GLUT-4 translocation in skeletal muscle studied with a cell-free assay: involvement of phospholipase D

SØREN KRISTIANSEN,1 JAKOB N. NIELSEN,1 SYLVAIN BOURGOIN,2 AMIRA KLIP,3 MICHEL FRANCO,4 AND ERIK A. RICHTER1

1Copenhagen Muscle Research Center, Department of Human Physiology, University of Copenhagen, DK-2100 Copenhagen, Denmark; 2Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du Pavillon CHUL, Sainte-Foy, Quebec G1V 4G2, 3Division of Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada; and 4Centre National de la Recherche Scientifique, Institut de Pharmacologie Moleculaire et Cellulaire, Sophia Antipolis, 06560 Valbonne, France

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Kristiansen, Søren, Jakob N. Nielsen, Sylvain Bourgoin, Amira Klip, Michel Franco, and Erik A. Richter. GLUT-4 translocation in skeletal muscle studied with a cell-free assay: involvement of phospholipase D. Am J Physiol Endocrinol Metab 281: E608–E618, 2001.—GLUT-4-containing membranes immunoprecipitated from insulin-stimulated rat skeletal muscle produce the phospholipase D (PLD) product phosphatidic acid. In vitro stimulation of PLD in crude membrane with ammonium sulfate (5 mM) resulted in transfer of GLUT-4 (3.0-fold vs. control) as well as transferrin receptor proteins from large to small membrane structures. The in vitro GLUT-4 transfer could be blocked by neomycin (a PLD inhibitor), and neomycin also reduced insulin-stimulated glucose transport in intact incubated soleus muscles. Furthermore, protein kinase Bb (PKBb) was found to associate with the GLUT-4 protein and was transferred to small vesicles in response to ammonium sulfate in vitro. Finally, addition of cytosolic proteins, prepared from basal skeletal muscle, and GTP nucleotides to an enriched GLUT-4 membrane fraction resulted in in vitro transfer of GLUT-4 to small membranes (6.8-fold vs. unstimulated control). The cytosol and nucleotide-induced GLUT-4 transfer could be blocked by neomycin and N-ethylmaleimide. In conclusion, we have developed a cell-free assay that demonstrates in vitro GLUT-4 transfer. This transfer may suggest release of GLUT-4-containing vesicles from donor GLUT-4 membranes involving PLD activity and binding of PKBb to GLUT-4.

Address for reprint requests and other correspondence: E. A. Richter, Copenhagen Muscle Research Center, Dept. of Human Physiology, Univ. of Copenhagen, 13 Universitetsparken, DK-2100 Copenhagen, Denmark (E-mail: Erichter@aki.ku.dk).

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GLUT-4-containing vesicles from large donor GLUT-4 membranes in skeletal muscle. In particular, the conversion of PIP$_2$ into PIP$_3$ by the insulin-sensitive PI 3-kinase may result in activation of GTP-exchange factors that activate ARF proteins and, thereby, PLD (10, 21). Thus insulin could activate PLD by activating ARF through formation of PIP$_3$. Indeed, insulin has been shown to activate both ARF and PLD (40, 43). Furthermore, GLUT-4-containing vesicles contain the adenosine-sensitive PI 4-kinase (23), which produces the PLD cofactor and PI 3-kinase substrate PIP$_2$. Furthermore, GLUT-4-containing vesicles isolated from adipocytes have been shown to be associated with acyl-CoA synthase (42), and the product of this synthase activity, “fatty acyl-CoA,” has been shown to be important for vesicle budding (14). Finally, GLUT-4 translocation process, we have examined the effect of insulin on the change in phospholipids in GLUT-4-containing membranes immunoprecipitated from a low-spin membrane fraction containing large membrane structures. Furthermore, we have developed an in vitro assay produced from rat skeletal muscle, by use of which we have examined the effect of stimulating PLD activity on the GLUT-4 membrane protein distribution between small and large membrane structures.

MATERIAL AND METHODS

Materials. Paramagnetic beads were from Dynal (Skøyen, Norway). The monoclonal anti-GLUT-4 IgG was from Genzyme (Cambridge, MA). The anti-spectrin IgG was from Sigma (St. Louis, MO). The B21(De3)pLyS strain was from Novagen (Madison, WI). The diethylaminoethyl-Sepharose column was from Pharmacia Biotech (Uppsala, Sweden). The Centricon-10 tubes were from Amicon (Bedford, MA). The goat polyclonal anti-GLUT-4 and rabbit polyclonal anti-ARF antibodies were from Santa Cruz (Santa Cruz, CA). The insulin and the sheep anti-protein kinase B antibody was from Upstate Biotechnology (Lake Placid, NY). The monoclonal anti-GLUT-4 IgG was from Affinity Bioreagents (Golden, CO).

Insulin stimulation. Male Wistar rats (250–300 g) were allowed unlimited access to standard rodent chow and water. Food was withdrawn 12–14 h before experiments. The abdominal cavity was opened in anesthetized rats, the portal vein was exposed, and 2 U of insulin (or saline) were injected as a bolus. After the designated time point, the hindlimb muscles were excised, quick-frozen in liquid N$_2$, and processed immediately.

Preparation of membranes. A postnuclear supernatant was used for GLUT-4 immunoprecipitation and detection of $^{32}$P-labeled lipids. It was produced by homogenization (2 × 20 s with a Polytron) of 1–2 g of basal or insulin-stimulated skeletal muscle in ice-cold buffer (20 mM NaHCO$_3$, 250 mM sucrose, 5 mM Na$_2$ATP, and 100 mM Na$_2$HPO$_4$, pH 7.4). The homogenate was centrifuged at 8,000 g for 10 min. Subsequently, 300 μg of protein from the supernatant were used for immunoprecipitation of GLUT-4-containing membranes (see Immunoprecipitation experiments).

We used two different membrane fractions for the in vitro GLUT-4 transfer from large to small membrane structures, a crude membrane fraction and an enriched GLUT-4 membrane fraction. The membrane fractions were always produced from nonfrozen rat skeletal muscle. For the preparation of crude membranes, hindlimb muscles (~8–10 g wet wt) were excised from anesthetized rats and transferred to a 2.5-fold volume (ml) of ice-cold buffer (4 mM EDTA, 0.25 mM PMSF, pH 7.0 with KOH) per gram of wet weight muscle. The muscles were homogenized immediately (1 min), and the homogenate was left on ice for 5 min and then centrifuged for 30 min at 4,000 g before the resulting postnuclear supernatant was pelleted for 1 h at 140,000 g. The crude membrane pellet was resuspended in buffer (50 mM Tris, 10% glycerol (vol/vol), 2 mM EDTA, 1 mM MgCl$_2$, pH 7.4) to 1.85 mg protein/ml final concentration. To the reaction medium was added ammonium sulfate (5 mM final concentration), and it was incubated for 45 min at 37°C before a differential centrifugation step that separates small membrane structures from fast-pelleting large membrane structures. The enriched GLUT-4 membrane fraction was produced by blending skeletal muscle for 20 s in 5× (vol/g muscle wet wt) ice-cold Tris buffer (10 mM Tris, pH 7.4) containing 1.4 M sucrose and 0.25 mM PMSF (1). The homogenate was centrifuged for 10 min at 1,500 g at 4°C. The postnuclear supernatant was added to 1 mM EDTA (final concentration) and prepared for density centrifugation (SW 41 rotor, 23,000 rpm, 2.5 h, 4°C) by overlaying the postnuclear supernatant with Tris buffer containing 1.2 and 0.8 M sucrose, respectively. The enriched GLUT-4 membrane fraction was harvested in the 0.8/1.2 M sucrose interphase. Two hundred microliters of the harvested interphase sample was resuspended in 200 μl buffer A (20 mM Tris, 25 mM KCl, 1.5 mM magnesium acetate, 100 μM ATP, and 100 μM ammonium sulfate, pH 7.4). Some preparations were stimulated by the addition of a cytosolic protein fraction (100 μl; see Preparation of the cytosolic protein fraction) and 50 μM GTP and 50 μM GTP-S (final concentrations, respectively). As a control, 100 μl of buffer B used for preparing the cytosolic protein fraction (see Preparation of the cytosolic protein fraction) were added to another preparation originating from the same harvested interphase sample. All samples were incubated together for 45 min at 37°C. The samples were cooled on ice before being mixed with 500 μl of buffer A containing 250 mM KCl, followed by a differential centrifugation step (10 min at 30,000 rpm at 4°C, rotor TLA, 120.2). The pellet contains the fast-pelleting, large membrane structures; the supernatant contains slow-pelleting, small membrane structures such as vesicles/microsomes. The enriched GLUT-4 membranes were pelleted at 100,000 rpm for 30 min. Both membrane pellets were mixed with Lactinl buffer (26) and stored at −20°C.

Preparation of the cytosolic protein fraction. Rat skeletal muscle from basal resting rats was homogenized in ice-cold buffer B (in mM: 20 HEPES, 70 KCl, 3 magnesium acetate, 2 CaCl$_2$, 1 EDTA, pH 7.4). The homogenate was centrifuged (4,000 g, 30 min), and the supernatant was spun for 1 h at 50,000 rpm (Ti 70.1 rotor). The cytosolic phase was quick-frozen in liquid N$_2$ and stored at −80°C. The cytosolic protein fraction did not contain any detectable GLUT-4 protein and was saved for no longer than 1 mo. The cytosolic protein...
fraction was thawed at 37°C and thereafter kept on ice before being added to the enriched GLUT-4 membrane preparation.

Production and purification of recombinant myristoylated ARF. The ARF1 (11) or ARF6 (12) cDNAs were cotransfected with the N-myristoyltransferase cDNA (7) in the BL21(DE3)pLysS strain of Escherichia coli and selected for chloramphenicol (34 µg/ml), kanamycin (50 µg/ml), and ampicillin (100 µg/ml) resistance. Transformed cells were grown at 37°C to 600 nm OD, and added to the myristate. At 0.6 and added to the myristate. At 600 nm OD = 1.0, protein expression was induced with the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The medium was left at room temperature overnight, whereafter the cells were harvested. After resuspension in lysis buffer (in mM: 20 Tris, 2 EDTA, 10 MgCl₂, 1 DTT, and 0.25 M glycerol), the solution was centrifuged. The supernatant was precipitated at 50% saturation of ammonium sulfate, and the precipitate was resuspended in dialysis buffer (20 mM Tris, 1 mM MgCl₂, pH 8.0) containing 10 µM GDP and dialysed overnight at 4°C against the same buffer. The preparation was applied to a diethylaminoethyl-Sepharose column equilibrated with dialysis buffer. Bound ARF was eluted with a linear gradient of 0–200 mM NaCl dissolved in dialysis buffer. Fractions containing ARF were pooled and concentrated in a Centricon-10 tube. The ARF was aliquoted, quick-frozen in liquid N₂, and stored at −80°C.

Western blotting. Frozen samples in Laemmli buffer were thawed at 37°C for 20 min, and solubilized proteins were separated on a SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane, as previously explained (23). The membranes were probed with antibodies raised against GLUT-4, PKBβ, transferrin receptor, spectrin (α₁-subunit), TGN38, or Rab4. The primary antibodies were detected with secondary antibodies conjugated with alkaline phosphatase and by the use of the ECF substrate kit (Amer sham Pharmacia Biotech). The resulting signal was detected and quantitated on a phosphoimager system (Storm 840, Molecular Dynamics).

Deoxyc glucose uptake in isolated soleus muscles. Resting rats (50 g body wt) were anesthetized with pentobarbital sodium (10 mg/100 g body wt), and soleus muscle was dissected out and trimmed of connective tissue. The muscles were incubated for 30 min in a shaking incubator at 32°C in 10 ml of Krebs-Henseleit bicarbonate buffer (KHBB) containing 10% BSA (prepared by diluting 10% BSA in KHBB dialysed against the same buffer overnight), 1 mM pyruvate, and 25 mM mannnit. All media were constantly gassed with 95% O₂–5% CO₂. After the recovery phase, the muscles were incubated for 60 min in 10 ml of the same medium added to 8 mM d-glucose and in the presence or absence of 20 mM neomycin and 100 mM insulin, respectively. The intracellular concentration of neomycin is unknown but may be considerably lower. The muscles were rinsed for 10 min in KHBB containing 32 mM mannitol and 2 mM deoxyc glucose (still in the presence or absence of neomycin and insulin) before glucose uptake was measured for 10 min by the addition of an isotopic stock solution in KHBB containing 0.1% BSA, 0.5 µCi/ml 2-³²P deoxyglucose, and 0.2 µCi/ml ¹⁴C mannnit (final concentrations). A sample of the incubation medium was saved for measurement of the specific radioactivity. The muscles were transferred to and dried on a filter paper and quick-frozen in liquid N₂. The frozen muscles were weighed, homogenized in 10% trichloracetic acid, and spun for 10 min in a high-speed centrifuge. The radioactivity in the supernatant was determined by liquid scintillation spectroscopy. The total radioactivity in the medium (specific activity) and in the muscle samples was calculated, and the radioactivity of the extracellular marker (¹⁴C mannnit) was used to calculate the intracellular content of 2-³²P deoxyglucose.

PLD activity assay. PLD activity was determined by mixing 200 µl of resuspended membranes with the PLD substrate phosphatidyl-¹³C choline, as previously explained (2). In brief, the reaction was stopped by the addition of perchloric acid, and the released enzyme product [³²P]choline was recovered in the upper waterphase after a short centrifugation step.

Statistics. All presented data are shown as means ± SE. Statistical evaluation was done by paired and unpaired t-tests with the level of significance set at P < 0.05.

RESULTS

GLUT-4-containing membranes were immunoprecipitated from a low-spin, postnuclear supernatant prepared from basal and insulin-stimulated (3 min) rat skeletal muscle. The immune pellet was incubated in the presence of phospholipid vesicles and [³²P]ATP. Extracted lipids were separated on a thin-layer chromatography plate. As shown in Fig. 1A, incubation of GLUT-4-containing membranes with lipid substrate and [³²P]ATP resulted in labeling of lipids. Interestingly, previous in vivo insulin stimulation resulted in
higher labeling of a lipid product with a migration pattern similar to that of phosphatidic acid. There was no detectable labeling of lipids in the same migration position as phosphatidic acid in the preimmune pellet, suggesting that it was a reaction specific for the GLUT-4 precipitate. This finding may suggest that PLD mediated the hydrolysis of phosphatidylcholine to phosphatidic acid, which may be converted to $^{32}$P-labeled phosphatidic acid by the phosphatidate phosphohydrolase and diacylglycerol kinase pathway (9).

To further investigate the presence of a PLD enzyme reaction, we took advantage of the specific transphosphatidyl reaction, a reaction catalyzed by PLD, whereby the phosphatidyl group of the phospholipid is transferred to a primary alcohol to produce phosphatidylethanol. As shown in Fig. 1B, adding 1% ethanol produced conversion of phospholipids into labeled phosphatidylethanol and reduced the amount of $^{32}$P-phosphatidic acid formed. Furthermore, the phosphatidylethanol accumulation was more pronounced in GLUT-4-containing membranes isolated from insulin-stimulated skeletal muscle, suggesting activation and/or binding of PLD in GLUT-4-containing membranes as a result of insulin stimulation.

In our further work, we used two different membrane preparations, a crude membrane and an enriched GLUT-4 membrane preparation. To characterize these two preparations, they were subjected to SDS-PAGE and immunoblotting with antibodies raised against spectrin (trans-Golgi membrane/skeleton marker), $\alpha_1$ subunit of the Na$^+/K^+$-ATPase (sarcolemmal marker), TGN38 (trans-Golgi membrane marker), and Rab4 (endosomal membrane marker). The signals per microgram of total protein were compared with a muscle homogenate, as shown in Fig. 2. Spectrin was recovered in all three different preparations, but it was not upregulated in the crude membrane or in the enriched GLUT-4 membrane preparation compared with the homogenate. An $\alpha_1$-signal was also found in all three different preparations, but with no significant difference between homogenate and crude membrane. However, the concentration of $\alpha_1$ subunit was significantly lower in the enriched GLUT-4 membrane preparation. The TGN38 signal was significantly lower in the crude membrane preparation compared with the muscle homogenate and was almost undetectable in the enriched GLUT-4 membrane preparation. Finally, the Rab4 signal was significantly higher in the crude membrane compared with the homogenate and the enriched GLUT-4 membrane preparation.

Because PLD is known to be involved in the formation and budding of post-Golgi vesicles, we were interested in determining whether PLD activation could lead to formation of GLUT-4-containing vesicles from large membrane structures containing GLUT-4. First, we used the isolated crude membrane fraction from basal rat skeletal muscle. Because we did not know which might be the physiological activator for PLD in skeletal muscle, we used low concentrations of ammonium sulfate to activate PLD in the crude membrane preparation (31). To the crude membranes a lipid mix containing $[^3H]$phosphatidylcholine was added in pres-
ence and absence of ammonium sulfate. The radioactivity in the water phase containing soluble \([3H]\)choline was significantly higher in the presence of ammonium sulfate (5,347 ± 66 vs. 6,645 ± 231 cpm; \(n = 4\), \(P < 0.05\), control vs. ammonium sulfate), suggesting that ammonium sulfate did stimulate PLD-mediated hydrolysis of \([3H]\)phosphatidylcholine. Interestingly, when the control and ammonium sulfate-stimulated crude membranes were separated by density-gradient centrifugation into 20 different membrane fractions, PLD activation with ammonium sulfate coincided with a massive transfer of GLUT-4 protein, as well as of the transferrin receptors, from large membrane structures to small membrane structures (Fig. 3). The in vitro transfer of GLUT-4 and transferrin receptors was not a consequence of a bulk flow of mass protein, since the protein concentrations in the fractions treated with ammonium sulfate were similar to those in the control fractions judged by Ponceau S staining of the PVDF membrane used for immunoblotting (data not shown) and by measuring the total protein concentration in the 20 different fractions (data not shown).

Instead of producing 20 different fractions by density centrifugation (as shown in Fig. 3), we next used a quick differential centrifugation step to produce two fractions, a supernatant containing small membrane structures and a pellet containing large membrane structures. We then undertook a number of experiments to examine whether the in vitro GLUT-4 translocation from large to small membrane structures could be an artifact. First, the in vitro GLUT-4 transfer could be induced with very low concentrations of ammonium sulfate known not to precipitate proteins, as shown in Fig. 4A. In particular, if the present observations were due to precipitation of membrane structures, it would cause pelleting of the GLUT-4 protein and not transfer to light membrane structures as in the present data. We also measured the protein content of spectrin in the two fractions in the control situation and after ammonium sulfate stimulation. As with the total protein content, we did not observe any change in the spectrin content.

Fig. 3. A crude membrane preparation was incubated in the absence (control) and presence of ammonium sulfate (which causes liberation of choline, a product of phospholipase D (PLD) enzymatic activity) before the membrane structures were separated into small and large membrane structures by density-gradient centrifugation. The reaction mixtures were loaded on the tops of 2 separate tubes containing a 10–30% (wt/vol) nycodenz gradient and centrifuged at 40,000 g (SW 41 rotor, 2 h, 4°C). Twenty different membrane fractions were harvested, and the content of the GLUT-4 transporters and the transferrin receptors was determined by Western blotting. One experiment is shown, representative of 1 experiment.

Fig. 4. Dependency of the in vitro transfer of GLUT-4 protein from large to small membrane structures by ammonium sulfate and magnesium concentrations. A: crude membrane preparations were incubated for 45 min at 37°C with 0 (control), 0.05, 0.5, and 5.0 mM final concentrations of ammonium sulfate, followed by a differential centrifugation step. GLUT-4 protein (open bars, left y-axis) and total membrane protein (filled bars, right y-axis) concentrations were determined in the fractions containing small membrane structures. *\(P < 0.05\) vs. control, means ± SE; \(n = 4\). B: crude membrane preparations were incubated for 45 min at 37°C in the presence (filled bars) or absence (open bars) of 5.0 mM ammonium sulfate and 0 mM MgCl₂ (control), 1 mM MgCl₂ + 2 mM EDTA (low Mg), or 1 mM MgCl₂ (high Mg), as indicated. The GLUT-4 protein concentration was determined in supernatant containing small membrane structures obtained by a differential centrifugation step. *\(P < 0.05\) vs. control, means ± SE; \(n = 4\).
content in the large membrane structures (control: 1.89 ± 0.2 arbitrary units; n = 4) or in the small membrane structures (control: 1.34 ± 0.1 arbitrary units; n = 4). Furthermore, the in vitro GLUT-4 transfer was sensitive to the magnesium concentration (Fig. 4B). Only absence and micromolar concentrations but not high concentrations (1 mM) of magnesium resulted in GLUT-4 membrane transfer from large to small vesicle structures. If the in vitro GLUT-4 formation of vesicles were a physical artifact, it would not likely depend on the magnesium concentration. Second, the in vitro GLUT-4 transfer could be blocked by binding of polyphosphatidylinositolides by neomycin (a PLD inhibitor), as shown in Fig. 5A. In addition, insulin-stimulated glucose transport in intact soleus was reduced by neomycin (Fig. 5B), suggesting that PLD activity is important for insulin-induced glucose transport and, thereby, GLUT-4 translocation in intact muscle. Taken together, the congruence of findings in vitro and in situ appears to rule out that the in vitro GLUT-4 transfer could be an artifact and strongly supports the involvement of PLD.

Furthermore, we wanted to find proteins that might be specifically associated with the in vitro GLUT-4 protein transfer. We validated a method by immunoprecipitating GLUT-4-containing membranes from a crude membrane preparation with beads coated with anti-GLUT-4 IgG. The proteins on the beads were eluted and subjected to a GLUT-4 Western blot. We found no signal in lanes corresponding to beads coated with preimmune IgG or with the coating IgG alone (data not shown). Thus the immunoprecipitation was specific for GLUT-4, and no cross-reactivity between IgGs could be detected. Moreover, no GLUT-4 signal could be detected in a PBS + 1% Triton X-100 eluate of beads coated with the GLUT-4 specific antibody. When the same beads were eluted the second time with Laemmli buffer containing 10 mM β-mercaptoethanol, a GLUT-4 signal appeared. Taken together, GLUT-4 proteins could be eluted with Laemmli buffer containing β-mercaptoethanol but not by washing in PBS containing 1% Triton X-100. Consequently, only proteins directly bound to GLUT-4 proteins can be detected in a Laemmli eluate after extensive washing of beads with PBS + 1% Triton X-100. This is because all other proteins found in the membrane but not directly bound to GLUT-4 are dissolved and washed away. Using this method, we immunoprecipitated the GLUT-4 protein from the crude membrane preparation with or without ammonium sulfate. After extensive washing of the Dynal beads with PBS + Triton X-100, the beads were thus eluted with Laemmli buffer + β-mercaptoethanol and subjected to SDS-PAGE. Total protein content was visualized by silver staining. Interestingly, a faint signal (50–60 kDa) appeared as a specific result of the PLD activation with ammonium sulfate (data not shown). By Western blotting, it was validated that the protein had a migration pattern different from that of the GLUT-4 protein. One possible candidate could be the 57-kDa PKBβ, because it has been found in adipocytes that PKBβ is found to bind to the GLUT-4 protein in response to insulin. Given that the 55- to 60-kDa protein was directly bound to GLUT-4, in vitro transfer of GLUT-4 must also result in transfer of the unknown protein. As shown in Fig. 6, ammonium sulfate, which resulted in in vitro GLUT-4 transfer, also resulted in PKBβ transfer from large to small membrane structures. Finally, we were able to communiosolate PKBβ from the crude membrane preparation with beads coated with the GLUT-4 specific antibody.
membrane preparation with beads coated with anti-spectrin IgG also resulted in the release of GLUT-4 protein from the beads (Fig. 8). Furthermore, the in vitro GLUT-4 protein transfer from the enriched GLUT-4 membrane preparation could be blocked with neomycin as well with the cysteine-alkylating agent N-ethylmaleimide (Fig. 9).

The most potent physiological activators of PLD are small GTP-binding proteins belonging to the ARF family (2). We hypothesized that ARF proteins could induce GLUT-4 transfer by activating PLD. We produced and isolated recombinant myristoylated ARF1 in E. coli. Protein expression in the presence of [3H]myristate resulted in induction of a major protein in the 21-kDa range (data not shown). Purification of this protein led to a copurification of 3H activity with the 21-kDa protein, suggesting both expression and in situ myristoylation of ARF1.

As shown in a representative immunoblot of the vesicle GLUT-4 content (Fig. 10), adding myristoylated ARF1 to the enriched GLUT-4 membrane alone, to
GLUT-4 membrane with GTP nucleotides, or to GLUT-4 membrane with nucleotides and cytosol did not result in an in vitro GLUT-4 transfer higher than the effect of cytosol alone. Furthermore, myristoylated ARF1 was not able to induce GLUT-4 transfer in the crude membrane preparation (data not shown). The same lack of effect on the in vitro GLUT-4 transfer was observed with ARF6 (data not shown). Thus neither ARF1 nor ARF6 was able to induce in vitro GLUT-4 transfer from large to small membrane structures to any greater extent than cytosol alone. Finally, adding cytosol together with an ARF1-inhibitory peptide to the enriched GLUT-4 membrane preparation did not inhibit the GLUT-4 transfer compared with the effect of cytosol alone (data not shown).

DISCUSSION

We have investigated whether PLD may be important for mobilization or formation of small GLUT-4-containing vesicles from large donor GLUT-4-containing membranes in skeletal muscles. This was examined by using several different methods. First, we showed that PLD stimulated production of labeled lipids in GLUT-4-containing vesicles isolated from a low-spin, postnuclear supernatant from basal and insulin-stimulated muscles by use of a unique PLD reaction. Second, studies showed a GLUT-4 transfer from a crude membrane preparation to small membrane structures under conditions shown to activate PLD. The transfer of GLUT-4 from large to small membrane structures resulted in a concomitant transfer of two different proteins, PKB and the transferrin receptors, proteins known to be closely associated with GLUT-4-containing membranes (3, 19, 25, 34). Third, neomycin, a PLD inhibitor, also inhibited the insulin-stimulated increase in glucose transport in incubated skeletal muscle. Fourth, addition of cytosol and GTP/GTPγS to an enriched GLUT-4 membrane fraction as well as the same preparation subjected to anti-spectrin beads also led to GLUT-4 transfer from large to small membrane structures. This in vitro GLUT-4 transfer was also inhibited by neomycin. Collectively, these data strongly suggest that, in skeletal muscle, activation of PLD may lead to mobilization or formation of small GLUT-4-containing vesicles from donor membranes originating from the larger membrane structures. It should be emphasized that the present data do not determine whether the GLUT-4 transfer from small to large membrane structures is due to vesiculation/budding or release of preformed vesicles from a donor compartment. Such data can be obtained only with electron microscopy in combination with GLUT-4 labeling of the membrane in which the process takes place.

Fig. 8. An enriched GLUT-4 membrane preparation was isolated from basal rat skeletal muscle and subjected to immunoprecipitation with Dynal paramagnetic beads coated with anti-spectrin IgG. The beads were incubated for 45 min at 37°C in the presence (+) or absence (-) of a cytosolic protein fraction and GTP/GTPγS nucleotides. Proteins in SN were separated from P, followed by GLUT-4 Western blotting. Shown is 1 experiment, representative of 3 individual experiments.

Fig. 9. An enriched GLUT-4 membrane preparation was resuspended and incubated for 45 min at 37°C in the absence (open bar) or presence (filled bars) of a cytosolic protein fraction and GTP/GTPγS nucleotides alone or with cytosol/nucleotides and neomycin (a PLD inhibitor) or with cytosol/nucleotides and N-ethylmaleimide (NEM), as indicated. The preparations were differentially centrifuged to separate small from large membrane structures. The GLUT-4 protein content in the small membrane structure fraction is shown in arbitrary units. Data are means ± SE; n = 6. *P < 0.05.

Fig. 10. An enriched GLUT-4 membrane preparation was isolated from basal rat skeletal muscle. Membranes were resuspended in the absence (control) or presence of a cytosolic protein fraction and nucleotides, cytosol, and nucleotides plus 100 μM murine ADP ribosylation factor (mARF1), or nucleotides plus mARF1, or mARF1 alone, as indicated. The preparations were incubated for 45 min at 37°C before small membrane structures were separated from large membrane structures by a differential centrifugation step. Small membrane proteins were subjected to SDS-PAGE and immunoblotting for determination of GLUT-4 protein content, as shown by a representative GLUT-4 Western blot. Shown are 2 experiments, representative of 8 individual experiments.
The measured PLD activity is very modest in the present study. This may be considered a problem. However, when exogenous substrate is offered to the PLD enzyme, low activity seems to be a general finding. In particular, in skeletal muscle and L6 myotubes, only ~1% of the offered phosphatidylcholine substrate was converted to free choline (20, 43). In contrast, when the endogenous lipid pool can be filled up with labeled phosphatidylcholine, a much higher PLD activity can be observed (33). Thus the measured PLD activity may not truly reflect the PLD-mediated conversion of the endogenous substrate.

A limitation of the use of an in vitro assay is the inherited homogenization of the tissue before the isolation of the donor membrane preparation. The homogenization may cause cross-contamination of membranes, which makes conclusions regarding the origin of the donor organelle speculative. The majority of GLUT-4 is associated with the trans-Golgi network/tubulovesicular structures (35), and so is the PLD enzyme (16). We have chosen a protocol to isolate an enriched GLUT-4 membrane fraction that has been used extensively by several independent research groups to isolate Golgi membrane structures (1) and have observed GLUT-4 transfer from this fraction to smaller membrane structures. Membrane marker analysis of the enriched GLUT-4 membrane and the crude membrane fractions showed that both membrane fractions are not enriched in the sarcosomial membrane marker α-subunit of the Na⁺/K⁺-ATPase, but the crude membrane may contain intracellular membrane compartments of endosomal origin and, to some degree, also Golgi membranes. The enriched GLUT-4 membrane fraction contained spectrin, but no enrichment in TGN38 or Rab4. Interestingly, spectrin has previously been reported to be associated with GLUT-4 proteins in 3T3-L1 adipocytes (44). To further substantiate our GLUT-4 in vitro transfer, we immunoprecipitated GLUT-4 proteins with beads coated with anti-spectrin (15) and also found transfer of GLUT-4 when fractions were separated by another separation technique (magnetic forces vs. centrifugation).

With the use of cell-free assays, a general finding is the requirement for cytosolic proteins for the PLD-mediated vesicle budding to occur. The required cytosolic factors are typically considered to be coat proteins (37), soluble N-ethylmaleimide-sensitive factors and/or its corresponding attachment proteins (5, 37), and ARFs (2, 24, 27, 41). Activation of GLUT-4 transfer by cytosolic factors in our assay may also suggest a requirement for an N-ethylmaleimide-sensitive protein (22, 45). Interestingly, adding N-ethylmaleimide completely blocked the cytosol-induced GLUT-4 transfer, consistent with a possible requirement for an N-ethylmaleimide-sensitive factor. However, N-ethylmaleimide is a cysteine-alkylating agent and therefore has a broad range of action; nevertheless, the cytosol may contain the protein(s) required for GLUT-4 budding. This protein is probably not the insulin receptor substrate-associated PI 3-kinase, because wortmannin did not block the in vitro GLUT-4 mobilization (data not shown). The finding, however, does not exclude the possibility that PI 3-kinase is part of the upstream insulin-signaling pathway leading to GLUT-4 mobilization. More likely, this finding suggests that the process that is facilitated by cytosolic proteins lies down-stream from the PI 3-kinase reaction. Isolation and characterization of such cytosolic proteins are now under investigation in our laboratory.

There is considerable interest in the possible involvement of an ARF-sensitive PLD in the GLUT-4 mobilization process (6, 8). Furthermore, ARF proteins are important regulators of membrane protein traffic, and ARF1 has been found to increase in streptozotocin-treated adipocytes (39), whereas introduction of ARF6 peptides has been found to block GLUT-4 translocation in adipocytes (29). Thus we wanted to investigate whether ARF1 or ARF6 could lead to in vitro transfer of GLUT-4. However, addition of ARF1 or ARF6 in the presence and/or absence of cytosol did not further increase the rate of GLUT-4 transfer. This may, of course, suggest that ARF1 and/or ARF6 are not important for GLUT-4 transfer. Alternatively, endogenous ARF proteins may be present in the cytosol and membrane, and adding exogenous ARF1 or ARF6 does not make any difference for the GLUT-4 transfer and/or budding. However, addition of an ARF1-inhibitory peptide (18) did not block the cytosol-mediated GLUT-4 transfer from the donor membrane (data not shown). Taken together, the present data may suggest that ARF1 and ARF6 do not play a role, at least in this assay, in the formation/mobilization of GLUT-4-containing vesicles. This interpretation must be made with care, because the isolated membrane and/or cytosol may contain other factors required for GLUT-4 budding, or the PLD in question may be a type that is not ARF sensitive. Finally, a cell-free assay may not fully reflect a biological system as an intact skeletal muscle, but the assay can be used as a tool to find potential important proteins, which must be later experimentally tested in other systems.

In conclusion, we have observed production of labeled lipids in GLUT-4-containing vesicles, which is most likely related to PLD activity. In vitro activation of PLD in crude membranes resulted in in vitro transfer of GLUT-4 proteins to small membrane structures. The GLUT-4 transfer could be blocked by the PLD inhibitor, neomycin, which also reduced insulin-stimulated glucose transport in incubated rat soleus muscle. We suggest that PLD is involved in the release or budding. However, addition of an ARF1-inhibitory peptide (18) did not block the cytosol-mediated GLUT-4 transfer from the donor membrane (data not shown). The finding, however, does not exclude the possibility that PI 3-kinase is part of the upstream insulin-signaling pathway leading to GLUT-4 mobilization. More likely, this finding suggests that the process that is facilitated by cytosolic proteins lies down-stream from the PI 3-kinase reaction. Isolation and characterization of such cytosolic proteins are now under investigation in our laboratory.

Dr. Sonia Paris, Centre National de la Recherche Scientifique, Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, Paris, France, and Dr. Jeffrey I. Gordon, Dept. of Biological Chemistry and Medicine, Washington University School of Medicine, St. Louis, MO, are gratefully acknowledged for providing the ARF1/ARF6 and NMT cDNA constructs, respectively. Dr. Bo Falck Hansen, Novo Nordisk, Bagsværd, Copenhagen, Denmark, is gratefully acknowledged for the gift of the antibody against PKBβ.
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