Tyrosine Phosphatase Inhibitors, Vanadate and Pervanadate, Stimulate Glucose Transport and GLUT Translocation in Muscle Cells by a Mechanism Independent of Phosphatidylinositol 3-Kinase and Protein Kinase C

Evangelia Tsiani, Elena Bogdanovic, Alexander Sorisky, Laura Nagy, and I. George Fantus

Vanadate and pervanadate (pV) are protein tyrosine phosphatase (PTP) inhibitors that mimic insulin to stimulate glucose transport. To determine whether phosphatidylinositol (PI) 3-kinase is required for vanadate and pV, as it is for insulin, cultured L6 myotubes were treated with vanadate and pV. The two compounds stimulated glucose transport to levels similar to those stimulated by insulin; however, while PI 3-kinase activity and the increase in the lipid products PI 3,4-bisphosphate and PI 3,4,5-trisphosphate were inhibited by wortmannin after stimulation by all three agents—insulin, vanadate, and pV—wortmannin blocked glucose transport stimulated by insulin but not vanadate or pV. Vanadate and pV stimulated the translocation of GLUTs from an intracellular compartment to the plasma membrane; this stimulation was not blocked by wortmannin, but insulin-induced GLUT translocation was inhibited. Similar results were obtained in cultured H9c2 cardiac muscle cells in which wortmannin did not inhibit glucose transport or the vanadate-induced translocation of GLUT4 in c-*myc*–GLUT4 transfected cells. The ser/thr kinase PKB (Akt/PKB/RAC-PK) is activated by insulin, acts downstream of PI 3-kinase, and has been implicated in signaling of glucose transport. Insulin and pV stimulated PKB activity, and both were inhibited by wortmannin. In contrast, vanadate, at concentrations that maximally stimulated glucose transport, did not significantly increase PKB activity. To determine the potential role of protein kinase C (PKC), L6 cells were incubated chronically with phorbol myristate acetate (PMA) or acutely with the PKC inhibitors calphostin C and bisindolylmaleimide. There was no inhibition of glucose transport stimulation by insulin, vanadate, or pV, and a combination of wortmannin and PKC inhibitors also failed to block the effect of vanadate and pV. In contrast, disassembly of the actin network with cytochalasin D blocked the stimulation of glucose transport by all three agents. In conclusion, vanadate and pV are able to stimulate glucose transport and GLUT translocation by a mechanism independent of PI 3-kinase and PKC. Similar to that by insulin, glucose transport stimulation by vanadate and pV requires the presence of an intact actin network. *Diabetes* 47:1676–1686, 1998

From the Department of Medicine (E.T., E.B., I.G.F.), Banting and Best Diabetes Centre, Mount Sinai Hospital, Toronto, Ontario; the Department of Physiology (E.T., E.B., I.G.F.), University of Toronto, Toronto, Ontario; the Department of Medicine (A.S.), Ottawa Civic Hospital, Ottawa, Ontario, Canada; and the Department of Nutrition (L.N.), School of Medicine, Case Western Reserve University, Cleveland, Ohio.

Address correspondence and reprint requests to Dr. I.G. Fantus, Mount Sinai Hospital, 600 University Ave., Suite 780, Toronto, Ontario, Canada M5G 1X5.

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ANOVA, analysis of variance; BIM, bisindolylmaleimide; BSA, bovine serum albumin; DAG, diacylglycerol; 2DG, 2-deoxy-[^{3}H]glucose; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HBSS, HEPES-buffered saline solution; HPLC, high-performance liquid chromatography; IM, internal membrane; IRS-1, insulin receptor substrate 1; MAPK, mitogen-activated protein kinase; α-MEM, α-minimum essential medium; MKP, MAPK phosphatase; P, pellet; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PI 3,4P_2, PI 3,4-bisphosphate; PI 3,4,5P_3, PI 3,4,5-trisphosphate; PKB, protein kinase B or Akt/PKB/RAC-PK; PKC, protein kinase C; PLC, phospholipase C; PM, plasma membrane; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonylfluoride; PTP, protein tyrosine phosphatase; p-tyr, phosphotyrosine; pV, pervanadate; PVDF, polyvinylidene difluoride; SN, supernatant; TLC, thin layer chromatography.

Vanadium is a trace element found at low concentrations in mammalian tissues; its physiological function remains unknown (1,2). Vanadate, which structurally resembles phosphate, has been demonstrated to interact with several enzymes in vitro and to have biological effects in cultured cells and animals in vivo. It inhibits the P-type phosphorylated ATPases (3,4) and protein tyrosine phosphatases (PTPs) (5,6). In vivo, it enhances protein tyrosine phosphorylation and has been documented to mimic a number of the metabolic actions of insulin (7,8), leading to its successful use as a therapeutic agent in various rodent models of diabetes (9–16) and to preliminary trials in human subjects with type 1 and type 2 diabetes (17,18). We previously reported that upon mixing hydrogen peroxide with vanadate, an insulin-mimetic aqueous peroxovanadium compound (pervanadate [pV]) was formed (19). pV is a powerful PTP inhibitor and also mimics many of insulin's biological effects in target tissues (20–22).

Insulin increases glucose uptake in both adipose tissue and skeletal muscle by inducing the translocation of GLUTs from an intracellular compartment to the cell surface (23,24). The insulin signaling cascade is initiated by the activation of the insulin receptor tyrosine kinase, leading to tyrosine phosphorylation of intracellular proteins such as insulin receptor substrate 1 (IRS-1), which acts as a docking site for proteins containing SH2 domains (25). Phosphatidylinositol (PI) 3-kinase, through the SH2 domain of its p85 regulatory subunit, binds to IRS-1, resulting in increased activity of the p110 cat-

alytic subunit, which phosphorylates inositol phospholipids on the D3 position (26). The fungal metabolite wortmannin inhibits both PI 3-kinase activity and the insulin-induced increase in glucose transport in rat adipocytes (27), 3T3L1 adipocytes (28), rat skeletal muscle (29), and L6 muscle cells (30), indicating that PI 3-kinase is essential for insulin-stimulated glucose transport. Similarly, overexpression of a mutant p85, which does not bind to the p110 catalytic subunit, abolished insulin-stimulated glucose uptake (31–33). Although the physiological role of the lipid kinase products of PI 3-kinase, namely PI 3,4-bisphosphate (PI 3,4P_2) and PI 3,4,5-trisphosphate (PI 3,4,5P_3), is not clear, they appear to partake in intracellular protein trafficking (34) and activation of certain enzymes, namely isoforms of protein kinase C (PKC) (35) and PKB (Akt/PKB/RAC-PK) (36,37).

L6 cells are derived from rat skeletal muscle and differentiate spontaneously from myoblasts to myotubes. The myotubes express three glucose transporter isoforms: GLUT1, GLUT3, and GLUT4 (37,38). Insulin stimulates glucose transport in these cells by a mechanism similar to that in muscle tissue, that is, via the recruitment of GLUTs from an intracellular donor pool to the plasma membrane (39). H9c2 cells are derived from rat heart muscle and respond to insulin with increased glucose uptake (40).

Given the well-established insulin-mimetic actions of vanadium compounds and their effectiveness at lowering glucose in insulin-resistant forms of diabetes, we compared the mechanism of action of their glucose-uptake stimulation to that of insulin. We found that in contrast with insulin, vanadate and pV were able to stimulate glucose uptake and translocation of GLUTs by a mechanism independent of PI 3-kinase and PKB. An intact actin network was required for stimulation by all the agents, however, indicating a convergence of the different signaling pathways.

RESEARCH DESIGN AND METHODS

Materials. α-Minimum essential medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco (Burlington, ON, Canada). HEPES, 2-deoxy-D-glucose, sodium orthovanadate (vanadate), cytochalasin B, PMA, wortmannin, and cytochalasin D were purchased from Sigma (St. Louis, MO). Human insulin was a gift from Eli Lilly (Indianapolis, IN). 2-Deoxy-[^{3}H]glucose (2DG; 8.2 Ci/mmol) was purchased from Du Pont-NEN (Boston, MA). Antiphosphotyrosine (anti–p-tyr) antibody coupled to agarose beads and antibodies against the PH domain of akt/PKBα were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-rat GLUT1 and GLUT4 antibodies were purchased from East Acres Biologicals (Southbridge, MA). Rabbit anti-mouse GLUT3 was a gift from Dr. I. Simpson (NIH, Bethesda, MD). Anti-*myc* antibody (9E10) was purified from serum-free hybridoma supernatant (ATCC, Rockville, MD). The antibody recognizing the ser 473 phosphorylated form of PKB was from New England Biolabs (Boston, MA), and that against native PKB was provided by Dr. J. Woodgett (Ontario Cancer Institute, Toronto, ON, Canada). The enhanced chemiluminescence (ECL) reagent, $^{32}P_i$ (10 mCi/ml), and ^{32}P-ATP (6,000 Ci/mmol) were obtained from Amersham (Oakville, ON, Canada). Oxalate-treated thin layer chromatography (TLC) plates were obtained from Analtech (Newark, DE), and purified L-α-phosphatidylinositol was purchased from Avanti Polar Lipids (Alabaster, AL). The PKB-specific peptide substrate RPRAATF (a modified version of crosstide) was from Upstate Biotechnology. DOTAP liposomal transfection reagent was from Boehringer Mannheim (Laval, QC, Canada). Calphostin C and bisindolylmaleimide (BIM) were obtained from Calbiochem (San Diego, CA).

Cell cultures. L6 cells were grown in α-MEM containing 2% (vol/vol) FBS and 1% (vol/vol) antibiotic-antimycotic solutions and allowed to fuse into myotubes as described (41). All experiments were carried out with fully differentiated myotubes. The cells were grown in 6-well plates for the transport experiments, in 10-cm-diameter dishes for immunoprecipitations and PI 3-kinase activity measurements, and in 15-cm diameter dishes for preparation of membrane fractions.

H9c2 cells (40) (wild-type from ATCC or c-*myc*–GLUT4 transfected) were cultured in 10% FBS in DMEM. For transport studies, cells were seeded at 4,000 cells/cm^2 in 24-well plates or LabTek slide chambers in 10% FBS. After 2 days, the medium was changed to 2% FBS, and cells were allowed to differentiate to myotubes for 12 days. H9c2 cells were stably transfected with a c-*myc* epitope–GLUT4 cDNA construct (a gift from Y. Ebina, University of Tokushima, Japan) that contains the coding sequence for the 14–amino acid sequence of the human c-*myc* epitope inserted in the first exofacial loop of GLUT4 (42). cDNA constructs for the tagged proteins were transfected into subconfluent myoblasts using the DOTAP liposomal transfection reagent (43).

Drug treatment. A stock solution of vanadate was prepared in 50 mmol/l HEPES buffer and subsequently diluted using HEPES-buffered saline solution (HBSS) (140 mmol/l NaCl, 5 mmol/l KCl, 20 mmol/l HEPES, 2.5 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, pH 7.4) containing 5 mmol/l glucose. Pervanadate solutions were prepared by mixing vanadate and H_2O_2 as described (19). Cells were exposed to the different agents and to insulin in HBSS containing 5 mmol/l glucose. In the experiments with wortmannin, cells were exposed to 100 nmol/l wortmannin for the time indicated in each figure before the addition of insulin, vanadate, or pV and throughout the stimulation period. For the PKC-downregulation experiments, cells were preincubated with or without 100 nmol/l PMA for 24 h in α-MEM. The PKC inhibitors calphostin C (0.2 μmol/l) and BIM (1 μmol/l) were added 30 and 60 min, respectively, before addition of vehicle, insulin, vanadate, or pV. H9c2 myotubes were cultured in DMEM with 0.5% FBS for 16 h, washed twice with phosphate-buffered saline (PBS) (137 mmol/l NaCl, 2.7 mmol/l KCl, 15 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 0.7 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, pH 7.4), and incubated with or without 100 nmol/l insulin in PBS or 2 mmol/l vanadate in phosphate-free buffer (137 mmol/l NaCl, 2.7 mmol/l KCl, 0.7 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 25 mmol/l HEPES, pH 7.4) for 20 min at 37°C. Wortmannin-treated cells were preincubated with 100 nmol/l wortmannin for 30 min at 37°C before and during agonist stimulation.

Glucose uptake assay. After incubation of L6 cells in the presence and absence of the various agents, the medium was removed, the cells were rinsed twice with HBSS, and glucose uptake (2DG uptake) was measured with 10 μmol/l 2DG (1 mCi/ml) in HBSS for 10 min at 23°C. For the non–carrier-mediated glucose transport, 10 mmol/l cytochalasin B was added in parallel wells. The glucose uptake assay was terminated by washing the cells three times with 3 ml ice-cold 0.9% saline solution followed by cell disruption with 0.05 N NaOH. Cell-associated radioactivity was determined by scintillation counting. Carrier-mediated glucose uptake was determined after subtraction of the nonspecific (presence of cytochalasin B) from the total (absence of cytochalasin B) uptake. The nonspecific glucose uptake was less than 3% of total. Glucose uptake was measured in a similar way in H9c2 myotubes, where the nonspecific uptake was determined using 10 mmol/l phloretin.

Immunoprecipitations. L6 myotubes were incubated for 24 h in α-MEM containing 0.1% FBS and were treated the next day with the different agents as described above. After treatment, the cells were immediately washed once with ice-cold PBS containing 100 μmol/l Na_3VO_4 and twice with buffer A (20 mmol/l HEPES, 137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 100 μmol/l Na_3VO_4, pH 7.5); finally, they were lysed in 1 ml of lysis buffer (buffer A containing 10% glycerol, 1% Nonidet P-40 [vol/vol], 2 mmol/l PMSF). The cell lysate was spun at 16,000 rpm for 10 min at 4°C, and the supernatants were incubated overnight with anti–p-tyr antibody coupled to agarose beads. The immunocomplexes were used for measurements of PI 3-kinase activity.

Anti–p-tyr antibody–associated PI 3-kinase activity. The immunocomplexes were washed and incubated for 5 min with 50 μl buffer B (10 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, 100 μmol/l Na_3VO_4, pH 7.5), 10 μl of 100 mmol/l $MgCl_2$, and 20 μg PI resuspended by sonication in 10 μl of 10 mmol/l Tris, pH 7.5, and 1 mmol/l EGTA. The reaction was initiated by the addition of 5 μl of 1 mmol/l γ-[^{32}P]ATP in 20 mmol/l $MgCl_2$ and terminated after 10 min at 23°C by the addition of 20 μl of 6 mol/l HCl. Lipids were extracted with 160 μl of $CHCl_3$:MeOH (chloroform:methanol) (1:1) followed by centrifugation for 10 min at 16,000 rpm (Eppendorff microfuge). The lower phase was extracted, and 50 μl was applied to TLC plates that had been prebaked at 110°C for 1 h. The lipids were separated with $CHCl_3$:MeOH:H_2O:NH_4OH (60:47:11.6:2), and detection and quantitation of [^{32}P]PI 3-phosphate was determined by densitometry using a Molecular Dynamics Phosphor Imager System.

Determination of PI 3-kinase lipid products in intact cells. Myotubes grown in 10-cm-diameter dishes were incubated overnight in medium containing 0.1% FBS and then for 4 h in phosphate-free medium supplemented with 0.5% bovine serum albumin (BSA) and containing $^{32}P_i$ to a final concentration of 250 μCi/ml. The cells were rinsed three times with HBSS containing 5 mmol/l glucose and were stimulated with insulin, vanadate, and pV at the concentrations and for the times indicated. At the end of the incubation, the buffer was rapidly aspirated, and 1 ml ice-cold 2.4 mol/l HCl was added to each dish. The cells were scraped and transferred to 5-ml polypropylene tubes. One milliliter ice-cold $CHCl_3$ was added, followed by 1.5 ml of $CHCl_3$:MeOH (1:2, vol/vol). The samples were vortexed thoroughly and spun at 500*g* at 4°C for 5 min to separate the organic and aqueous phases. The lower organic phase was aspirated, the remaining aqueous phase was similarly extracted twice more, and all three lipid extracts were pooled and dried

nder nitrogen (N_2). The dried lipids were redissolved in 50 µl $CHCl_3$:MeOH (1:1, ol/vol) and resolved by TLC. ^{32}P-labeled lipids were visualized by autoradiogra-hy and deacylated without elution from silica (44). The resulting glycerol phos-hoinositol polyphosphate species were separated by high-performance liquid romatography (HPLC) on a Whatman Partisphere 5 SAX 25 cm column. Quan-tation of eluting ^{32}P was performed using a radioisotope flow detector (Beckman odel 171). Lipid species were identified by comparison of retention times with d standards. Incorporated radioactivity was determined with a beta-counter.

reparation of membrane fractions. Cells grown in 15-cm–diameter dishes ere incubated with vanadate or pervanadate in the same manner as for the glu-ose uptake assay. After incubation, myotubes were gently scraped in their own cubation medium with a rubber policeman, centrifuged (700*g* for 8 min), and aced on ice. All subsequent steps were performed at 4°C. The cell pellet was suspended in homogenization buffer (250 mmol/l sucrose, 20 mmol/l HEPES, H 7.4, 2 mmol/l EGTA, 3 mmol/l NaN_3, and freshly added protease inhibitors: 00 µmol/l PMSF, 10 µmol/l E-64 [trans-epoxysuccinyl-L-leucyl amido(4-guani-no)butane], 1 µmol/l leupeptin, and 1 µmol/l pepstatin A and homogenized ith 20 strokes in a 40-ml glass Dounce type A homogenizer. That pellet (P1) was scarded, the supernatant (SN1) was centrifuged at 31,000*g* for 60 min, and the sulting supernatant (SN2) was used to collect internal membranes (IM) by ntrifugation at 177,000*g* for 60 min (P3). The 31,000*g* pellet (P2) was gently rinsed ithout disturbing with homogenization buffer and resuspended in the same uffer to a final volume of 3 ml using a Wheaton 5-ml Teflon glass homogenizer. 2 was placed on a discontinuous sucrose gradient of 3 ml each of 32, 40, and 50% wt/wt) sucrose solution in 20 mmol/l HEPES, pH 7.4. The membranes that anded on top of the 32% sucrose layer at the 32/40% and at the 40/50% sucrose terfaces were collected and pelleted. Membranes isolated atop the 32% sucrose yer are enriched in plasma membrane markers; therefore that fraction is enoted plasma membrane (PM) (41).

mmunoblotting. Samples (15 µg) of plasma membrane and intracellular mem-ane fractions were solubilized in electrophoresis sample buffer and separated y SDS-PAGE. The samples were subsequently transferred electrophoretically to olyvinylidene difluoride (PVDF; BIORAD) membranes. The membranes were cubated for 1 h at room temperature with 3% (wt/vol) BSA in Tris-buffered saline ontaining 0.04% NP-40 and incubated overnight at 4°C with anti-GLUT4 polyclonal ntibody R820 (1:500 dilution), anti-GLUT1 polyclonal antibody (1:1,000 dilu-on), or anti-GLUT3 antibody (1:500 dilution) in the same buffer. Primary anti-odies were detected with the ECL method, visualized by autoradiography using odak XAR-5 film, and quantified by laser scanning densitometry.

KB immunoprecipitation and kinase assay. After stimulation for the indicated mes at the indicated concentrations of insulin, vanadate, and pV, L6 myotubes ere washed twice with ice-cold PBS, scraped, and lysed in buffer C (50 mmol/l ris-HCl, pH 7.5, 1 mmol/l EDTA, 1 mmol/l EGTA, 0.5 mmol/l Na_3VO_4, 0.1% 2-mer-aptoethanol, 1% Triton X-100, 50 mmol/l NaF, 5 mmol/l Na pyrophosphate, 10 mmol/l sodium β-glycerophosphate, 0.1 mmol/l phenylmethylsulfonylfluoride PMSF), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µmol/l micro-ystin). The lysates were centrifuged and frozen at –80°C until assay. PKB activ-y was determined according to the instructions of the immunoprecipitation nase assay kit (Upstate Biotechnology). Briefly, PKBα was immunoprecipi-ted from 200 µg cell lysate with 4 µg antibody against the PH domain of PKBα ound to protein G. After 90 min, the samples were centrifuged, and the immuno-omplexes were washed once with buffer C containing 0.5 M NaCl, twice with uffer D (50 mmol/l Tris-HCl, pH 7.5, 0.03% [wt/vol] Brij-35, 0.1 mmol/l EGTA, 0.1% mercaptoethanol), and finally once with assay buffer (20 mmol/l MOPS, pH 7.2, 5 mmol/l β-glycerol phosphate, 5 mmol/l EGTA, 1 mmol/l Na orthovanadate, 1 mmol/l dithiothreitol). The kinase assay was carried out for 10 min at 30°C in assay uffer with 10 µCi γ-[^{32}P]ATP and unlabeled ATP (100 µmol/l final concentration) ontaining 10 µmol/l cAMP-dependent protein kinase inhibitor and 100 µmol/l of modified version of crosstide. The latter peptide RPRAATF is a relatively spe-fic substrate with a thr phosphorylation site analogous to the ser on GSK-3 (ser-phosphorylated by PKB (45). Crosstide is not phosphorylated by p70 S6 kinase 5). The assay was terminated by centrifugation and blotting aliquots of the super-atant on phosphocellulose paper (Whatman). Radioactivity was determined by cintillation counting.

nmunoblotting of total and phosphorylated PKB. Cell lysates (60 µg) were ubjected to SDS-PAGE (10%) followed by electrotransfer to nitrocellulose mem-anes (Schleicher & Schuell). After blocking for 1 h at 22°C in Tris-buffered saline, H 7.5, containing 0.1% Tween-20 and 5% nonfat dry milk, the membranes were cubated overnight with a 1:1,000 dilution of either the anti–phospho-PKB anti-ody or anti-PKB. The membranes were washed and treated for 1 h with sec-ndary antibody (1:10,000) conjugated to horseradish peroxidase (Amersham). roteins were visualized by ECL (Amersham).

nmunofluorescence. c-*myc*–GLUT4 transfected H9c2 myotubes were incubated DMEM with 0.5% FBS for 16 h, washed twice in PBS, and treated with or with-ut 100 nmol/l insulin or 2 mmol/l vanadate for 30 min at 37°C. Wortmannin-treated ells were preincubated in 100 nmol/l wortmannin for 30 min at 37°C before and during agonist stimulation. Cells were then fixed in freshly prepared 4% paraformaldehyde for 10 min on ice and 20 min at room temperature, quenched in glycine, and blocked for 1 h in PBS containing 2% BSA and 5% fish gelatin for at least 2 h. Slides were then incubated with a 1:200 dilution of monoclonal antibody to c-*myc* (9E10), washed three times for 15 min each in fresh blocking buffer, and incubated for 1 h with Texas red–conjugated anti-mouse IgG (1:200; Molecular Probes) in blocking buffer. Slides were finally washed three times for 15 min each in blocking buffer and twice for 5 min each in PBS and mounted with Vectashield anti-fade. Cells were examined under a 60× lens on a BioRad confocal microscope by first localizing cells with transmitted light and then examining them by fluorescence with zoom set at 1.5.

Statistical analysis. Results are presented as means ± SE. The significance of the differences between groups was determined using analysis of variance (ANOVA).

RESULTS

Wortmannin does not inhibit vanadate- or pV-stimulated glucose uptake. We found that incubation of myotubes with vanadate and pV stimulated the rate of glucose transport in a dose- and time-dependent manner to a maximum similar to that achieved with insulin (46 and data not shown). To determine the role of PI 3-kinase, L6 myotubes were exposed to 100 nmol/l insulin (30 min), 5 mmol/l vanadate (60 min), or 100 µmol/l pV (60 min) in the absence and presence of 100 nmol/l wortmannin, which was added for 35 min before insulin and 5 min before vanadate and pV so that the duration of wortmannin treatment was identical in all groups. Wortmannin significantly inhibited basal glucose transport to 59 ± 2.0% of control ($P < 0.001$), as previously reported (30). Thus the ability of the agents to stimulate glucose uptake in the presence of wortmannin was expressed as a percent of the basal transport in the presence of wortmannin. Stimulation of glucose transport by insulin was inhibited by wortmannin (117 ± 5.3%, NS vs. basal), while stimulation by vanadate (152 ± 12.3%, $P < 0.001$) and pV (240 ± 9.2%, $P < 0.001$) was not affected (Fig. 1). In paired experiments, all three agents significantly stimulated glucose uptake in the absence of wortmannin; the extent of stimulation by vanadate and pV was not different from that of insulin (insulin 178 ± 5.8% of basal; vanadate 144 ± 8.5%; pV 179 ± 3.9%; $P < 0.001$ vs. basal). It should be noted that increasing concentrations of wortmannin to 1 µmol/l did not inhibit vanadate- or pV-stimulated glucose transport (data not shown), nor did longer incubation time (wortmannin added 30 min instead of 5 min before the addition of vanadate or pV) (data not shown). These results suggested that, in contrast with insulin, vanadate and pV could stimulate glucose transport in L6 muscle cells without activating PI 3-kinase.

Wortmannin blocks PI 3-kinase activity. To determine whether vanadate and pV were in fact activating PI 3-kinase in myotubes and whether any activation was blocked by wortmannin, PI 3-kinase activity was measured in vitro in anti–p-tyr immunoprecipitates. L6 myotubes were treated with 100 nmol/l insulin, 5 mmol/l vanadate, or 100 µmol/l pV for 15 min, after which the cells were solubilized, total cell lysates were subjected to immunoprecipitations with anti–p-tyr antibody, and PI 3-kinase activity was measured. Insulin, vanadate, and pV increased PI 3-kinase activity to a similar extent, 2- to 2.5-fold of basal (Fig. 2). Pervanadate increased PI 3-kinase activity with a time-course similar to that of insulin, reaching maximum levels within 5 min. Activation by vanadate was somewhat slower: maximum levels were reached within 15 min (data not shown). Pretreatment with 100 nmol/l wortmannin inhibited basal PI 3-kinase activity and abolished the increase induced by insulin

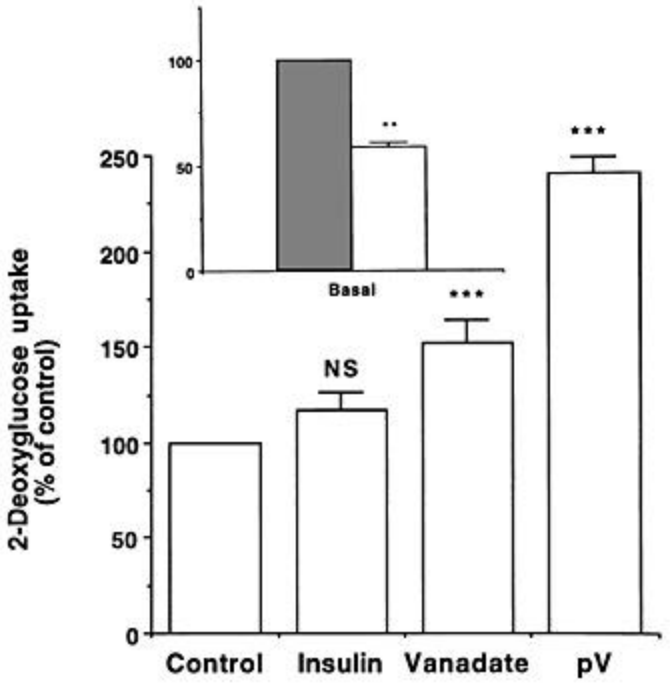


FIG. 1. Effect of wortmannin on insulin, vanadate, and pV stimulation of glucose uptake in myotubes. Fully differentiated L6 myotubes were incubated with and without 100 nmol/l wortmannin for 35 min followed by the addition of insulin (10^{-7} mol/l) for 30 min or 5 min with wortmannin followed by addition of vanadate (5 mmol/l) or pV (100 µmol/l) for 60 min. At the end of the incubation, 2DG uptake was measured as described in METHODS. The results are the mean ± SE of three to four experiments performed in duplicate and are expressed as percent of basal uptake in the presence of wortmannin. The effect of wortmannin on basal glucose transport is shown in the inset (■, control; □, wortmannin). **P < 0.01 compared with control in the absence of wortmannin; ***P < 0.001 compared with wortmannin-treated basal.

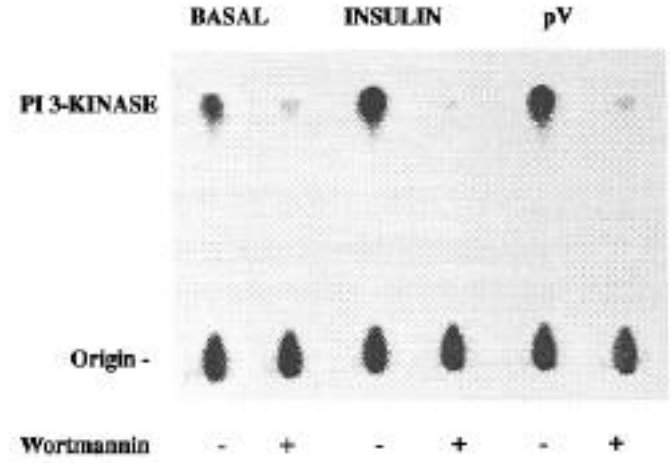


FIG. 2. Effect of wortmannin on PI 3-kinase activity stimulated by insulin and pV. L6 myotubes were incubated with and without 100 nmol/l wortmannin for 15 min followed by stimulation with insulin (100 nmol/l, 5 min) or pV (100 µmol/l, 15 min). After cell lysis, immunoprecipitations were performed with anti–p-tyr antibody, and PI 3-kinase activities were measured as described in METHODS. Results shown are the densitometric scans of the TLC plate of one of three experiments with similar results.

and pV (Fig. 2). Wortmannin also abolished the increase in PI 3-kinase activity in response to vanadate (not shown).

Generation of the PI 3-kinase lipid products in intact cells. To confirm the results of the PI 3-kinase assays described above and to be certain that no PI 3-kinase was activated but not immunoprecipitated with the anti–p-tyr antibody, we assessed the effects of insulin, vanadate, and pV on the generation of lipid products. Myotubes were labeled with $^{32}P_i$ for 4 h followed by stimulation with insulin, vanadate, and pV in the absence and presence of 100 nmol/l wortmannin as described above. Insulin, vanadate, and pV increased the levels of PI 3,4,5P_3 and PI 3,4P_2 (Fig. 3). Wortmannin blocked the effect of all three agents on the generation of the PI 3-kinase lipid products in intact cells, consistent with an inhibition of the stimulation of PI 3-kinase activity.

Vanadate and pV stimulate GLUT translocation independent of PI 3-kinase. To investigate the mechanism of action of vanadate and pV, we examined their effects on subcellular distribution of GLUTs. Internal membranes and plasma membranes from control and vanadate- and pV-treated myotubes were prepared as described in METHODS. The proteins in each fraction were separated by SDS-PAGE, transferred to PVDF membranes, and examined by immunoblotting with specific antibodies against the different GLUT isoforms. Representative immunoblots are shown for GLUT1 (Fig. 4*A* and *C*) and GLUT4 (Fig. 5*A* and *C*), which demonstrate an increase in PM and a decrease in IM fractions of both GLUTs in response to vanadate treatment. There was an approximate doubling of PM GLUT1 and GLUT4 after insulin or vanadate stimulation and a concomitant decrease in IM of GLUT1 (~65%) and GLUT4 (~55%). These results are consistent with translocation of GLUTs that is identical for vanadate and insulin. Similar results were observed for GLUT3 (data not shown).

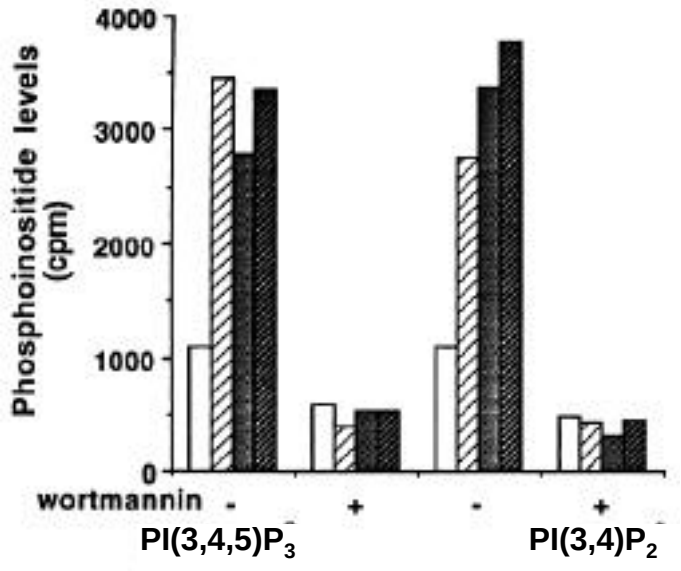
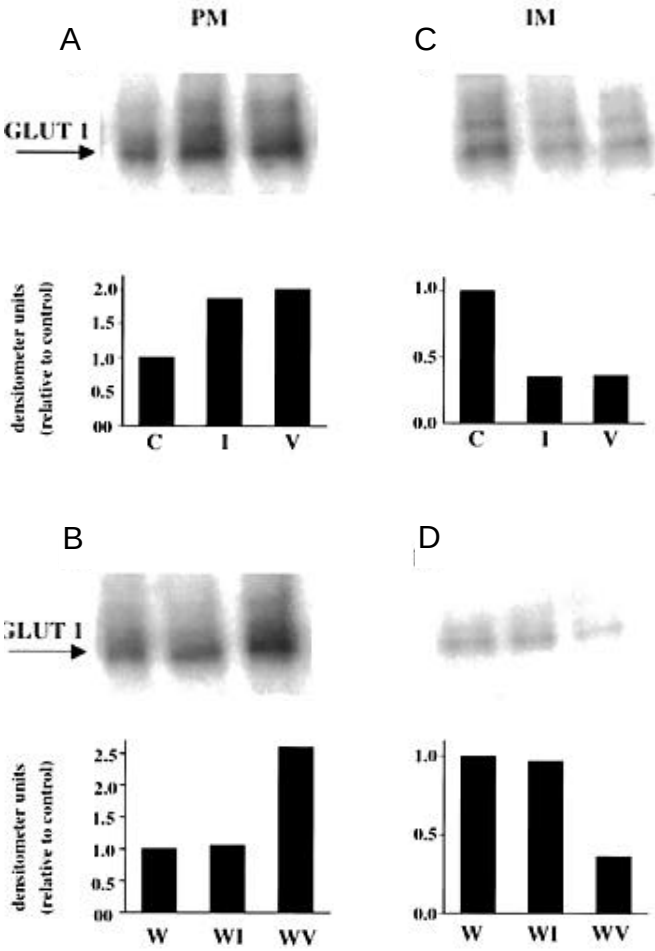


FIG. 3. Effect of wortmannin on insulin, vanadate, and pV stimulation of synthesis of PI 3-kinase lipid products in situ. L6 myotubes were incubated for 4 h in phosphate-free medium containing $^{32}P_i$ followed by incubation with (+) and without (–) 100 nmol/l wortmannin for 15 min and stimulation with 100 nmol/l insulin for 5 min and 5 mmol/l vanadate and 100 µmol/l pV for 15 min. Lipids were extracted, the phospholipids PI 3,4,5P_3 and PI 3,4P_2 were separated by HPLC, and ^{32}P incorporation was determined as described in METHODS. The results shown are from one of three experiments with similar results. □, unstimulated control; ▨, insulin; ■, vanadate; ▨, pV.



IG. 4. Effect of insulin and vanadate on the distribution of GLUT1 in 6 skeletal muscle cells. L6 myotubes were cultured and treated as escribed in the legend to Fig. 1. At the end of the incubation, PM (*A* nd *B*) and IM (*C* and *D*) fractions were prepared as described in ETHODS, separated by SDS-PAGE, transferred to PVDF membranes, and mmunoblotted with antibodies against GLUT1. The intensities of the ands were determined by laser densitometry and are plotted in units elative to unstimulated controls in the absence (*A* and *C*) and pres-nce (*B* and *D*) of wortmannin. Data shown are representative of four xperiments with similar results. C, control; I, 10^{-7} mol/l insulin; V, 5 mmol/l vanadate; W, 100 nmol/l wortmannin; WI and WV, wortmannin in ombination with insulin and vanadate, respectively.

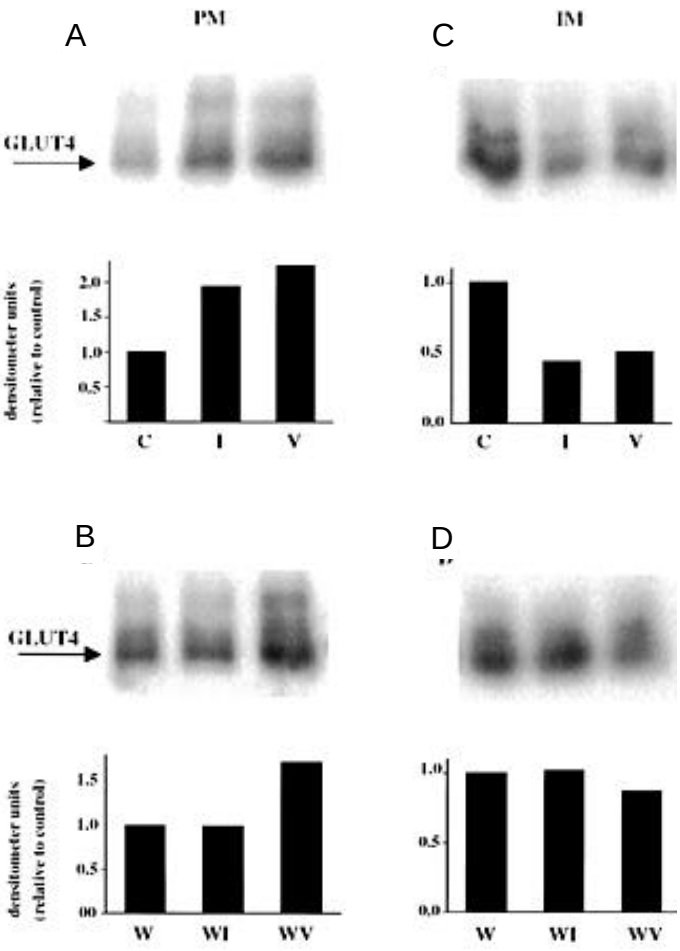


FIG. 5. Effect of insulin and vanadate on the distribution of GLUT4 in L6 skeletal muscle cells. L6 myotubes were cultured and treated as described in the legend to Fig. 1. At the end of the incubation, PM (*A* and *B*) and IM (*C* and *D*) fractions were prepared as described in METHODS, separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against GLUT4. The intensities of the bands were determined by laser densitometry and are plotted in units relative to unstimulated controls in the absence (*A* and *C*) and presence (*B* and *D*) of wortmannin. Data shown are representative of four experiments with similar results. C, control; I, 10^{-7} mol/l insulin; V, 5 mmol/l vanadate; W, 100 nmol/l wortmannin; WI and WV, wortmannin in combination with insulin and vanadate, respectively.

It is well established that inhibition of PI 3-kinase blocks nsulin-stimulated glucose uptake at a step proximal to GLUT ranslocation (33). To determine whether the stimulation of lucose uptake by vanadate and pV in the absence of PI 3-inase activity is associated with GLUT translocation, cellu-ar fractionation experiments were performed in the presence nd absence of wortmannin. As expected, wortmannin locked the ability of insulin to stimulate translocation of GLUT1 and GLUT4 but did not alter translocation stimulated y vanadate (Fig. 4*C* and *D*; Fig. 5*C* and *D*). Similar results vere obtained for GLUT3 and after stimulation by pV (data ot shown).

Effect of vanadate on the glucose transport system in H9c2 myotubes. Insulin (10^{-7} mol/l) stimulated 2DG uptake twofold in H9c2 wild-type and c-*myc*–GLUT4 transfected ells (Fig. 6). Stimulation of cells with 2 mmol/l vanadate also increased uptake by twofold over basal in both cell lines. Pretreatment with 100 nmol/l wortmannin completely blocked insulin's stimulation of 2DG uptake but had no effect on vanadate-mediated increases (Fig. 6). We further examined the effect of vanadate on the distribution of the *myc* epitope–tagged GLUT4 transfected into H9c2 cells using indirect immunofluorescence as described in METHODS. Insulin treatment increased the surface-accessible c-*myc* GLUT4 in transfected H9c2 cells, consistent with translocation of GLUT4 to the plasma membrane (Fig. 7*A* and *B*). Treatment with 2 mmol/l vanadate also increased surface-accessible c-*myc* GLUT4 (Fig. 7*C*). Pretreatment with 100 nmol/l wortmannin prevented the insulin-induced increase in surface labeling (Fig. 7*D*), but not that stimulated by vanadate (Fig. 7*E*). These results are in agreement with the data obtained in L6 myotubes; together they indicate that vanadate and pV are

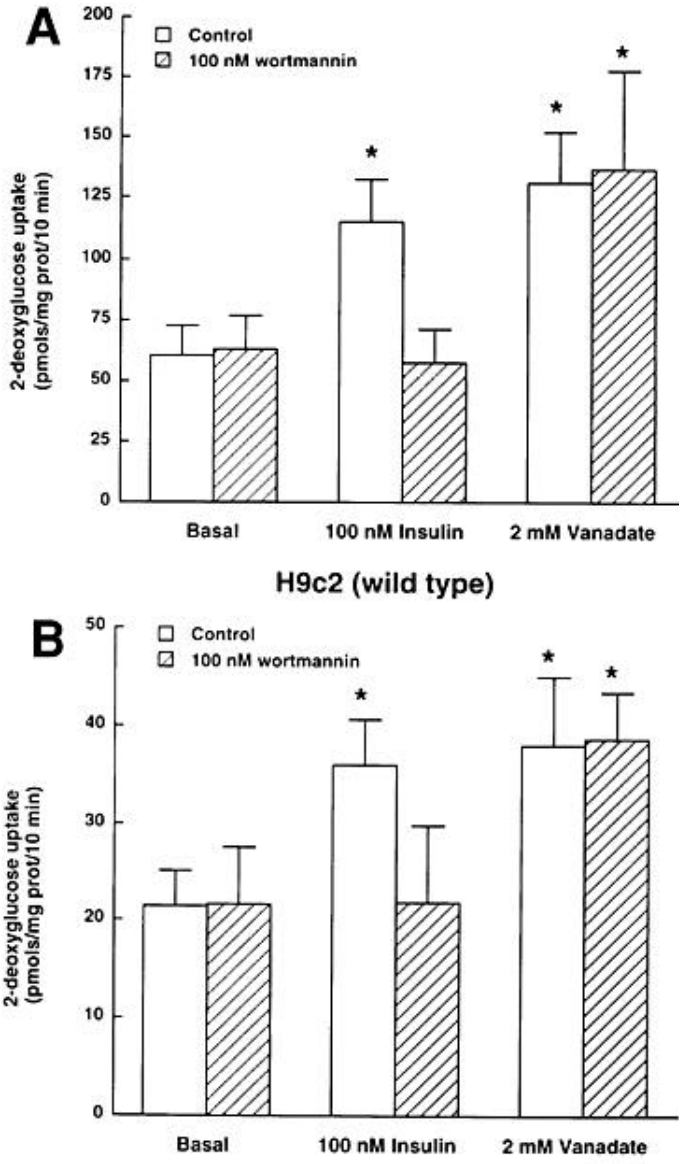


FIG. 6. Uptake of 2-deoxyglucose in H9c2 wild-type and c-*myc*–GLUT4 transfected cells. Cells were differentiated to myotubes for 14 days, and uptake of 10 µmol/l 2-DG was measured over 10 min. Cells were preincubated with (▨) and without (□) 100 nmol/l wortmannin and stimulated with and without 100 nmol/l insulin or 2 mmol/l vanadate. *A*: H9c2 wild-type cells. *B*: H9c2 c-*myc*–GLUT 4 transfected cells. Values represent mean ± SE, n = 5–12. *P < 0.05 compared with basal.

able to stimulate glucose transport and GLUT translocation by a mechanism independent of PI 3-kinase.

Effect of vanadate and pV on phosphorylation and activation of PKB. Protein targets downstream of the PI 3-kinase reaction include ser/thr kinases such as p70 S6 kinase and akt/PKB (36,37,47,48). The latter has been implicated in some studies as the direct target of the PI 3-kinase lipid products mediating insulin-stimulated glucose transport (49–51). At the same time, it has been reported that akt/PKB can be activated by a PI 3-kinase–independent mechanism (52,53). To determine whether vanadate or pV could stimulate PKB in L6 myotubes, and if so, whether they were dependent on PI3-kinase, cells were preincubated with and without 100 nmol/l wortmannin followed by treatment with 100 nmol/l insulin for 5 min, 5 mmol/l vanadate for 30 and 60 min, or 100 µmol/l pV for 15 min. Cell lysates were subjected to immunoprecipitations with anti-PKB antibodies, and the immunocomplexes were assayed as described in METHODS. Insulin and pV stimulated PKB activity to 2.3 ± 0.4- and 17 ± 2.1-fold of basal, respectively. On the other hand, 5 mmol/l vanadate had a minimal effect (1.15 ± 0.18 of basal at 30 min, NS) or no effect (0.9 ± 0.1 at 60 min) (Fig. 8). Neither did treatment with vanadate for 15 min alter PKB activity (data not shown). Similar results were obtained by immunoblotting with a phospho-specific anti-PKB antibody that recognizes only the ser-473 phosphorylated active form (Fig. 9). Preincubation with wortmannin blocked the stimulation of PKB activity by insulin (1.08 ± 0.3) and pV (1.8 ± 0.6), while activity in the presence of 5 mmol/l vanadate and wortmannin was even lower than basal (0.87 ± 0.12) (Fig. 8). A higher concentration of vanadate, 10 mmol/l, was able to stimulate PKB activity to 1.8-fold and was completely inhibited by wortmannin (data not shown). Immunoblotting confirmed these results: ser-phosphorylated PKB was undetectable in the presence of wortmannin (Fig. 9). These data indicate that the stimulation of glucose transport by vanadate and pV is independent of PKB activity.

Vanadate- and pV-stimulated glucose transport does not require PKC. It has been reported that acute activation of PKC by PMA leads to an increase in glucose uptake (54). To determine whether the mechanism of action of vanadate and pV to increase glucose transport could involve PKC, the glucose transport response of L6 cells to insulin, vanadate, and pV was examined after downregulation of PKC and in the presence of PKC inhibitors. Acute exposure of L6 myotubes to 1 µmol/l PMA resulted in stimulation of glucose transport (171 ± 2.7% of control) to levels similar to those of insulin (182 ± 2.1% of control) (Table 1). After 24-h exposure to 100 nmol/l PMA to downregulate PKC (55), the cells were washed and restimulated with 1 µmol/l PMA. Glucose uptake was no longer stimulated by PMA, indicating that PKC activity was downregulated. In contrast, the responses of the cells to insulin as well as to pV and vanadate were not altered (Table 1). In a second approach, L6 myotubes were preincubated with the PKC inhibitors calphostin C, 0.2 µmol/l, for 30 min or BIM, 1 µmol/l, for 60 min followed by stimulation with insulin, vanadate, or pV. The PKC inhibitors did not affect glucose transport stimulation by any of the agents (Table 1). Thus PKC does not appear to play a significant role in the insulin-, vanadate-, or pV-induced increase in glucose transport.

We considered the possibility that vanadate could signal via both pathways, that is, PI 3-kinase and PKC. If such redundancy occurred, inhibiting either one alone would not block glucose transport. The combination of wortmannin and the PKC inhibitor BIM did not alter glucose transport stimulation by pV (Table 2) or vanadate (not shown), confirming that neither pathway was involved.

Cytochalasin D inhibits insulin-, vanadate-, and pV-stimulated glucose transport. Cytochalasin D, which disrupts the actin network, has been documented to inhibit insulin-stimulated glucose transport in L6 myotubes (56). To examine whether an intact actin network is also required for glucose uptake stimulated by vanadate and pV, myotubes were preincubated for 1 h in the presence of 1 µmol/l cytochalasin D, followed by stimulation with the different agents. Cytochalasin D abolished the insulin, vanadate, and pV stimulation of glucose uptake, indicating that the actin network is important for the action of all these agents (Fig. 10).

ABLE 1
ffect of PKC inhibitors on insulin-, vanadate-, and pV-stimulated glucose transport

	2DG uptake (% of control)			
	Control	Calphostin C (0.2 µmol/l)	BIM (1 µmol/l)	PMA (24 h)
asal	100	90 ± 0.6	98 ± 0.6	103 ± 5.3
nsulin	188 ± 11.2‡	181 ± 12.5‡	183 ± 10.7‡	186 ± 7.8‡
anadate	138 ± 2.7*	154 ± 3.8†	134 ± 2.6‡	131 ± 1.9*
V	175 ± 7.0‡	174 ± 6.3‡	168 ± 5.4‡	188 ± 5.4‡
MA	171 ± 2.7‡	ND	104 ± 1.8§	96 ± 2.5

D, not determined. *P < 0.05; †P < 0.01; ‡P < 0.001 compared with basal; §NS.

ISCUSSION

critical component of glucose transport stimulation by nsulin immediately downstream of the insulin receptor and s substrate IRS-1 is the enzyme PI 3-kinase. The signaling echanism by which vanadium compounds stimulate glu- ose transport is not known. Hadari et al. (57) demonstrated hat portal vein injection of pV in rats activated PI 3-kinase in nti–p-tyr and anti–IRS-1 immunoprecipitates from liver omogenates. Similarly, pV stimulated PI3-kinase activity ssociated with IRS-1 in cultured CHO cells overexpressing nsulin receptors (58). In those studies, there was no correla- on of activation with any insulin-like metabolic or mitogenic ctivity. In this study, we demonstrated that vanadate and pV oth activate PI 3-kinase in L6 myotubes. In contrast with nsulin, however, the PI 3-kinase inhibitor wortmannin did ot abrogate the stimulation of glucose transport by these gents. The inhibition of PI 3-kinase activity by wortmannin as demonstrated in anti–p-tyr immunoprecipitates as well as y the inhibition of the incorporation of ^{32}P into the PI 3- inase lipid products, PI 3,4,5P_3 and PI 3,4P_2, in intact cells.

Insulin increases glucose uptake in its target tissues, muscle nd fat, by inducing the translocation of GLUTs from an intra- ellular pool to the plasma membrane (23,24,33). To investigate hether vanadate and pV act by a similar mechanism, we xamined their effect on GLUT distribution. We found that in 6 myotubes, both agents induced the translocation of glucose ransporters (GLUTs 1, 4, and 3) exactly as insulin would, as as been suggested for vanadate in adipocytes (59). In contrast ith insulin, however, and similar to the results obtained for lucose transport, this process was not inhibited by wort- nannin. The translocation of GLUTs in L6 cells was deter- nined using subcellular fractionation and immunoblotting. he same results were obtained using indirect immunofluo- escence in a cardiac myocyte–derived cell line, H9c2, which displays insulin-sensitive glucose transport and was engineered to express a c-*myc* epitope–tagged GLUT4 protein. Thus, wortmannin did not inhibit vanadate-stimulated glucose transport or c-*myc* GLUT4 plasma membrane localization in these cells. Taken together, these data strongly support the conclusion that vanadate and pV can stimulate the glucose transport system independent of PI 3-kinase.

The cellular events mediating insulin-stimulated glucose transport between PI 3-kinase and GLUT translocation are not clear. Recent studies have implicated PKB, an enzyme activated by direct interaction with the PI 3-kinase lipid products and phosphorylation by two upstream 3-phosphoinositide–dependent kinases, PDK1 and a putative PDK2, on thr-308 and ser-473 (human PKBα) (36,37,47,48,60). Thus, overexpression of constitutively active PKB resulted in increased glucose transport in 3T3-L1 adipocytes (49,50) and L6 cells (51). To determine whether PI 3-kinase–independent PKB activation could explain the glucose transport stimulation by vanadate and pV, its extent of activation was measured in response to those agents in the absence and presence of wortmannin. Vanadate had a very minimal effect on PKB activity, with no significant stimulation at a concentration (5 mmol/l) that induced maximum stimulation of glucose transport. In addition, while pV had a marked stimulatory effect on PKB—much greater than that of insulin—it was almost completely (95%) inhibited by wortmannin. These data indicate that the agents activate PKB in a PI 3-kinase–dependent manner but that their ability to activate the glucose transport system in the presence of wortmannin is mediated by a PKB-independent mechanism.

In an attempt to define the mechanism of glucose transport signaling by vanadate and pV, we examined the potential role of the family of PKC isoenzymes. The diacylglycerol analog PMA activates PKC and stimulates glucose uptake in both insulin target tissues, fat and muscle (54,61,62). Furthermore, although phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), may not be activated by insulin, it is activated by other growth factor receptors such as platelet-derived and epidermal growth factors (PDGF and EGF) (63), as well as by vanadate and pV (64–66). Since PTP inhibition by vanadate and pV is not specific, it is conceivable that tyrosine phosphorylation of other receptors or even nonreceptor tyrosine kinases may result in the generation of DAG and activation of PKC. Depletion of PKC by chronic preincubation with a high concentration of PMA, as well as inhibition of PKCs with two different phar-

ABLE 2
ffect of wortmannin in combination with PKC inhibitor on nsulin and pV-stimulated glucose transport

	2DG uptake (% of control)	
	Control	BIM + wortmannin
asal	100	70 ± 16.3 (100)
nsulin	178 ± 13.2†	101 ± 17.2
V	250 ± 71.4†	260 ± 35.2*

P < 0.05; †P < 0.01 compared with basal.

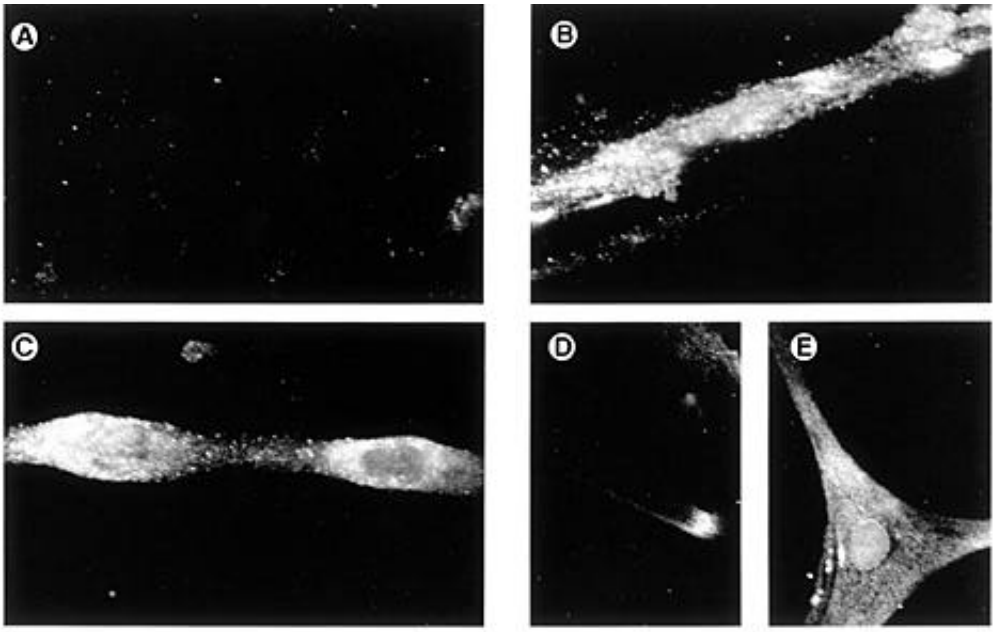


FIG. 7. Effect of insulin and vanadate on cell-surface expression of c-*myc*–tagged GLUT4 in H9c2 cells. H9c2 cells stably expressing c-*myc*–tagged GLUT4 were incubated with and without 100 nmol/l wortmannin for 30 min and then stimulated with or without 100 nmol/l insulin or 2 mmol/l vanadate for 30 min. Cell surface–accessible c-*myc* was visualized by confocal fluorescence microscopy as described in METHODS. Micrographs shown are representative of four experiments. *A*, basal; *B*, insulin; *C*, vanadate; *D*, insulin with wortmannin; *E*, vanadate with wortmannin.

macological agents, failed to block vanadate- or pV-stimulated glucose transport. At the same time, PMA stimulation was completely inhibited. Thus it appears that PKC is not part of the signaling mechanism of the PTP inhibitors for stimulation of glucose transport. It should be noted that the atypical PKC isoforms that are not phorbol ester sensitive may not have been completely blocked by the PKC inhibitors (67). One of them, PKC-ζ, has been implicated in mediating glucose trans-

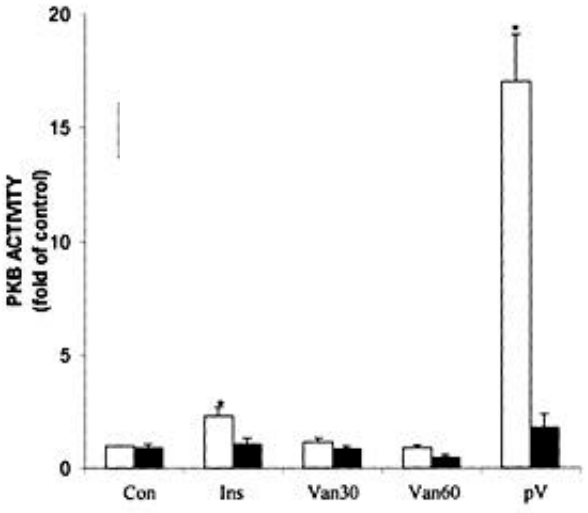


FIG. 8. Effect of insulin, vanadate, and pV on akt/PKB activity. L6 myotubes were cultured and pretreated (■) or not (□) with 100 nmol/l wortmannin for 10 min followed by addition of 100 nmol/l insulin for 5 min (Ins), 5 mmol/l vanadate for 30 min (Van30) or 60 min (Van60), or 100 μmol/l pV for 15 min (pV). PKB activity was determined using an immunoprecipitation assay as described in METHODS. Insulin and pV augmented PKB activity while vanadate had no significant effect. Wortmannin inhibited the stimulation by both insulin and pV. Results are means ± SE of three to five experiments performed in duplicate. *P < 0.05 compared with untreated control.

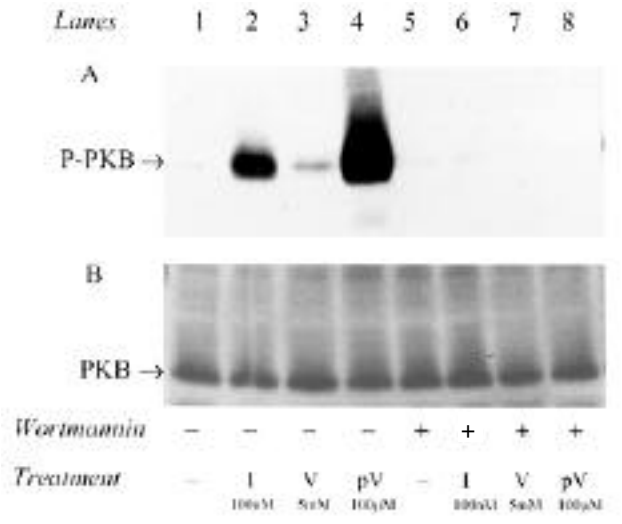
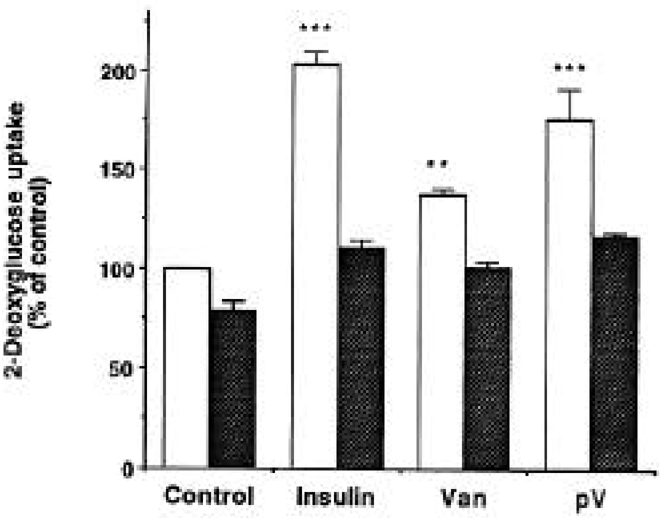


FIG. 9. Wortmannin inhibits ser phosphorylation of akt/PKB. L6 myotubes were cultured and pretreated or not with wortmannin followed by insulin, vanadate, pV, or medium alone as described in the legend to Fig. 8. Cells were lysed, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phospho-PKB antibody (*A*) or anti-PKB (*B*). Insulin and pV strongly stimulated ser phosphorylation of PKB while vanadate had a minimal effect. Relative intensities of stimulation corrected for total PKB determined by densitometry in arbitrary units; insulin, 0.64; vanadate, 0.065; pV, 1.32. Phosphorylated PKB was undetectable in the basal state and upon stimulation in the presence of wortmannin. Similar results were obtained in three experiments.



IG. 10. Cytochalasin D inhibits insulin-, vanadate-, and pV-stimulated glucose transport. Myotubes were incubated in the absence (□) nd presence (▨) of 1 mmol/l cytochalasin D for 1 h before stimulaon with 100 nmol/l insulin, 5 mmol/l vanadate (Van), or 100 μmol/l V for 60 min. At the end of treatment, 2DG uptake was measured as escribed in METHODS. The results are the means ± SE of three expernents performed in duplicate. **P < 0.01; ***P < 0.001 compared with ntreated control.

ort (68). However, since these isoforms have been sugested to be targets of the PI 3-kinase lipid products (35), nother mechanism of activation would be required in the resence of wortmannin, making it unlikely that PKC-ζ actiation explains our results.

Recent studies suggest that the actin network is involved in ne process of GLUT recruitment to the plasma membrane 56). The hypothesis that this requires close, if not direct, nteraction between actin filaments and the GLUTs suggests nat the site of this interaction is downstream of PI 3-kinase. ndeed, the actin-binding protein spectrin is localized on the LUT4 vesicle (56). Our results support this concept. Disuption of the actin network by cytochalasin D blocked gluose transport stimulation by insulin as well as by vanadate nd pV. These data, along with our previous findings of a lack f stimulation of glucose transport by vanadate or pV when dded to maximum insulin (46), suggest that the signaling athway activated by the PTP inhibitors converges with that ctivated by insulin.

A previous study showed that wortmannin inhibited vanaate-stimulated glucose transport in L6 cells by 60% (69). In nat study, however, the effect of wortmannin on basal glucose ansport was not measured or considered. The inhibition of asal glucose transport by wortmannin in L6 cells has been vell documented (30 and this study). In a study in 3T3-L1 dipocytes, there was a partial inhibition of pV-stimulated gluose transport by wortmannin (70). Our results differ and emonstrate that glucose transport stimulation by both vanaate and pV was unaltered by wortmannin under conditions n which activation of PI 3-kinase and the generation of lipid roducts were inhibited. It is known that nonspecific inhibition of tyrosine kinases blocks pV-stimulated glucose transport. As well, Shisheva and Shechter (71) have previously demonstrated the activation of a 54-kDa cytosolic tyr kinase (cyt PTK) in rat adipocytes by vanadate. Selective inhibition of this cyt PTK by staurosporine resulted in inhibition of several biological effects not including glucose transport. More recently, a second 55–60-kDa tyrosine kinase, which is membrane associated and activated by vanadate, has been suggested to be involved in the stimulation of glucose uptake in adipocytes (72). The identity of these putative tyrosine kinase mediators of vanadate action remains unknown.

Apart from vanadium compounds, glucose transport stimulation by mechanisms independent of PI 3-kinase has been demonstrated for muscle contraction and hypoxia (29), okadaic acid (73), growth hormone (74), and most recently, hyperosmolar stress (74,75). Hyperosmolar stress is an efficient stimulator of the mitogen-activated protein kinase (MAPK) family member p38 (HOG1) (75,76). Since vanadate and pV also inhibit the dual specificity phosphatases such as MAPK phosphatase-1 (MKP-1) (77), one might expect activation of MAPK and possibly p38. Although MAPK has been found to be activated by vanadate (78), several studies have determined that MAPK activation does not participate in stimulation of glucose transport (33,74). We have determined that inhibition of p38 with SB 203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole] does not block the stimulation of glucose transport by vanadate or pV (data not shown). Whether any of the other stimuli of PI 3-kinase–independent glucose transport share signaling pathways with the PTP inhibitors remains to be determined.

Vanadium compounds have been proposed as potential therapeutic agents for diabetes (7,8), particularly for type 2 diabetes, which is associated with insulin resistance (17,18). The effectiveness of these agents may be related, at least in part, to their ability to bypass some of the biochemical defects in insulin signal transduction.

ACKNOWLEDGMENTS

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Author Queries (please see Q in margin and underlined text)

Q1: Please clarify the definition of PKB.
Q2: Neither “crosstide” nor “cross-tide” is in the medical dictionary; please double-check usage.
Q3a: Please provide a key to the bar graph in Figure 3.
Q3: Please clarify “PKB kinase”—should it be just “PKB”?
Q4: How many experiments?
Q5: How many experiments?
Q6:Definition of IP_3 correct?
Q7: Please define SB 203580.