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

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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 transport of 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 the percentage of GLUT-4 in intact incubated soleus muscles. Furthermore, protein kinase B (PKB), which is involved in glucose transport; adenosine diphosphate ribosylation factors; protein kinase B; neomycin

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GLUT-4-containing vesicles from large donor GLUT-4 membranes in skeletal muscle. In particular, the conversion of PIP2 into PIP3 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 PIP3. 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 PIP2. 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 is stimulated by the nonhydrolyzable GTP analogue GTPγS (19), indicating the involvement of a GTP-binding protein. Taken together, these results suggest that GLUT-4-containing vesicles may be formed as a result of PLD activation. To study the GLUT-4 mobilization 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 BL21 (DE3) pLysS 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-Rab4 antibodies were from Santa Cruz (Santa Cruz, CA). The insulin and the sheep anti-protein kinase Bα (PKBα) IgG were from Novo Nordisk (Bagsværd, Denmark). The rabbit anti-transferrin receptor IgG was from Zymed Laboratories (San Francisco, CA). The anti-α1-ATPase (α1-subunit) antibody was from Upstate Biotechnology (Lake Placid, NY). The monoclonal anti-TGN38 antibody 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 food was withdrawn 12–14 h before experiments. The abdominal cavity was opened in anesthetized rats, the portal capsule was excised, quick-frozen in liquid N2, and stored at −80°C. The 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 100.2). The pellet contains the fast-pelleting, large membrane structures; the supernatant contains slow-pelleting, small membrane structures such as vesicles/microsomes. The supernatants were pelleted at 100,000 rpm for 30 min. Both membrane pellets were mixed with Laemmli 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 CaCl2, 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 quickly frozen in liquid N2 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.

**Immunoprecipitation experiments.** Paramagnetic beads were coated with monoclonal anti-GLUT-4 IgG or anti-spectrin, as described previously (23). For the detection of labeled lipids in GLUT-4-containing membranes, the membranes were immunosorbed with a monoclonal anti-GLUT-4 antibody from a low-spin, postnuclear supernatant (23) (see Preparation of membranes) and transferred to 50 μl of buffer [in mM: 50 HEPES, 80 KCl, 3 EGTA, 1 dithiothreitol (DTT), 3 MgCl2, 1 CaCl2, pH 7.4]. This solution was mixed with 10 μl of sonicated phospholipid vesicles made in PI buffer (10 mM HEPES, 1 mM EGTA, pH 7.4), 1 mM ATP, and 1 μCi [32P]ATP (final concentrations). The samples were incubated in the presence of 300 μM adenosine and 300 nM wortmannin to inhibit any possible interference from PI 4-kinase and/or PI 3 kinase activities (23). The samples were incubated for 30 min at 37°C with constant shaking before the lipids were extracted and separated as explained previously (23). The [32P]-labeled lipids were detected on a phosphoimager system (Molecular Dynamics). For the detection of PKBα associated with GLUT-4 protein, the crude membrane preparation was resuspended in PBS in the presence (stimulated) or absence (control) of 5 mM ammonium sulfate and 1 mM MgCl2. After an overnight end-over-end rotation at 4°C with beads coated with anti-GLUT-4 IgG or preimmune IgG, the beads were isolated and washed extensively for 1 h in PBS containing 1% (vol/vol) Triton X-100. The supernatant was pelleted for 30 min at 50,000 rpm (TLA 20.2). Both pellets were solubilized in Laemmlı buffer with β-mercaptoethanol before being loaded on an SDS polyacrylamide gel. For the in vitro release of GLUT-4 vesicles from Dynal beads coated with anti-spectrin IgG, 200 μl of nonfrozen enriched GLUT-4 membrane preparations were diluted in 700 μl of buffer A containing 50 μM GTP and 50 μM GTP-γ-S. The samples were added to 100 μl of cytosolic proteins in buffer B (stimulated) or 100 μl buffer B (control). The beads were rotated at 37°C for 45 min before the beads and supernatant were separated. The GLUT-4 protein content was measured by Western blotting in the supernatant and in the eluate of the beads, as explained below.

**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 = 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 PMSF and (in μM) 100 aprotinin, 100 benzamidine, 10 leupeptin, 10 pepstatin A, and 50 GDP and 10 mg/ml lysozyme, pH 7.4], 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, PKBα, 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).

**Deoxyglucose 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 0.1% BSA (prepared by diluting 10% BSA in KHBB dialyzed against the same buffer overnight), 1 mM pyruvate, and 25 mM mannositol. 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 mannositol and 2 mM deoxyglucose (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-[3H]deoxyglucose, and 0.2 μCi/ml [14C]mannositol (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% trichloroacetic 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]mannositol) was used to calculate the intracellular content of 2-[3H]deoxyglucose.

**PLD activity assay.** PLD activity was determined by mixing 200 μl of resuspended membranes with the PLD substrate phosphatidyl-[3H]choline, as previously explained (2). In brief, the reaction was stopped by the addition of perchloric acid, and the released enzyme product [3H]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...
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/cytoskeleton marker), α1 subunit of the Na+/K+-ATPase (sarcosomal 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 upconcentrated in the crude membrane or in the enriched GLUT-4 membrane preparation compared with the homogenate. An α1-subunit signal was also found in all three different preparations, but with no significant difference between homogenate and crude membrane. However, the concentration of α1-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-

Fig. 1. Formation of 32P-labeled lipids in immunoprecipitated GLUT-4-containing membranes. In brief, GLUT-4 containing membranes (GLUT-4) or control preimmune (IgG) were immunoprecipitated from a low-spin, postnuclear supernatant prepared from basal (B) or in vivo insulin (I)-stimulated (3 min) rat skeletal muscle. A: washed immune pellets were sonicated in the presence of [32P]ATP, ATP, a mix of phosphatidylinositols (PI), 300 μM adenosine (a PI 4-kinase inhibitor), and 300 μM wortmannin (a PI 3-kinase inhibitor) and incubated for 30 min at 37°C. Lipids were extracted by addition of chloroform-methanol-HCl, and 32P-labeled assay products were analyzed by TLC. Radioactivity on the TLC plate was transferred to a primary alcohol to produce phosphatidylethanol and reduced the amount of [32P]phosphatidic acid. 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.

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 phosphatidylincholine to phosphatidic acid, which may be converted to 32P-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 transphosphatidylation 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 32P-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.

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Fig. 2. Membrane marker analysis of the crude membrane (filled bars) and enriched GLUT-4 membrane (hatched bars) preparations compared with a total muscle homogenate (open bars). Proteins from the 3 different preparations were separated by SDS-PAGE and immunoblotted with antibodies raised against spectrin, α1-subunit (Na+/K+-ATPase), TGN38 (trans-Golgi membrane marker), and Rab4. The density of each signal was quantitated and expressed relative to the total amount of protein loaded in each lane on the gel.

*P < 0.05; n = 4.
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 ± 12 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

![Diagram](http://ajpendo.physiology.org/)

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 vs. ammonium sulfate: 1.77 ± 0.2 arbitrary units; n = 4) or in the small membrane structures (control: 1.34 ± 0.1 vs. ammonium sulfate: 1.53 ± 0.2 arbitrary units; n = 4). Furthermore, the in vitro GLUT-4 transfer was sensitive to the magnesium concentration (Fig. 4B). Only absence and micromolar concentration (Fig. 4B) GLUT-4 transfer was sensitive to the magnesium concentration. Second, the in vitro GLUT-4 transfer could be blocked by binding of polyphosphatidylinositides 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 (3, 25). 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 communioslate PKBβ from the crude

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**Fig. 5. Effect of the PLD inhibitor neomycin on in vitro GLUT-4 protein transfer and 2-deoxyglucose uptake in incubated soleus muscle.**

A: a crude membrane preparation was incubated for 45 min at 37°C in the absence [control (C)] and presence of ammonium sulfate (AM) or ammonium sulfate + 1 mM neomycin (AM+N). After a differential centrifugation step, the GLUT-4 protein concentration in the small membrane structure fraction was determined by Western blotting (top), and the visualized signals were quantitated (bottom). *P < 0.05, means ± SE; n = 4. B: basal (B) and insulin-stimulated (I) 2-[3H]deoxyglucose uptake were determined in incubated rat soleus muscle in the absence (B or I) or presence (B+N or I+N) of neomycin. Data are means ± SE of 5 independent experiments. †P < 0.05 vs. B; ††P < 0.05 vs. I.
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 and GTP nucleotides. 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/GTP_\gamma S 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.
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 were not enriched in the sarcosomal 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 downstream 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, 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.

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