and streptozotocin-induced diabetic rats.
Methods: Membrane fractions were isolated with subcellular fractionation and with cell surface biotinylation technique. Na^+/K^+-ATPase subunit isoforms were analysed with ouabain binding assay and Western blotting. Enzyme activity was measured using 3-*O*-methylfluorescein-phosphatase activity.
Results: In control rat heart muscle α1 isoform of Na^+/K^+ ATPase resides mainly in the plasma membrane fraction, while α2 isoform in the intracellular membrane pool. Diabetes decreased the abundance of α1 isoform (25 %, $P<0.05$) in plasma membrane and α2 isoform (50%, $P<0.01$) in the intracellular membrane fraction. When plasma membrane fractions were isolated by discontinuous sucrose gradients, insulin-stimulated translocation of α2- but not α1-subunits was detected. α1-Subunit translocation was only detectable by cell surface biotinylation technique. After insulin administration protein level of α2 increased by 3.3-fold, α1 by 1.37-fold and β1 by 1.51-fold ($P<0.02$) in the plasma membrane of control, and less than 1.92-fold ($P<0.02$), 1.19-fold (not significant) and 1.34-fold ($P<0.02$) in diabetes. The insulin-induced translocation was wortmannin sensitive.
Conclusion: This study demonstrate that insulin influences the plasma membrane localization of Na^+/K^+-ATPase isoforms in the heart. α2 isoform translocation is the most vulnerable to the reduced insulin response in diabetes. α1 isoform also translocates in response to insulin treatment in healthy rat. Insulin mediates Na^+/K^+-ATPase α1- and α2-subunit translocation to the cardiac muscle plasma membrane via a PI3-kinase-dependent mechanism.

Keywords: rat cardiac muscle; streptozotocin-diabetes; Na^+/K^+-ATPase isoenzyme; redistribution

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Introduction

Diabetes mellitus is one of the most frequent metabolic diseases[1, 2]. Both insulin-dependent (Type 1) and non-insulin-dependent (Type 2) diabetes are known to lead to cardiac dysfunction[3], which is partially caused by impaired function of the Na^+/K^+-ATPase[4]. Present in all eukaryotic cells Na^+/K^+-ATPase maintains the electrochemical gradient of Na^+ and modulates cardiac contractility by influencing the Na^+/Ca^{2+} exchange[1, 2]. Its functional unit is composed of two catalytic α and two glycosylated β subunits[3]. There are at least four known isoforms of the α subunit, with different kinetic properties and cardiac glycoside binding affinities[4]. The existence of multiple catalytic isoforms, each with tissue-specific expression, suggests a special function[4]. Three different β subunit isoforms have been identified, which lack enzymatic activity, but may play a role in ensuring the appropriate orientation of the α subunits in the membrane. The α/β ratio might modify enzyme activity[5] or even stability[6]. It has been suggested that different isoforms may function differently in the cell, depending on their different localization[7]. In resting skeletal muscle the α2β1 isoforms of Na^+/K^+-ATPase are found almost exclusively in intracellular vesicles and are translocated to the

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plasma membrane upon insulin stimulation, resulting in a substantial increase in potassium influx and sodium efflux[8, 9]. The rat heart expresses α1, α2, β1, and β2 isoforms[10].

Several studies have shown that diabetes is associated with a reduction in Na^+/K^+-ATPase activity in different tissues[11–14]. Both insulin-dependent (Type 1) and non-insulin-dependent (Type 2) diabetes are known to lead to cardiac dysfunction[15, 16], which is partially caused by the impaired function of Na^+/K^+-ATPase[17, 18]. We have previously reported that in the diabetic heart high affinity ouabain binding sites corresponding to the α2 isoform of Na^+/K^+-ATPase are the ones that are mainly decreased and chronic insulin administration almost completely restored diabetes-induced abnormalities of the sodium pump system[10]. The acute administration of insulin causes translocation of GLUT4 from intracellular store to the plasma membrane[19].

No data is available with regard to the acute effect of insulin on the insulin-induced redistribution of Na^+/K^+-ATPase isoforms in cardiac muscle, especially in diabetes. The aim of our present study was to examine whether acute insulin administration affected the translocation of the isoforms of Na^+/K^+-ATPase in the healthy and diabetic heart.

Materials and methods

Materials

Streptozotocin, ovalbumin, phenylmethylsulphonyl-fluorid and dithiotreitol were purchased from Sigma-Aldrich (Budapest, Hungary), regular insulin from Fluka (Buchs, Switzerland), blood glucose assay kit from Boehringer (Mannheim, Germany), sucrose from Merck (Darmstadt, Germany), [^{3}H] ouabain from Amersham (Buckinghamshire, UK), Sulfo-*N*-hydroxysuccinimide-*S*-*S*-biotin from Pierce (Rockford, IL, USA), cell culture medium and supplements were purchased from Invitrogen. All other reagents were of analytical grade.

Anti-GLUT4 antibody was purchased from Calbiochem (Darmstadt, Germany) and anti-Na^+/K^+-ATPase subunit isoform antibodies were obtained from Santa Cruz Biotechnology (California, USA) and Upstate Biotechnology (New York, USA). All other primary antibodies were from Santa Cruz Biotechnology (California, USA). Secondary antibodies were either purchased from Santa Cruz Biotechnology (anti-goat, anti-mouse and anti-rabbit IgGs) or from Sigma (anti-rat IgG).

Experimental animals

Six-week-old, male Sprague Dawley rats (LATI, Gödöllő, Hungary) weighing 190 to 260 g were randomly assigned into two groups: C (age matched controls), D (untreated diabetes for 4-weeks). Each experimental group for subcellular fractionation and biotinylation studies (untreated C, insulin treated C, untrated D and insulin treated D) contained 5 animals (n=5).

Diabetes was induced by streptozotocin (65 mg/kg body weight via tail vein) and verified 72 h later by detecting hyperglycaemia (blood glucose level>16 mmol/L) and glucosuria[10]. For insulin stimulation studies, rats received tail vein injections of regular insulin (1.5 units/100 g body weight) or saline (0.01 mL/100 g body weight) 20 min before tissue removal. Blood samples were obtained from tail tips and analyzed for glucose levels. Animals were anaesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight), then killed by decapitation. All experimental protocols were in compliance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary. Rats were fed a standard laboratory diet and water *ad libitum*.

Tissue preparation and subcellular fractionation

Plasma and intracellular membranes from cardiac left ventricle were prepared as previously described[19]. Left ventricular tissue (1 g) was homogenized in 5 mL solution containing 10 mmol/L Tris/HCl, pH 7.4, 0.1 mmol/L phenylmethylsulphonyl-fluoride and 2.6 mmol/L dithiotreitol by a homogenizer (Janke and Kungel, Germany) for three times 20 s at half maximal speed. After centrifugation at 3000×*g*, 10 min, the supernatant was centrifuged in a Beckman TLA 100.4 rotor at 200 000×*g*, 90 min. The pellet (crude membranes) was suspended in the homogenizing solution and layered on discontinuous sucrose gradient containing 0.57, 0.72, 1.07, and 1.43 mol/L sucrose, then centrifuged in a Beckman TLS-55 rotor (40 000×*g*, 16 h). Membranes collected from sucrose layers were diluted by the homogenizing medium and recovered by centrifugation at 100 000×*g*, 60 min in a Beckman TLA 100.4 rotor. All procedures were carried out at 2−4 °C. Samples were stored at -80 °C and used within two weeks.

Isolation of cardiomyocytes

Cardiomyocytes were isolated from the hearts of C and D rats[20]. Animals were anesthetized by pentobarbital sodium (5 mg/100 g body weight), and the heart was excised and perfused with buffer 126 mmol/L NaCl, 4.4 mmol/L KCl, 1.0 mmol/L $MgCl_2$, 4.0 mmol/L $NaHCO_3$, 10.0 mmol/L HEPES 30.0 mmol/L 2,3-butanedione monoxime, 5.5 mmol/L glucose, 1.8 mmol/L pyruvate, 0.04 mmol/L $CaCl_2$ (pH 7.3), and type I collagenase 0.9 mg/mL, for 8–10 min. The heart was minced and washed in buffer with increasing calcium concentration until 1 mmol/L. The cells were pelleted, resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Invitrogen). Half of the cells were stimulated with 120 nmol/L insulin with and without wortmannin (200 nmol/L; Sigma) for 30 min. Plasma and intracellular membranes of cardiomyocytes were prepared as previously described[19].

Cell surface biotinylation

Cardiomyocytes were then biotinylated with sulfo-N-hydroxysuccinimide-*S*-*S*-biotin (Pierce, Rockford, IL) at a final concentration of 0.5 mg/mL for 60 min at 4 °C. Cells were then washed with ice-cold PBS and homogenized in solution containing HEPES 10 mmol/L, EDTA 5 mmol/L, sucrose 250 mmol/L, aprotinin 10 μg/mL, leupeptin 10 μg/mL, and 0.1% Triton X-100. The homogenate was then incubated overnight with streptavidin beads (Pierce). Beads were washed four

times with PBS containing 0.1% Triton X-100, and twice with PBS[19]. Laemmli buffer was added to each sample and heated for 56 °C for 20 min. Samples were subjected to SDS–PAGE to determine the surface abundance of Na^+/K^+-ATPase subunit isoforms and PMCA.

Enzyme activities

Na^+/K^+-ATPase activity was assayed by measuring the strophanthidin sensitive K^+-dependent 3-*O*-methylfluoresceinphosphatase activity[21]. Ca^{2+}/K^+-ATPase and 5′-nucleotidase activity[22] and [^{3}H]ouabain binding were performed as described previously[23]. Protein was assayed according to Bradford[24] using ovalbumin as a standard.

Western blot analysis

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels with a BioRad Miniprotean II (Hercules, CA, USA) and transferred onto nitrocellulose filters (Bio Rad)[10]. Filters were blocked and then incubated with different primary antibodies: NKA α1 (Santa Cruz Biotechnology, CA, USA) diluted to 1:1000 and NKA α2 (Santa Cruz Biotechnology, CA, USA), diluted to 1:2000, β1 and β2 (Upstate Biotechnology, NY, USA) diluted to 1:750, PMCA (Santa Cruz Biotechnology, CA, USA) diluted to 1:500, GLUT4 (Calbiochem, Darmstadt, Germany), diluted to 1:4000, washed and then incubated with peroxidase-conjugated secondary antibodies (anti mouse Transduction Laboratories, CA, USA, diluted to 1:15 000, anti rabbit Santa Cruz Biotechnology, CA, USA, diluted to 1:6000). After subsequent washing, blots were developed by the enhanced chemiluminescence Western blotting detection reagent (Amersham, Buckinghamshire, UK). The film negatives were analysed by laser densitometry (Pharmacia, Uppsala, Sweden). The values were normalized to β-actin (Sigma Chemical Co) as an internal standard and expressed as relative optical density.

Statistical analysis

Multiple comparisons were evaluated by ANOVA followed by Fisher-correction or in the case of Western blots by the Kruskal-Wallis ANOVA on ranks for non-parametrical data. Criterion for significance was $P<0.05$ in all experiments.

Results

General characteristics of experimental animals

The streptozotocin-induced diabetic state in group D animals was reflected by an elevation of plasma glucose concentration from 8.25±0.52 mmol/L to 21.2±3.7 mmol/L ($P<0.05$). Insulin administration decreased the blood glucose level to 5.15±0.42 mmol/L in control and to 19.8±3.7 mmol/L in diabetic animals. Heart/body weight ratio did not change with the diabetic state (Table 1).

Table 1. Body, heart weight, and blood glucose levels of control and streptozotocin-diabetic rats. Mean±SEM. n=15. $^bP<0.05$ between the age-matched controls and streptozotocin-diabetic rats.

Group	C	D
Initial body weight (g)	190.5±24.3	194.6±20.2
Body weight at 4 weeks	288.8±20.3	235.7±19.8^b
Heart weight(g)	0.82±0.04	0.65±0.04^b
Heart/Body weight×10^3	2.84±0.3	2.75±0.3
Non fasting blood glucose (mmol/L)	8.25±0.52	21.2±3.7^b
Blood glucose 20 min after insulin injection (mmol/L)	5.15±0.42	19.8±3.7^b

For insulin stimulation studies, rats received tail vein injections of regular insulin (1.5 units/100 g body weight) or saline (0.01 mL/100 g body weight) 20 min before tissue removal.

Characterization of subcellular fractions

5′-Nucleotidase, Ca^{2+}/K^+-ATPase, strophanthidin-insensitive 3-*O*-methylfluorescein-phosphatase and strophanthidin-sensitive 3-*O*-methylfluorescein-phosphatase (SS-OMFPase, representing Na^+/K^+-ATPase) activities were compared in crude membranes from C and D animals. No changes in the protein yield were apparent in the D animals (15.7±2.1 mg/g wet weight) compared to the C (17.7±2.2 mg/g wet weight). The SS-OMFPase and 5′-nucleotidase activity were equal in the two groups, however, a marked reduction in the activity of SS-OMFPase (representing Na^+/K^+-ATPase) and Ca^{2+}/K^+-ATPase was observed in the diabetic heart (31.9% and 35.7%, $P<0.05$, respectively).

Subcellular distribution of Na^+/K^+-ATPase activity, ouabain binding sites and subunit isoforms in control and diabetic cardiac muscle; the effect of insulin

After separation on sucrose gradient, membrane vesicles recovered from 0.72 mol/L sucrose, considered to be mainly derived from the plasma membrane were enriched 5.4 and 5.37-fold with respect to 5′-nucleotidase in C and D rat hearts, respectively. Membrane vesicles obtained from the 1.07 mol/L sucrose layer, considered to be mainly derived from intracellular membranes were enriched 4.07 and 4.33-fold with respect to Ca^{2+}/K^+-ATPase in C and D rat hearts, respectively. The SS-OMFPase activity was represented in both membrane fractions and was lower both in the plasma membrane and in intracellular membranes of the D heart compared with the C group by 21.01% ($P<0.05$) and 44.27% ($P<0.01$), respectively (Table 2). The low affinity ouabain binding site reflecting the α1 isoform of Na^+/K^+-ATPase was found predominantly in the plasma membranes of both C and D hearts, but B_{max} was lower in diabetes by 25.39% ($P<0.05$) in the plasma membrane and 25.06% ($P<0.05$) in the intracellular membrane. The high affinity ouabain-binding sites reflecting the α2 isoform were found mainly in intracellular membranes in both C and D, but B_{max} was decreased by 50.0% ($P<0.01$) in D (Table 3). Insulin administration caused a 3.33-fold increase in the high affinity ouabain-binding site in the plasma membrane fraction in the C, while only a 1.99-fold increase was observable in the D group. Insulin did not influence the distribution of low affinity ouabain binding sites.

Table 2. General characteristics of microsomes from the left cardiac ventricles of control and streptozotocin-diabetic rats. Mean±SEM. *n*=5 experiments. [b]*P*<0.05 between control (C) and streptozotocin-diabetic (D) group.

Group	C	D
Yield (mg/g heart)	17.7±2.2	15.7±2.1
5′-nucleotidase (μmol phosphate ·mg^{-1}·h^{-1} protein)	4.31±0.59	4.6±0.58
Strophanthidin-insensitive-OMFPase (μmol fluorescein·mg^{-1}·h^{-1} protein)	3.0±0.39	2.99±0.35
Strophanthidin-sensitive OMFPase (μmol fluorescein·mg^{-1}·h^{-1} protein)	2.35±02.6	1.6±0.23[b]
Ca^{2+}/K^+ -ATPase (μmol phosphate ·mg^{-1}·h^{-1} protein)	11.2±1.7	7.2±0.7[b]

The strophanthidin-sensitive 3-*O*-methylfluorescein-phosphatase (SS-OMFPase) represents the Na^+/K^+-ATPase activity.

Acute effect of insulin on the subcellular distribution of Na^+/K^+-ATPase subunit isoforms

In crude membrane (CM) preparations diabetes decreased, while acute insulin treatment did not affect total Na^+/K^+-ATPase subunit isoform and GLUT4 content (Figure 1A, Figure 2). The effect of insulin on the distribution of subunit isoforms of Na^+/K^+-ATPase among plasma and internal membranes was also studied by Western blot analysis (Figure 1B). The α1 isoform was found mostly in the plasma membrane and was reduced by 17.3% in D rats. No detectable effect of insulin could be observed. The α2 isoform was found in the intracellular membrane fractions both in C and D hearts, and the amount of the α2 isoform decreased by 40.2% (P<0.01) in diabetes.

The β subunit isoforms of Na^+/K^+-ATPase were present in both membrane fractions, with β1 being predominant over β2. The level of the β1 isoform decreased in the diabetic group by 25.9% (P<0.05) in the plasma membrane fraction and by 51.8% (P<0.01) in the intracellular membrane fraction (Figure 3). In the diabetic group, there was also a significant decrease in the β2 isoform in both membrane fractions (21.2%±2.9%, P<0.05) and 23.7% (P<0.05, data not shown).

Insulin-induced mobilisation of Na^+/K^+-ATPase isoforms from the intracellular pool was also investigated 20 min after insulin injection. Insulin injection caused a 3.3-fold (P<0.01) and 1.51-fold (P<0.01) increase in α2 and β1 subunits respec-

Table 3. The effect of insulin on the localization of different membrane proteins in control and diabetic hearts. Mean±SEM. *n*=number of experiment. [b]*P*<0.05, [c]*P*<0.01 differences between control (C) and streptozotocin-diabetic (D) group. [e]*P*<0.05 differences between insulin-treated and non-treated group.

Groups	Control (*n*=10)		Diabetic (*n*=10)	
Basal (*n*=5) Insulin (20 min) (*n*=5)	Plasma membrane	Intracellular membrane	Plasma membrane	Intracellular membrane
Yield mg/g heart				
Basal	0.29±0.03	1.78±0.15	0.30±0.036	1.81±0.2
Insulin (20 min)	0.31±0.03	1.76±0.15	0.29±0.04	1.79±0.19
5′-Nucleotidase (μmol phosphate·mg^{-1}·h^{-1})				
Basal	23.34±3.22	2.12±0.21	24.74±3.7	1.95±0.24
Insulin (20 min)	23.31±3.22	2.10±0.21	23.81±3.5	1.81±0.35
Ca^{2+}/K^+ ATPase (μmol phosphate·mg^{-1}·h^{-1})				
Basal	3.25±0.40	45.62±4.09	2.76±0.33	31.2±3.6[b]
Insulin (20 min)	2.97±0.34	47.82±4.81	2.97±0.40	32.7±3.4[b]
SS-OMFPase (μmol fluorescein·mg^{-1}·h^{-1})				
Basal	13.82±2.2	3.75±0.4	10.91±1.5[b]	2.09±0.2[c]
Insulin (20 min)	16.05±2.1	2.98±0.3[e]	11.91±1.7[b]	1.29±0.16[be]
B_{max} high affinity (pmol/mg protein)				
Basal	34.22±3.21	82.57±7.2	31.14±3.21	41.28±4.2[c]
Insulin (20 min)	113.85±10.4[e]	51.93±5.2[e]	61.93±6.22[be]	14.96±3.1[be]
B_{max} low affinity (pmol/mg protein)				
Basal	324.20±31.8	36.36±4.2	241.92±30.8[b]	27.26±3.2[b]
Insulin (20 min)	342.54±31.8	27.45±4.3[e]	255.82±28.7[b]	24.15±3.17

The strophanthidin-sensitive 3-*O*-methylfluorescein-phosphatase (SS-OMFPase) represents the Na^+/K^+-ATPase activity.

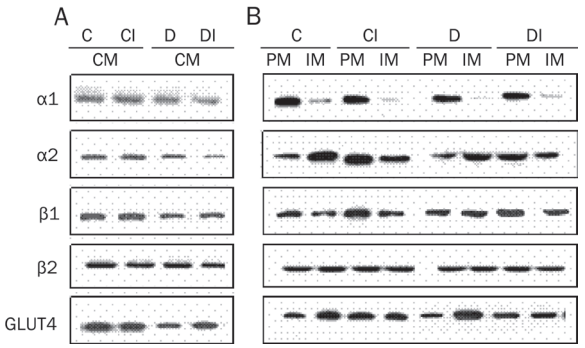

Figure 1. Representative blots of the effect of insulin on the distribution of Na^+/K^+-ATPase α1, α2, β1, β2 subunits and GLUT4 in control and STZ diabetic rat hearts. Control (C), control+insulin (CI), STZ diabretic (D), STZ diabetic+inzulin (DI). A: Protein content of crude membrane, B: Protein content of separated plasma membrane and intracellular membranes. Crude membrane (CM), plasma membrane (PM) and intracellular membranes (IM) were prepared as described in material and methods.

tively in the plasma membrane fraction in the control group, and a less pronounced increase (1.92-fold, $P<0.01$) and 1.34-fold ($P<0.01$) in α2 and β1 subunits respectively in diabetes. The level of α2 and β1 subunits of Na^+/K^+-ATPase in the intracellular membrane fraction decreased concomitantly in control rats. However, in diabetic animals the decrease seen in the intracellular membrane was higher than the increase seen in the plasma membrane (Figure 1B and Figure 3). Similarly to that observed in Na^+/K^+-ATPase, the amount of GLUT4 was increased by 2.52-fold ($P<0.01$) in the plasma membrane in the control group after insulin administration. In comparison it increased only 1.61-fold ($P<0.01$) in diabetic cardiac muscle (Figure 1B and Figure 3).

EZ-link Sulfo-NHS-SS-biotinylation

Similarly to the results obtained using subcellular fractionation methods, insulin markedly increased the presence of α2 and β1 subunits of Na^+/K^+-ATPase in the cell surface of control (2.89-fold, 1.52-fold, $P<0.01$) and diabetic (1.75-fold; 1.42-fold, $P<0.03$) rat heart muscle using the biotinylation technique (Figure 4, 5). The insulin dependent α1 translocation to the cell surface was also detectable (1.37-fold) in control hearts. Translocation of Na^+/K^+-ATPase subunits was abolished by the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin (Figure 4, 5). To validate our results, we investigated the cell surface abundance of PMCA. Unlike the cell surface abundance of Na^+/K^+-ATPase subunits, PMCA cell surface abundance was not affected by insulin and wortmannin administration. The insulin dependent translocation of GLUT4 was investigated in plasma membrane fraction of cardiomyocytes isolated from heathy and diabetic animals. In response to insulin there was a significant increase in the GLUT4 content of plasma membrane both in control and diabetic heart muscle (4.83-fold, 3.6-fold, $P<0.01$ respectively). Translocation of GLUT4 was abolished by wortmannin (Figure 4, 5).

Discussion

The purpose of this study was to analyse the subcellular distribution of some membrane proteins that are known to be involved in the acute response to insulin, namely Na^+/K^+-

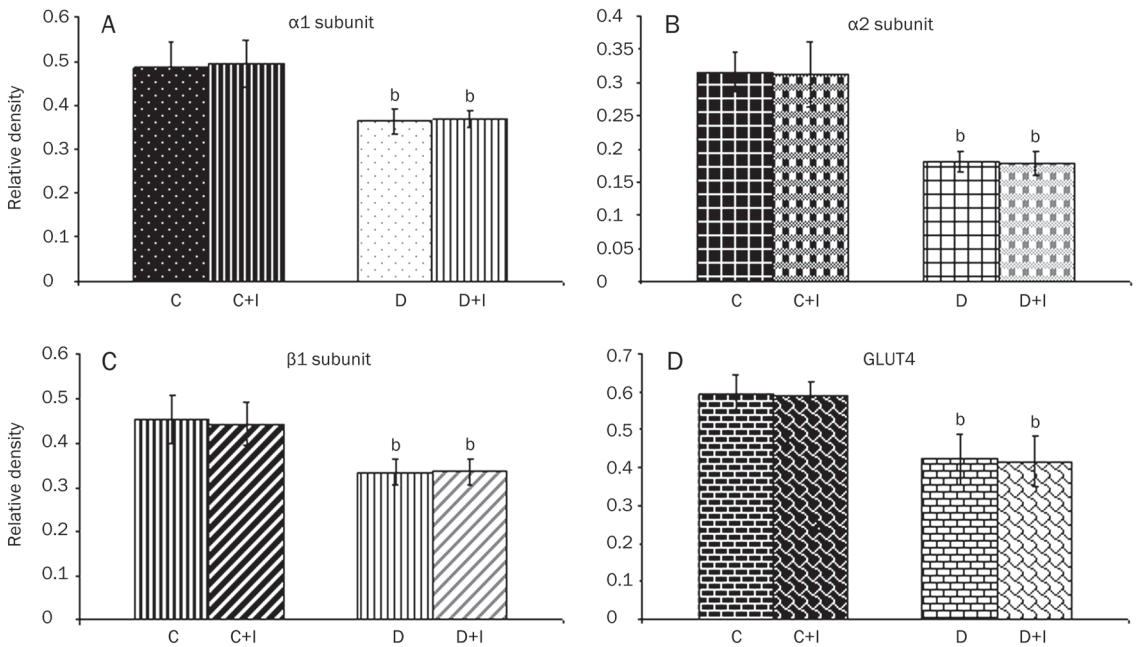

Figure 2. The relative amount of Na^+/K^+-ATPase α1 (A), α2 (B), β1 (C) subunits and GLUT4 (D) in control and STZ diabetic rat hearts with or without insulin treatment. Crude membranes (CM) were prepared as described in material and methods. Values are mean±SEM of 5 experiments. Relative density was calculated from optical density film negative of Western blot corrected for the amount of total protein blotted onto the nitrocellulose for each gel lane. Results are expressed in arbitrary units relative to the value in crude membrane. $^bP\leq0.05$ *vs* control (C) and streptozotocin-diabetic rats (D).

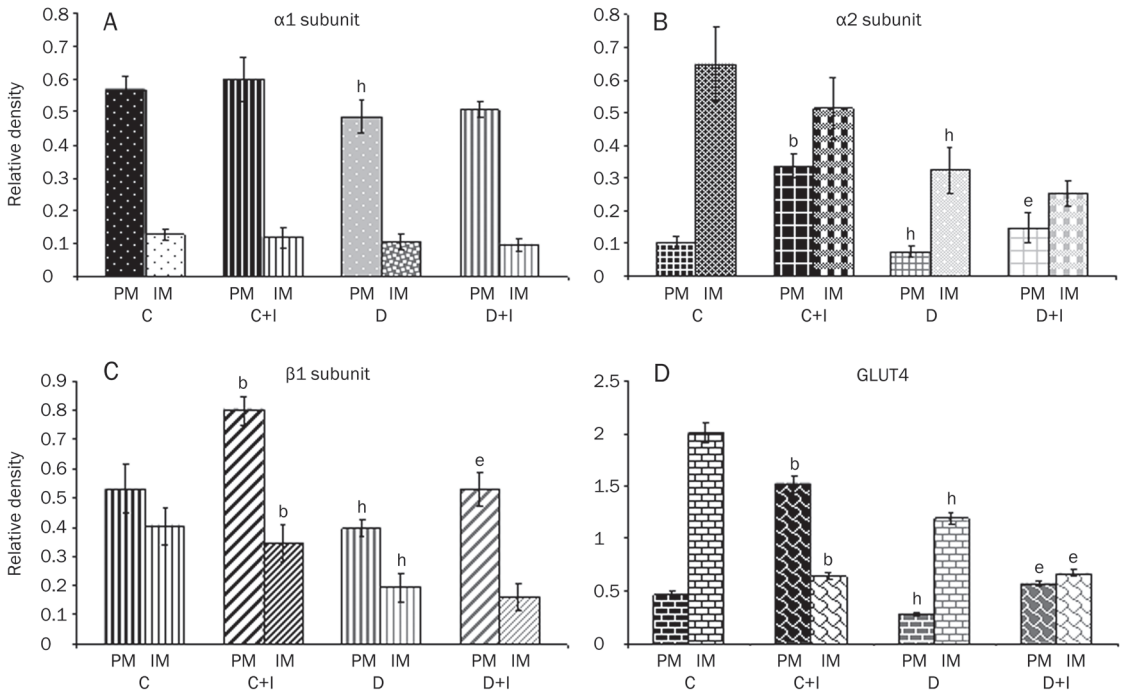

Figure 3. The effect of insulin on the distribution of Na^+/K^+-ATPase α1 (A), α2 (B), β1 (C) subunits and GLUT4 (D) in control and STZ diabetic rat hearts. Bar graphs represent the quantitative data from the experiments (mean±SEM) in Figure 1B. Plasma membrane (PM) and intracellular membranes (IM) were prepared as described in material and methods. Values are mean±SEM of 5 experiments. Relative density was calculated from optical density film negative of Western blot corrected for the amount of total protein blotted onto the nitrocellulose for each gel lane and further multiplied by the total amount of protein recovered in each fraction. Results are expressed in arbitrary units relative to the value in fraction plasma membrane (PM) basal state. $^bP<0.05$ differences between control (C) and insulin treated control (C+I). $^eP<0.05$ differences between streptozotocin-diabetic rats (D) and streptozotocin-diabetic insulin treated rats (D+I). $^hP<0.05$ between control (C) and streptozotocin-diabetic rats (D).

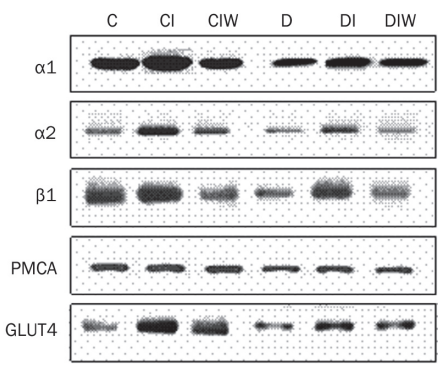

Figure 4. Translocation of Na^+/K^+-ATPase subunits to the cell surface in the presence of insulin and PI3-kinase inhibitor. Representative Western blot images of Na^+/K^+-ATPase subunits, PMCA and GLUT4 are shown. Rat cardiomyocytes were incubated without or with 120 nmol/L insulin and in the absence or presence of 100 nmol/L wortmannin. Cell surface Na^+/K^+-ATPase and PMCA abundance was determined by biotinylation with EZ-link Sulfo-NHS-SS-biotin and streptavidin-precipitation as described in Materials and Methods. GLUT4 was determined after separation of the plasma membrane from cardiomyocytes as described in material and methods.

ATPase subunit isoforms, and GLUT4 in normal and diabetic rat heart muscle. Along with others we previously showed that ouabain binding sites, Na^+/K^+-ATPase activity and protein levels are decreased in streptozotocin-diabetic hearts compared to control[10, 25–27]. Limited studies have addressed the consequences of acute insulin administration on cardiac muscle. Eckel and Reinauer measured a hyperpolarizing effect of insulin on cardiac muscle using a voltage-sensitive dye[28]. Acute addition of insulin resulted in the hyperpolarization of the membrane potential in ventricular tissue, which was abolished by cardiotonic steroids[29]. The directly measured Na^+/K^+ current that supposedly involves Na^+/K^+-ATPase was also affected by the acute addition of insulin[30, 31]. However, no data is available on the insulin-induced redistribution of Na^+/K^+-ATPase subunit isoforms in heart. We employed subcellular fractionation and gradient centrifugation and cell surface biotinylation methods to detect changes in subcellular distribution of proteins. Subcellular fractionation and gradient centrifugation are essential methods to study membrane protein trafficking. One major disadvantage of this method that proper separation of membrane fractions in skeletal and heart muscle is limited since surface membranes include both sarcolemma and T-tubules, and the large content of myofibrills and connective tissue. This method also has a low yield of (0.7%−8%) of PM/sarcolemma recovery, which makes difficult to detect small changes in PM protein abundance[32]. The use of cell surface biotinylation provides a useful and

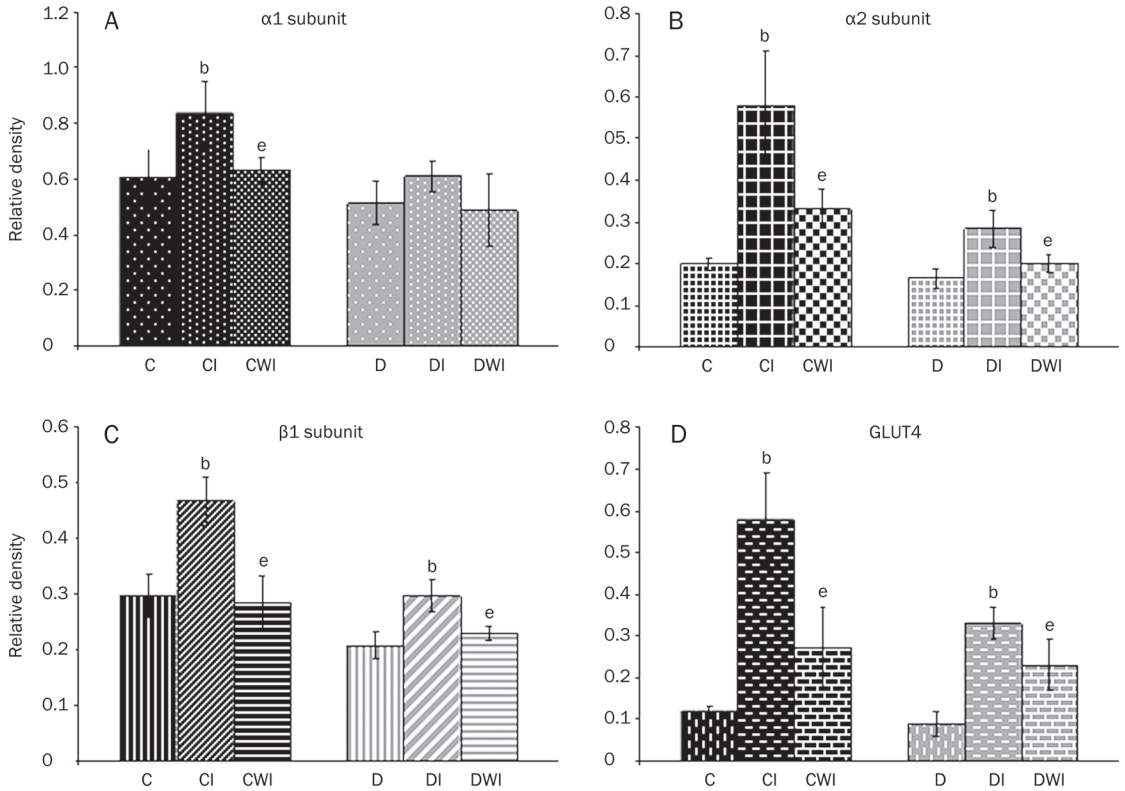

Figure 5. Translocation of Na^+/K^+-ATPase subunits and GLUT4 to the cell surface in the presence of insulin and PI3-kinase inhibitor. Bar graphs represent the quantitative data from the experiments (mean±SEM) in Figure 4. Rat cardiomyocytes were incubated without or with 120 nmol/L insulin and in the absence or presence of 100 nmol/L wortmannin. Na^+/K^+-ATPase α1 (5A)- and α2- (5B), β1- (5C) subunits and GLUT4 (5D) were determined as described under Figure 4. $^bP<0.05$ without insulin *vs* insulin addition, $^eP<0.05$ insulin addition *vs* insulin and wortmannin addition.

sensitive tool to detect PM associated changes and supplements well the subcellular fractionation method. Biotinylation technique is well suited and widely used for the detection of the cell surface abundance of Na^+/K^+-ATPase subunits as well as PMCA[32, 33]. However, GLUT4 is biotinylated to a very low degree, most probably because there is only one lysine in its extracellular portion that is minimally accessible for reaction[34]. For that reason we employed subcellular fractionation followed by gradient centrifugation when measuring GLUT4 content in the plasma membrane of untreated and insulin treated cardiomyocytes with or without wortmannin.

Concerning our present results, the two distinct α isoforms of the catalytic subunit of Na^+/K^+-ATPase that differ in their [^{3}H]ouabain binding affinity showed a specific subcellular localization in the left cardiac ventricle. Both in control and diabetic rat hearts low affinity [^{3}H]ouabain binding sites reflecting the α1 subunit isoform were localized in the plasma membrane and the high affinity [^{3}H]ouabain binding sites reflecting the α2 isoform were localized in the intracellular membranes, similar to that seen in skeletal muscle[35]. The activity of Na^+/K^+-ATPase was present in both membrane fractions, however, the turnover rate (Na^+/K^+-ATPase activity/[^{3}H]ouabain binding sites) was different. The activity of Na^+/K^+-ATPase was lower both in the plasma- and intracellular membranes of diabetic hearts. This decrease correlated with changes in ouabain-binding sites, however the turnover rate did not change significantly in diabetes (the values are: 642±86 min^{-1} and 626±78 min^{-1} for plasma membranes and 553±74 min^{-1} and 519±83 min^{-1} for internal membrane fractions in the control and diabetic groups, respectively). This suggests a decrease in the amount of ATPase molecules, rather than the inactivation or modification of the enzyme. These findings were also supported by Western blot analysis. In the diabetic heart we observed a significant decrease in the level of both the α1 and α2 catalytic subunits, and the non-catalytic β subunits similarly to that described previously[10]. However, the level of intracellular membrane-localized α2 and β1 subunit isoforms decreased to a larger extent in diabetes as compared to the plasma membrane-localized α1 and β1 subunits. In agreement with our previous results the α1 isoform was predominant over the α2[36]. Acute administration of insulin enhanced the α2 and β1 subunit isoforms of Na^+/K^+-ATPase in plasma membrane with a concomitant decrease in the intracellular membrane similar to GLUT4 in cardiac muscle[37]. This was in agreement with the insulin-induced changes of the high affinity ouabain-binding site. Our results demonstrate for the first time the insulin-induced recruitment of α2 catalytic and β1 non-catalytic subunits of Na^+/K^+-ATPase in healthy and

diabetic cardiac muscle. These are expected results based on numerous studies on skeletal muscle[9, 38-40], albeit no information was previously available on cardiac muscle. The current study is unique in its demonstration of the insulin-mediated redistribution of these insulin responsive proteins in diabetic cardiac muscle, and in proving that insulin-induced translocation of Na^+/K^+-ATPase subunits as well as that of GLUT4 is considerably reduced in diabetes. However, further research is needed to clarify the functional distribution of Na^+, K^+-ATPase isoforms.

One possible explanation for the reduced translocation could be the originally lower intracellular pool of Na^+/K^+-ATPase subunit isoforms in the diabetic heart. However, this assumption is contrary to previous results in skeletal muscle of transgenic mice showing an overexpression of the GLUT4 glucose transporter[36]. In that model, the net Na^+/K^+-pump subunit content was lower in the intracellular membranes of the diabetic group as compared to the control group. Despite the reduced expression in pump subunits, the residual Na^+/K^+-pump showed an enhanced response to insulin[36]. However, contrary to transgenic mice overexpressing the GLUT4 glucose transporter, the streptozotocin-induced diabetic state is associated with low expression and reduced insulin-induced recruitment of GLUT4 in skeletal and cardiac muscle[39]. We found that the insulin-induced redistribution of Na^+/K^+-pump subunits is also reduced in diabetes. However, at least in skeletal muscle of healthy rats, the α2 subunit of Na^+/K^+-ATPase and the GLUT4 glucose transporter do not colocalize to a significant extent in intracellular membranes[41]. On the other hand, as our results shown, the insulin-induced decrease of Na^+/K^+-ATPase subunits in the intracellular pool is higher than the increase in the plasma membrane in diabetes, thus it appears that a part of the Na^+/K^+-ATPase activity "gets lost" during the translocation.

The possible cause of this could be that in diabetes the amount of the β subunit is decreased to a greater extent as compared to the α subunit in intracellular membranes. However, the exact role of the β subunits is still questionable; it is also debated whether the α/β ratio might modify enzyme activity or stability as well as the recruitment of the enzyme[5-6]. On the other hand, according to our experiments with insulin and PI3 kinase inhibitor, wortmannin, the insulin induced translocation of Na^+/K^+-ATPase, α2, β1 subunits in rat heart muscle was observable and preventable, similarly to the findings in skeletal muscle[32]. However, according to our data α1 isoform was less insulin sensitive.

In summary, we have documented that the mechanism of short-term insulin-dependent regulation of Na^+/K^+-ATPase occurs through the recruitment of pump subunit isoforms (α2 and β1) to the plasma membrane from an intracellular pool in cardiac muscle, similar to that observed in skeletal muscle, and the insulin-induced redistribution of the pump subunits is reduced in diabetes. We suggest that the reduced response of Na^+/K^+-ATPase to insulin in diabetic cardiac muscle should be considered a consequence of the impaired expression of the α2 and β1 subunit isoforms of the enzyme.

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Author contribution

Klara ROSTA, Eszter TULASSAY, Anna ENZSOLY and Katalin RONAI performed research; Ambrus SZANTHO wrote paper; Tamas PANDICS analysed data; Andrea FEKETE contributed analytical tools; Peter MANDL performed research, wrote paper and analysed research; Agota VER designed research, wrote paper, analysed data and contributed analytical tools.

Abbreviations

Na^+/K^+-ATPase, Na^+- and K^+-dependent ATPase; OMFPase, 3-*O*-methylfluorescein-phosphatase; SS-OMFPase, Strophantidine-sensitive; PMCA, plasma membrane Ca^{2+}-ATPase; C, control rat; CI, insulin-treated control rat; D, diabetic rat; DI, insulin-treated diabetic rat; PM, plasma membrane fraction; IM, internal membranes fraction; SDS, sodium dodecylsulphate; ECL, enhanced chemiluminescence.

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