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The GLUT4 glucose transporter and the α_2 subunit of the Na^+,K^+-ATPase do not localize to the same intracellular vesicles in rat skeletal muscle

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Abstract The GLUT4 glucose transporter and the α_2 subunit of the Na^+,K^+-ATPase of rat skeletal muscle are two proteins which redistribute from intracellular membranes to plasma membranes following in vivo insulin stimulation. Here we show that although both proteins co-segregate after subcellular fractionation of unstimulated rat hindlimb muscles, they do not share the same intracellular residence inside the muscle fibre. By immunogold single- and double-labeling on ultrathin muscle cryosections with specific antibodies, the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit were observed on different vesicular structures within the cell. GLUT4 was detected on subsarcolemmal and perinuclear membranes, and at the junction between myofibrillar A and I bands where triads are localized. The α_2 subunit of the Na^+,K^+-ATPase was observed at the plasma membrane and in distinct subsarcolemmal vesicles and intermyofibrillar membranes. Quantitative analysis of double-labeling of GLUT4 and Na^+,K^+-ATPase α_2 subunit revealed that less than 6% of the two proteins co-localize in the same continuous vesicular structures. The differential intracellular localization of the two proteins was further confirmed by immunopurification of GLUT4-containing membranes from muscle homogenates, in which the α_2 subunit of the Na^+,K^+-ATPase was found only at the same extent as the α_1 subunit of the enzyme, a protein exclusively present at the plasma membrane.

Key words: Immunogold labeling; Muscle membrane

1. Introduction

It is well established that insulin stimulates glucose uptake in muscle by inducing redistribution of glucose transporters from an intracellular pool to the plasma membrane [1–4]. Over the past 10 years, several glucose transporter isoforms have been identified and named GLUT1 to GLUT7 in order of their discovery [5]. We [6] and others [4] have shown by subcellular fractionation and immunoblotting using isoform-specific antibodies that the insulin-induced translocation process from internal membranes to the plasma membrane in rat skeletal muscle involves only the GLUT4 isoform. However, the intracellular residence of this protein has not been clearly defined and continues to be the subject of intense investigation. Immunocytochemical studies in skeletal muscle have shown that GLUT4 protein is present in tubulo-vesicular structures near the plasma membrane and the *trans* Golgi region [7–9], and at the triads where the transverse tubules abut the sarcoplasmic reticulum [10]. The glucose transporter has also been localized in clathrin-coated structures in brown fat [11], cardiomyocytes [12] and 3T3-L1 adipocytes [13].

Insulin rapidly increases Na^+,K^+ pump activity at the muscle cell surface [14–16] to provide for effective K^+ retrieval from the blood. The responsible enzyme, the Na^+,K^+-ATPase (EC 3.6.1.37), is composed of a catalytic α-subunit and a glycosylated β-subunit [17]. Three isoforms of the α (α_1, α_2, and α_3) and β (β_1, β_2, and β_3) subunits have been described [17,18]. Rat skeletal muscle expresses α_1, α_2, β_1, and β_2 isoforms, based on subcellular fractionation and immunoblotting with isoform-specific antibodies [19]. By this procedure, the α_1 isoform is recovered mainly in a plasma membrane-enriched fraction, whereas α_2, β_1 and β_2 isoforms are recovered both in plasma membrane and intracellular membrane fractions [19]. We have shown that the content of the Na^+,K^+-ATPase α_2 subunit is reduced in intracellular membranes and augmented in plasma membranes isolated from rat skeletal muscle following in vivo acute insulin stimulation, resembling the GLUT4 translocation process [19]. By immunoelectron microscopy, we recently detected a significant increase in the Na^+,K^+-ATPase α_2 subunit at the plasma membrane of the muscle fibre upon insulin stimulation [20]. The Na^+,K^+-ATPase α_2 subunit protein was also visualized within the muscle fibre, principally in subsarcolemmal vesicles and near the triads [20]. Whether there is any relationship, temporal or in magnitude, between the recruitment of the GLUT4 glucose transporter and of the Na^+,K^+-ATPase α_2 subunit has not been explored.

In the present study we investigated whether the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit reside in the same intracellular structures in unstimulated skeletal muscle, which could allow insulin to recruit both proteins together within same vesicles. To this end, we used quantitative immunogold double-labeling of ultrathin muscle cryosections and chemical analysis of immunopurified GLUT4-containing membranes from rat hindlimb muscles.

2. Experimental

2.1. Subcellular fractionation of rat skeletal muscle and immunoblotting

Overnight fasted male Sprague–Dawley rats (250 g) were used. Red muscles (soleus, red gastrocnemius, red rectus femoris and red vastus lateralis) and white muscles (white gastrocnemius, white rectus femoris, white vastus lateralis and tensor fasciae latae) were isolated. Membrane fractions from the separated muscle homogenates were prepared by sucrose gradient centrifugation and characterized as previously described [3,21–23]. Membranes floating atop the 25% sucrose layer (here called the 25% sucrose fraction) are enriched in plasma membranes; the 30% sucrose fraction also contains surface markers albeit to a lower degree of purity; the 35% sucrose fraction contains the internal insulin-

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regulatable pools of GLUT4 glucose transporters [3,21] and Na^+,K^+-ATPase α_2 subunits [19], and the pellet of the sucrose gradient is enriched in sarcoplasmic reticulum [3]. Muscle membrane fractions (15 μg of protein) were subjected to SDS-PAGE and immunoblotting as previously described [21] using antibodies specific to the GLUT4 glucose transporter (polyclonal antibody from East Acres Biologicals, MA) or the α_2 subunit of the Na^+,K^+-ATPase (monoclonal antibody McB2, kindly provided by Dr K. Sweadner, Harvard University). Autoradiographs of immunoblot membranes were quantitated by a PDI DNA scanner with one-dimensional gel analysis software (1.3 DISCOVERY). Scanning densitometric values were analysed by Student's *t*-test for unpaired data.

2.2. Immunogold single- and double-labeling and quantitative analysis

Fasted and anaesthetized rats were perfused with 2% paraformaldehyde in PBS as described before [20]. Soleus and white gastrocnemius muscles were excised, fixed by immersion in 2% paraformaldehyde in PBS for 2 h, infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.2) and processed for ultracryosectioning as previously described [20]. For single antigen labeling, ultrathin muscle cryosections were labeled separately with antibodies specific for the GLUT4 glucose transporter, the α_2 subunit of the Na^+,K^+-ATPase or the α_1 subunit of the enzyme, the latter using the monoclonal McK1 (kindly provided by Dr K. Sweadner), essentially following protocol 1 described in [20]. The same labeling protocol was adapted to allow for double labeling of the sections. Briefly, after labeling with anti-GLUT4 antibody and 10-nm gold-labeled Fab fragments from goat anti-rabbit IgG, sections were washed with PBS (3 × 5 min), fixed with 2% paraformaldehyde in PBS (1 × 5 min), and washed again with PBS (3 × 5 min). The remaining reactive aldehydes were blocked with 80 mM ammonium chloride (2 × 5 min) and grids were further processed for labeling with anti-Na^+,K^+-ATPase α_2 subunit monoclonal antibody and 3-nm gold-labeled Fab fragments from goat anti-murine IgG. Controls included the use of pre-immune serum or PBS/BSA instead of primary antibody. In addition, single labeling of either GLUT4 or Na^+,K^+-ATPase α_2 subunit were run in parallel to verify that double-labeling did not interfere with the recognition of each epitope. Specimens were observed through a Jeol 1200 EXII transmission electron microscope.

Quantitative analysis of double labeling was used to calculate the colocalizing GLUT4 and Na^+,K^+-ATPase α_2 subunit gold particles relative to the total particles for each protein. Images were digitized using a scanning transmission electron microscope interfaced to a Noran TN 5500 series II microanalyser, then converted to PC IFF image files and transferred to a silicon graphics image analysis work station. Grey levels were adjusted to enhance cellular ultrastructure. Images were thresholded, binarized, and the gold particles separated from the rest of the field. Gold particles were sorted by size and counted to determine the total number of 10-nm and 3-nm particles. These two particle sizes were easily distinguished even though preparation of gold conjugates implies a certain degree of variability. Binary images of the gold particles were overlayed on the original image and co-localizations manually counted. Three-nm and 10-nm gold particles were considered colocalizing if they shared the same continuous vesicular structures. Four independent muscle specimens (2 soleus and 2 white gastrocnemius) and a minimum of 25 fields per sample were analysed. The ratio of colocalizing particles to the total 3-nm or 10-nm particles was determined in each muscle sample.

2.3. Immunoprecipitation of GLUT4-containing membranes from rat skeletal muscle

GLUT4-containing membranes were immunoprecipitated from a low-speed supernatant of rat hindlimb muscle homogenates using a method described for fat cells [24] with the following modifications. Magnetic beads combined to anti-rabbit IgG (Dynal 280, Norway) were washed with buffer A (100 mM KH_2PO_4, pH 7.4) containing 10 mg/ml BSA and incubated overnight at 4°C with either 3 μl of an anti-GLUT4 antibody raised in our laboratory [25] or 3 μl of the corresponding pre-immune serum in 1 ml of buffer A containing 0.5 mg/ml BSA. Beads were then washed with buffer A before incubating with muscle samples. Muscles (1 g) were cleaned of all visible fat and nerve tissues, cut in small pieces (1 mm^3) in 5–7 ml of ice-cold buffer B (10 mM $NaHCO_3$, 0.25 M sucrose, 5 mM NaN_3 and 100 μM phenylmethylsulfonyl fluoride, pH 7.3), homogenized with a Polytron (setting 5.1) for 2 × 20 s and then with a motor-driven teflon pestle (20 strokes). The resulting homogenate was diluted with 2 ml of buffer B and centrifuged at low speed (1,200 × *g*) for 20 min. Five-hundred μg of protein of the supernatant were diluted to 1 ml of buffer A, added to antibody-laden beads, vortexed and incubated for 4 h at 4°C. The loaded beads were washed 5 times with 0.5 ml buffer A and the supernatants were pooled and centrifuged at 200,000 × *g* for 1 h at 4°C. Solubilizing sample buffer [26] (20 μl) was added to the resulting pellet ('supernatant sample') and to the washed beads ('precipitate sample') for SDS-PAGE and immunoblotting with commercial anti-GLUT4, McB2 anti-Na^+,K^+-ATPase α_2 subunit, and McK1 anti-Na^+,K^+-ATPase α_1 subunit antibodies.

3. Results and discussion

3.1. Distribution of the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit in subcellular fractions

We have previously reported the distribution of the GLUT4 glucose transporter [6] and of the Na^+,K^+-ATPase α_2 subunit [19] using pooled red and white muscle membranes from con-

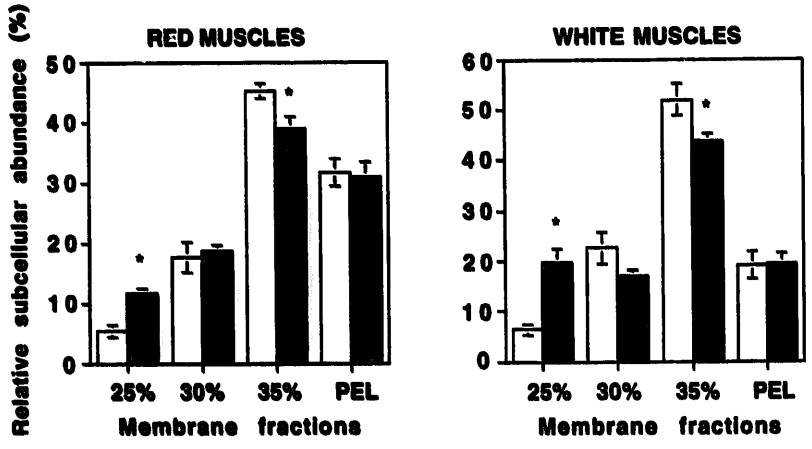


Fig. 1. Relative subcellular abundance of GLUT4 (open bars) and Na^+,K^+-ATPase α_2 subunit (cross-hatched bars) proteins after fractionation of red and white muscles. Results are from densitometric scanning values from 4 immunoblots in each kind of muscle. For each fraction (25%, 30%, 35% sucrose fractions and pellet (PEL)), the densitometric value of SDS-PAGE immunoblots detecting each protein was multiplied by the corresponding protein yield and is expressed as percentage of the total value calculated from the sum of all the fractions. Values are expressed as means ± S.E.M. *$P < 0.05$, GLUT4 vs. Na^+,K^+-ATPase α_2.

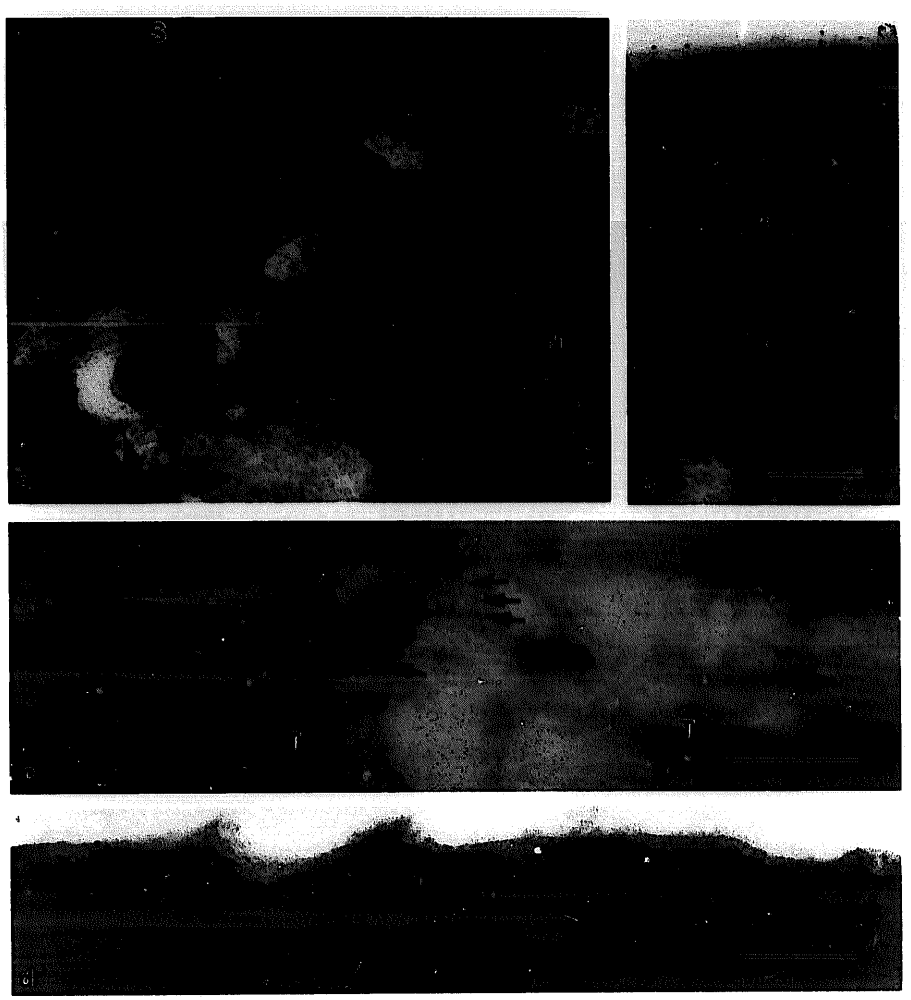


Fig. 2. Electron micrographs of ultracryosectioned white gastrocnemius muscles labeled with either anti-GLUT4, anti-Na^+,K^+-ATPase α_2 or α_1 subunits antibodies. (a) Perinuclear labeling of GLUT4 (arrowheads) using 10-nm gold-labeled goat anti-rabbit IgG (Amersham, IL) as secondary reagent. Numerous GLUT4 gold particles are seen near the nucleus (N) beneath the sarcolemma (S). (b) Sarcolemmal labeling of the Na^+,K^+-ATPase α_2 subunit (arrows) using 10-nm gold-labeled goat anti-murine IgG (Amersham, IL) as secondary reagent. (c) Intermyofibrillar labeling of the Na^+,K^+-ATPase α_2 subunit (arrows) using 3-nm gold-labeled Fab fragments from goat anti-murine IgG as secondary reagent. Na^+,K^+-ATPase α_2 gold particles are associated with the sarcotubular network. T = transverse tubules. (d) Sarcolemmal labeling of the Na^+,K^+-ATPase α_1 subunit (arrows) using 10-nm gold-labeled goat anti-murine IgG (Amersham, IL) as secondary reagent. The α_1 subunit of the Na^+,K^+-ATPase was detected only at the plasma membrane. All bars = 0.25 μm.

trol and insulin-injected rats. In the present study we reasoned that if GLUT4 and the α_2 subunit of the Na^+,K^+-ATPase are recruited together to the plasma membrane by insulin they should share the same location inside the cell prior to the action of the hormone. To this end it was important to compare first the distribution of the two proteins in separate red and white muscle membranes. Fig. 1 shows the percentages of GLUT4 and α_2 subunit proteins recovered in the membrane fractions isolated by sucrose gradient centrifugation. Whereas the overall profile of distribution was similar for red and white muscles, both proteins were more abundant in the pellet fraction isolated from red muscles than from white muscles (Fig. 1). In both muscle types, the two proteins were highly abundant in the intracellular membrane fraction (35% sucrose), accounting for several times higher yield than in the 25% and 30% sucrose fractions containing plasma membranes. Importantly, the α_2 subunit of the Na^+,K^+-ATPase was 2–3 times ($P < 0.05$) more abundant in the plasma membrane-enriched fraction (25% sucrose) and significantly less abundant in the intracellular membrane fraction (35% sucrose) than the glucose transporter. This suggests that the two proteins are differentially sorted out of the endoplasmic reticulum/Golgi apparatus to the diverse domains of the cell. Accordingly, it has been suggested that the intracellular sequestration of GLUT4 involves specific retention motifs in its amino acid sequence [27]. This analysis of the relative subcellular distribution of the GLUT4 glucose transporter and the α_2 subunit of the Na^+,K^+-ATPase does not allow, however, to assess their precise localization within the cell. Therefore, a morphological approach using immunogold double-labeling of ultrathin muscle cryosections was chosen to study directly the relative cellular localization of the two proteins.

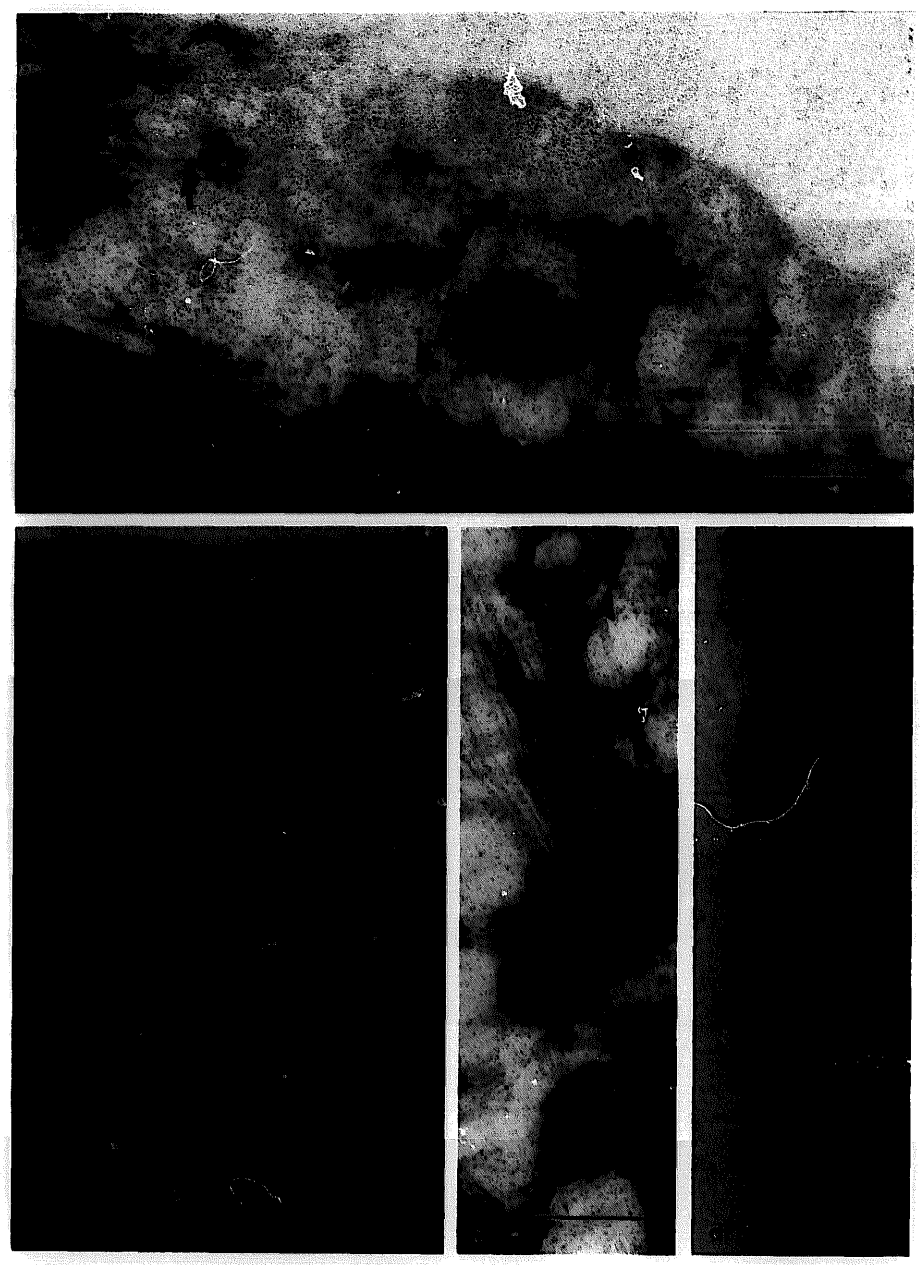
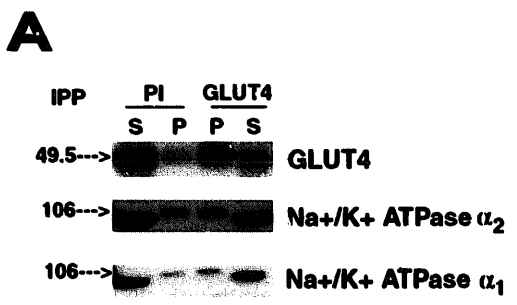


Fig. 3. Electron micrographs of ultracryosectioned soleus and white gastrocnemius muscles labeled sequentially with anti-GLUT4 (10-nm gold particles; arrowheads) and anti-Na^+,K^+-ATPase α_2 subunit (3-nm gold particles; arrows) as described in section 2. (a) Subsarcolemmal labeling of GLUT4 and Na^+-K^+-ATPase α_2 subunit in soleus muscle. There are no co-localizing particles in the field. Z = myofibrillar Z line. (b) Sarcolemmal labeling of the Na^+,K^+-ATPase α_2 subunit and intracellular labeling of GLUT4 in white gastrocnemius muscle. GLUT4 gold particles are localized at the junction between myofibrillar A and I bands. (c) Intracellular labeling of GLUT4 and Na^+,K^+-ATPase α_2 subunit in white gastrocnemius muscle. GLUT4 and Na^+,K^+-ATPase α_2 gold particles were found in different areas of the sarcotubular network. T = transverse tubules. (d) Sarcolemmal labeling of the Na^+,K^+-ATPase α_2 subunit and subsarcolemmal labeling of GLUT4 in white gastrocnemius muscle. No GLUT4 gold particle is seen at the plasma membrane. All bars = 0.25 μm.

3.2. *Immunogold labeling of the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit in ultrathin muscle cryosections*

Fig. 2 shows micrographs of ultrathin muscle cryosections labeled for separate detection of the GLUT4 glucose transporter and the α_1 and α_2 subunits of the Na^+,K^+-ATPase. GLUT4 was abundant in vesicular structures near the nucleus (Fig. 2a) and was found less extensively in deep areas of the fibre. The α_2 subunit of the Na^+,K^+-ATPase was detected at the plasma membrane (Fig. 2b) and intracellularly (Fig. 2c) consistent with our previous immunoelectron microscopy study in rat skeletal muscle [20] and immunofluorescence study in human skeletal muscle [28]. In contrast, the α_1 subunit of the enzyme, a plasma membrane marker, was detected exclusively at the



B

Antigen	Percentage immunoprecipitated with anti-GLUT4 antibody	pre-immune serum	Specific immunoprecipitation
GLUT4	65.8 ± 2.3	19.3 ± 2.0	46.5 ± 1.3
Na^+-K^+-ATPase α_2	39.6 ± 1.0	31.2 ± 1.6	8.4 ± 0.8
Na^+-K^+-ATPase α_1	28.0 ± 3.1	21.0 ± 5.5	7.0 ± 2.5

Fig. 4. Immunoprecipitation of GLUT4-containing membranes. GLUT4-containing membranes were immunoprecipitated from a low-speed supernatant of rat muscle homogenates as detailed in section 2. Supernatant (S) and precipitate (P) samples from pre-immune serum (PI) and anti-GLUT4 antibody (GLUT4) precipitations (IPP) were examined for the presence of GLUT4, Na^+,K^+-ATPase α_2 and α_1 subunits proteins by immunoblotting using East Acres anti-GLUT4 antibody, McB2 and McK1 anti-Na^+,K^+-ATPase α_2 and α_1 subunits antibodies, respectively. (A) Representative immunoblots. Numbers on the left represent the molecular weight of reference standard proteins, in kDa. (B) The percentage of pelleted GLUT4 and Na^+,K^+-ATPase α_2 and α_1 subunits was calculated from densitometric values after immunoblot scanning, dividing the signal in the precipitate sample by the sum of the signals of precipitate and supernatant samples. Percentages reported are means ± S.E.M. for 3–6 independent immunoprecipitation experiments.

plasma membrane of the fibre (Fig. 2d) and no fields were found containing intracellular immunolabeling (results not shown).

Fig. 3 shows micrographs of ultrathin cryosections of rat soleus and white gastrocnemius muscles subjected to double-labeling with anti-GLUT4 and anti-Na^+,K^+-ATPase α_2 subunit antibodies. Both GLUT4 and the Na^+,K^+-ATPase α_2 subunit were localized in the subsarcolemmal area but in different vesicular structures (Fig. 3a). The Na^+,K^+-ATPase α_2 subunit was also observed extensively at the plasma membrane, where GLUT4 was not detected (Fig. 3b and d). The subsarcolemmal labeling of GLUT4 may explain the significant recovery of the protein in the isolated 25% sucrose fraction which in all likelihood is not a pure plasma membrane fraction, in spite of its enrichment in plasma membrane markers [3,22]. This observation is in agreement with previous immunolocalization studies showing GLUT4 in subsarcolemmal vesicles near the plasma membrane and the *trans*-Golgi region in rat skeletal muscle [7–9].

The GLUT4 glucose transporter was also observed intracellularly, mostly in regions corresponding to the location of triads at the junction between myofibrillar A and I bands (Fig. 3b). The α_2 subunit of the Na^+,K^+-ATPase was also detected in deep areas of the cell but on different membranes than GLUT4 (Fig. 3c).

To strengthen these observations we performed an extensive quantitative analysis of the double-labeling as detailed in the Experimental section. Quantification of colocalizing GLUT4 and α_2 subunit particles relative to their respective total particles indicated a colocalization ranging from 0.5% to 6.0% in 4 independent muscle samples (Table 1). Taken together, these results indicate that although the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit cosegregate upon subcellular fractionation of rat skeletal muscle, they do not share the same vesicular membranes inside the muscle fibre based on morphological criteria.

3.3. *GLUT4 and Na^+,K^+-ATPase α_2 subunit in immunopurified GLUT4 membranes from rat skeletal muscle*

The potential co-localization of the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit was assessed biochemically, using immunopurified GLUT4-containing membranes from a low-speed supernatant of rat hindlimb muscle homogenates. Fig. 4A shows representative immunoblots detecting the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 and α_1 subunits in the supernatants and precipitates following immunoprecipitation of membranes with pre-immune serum or anti-GLUT4 antibody. The top immunoblot illustrates the immunoprecipitation of GLUT4-containing membranes. The middle and bottom panels illustrate the extent of comigration of the Na^+,K^+-ATPase α_2 and α_1 subunits with GLUT4 vesicles. Both α isoforms comigrated to a small extent with membranes sedimented using either pre-immune serum or anti-GLUT4 antibody. In precipitate samples, all proteins migrated somewhat slower than in the supernatants samples, probably due to the large amount of heavy chain IgG which retards overall sample migration. Quantitation of six similar but independent preparations indicated that 66% of the total GLUT4 protein was recovered in the membranes sedimented with the anti-GLUT4 antibody, compared to only 19% when using pre-immune serum (Fig. 4B). This suggests that 47% of the signal in the anti-GLUT4 immunoprecipitates is specific. Concomitantly, 8% of Na^+,K^+-ATPase α_2 subunit molecules and 7% of Na^+,K^+-ATPase α_1 subunit molecules specifically co-immunoprecipitated with GLUT4 vesicles (Fig. 4B). If it is assumed that the specifically immunoprecipitated GLUT4-con-

Table 1
Quantification of co-localization of GLUT4 and Na^+,K^+-ATPase α_2 subunit gold particles in ultracryosections of rat skeletal muscle

Muscle	GLUT4		Na,K-ATPase α_2 subunit	
	% co-localizing particles	*n*	% co-localizing particles	*n*
RM1	6.00	416	0.49	610
RM2	0.54	302	0.58	358
WM1	0.46	401	6.10	524
WM2	0.54	321	0.47	489

Ultrathin muscle cryosections were sequentially labeled with a polyclonal anti-GLUT4 antibody and 10-nm gold-labeled Fab fragments from goat anti-rabbit IgG and then with a monoclonal anti-Na^+,K^+-ATPase α_2 isoform antibody (McB2) and 3-nm gold-labeled Fab fragments from goat anti-murine IgG. Quantification of co-localizing gold particles was done in 2 red muscle (soleus) samples (RM1 and RM2) and 2 white muscle (white gastrocnemius) samples (WM1 and WM2) each providing 25 fields (see section 2 for details). Results are expressed as the percentage (%) of co-localizing particles out of the total number (*n*) of particles of the same size counted in all fields of each muscle sample.

taining membranes comprising 47% of the total GLUT4 content are representative of all the membranes containing GLUT4 protein, then it can be extrapolated that 100% of the GLUT4-containing membranes would contain 17% of the total Na^+,K^+-ATPase α_2 subunit molecules and 15% of the total Na^+,K^+-ATPase α_1 subunit molecules. At first glance these values are at odds with the immungold localization results, which showed a co-localization between the *intracellular* Na^+,K^+-ATPase α_2 subunit and GLUT4 proteins of less than 6%. The biochemical and morphological results can be reconciled, however, if it is considered that some of the precipitating GLUT4 and pump subunits may be present in membranes of plasma membrane origin. In support of this tenet is the observation that similar fractions of Na^+,K^+-ATPase α_1 and α_2 precipitated with GLUT4 protein, and by immunogold localization the Na^+,K^+-ATPase α_1 subunit was found only at the plasma membrane. It is possible, though, that a small fraction of the α_2 subunits co-localizes with the GLUT4 protein, and that tis fraction is insulin-responsive. However, the fact that about 50% of the intracellular pool of α_2 subunits translocates to the cell surface in response to insulin [19], together with the results presented in this study, argues against the possibility that the majority of the insulin-recruitable pump α_2 subunits would co-localize with the GLUT4 protein.

In summary, we have demonstrated by two different approaches that the GLUT4 glucose transporter and the Na^+,K^+-ATPase α_2 subunit do not share their intracellular location in unstimulated rat skeletal muscle. This suggests that different kind of vesicles may be involved in the insulin-stimulated redistribution of the two proteins from their respective intracellular locations to the plasma membrane. This further raises the possibility that different signals and temporary courses may affect traffic of membranes containing glucose transporters and Na^+,K^+-ATPase molecules.

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