Phosphatidylinositol 4-kinase, but not phosphatidylinositol 3-kinase, is present in GLUT4-containing vesicles isolated from rat skeletal muscle

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Insulin stimulates the rate of glucose transport into muscle and adipose cells by translocation of glucose transporter (GLUT4)-containing vesicles from an intracellular storage pool to the surface membrane. This event is mediated through the insulin receptor substrates (IRSs), which in turn activate phosphatidylinositol (PI) 3-kinase isoforms. It has been suggested that insulin causes attachment of PI 3-kinases to the intracellular GLUT4-containing vesicles in rat adipose cells. Furthermore, it has also been shown that GLUT4-containing vesicles in adipose cells contain a PI 4-kinase. In the present study we investigate whether GLUT4-containing vesicles isolated from rat skeletal muscle display PI 3-kinase and/or PI 4-kinase activities. Insulin stimulation caused a rapid increase (5–15-fold increase compared with control) in the intracellular cytosolic IRS-1-associated PI-3 kinase activity. This PI 3-kinase activity was also present in a membrane preparation containing the insulin-regulatable pool of GLUT4 transporters. However, when GLUT4-containing vesicles were isolated by immunoprecipitation from basal and insulin-stimulated (3 min) skeletal muscle, the vesicles displayed PI 4-kinase, but not PI 3-kinase, activity. Insulin did not regulate the PI 4-kinase activity in the GLUT4-containing vesicles. In conclusion, GLUT4-containing vesicles from rat skeletal muscle contain a PI 4-kinase, but not a PI 3-kinase. It is suggested that, in skeletal muscle, insulin causes activation of the IRS/PI 3-kinase complex in an intracellular membrane compartment associated closely with the GLUT4-containing vesicles, but not in the GLUT4-containing vesicles themselves.

INTRODUCTION

Insulin binding to its receptor causes autophosphorylation of the receptor by the intrinsic receptor tyrosine kinase, leading to binding and activation of intracellular insulin receptor substrates (IRSs). Phosphorylation of phosphotyrosine residues on the IRS proteins creates binding sites for several Src homology 2 (SH2) proteins [1] such as the phosphatidylinositol (PI) 3-kinase complexes [2]. The PI 3-kinase complex consists of 2 subunits: a catalytic subunit that phosphorylates phosphoinositides at the D3 position of the inositol ring; and an adaptor component that, on the one hand, binds to IRS via its SH2 domain and, on the other hand, binds to the PI 3-kinase catalytic subunit. The increase in PI 3-kinase activity may be part of the mechanism leading to translocation of insulin-responsive glucose transporter (GLUT4)-containing vesicles to the surface membrane in skeletal muscle and adipose tissue. This is because first, the PI 3-kinase inhibitor wortmannin blocks insulin-stimulated muscle glucose uptake [3–5]. Secondly, insulin does not stimulate glucose uptake in cells microinjected with a dominant negative mutant of the p85 adaptor subunit, which fails to bind the catalytical p110 subunit [6,7]. Thirdly, overexpression of a constitutively active form of PI 3-kinase and IRS stimulates GLUT4 translocation in rat adipose cells [8,9]. Finally, the PI 3-kinase activity was shown to increase in intracellular GLUT4-containing vesicles after a few minutes of insulin stimulation in 3T3-L1 adipocytes [10]. Taken together, these findings [3–10] suggest that insulin could recruit the intracellular GLUT4-containing vesicles in muscle and adipose cells via activation of a PI 3-kinase associated with the GLUT4-containing vesicles [10]. Interestingly, it has also been reported that the GLUT4-containing vesicles isolated from rat adipocytes contain a PI 4-kinase [11]. Consequently, it is suggested that GLUT4-containing vesicles isolated from adipose cells contain both a PI 3-kinase and a PI 4-kinase, and that the PI 3-kinase, but not the PI 4-kinase, is activated by insulin [10,11]. It is not known whether a PI 3-kinase and/or a PI 4-kinase are associated with GLUT4-containing vesicles isolated from rat skeletal muscle. Hence, the present study was carried out to determine whether GLUT4-containing vesicles isolated from rat skeletal muscle display PI 3-kinase and/or PI 4-kinase activity and whether this activity could be regulated by insulin.

EXPERIMENTAL

Materials

Reagents for SDS/PAGE and immunoblotting were from Bio-Rad (Mississauga, ON, Canada). Human insulin was obtained from Eli Lilly Co. (Scarborough, ON, Canada). α-L-PI was from Avanti Polar Lipids Inc. (Atlanta, GA, U.S.A.). Poly- and monoclonal antibodies to GLUT4 were purchased from East Acres (Southbridge, MA, U.S.A.). Polyclonal antibodies to IRS-1 was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Goat anti-mouse IgG and ^{125}I-Protein A was obtained from ICN (St. Laurent, Quebec, Canada). Sepharose conjugated to Protein A were purchased from Pharmacia (Uppsala, Sweden). M-500 magnetic beads were from Dynal (Oslo, Norway).

Animals

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

Abbreviations used: GLUT4, insulin-responsive glucose transporter; IRS, insulin receptor substrate; PI, phosphatidylinositol; SH2, Src homology 2; PtdIns3*P* and PtdIns4*P*, phosphatidylinositol 3- and 4-phosphate respectively.

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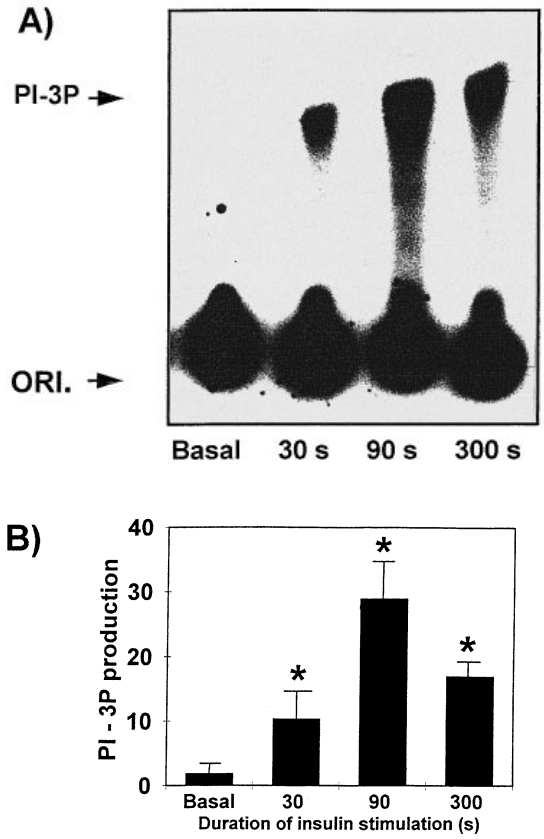

Figure 1 Effect of insulin on the IRS-1-associated PI 3-kinase activity

(**A**) Time course of IRS-1-associated PI 3-kinase activities in basal skeletal muscle and after insulin stimulation *in vivo* (30 s, 90 s and 300 s). Skeletal muscle lysates were incubated with anti-IRS-1 antibodies followed by Protein A–Sepharose. PI and [^{32}P]ATP were added to the washed immunoprecipitates and total lipids were extracted and separated by TLC. ORI, origin of the lipids; PI-3P, the position of migration of PtdIns3*P*. (**B**) Quantification of the radiolabelled phosphate incorporated into PtdIns3*P* (PI-3P). The PtdIns3*P* signals are expressed in arbitrary units relative to the basal activity. The values are expressed as mean ± S.E.M. of three separate experiments. $^{*}P < 0.05$ compared with basal.

Membrane subfractionation of rat skeletal muscle

Membrane fractions from the muscle homogenates were separated by sucrose-gradient centrifugation, as described previously [12,13]. In brief, approx. 1–2 g of muscle was homogenized for 2 × 20 s with a Polytron in ice-cold homogenization buffer (20 mM $NaHCO_3$/250 mM sucrose/5 mM NaN_3/100 μM PMSF/1 μM leupeptin/1 μM pepstatin A/2 mM orthovanadate, pH 7.4), transferred to a Wheaton 30 ml tissue grinder and homogenized in a motor-driven homogenizer (10 strokes). The homogenate was centrifuged at 1200 ***g*** for 10 min. The pellet was discarded and the supernatant was centrifuged at 8000 ***g*** for 10 min. The pellet was discarded and the supernatant was layered on a 25 % and 50 % sucrose (w/w) gradient and centrifuged at 210000 ***g*** for 2 h. It should be noted that the GLUT4-containing vesicles were immunoadsorbed from non-sedimented material, since sedimentation leads to aggregation of membrane materials of different organelle origin [14].

Immunoprecipitation of GLUT4-containing vesicle and IRS-1

The 50 % sucrose interphase were used for immunoadsorption of GLUT4-containing vesicles, as described earlier [13]. This fraction is enriched in intracellular membranes and largely devoid of plasma membranes, which are recovered in the 25 % succrose layer. Briefly, the Dynal M-500 beads were washed with PBS, and incubated with specific-affinity purified goat anti-mouse IgG in 0.1 M borate buffer, pH 9.4, at 37 °C for 12–15 h. The beads were rinsed twice with PBS containing 0.1 % BSA (PBS/BSA), blocked with 0.2 M Tris/HCl, pH 8.5, with 0.1 % BSA for 6 h at 37 °C, and washed in PBS/BSA for 5 min at 4 °C. The coated beads were incubated with monoclonal GLUT4 IgG or control-mouse IgG at 4 °C for 16–18 h under constant rotation, and washed 4 × 5 min with PBS/BSA. Subsequently, 300 μg of protein from the 50 % interphase were added to the beads and incubated in PBS + 1 mM sodium vanadate for 16–18 h at 4 °C under constant rotation. The magnetic beads were pelleted and the supernatant was removed. The immune pellet was washed 5 × 5 min in ice-cold PBS. The resulting supernatants were pooled and centrifuged at 200000 ***g*** for 60 min. The sedimented membranes and immune pellets were either solubilized in 2 × Laemmli's sample buffer without β-mercaptoethanol and stored at −80 °C, or used immediately for PI kinase activity or IRS-1 immunoprecipitation followed by PI kinase activity, as indicated. IRS-1 was immunopurified essentially as described previously [15]. In brief, the frozen muscles were homogenized in ice-cold lysis buffer (50 mM Hepes/137 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/2 mM Na_3VO_4/10 mM sodium pyrophosphate/10 mM sodium fluoride/2 mM EDTA/1 % Nonidet P-40/10 % glycerol/1 μM leupeptin/1 μM pepstatin A/34 μg/ml PMSF, pH 7.4) with a Polytron (Brinkman Instruments, Westbuty, NY, U.S.A.) operated at maximum speed for 30 s. The homogenate incubated on ice for 30 min before insoluble material was removed by centrifugation at 15000 revs./min in a Ti 70 rotor (Beckman, Mississauga, ON, Canada) for 15 min. IRS-1 was immunoprecipitated from the supernatant or other fractions, as indicated, with an anti-IRS-1 polyclonal antibody or preimmune rabbit IgG followed by Protein A–Sepharose 6MB for 2 h, sedimentation and washing. The immune pellets and supernatants were used for PI kinase activity or immunoblotting.

PI kinase activities

The PI 3- and PI 4-kinase activity assays were performed as described earlier [15,16]. Briefly, to anti-IRS-1 immunoprecipitates or immunopurified GLUT4-containing vesicles (300 μg of total protein from the 50 % fraction) were added 20 μg of PI. The reaction was started by the addition of 5 μl of 2 mM ATP/8.8 mM $MgCl_2$ containing 5 μCi of [^{32}P]ATP. After 10 min at 30 °C with constant shaking the reaction was stopped by the addition of 20 μl of 8 M HCl and 160 μl of $CHCl_3$:methanol (1:1, v/v). The samples were centrifuged, and the lower organic phase containing the labelled lipids was separated on a TLC silica plate. In some cases the TLC plate was treated with *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, and the PtdIns3*P* and PtdIns4*P* (phosphatidylinositol 3- and 4-phosphate, produced by PI 3-kinase and PI 4-kinase, respectively) were separated in the presence of boric acid as described by Walsh et al. [16]. The [^{32}P]PtdIns3*P* and [^{32}P]PtdIns4*P* signals on the silica plates were quantificated using the Molecular Dynamics PhosphoImager system (Sunnyvale, CA, U.S.A.).

Immunoblotting

The sedimented membranes and immune pellets were subjected to SDS/PAGE and immunoblotted with the use of a polyclonal anti-GLUT4 antibody (East Acres Biologicals, Southbridge, MA, U.S.A.), as described previously [13].

RESULTS

The time course of insulin-stimulated PI 3-kinase was estimated in anti-IRS-1 immunoprecipitates from muscle lysates. The PtdIns3*P* production was significantly increased 5-, 15- and 9-fold at 30, 90 and 300 s after the insulin *in vivo* injection, respectively, and displayed peak activity at 90 s (Figure 1).

To assess whether the PI 3-kinase activity increased in response to insulin stimulation *in vivo* in a membrane subfraction containing the majority of the intracellular GLUT4 protein, 50 μg of membrane protein from the 50% fraction was assayed directly for production of PtdIns3*P* and/or PtdIns4*P* by the use of a borate-treated TLC plate. As shown in Figure 2, the exogenously added [^{32}P]ATP was incorporated into PtdIns4*P*, but not PtdIns3*P*. Prior insulin stimulation *in vivo* (3 min) did not induce any detectable change in the PtdIns4*P* production (Figure 2B). We considered the possibility that the activity of PI 3-kinase in these membranes might be lower than that of PI 4-kinase, requiring further purification for its detection. Accordingly, when 300 μg of the 50% membrane fraction were pelleted, solubilized and subsequently immunoprecipitated with an anti-IRS-1 antibody, the IRS-1 immune pellet did produce PtdIns3*P* (Figure 3A). Furthermore, there was a significant 5-fold increase in PtdIns3*P* production in response to a prior insulin stimulation *in vivo* (3 min), as shown in Figure 3(B).

As predicted, biochemical characterization of the IRS-1-associated PI 3-kinase activity revealed that the PI 3-kinase

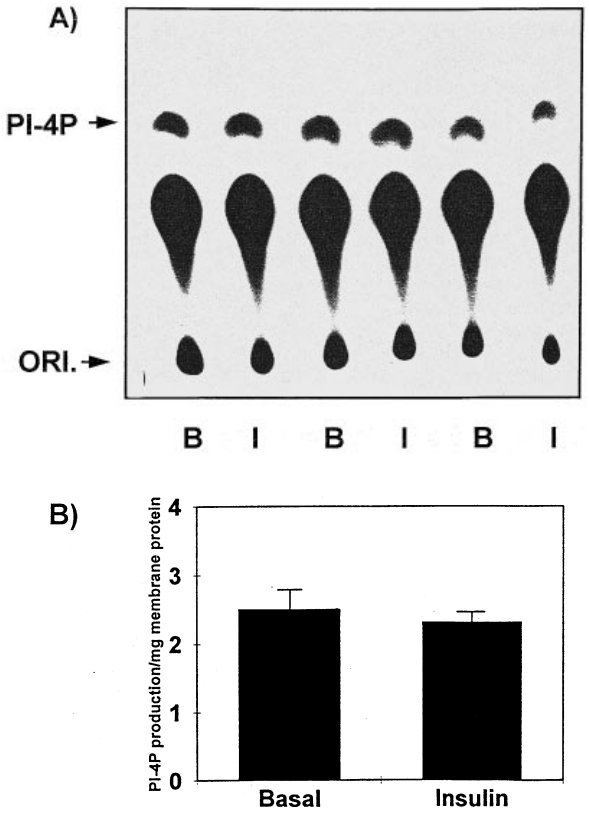

Figure 2 Effect of insulin on the PI 4-kinase activity in the membrane subfraction

(**A**) Basal skeletal muscle (B) and *in vivo* insulin-stimulated skeletal muscle (I) were homogenized and the post-nuclear supernatant was subfractionated on a 25% and 50% sucrose density gradient. Membrane protein (50 μg) from the 50% membrane fraction was pelleted and PI and [^{32}P]ATP added. The lipids were extracted and separated on a borate-treated TLC plate. ORI, origin of the lipid sample; PI-4P, the migration of a PtdIns4*P* standard. (**B**) The ^{32}P-phosphate incorporated into PtdIns4*P* was quantificated in arbitrary units and expressed as mean $\pm$ S.E.M. from 3 different experiments.

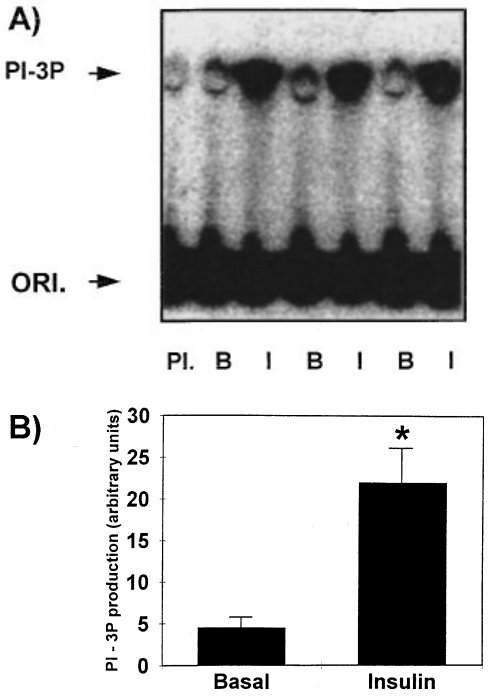

Figure 3 Effect of insulin on the IRS-1-associated PI 3-kinase activity immunoprecipitated from a membrane lysate

(**A**) Basal skeletal muscle (B) and *in vivo* insulin-stimulated muscle (I) were homogenized and subfractionated. Membrane protein (200 μg) from the 50% fraction were pelleted and resuspended in lysis buffer and incubated with anti-IRS-1 antibodies followed by immunoadsorption with Protein A–Sepharose. To the washed IRS-1 immune pellet was added PI in the presence of [^{32}P]ATP and the lipids were separated on a TLC plate. ORI, origin of the added sample; PI-3P, migration of the PtdIns3*P*. PI, to the membrane lysate was added preimmune rabbit IgG followed by Protein A–Sepharose. (**B**) The quantification of ^{32}P-phosphate incorporated into PtdIns3*P*. The values are expressed in arbitrary units expressed as mean $\pm$ S.E.M. from 3 different experiments. $^{*}P < 0.05$ compared with basal.

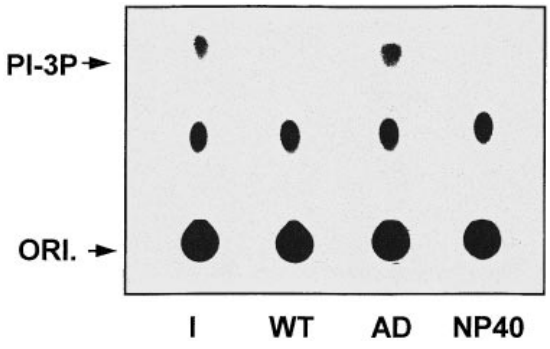

Figure 4 Effects of wortmannin, adenosine and Nonidet P40 on the IRS-1-associated PI 3-kinase activity

IRS-1 was immunoprecipitated from an insulin-stimulated (I) muscle lysate followed by Protein A–Sepharose. The washed immune pellets were incubated in the presence of PI and ^{32}P-ATP (I) or added 100 nM wortmannin (WT), 300 μM adenosine (AD) or 1% Nonidet P40 (NP40). The lipids were separated on a borate-treated TLC plate. PI-3P, migration of PtdIns3*P*.

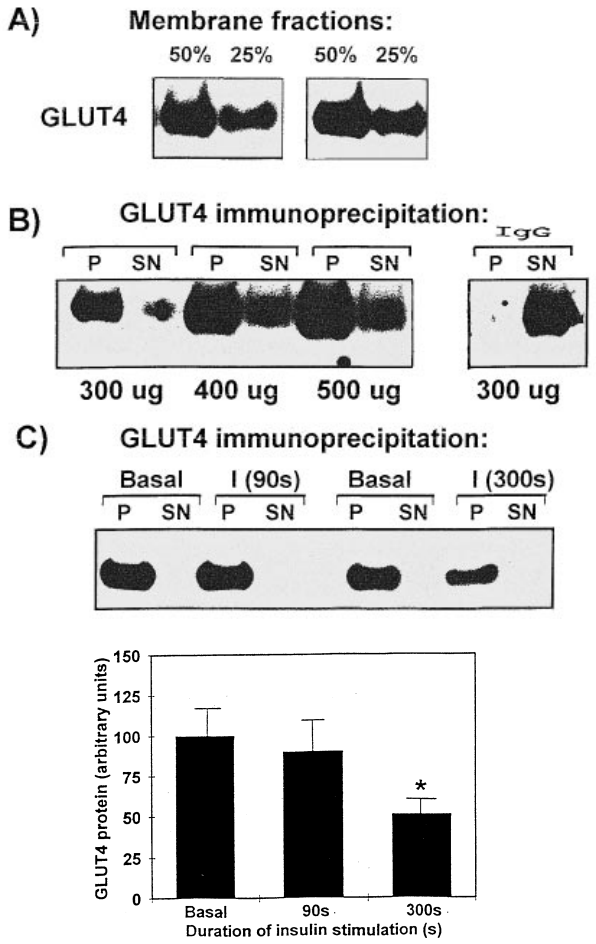

Figure 5 Immunoprecipitation of the GLUT4-containing vesicles from a membrane subfraction

(**A**) Basal skeletal muscle was homogenized and the post-nuclear supernatant containing the crude membranes was subfractionated into a 25% and 50% fraction. Membrane protein (30 μg) from the 25% and 50% fractions, respectively, was pelleted and immunoblotted for the amount of GLUT4 protein. (**B**) GLUT4-containing vesicles were immunoadsorbed from the 50% membrane fraction with equals amounts of Dynal Magnetic beads and with increasing amounts of membrane material (300–500 μg as indicated). IgG: to the beads were added preimmune mouse immunoglobulins. (**C**) Basal skeletal muscle and *in vivo* insulin-stimulated (90 and 300 s) skeletal muscle (I) were homogenized and subfractionated. GLUT4-containing vesicles were immunoadsorbed from 300 μg of membrane protein (50% fraction). The resulting GLUT4 immune pellet (P) and pelleted supernatant (SN) were immunoblotted for the amount of GLUT4 protein, which is expressed in arbitrary units on the histogram as means $\pm$ S.E.M. from 3 different experiments. $^*P < 0.05$ compared with basal.

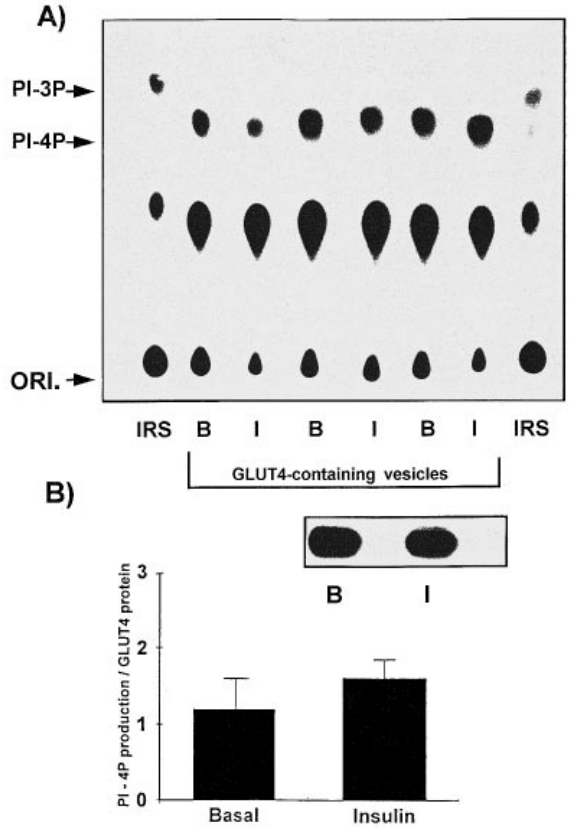

Figure 6 Detection of PtdIns3*P* and PtdIns4*P* in the IRS-1-associated Pi 3-kinase and GLUT4 immune pellets respectively

(**A**) Basal skeletal muscle (B) and *in vivo* insulin-stimulated (I; 3 min) skeletal muscle were homogenized and the post-nuclear supernatant subfractionated. GLUT4-containing vesicles were immunoadsorbed from 300 μg of membrane protein (50% fraction) and added PI and [^{32}P]ATP. The lipids were separated on a borate-treated TLC plate and the labelled lipids were detected. ORI, origin of the added lipid sample. PI-3P/PI-4P, migration of PtdIns3*P*/PtdIns4*P*; IRS, IRS-1 was immunoprecipitated from an *in vivo* insulin-stimulated muscle lysate and used as a PtdIns3*P* standard. (**B**) The quantification of the ^{32}P-phosphate incorporated into PtdIns4*P* in immunoadsorped GLUT4-containing vesicles isolated from basal and *in vivo* insulin-stimulated rat skeletal muscle. The arbitrary units were calculated by dividing the PtdIns4*P* signal (**A**) with the amount of GLUT4 protein in the corresponding sample (see insert of representative GLUT4 immunoblot: B, amount of GLUT4 proteins in the immune pellet produced from basal skeletal muscle; I, amount of GLUT4 proteins in the immune pellet isolated from insulin-stimulated skeletal muscle). The arbitrary units are means $\pm$ S.E.M. from 3 different experiments.

activity was effectively blocked by 100 nM of wortmannin (Figure 4). However, the PI 3-kinase activity was insensitive to 300 μM adenosine, but inhibited by 1% of the non-ionic detergent Nonidet P40.

As shown in Figure 5(A), the 50% sucrose membrane fraction contained the majority of the GLUT4 protein when compared with the 25% fraction. Furthermore, when GLUT4 was immunoprecipitated from 300, 400 and 500 μg of the 50% membrane preparation, the majority of the GLUT4-containing vesicles could be found in the immune pellet. However, increasing amounts of GLUT4 were found in the supernatant with increasing amounts of starting membrane material. Very little, if any, GLUT4 protein was detected in the preimmune (IgG) pellet (Figure 5B). Finally, insulin stimulation *in vivo* of the rats for 300 s, but not 90 s, induced a significant decrease in the amount of GLUT4-containing vesicles that could be immunoadsorbed from 300 μg of the 50% membrane fraction, demonstrating that the 50% membrane pool of glucose transporters was sensitive to insulin and partly depleted of translocated vesicles (Figure 5C).

Based on the observations shown in Figures 1, 2, 4 and 5, rats were insulin-stimulated for 3 min and GLUT4-containing vesicles were immunoadsorbed from 300 μg of the 50% membrane fraction. The washed immune pellets were incubated with PI and [^{32}P]ATP. The resulting ^{32}P-labelled lipids were separated on a borate-treated TLC plate. As shown in Figure 6(A), there was a clear and distinct separation of PtdIns3*P* from PtdIns4*P*. This separation is due to the borate-binding of vicinal diols (present in PtdIns4*P*), which causes slower migration of PtdIns4*P*, but not PtdIns3*P*. Interestingly, the GLUT4-containing vesicles produced a clear PtdIns4*P* signal, but there was no detectable PtdIns3*P* signal. When the PtdIns4*P* signal was expressed relative

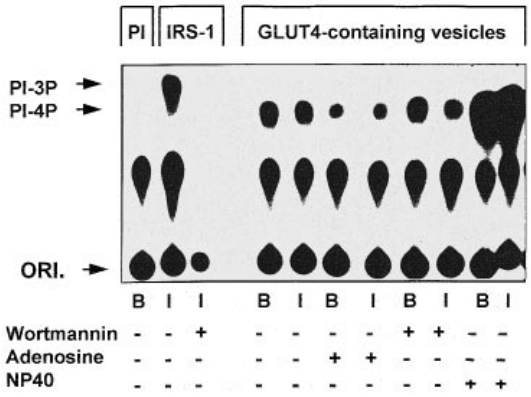

Figure 7 Effects of wortmannin, adenosine and Nonidet P40 on the PI 4-kinase activity in the GLUT4-containing vesicles

Basal skeletal muscle (B) and insulin-stimulated (I) skeletal muscle were homogenized and the post-nuclear supernatants were subfractionated. GLUT4-containing vesicles were immunoadsorbed from the 50% membrane subfraction. The washed immune pellets were added to PI and [^{32}P]ATP and incubated in the presence of 100 nM wortmannin, 300 μM adenosine or 1% Nonidet P40 (NP40) as indicated. The lipids were separated on a borate-treated TLC plate and the labelled lipids were detected. ORI, originate of the added sample; positions of PtdIns3*P* and PtdIns4*P* are indicated; PI, Dynal beads were added to preimmune mouse IgG instead of GLUT4-specific mouse IgG. IRS-1 was immunoprecipitated from an insulin-stimulated muscle lysate. The washed IRS-1 immune pellets were incubated with PI and [^{32}P]ATP and the resulting labelled PtdIns3*P* used as a standard.

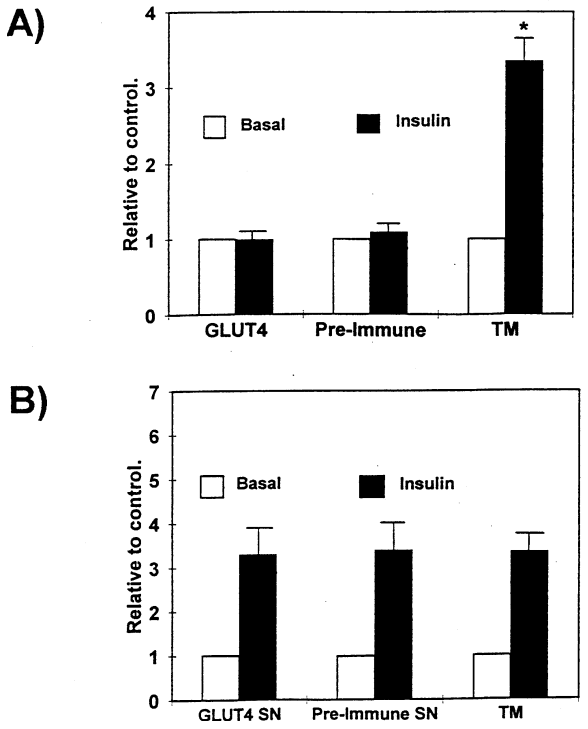

Figure 8 Immunoprecipitated IRS-1-associated PI 3-kinase activity after solubilization of total membrane, GLUT4-containing vesicles and supernatant devoid of GLUT4

GLUT4-containing vesicles were immunoprecipitated from a membrane fraction produced from basal (white bars) and insulin-stimulated (black bars) rat skeletal muscle. The GLUT4 or preimmune pellets (**A**), the supernatants (SN; **B**) and the total-membrane (TM; **A** and **B**) were solubilized and immunoprecipitated with an IRS-1 antibody and subjected to a PI 3-kinase activity assay; activity is expressed relative to the control value. The values are means ± S.E.M. from 3 different experiments. $^*P < 0.05$ compared with basal.

to the amount of GLUT4 protein in the same sample (Figure 6B, insert), there was no significant change in PI 4-kinase activity per GLUT4 protein content after 3 min of insulin stimulation (Figure 6B).

To further establish that the PtdIns4*P* signal was the consequence of the presence of a PI 4-kinase in the GLUT4-containing vesicles, the GLUT4 immune pellet was incubated in the presence of 300 μM adenosine, 100 nM wortmannin or 1% Nonidet P40 (Figure 7). First, the PtdIns4*P* production was inhibited by adenosine, an inhibitor of PI 4-kinases [17,18]. Secondly, 100 nM wortmannin did not inhibit PtdIns4*P* production in the washed immune pellet. It should be noted that the wortmannin was fully active, since the same concentration fully inhibited PtdIns3*P* production in the IRS-1 immune pellet. Thirdly, 1% of the non-ionic detergent Nonidet P40 caused a marked stimulation of PtdIns4*P* production in the GLUT4 immune pellet, a characteristic of PI 4-kinases [17]. Thus, it was found that the PtdIns4*P* production was inhibited by adenosine, insensitive to wortmannin and activated by the non-ionic detergent (these findings are in contrast to the effects of adenosine, wortmannin and Nonidet P40 on PtdIns3*P* production in the IRS-1-associated PI 3-kinase immune pellet, as previously shown in Figure 4).

Finally, GLUT4-containing vesicles were immunoprecipitated from the 50% membrane fraction produced from basal and insulin-stimulated (3 min) skeletal muscle. The immunoadsorbed GLUT4-containing vesicles (Figure 8A), the supernatant devoid of the GLUT4-containing vesicles (Figure 8B) and the 50% membrane fraction (total activity) were solubilized and subjected to IRS-1 immunoprecipitation followed by the PI 3-kinase assay. As shown in Figure 8(A), no IRS-1-associated PI 3-kinase activity could be immunoadsorbed from the solubilized GLUT4 immune pellet when compared with the preimmune control. However, insulin increased the IRS-1-associated PI 3-kinase activity in the solubilized total-membrane preparation. As expected, the insulin-stimulated IRS-1-associated PI 3-kinase activity (3.5-fold increase compared with basal) remained in the supernatant devoid of the GLUT4-containing vesicles and in the preimmune supernatant (Figure 8B). The PI 3-kinase activity found in the two supernatants completely matched the PI 3-kinase activity found in the total-membrane preparation.

DISCUSSION

We have investigated whether GLUT4-containing vesicles isolated from rat skeletal muscle display any PI 3-kinase and/or PI-4 kinase activity. First, as expected, insulin induced a rapid increase (5–15-fold) in the IRS-1-associated PI 3-kinase activity and this increase in PI 3-kinase activity was also present in a membrane fraction containing the majority of the insulin-regulatable pool of GLUT4 transporters. However, GLUT4-containing vesicles isolated from basal and 3-min insulin-stimulated rat skeletal muscle produced PtdIns4*P*, but not PtdIns3*P*. Furthermore, the PI 4-kinases can easily be distinguished from the PI 3-kinases, since they have different enzymic characteristics. The PI 3-kinases are inhibited by wortmannin and non-ionic detergents, but are relatively insensitive to adenosine [3–5,17]. The type-2 PI 4-kinases are inhibited by adenosine, activated by non-ionic detergents, and the membrane-bound PI 4-kinases, but not cytosolic PI 4-kinases

[19,20], are insensitive to wortmannin in the low-nanomolar range [17]. Using these biochemical characteristics of the two distinct kinase activities (Figures 4 and 7), the present findings indicate that GLUT4-containing vesicles in skeletal muscles display PI 4-kinase activity, but not PI 3-kinase activity.

Although, PtdIns3*P* production was not detected in the total-membrane fraction (Figure 2), it was clearly detectable after immunoprecipitation of IRS-1/PI 3-kinase from the same membrane fraction (Figure 3). The reason for this could be the presence of PtdIns4*P* and PtdIns4,5P_2 in the total membrane fraction. The p85-p110 PI 3-kinase predominantly utilizes PtdIns4*P* and/or PtdIns4,5P_2 as substrates *in vivo* [21], but these lipids may be absent after immunoprecipitation due to the washing procedure. Given this, it is likely that the PI was not used as a substrate (and therefore not phosphorylated by the PI 3-kinases) in the total-membrane fractions due to the presence of the more natural substrate choices. It could then be argued that the PI 3-kinase activity could not be detected in the immunoprecipitated GLUT4-containing vesicles due to the presence of PtdIns4*P* (produced by the PI 4-kinase). This possibility was tested. PtdIns3*P* production was only detected when IRS-1 was immunoprecipitated from the solubilized GLUT4 supernatant and total membrane, but not from the solubilized GLUT4 immune pellet (Figure 8). This indicates that the IRS-1/PI 3-kinase complex could not be part of the GLUT4 vesicle membrane.

Clark et al. [22] have recently shown that the PI 3-kinase in adipocytes is redistributed to a high-speed membrane pellet containing the GLUT4 transporter. However, using sucrose density-gradient sedimentation the high-speed membrane pellet could be resolved into two discrete membrane compartments; one containing the GLUT4 transporter and the other containing the PI 3-kinase activity. Furthermore, Kelly and Ruderman [21] reported that the IRS-1/PI 3-kinase complex was localized in a very low-density subpopulation in rat adipocytes that could be distinguished from vesicles containing the GLUT4 transporter. In contrast, insulin increased the IRS-1-associated PI 3-kinase activity in GLUT4-containing vesicles isolated from 3T3-L1 adipocytes treated with insulin for 3–3.5 min [10,23]. The reason for this discrepancy is not known. One possibility could be tissue-specific differences in the membrane traffic of the GLUT4 transporter. However, detailed analysis of insulin-sensitive GLUT4-containing vesicles from unstimulated rat skeletal muscle has demonstrated that they are almost identical to the analogous vesicles from adipocytes in terms of size, sedimentation coefficient, bouyant density and total polypeptide composition [24–26]. A second possibility is that the association of PI 3-kinase with the GLUT4 vesicle is transient. Criticial time-course analyses are required to examine this possibility in various tissues. Thirdly, it is possible that cytoskeletal elements participate in bringing together PI 3-kinase and membrane compartments [23]. Subcellular fractionation and vesicle immunoprecipitation would show co-migration of PI 3-kinase and GLUT4 vesicle only when cytoskeletal networks would co-purify. This possibility is in line with recent observations that solubilization of membranes by non-ionic detergents leaves a cytoskeletal residue containing PI 3-kinase [22]. We hypothesize that the GLUT4 vesicles isolated from 3T3-L1 adipocytes, but not from muscle, retain cytoskeletal elements that bind PI 3-kinase. Indeed, GLUT4 vesicles of 3T3-L1 adipocytes were shown to contain spectrin [23].

In conclusion, the present finding may suggest that the activity of the IRS/PI 3-kinase complex is stimulated in a membrane compartment containing the GLUT4 transporter. However, GLUT4-containing vesicles in rat skeletal muscle display PI 4-kinase, but not PI 3-kinase, activity. The PI 4-kinase activity in the GLUT4-containing vesicles was not stimulated by insulin.

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