Sedimentation and immunological analyses of GLUT4 and α2-Na,K-ATPase subunit-containing vesicles from rat skeletal muscle: evidence for segregation

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Abstract **In skeletal muscle insulin induces the translocation of both the GLUT4 glucose transporter and the α2 subunit of the Na,K-ATPase from an intracellular membrane (IM) compartment to the plasma membrane (PM). Fractionation studies of rat skeletal muscle using a discontinuous sucrose gradient have indicated that the insulin-induced loss of both proteins occurs from a fraction containing intracellular membranes (IM) of common density. This raises the possibility that both proteins may be colocalized in a single intracellular compartment or are present in separate membrane vesicles that are of similar buoyant density. In this study we report that membrane vesicles from the insulin-responsive IM fraction can in fact be separated on the basis of differences in their sedimentation velocities; immunoblot analyses of fractions collected from a sucrose velocity gradient revealed the presence of two separate peaks for GLUT4 and the α2 subunit of the Na,K-ATPase. One of these peaks representing a fast sedimenting population of vesicles (with a sedimentation coefficient of 2697 ± 57 S) reacted against antibodies to the α2 subunit of the Na,K-ATPase, whereas, the second peak contained a population of much slower sedimenting vesicles (with a sedimentation coefficient of 209 ± 4 S) were practically devoid of the α2-subunit. By contrast, the slow sedimenting vesicles were enriched by ~32-fold in GLUT4 relative to the starting IM fraction when the fractional protein content was taken into account. Immunoprecipitation of GLUT4-containing vesicles from the insulin-sensitive IM fraction revealed that no immunoreactivity towards either the α2 or the β1 subunits of the Na,K-ATPase could be observed, signifying that the insulin-responsive subunits of the Na,K-ATPase and GLUT4 were present in different membrane vesicles and that it was unlikely, therefore, that the insulin-induced redistribution of these proteins to the PM occurs from a common intracellular pool.**

Key words: Membrane; Transport; Na pump; Insulin

1. Introduction

Among the best-documented responses in skeletal muscle to insulin are the activation of glucose transport and the Na,K-ATPase [1–4]. Skeletal muscle expresses both the GLUT1 and GLUT4 glucose transporters but it is the mobilization of GLUT4, from an intracellular store to the plasma membrane (PM), that explains the observed increase in glucose transport in response to insulin binding [5–8]. In contrast, GLUT1 expression is largely restricted to the PM where, under normal physiological circumstances, its primary function may be that of helping to maintain basal glucose uptake [9].

As with the facilitative glucose transporter family, different isoforms of the Na,K-ATPase are also known to exist [10,11]. The Na,K-ATPase is a heterodimeric protein consisting of a catalytic α-subunit of which there are three known isoforms (α1, α2 and α3) and a glycosylated β-subunit of which two mammalian isoforms (β1 and β2) have thus far been identified [10,11]. Skeletal muscle expresses the α1, α2, β1 and β2 subunits of the Na,K-ATPase but it is likely that only the α2 and β1 subunits constitute the insulin-responsive enzyme according to the observation that after exposure to insulin these subunits are recruited, by a mechanism analogous to the translocation of the GLUT4 transporter, to the PM from intracellular stores [12]. Little is known regarding the nature and identity of the intracellular compartments containing the GLUT4 transporter and the subunits of the Na,K-ATPase. However, subcellular fractionation studies have revealed that the insulin-induced loss of both GLUT4 and the α2-Na,K-ATPase subunit occurs from intracellular membranes (IM) of the same buoyant density [7,12]. This observation may signify that these proteins are colocalized in a single intracellular pool enabling them to be recruited to the PM simultaneously upon insulin stimulation. Such a proposition is not without precedent given that other proteins, such as the transferrin receptor and the mannose 6-phosphate receptor, whose cellular distribution is also affected by insulin, have been colocalized with intracellular GLUT4 in 3T3-L1 adipocytes [13]. In an attempt to address this question and gain further insights into the nature of the intracellular pools containing the insulin-senstive GLUT4 transporter and the subunits of the Na,K-ATPase we have, in this investigation, studied the sedimentation and immunological characteristics of intracellular membranes containing these proteins.

2. Materials and methods

2.1. Animals and experimental procedures

Male Sprague Dawley rats (250 g, Bantin and Kingman, Hull, UK) were studied in the post-absorptive state. Animals were placed under terminal anaesthesia using a combination of Hipnorm (Fentanyl Citrate, 0.2 mg/kg; Fluanisone, 7 mg/kg i.p) and Hipnoval (a muscle relaxant, Midazolam Hydrochloride, 1.5 mg/kg i.p, Janssen Pharmaceuticals Ltd, Oxford). Hindlimb muscles were rapidly excised, frozen in liquid N_2 and subsequently stored at −80°C until required for study.

2.2. Subcellular fractionation of rat skeletal muscle

The procedure for isolating rat muscle membrane fractions enriched with plasma membranes (PM) and intracellular membranes (IM) endowed with the insulin sensitive pool of GLUT4 and α2-Na,K-ATPase subunit has been reported in detail [9,12,14]. Briefly, 10–15 g of skeletal muscle tissue was isolated from rat hindlimbs, homogenized, and sub-

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jected to a series of differential centrifugation steps to obtain crude or total membranes. Crude membranes (CM) were subsequently applied to a discontinuous sucrose gradient (25, 30 and 35% sucrose wt/wt) and centrifuged at 190,000 × *g* for 16 h. Membranes isolated on each sucrose face were washed by centrifugation prior to further analyses. Potassium-stimulated *p*-nitrophenyl phosphatase (KpNPPase) and 5′ nucleotidase activities were assayed using standard enzymatic methods [15]. Protein content of each fraction was assayed using the Bradford method [16]. Membranes isolated from the top of the 35% were used either immediately for sedimentation or immunoprecipitation analyses or alternatively stored at −80°C until required for further study.

2.3. Sedimentation analyses of native intracellular membrane vesicles

Membranes removed from the top of the 35% sucrose fraction, which are typically intracellular in nature and possess the insulin-sensitive pools of the GLUT4 glucose transporter and the α2-subunit of the Na,K-ATPase [9,12,20], were pelleted by centrifugation at 190,000 × *g* for 1 h in sucrose free buffer (10 mM $NaHCO_3$ pH 7.0, 5 mM NaN_3 and 100 μM phenylmethylsulfonyl fluoride). The pellet was washed three times prior to resuspending in a small volume of homogenizing buffer (0.25 M sucrose, 10 mM $NaHCO_3$ pH 7.0, 5 mM NaN_3 and 100 μM phenylmethylsulfonyl fluoride). The protein concentration was adjusted to 1 mg/ml with homogenization buffer before 0.5 mg protein was applied onto a 4 ml 20–40% pre-formed continuous sucrose gradient. After a 20 min spin at 100,000 × *g* 0.2 ml fractions were collected in a sequential manner by piercing the bottom of the centrifuge tube. The protein content and refractive index of each fraction was determined and fractions subsequently subjected to SDS-PAGE and immunoblotting using antibodies to the GLUT4 transporter and the α2-Na,K-ATPase subunit as described below. Sedimentation coefficients for the peaks of vesicles containing GLUT4 and the α2-subunit were calculated using a on-line computational program written for the T-2190 Kontron ultracentrifuge (Kontron Instruments Ltd) which takes account of the volume and density of individual fractions enriched with the appropriate protein as well as the volume and density of the sample applied to the gradient and all known variables regarding the nature of the centrifugation spin.

2.4. Western-blot analyses

Muscle membrane fractions (10 μg) were subjected to SDS-PAGE on either 9% or 7% resolving polyacrylamide gels [17]. Separated proteins were electrophoretically transferred onto PVDF (polyvinylidene difluoride, Biorad, UK) and then blocked with 3% BSA/Tris-saline-Tween 20 prior to incubating overnight at 4°C with a monoclonal antibody to the α2 (McB2) subunit of the Na,K-ATPase kindly provided by Dr Kathleen Sweadner (Harvard University, Boston, USA) [18] used at a dilution of 1:100. Anti-GLUT4 antibodies were purchased from East Acres Biologicals, (Southbridge, MA, USA) and antibodies to the β1 subunit of the Na,K-ATPase were obtained from UBI (Lake Placid, NY) both being used at a final dilution of 1:500. Following primary antibody incubation, PVDF membranes were washed in Tris-saline- Tween 20-NP40 and primary antibody detected using 0.1 μCi/ml of either [^{125}I]protein A (for GLUT4 and β1) or ^{125}I-labelled sheep anti- mouse IgG (for anti-α2). The PVDF membranes were subsequently washed three times in Tris-saline-Tween 20 for 15 min, air dried and autoradiography performed by exposure to XAR-5 Kodak film at −80°C. Autoradiographs were quantitated using a Molecular Dynamics laser scanner with Image Quant 3 software.

2.5 Immunoisolation of intracellular GLUT4-containing vesicles

Cyanogen-bromide activated Sepharose beads (90 μm diameter) were used for immunoadsorption studies (Pharmacia). Beads were conjugated to Protein A as per manufactures instructions. Beads were finally resuspended in PBS containing 0.4% BSA. Membranes from the IM fraction were washed as above and resuspended at 1 mg/ml in homogenization buffer and were then used to immunoisolate GLUT4-containing vesicles. 100 μg of IM protein was incubated for 2 h at 4°C with 10 μl of anti-GLUT4 in a final volume of reaction of 0.25 ml and with mixing by rotation. The immune complexes were collected by addition of 50 μl of Protein A-Sepharose beads (100 μl of a 50% suspension of beads previously conjugated to Protein A). After 2 h incubation with end-over-end mixing at 4°C, the immunoprecipitate was pelleted by centrifugation at 12,000 × *g* for 30 s at room temperature. The resulting supernatant was carefully removed and retained. The pelleted beads were washed four times by successive resuspension and recentrifugation in PBS. The supernatant from each step was pooled with the original supernatant and finally centrifuged at 190,000 × *g* for 30 min. The pellet from this step represented the immune supernatant. The immune pellet and the immune supernatant were resuspended in Laemmli buffer [17]. In control samples, 100 μg of IM protein was treated with 10 μl of an irrelevant rabbit serum and the immunoprecipitation performed in a identical manner as that described above. Immunoblotting was performed with antibodies specific for GLUT4, the α2 and β1 subunits of the Na,K-ATPase as described in section 2.4. above.

3. Results and discussion

3.1. Membrane characterization

Membranes isolated on top of the 25% sucrose layer were enriched by ~5.5-fold in 5′ nucleotidase activity and by 2.5 fold in KpNPPase activity relative to the starting muscle homogenate (Table 1). The fold enrichments of both plasma membrane enzymes in the 25% sucrose fraction compare well with those reported originally by Klip and coworkers [14]. The specific activity of both enzymes was lower in membranes recovered on top of the 30 and 35% sucrose fractions and in the membrane pellet (Table 1) relative to those recovered from the 25% sucrose fraction indicating that the latter fraction was enriched with plasma membranes. This is in line with the finding that membranes isolated from top of the 25% sucrose fraction react against antibodies to GLUT1 and the α1 subunit of the Na,K-ATPase which have been shown to be localised to the plasma membrane [9,19]. This fraction was therefore termed the plasma membrane (PM). The 30% sucrose fraction most likely represents a mixture of PM and intracellular membranes given that both KpNPPase and 5′ Nucleotidase were measurable in this fraction (Table 1). By contrast, membranes isolated from the 35% sucrose fraction were depleted in KpNPPase and 5′ nucleotidase specific activity and have also been shown to be devoid in immunoreactive GLUT1 and α1 subunits of the Na,K-ATPase [9,12]. This fraction, however, contains both

Table 1
Marker enzyme activities of the rat muscle membrane fractions

Fraction	Protein (μg/g tissue)	5′-Nucleotidase (nmol/min/mg protein)	KpNPPase (nmol/min/mg protein)
PM (25% sucrose fraction)	30 ± 2	265 ± 38 (5.5)	50 ± 9 (2.5)
30% sucrose fraction	50 ± 4	91 ± 59 (1.8)	35 ± 5 (1.7)
IM (35% sucrose fraction)	150 ± 22	57 ± 19 (1.1)	31 ± 3 (1.5)
Membrane pellet	300 ± 31	67 ± 14 (1.3)	23 ± 9 (1.1)

Results are means ± S.E.M. of 6–9 independent muscle membrane preparations. The purification index for both enzymes in the different fractions is shown in brackets and has been calculated with respect to the specific activity found in crude unfractionated muscle membranes.

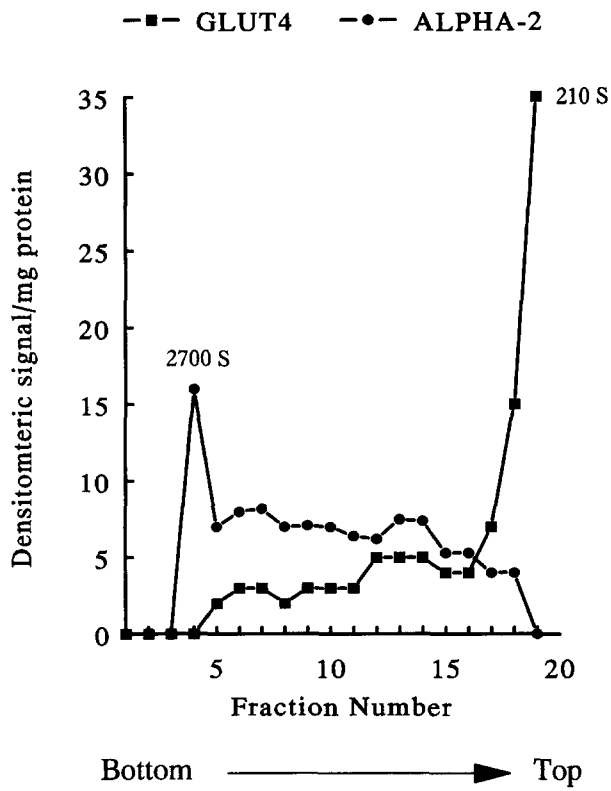

Fig. 1. Sucrose velocity grandient analyses of GLUT4 and the α2-Na,K-ATPase subunit. 0.5 mg of protein from the IM fraction was applied onto a 20–40% continous sucrose gradient and centrifuged for 20 min. Fractions were collected and analyzed for GLUT4 and α2-Na,K-ATPase by Western blotting as decribed in section 2. GLUT4 and α2-subunit immunoreactivity were quantitated and plotted as densitometric units per mg protein. The sedimentation coefficients (S) for fractions showing peak reactivity for either the α2-Na,K-ATPase subunit or GLUT4 are indicated.

GLUT4 glucose transporters and α2 subunits of the Na,K-ATPase which redistribute to the PM following insulin treatmen [12,20]. Membranes isolated from the 35% sucrose fraction most likely represent the intracellular stores of these proteins based on the observation that in the basal state this fraction contained 7.2- and 3.5-fold more GLUT4 and α2-subunits, respectively, than the PM fraction (data not shown). Since these membranes were depleted in PM markers and appear to donate GLUT4 and the α2-subunits to the PM following insulin treatment this fraction was denoted as the intracellular membrane (IM) fraction [12,20]. Membranes isolated from the IM were subsequently used either for sedimentation analyses or for immunprecipitation of GLUT4-containing vesicles.

3.2. *Sedimentation analyses of intracellular membrane (IM) vesicles*

One physical parameter which can be used to characterize biological particles is their sedimentation coefficient (or *Svedberg* (S) value) which is a measure of the velocity at which a particle sediments within a centrifugal field. We exploited likely differences in sedimentation rates between GLUT4 and α2-subunit-containing vesicles to design a sucrose velocity gradient experiment allowing GLUT4 and α2-subunit proteins to be segregated. We found that when membranes from the IM fraction were separated on a sucrose gradient, then subjected to immunoblotting, distinct immunoreactivity patterns for both GLUT4 and the α2-Na,K-ATPase subunit were observed. Fig. 1 shows a representative scanning profile of GLUT4 and α2-reactivity in the various fractions collected from the sucrose gradient. Fractions collected from the bottom half the gradient contained the bulk (96%) of the microsomal protein initially applied to the top of the gradient. The particles recovered in these fractions showed sedimentation coefficients ranging between 400 and 9,000 S as would be expected for microsomal membranes [21]. Immunoblot analyses of all the fractions collected from the sucrose velocity gradient revealed that the α2-subunit and GLUT4 peaked at opposite ends of the gradient. The peak α2-immunoreactivity was detected in the initial fractions collected from the bottom of the gradient which contained membrane particles with a sedimentation coefficient of 2,697 ± 57 S (values are means ± S.E.M. from three separate muscle preparations). By contrast, the strongest GLUT4 reactivity was observed in a fraction localized to the top of the sucrose gradient which housed a population of slow sedimenting vesicles (209 ± 4 S, values are means ± S.E.M. from three separate muscle preparations) and which were practically devoid of any α2-subunits. The observation that the peak immunoreactivities of GLUT4 and the α2-Na,K-ATPase subunit were present in membranes whose sedimentation coefficients differed by one order of magnitude but whose densities were similar may signify that the vesicular structures housing the α2-subunit may be larger than those containing GLUT4. However, no direct experimental evidence for this proposition is currently available. It is also noteworthy that the GLUT4 enriched fraction contained less than 2% of the total protein initially applied to the gradient. Our findings therefore indicate that fractionation of IM on a continuous sucrose velocity gradient had not only resolved two major peaks; one for GLUT4-containing vesicles and the other for the α2-Na,K-ATPase subunit, but that GLUT4 vesicles from the intracellular compartment had been highly purified on the basis that they were enriched by ~32-fold in GLUT4 relative to the starting IM fraction when the fractional protein content was taken into account. This finding indicates that this procedure may offer a useful means of obtaining GLUT4 vesicles in a highly enriched

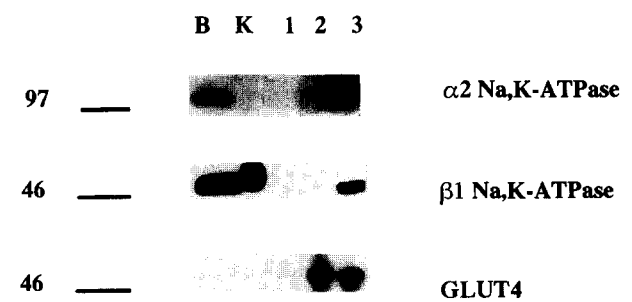

Fig. 2. Analysis of immunoprecipitated GLUT4-containing vesicles. GLUT4-containing vesicles were immunoprecipitated from the IM fraction as described in section 2. Lane 1 represents the immunoprecipitate obtained using a irrelevant control serum. Lane 2 represents immunoprecipitated GLUT4 vesicles obtained using a GLUT4 specific antibody and Lane 3 is the resulting immunosupernantant from the GLUT4 precipitation. The immunoreactivity of these samples towards antibody probes to the α2- and β1-Na,K-ATPase subunits and GLUT4 is shown. Rat brain (B) and kidney (K) microsomes (2 μg) were run in adjacent lanes as appropiate controls.

form without having to resort to immunoprecipitation techniques. However, the observation that some overlap in GLUT4 and the α2-subunit reactivity was observed in the intermediary fractions from the sucrose velocity gradient may signify some colocalization of these proteins outwith of the membranes contained within the two major peaks. To test this possibility we subsequently carried out immunoprecipitation analyses of GLUT4-containing vesicles isolated from the whole IM fraction.

3.3. *Immunoisolation of GLUT4-containing vesicles from the intracellular membrane fraction*

Lavoie et al. recently attempted to address the question of whether GLUT4-containing vesicles express the α2-Na,K-ATPase subunit by screening GLUT4 vesicles immunoprecipitated from whole muscle homogenates [22]. However, these authors found that immunoprecipitation of GLUT4 vesicles resulted in coprecipitation of the α2 subunit. The observed coprecipitation was suggested to have arisen as a result of non-specific precipitation and that due to the fractional colocalization of these proteins in the PM. Moreover, given that muscle homogenates were used for immunoprecipitation analyses and that both GLUT4 and the α2 subunit could also be precipiated by the irrelevant control serum the extent of colocalization of GLUT4 and the α2 subunit in the IM could not be determined with any strong degree of confidence.

In order to clarify this issue we immunoprecipitated GLUT4-containing vesicles from the IM pool which characteristically shows an insulin-dependent fall in GLUT4 and the α2-Na,K-ATPase subunit [9,12] using antibodies to both the α2 and β1 subunits of the Na,K-ATPase. Fig. 2 shows a representative Western blot analyses of immunoisolated GLUT4-containing vesicles. The efficiency of the GLUT4-immunoprecipitation was 45 ± 5% (mean ± S.E.M. from three experiments). We found that GLUT4 vesicles (lane 2) contained no detectable reactivity towards either the α2- or β1-subunits of the Na,K-ATPase. These proteins were, however, detectable in the immunesupernatant derived from the GLUT4 precipitation step (Fig. 2, lane 3). The use of an irrelevant control serum did not result in precipitation of GLUT4 vesicles or of subunits of the Na pump (Fig. 2, lane 1) indicating that there was no detectable non-specific precipitation of these proteins. By contrast, GLUT4, α2- and β1-subunits were fully recovered in the immunesupernatant obtained using the irrelevant serum. Given that we were unable to precipitate 100% of the IM GLUT4 we are unable to fully exclude the possibility that some α2 subunits may be colocalised with the glucose transporter. However, if we assume that the GLUT4 vesicles isolated in this study (which approximate to nearly 50%) are representative of the whole IM sample then it is highly unlikely that the observed overlap of these proteins in the intermediary fractions on the sucrose velocity gradient is due to their colocalisation. This suggestion is supported by recent electron-microscopic data showing that whilst an increase in the α2 Na,K-ATPase subunit can be shown to occur in the PM of rat muscle after insulin treatment, α2 subunits cannot be colocalised intracellularly with GLUT4 in the basal state when double-labelling methods are employed [22,23].

Trafficking of membrane proteins from intracellular organelles to the plasma membrane forms an integral part of the mechanism by which insulin acutely activates membrane events such as glucose transport and Na/K transport. Over the past few years considerable interest has focused on characterizing the intracellular compartment containing the GLUT4 glucose transporter [24,25]. Identification of proteins resident in GLUT4-containing vesicles has been taken as prima facie evidence that such proteins may play some functional role in the sorting and delivery of the GLUT4 transporter to the cell surface in response to insulin binding. Several candidate proteins have indeed been identified by this approach and these include, for example, secretory carrier associated membrane proteins (SCAMPS) [26], vesicle-associated membrane proteins (VAMPS) [27], cellubrevin [28], gp160/vp165, a glycoprotein with aminopeptidase activity [25,29] and rab4, a low molecular weight GTP-binding protein implicated in vesicular trafficking [30,31]. The GLUT4-containing vesicles investigated in this study are, we believe, of non-endosomal nature given that we have recently shown that they lack immunoreactive transferrin receptors, generally accepted as a good marker of the endosomal/PM recyclying pathway [31]. By contrast little attention has focussed on the nature and identity of the intracellular compartment housing subunits of the Na,K-ATPase: we believe that our sedimentation data provides the first report documenting biophysical aspects of the α2-subunit containing compartment and has highlighted the significant differences that are likely to exist, in terms of vesicle size, between membrane structures containing GLUT4 or the α2-Na,K-ATPase subunit, respectively. The observed difference in the sedimentation coefficients of the membranes containing these proteins is consistent with the notion that they are present in separate vesicle populations; a suggestion strengthened by the lack of α2-reactivity in GLUT4 vesicles immunoisolated from the insulin-sensitive IM compartment.

Whether the intracellular α2-subunit containing vesicles rely upon the same trafficking proteins as those involved in GLUT4 translocation remains presently unknown. Progress on this point has been hampered due to the fact that current antibodies to the α2-subunit have proved highly inefficient for use in immunoprecipitation studies and consequently the strategy that has enabled various protein components of GLUT4-vesicles to be identified cannot be used at present. Such information would clearly be of importance in establishing whether the recruitment of membrane transport proteins from different compartments, in response to insulin, depend upon a common group of proteins whose expression and regulation may have important implications for the uptake of a variety organic and inorganic nutrients under physiological and pathological circumstances.

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