Regulation of Protein Kinase B in Rat Adipocytes by Insulin, Vanadate, and Peroxovanadate
MEMBRANE TRANSLOCATION IN RESPONSE TO PEROXOVANADATE

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Protein kinase B (PKB) (also referred to as RAC/Akt kinase) has been shown to be controlled by various growth factors, including insulin, using cell lines and transfected cells. However, information is so far scarce regarding its regulation in primary insulin-responsive cells. We have therefore used isolated rat adipocytes to examine the mechanisms, including membrane translocation, whereby insulin and the insulin-mimicking agents vanadate and peroxovanadate control PKB. Stimulation of adipocytes with insulin, vanadate, or peroxovanadate caused decreased PKB mobility on sodium dodecyl sulfate-polyacrylamide gels, indicative of increased phosphorylation, which correlated with an increase in kinase activity detected with the peptide KKRNRTLTK. This peptide was found to detect activated PKB selectively in crude cytosol and partially purified cytosol fractions from insulin-stimulated adipocytes. The decrease in electrophoretic mobility and activation of PKB induced by insulin was reversed both in vitro by treatment of the enzyme with alkaline phosphatase and in the intact adipocyte upon removal of insulin or addition of the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin. Significant translocation of PKB to membranes could not be demonstrated after insulin stimulation, but peroxovanadate, which appeared to activate PI 3-kinase to a higher extent than insulin, induced substantial translocation. The translocation was prevented by wortmannin, suggesting that PI 3-kinase and/or the 3-phosphorylated phosphoinositides generated by PI 3-kinase are indeed involved in the membrane targeting of PKB.

In recent years, the recognition of phosphatidylinositol 3-kinase (PI 3-kinase) as an important link in insulin signaling has facilitated understanding of new signaling mechanisms. Activation of PI 3-kinase by insulin and growth factors leads to generation of 3-phosphorylated phosphoinositides such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (1, 2), which are believed to act as second messengers. In a number of cultured cells the serine/threonine protein kinase B (PKB), also known as RAC or Akt, has been shown to be a target for PI 3-kinase-generated signals (3–8). PKB is expressed as three isoforms (9–13), all of which contain an amino-terminal pleckstrin homology (PH) domain (14), which may be involved in protein-protein or protein-lipid interactions (15). A proposed mechanism for the PI 3-kinase-dependent activation of PKB involves translocation of PKB from the cytosol to membranes and subsequent phosphorylation by one or several membrane-associated kinase(s) (16). It has been demonstrated that PKB can bind to phosphatidylinositol 3,4-bisphosphate (17, 18) and phosphatidylinositol 3,4,5-trisphosphate in vitro (18), most likely through its PH domain, suggesting that in vivo the formation of such lipids by activated PI 3-kinase could have a role in the translocation of PKB. It is a matter of controversy whether the 3-phosphorylated phosphoinositides not only bind but also to some extent activate PKB (3, 17–19).

Because almost all of the studies concerning the proposed mechanism(s) for insulin regulation of PKB have been carried out using cell lines or transfected cells and some of the findings are conflicting, there is obviously need for such information also from insulin-responsive target tissues such as liver, muscle, or adipose tissue. Therefore, we have performed such studies in the isolated rat adipocyte. Major findings reported in this paper are that PKB in the intact adipocyte is rapidly and reversibly activated in response to physiological concentrations of insulin and upon stimulation with the insulin-mimicking agent peroxovanadate is translocated to membranes via a wortmannin-sensitive mechanism.

EXPERIMENTAL PROCEDURES
Materials—Insulin and PD 98059 were gifts from Novo Nordisk, Gentofte, Denmark, and Parke Davis, Michigan. 4-β-Phorbol 12-myristate 13-acetate (PMA), wortmannin, sodium orthovanadate, &AMP; protein kinase inhibitor, and alkaline phosphatase (from bovine intestinal mucosa, type VII-T) were from Sigma. Rapamycin was from ICN Bio-medical, and protein A-Sepharose from was Pharmacia Biotech Inc. Peptides KKRNRTLTK (K9), KKRNRTLSK, and Crosstide (6) were synthesized as described (20). [γ-32P]ATP was synthesized as described (20).

Preparation and Stimulation of Rat Adipocytes—Adipocytes prepared from epididymal adipose tissue of 36–38-day-old male Harlan Sprague Dawley rats (B&K Universal, Stockholm) (21, 22) were suspended (1 ml of packed cells/10 ml of medium) in Krebs-Ringer medium, pH 7.4, 25 mM Hepes, 200 mM ascorbine, 2 mM glucose, and 1% bovine serum albumin. Vanadate (300 mM) was dissolved in water and boiled before use. Peroxovanadate was prepared fresh by incubating vanadate and H2O2 (12 mM each) at 20 °C in 40 mM Hepes, pH 7.4, for 15 min

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§ The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PH, pleckstrin homology; PMA, 4-β-phorbol 12-myristate 13-acetate; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MAP kinase, mitogen-activated protein kinase.
prior to use. PMA, wortmannin, rapamycin, and PD 98059 were dissolved in Me2SO and added to cells, resulting in a final Me2SO concentration of ≤ 0.15%. Adipocytes (1.5 ml of 10–12% (v/v) cell suspension, unless otherwise stated) were incubated at 37 °C with the indicated additions. The incubations were terminated by the addition of 7 ml of homogenization buffer consisting of 50 mM Tris, pH 7.4, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 1 mM dithioerythritol, 40 mM phenyl phosphosphate, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM sodium orthovanadate, antipain (10 μg/ml), leupeptin (10 μg/ml), and peptatin A (1 μg/ml). Cells were centrifuged, resuspended in 1 ml of homogenization buffer, and homogenized (10 strokes) at room temperature. The homogenates were centrifuged at 35,000 g for 6 min at 4 °C, supernatants (referred to as cytosol fractions) withdrawn, and the pellets (referred to as membrane fractions) resuspended in 0.5 ml of homogenization buffer. Cytosol and membrane fractions were usually assayed for kinase activity directly; they could, however, be stored at −20 °C for more than 3 months without detectable loss of kinase activity.

Cloning and Expression of PKB—A fragment of 1,440 base pairs, encompassing the coding sequence of PKBα, was amplified from a rat adipocyte cDNA library in the pGAD 10 yeast expression vector (CLONTECH). The primers for polymerase chain reaction were complementary to nucleotides 1–24 and 1420–1440, respectively, of PKBα (13). Amplification was performed with 35 cycles of denaturation (1 min at 94 °C), annealing (2 min at 50 °C), and polymerization (2 min at 72 °C). The amplified cDNA was subcloned into the PCR-Script vector (Stratagene). When the sequence was verified by automated sequencing, full-length PKBα (amino acids 1–480) and a PH-domain-containing fragment (PH-PKB, amino acids 1–110) were obtained as histidine-tagged proteins from the BL23/pLysS strain using 1 mM isopropyl β-D-thiogalactopyranoside for 3 h at 37 °C (full-length PKB) or 6 h at 28 °C (PH-PKB).

Yeast expression of PKB α fused to a GAL4 DNA binding domain was accomplished through subcloning the full-length coding cDNA of PKBα into the pGAL2–1 vector (CLONTECH). pGAL2/PKBα was used to transform S. cerevisiae strain Y190, employing the lithium acetate method (23). For analysis of expressed protein, yeast extracts were prepared from mid-log phase cultures using a glass bead cell disruption procedure as described (24).

Antibodies—Antibodies against amino- and carboxyl-terminal peptides of PKBα (anti-NT-PKBα and anti-CT-PKBα, respectively) were from Upstate Biotechnology Inc., Lake Placid, NY. Antibodies against the PH domain of PKBα were from Transduction Laboratories, Lexington, KY. Antibodies against full-length PKB and PH-PKB were raised in rabbits using the E. coli-expressed, histidine-tagged proteins, purified from inclusion bodies and eluted from SDS-polyacrylamide gel electrophoresis (PAGE) gels. The antisera were affinity purified as described (25).

Immunoblot Analysis and Immunoprecipitation of PKB—Adipocyte cytosol and membrane fractions and column fractions were mixed with Laemmli sample buffer (26) and subjected to SDS-PAGE (8% polyacrylamide) followed by electrophoretic transfer of protein onto polyvinylidene difluoride membranes (Millipore). After blocking with 0.5% gelatin in phosphate-buffered saline the membranes were incubated for 4–16 h with a 1:5,000 dilution of the anti-NT-PKBα antibody. Immunoblot analysis was performed using the enhanced chemiluminescence system (Amer sham) or the Super Signal System (Pierce).

Numerous attempts to immunosolubilize PKB from adipocytes using both anti-PH-PKB and anti-full-length-PKB antibodies and also a set of commercially available antibodies were made, but these attempts have failed consistently. This is in contrast to experiments using lysates from liver and 3T3-L1 cells, from which endogenous PKB was successfully immunosolubilized employing the anti-PH-PKB antibody. Furthermore, the normally obtained, quantitative immunoprecipitation of recombinant PKB from yeast extracts was blocked completely by the addition of adipocyte cytosol fraction or even partially purified cytosol (Mono Q fractions) to the extracts (Fig. 1). This observation strongly suggests the presence of components interfering with the PKB-antibody interaction in adipocytes.

The immunoprecipitation protocol employed made use of affinity purified anti-PH-PKB antibodies raised by us (see above). Antibodies and protein A-Sepharose beads were preincubated for 1 h at 4 °C, washed with phosphate buffer, and added to tissue/cell extracts. After overnight incubation at 8 °C, the beads were pelleted, washed with phosphate buffer, and subjected to SDS-PAGE and immunoblot analysis using the anti-PH-PKB antibody.

Protein Kinase Assay—Membrane fractions (5 μl), cytosol fractions (10 μl), and chromatography fractions (10 μl) were incubated with 5 μl of a mixture containing 150 μM γ-32P-ATP (4–10 μCi), 150 mM TES, pH 7.5, 40 mM MgCl2, 250 mM sucrose, 4 mM dithioerythritol, 5 μM cAMP protein kinase inhibitor (final incubation volume, 15 μl). Substrates included were either peptide KRRNTTLTK (K9) (13 μg), KRRNTLSK (K9+K12) (13 μg), or Crostide (1 μg). Incubations were terminated after 20 min at 30 °C by addition of 10 μl of 1% bovine serum albumin, 1 mM ATP, pH 3.0, and 5 μl of 30% trichloroacetic acid. Samples were centrifuged and supernatants applied onto phosphocellulose paper (Whatman P81), washed three times with 75 mM phosphoric acid and once with water. The amount of 32P incorporated into peptide substrates was determined by scintillation counting. The kinase assay was linear with respect to time of incubation and protein concentration.

RESULTS

Insulin-induced Shift in the Electrophoretic Mobility and Activation of PKB in Adipocytes—Activation of PKB has previously been shown to be associated with decreased mobility of the protein on SDS-polyacrylamide gels, indicative of phosphorylation of the enzyme (4, 5, 7). As shown in Fig. 2A, stimulation of adipocytes with insulin resulted in reduction in the electrophoretic mobility of PKB demonstrated by immunoblot analysis of cytosol fractions using an anti-NT-PKBα antibody. Treatment of adipocytes with the PI 3-kinase inhibitor wortmannin (27), added prior to or after stimulation with insulin, blocked or reversed the shift in mobility of PKB. In contrast, rapamycin, an agent that blocks activation of p70 S6 kinase, had no effect on the insulin-induced shift in mobility. Stimulation of adipocytes with PMA, which results in activation of PKB, failed consistently. This is in contrast to experiments using lysates from adipocytes, either cytosol fractions or chromatography fractions, which has not been successful (see “Experimental Procedures”). To study activation of PKB it was therefore necessary to find a substrate that could selectively detect PKB in crude adipocyte fractions. We noted that after quantitative immunoprecipitation of p70 S6 kinase from adipocytes,2 96% ± 11% (mean ± S.D., n = 13) of the insulin-induced kinase activity detected with the p70 S6 kinase substrate KRRNNTTLTK (K9) remained in the immunosupernatants. Furthermore, rapamycin had no effect on the insulin-induced activation of K9 kinase (Fig. 2B). These results show that p70 S6 kinase does not contribute significantly to the insulin-induced kinase activity detected in cytosol fractions. Initial characterization of the insulin-stimulated kinase activity indicated that it could be PKB. Therefore, we proceeded with a more detailed characterization and found, as is shown below, that this kinase activity to a large extent represented PKB.

As shown in Fig. 2B, activation/deactivation of K9 kinase
activity correlated well with the appearance/disappearance of the PKB species with lower electrophoretic mobility. Wortmannin, added either before or after stimulation with insulin, completely inhibited the insulin-induced activation of K9 kinase. Direct addition of wortmannin (1 μM) to the assay had no effect on the K9 kinase activity. PMA stimulation of adipocytes (150 nM (Fig. 2B) or 500 nM (data not shown)) only marginally altered K9 kinase activity, which amounted to no more than 15% of the response to insulin. Furthermore, pretreatment of adipocytes with the MAP kinase kinase inhibitor PD 98059, which inhibits insulin-induced activation of MAP kinase, had no effect on the insulin-induced activation of K9 kinase. These results suggest that MAP kinase and kinases downstream of MAP kinase, such as p90 rsk, are not involved in the insulin-induced activation of the kinase.

As shown in Fig. 2C, there was substantial activation of K9 kinase in cytosol fractions from adipocytes stimulated with 1 nM insulin for 1 min, and a 5-fold activation was reached after 5 min in the presence of insulin. No further increase in activity was seen using 100 nM insulin (not shown). Partial activation of the kinase was seen with 0.1 nM insulin (15 ± 5% (mean ± S.D., n = 3) of the activity in response to 1 nM insulin), which was accompanied by a partial shift in the electrophoretic mobility of PKB (not shown). Although the kinase activity remained elevated for more than 30 min in the presence of insulin, the activity returned to control values when insulin-stimulated cells were washed and incubated without insulin. This coincided with reversal of the insulin-induced shift in the electrophoretic mobility of PKB as well as reduction of the kinase activity to that seen in cytosol fractions from control cells (Fig. 3).

The results presented above are consistent with activated PKB as the kinase phosphorylating K9 in our assay. To establish that insulin-stimulated K9 kinase activity represents PKB we investigated the elution profile of K9 kinase activity and PKB protein on Mono Q and Superdex columns. K9 kinase activity quantitatively bound to the Mono Q column and was eluted as two peaks of activity (Fig. 4A). Increased K9 kinase activity from insulin-stimulated cells was found in the first peak eluting at 0.15–0.20 M NaCl. Immunoblot analysis of Mono Q fractions with anti-NT-PKB antibodies (Fig. 4A, inset) showed that PKB protein from insulin-stimulated cells was present in this first peak (fractions 19–21) and coeluted with the insulin-stimulated K9 kinase activity. In contrast, PKB protein from control cells was detected in fractions 17–19, indicating that the insulin-stimulated form of PKB (phosphorylated) binds more tightly to the Mono Q column than does PKB from control cells. A peak of insulin insensitive kinase activity, eluting at about 0.3 M NaCl, was also detected consistently (four out of four experiments). No protein cross-reacting with either anti-NT-PKB or anti-CT-PKB antibodies (not shown) could be detected in this second peak. We do not know the identity of the kinase(s) in this second peak; however, the kinase activity is not to any major extent the

![Fig. 2. Regulation of PKB in adipocytes.](image-url)

Adipocytes were treated and cytosol fractions prepared as described under "Experimental Procedures." **Panel A**, cytosol fractions (100 μl) were subjected to SDS-PAGE and immunoblot analysis with the anti-NT-PKB antibody. The immunoblot is representative of three or more experiments. The positions of PKB are indicated to the right. The band above PKB represents nonspecific interaction with bovine serum albumin originating from the incubation medium. **Panel B**, cytosol fractions were assayed for kinase activity with K9 as substrate. Results are mean values ± S.D. from several different adipocyte preparations (n) and expressed as percent of the activity in cytosol fraction from control cells (taken as 100%). Cytosol from control cells gave 32P incorporation of 28,000 ± 2,500 (n = 4) cpm/20 min of incubation with 6 μCi (0.75 nmol) of [γ-32P]ATP. Cells were treated with 1 nM insulin (ins), 150 nM PMA, 100 nM wortmannin (wort), 20 nM rapamycin (rap), or 50 μM PD 98059 (PD). Wortmannin and rapamycin were added 10 and 15 min, respectively, before insulin. **Panel C**, adipocytes were stimulated with 1 nM insulin for the indicated times (solid line) or stimulated with 1 nM insulin for 15 min, washed with 7 ml of Krebs-Ringer medium containing 1% bovine serum albumin, and the incubation continued for an additional 15 min (dotted line) without (open circle) or with 1 nM insulin (filled circles). Results are mean values ± S.D. from three or more adipocyte preparations and expressed as percent of the activity in cytosol fraction from control cells (taken as 100%). Inset, immunoblot analysis with the anti-NT-PKB antibody of cytosol fractions from, control cells (lane 1); cells stimulated with 1 nM insulin for 15 min (lane 2); cells stimulated with 1 nM insulin for 15 min, washed, and the incubation continued without (lane 3) or with 1 nM insulin for 15 min (lane 4).
result of p70 S6 kinase as this kinase elutes in fractions 24 and 25 as determined by immunoblot analysis (not shown). The kinase activity recovered in the first peak (PKB) varied among different experiments and accounted for 40–65% of the total kinase activity eluting from the column. The 4–5-fold activation of K9 kinase observed in cytosol fractions (Fig. 2B) is higher than one would expect when taking into account the relatively large peak of insulin-insensitive kinase activity observed after Mono Q chromatography. The reason could be that the insulin-insensitive kinase is suppressed in cytosols or that the insulin-stimulated K9 kinase is partially lost during the purification procedure.

Subsequent chromatography of PKB-containing Mono Q fractions (fractions 17–20 from control and 19–22 from insulin-stimulated cells) on a gel filtration column revealed coelution of K9 kinase observed in cytosol fractions (Fig. 2B) is higher than one would expect when taking into account the relatively large peak of insulin-insensitive kinase activity observed after Mono Q chromatography. The reason could be that the insulin-insensitive kinase is suppressed in cytosols or that the insulin-stimulated K9 kinase is partially lost during the purification procedure.

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Vanadate- and Peroxovanadate-induced Activation of PKB—Vanadate and peroxovanadate are well known inhibitors of phosphotyrosine phosphatases (28) and have been shown to mimic several of the actions of insulin such as stimulation of glucose uptake (29), lipogenesis (30), and inhibition of lipolysis (30). Stimulation of adipocytes with either vanadate or peroxovanadate induced activation of PKB; peroxovanadate was approximately 1,000-fold more potent than vanadate and active at micromolar concentrations (Fig. 5). In addition, both vanadate and peroxovanadate, at concentrations exerting no or little activation of PKB, potentiated the activation induced by 0.25 mM insulin (Fig. 5) and 1 nM insulin (not shown). Although treatment with 5 mM vanadate for 20 min resulted in only a partial activation of PKB (Fig. 5), a 4.5 ± 0.9-fold (n = 7) increase in kinase activity was seen when the incubation was extended to 40 min. No activation of PKB was seen when adipocytes were treated with 0.25 mM H2O2 (not shown). Wortmannin pretreatment inhibited the PKB activation induced by either vanadate or peroxovanadate. The peroxovanadate-induced activation of PKB was not affected by pretreatment with.
The increase in PKB activity in these experiments. It has been proposed that formation of 3-phosphorylated phosphoinositides by activated PI 3-kinase may lead to recruitment of PKB to membranes where it is activated by membrane-associated kinase(s) (7, 16). PKB binds to vesicles containing phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (17, 18); however, there is no direct evidence for translocation of PKB to membranes in response to stimulation of intact cells with agents that activate PKB. Therefore, adipocytes were stimulated with insulin, homogenized, and the amount of PKB in the cytosol and membrane fractions was determined.

Despite repeated attempts and the use of different insulin concentrations (up to 100 nM) we were not able to demonstrate any significant amount of PKB protein in membrane fractions after incubation with insulin (Fig. 6A). Similarly, vanadate stimulation did not result in translocation of PKB (not shown). Because it appeared possible that translocation of PKB was transient or could have been reversed during homogenization and therefore difficult to detect, studies with peroxovanadate were performed. We have observed that this insulin-mimicking agent is more effective than insulin in recruiting PI 3-kinase to insulin receptor substrate-1,2 and thus presumably generates more 3-phosphorylated phosphoinositides. As shown in Fig. 6A, treatment with peroxovanadate (≥50 μM) resulted in substantial translocation of PKB to the membrane fraction. The increase in PKB protein in the membrane fraction was accompanied by a corresponding decrease in the cytosol fraction. The translocation of PKB was blocked completely by wortmannin. Furthermore, the addition of wortmannin subsequent to stimulation with peroxovanadate (50 μM, 40 min) reversed the membrane translocation of PKB (not shown). An increase in kinase activity was also detected in membrane fractions from peroxovanadate-stimulated cells (Fig. 6B). This increase in activity was inhibited by pretreatment of the cells with wortmannin. However, the kinase activity in the membrane fraction was lower than expected as judged from the amount of PKB protein in this fraction. Kinase assay of membrane fractions (either from control or stimulated cells) mixed with an equal volume of difluoride membranes with adipocyte membrane and cytosol fractions were processed together. The band detected in all lanes of the membrane fractions has a higher mobility compared with PKB in the cytosol fraction from control cells and may therefore represent nonspecific interaction with the antibodies. Panel B, 5 μl of the membrane fractions were assayed for kinase activity with K9 as substrate, and results are expressed as 32P incorporated (cpm) into the substrate/20 min and represent mean ± S.D. of several individual experiments (n). Wortmannin (100 nM) was added 10 min before stimuli, C; control; ins, insulin; pv, peroxovanadate; wort, wortmannin; van, vanadate.

Fig. 5. Effect of vanadate and peroxovanadate on PKB activity. Adipocytes were treated with the indicated concentrations of vanadate (open squares) or peroxovanadate (open circles) for 20 min (solid line), with vanadate or peroxovanadate for 10 min followed by 0.25 nM insulin for 10 min (broken line), or with 100 nM wortmannin for 10 min followed by vanadate (filled square) or peroxovanadate (filled circles) for 20 min. Cytosol fractions were prepared and assayed for kinase activity with K9 as substrate. Results are mean values ± S.D. from three or more adipocyte preparations and expressed as percent of control (taken as 100%). For comparison, 1 nM insulin gave a 4.5 ± 0.5-fold (n = 4) increase in PKB activity in this set of experiments.

Fig. 6. Immunoblot analysis (panel A) and kinase activity (panel B) of PKB from vanadate and peroxovanadate-stimulated adipocytes. Adipocytes were treated as indicated and cytosol and membrane fractions prepared. Panel A, equivalent portions (corresponding to 25 μl of packed cells) of cytosol fractions (upper panel) and membrane fractions (lower panel) were subjected to SDS-PAGE and immunoblot analysis with the anti-NT-PKB antibody. Polyvinylidene difluoride membranes with adipocyte membrane and cytosol fractions were processed together. The band detected in all lanes of the membrane fractions has a higher mobility compared with PKB in the cytosol fraction from control cells and may therefore represent nonspecific interaction with the antibodies. Panel B, 5 μl of the membrane fractions were assayed for kinase activity with K9 as substrate, and results are expressed as 32P incorporated (cpm) into the substrate/20 min and represent mean ± S.D. of several individual experiments (n). Wortmannin (100 nM) was added 10 min before stimuli, C; control; ins, insulin; pv, peroxovanadate; wort, wortmannin; van, vanadate.
DISCUSSION

The major findings in this paper are that PKB is rapidly and reversibly activated via a wortmannin-sensitive mechanism by physiological concentrations of insulin in adipocytes, a major target cell for insulin, and that PKB is recruited to membranes via a wortmannin-sensitive mechanism in the intact cell. Kohn et al. (4) observed that stimulation of adipocytes with insulin resulted in decreased electrophoretic mobility of PKB on SDS-polyacrylamide gels. Here we show that the decreased electrophoretic mobility of PKB is linked to activation of the kinase and that the activation is wortmannin-sensitive and also appears to involve phosphorylation. Furthermore, the activation is rapid (detectable within 1 min), reversible, and occurs in response to physiological concentrations of insulin (detectable with 100 pM). Therefore, the insulin-induced activation occurs in response to physiological concentrations of insulin and is rapid (detectable within 1 min), reversible, and also appears to involve phosphorylation. Furthermore, the activation of PKB by insulin could well be important in metabolic actions of insulin. Insulin and insulin-like growth factor-1-mediated activation of PKB have been demonstrated using different cell lines and transfected cells, sometimes in the presence of as much as 100–1000 nM insulin (4–6, 16, 31–33). At high concentration of insulin it cannot be excluded that the effects observed are mediated via the insulin-like growth factor-1 receptor and not by the insulin receptor (34). Our results show that in primary rat adipocytes there is a physiological regulation of PKB by insulin.

The K9 peptide (KKRRNTLTK) was originally designed as a substrate for p70 S6 kinase (35). Our results show that K9 can be used to detect PKB activity selectively in cytosolic fractions from stimulated adipocytes. The peptide Crosstide (GRPRTTSS-FAEG), which closely resembles the sequence containing the site phosphorylated by PKB in glycogen synthase kinase-3, has been used to determine PKB activity in immunoprecipitates as well as in partially purified fractions of PKB (6). Both Crosstide and K9 have basic residues at positions n-3 and n-5 (where n is the site of phosphorylation). In the case of Crosstide the phosphate acceptor is a serine, and in K9 it is a threonine. In a set of experiments (results not shown) we compared kinase activity in adipocytes using three different peptide substrates; Crosstide, K9, and a K9 peptide with the most carboxyl-terminal threonine replaced by a serine (K9 Thr → Ser). In contrast to the results in Fig. 2B with K9 as substrate, Crosstide as well as K9 Thr → Ser detected PMA-stimulated kinase activity in the cytosol fraction to the same extent as insulin-stimulated kinase activity. Because PMA stimulation does not result in activation of PKB in adipocytes (Fig. 2) or other cells (3–5) this indicates that kinase assay of crude adipocyte preparations with peptides containing a serine as phosphate acceptor (Crosstide and K9 Thr → Ser) are less selective for PKB and will also detect other kinases such as p90 rsk. This conclusion is supported by a recent study where the substrate specificity of PKB, p90 rsk, and p70 S6 kinase were compared (36).

The importance of phosphorylation as a mechanism to activate PKB was demonstrated recently by Alessi et al. (32). Site-directed mutagenesis studies of PKB revealed two activity-controlling phosphorylation sites. However, the protein kinase(s) and phosphatase(s) responsible for the phosphorylation of PKB in vivo have not yet been identified. In agreement with findings by others (4–6), alkaline phosphatase treatment of PKB from insulin-stimulated adipocytes resulted in its deactivation as well as reversal of its electrophoretic mobility to that of PKB from control cells. A role for protein phosphatases in the regulation of PKB in intact cells is supported by the observation that treatment of Swiss 3T3 cells with okadaic acid induces activation and reduction in the electrophoretic mobility of PKB (7). Our results demonstrated that the insulin-induced activation of PKB, as well as the corresponding decrease in electrophoretic mobility, was reversed upon removal of insulin or addition of wortmannin subsequent to insulin stimulation, indicating that the activation of PKB is reversed rapidly by protein phosphatase(s) in the intact cell.

The finding that wortmannin blocked the peroxovanadate-induced translocation of PKB to membranes in intact cells is of considerable interest since it has been proposed that PKB can be targeted to membranes where it can be phosphorylated and activated by membrane-associated kinase(s) (7, 16). However, membrane translocation of PKB has not been demonstrated in intact cells but is supported by observations that PKB binds to lipid vesicles containing phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (17, 18) and that targeting of PKB to membranes by adding the src myristoylation sequence results in constitutive activation of PKB (5, 8, 31). In contrast to the results with peroxovanadate, we were unable to find significant amounts of PKB in membrane fractions from insulin-stimulated cells. We have not identified the factor responsible for the marked difference in translocation of PKB to membranes during stimulation of adipocytes with insulin or peroxovanadate. It is possible that higher concentrations of 3-phosphorylated phosphoinositides are generated in the presence of peroxovanadate, since several-fold greater amounts of PI 3-kinase were found in association with insulin receptor substrate-13 after stimulation of adipocytes with peroxovanadate than after treatment with insulin. The amount of phosphatidylinositol 3,4,5-trisphosphate could also have been increased by inhibition of its degradation. Since vanadate has been shown to inhibit phosphatidylinositol 3,4,5-trisphosphate 5'-phosphatase in vitro (37), it is tempting to speculate that peroxovanadate could inhibit this phosphatase in the intact cell and thereby increase the level of phosphatidylinositol 3,4,5-trisphosphate.

Little is known regarding the physiologically important downstream targets for PKB. Recently, it was demonstrated that the insulin-induced phosphorylation and inhibition of glycogen synthase kinase-3 in myotubes are mediated by PKB, indicating an important role for PKB in the regulation of glycogen synthesis (6). In 3T3-L1 adipocytes the expression of constitutively active PKB resulted in increased glucose uptake, which was associated with increased translocation of GLUT4 to plasma membranes and increased expression of GLUT1 (31). In these cells, increased glucose uptake in the presence of constitutively active PKB was associated with increased lipid synthesis. Another important effect of insulin is to counteract catecholamine-induced hydrolysis of stored adipose tissue triglycerides. A key enzyme in this action of insulin is phosphodiesterase 3B whose activation causes reduction of cellular cAMP (38). We are currently investigating the role of PKB in the insulin-induced phosphorylation and activation of phosphodiesterase 3B. Preliminary results indicate that phosphodiesterase 3B could be a substrate for PKB in vivo.4,5

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