Stimulation of Glucose Transport and Glucose Transporter Phosphorylation by Okadaic Acid in Rat Adipocytes*

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Okadaic acid, an inhibitor of Type I and IIa protein phosphatases, was recently found to stimulate 2-deoxyglucose uptake in rat adipocytes (Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P., and Hardie, D. G. (1989) Nature 337, 78-81). In the present experiments the effect of okadaic acid on the phosphorylation and subcellular distribution of the insulin-regulatable glucose transporter (IRGT) was investigated. At maximally effective concentrations, insulin and okadaic acid increased the amount of IRGT in the plasma membrane by 10- and 4-fold, respectively. Thus, the stimulation of glucose transport by okadaic acid was apparently due to an increase in the surface concentration of the IRGT. However, despite its stimulatory actions, okadaic acid partially inhibited the ability of insulin to enhance glucose transport and translocation of the transporter. When cells were incubated with okadaic acid alone or in combination with insulin, phosphorylation of the IRGT in the plasma membrane was increased by approximately 3-fold relative to the intracellular pool of transporters in control cells. Phosphorylation of the IRGT was confined to the presumed cytoplasmic domain at the COOH terminus of the protein. Glucose transporters were dephosphorylated in vitro by Type I or Type IIa protein phosphatases, indicating that inhibition of one or both of these phosphatases could account for the increased phosphorylation produced by okadaic acid. The observation that okadaic acid stimulated translocation of the IRGT implicated a serine/threonine phosphorylation event in triggering movement of the Intracellular IRGT-containing vesicles (GTV) to the cell surface. Immunoadsorption of GTV from 32P-labeled adipocytes revealed that the IRGT was the major phosphoprotein in these vesicles. The phosphorylation of at least three other GTV proteins was increased by okadaic acid, and these species would appear to be candidates for regulators of GTV movement to the plasma membrane. It is unlikely that phosphorylation of the IRGT is the signal for translocation because insulin did not increase phosphorylation of the protein. Rather, the inhibitory effect of okadaic acid on insulin-stimulated translocation is consistent with the hypothesis that phosphorylation of the IRGT promotes its internalization.

Insulin stimulates the transport and metabolism of glucose in muscle and fat cells. The stimulation of transport involves the translocation of transport proteins from an intracellular store to the plasma membrane (1-7). Although the mechanism for this insulin-dependent translocation has not been determined, considerable advances have been made in defining the structure of the glucose transporter itself. cDNAs encoding at least five different transporter species have been cloned (8-17). The transporter proteins appear to be highly homologous, with overall amino acid sequence identity as high as 76% (17). Computer modeling based on hydrophobicity of the predicted amino acid sequences indicates that all five species have 12 membrane-spanning domains (8, 17), with intracellular domains located at the beginning, the middle, and the end of the proteins. Skeletal muscle fibers, cardiac myocytes, and adipocytes, the three cell types in which glucose transport is most dramatically stimulated by insulin, have a unique transporter referred to as the insulin-regulated glucose transporter (IRGT)† (18) or Glit4 (17). In fat cells the concentration of the IRGT in the plasma membrane increases 10-fold or more with insulin (18). This is likely to be the major mechanism by which insulin stimulates glucose transport in these cells although little is known about the biochemical signals that cause movement of transporters to the plasma membrane.

Many of the metabolic effects of insulin are mediated by dephosphorylation of rate-limiting enzymes in various metabolic pathways. For example, the stimulation of glycogen synthesis involves dephosphorylation and activation of glycogen synthase. On the other hand, insulin stimulates the phosphorylation of a number of proteins in fat cells (19, 20). Several protein kinases have been shown to be activated when cells are incubated with insulin (21) although the function of these insulin-stimulated phosphorylation reactions has not been established. We found recently that the IRGT was phosphorylated in muscle and fat cells but that its phosphorylation was not increased by insulin (22, 23). This was not unexpected since the effect of insulin on stimulating translocation of the IRGT is presumably mediated by elements that regulate the movement of the intracellular vesicles containing the IRGT. In contrast to insulin, isoproterenol and cAMP derivatives stimulated IRGT phosphorylation and inhibited insulin-dependent glucose transport (22). The effects of isoproterenol were not associated with a decrease in the amount of the IRGT in the plasma membrane, suggesting that phosphorylation may regulate the "intrinsic activity" of the transporter.

Okadaic acid is a tumor promoter originally isolated from the sea sponge, Halichondria okadaii (24). It has been found

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1 The abbreviations used are IRGT, insulin regulatable glucose transporter; EGTa, [ethylmethylisoxethane]tricarboxylic acid; GTV, glucose transporter-containing vesicles; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDM, low density microsomes; SDS, sodium dodeyl sulfate; PAGE, polyacrylamide gel electrophoresis; IGF-II, insulin-like growth factor II.

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Subcellular Fractionation—In experiments not involving $^{32}$P, cells were solubilized, and subcellular fractionation was performed as described previously (30). Under these conditions we have found that the transporter is completely dephosphorylated during the isolation of membrane fractions. Consequently, to investigate the effects of okadaic acid on transporter phosphorylation, it was necessary to incorporate phosphate into all of the solutions used during subcellular fractionation. In these experiments cells were rinsed as described under these conditions, and the cells were then homogenized immediately at 0°C in Buffer A (3 ml/g of adipose tissue) by using a glass homogenization tube and Teflon pestle (six strokes) driven at 1,500 rpm. Buffer A contained 250 mM sucrose, 25 mM NaF, 1 mM EDTA, 0.5 mM EGTA, 0.1 mM sodium molybdate, and 1 mM NaPPi (pH 7.4). Plasma membranes, high density microsomes, low density microsomes (LDM), and mitochondria/nuclei were prepared as described previously (22).

Immunoprecipitation and Electrophoretic Analyses—Glucose transporters were immunoprecipitated using the monoclonal antibody 1F8, which is specific for the IRGT (18), exactly as described previously (22). The immunoprecipitated glucose transporters were subjected to SDS-PAGE by the method of Laemmli (31). Samples were incubated for 37°C for 10 min before electrophoresis was conducted using 10% resolving gels. CNBr fragments (see below) of the transporter were dissolved in SDS sample buffer and subjected to electrophoresis using resolving gels formed with a linear gradient (10–20%) of acrylamide. After electrophoresis, gels were dried onto filter paper and then placed on X-ray films. Protein standards (Kodak XAR-5) that were exposed at −80°C for 24 h were quantitated either by scintillation counting after slicing the radioactive bands from the gels or by optical density scanning of autoradiographs. Apparent molecular weights were estimated from the mobilities of the following protein standards: aprotinin (6,500), cytochrome c (12,400), myoglobin (17,200), soybean trypsin inhibitor (21,000), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase b (97,000), and β-galactosidase (116,000).

Immunoblotting was performed to allow estimation of the relative amounts of transporters in different fractions. Proteins were electrophoretically transferred from SDS gels to nitrocellulose sheets (32). To identify IRGT, sheets were incubated with the polyclonal antibody 1F8, which was generated by immunizing rabbits with a synthetic peptide having the same sequence as the last 12 amino acids in the COOH terminus of the IRGT (12). The Na+–K+–ATPase was identified by incubating sheets with a polyclonal antibody (provided by Dr. Robert Mercer, Washington University) raised against the purified ATPase from rat kidney. Antibody binding was detected by autoradiography using sheets incubated with $^{32}$P-labeled protein A (2 μCi/ml) as described previously (23).

When samples of IRGT that had been immunoprecipitated from $^{32}$P-labeled cells were transferred to nitrocellulose sheets, autoradiograms were prepared before the sheets were incubated with antibody. This enabled detection of the amount of $^{32}$P-labeled IRGT present. Electrophoresis times of 15 min were generally required. Sheets were then incubated with the appropriate IgG in buffer containing Triton X-100 and 1% powdered milk (Carnation) followed by an incubation with $^{125}$I-labeled protein A as described previously (23). Incubation with the milk resulted in almost complete dephosphorylation of the transporter. Consequently, $^{32}$P did not interfere with detection of $^{125}$I-labeled protein A. Typically autoradiograms of $^{125}$I were developed after 3–4 h of exposure.

Clueage of the IRGT with CNBr—The $^{32}$P-labeled glucose transporter was eluted from gel slices and precipitated with acetone (23). The samples were then dissolved in 200 μl of 70% formic acid and incubated for 18 h with 3 mg/ml CNBr (Pierce). CNBr and formic acid were removed under vacuum as described previously (22).

Dephosphorylation of IRGT in LDM with the Catalytic Subunit of Type I and Type IIa Protein Phosphatases—LDM (5 μg) were prepared from $^{32}$P-labeled fat cells as described previously (22) except that cells were homogenized in 10 mM NaF, 1 mM EDTA, and 0.1 mM NaPPi (pH 7.4), and these concentrations of NaF and NaPPi, dissolved in the freeze-thaw buffers, inhibit dephosphorylation of the transporter. The membranes were washed in a solution of 250 mM sucrose, 1 mM EDTA, and 10 mM HEPES (pH 7.4) to remove the F− and P3. Phosphatases were then added, and samples (approximate 100 μg of LDM protein) were incubated at 30°C in a final volume of 66 μl. Aliquots (10 μl) were removed after increasing times

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EXPERIMENTAL PROCEDURES

Preparation of Adipocytes—Adipocytes were isolated by incubating epididymal adipose tissue of rats (Sprague-Dawley, 180–220 g) in medium containing 1 mg/ml crude collagenase (Clistroid histolyticum, Worthington Biochemical, lot no. 47JO57) (28). The medium was composed of Krebs-Ringer phosphate buffer containing 3% bovine serum albumin (Fraction V, Sigma, lot no. 58P0581). After 1 h, the cells were strained through nylon mesh and washed to remove the collagenase.

Incubation with Insulin and Okadaic Acid—In experiments not involving labeling with $^{32}$P, cells were washed twice and suspended in medium (1 g of cells/10 ml of medium) before additions of insulin and/or okadaic acid. For labeling with $^{32}$P, the cells were washed two times and then incubated for 20 min after incubation with $^{32}$P (0.1–0.3 mCi/ml) for 2 h before the addition of insulin or okadaic acid. Okadaic acid was prepared as a 1 mM HEPES (pH 7.4) and phosphate at 0.2 mM of NaF and phosphate at 0.2 mM of NaPPi (pH 7.4). Cells were suspended in low Pi medium and incubated at 37°C with insulin and/or okadaic acid for 20 min. 2-Deoxy[1-14C]glucose was added prior to the 2-deoxy[1-14C]glucose.

Dephosphorylation experiments were performed to determine whether okadaic acid had an insulin-like effect of stimulating movement of transporters to the plasma membrane. In addition, the effects of insulin and okadaic acid on the phosphorylation states of the IRGT and other proteins associated with the intracellular vesicles containing the transporter were examined.

Measurements of 2-Deoxyglucose Uptake—Adipocytes were washed two times and suspended (1 g of original tissue/10 ml of medium) in either normal or low Pi medium (see the legends to Figs. 1 and 2). To assess the effects of insulin and okadaic acid on the initial rate of 2-deoxyglucose uptake, suspensions (200 μl, 50,000–75,000 cells) were incubated at 37°C with insulin and/or okadaic acid for 20 min. 2-Deoxy[1-14C]glucose (50 μl, approximately 2.5 × 106 cpm) was added to a final concentration of 100 μM. After 15 s, 50 μl of 80 mM cytochalasin B was added, and the cells were separated immediately from the medium by centrifugation through oil as described previously (29). The amount of 2-deoxyglucose taken up via the glucose transporter was determined after subtracting the uptake measured when cytochalasin B (10 μM) was added prior to the 2-deoxy[1-14C]glucose. Uptake measurements under these conditions reflect the initial rate and provide an accurate index of glucose transport.

Cells in low Pi medium were incubated for 2 h before treatment with insulin and/or okadaic acid so that transport measurements could be made under conditions approximating those used in the $^{32}$P-labeling experiments. The relatively low incubation times before the measurements were performed probably explains why the fold stimulation with insulin is somewhat lower than that observed under more optimal conditions.
and added to tubes containing SDS (1%) to terminate the phosphatase reaction. The IRGT was immunoprecipitated as described previously (22). The catalytic subunit of Type I protein phosphatase purified from rabbit skeletal muscle (33) was supplied by Dr. Anna DePaoli-Roach (Indiana University School of Medicine). Type IIa catalytic subunit was purified from beef heart (34) and was provided by Dr. Marc Mumber (University of Texas Health Science Center at Dallas). The activities of the respective catalytic subunits were compared by using 32P-labeled phosphorylase a as substrate. A unit is defined as the amount of enzyme that released 1 nmol of 32P from phosphorylase a in 1 min.

Vesicle Immunoadsorption—The epitope of the monoclonal antibody 1F8 is within the cytoplasmic domain of the IRGT and thus provides accessibility to the intact intracellular glucose transporter-containing vesicles (GTG). Acrylamide beads (Bio-Rad) were incubated with 1 mg/ml goat anti-mouse IgG (Fast Access Biologicals). In experiments involving immunoadsorption of GVT from 32P-labeled cells, beads (200 μl) were incubated with 1% bovine serum albumin in phosphate buffered saline for 60 min at 20°C. Beads were then incubated with GVT-depleted LDM (200 μg) from nonlabeled cells for 30 min at 4°C. Beads were washed three times with Buffer A supplemented with 100 mM NaCl (Buffer B). The suspensions were divided in halves and incubated with either 1F8 (50 μg/100 μl of beads) or nonimmune mouse serum (10 μl) for 60 min at 20°C. The beads were then washed three times with Buffer A and added to tubes containing 32P-labeled LDM (10 μg) and bovine serum albumin (0.1%) in Buffer B. After a 2-h incubation at 4°C, beads were pelleted through a 0.4 M sucrose cushion and washed three times with Buffer B. Beads were subjected to SDS-PAGE following addition of Lasemmli sample buffer.

Sucrose Density Gradient Sedimentation—Adipocytes were incubated in the absence or presence of insulin following labeling with 32P as described above. Homogenates were centrifuged at 48,000 × g for 20 min at 4°C to remove the plasma membrane, high density microsomes, and mitochondrial/nuclear fractions. The supernatants were concentrated (2 mg/ml, final protein concentration) by using Aquacide (Calbiochem) and then layered gently onto continuous sucrose gradients (12-40%, w/v) prepared in Buffer A. Gradients were centrifuged at 100,000 × g in a Beckman SW 41 rotor for 18 h at 4°C. Fractions (0.7 ml) were collected at 4°C by puncturing the bottom of each tube with a needle. The IRGT was immunoprecipitated from each fraction as described above.

Other Materials—Okadaic acid was generously provided by Dr. Philip Cohen (University of Dundee) and by Dr. M. Gibbs (Pfizer Inc, originally supplied by Professor Takeshi Yashimoto and Dr. Michio Murata, Tohoku University, Sendai). 32P- and Na[32P] were obtained from Du Pont-New England Nuclear. 125I-labeled protein A was prepared by nonenzymatic iodination of protein A as described previously (35). Highly purified porcine insulin (27 units/mg) was a gift from Lilly.

RESULTS

Stimulation of 2-Deoxyglucose Uptake by Okadaic Acid—The effect of okadaic acid on the uptake of 2-deoxyglucose by rat adipocytes was measured initially using the experimental design described by Haystead et al. (27) (Fig. 1). Cells were incubated for increasing times in medium containing 0.5 mM 2-deoxy[1-14C]glucose. Where indicated, insulin or okadaic acid was added to the medium at the same time as the 2-deoxy[1-14C]glucose (Fig. 1). Okadaic acid increased uptake after a lag of approximately 5 min, and between 10 and 20 min of incubation, the rate of uptake in the presence of okadaic acid was comparable to the maximum rate of uptake observed by insulin. The present results agree with the earlier findings of Haystead et al. (27) and confirm their observation that okadaic acid increases glucose transport.

From the results in Fig. 1, it was not clear if okadaic acid stimulated glucose transport to the same extent as insulin because the 2-deoxy[1-14C]glucose was added to the cells together with the insulin and okadaic acid. This is because several minutes of incubation are needed for insulin to exert its full effect on transport (36). Therefore, additional experiments were performed to compare the effects of insulin and okadaic acid on stimulating glucose transport. Cells were incubated with the two agents for 20 min, a time sufficient to elicit their respective maximum effects. The uptake of 2-deoxy[1-14C]glucose was then measured after a 15 s incubation, which provides an index of the initial rate of sugar uptake. Insulin and okadaic acid stimulated 2-deoxy[1-14C]glucose uptake by approximately 8- and 4-fold, respectively (Fig. 2).

To determine whether the effects of insulin and okadaic acid were additive, cells were incubated with the combination of both agents (Fig. 2). With insulin plus okadaic acid, the rate of 2-deoxyglucose uptake was actually less than that observed with insulin alone (P < 0.05, paired comparison). Similar results were obtained when glucose transport was assessed by measuring the initial rates of 3-O-methylglucose uptake. Thus, not only was okadaic acid less effective than insulin in stimulating glucose transport, but it also inhibited insulin-stimulated glucose transport.

Effect of Okadaic Acid on the Subcellular Distribution of Glucose Transporters—To determine whether okadaic acid...
mimicked insulin by increasing the number of glucose transporters in the plasma membrane, the relative amounts of IRGT were determined in various subcellular fractions (Figs. 3 and 4). Samples of different membrane fractions were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose sheets. The IRGT was identified by using a polyclonal antibody (R820) and 125I-labeled protein A. The subcellular distribution of the IRGT among the four membrane fractions obtained from adipocytes incubated either in the absence or presence of insulin was similar to that described previously (18). Briefly, in the absence of insulin, most of the transporter was found in the LDM fraction with negligible amounts found in the plasma membrane or the mitochondrial/nuclear fractions. In the presence of insulin, transporters in the plasma membrane increased, and those in the LDM decreased. Okadaic acid also stimulated translocation of the IRGT from the LDM to the plasma membrane, but this effect was significantly less than that of insulin. It should be noted that at the concentrations used in these experiments, insulin and okadaic acid produce their respective maximum effects on IRGT translocation.2

When adipocytes were incubated with insulin together with okadaic acid, additivity of the effects was not observed. In fact, okadaic acid impaired the ability of insulin to stimulate translocation (p < 0.05, insulin versus insulin plus okadaic acid). The protein recovery in the different membrane fractions was not affected by insulin or okadaic acid, and the distribution of the Na+-K+-ATPase, a plasma membrane marker, was not significantly different among the treatment groups (Fig. 3). Therefore, it is unlikely that the effects of okadaic acid on opposing insulin-stimulated translocation result from a fractionation artifact.

Stimulation of Protein Phosphorylation by Okadaic Acid—Okadaic acid stimulated the phosphorylation of proteins in all subcellular fractions except the mitochondrial/nuclear fraction. Phosphorylation of most of the major phosphoproteins in the plasma membrane, LDM, and soluble fractions was increased by okadaic acid (Fig. 5). These species appeared to include proteins whose phosphorylation was also increased by insulin. An example is the Mr = 130,000 species, which is probably ATP-citrate lyase (37), found in the LDM and soluble fractions. However, okadaic acid also increased the phosphorylation of proteins not significantly phosphorylated in response to insulin, such as the Mr = 90,000 species present in the soluble fraction.

To determine whether the IRGT was phosphorylated in response to okadaic acid, the protein was immunoprecipitated from subcellular fractions of 32P-labeled adipocytes (Fig. 6). Samples of the immunoprecipitate were then subjected to SDS-PAGE, and proteins were transferred to nitrocellulose.
Acid. Glucose transporters were immunoprecipitated from the LDM and plasma membrane (PM) fractions prepared as described in the legend to Fig. 5. The proteins were transferred to nitrocellulose, and an autoradiogram was prepared to allow detection of $^{32}$P associated with the IRGT (A). The sheets were then incubated with R820 followed by $^{125}$I-labeled protein A to estimate the relative amount of glucose transporters present (A).

The amount of $^{32}$P-labeled transporter was determined by autoradiography (Fig. 6B). Okadaic acid increased phosphorylation of the IRGT in both the LDM and plasma membrane fractions. However, because okadaic promotes translocation of the transporter, it was necessary to measure the amount of transporter in the fractions in order to assess the magnitude of the effect of okadaic acid on increasing phosphorylation. Therefore, the nitrocellulose sheets were incubated with R820 and $^{125}$I-labeled protein A to enable estimation of the relative amounts of IRGT present (Fig. 6A). Values for specific activity in arbitrary units could then be determined by dividing the amount of $^{32}$P by the amount of $^{125}$I. To allow comparison among experiments, the specific activities in the different fractions were expressed relative to that in the LDM from control cells (22).

A potential problem with expressing specific activity relative to the control LDM is that the specific activity of the IRGT in the precursor pool from which the transporters move to the plasma membrane may differ from that of the whole LDM population. However, we found no evidence that the $^{32}$P-labeled IRGT was found in a subset of the GTV within the LDM when sucrose gradient analyses were performed on LDM from control and insulin-treated cells. The $^{32}$P-labeled IRGT was found in gradient fractions identical to those containing the bulk of the intracellular IRGT (Fig. 7). Furthermore, the density of the GTV isolated from control cells was indistinguishable from those of insulin-treated cells.

In agreement with our previous results (22, 23), the specific activity of $^{32}$P-labeled IRGT was significantly higher in the plasma membranes of control cells than in the membranes of insulin-treated cells (Fig. 8B). It seems likely that translocation per se accounts at least in part for the decrease in specific activity of the plasma membrane transporters produced by insulin. The transporters that are translocated from the LDM to the plasma membrane in response to insulin have a lower specific activity than those transporters already in the plasma membrane. Consequently, the specific activity of the plasma membrane fraction of IRGT would be expected to resemble more closely that of these newly inserted transporters because of their relative abundance. For this reason, we believe it is most appropriate to make all comparisons of $^{32}$P-labeled IRGT relative to the specific activity of the $^{32}$P-labeled transporters in the donor compartment (i.e. the control LDM fraction) of control cells. It is possible that changes in $^{32}$P-labeled transporters may occur in the plasma membrane fraction in an insulin-dependent manner. However, such changes should still be evident when the specific activities are expressed relative to that of the transporters in the control LDM.

Okadaic acid increased the phosphorylation of IRGT in the LDM fraction by only 60%. In contrast, in the presence of okadaic acid alone or okadaic acid plus insulin, there was a 3-4 fold increase in the phosphorylation of transporters in the plasma membrane fraction (Fig. 6A). Assuming that the specific activity of $[^{32}P]$phosphate in the transporter was equal to that of intracellular $[\gamma^{32}P]$ATP and by using measurements of glucose-inhibitable cytochalasin B binding sites as an estimate of transporter number, we have calculated that there is approximately 0.2 mol of phosphate per mol of IRGT in the LDM of control cells incubated under these conditions (22). Although this value is likely to be lower than the actual stoichiometry (22), it indicates that there is at least 0.6-0.8 mol of phosphate per mol of plasma membrane IRGT after okadaic acid treatment.

Essentially all of the $^{32}$P in transporters from control or insulin-treated cells is recovered in a single CNBr fragment, denoted CB-T (23). This fragment appears to encompass the COOH terminus of the IRGT because it binds to the COOH-terminal antibody, R820 (12). As shown in Fig. 9, CB-T was the only $^{32}$P-labeled CNBr fragment evident in transporter immunoprecipitated from okadaic acid-treated cells. Thus, even after inhibiting protein phosphatases with okadaic acid, phosphorylation of the IRGT was restricted to a relatively small region in the COOH-terminal intracellular domain.

Phosphoproteins Associated with Intracellular Glucose Transports.
Comparison of the effects of insulin (INS) and okadaic acid (OKA) on the relative specific activity of glucose transporters in LDM and plasma membranes (PM). Autoradiograms from experiments performed as described in the legend to Fig. 6 were scanned for optical density, and areas beneath the peaks corresponding to the IRGT were determined. Specific activities in arbitrary units were calculated by dividing the values for $^{32}$P peaks by those of $^{14}$C peaks. To compare results from different experiments, the specific activity of the transporter in the LDM of control cells was assigned a value of 1. The results presented are mean values ($\pm$ S.E.) of four experiments. CON, control.

Transporter Vesicles—The GTV appear to be correctly oriented following homogenization because they can be isolated quantitatively using antibodies directed against cytoplasmic domains of the glucose transporter (38). Using this technique, GTV from $^{32}$P-labeled cells were isolated, and the major phosphoprotein constituents of the vesicles were identified (Fig. 10). An $M_r = 45,000$ protein that we have identified as the IRGT is the major $^{32}$P-labeled protein in the GTV isolated from control cells. At least three other phosphoproteins (denoted PGTV-1 to PGTV-3 in Fig. 10) were identified which underwent increased phosphorylation in response to okadaic acid. These proteins were not evident in the supernatant fractions from the vesicle immunoprecipitation or when immunoadsorption was performed using a nonimmune IgG (Fig. 10). The phosphorylation of an $M_r = 32,000$ protein in the immunoprecipitates was increased markedly after insulin and okadaic acid. However, this species is probably not associated with the GTV because it was recovered using nonimmune IgG.

Dephosphorylation of IRGT by the Catalytic Subunits of

Type I and Type IIa Protein Phosphatases—Because the GTV are isolated with the cytoplasmic side facing outward, sites of phosphorylation should be accessible to protein phosphatases added to the LDM. This enabled us to determine whether the IRGT could be dephosphorylated by Type I and Type IIa phosphatases, the enzymes inhibited by okadaic acid (25, 26). LDM were isolated from $^{32}$P-labeled cells in the presence of the phosphatase inhibitors $F$- and $PP_\alpha$. The membranes were washed to remove the inhibitors and then used as substrate for exogenously added protein phosphatases. The amounts of $^{32}$P-labeled transporter were determined by autoradiography after immunoprecipitation and SDS-PAGE. Phosphatase ac-
transporter. Type I and Type IIa phosphatases dephosphorylate the transporter. Therefore, all of the sites in the transporter per se is not required for its translocation to the cell surface.

Even though the action of okadaic acid resembles that of insulin, and it seems reasonable to conclude that the increase in plasma membrane transporters produced by okadaic acid is primarily responsible for the observed increase in glucose transport. Our results also indicate that okadaic acid increases the phosphorylation of the IRGT (Fig. 6), suggesting that the transporter is dephosphorylated in cells by either Type I or Type IIa protein phosphatases. Further support for this hypothesis is provided by the findings that the transporter could be dephosphorylated in vitro by the catalytic subunit of either of these phosphatases (Fig. 11).

Several differences were noted between the effects of okadaic acid and insulin on glucose transport. Unlike okadaic acid, insulin did not stimulate phosphorylation of the IRGT (Fig. 8). This suggests that phosphorylation of the transporter per se is not required for its translocation to the cell surface. With okadaic acid, but not with insulin, there was a pronounced lag before increased transport was observed (Fig. 1). This lag might be because of the delay in diffusion of okadaic acid to the plasma membrane. In this respect alone, okadaic acid has at least two actions, an insulin-like action that opposes the first. The second might be at the level of the insulin receptor since there is evidence that increased serine/threonine phosphorylation of the receptor inhibits its activity (41). However, because insulin does not stimulate IRGT phosphorylation, it is tempting to propose that it is the action of okadaic acid on increasing transporter phosphorylation which is involved in opposing the effect of insulin.

The role of phosphorylation in modulating transporter function has not been defined. However, there is evidence that the phosphorylation state of the IRGT is altered under various experimental conditions. We have shown previously that phosphorylation of the IRGT is increased by β-adrenergic agonists and CAMP derivatives (22, 23). Incubation of adipocytes with the tumor promoter, phorbol 12-myristate 13-acetate, also stimulates phosphorylation of the IRGT. The results with isoproterenol were of particular interest because β-adrenergic receptor stimulation inhibits insulin-stimulated glucose transport without decreasing the numbers of transporters in the plasma membrane. Based on these observations we proposed that phosphorylation of the transporter by CAMP-dependent protein kinase decreased the ability of the IRGT to transport glucose. Presumably this is not the case with okadaic acid, whose inhibition of insulin-stimulated glucose transport appears to involve inhibition of transporter translocation. The actual site of phosphorylation increased in

![Fig. 11. Dephosphorylation of the IRGT by Type I and Type IIa protein phosphatases. LDM (100 μg) from 32P-labeled adipocytes were incubated at 30°C for increasing times without added phosphatase (no P'ase), with 0.5 milliunits/ml of the catalytic subunit of protein phosphatase I (Type I), and either 0.5 or 40 milliunits/ml of the catalytic subunit of protein phosphatase IIa (Type IIa). Immunoprecipitations were performed using monoclonal antibody 1F9, and samples were subjected to SDS-PAGE. An autoradiogram of the dried gel was scanned for optical density to estimate the relative activity of the phosphatase. The autoradiograms indicated that there was no decrease in the amount of glucose transporters during the 60-min incubation. Therefore, the decrease in 32P is because of phosphatase activity rather than a decrease in the number of transporters immunoprecipitated. The results are expressed as the percentage of the 32P content of the transporters before incubation at 30°C.](http://www.jbc.org/content/272/35/19774)

DISCUSSION

The present results indicate that okadaic acid stimulates translocation of glucose transport proteins from an intracellular store to the plasma membrane (Fig. 3). In this respect the action of okadaic acid resembles that of insulin, and it seems reasonable to conclude that the increase in plasma membrane transporters produced by okadaic acid is primarily responsible for the observed increase in glucose transport. Our results also indicate that okadaic acid increases the phosphorylation of the IRGT (Fig. 6), suggesting that the transporter is dephosphorylated in cells by either Type I or Type IIa protein phosphatases. Further support for this hypothesis is provided by the findings that the transporter could be dephosphorylated in vitro by the catalytic subunits of either of these phosphatases (Fig. 11).

Dephosphorylation of the IRGT by Type I and Type IIa protein phosphatases. LDM (100 μg) from 32P-labeled adipocytes were incubated at 30°C for increasing times without added phosphatase (no P'ase), with 0.5 milliunits/ml of the catalytic subunit of protein phosphatase I (Type I), and either 0.5 or 40 milliunits/ml of the catalytic subunit of protein phosphatase IIa (Type IIa). Immunoprecipitations were performed using monoclonal antibody 1F9, and samples were subjected to SDS-PAGE. An autoradiogram of the dried gel was scanned for optical density to estimate the relative activity of the phosphatase. The autoradiograms indicated that there was no decrease in the amount of glucose transporters during the 60-min incubation. Therefore, the decrease in 32P is because of phosphatase activity rather than a decrease in the number of transporters immunoprecipitated. The results are expressed as the percentage of the 32P content of the transporters before incubation at 30°C. The ability of insulin and okadaic acid to stimulate translocation of the IRGT raises the possibility that both agents stimulate the phosphorylation of the same regulatory protein responsible for triggering movement of vesicles to the cell surface. A number of proteins whose phosphorylation was increased by both insulin and okadaic acid were evident in the cell extract (Fig. 5). However, the effects of okadaic acid on protein phosphorylation were far more widespread than those of insulin. Thus, establishing a link between phosphorylation of one of the extract phosphoproteins and augmented glucose transport will be difficult. Therefore, as an initial step in identifying phosphoproteins involved in regulating glucose transport, it seemed reasonable to focus on those associated with the GTV. The three phosphoproteins that were identified in the vesicles (Fig. 10) are attractive candidates for regulators of movement of the GTV to the plasma membrane.

One interpretation of the differences between the extent of glucose transport activation by insulin and okadaic acid is that okadaic acid has at least two actions, an insulin-like action to stimulate translocation of the IRGT and a second action that opposes the first. The second might be at the level of the insulin receptor since there is evidence that increased serine/threonine phosphorylation of the receptor inhibits its activity (41). However, because insulin does not stimulate IRGT phosphorylation, it is tempting to propose that it is the action of okadaic acid on increasing transporter phosphorylation which is involved in opposing the effect of insulin. The roles of phosphorylation in modulating transporter function have not been defined. However, there is evidence that the phosphorylation state of the IRGT is altered under various experimental conditions. We have shown previously that phosphorylation of the IRGT is increased by β-adrenergic agonists and CAMP derivatives (22, 23). Incubation of adipocytes with the tumor promoter, phorbol 12-myristate 13-acetate, also stimulates phosphorylation of the IRGT. The results with isoproterenol were of particular interest because β-adrenergic receptor stimulation inhibits insulin-stimulated glucose transport without decreasing the numbers of transporters in the plasma membrane. Based on these observations we proposed that phosphorylation of the transporter by CAMP-dependent protein kinase decreased the ability of the IRGT to transport glucose.
response to okadaic acid has not been determined although it is located in the same CNBr fragment as the isoproterenol-stimulated phosphorylation site(s). It should be noted that there are several serine and threonine residues that are potential sites of phosphorylation in this fragment, and it seems possible that the IRGT tail contains multiple phosphorylation sites that have different functional roles.

Phosphorylation has been proposed to regulate endocytosis of a number of membrane proteins, including the receptors for epidural growth factor (42), insulin (43), and insulin-like growth factor II (IGF-II) (44). The IGF-II receptor is of particular interest because like the glucose transporter, its cell surface concentration is increased following incubation of cells with insulin (45). Another similarity between the proteins is that the phosphorylation state of the IGF-II receptor in the plasma membrane from control cells is significantly higher than that in the plasma membrane of insulin-treated cells (44). It has been proposed that the phosphorylated IGF-II receptor recycles more rapidly than the nonphosphorylated receptor and that insulin causes dephosphorylation of the receptor at the cell surface (44).

We suggest that phosphorylation of the IRGT triggers its internalization. In order for this hypothesis to accommodate the effects of insulin and okadaic acid on glucose transport, it is necessary to assume that the IRGT recycles between the intracellular compartment and the cell surface. This is likely because the glucose transporter repopulates the intracellular compartment following insulin removal (46). The difference between the maximum effects of insulin and okadaic acid would be consistent with an action of phosphorylation to increase the rate of IRGT internalization since okadaic acid increased, and insulin decreased, the phosphorylation state of transporters in the plasma membrane. Furthermore, it might be significant that transporters in the plasma membrane of control cells are more highly phosphorylated than those in the LDM. It is an intriguing possibility that the high basal phosphorylation is a mechanism for maintaining a low number of the IRGT proteins in plasma membrane in the absence of insulin. It may be noteworthy that the $^{32}$P-labeled IRGT in the basal and insulin-treated plasma membrane fraction was not recovered in a Triton X-100-insoluble fraction of the plasma membrane. This result is in contrast to previous findings with the IGF-II receptor (47). However, the IRGT may not cluster in coated pits to the same extent as the IGF-II receptor.

Further characterization of the phosphorylation sites within the IRGT and establishing their functional roles may yield important information about insulin action. A working hypothesis is that insulin has two actions in regulating the increased expression of the IRGT at the cell surface. One is to increase movement of the intracellular vesicles to the plasma membrane. The second is to promote retention of the transporter in the plasma membrane. The latter effect might involve maintaining the IRGT in a relatively dephosphorylated state, an action that does not occur with okadaic acid and possibly other agents such as phorbol 12-myristate 13-acetate. The more potent stimulatory effect of okadaic acid on phosphorylation of transporters in the plasma membrane fraction compared to those in the LDM suggests that phosphorylation/dephosphorylation occurs either within or close to the plasma membrane.

In summary, our results lend further support to the hypothesis that translocation is the major mechanism for acutely regulating glucose transport in rat adipocytes. We have demonstrated that the IRGT is a target for cellular protein kinases and protein phosphatases although the physiological role of transport phosphor phosphorylation is yet to be determined. Our findings are consistent with the hypothesis that increased serine/threonine phosphorylation of a protein, possibly associated with the GTP, stimulates movement of transporters to the plasma membrane whereas phosphorylation of the glucose transporter itself accelerates its internalization.

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