

Insulin but not PDGF relies on actin remodeling and on VAMP2 for GLUT4 translocation in myoblasts

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Summary

Insulin promotes the translocation of glucose transporter 4 (GLUT4) from intracellular pools to the surface of muscle and fat cells via a mechanism dependent on phosphatidylinositol (PtdIns) 3-kinase, actin cytoskeletal remodeling and the v-SNARE VAMP2. The growth factor PDGF-BB also robustly activates PtdIns 3-kinase and induces actin remodeling, raising the question of whether it uses similar mechanisms to insulin in mobilizing GLUT4. In L6 myoblasts stably expressing Myc-tagged GLUT4, neither stimulus affected the rate of GLUT4 endocytosis, confirming that they act primarily by enhancing exocytosis to increase GLUT4 at the cell surface. Although surface GLUT4myc in response to insulin peaked at 10 minutes and remained steady for 30 minutes, PDGF action was transient, peaking at 5 minutes and disappearing by 20 minutes. These GLUT4myc translocation time courses mirrored that of phosphorylation of Akt by the two stimuli. Interestingly, insulin and PDGF caused distinct

manifestations of actin remodeling. Insulin induced discrete, long (>5 μm) dorsal actin structures at the cell periphery, whereas PDGF induced multiple short (<5 μm) dorsal structures throughout the cell, including above the nucleus. Latrunculin B, cytochalasin D and jasplakinolide, which disrupt actin dynamics, prevented insulin- and PDGF-induced actin remodeling but significantly inhibited GLUT4myc translocation only in response to insulin (75-85%, $P<0.05$), not to PDGF (20-30% inhibition). Moreover, transfection of tetanus toxin light chain, which cleaves the v-SNAREs VAMP2 and VAMP3, reduced insulin-induced GLUT4myc translocation by >70% but did not affect the PDGF response. These results suggest that insulin and PDGF rely differently on the actin cytoskeleton and on tetanus-toxin-sensitive VAMPs for mobilizing GLUT4.

Key words: Insulin, PDGF, Actin, VAMP2, GLUT4

Introduction

The glucose transporter GLUT4 is responsible for most of the glucose uptake into muscle and fat cells when stimulated by insulin (Rudich et al., 2003). Using the immortalized L6 muscle cell line transfected with Myc-tagged GLUT4 (GLUT4myc), we have shown that GLUT4 continuously cycles between the cell surface and intracellular sites, and that insulin increases the rate of GLUT4 exocytosis without affecting its rate of endocytosis (Foster et al., 2001; Li et al., 2001). The insulin-dependent gain in surface GLUT4 functionally requires the fusogenic v-SNARE VAMP2 (Randhawa et al., 2000). Importantly, the entire intracellular complement of GLUT4 eventually recycles to the cell surface, even in unstimulated cells, and the surface levels of GLUT4 in this condition is unaffected by interfering with VAMP2 (Li et al., 2001; Randhawa et al., 2000). These two observations suggest that VAMP2 defines a specific, insulin-induced mechanism of GLUT4 traffic, possibly by acting as the fusogen of a distinct pool of GLUT4.

Insulin-dependent exocytosis of GLUT4 requires the activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase),

primarily class I and its major product PtdIns-(3,4,5)- P_3 (Cheatham et al., 1994; Evans et al., 1995; Kotani et al., 1995; Okada et al., 1994; Patel et al., 2003). PtdIns-(3,4,5)- P_3 generation is required in muscle cells for cortical actin remodeling (Patel et al., 2003), which is in turn necessary for effective targeting and/or fusion of GLUT4-containing vesicles with the plasma membrane (Khayat et al., 2000). Over the years, it has been debated whether activation of PtdIns 3-kinase by other stimuli suffices to translocate GLUT4 to the plasma membrane. Whereas interleukin 4 or integrin cross-linking robustly activate PtdIns 3-kinase, they do not cause significant GLUT4 translocation in adipocytes (Guilherme and Czech, 1998; Isakoff et al., 1995). Platelet-derived growth factor (PDGF-BB), which also activates PtdIns 3-kinase, was initially reported to have no effect on GLUT4 distribution (Clark et al., 1998; Ricort et al., 1996; Wiese et al., 1995), but a transient PDGF-induced GLUT4 gain at the cell surface was subsequently documented (Wang et al., 1999). In cells overexpressing PDGF receptors, PDGF-BB induces GLUT4 translocation along with stimulation of PtdIns 3-kinase (Quon et al., 1996; Whiteman et al., 2003). The emerging questions

are whether PDGF engages actin remodeling to translocate GLUT4, whether it uses VAMP2-dependent or -independent modes of GLUT4 traffic, and whether it promotes GLUT4 exocytosis or reduces its endocytosis. Here, we take advantage of GLUT4myc-expressing L6 myoblasts that express endogenous levels of PDGF and insulin receptors to examine the cytoskeleton and v-SNARE dependence of GLUT4 traffic in response to the cognate agonists PDGF-BB and insulin. Using this system, we report that both PDGF and insulin induce GLUT4 translocation (albeit with distinct time courses), that actin remodeling and tetanus-toxin-sensitive VAMPs like VAMP2 are required for insulin but not PDGF action, and that the GLUT4 endocytic rate is similar in cells stimulated with either agent. We also report that PDGF elicits actin patterns distinct from those provoked by insulin. We propose that insulin and PDGF recruit GLUT4 to the cell surface through distinct mechanisms, differentiated by their v-SNARE dependence and their reliance on actin remodeling.

Materials and Methods

Reagents

All tissue-culture reagents were supplied from Life Technologies / GIBCO (Burlington, Ontario, Canada). Human insulin was from Eli Lilly (Toronto, Ontario, Canada). Rat PDGF-BB, *o*-phenylenediamine dihydrochloride (OPD) reagent, Latrunculin B and cytochalasin D were from Sigma-Aldrich (St Louis, MO). Monoclonal and polyclonal antibodies against c-Myc were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal antibodies against Akt and phospho-serine Akt were obtained from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Jaspalinolide, Alexa-488-conjugated goat anti-mouse antibody and rhodamine-conjugated phalloidin were from Molecular Probes (Eugene, OR). The mammalian expression vector for enhanced green fluorescent protein (eGFP), pEGF-N1, was purchased from Clontech (Palo Alto, CA). pcDNA3 was from Invitrogen (Carlsbad, CA). Plasmid encoding the light chain of tetanus toxin (TeTx) was obtained from H. Niemann (Medizinische Hochschule, Hannover, Germany) and plasmid encoding eGFP-tagged VAMP2 was a gift from W. Trimble (The Hospital for Sick Children, Toronto, Ontario, Canada). Plasmid purification Maxi kits and Effectene transfection reagent were from Qiagen (Mississauga, Ontario, Canada).

Cell culture and transfection

L6 muscle cells expressing c-Myc-epitope-tagged GLUT4 (GLUT4myc) were maintained in a myoblast monolayer culture as described earlier (Wang et al., 1998). Transfection of L6 GLUT4myc myoblasts grown on 25-mm-diameter glass coverslips (VWR Scientific, West Chester, PA) was performed in six-well plates as described previously (Randhawa et al., 2000).

Determination of 2-deoxy-³H-D-glucose uptake

Cells grown in 24-well plates were serum starved (3-5 hours) and stimulated without or with 100 nM insulin or 50 ng ml⁻¹ PDGF, and 2-deoxyglucose uptake was measured as described earlier (Rudich et al., 2003) using 10 μmol l⁻¹ 2-deoxy-(³H)-glucose (PerkinElmer, Boston, MA).

Colorimetric assay of cell-surface GLUT4myc

Colorimetric detection of cell-surface GLUT4myc was carried out as

previously described (Ueyama et al., 1999; Wang et al., 1998). Briefly, after serum deprivation and stimulation without or with 100 nM insulin or 50 ng ml⁻¹ PDGF, cells were washed twice with ice-cold PBS, blocked with 3% (v/v) goat serum (20 minutes at 4°C) and incubated with polyclonal anti-Myc antibody (1:200) for 60 minutes at 4°C. Cells were then fixed (3% paraformaldehyde for 10 minutes at 4°C), and incubated with HRP-conjugated goat anti-rabbit antibody (1:2000) for 60 minutes at 4°C. The cells were washed six times with PBS followed by incubation with OPD for 20-30 minutes at room temperature. The reaction was stopped by adding 3 N HCl. The supernatant was collected and absorbance was measured at 492 nm. Background absorbance obtained in the absence of anti-Myc antibody was subtracted from all values. For GLUT4 internalization studies, serum-deprived GLUT4myc cells were stimulated with 100 nM insulin for 20 minutes, washed, and incubated for 60 minutes at 4°C in the absence of insulin with polyclonal anti-Myc antibody. Cells were washed and then incubated with medium prewarmed to 37°C containing no insulin, 100 nM insulin, 450 mM sucrose or 50 ng ml⁻¹ PDGF for 3 minutes or 7 minutes. At the indicated times, cells were placed on ice, washed once with ice-cold PBS, fixed and analysed for the remaining GLUT4myc signal using HRP-conjugated antibody as described above. To measure GLUT4myc recycling (Foster et al., 2001), serum-starved cells were exposed to anti-Myc antibody at 37°C for the times indicated, in the presence of 0.025% dimethyl sulfoxide (vehicle) or 250 nM cytochalasin D. The cells were then washed, fixed, permeabilized and assayed for GLUT4myc signal within the cell as described above. Total GLUT4myc signal was determined from cells labeled with anti-Myc antibody after permeabilization.

Immunofluorescence assay of cell-surface and intracellular GLUT4myc

Indirect immunofluorescence for cell-surface GLUT4myc was carried out on intact cells as previously described (Randhawa et al., 2000) with slight modifications. Briefly, serum-deprived L6 myoblasts were stimulated without or with 100 nM insulin or 50 ng ml⁻¹ PDGF at 37°C, followed by two washes with ice-cold PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ on ice. The subsequent steps were performed at 4°C, unless otherwise indicated. The cells were mildly fixed for 3 minutes with 3% paraformaldehyde and then incubated with the monoclonal anti-Myc antibody (1:100) in 3% (v/v) goat serum for 1 hour. The cells were extensively washed and incubated with secondary antibody (Cy3-conjugated goat anti-mouse IgG, 1:750) in the dark for 1 hour. The coverslips were fixed with 3% paraformaldehyde, quenched with 0.1 M glycine and then mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA). To assess the intracellular localization of GLUT4myc relative to the actin cytoskeleton, insulin- and PDGF-stimulated L6GLUT4myc myoblasts were immediately fixed at 4°C with 3% paraformaldehyde. The cells were then permeabilized with 0.1% (vol/vol) Triton X-100 for 3 minutes in order to preserve actin morphology, followed by incubation with monoclonal anti-Myc antibody (1:150) for 1 hour. To label actin filaments, the cells were incubated for 1 hour at room temperature with the rhodamine-conjugated phalloidin (0.01 U per coverslip) during the incubation with the Alexa-488-conjugated goat anti-mouse secondary antibody. For confocal fluorescence microscopy, cells were examined with a Zeiss LSM 510 laser-scanning confocal microscope. Acquisition parameters were adjusted to exclude saturation of the pixels. For quantification, such parameters were kept constant across the various conditions.

Lysate preparation and immunoblotting

After serum deprivation, cells were left untreated or treated with 100 nM insulin or 50 ng ml⁻¹ PDGF. Cells were quickly rinsed with ice-cold PBS containing 1 mM sodium vanadate and whole cell lysates were prepared by solubilizing the cells with 2× Laemmli sample

buffer containing freshly added inhibitors [1 mM Na_3VO_4 , 100 nM okadaic acid and 1:500 of the protease inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario, Canada)]. The lysates were then homogenized by passing five times through a 25 G⁵/8 needle and heated at 65°C for 15 minutes. The protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985) and equal amounts of protein from each sample were resolved by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and subjected to immunoblotting for Akt or anti-phosphoserine 473-Akt.

Quantification and statistical analysis

To score the distinct actin morphologies throughout the cell, the whole cell was scanned along the z -axis by taking optical slices 0.4 μm apart, and a single composite image (collapsed xy projection) was generated using LSM5 Image software (Carl Zeiss, Thornwood, NY) and used for subsequent analyses. The morphology of actin was classified into three types: (1) no ruffles; (2) short ruffles (<5 μm); and (3) long ruffles. The number of cells exhibiting these morphologies is expressed as a proportion of the total number of cells analysed. To measure the surface GLUT4myc content by indirect immunofluorescence, images from at least three independent experiments were acquired. The single composite image (collapsed xy projection) was quantified using Image J software (National Institutes of Health). Raw data for GLUT4myc translocation were converted to fold stimulation by insulin and PDGF above basal levels. Statistical analyses were carried out by analysis of variance and post-hoc analysis using Prism software (Graph Pad Software, San Diego, CA).

Results

PDGF and insulin increase surface GLUT4myc content and stimulate glucose uptake in myoblasts with distinct time courses

Cellular responses to PDGF are frequently lost during cell differentiation, including during the transition from myoblasts to myotubes (Fiaschi et al., 2003). However, myoblasts display biological responses to both insulin and PDGF through their respective endogenous receptors. This property renders myoblasts an ideal experimental system in which to study elements of specificity in cellular response to insulin through comparison to PDGF, without artificially manipulating the expression level of their receptors. L6 myoblasts stably expressing GLUT4myc also allow the detection and quantification of GLUT4 at the surface through the Myc epitope that becomes externalized upon full fusion of the transporters with the plasma membrane (Kanai et al., 1993; Wang et al., 1998). To begin comparing the effects of insulin and PDGF on GLUT4 traffic, the amount of GLUT4myc at the cell surface was quantified after stimulation with insulin (100 nM) or PDGF-BB (50 ng ml^{-1}). Fig. 1A shows that PDGF caused GLUT4myc translocation that peaked at 5.0-7.5 minutes and thereafter declined within 20 minutes (Fig. 1A). This time course differed from that observed in response to insulin, for which GLUT4myc translocation peaked at 10 minutes and remained constant for up to 20 minutes. The transient response caused by PDGF is reminiscent of previous observations made in 3T3-L1 adipocytes (Tengholm and Meyer, 2002; Wang et al., 1999).

In principle, an increased number of glucose transporters at the cell surface can arise from enhanced rate of exocytosis

and/or from inhibition of GLUT disappearance from the surface by endocytosis. In L6 GLUT4myc cells, insulin increases GLUT4myc exocytosis but does not significantly affect the initial endocytic rate (Foster et al., 2001; Li et al., 2001). Conversely, hyperosmotic stimulus increases surface GLUT4myc by a compound effect on exocytosis and endocytosis (Li et al., 2001). To assess the underlying mechanism for GLUT4myc recruitment by PDGF, we measured GLUT4 endocytosis. In this assay, transporters are brought to the cell surface by insulin stimulation for 20 minutes, followed by washing and incubation at 4°C with anti-Myc antibody. Cells are then again warmed to 37°C and the proportion of GLUT4myc signal remaining at the cell surface is compared with the time 0 of internalization. In unstimulated cells, approximately one-half of pre-labeled surface GLUT4myc internalized by 3 minutes (Table 1). This time course was not altered in insulin- or PDGF-stimulated cells, whereas hyperosmolarity virtually obliterated GLUT4myc internalization. These results imply that, like insulin, PDGF recruits GLUT4 to the cell surface largely by enhancing its exocytosis.

To assess whether the GLUT4myc transporters appearing at the cell surface following PDGF stimulation are fully functional, 2-deoxyglucose uptake was measured. Consistent with the time course of GLUT4myc translocation (Fig. 1A), the maximal response was observed 5.0-7.5 minutes after exposure to PDGF (Fig. 1B), after which 2-deoxyglucose uptake activity decreased. In response to insulin a more gradual increase in 2-deoxyglucose was observed, peaking at 10 minutes and sustained thereafter. This distinct time course of GLUT4myc translocation and 2-deoxyglucose uptake elicited by PDGF and insulin was also reflected in the time course of Akt phosphorylation by the two stimuli (Fig. 1C). Mild phosphorylation of Akt was detected as early as 1 minute after exposure to PDGF, followed by a marked phosphorylation at 7 minutes that rapidly returned to near-basal levels by 30 minutes. By contrast, Akt phosphorylation in response to insulin peaked by 7 minutes and was sustained for up to 30 minutes (Fig. 1C). These results illustrate that insulin and PDGF share common signaling components and induce similar biological responses, but differ in the time courses of these effects.

Table 1. GLUT4myc endocytosis

	3 minutes	7 minutes
Unstimulated	42±7%	27±5%
Insulin (100 nM)	37±6%	31±6%
PDGF (50 ng ml^{-1})	42±11%	33±3%
Hyperosmolarity (450 mM sucrose)	107±15%*	86±14%*

The table shows the percent of GLUT4myc remaining at the cell surface. L6 GLUT4myc myoblast monolayers in 24-well plates were stimulated with 100 nM insulin for 20 minutes. Surface GLUT4myc was then labeled with anti-Myc antibody by incubation for 1 hour at 4°C. Cells were then washed and rewarmed to 37°C to allow GLUT4myc endocytosis for 3 or 7 minutes with or without insulin, PDGF or hyperosmolar sucrose. Cells were then fixed and reacted with HRP-conjugated anti-IgG antibody, and the amount of signal remaining at the surface was quantified spectrophotometrically (see Materials and Methods). Results are the means±s.e.m. of the percent of the signal at time 0, derived from three independent experiments, each performed in triplicate.

* $P<0.05$ against unstimulated, insulin and PDGF.

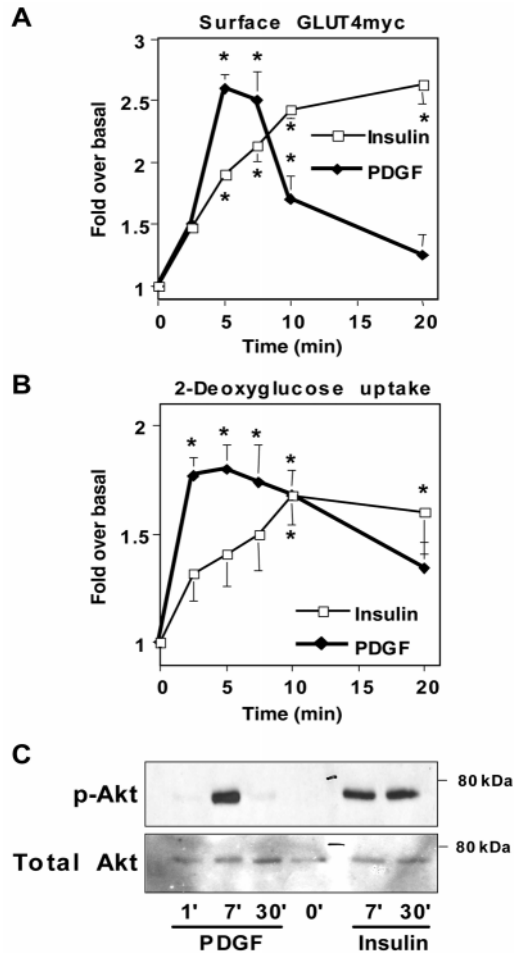


Fig. 1. Insulin and PDGF increase the amount of GLUT4myc at the cell surface and stimulate glucose uptake with different time courses that correlate with Akt phosphorylation. Confluent GLUT4myc myoblasts were incubated with either 100 nM insulin or 50 ng ml⁻¹ PDGF-BB at 37°C for the times indicated, and assayed for (A) GLUT4myc translocation or (B) 2-deoxyglucose uptake; *, $P < 0.05$ against time 0. The means \pm s.e.m. of at least three independent experiments are shown per condition, each done in triplicate. (C) Total cell lysates were separated by 10% SDS-PAGE and immunoblotted with anti-phospho-Akt (Ser 473) antibody. The same membrane was stripped and reblotted for total Akt.

Distinct actin remodeling patterns are observed in response to insulin and PDGF

Insulin causes a characteristic actin remodeling in L6 myoblasts, forming cortical ruffles that are required for GLUT4 translocation (Patel et al., 2003). In many cell types, PDGF induces a characteristic actin remodeling that is considered to be necessary for promoting its biological functions, such as cell migration (for a review, see Heldin and Westermark, 1999). We therefore examined the morphological aspects of PDGF-induced actin remodeling in L6 GLUT4myc myoblasts and whether it is required for GLUT4myc translocation. The time course of actin remodeling in response to insulin or PDGF is depicted in Fig. 2. Actin reorganization was already notable at 3 minutes after exposure to either stimuli. Yet, although the dorsal structures appeared to

originate peripherally in response to insulin, PDGF tended to initiate newly formed structures throughout the cell. At later time points, peripheral structures could also be observed in response to PDGF but they were less dense and more branched than those formed in response to insulin. In order to quantify the different actin manifestations, four types of F-actin structures were identified. (1) Long filamentous structures organized along the longer axes of the cell (stress fibers; Fig. 3A, arrows). (2) Large (>5 μ m long) mesh-like structures, mostly at the cell periphery close to the dorsal surface (long ruffles; Fig. 3A, asterisk). (3) Many short (<5 μ m long) structures evenly distributed on the dorsal cell surface, including above the nucleus (short ruffles; Fig. 3A, open arrowheads). (4) Finger-like protrusions at the cell periphery containing tight parallel arrays of filamentous actin (microspikes), often connected by a long actin bundle at the base (filopodia; Fig. 3A, closed arrowheads). Stress fibers were observed in unstimulated cells and in both insulin- and PDGF-stimulated cells. Filopodia were inconsistently observed in stimulated cells, mostly in response to PDGF (in two out of three experiments and in fewer than half of the cells). Long and short ruffles were the major stimulus-induced filamentous actin structures (Fig. 2). The proportion of cells exhibiting long (>5 μ m) or short (<5 μ m) ruffles was determined in at least 60 individual cells per condition from three independent experiments. As shown in Fig. 3B, insulin (100 nM, 7 minutes) predominantly induced long ruffles, in nearly 80% of the cells ($P < 0.01$). By contrast, short ruffles predominated in response to PDGF (50 ng ml⁻¹, 7 minutes) and were observed in nearly 90% ($P < 0.001$) of the cells, confirming that most cells harbor functional, endogenous PDGF receptors. These results demonstrate that insulin and PDGF induced structurally distinct actin remodeling.

Unlike with insulin, PDGF-induced GLUT4myc translocation is resistant to actin disruption

We have previously reported that insulin-induced actin structures localize with a subpopulation of intracellular GLUT4myc vesicles (Khayat et al., 2000; Tong et al., 2001). In response to insulin or PDGF, GLUT4myc partially localized with both short and long ruffles, but not to a discernable level with either stress fibers or microspikes (Fig. 3C). We therefore assessed the dependence of GLUT4myc translocation in response to insulin or PDGF on actin dynamics and remodeling using F-actin-disrupting and -stabilizing agents. A 20 minute pretreatment of the cells with 250 nM cytochalasin D had only a minimal effect on stress fibers, yet markedly decreased both insulin- and PDGF-induced actin remodeling (Fig. 4A). Under these conditions and also in response to higher concentrations of cytochalasin D that disrupt stress fibers (data not shown), surface GLUT4myc content in the unstimulated basal state was unaffected. In addition, we assessed the effect of 250 nM cytochalasin D on basal GLUT4 recycling. Myoblasts were incubated at 37°C for 2 hours or 6 hours in the absence or presence of cytochalasin D along with anti-Myc antibody, so that any GLUT4myc molecule arriving at the cell surface was labeled with the antibody. As previously reported (Foster et al., 2001), approximately 51% of total GLUT4myc molecules were labeled within 2 hours and 91% of the transporters were labeled within 6 hours. Treatment with cytochalasin D during

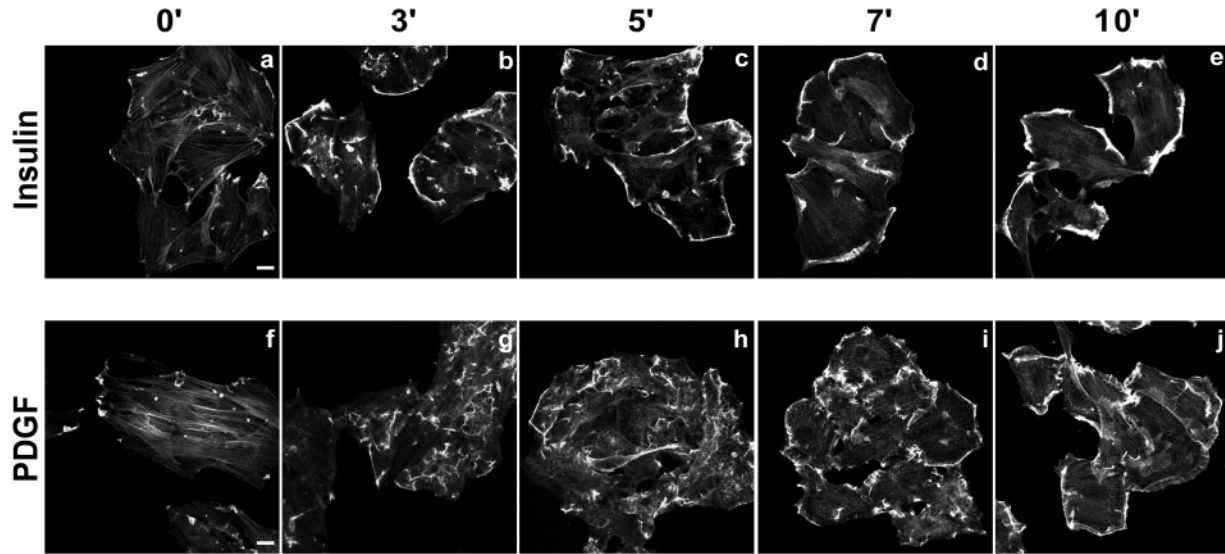
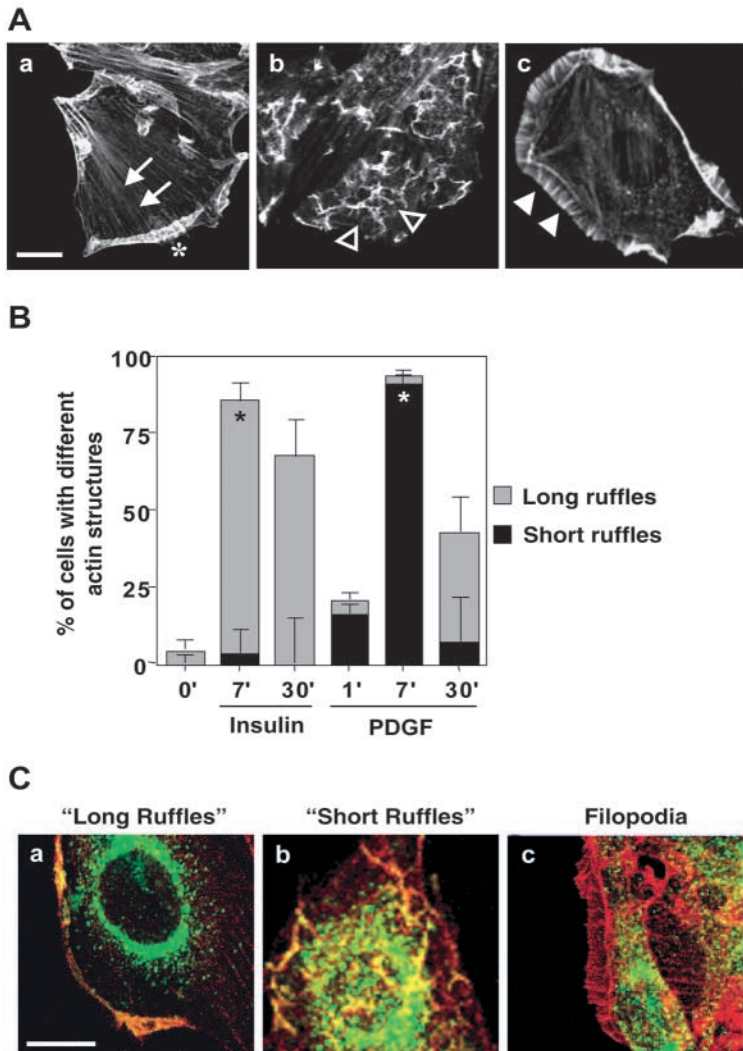


Fig. 2. Time course of insulin- and PDGF-induced actin remodeling in myoblasts. Serum-deprived GLUT4myc myoblasts were left untreated (a,f) or stimulated with 100 nM insulin (b-e) or with 50 ng ml⁻¹ PDGF-BB (g-j) for the times indicated. The cells were then fixed, permeabilized and labeled with rhodamine-conjugated phalloidin. Representative images are shown of a single focal plane near the dorsal surface of the cell. The images shown are representative of five experiments. Scale bar, 10 μm.



the live-cell-labeling period did not affect GLUT4myc labeling (54% and 97% at 2 hours and 6 hours, respectively). These findings suggest that basal GLUT4 recycling was not dependent on filamentous actin and/or on actin dynamics. By contrast, the insulin-induced increase in surface GLUT4myc was 80% inhibited compared with control cells and, strikingly, PDGF-induced GLUT4 translocation was only inhibited by 30% ($P=0.19$; Fig. 4B). Similar results were obtained with latrunculin B and the F-actin-stabilizing agent jasplakinolide (Table 2). With the latter, insulin-stimulated GLUT4myc translocation was fully inhibited, whereas basal GLUT4myc content (not shown) and the response to PDGF (Table 2) were not

Fig. 3. Insulin and PDGF promote morphologically distinct actin structures. (A) Serum-deprived L6 GLUT4myc myoblasts were stimulated with either insulin or PDGF-BB for the times indicated in B, after which cells were fixed and stained for actin. Collapsed *xy* projections were obtained by confocal microscopy to reveal distinct filamentous actin structures such as stress fibers and long ruffles (a, arrows and asterisk, respectively), short ruffles (b, open arrowheads), and filopodia (c, closed arrowheads). (B) Images were obtained from three independent experiments and the presence of long and short actin ruffles was scored in at least 60 cells per condition. The means±s.e.m. are given of the proportion of cells exhibiting either of the two types of actin structure at each time point after insulin or PDGF stimulation. *, $P<0.001$ against time 0. (C) GLUT4myc proteins were stained with anti-Myc antibody followed by FITC-conjugated secondary antibody. Composite images of F-actin labeled with rhodamine-conjugated phalloidin and GLUT4myc protein staining are presented in cells that exhibit long ruffles (a), short ruffles (b) and filopodia (c); yellow indicates regions of co-localization. The images shown are representative of five individual experiments. Scale bar, 10 μm.

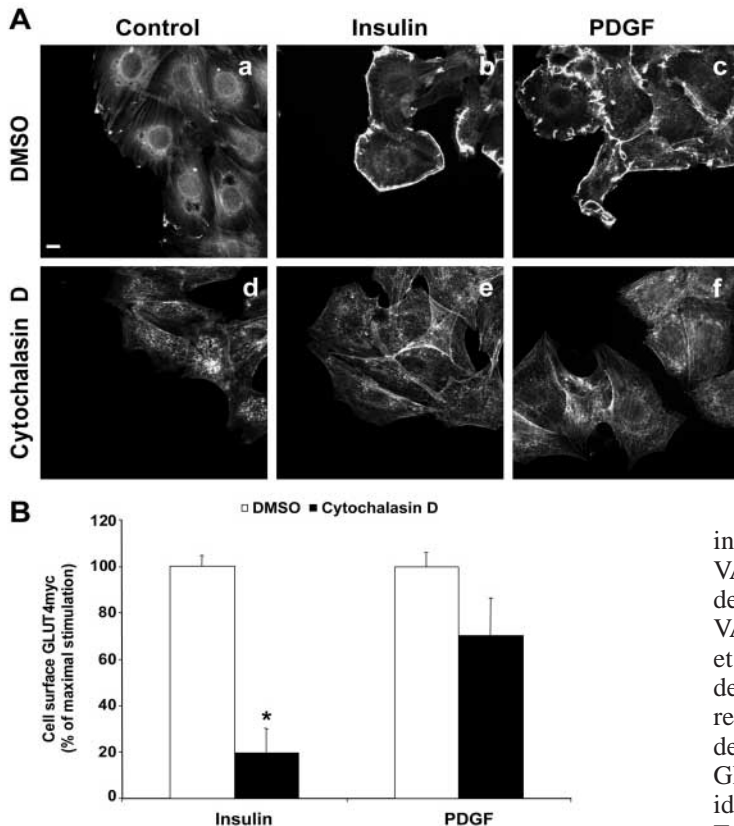


Fig. 4. The actin-disrupting agent cytochalasin D affects GLUT4myc translocation differently in response to insulin and PDGF. Serum-deprived GLUT4myc myoblasts were treated without (a-c) or with (d-f) 250 nM cytochalasin D for 20 minutes before and during incubation with either 50 ng ml⁻¹ PDGF-BB or 100 nM insulin for 7 minutes at 37°C. The cells were then either (A) fixed, permeabilized and stained for filamentous actin with rhodamine-phalloidin or (B) left intact and reacted with an anti-Myc antibody and subjected to the colorimetric assay for cell surface GLUT4myc. The increase in surface GLUT4myc in response to each stimulus over basal (unstimulated) conditions was calculated and presented as the proportion of the response in the absence of cytochalasin D. The means±s.e.m. are shown of four independently performed experiments, each performed in triplicate. *, *P*<0.05 compared with the respective control in the absence of the drug. Scale bar, 10 μm.

significantly affected. Collectively these results demonstrate a greater reliance of GLUT4 translocation on actin remodeling in response to insulin than to PDGF.

PDGF-mediated GLUT4myc translocation does not require TeTx-sensitive VAMPs

We have previously shown that the insulin-induced gain in surface GLUT4myc in L6 myoblasts functionally depends on VAMP2 (Randhawa et al., 2000), based on the inhibitory effect of transient transfection of TeTx light chain, a toxin that cleaves certain v-SNAREs (TeTx-sensitive VAMPs) such as VAMP2 and VAMP3. TeTx-induced inhibition of GLUT4myc translocation was rescued by co-transfection of a TeTx-

insensitive VAMP2 mutant (but not a similar mutant of VAMP3) (Randhawa et al., 2000). This conclusion, of the dependency of insulin-induced GLUT4 translocation on VAMP2, was also supported by studies in adipocytes (Malide et al., 1997; Martin et al., 1998; Ramm et al., 2000). To determine whether PDGF-induced GLUT4 translocation also relies on TeTx-sensitive VAMP, surface GLUT4myc was detected in myoblasts transiently co-transfected with TeTx and GFP, the latter used so that transfected cells could be easily identified (Fig. 5A). Consistent with previous observations, TeTx did not affect the cell-surface GLUT4myc content in the basal state, suggesting that basal GLUT4myc recycling does not functionally rely on v-SNAREs cleavable by the toxin, including VAMP2. By contrast, in response to insulin an 82% inhibition (*P*<0.05) in the gain in surface GLUT4myc was observed. Strikingly, the PDGF-induced gain in surface GLUT4myc was not significantly reduced in cells expressing TeTx (9% decrease; *P*=0.42; Fig. 5B,C). This result argues against a significant reliance of PDGF on TeTx-sensitive fusion for the recruitment of GLUT4 to the myoblast surface.

Collectively, the above results highlight marked differences between insulin- and PDGF-induced GLUT4 translocation (at the level of TeTx sensitivity and reliance on actin remodeling), even though both engage PtdIns 3-kinase. To explore whether the two stimuli involve totally independent pools of GLUT4 and distinct PtdIns-3-kinase-mediated signals, we examined their combined effect. Insulin and PDGF treatment for 7 minutes did not produce a significantly different gain in cell surface GLUT4myc than either stimulus alone (insulin, 1.6±0.1-fold above basal; PDGF, 1.8±0.2-fold; insulin + PDGF, 2.0±0.3). Because GLUT4 recruitment to the plasma membrane in response to insulin and PDGF is not additive, the mechanisms and/or GLUT4 pools mobilized by both stimuli are not fully separable.

Discussion

In the present study, we compare the cellular mechanisms used by insulin and PDGF in recruiting GLUT4 to the surface of muscle cells. The gain in cell surface GLUT4 directly corresponds to an increase in glucose uptake, because previous work has shown that regulation of GLUT4 activity is largely absent from myoblasts (Niu et al., 2003). Here, we demonstrate

Table 2. Insulin and PDGF-induced GLUT4myc translocation in the presence of actin-disrupting agents

Actin-disrupting agent	Insulin	PDGF
Latrunculin B	21±6%*	61±12%
Jasplakinolide	0±15%*	78±30%

The table shows the stimulus-induced increase in surface GLUT4myc as a proportion of the effect in control (vehicle-treated) cells. Serum-starved GLUT4myc myoblasts were treated with vehicle (dimethyl sulfoxide), latrunculin B (250 nM for 20 minutes) or jasplakinolide (2 μM for 30 minutes) before and during stimulation without or with either insulin (100 nM for 7 minutes) or PDGF-BB (50 ng ml⁻¹ for 7 minutes). The increase in surface GLUT4myc in response to each stimulus over basal (unstimulated) conditions was calculated and the percentage stimulation of this value induced in latrunculin-B- or jasplakinolide-treated cells was calculated from three independent experiments, each performed in triplicate.

**P*<0.05 against PDGF.

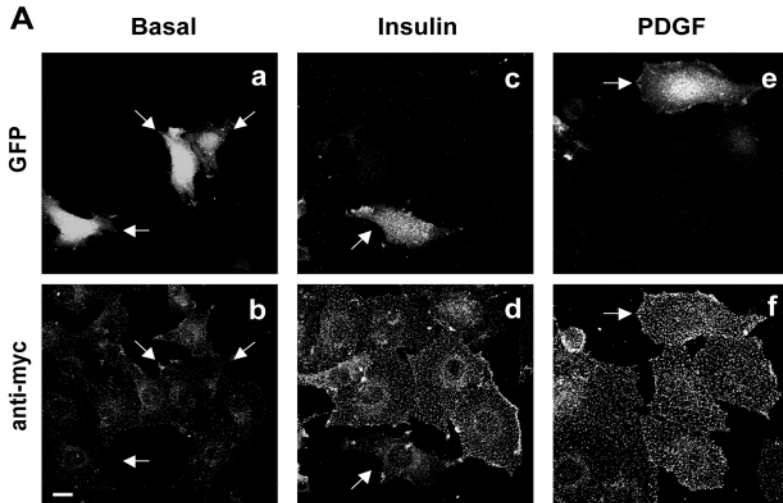
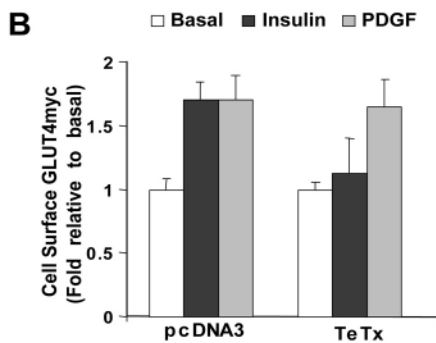


Fig. 5. Tetanus toxin (TeTx) reduces the insulin-dependent, but not the PDGF-dependent, gain in GLUT4myc at the surface. GLUT4myc myoblasts were co-transfected with 0.6 μg of eGFP-encoding cDNA and 0.9 μg of either pcDNA3 (empty vector) or TeTx-encoding cDNA, as indicated. (A) After 24 hours, cells were serum starved and then stimulated without insulin (a,b), with 100 nM insulin (c,d) or with 50 ng ml⁻¹ PDGF (e,f). Cells co-expressing TeTx and eGFP are shown at the top, and the corresponding anti-Myc surface staining from the same field of view is shown at the bottom. All images are collapsed xy projections. (B) Quantification of cell-surface GLUT4myc labeling in empty vector and TeTx-transfected cells, showing the means \pm s.e.m. of three independent experiments. Scale bar, 10 μm .



that neither stimulus had any appreciable effect on the rate of GLUT4 endocytosis (Table 1), and thus probably rely primarily on enhancing exocytosis to achieve the GLUT4 gain at the surface. However, although a large proportion of the insulin response functionally depends on the TeTx-cleavable VAMP2 (Randhawa et al., 2000) (Fig. 5), PDGF response does not require such TeTx-sensitive VAMPs. Moreover, although the insulin response requires actin dynamics (Tsakiridis et al., 1994) (Fig. 3), GLUT4 recruitment to the cell surface in response to PDGF still occurs when actin remodeling is largely prevented (Fig. 3, Table 2). Because, in response to insulin, VAMP2-containing vesicles partially localize with the actin structures induced by the hormone (Randhawa et al., 2000), it appears that insulin-induced actin remodeling is required to segregate VAMP2-dependent GLUT4 vesicles specifically and to allow their recruitment to the plasma membrane. VAMP2 would act as a fusogen either at the plasma membrane or through additional intermediate fusion (and fission) steps.

In an effort to understand specific mechanisms that promote GLUT4 translocation, many investigators have compared PDGF and insulin stimulations at the level of PtdIns 3-kinase, an enzyme essential for insulin-mediated GLUT4 translocation. Using this comparative analysis approach, various mechanisms of specificity have been proposed. Focusing on the enzyme itself, it was suggested that the two agents engage different isoforms of the p110 catalytic subunits of PtdIns 3-kinase (Hooshmand-Rad et al., 2000) or that insulin can activate the enzyme in unique subcellular compartments (Clark et al., 1998; Ricort et al., 1996). Importantly, PtdIns 3-

kinase is activated directly by the tyrosine-phosphorylated PDGF receptor, unlike the insulin receptor, which requires the tyrosine-phosphorylated insulin-receptor substrates (Whiteman et al., 2003). This difference might translate into distinct localization of PtdIns 3-kinase and its products within the cell. Moreover, a recent study reported that the two stimuli elevate total cellular levels of different 3'-phosphoinositide products (Maffucci et al., 2003). Alternatively, it was suggested that the signal specificity of insulin lies in the threshold of duration and/or intensity of PtdIns-(3,4,5)-P₃ produced (Oatey et al., 1999; Tengholm and Meyer, 2002). However, rather than explaining the specificity of signals at the level of PtdIns 3-kinase, failure of PDGF to reach such threshold could arise from the tendency of cells to lose PDGF responsiveness during differentiation, a process attributed to receptor downregulation and/or to post-receptor modulation (Fiaschi et al., 2003; Summers et al., 1999; Vaziri and Faller, 1996; Whiteman et al., 2003). Consistent with this possibility was the observation that 3T3-L1 adipocytes, a common cellular model used to study insulin-specific metabolic effects, constitute a heterogeneous population of cells: A PDGF-responsive subpopulation that is less differentiated expresses less insulin receptor and GLUT4, and more PDGF receptor than the insulin-responsive subpopulation (Shigematsu et al., 2001). The above-proposed explanations for insulin versus PDGF specificity at the level of PtdIns 3-kinase are not mutually exclusive. In fact, they highlight the need to re-examine the effects of insulin and PDGF in a homogeneous cell population that retains the capacity to respond to both stimuli through endogenous levels of both receptors.

The novelty of the present study lies in that: (1) it tests the responses to insulin and PDGF mediated through endogenous levels of the two respective receptors; and (2) it identifies elements downstream of PtdIns 3-kinase unique to each stimulus, specifically at the levels of actin remodeling, reliance of GLUT4 mobilization on actin dynamics and functional dependence on VAMP2.

Actin remodeling in response to insulin and PDGF might regulate very different cellular functions. PDGF is well known to induce cell migration, largely in cells that have not completed their terminal differentiation. Because any cell migration depends on well-coordinated actin remodeling, it is

conceivable that the response observed in myoblasts represents part of the mechanisms induced by PDGF to promote cell movement. An additional element in cell migration is the capacity to recruit and mobilize membrane structures to the leading edge of the cell. It is likely that, in this context, GLUT4-containing vesicles are recruited to the plasma membrane in response to PDGF, independent of actin remodeling. In this regard, PDGF might be enhancing the constitutive GLUT4 recycling in the basal state, which is also unaffected by actin-disrupting agents. In contrast to PDGF, insulin has well-established regulatory metabolic effects, such as the stimulation of glucose uptake in muscle. We have previously shown that actin structures formed in response to insulin create cellular microdomains in which signaling molecules and VAMP2-containing intracellular vesicles concentrate (Khayat et al., 2000; Patel et al., 2003; Tong et al., 2001). This process was required for downstream signal propagation, as well as for the ultimate recruitment of GLUT4 to the cell surface. Furthermore, in addition to PtdIns-(3,4,5)- P_3 generation at the plasma membrane (Patel et al., 2003; Tengholm and Meyer, 2002), vesicles trapped within the insulin-induced actin mesh seem to provide substrates for localized PtdIns-(3,4,5)- P_3 production in response to the hormone (Patel et al., 2003). This is consistent with biochemical analyses of PDGF and insulin-induced PtdIns-3-kinase activation: insulin-induced, but not PDGF-induced, PtdIns-3-kinase activity is recovered in cytoskeleton-rich cellular fractions (Clark et al., 1998). Therefore, it appears that actin structures induced by insulin are not only the consequence of specific upstream insulin signaling events but are in fact required to confer signal specificity. VAMP2-dependent membrane fusion events are a downstream target for such insulin-specific signals, differentiating insulin-induced GLUT4 traffic from the mobilization of GLUT4 in the basal state or in response to PDGF. The specificity of the insulin response might lie in acquisition of VAMP2 after the GLUT4 vesicles segregate away from the recycling pool.

Understanding insulin signal specificity and identifying alternative mechanisms by which muscle cells can recruit GLUT4 and increase glucose uptake are of utmost clinical relevance. Insulin resistance (i.e. the decreased capacity of insulin to induce its metabolic effects such as stimulation of glucose uptake in muscle) is frequently seen in obesity, hypertension and ageing. Moreover, impaired insulin-stimulated GLUT4 translocation and glucose uptake play a key role in the pathogenesis of Type 2 diabetes (Garvey et al., 1998; Kahn et al., 1991; Zierath et al., 1998). Remarkably, although, in muscle of affected individuals, these responses to insulin are blunted, stimulation of glucose uptake by other stimuli such as muscle contraction might be intact (Kennedy et al., 1999). Here, PDGF was used as an experimental tool to dissect out specific elements in insulin-induced GLUT4 translocation. Intriguingly, a recent report on large-scale gene expression profiling of skeletal muscle found the PDGF receptor to be regulated in response to insulin-dependent input as well as to a diabetic milieu (C. R. Kahn, personal communication). Based on the data presented in the present study, it is tempting to speculate that such regulation of the PDGF receptor might affect GLUT4 traffic in the muscle. This adds a hitherto-unrecognized mode of (dys)regulated GLUT4 traffic in diabetes and/or insulin resistance.

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