Comparative Effects of GTPγS and Insulin on the Activation of Rho, Phosphatidylinositol 3-Kinase, and Protein Kinase N in Rat Adipocytes

RELATIONSHIP TO GLUCOSE TRANSPORT

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Electroporation of rat adipocytes with guanosine 5′-3-O-(thio)triphosphate (GTPγS) elicited sizable insulin-like increases in glucose transport and GLUT4 translocation. Like insulin, GTPγS activated membrane phosphatidylinositol (PI) 3-kinase in rat adipocytes, but, unlike insulin, this activation was blocked by Clostridium botulinum C3 transferase, suggesting a requirement for the small G-protein, RhoA. Also suggesting that Rho may operate upstream of PI 3-kinase during GTPγS action, the stable overexpression of Rho in 3T3/L1 adipocytes provoked increases in membrane PI 3-kinase activity. As with insulin treatment, GTPγS stimulation of glucose transport in rat adipocytes was blocked by C3 transferase, wortmannin, LY294002, and RO 31-8220; accordingly, the activation of glucose transport by GTPγS, as well as insulin, appeared to require Rho, PI 3-kinase, and another downstream kinase, e.g. protein kinase C-ζ (PKC-ζ) and/or protein kinase N (PKN). Whereas insulin activated both PKN and PKC-ζ, GTPγS activated PKN but not PKC-ζ. In transfection studies in 3T3/L1 cells, stable expression of wild-type Rho and PKN activated glucose transport, and dominant-negative forms of Rho and PKN inhibited insulin-stimulated glucose transport. In transfection studies in rat adipocytes, transient expression of wild-type Rho and PKN provoked increases in the translocation of hemagglutinin (HA)-tagged GLUT4 to the plasma membrane; in contrast, transient expression of dominant-negative forms of Rho and PKN inhibited the effects of both insulin and GTPγS on HA-GLUT4 translocation. Our findings suggest that (a) GTPγS and insulin activate Rho, PI 3-kinase, and PKN, albeit by different mechanisms; (b) each of these signaling substances appears to be required for, and may contribute to, increases in glucose transport; and (c) PKC-ζ may contribute to increases in glucose transport during insulin, but not GTPγS, action.

GTPγS, like insulin, has been found to activate GLUT4 translocation and/or glucose transport in rat adipocytes (1, 2) and 3T3/L1 adipocytes (3). The mechanisms whereby GTPγS and insulin activate GLUT4 translocation and glucose transport, however, are unclear. In 3T3/L1 cells, unlike insulin, GTPγS was not found to activate cytosolic phosphatidylinositol (PI) 3-kinase (3), and this suggested that (a) PI 3-kinase was not essential for the activation of glucose transport and (b) GTPγS may operate through different or more distal processes. In keeping with the latter possibility, small G-proteins in the Rho group are present in GLUT4 vesicles, appear to translocate or mobilize in response to insulin stimulation (4), and could act as direct mediators for GTPγS stimulation of glucose transport; accordingly, GTPγS-stimulated glucose transport is only partly inhibited by the PI 3-kinase inhibitor, wortmannin, in 3T3/L1 adipocytes (5), and GTPγS therefore appears to act, at least partially, independently of PI 3-kinase in 3T3/L1 cells. On the other hand, we have recently found that the small G-protein, RhoA, is activated by insulin in rat adipocytes, and, based upon Clostridium botulinum C3 transferase sensitivity, Rho appears to be required for insulin-stimulated glucose transport in these cells (6). Further, it seems clear that Rho is directly activated by GTPγS in rat adipocytes, since it was found that the addition of GTPγS to rat adipocyte homogenates stimulates the translocation of Rho to plasma membranes and Rho-dependent activation of phospholipase D (6). Of further note, it has been reported that GTP-Rho directly activates PI 3-kinase in some (7), but not all (8, 9), cell-free systems. Presently, we examined the possibility that GTPγS activates PI 3-kinase via Rho in rat adipocytes. We also examined the role of Rho, PI 3-kinase, and protein kinases that are known to be downstream of Rho and PI 3-kinase (e.g. PKN and protein kinase C-ζ (PKC-ζ)) in the activation of glucose transport during treatment of rat adipocytes with GTPγS or insulin. Our findings suggested that (a) both GTPγS and insulin, albeit by different mechanisms, activate Rho, PI 3-kinase, and PKN; and (b) each of these factors may be required for, and may contribute to, the activation of GLUT4 translocation and glucose transport in rat adipocytes.

EXPERIMENTAL PROCEDURES

Rat Adipocytes (Preparation, Incubation, and Electroporation)—Rat adipocytes were prepared by collagenase digestion of epididymal fat pads obtained from male Sprague-Dawley rats weighing approximately 200–250 g, as described previously (6). The adipocytes were suspended
in glucose-free Krebs Ringer phosphate (KRP) buffer containing 1% bovine serum albumin for acute incubations, or in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.) containing 1% bovine serum albumin for overnight incubations. GTP\(\gamma\)S (Sigma or ICN) and C. botulinum C9 transferase (List) were introduced into adipocytes (in 25% serum-free KRP buffer in 3T3-L1 fibroblasts) by transfection (Bio-Rad Gene Pulsar; 350 V and 960 microfarads with a time constant of 12 ms) in either an intracellular buffer (118 mM KCl, 5 mM NaCl, 30 mM CaCl\(_2\), 1 mM EGTA, 1.2 mM Mg\(_2\)SO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 3 mM sodium pyruvate, 25 mM HEPES, and 20 mg/ml bovine serum albumin) or in DMEM, respectively, as described in the text.

GLUT4 Translocation Studies in Transiently Transfected Rat Adipocytes—Glucose transport was measured in adipocytes suspended (6%, cell volume/total volume) in glucose-free KRP buffer as described by Karnam et al. (6). Where indicated, inhibitors (wortmannin, Sigma; LY294002, Biomol; RO 31-8220, Alexis) was added to the incubation 15 min prior to agonist addition. The cells were then treated with vehicle alone (control), insulin (Eli Lilly), or the indicated concentrations of GTP\(\gamma\)S. In the case of GTP\(\gamma\)S treatment, immediately following electroporation in intracellular buffer (see above), the cells were diluted with glucose-free KRP buffer containing indicated inhibitor concentrations. After 30 min of treatment with vehicle (controls), 10 nM insulin, or 0–500 \(\mu\)M GTP\(\gamma\)S, the uptake of 2-[\(\text{H}\)]deoxyglucose (2-DOG; 0.1 mM; NEN Life Science Products) was measured over a 1-min period as described (6). In these assays, the data indicated that cytosoles of 3T3-L1 adipocytes were likely to reflect trapping of medium or nonspecific uptake, were relatively small (approximately 10% of stimulated values) and were not influenced significantly by electroporation or overnight incubation. In cells that were assayed directly without electroporation, insulin-induced increases in the uptake of 2-DOG generally ranged from 4- to 10-fold. In cells that were electroporated and then immediately assayed, there usually was a small, but variable, increase in basal 2-DOG uptake, with little or no change in maximal insulin-stimulated values; consequently, the relative effect of insulin on 2-DOG uptake generally tended to be slightly less, i.e. approximately 2-3-fold, in electroporated adipocytes (this is illustrated in Fig. 8). Overnight incubation of adipocytes also increased basal transport activity, and the relative insulin effect was similarly diminished to approximately 2-3-fold in these cells. However, the effect of combined electroporation and overnight incubation on 2-DOG uptake was not significantly different from that of either electroporation or overnight incubation alone, since insulin effects on 2-DOG uptake were also approximately 2-3-fold in these cells.

GLUT4 Translocation Studies in Transiently Transfected Rat Adipocytes—Effects of transiently transfected Rho and PKN on GLUT4 translocation were assessed in rat adipocytes that express HA-tagged GLUT4, as described previously (13). Immunoprecipitable PKC-\(\varepsilon\) enzyme activity was measured as described (12, 14). To measure immunoprecipitable PKN enzyme activity, cells were lysed in buffer containing 150 mM NaCl, 250 mM sucrose, 20 mM Tris/HC1 (pH 7.5), 1 mM EDTA; 5 mM MgCl\(_2\), 20 mM \(\beta\)-mercaptoethanol, 25 mM NaF, 3 mM Na\(_3\)P\(_2\)O\(_7\), 10 mM Na\(_4\)VO\(_4\), 20 \(\mu\)g/ml aprotinin, 125 \(\mu\)g/ml leupeptin, 1 mM PMSF, 1% Triton X-100, and 2 \(\mu\)g Microcystin-LR (Calbiochem). Rabbit anti-PKN polyclonal antibody (raised in Dr. Ono’s laboratories) was added in sufficient amounts to quantitatively precipitate PKN from 300 \(\mu\)g of lystate protein. After overnight incubation at 0–4°C, the precipitate was collected on Sepharose A/G beads (Santa Cruz Laboratories), washed three times with lysis buffer and twice with assay buffer (50 mM Tris/HC1, pH 7.5, 1 mM EDTA, 1 mM MgCl\(_2\), 10 mM Na\(_4\)VO\(_4\), 10 mM Na\(_3\)P\(_2\)O\(_7\), 10 mM \(\beta\)-glycerophosphate, 100 \(\mu\)M phenylmethylsulfonyl fluoride), and then incubated at 30°C for 6 min in the presence of 40 \(\mu\)g serine-25-2PKC-\(\alpha\) pseudosubstrate (Life Technologies) and 50 \(\mu\)M ATP containing 2 \(\mu\)Ci of \(\gamma\)-32P-ATP (NEN Life Science Products). Aliquots of the reaction mixture were spotted on ps81 filter paper, washed in 30% acetic acid, and counted for 32P. It may be noted that we probably did not reach the maximal transport rates with 500 \(\mu\)M GTP\(\gamma\)S, but it may be noted that we probably did not reach the maximal transport rates with 500 \(\mu\)M GTP\(\gamma\)S, electroporation opens membrane pores only fleetingly, and intracellular GTP\(\gamma\)S concentrations were most likely less than those present in the electroporator buffer. In addition to increasing glucose transport, 500 \(\mu\)M GTP\(\gamma\)S provoked increases in the translocation of HA-tagged GLUT4 to the plasma membrane (cell surface). HA-tagged anti-HA antibody (reflecting the level of exofacial HA-tagged GLUT4) was 1772 ± 72 (n = 4) versus 1748 ± 321 (n = 6) cpm/10\(^6\) cells (< 0.001 t test), control versus GTP\(\gamma\)S, respectively); these increases in HA-GLUT4 translocation were in some experimental groups similar to those provoked by insulin (viz. approximately 2-fold) or, in some groups (e.g. see below), slightly less.

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Since PI-3 kinase is required for insulin effects on glucose transport, it was of interest to see if inhibitors of PI-3 kinase
altered the effects of GTPγS on 2-DOG uptake. As shown in Fig. 1, A and B, both 100 nM wortmannin and 100 μM LY294002, (concentrations that fully inhibit PI 3-kinase) fully inhibited GTPγS-stimulated, as well as insulin-stimulated, 2-DOG uptake. These findings suggested that GTPγS, like insulin, requires PI 3-kinase for the activation of glucose transport in rat adipocytes.

We have previously reported that the effects of insulin on glucose transport are blocked by \textit{C. botulinum} C3 transerase, which specifically ADP-ribosylates, inhibits, and, after overnight incubation of rat adipocytes, leads to a complete loss of immunoreactive RhoA in these cells (see Ref. 6). As shown in Fig. 1C, overnight C3 transerase treatment blocked subsequent effects of both GTPγS and insulin on 2-DOG uptake in rat adipocytes. These results (as well as results from transfection studies; see below) suggested that Rho is required for the effects of GTPγS, as well as insulin, on glucose transport in rat adipocytes.

\textit{Studies of PI 3-Kinase Activation—}Since wortmannin and LY294002 fully blocked the effects of both GTPγS and insulin on 2-DOG uptake in the rat adipocyte, we questioned whether GTPγS activates PI 3-kinase. As shown in Fig. 2, both GTPγS and insulin provoked increases in membrane-associated PI 3-kinase activity in rat adipocytes. Interestingly, C3 transerase blocked the activating effect of GTPγS, but not insulin, on membrane-associated PI 3-kinase (Fig. 2C). Also, insulin-induced increases in PI 3-kinase immunoreactivity and enzyme activity that are specifically associated with IRS-1, were not blocked by C3 transerase treatment (Fig. 3). These findings suggested that activating effects of insulin on PI 3-kinase, both in total membranes and as specifically activated through IRS-1 in the rat adipocyte, were not dependent upon Rho. On the other hand, activating effects of GTPγS on membrane-associated PI 3-kinase in the rat adipocyte appeared to be fully dependent upon Rho. In keeping with a role for Rho in PI 3-kinase activation, as described below, stable overexpression of Rho in 3T3/L1 adipocytes led to increases in membrane-associated PI 3-kinase activity.

\textit{Studies on Effects of RO 31-8220 on PKN Activity and Glucose Transport—}Considerable evidence suggests that one or more protein kinases, apparently distal to PI 3-kinase, is (are) required for insulin stimulation of glucose transport. For example, we have previously shown that the bisindolemaleimide-type PKC inhibitor, RO 31-8220, inhibits insulin-stimulated glucose transport, without inhibiting the activation of PI 3-kinase by insulin (15). Recently, we have found (14) that (a) RO 31-8220 inhibits recombinant PKC-α, βI, βII, γ, δ, ε, η, and ι with IC\textsubscript{50} values of approximately 40, 20, 15, 15, 30, 100, 20 and 1000–4000 nM, respectively, and that (b) these PKCs inhibition of insulin-stimulated 2-DOG uptake in intact adipocytes by RO 31-8220 correlates best with the inhibition of PKC-ζ (IC\textsubscript{50} of approximately 4 μM, with nearly full inhibition at 20–30 μM). Presently, we found that RO 31-8220 inhibited immunoprecipitated PKN with an IC\textsubscript{50} of approximately 30 nM (Fig. 4). Although the exact identity of the RO 31-8220-sensitive kinase that is required for insulin-stimulated glucose transport is not certain, it was of interest to find that RO 31-8220 inhibited the effects of GTPγS, as well as insulin, on 2-DOG uptake in intact rat adipocytes (Fig. 5). However, the concentrations of RO 31-8220 that were required to inhibit GTPγS effects on 2-DOG uptake were considerably less (IC\textsubscript{50} < 1 μM) than those required for inhibition of insulin effects (IC\textsubscript{50} = 5 μM) on 2-DOG uptake in the rat adipocyte (Fig. 5). It therefore seems likely that different RO 31-8220-sensitive protein kinases are required for glucose transport effects of GTPγS and insulin in the rat adipocyte.

\textit{Studies on the Activation PKC-ζ and PKN—}Inasmuch as RO 31-8220 does not inhibit the activity or activation of PI 3-kinase

**Fig. 1. Effects of wortmannin (WORT) (A), LY294002 (B), and C3 transerase (C) on GTPγS- and insulin-stimulated 2-DOG uptake in rat adipocytes.** A and B, cells were treated with 100 nM wortmannin or 100 μM LY294002 for 15 min, electroporated in intracellular buffer with or without the indicated concentrations of GTPγS, and then diluted with glucose-free KRP medium and treated with or without 10 nM insulin as indicated. After 30 min of treatment with GTPγS or insulin, the uptake of 2-DOG uptake was measured over 1 min. C, cells were electroporated in DMEM with or without C3 transerase (0.5 μg/ml), and incubated overnight to deplete immunoreactive Rho in C3 transerase-treated cells (see Ref. 6). The cells were then electroporated a second time in intracellular buffer with or without 500 μM GTPγS and then diluted with glucose-free KRP medium and treated with or without 10 nM insulin, as indicated. After 30 min of treatment with GTPγS or insulin, 2-DOG uptake was measured over 1 min. Values are mean ± S.E. of four determinations.
in the rat adipocyte (15), as alluded to above, it may be surmised that one or more protein kinases, distinct from PI 3-kinase, is required during the activation of glucose transport. Accordingly, we questioned whether GTP\(\gamma S\) or insulin activates PKC-\(\zeta\) or PKN, i.e. kinases that appear to be downstream of PI 3-kinase and/or Rho. Whereas insulin (see Ref. 14 for more detailed studies) activated PKC-\(\zeta\), GTP\(\gamma S\), if anything, diminished the activity of immunoprecipitable PKC-\(\zeta\) in intact adipocytes (Table I). In addition, GTP\(\gamma S\), unlike insulin, failed to activate PKB (data not shown). On the other hand, GTP\(\gamma S\) activated immunoprecipitable PKN mildly (23%), but significantly, in intact adipocytes (Table II) and more dramatically in adipocyte homogenates (Table III). (Note that the in vitro incubation of adipocyte homogenate in low Mg\(^{2+}\) conditions prior to immunoprecipitation markedly lowered basal immunoprecipitable PKN activity, and this probably facilitated the observance of greater relative effects of GTP\(\gamma S\) in the cell-free system.) Similarly, insulin provoked approximately 60% increases in immunoprecipitable PKN activity throughout a 1–10-min treatment period in intact rat adipocytes, and these increases were blocked by C3 transferase, but not by wortmannin (Fig. 6). Thus, in keeping with our previous report that PI 3-kinase is not required for insulin stimulation of GTP-loading of Rho (6), PI 3-kinase did not appear to be required for insulin-induced activation of PKN. Also, in keeping with the possibility that PKN is downstream of Rho, Rho appeared to be required for insulin-induced activation of PKN.

**Transfection Studies**—Since our findings with C3 transferase suggested that Rho is required for (a) GTP\(\gamma S\)- and insulin-induced increases in glucose transport and (b) GTP\(\gamma S\)-induced increases in PI 3-kinase, we questioned whether Rho itself...
could provoke increases in PI 3-kinase activity and glucose transport and/or GLUT4 translocation. To pursue these questions and to further examine the requirement for Rho in glucose transport, we used several transfection approaches. First, in both 3T3/L1 fibroblasts and adipocytes, the stable overexpression of wild-type Rho led to increases in both immunoreactive Rho and basal and insulin-stimulated glucose transport (Fig. 7); in addition, stably transfected Rho provoked a 73 ± 10% increase (mean ± S.E.; n = 7; p < 0.001, paired t test, Rho transfecants versus untransfected and vector-transfected controls) in membrane-associated PI 3-kinase enzyme activity in 3T3/L1 adipocytes. Second, in rat adipocytes, transient transfection of wild-type and, even more so, constitutive, Rho led to increases in the translocation of transiently co-transfected HA-tagged GLUT4 to the plasma membrane (Fig. 8); in contrast, dominant-negative Rho largely inhibited the effects of insulin (Fig. 8) and fully inhibited the effects of GTPγS (Fig. 8) on activated Rho and basal and insulin-stimulated 2-DOG uptake (Fig. 7); this approach avoided having Rho overexpressed until 72 h prior to
Cated concentrations (0, 5, 100 nM) of insulin, 5-min uptake of 2-DOG glucose-free KRP medium and, after treatment for 30 min, with insulin were assayed in parallel with untransfected (Clones were selected by G418 resistance, and grown, differentiated, and assayed in parallel with untransfected (b) cells. Cells were incubated in glucose-free KRP medium and, after treatment for 30 min, with indicated concentrations (0, 5, 100 nM) of insulin, 5-min uptake of 2-DOG was measured. Values are the mean ± S.E. of (n) clones, each assayed in triplicate at each insulin concentration. Insets show increases in immunoreactivity in cells transfected (TX) with Rho (R). B, cells were stably transfected with (a) a plasmid (pTet-On) that contains cDNA encoding a mutated tetracycline repressor fused to the VP16 activation domain of a herpes simplex virus controlled by a constitutive Pcmv promoter and (b) a plasmid (pTRE) containing cDNA encoding wild-type Rho. In keeping with the possibility that PKN may operate downstream of Rho during glucose transport activation, we found that transient expression of wild-type PKN in rat adipocytes resulted in increases in the translocation of co-transfected HA-tagged GLUT4 to the plasma membrane of rat adipocytes. A, cells were co-transfected by electroporation in DMEM in the presence of (a) pCIS2 containing cDNA encoding HA-tagged GLUT4 and (b) pCDNA3 vector alone (V) or pCDNA3 containing cDNA encoding wild-type (WT), constitutive (CONSTIT), or dominant negative (DN) Rho. B, adipocytes were co-transfected with (a) pCIS2 containing cDNA encoding HA-GLUT4, along with (b) pCDNA3 containing cDNA encoding dominant negative Rho, pTB701 containing cDNA encoding dominant-negative PKN, or vectors. After overnight incubation to allow time for expression, cells were equilibrated in glucose-free KRP medium for 30 min and treated with or without 10 nM insulin (A) or 500 μM GTPγS (B) as indicated, prior to the addition of 2 mM KCN (to immobilize GLUT4) and measurement of cell number and cell surface HA-GLUT4 (reflected by 125I labeling). Values are mean ± S.E. of (n) shown in parentheses determinations. A, single asterisks indicate p < 0.05 (t test), Rho transfectants versus control, on transfection of dominant-negative Rho partially (25–35%) inhibited insulin-stimulated 2-DOG uptake in 3T3/L1 fibroblasts (Fig. 9). From these transfections studies, it appears that (a) Rho itself can activate PI 3-kinase, GLUT4 translocation, and glucose transport; and (b) in keeping with studies using C3 transferase, Rho is required for effects of both insulin and GTPγS on GLUT4 translocation and glucose transport in rat adipocytes. In keeping with the possibility that PKN may operate downstream of Rho during glucose transport activation, we found that transient expression of wild-type PKN in rat adipocytes resulted in increases in the translocation of co-transfected HA-tagged GLUT4 to the plasma membrane (Fig. 9); in contrast, dominant-negative PKN partially inhibited the effects of insulin (Fig. 9) and fully inhibited the effects of GTPγS (Fig. 8) on HA-GLUT4 translocation. Similarly, we found that stable over-
DISCUSSION

Our findings suggested that GTPγS activates PI 3-kinase through a Rho-dependent mechanism in intact rat adipocytes. GTP-Rho has also been found to activate PI 3-kinase in platelet homogenates (7) but, for uncertain reasons, not in other cell-free systems (8, 9). Our findings with wortmannin and LY294002 also suggested that PI 3-kinase is required for the activation of glucose transport during GTPγS stimulation of rat adipocytes. Thus, GTPγS, as presently used, did not appear to activate glucose transport in rat adipocytes simply by activating small G-proteins such as Rab that are thought to function distal to PI 3-kinase in regulating Glut4 translocation (4).

Whereas GTPγS appeared to activate PI 3-kinase through Rho, insulin effects on PI 3-kinase were largely independent of Rho. Thus, although insulin activates Rho (6), Rho was not a major contributor to insulin-stimulated PI 3-kinase activation, which probably occurs largely through tyrosine phosphorylation of IRS-1 and/or other proteins (16). Along these lines, it is pertinent to note that PI 3-kinase is required for the translocation, but not GTP loading, of Rho during insulin action (6); thus, PI 3-kinase can operate upstream (e.g. during insulin action), as well as downstream (e.g. during GTPγS activation), of Rho. During insulin action, Rho may translocate to specific sites of PI 3-kinase-induced increases in polyphosphoinositides (accordingly, we have found that Rho avidly binds to artificial phosphatidylcholine vesicles containing 5% PI-3, 4-(PO4)2, PI-3,4,5-(PO4)3, or PI-4,5-(PO4)2, and this may coordinate certain actions of PI 3-kinase and Rho. During GTPγS action, GTPγS stimulates the translocation of Rho to plasma and microsomal membranes (6), and this may explain how membrane-activated PI 3-kinase is activated by GTP-Rho.

In addition to requirements for Rho and PI 3-kinase, our findings with RO 31-8220 suggested a requirement for one or more protein kinases in the activation of glucose transport by GTPγS as well as by insulin. In the case of insulin, the required protein kinase(s) appears to operate distally to, or in parallel with, PI 3-kinase, since RO 31-8220 does not inhibit insulin-induced activation of either PI 3-kinase (15) or PI 3-kinase-dependent PKB activation2; presumably, the same situation pertains during GTPγS action, i.e. the RO 31-8220-sensitive protein kinase(s) required for glucose transport is distal or parallel to PI 3-kinase. Although the identity of the protein kinase is uncertain, note that both PKC-ζ and PKN are activated by PI 3-kinase lipid products (i.e. polyphosphoinositides) (17–19), and PKN is directly activated by GTP-Rho (20, 21). Also, as reported for other bisindolemaleimides (see Refs. 22 and 23), we have found (14) that RO 31-8220 inhibits recombinant conventional (α, β, and γ) and novel (δ, ε, and η) PKCs at relatively low concentrations (IC50 values of 15–100 nM) and the atypical PKC, PKC-ζ, at relatively high concentrations (IC50 of 1–4 μM). Presently, we found that RO 31-8220, at relatively low concentrations (IC50 = 30 nM), inhibited immunoprecipitated PKN. Presumably, inhibitory effects of RO 31-8220 on PKC and PKN reflect homology in the catalytic domains of PKN and most PKCs (24).

With respect to PKC-ζ and PKN as RO 31-8220-inhibitable protein kinases that may be required for glucose transport during the actions of GTPγS and insulin, the following are germane. First, PKC-ζ was activated by insulin, but not by GTPγS; thus, PKC-ζ seems unlikely to be involved in the action of GTPγS but may play a role during insulin action. Second, as in other systems in which GTP-Rho directly activates PKN (20, 21), we found that GTPγS activated both Rho and PKN, and insulin activated PKN by a Rho-dependent mechanism; accordingly, PKN is probably activated via Rho during the actions of both GTPγS and insulin in rat adipocytes. On the other hand, our studies suggested that different RO 31-8220-sensitive protein kinases were required for glucose transport effects of GTPγS and insulin in rat adipocytes; thus, insulin required a protein kinase sensitive to higher (IC50 of 4–5 μM) concentrations of RO 31-8220, e.g. PKC-ζ, whereas GTPγS required a protein kinase sensitive to lower (IC50 < 1 μM) concentrations of RO 31-8220, e.g. PKN. Although these findings with RO 31-8220 might suggest that PKN is required for glucose transport effects of GTPγS, but not insulin, note that expression of dominant-negative PKN partially inhibited (a) the effects of insulin as well as GTPγS on the translocation of HA-GLUT4 to the plasma membrane (Fig. 9), and this may be related to the effects of insulin on PI 3-kinase activation (4).

Fig. 9. Effects of stable expression of wild-type and dominant negative forms of Rho and PKN on glucose transport in 3T3/L1 fibroblasts (A) and transient expression of wild-type and dominant negative PKN on translocation of HA-tagged GLUT4 in rat adipocytes (B). A, fibroblasts were stably transfected as in Fig. 7A, except that pcDNA3 containing cDNAs encoding dominant negative (ΔN) forms of Rho and PKN were used, along with wild-type (WT) Rho and PKN. Shown here are data from clones containing easily discernible increases in total immunoreactive Rho or PKN and/or expression of HA epitope-tagged Rho or PKN. Values are mean ± S.E. of N clones, each assayed in triplicate at each insulin concentration (0, 5, and 100 nM). B, adipocytes were transiently transfected as in Fig. 8 with (a) pcIS2 containing cDNA encoding HA-GLUT4 and either (b) pTB701 vector (V) or (c) pTB701 containing cDNA encoding wild-type (WT) or dominant negative (ΔN) PKN. See the legend to Fig. 8 for other details of incubation and assay. Values are the mean ± S.E. of n (shown in parentheses) determinations. Double asterisk, p < 0.05 (t test) versus control, non-insulin-treated, vector-transfected cells (V). Double asterisk, p < 0.05 versus insulin-treated, vector-transfected cells.

expression of PKN enhanced, and dominant negative PKN partially (55%) inhibited, insulin effects on glucose transport in 3T3/L1 fibroblasts (Fig. 9).

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the plasma membrane in rat adipocytes and (b) insulin effects on glucose transport in 3T3/L1 fibroblasts. It is presently uncertain if these seemingly divergent findings reflect shortcomings in our experimental approaches (e.g., effective local concentrations of inhibitors such as RO 31-8220 at specific enzyme sites in situ are uncertain, and transfections of dominant-negative proteins may cause untoward effects).

As discussed, our findings suggested that Rho and PKN contributed to the activation of glucose transport during GTPγS action. However, activating effects of GTP-Rho on PKN would not explain the sensitivity of GTPγS-stimulated 2-DOG uptake to wortmannin and LY294002, unless PI 3-kinase, as well as Rho, needed to be co-activated, perhaps to further activate or correctly localize PKN. Accordingly, (a) PI-4,5-(PO4)2 and PI-3,4,5-(PO4)3 directly activate PKN (also known as PKC-related kinase-1 or 2 (PRK-1 or 2)) (18); and (b) Rho is translocated by PI-4,5-(PO4)2 (Ref. 6) and PI-3,4-(PO4)2 and PI-3,4,5-(PO4)3 (see above), and PI 3-kinase lipid products may correctly localize Rho and, therefore, PKN to specific membrane compartments during the actions of both GTPγS and insulin.

Our observation of activation of membrane-associated PI 3-kinase by GTPγS in rat adipocytes appears to differ from that of a previous report in which GTPγS failed to activate cytosolic PI 3-kinase in 3T3/L1 adipocytes (3); this may reflect differences in cell types or the fact that we measured membrane, rather than cytosolic, PI 3-kinase activity. Along these lines, note that (a) we found that insulin and GTPγS activated membrane, but not cytosolic, PI 3-kinase in rat adipocytes; and (b) our failure to observe increases in cytosolic PI 3-kinase may reflect the large pool of insulin-independent PI 3-kinase that is not activated indistinguishably during our assays of crude rat adipocyte cytosol. Although we did not examine the effects of GTPγS on membrane PI 3-kinase activity in 3T3/L1 adipocytes, we did find that overexpression of Rho activated membrane PI 3-kinase in these cells. Accordingly, it may be surmised that, as in rat adipocytes, GTPγS, by activating Rho, may activate PI 3-kinase in 3T3/L1 adipocytes; this could explain why GTPγS, at least partly (approximately 50%), as per wortmannin studies in Ref. 5) requires PI 3-kinase for the activation of glucose transport in 3T3/L1 adipocytes; on the other hand, glucose transport effects of GTPγS that are independent of PI 3-kinase (also approximately 50%; see Ref. 5) may be explained by direct activating effects of GTPγS on Rab (4) or other G-proteins that act distally to PI 3-kinase.

Finally, it was of interest to find that, in addition to inhibitory effects of C3 transferase and dominant-negative forms of Rho and PKN on GTPγS- and insulin-stimulated glucose transport and/or GLUT4 translocation, transfected Rho (particularly if constitutively activated) and its downstream kinase, PKN, provoked increases in GLUT4 translocation and/or glucose transport in rat adipocytes and 3T3/L1 cells. It therefore may be conjectured that Rho is not only required for, but may actively participate in, the activation of GLUT4 translocation and glucose transport in the actions of insulin, GTPγS, and other agonists.

In summary, like insulin, GTPγS provoked increases in 2-DOG uptake and HA-GLUT4 translocation in rat adipocytes. Also, like insulin, (a) GTPγS provoked increases in membrane-associated PI 3-kinase, and PI 3-kinase appeared to be required for GTPγS-induced activation of glucose transport; and (b) both Rho and an RO 31-8220-sensitive protein kinase appeared to be required for GTPγS-induced activation of glucose transport. In studies of RO 31-8220-sensitive protein kinases, both GTPγS and insulin activated PKN, and PKN appeared to be required for activation of GLUT4 translocation by GTPγS and insulin. Unlike insulin, however, GTPγS appeared to activate PI 3-kinase primarily through Rho, rather than through IRS-1; PKCζ was activated by insulin but not by GTPγS; and effects of GTPγS on glucose transport were inhibited by lower concentrations of RO 31-8220 than were effects of insulin. It may therefore be surmised that, although there are similarities in the signaling factors (i.e., Rho, PKN, and PI 3-kinase) that are used by GTPγS and insulin to activate glucose transport, these agents activate Rho and PI 3-kinase by different mechanisms and appear to use different distal protein kinases to activate glucose transport.

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