Insulin-mimetic signalling of synthetic phosphoinositolglycans in isolated rat adipocytes

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A set of synthetic phosphoinositolglycan (PIG) compounds has been demonstrated to exert insulin-mimetic activity on glucose and lipid metabolism in rat adipocytes differing considerably in potency [compound 41 > 37 > 45 > 7 > 1; W. Frick, A. Bauer, J. Bauer, S. Wied and G. Müller, G. (1998) *Biochemistry* 37, 13421–13436]. In the present study we examine whether these differences are based on the capability of the PIG compounds to stimulate signalling components which are thought to mediate metabolic insulin action. Studies using a tyrosine kinase inhibitor and introduction into adipocytes of anti-phosphotyrosine or inhibitory anti-insulin receptor β -subunit antibodies demonstrated dependence on tyrosine phosphorylation but independence of insulin receptor kinase activation of the insulin-mimetic signalling and metabolic activity of the PIG compounds. The five compounds elicited in rat adipocytes a significant increase in tyrosine phosphorylation of both insulin receptor substrate 1 (IRS-1) and IRS-3 and, to a minor degree, IRS-2, in IRS-1/3-associated phosphatidylinositol 3-kinase (PI 3-K) protein as well as activity, and in protein kinase B (PKB) activity as well as phosphorylation. This was most pronounced for compound 41, approaching 65–95% of the maximal insulin response (MIR) at 20 μ M, and declined in the order of compounds 37, 45, 7 and 1.

The same ranking was true for the maximal inhibition of glycogen synthase kinase 3 activity (GSK-3) (41, 75% of MIR; compound 37, 65%; compound 7, 25%; compound 1, 10%) and GSK-3 autophosphorylation. The half-maximal concentrations effective for signalling (compound 41, 2–5 μ M; compound 37, 10–20 μ M) corresponded well to those stimulating glucose and lipid metabolism. Interestingly, compounds 37 and 41 stimulated mitogen-activated protein kinase (MAPK) and protein synthesis in rat adipocytes to only about 20–30% (at 50 μ M) of MIR. We conclude that in rat adipocytes: (i) the potency of PIG compounds to regulate glucose/lipid metabolism depends on the activation of PI 3-K and PKB and inhibition of GSK-3; (ii) initiation of tyrosine phosphorylation of IRS-1/3 is sufficient and activation of the PI 3-K cascade is required for insulin-mimetic metabolic signalling; and (iii) PIG compounds are quite selective for the PI 3-K compared to the MAPK cascade, (iv) PIG compounds seem to use the same signalling components downstream of PI 3-K (including Rab4) for stimulation of glucose transport as does insulin. Thus the early signalling step(s) used by PIG, but not by insulin, may represent a target for the treatment of insulin-resistant states.

INTRODUCTION

Many of the proteins involved in insulin action have been identified at the molecular level (for reviews see [1–4]). The insulin receptor is a transmembrane tyrosine kinase, which when activated by insulin binding, undergoes rapid autophosphorylation and phosphorylates a number of intracellular substrates, among them one or more 50–60 kDa proteins, including the Shc, a 15 kDa fatty acid binding protein and several so-called insulin receptor substrate proteins, IRS-1/2/3. After tyrosine phosphorylation, the IRS polypeptides act as docking proteins for several Src homology 2 domain-containing adaptor molecules and enzymes, including phosphatidylinositol 3-kinase (PI 3-K), Grb2, SHP2, Nck, and Fyn. The interaction between the IRS proteins and PI 3-K occurs through the p85 regulatory subunit of the enzyme and results in an increase in catalytic activity of the p110 subunit. PI 3-K is essential for many insulin-sensitive metabolic processes, including stimulation of glucose transport and glycogen synthesis. In all cases in which there is stimulation of tyrosine phosphorylation of IRS proteins, there is concomitant docking of these proteins to the p85 subunit of PI 3-K and, with

the exception of the cross-talk between the insulin and angiotensin signalling systems [5], this docking was associated with stimulation of the PI 3-K activity.

It is generally accepted that one branch of acute metabolic insulin signalling is initiated by activation of PI 3-K (for reviews see [1–4,6]). This may be necessary for insulin stimulation of glycogen synthase (GS) [7–9] and glucose transport, and may even be sufficient for the latter ([10–12]; for a review see [1]). Furthermore, there is experimental evidence for protein kinase B (PKB) being an element of the signalling cascade leading from PI 3-K to activation of GS and glucose transport in isolated rat adipocytes, various cell culture models and human skeletal muscle [13–15]. The experimental data available favour a model for a cascade which assumes the following sequence of events (for a review see [16]): PI 3-K activation results in the production of PtdIns(3,4,5) P_3 and PtdIns(3,4) P_2 . The binding of the pleckstrin-homology domain of PKB to these phosphoinositides both recruits PKB to the plasma membrane and directly and/or indirectly (via phosphorylation by the membrane-associated protein kinases, PDK1/2) stimulates its kinase activity. Activated PKB phosphorylates substrate proteins [e.g. glycogen synthase

Abbreviations used: DTT, dithiothreitol; GS, glycogen synthase; GSK-3, glycogen synthase kinase 3; IRS-1/2/3, insulin receptor substrate 1/2/3; KRP, Krebs–Ringer phosphate buffer; MAPK, mitogen-activated protein kinase; MBP(K), myelin basic protein (kinase); MIR, percentage of maximal insulin response; P-GS1, GS peptide with phosphorylated priming site; PIG(-P), phosphoinositolglycan(-peptide); PI 3-K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PKB, protein kinase B; PP2AI, protein phosphatase 2A inhibitor protein; TX-100, Triton X-100.

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kinase 3 (GSK-3), which is thereby inhibited, leading to dephosphorylation and activation of GS], resulting in a variety of biological effects, possibly including stimulation of glycogen synthesis and GLUT4 translocation.

GSK-3 was discovered and named for its involvement in the control of glycogen metabolism (for reviews see [17,18]) and has been implicated in phosphorylating, and thereby modulating, the regulatory subunit of cAMP-dependent protein kinase, the transcription factors, *c-jun*, *c-myc*, *c-myb*, the microtubule-associated tau proteins, the largest subunit of eIF-2B and the regulatory subunits of the cytosolic and glycogen-associated forms of the type I inhibitor protein for the protein Ser/Thr phosphatase PP2A (PP2AI). GSK-3 also phosphorylates metabolic enzymes such as ATP citrate lyase and GS. However, the importance of GSK-3 in insulin activation of GS remains unclear since: (i) inactivation of GSK-3 by isoproterenol in rat adipocytes equalled that of insulin, and yet had no effect on GS activity [19]; (ii) overexpression of active PI 3-K had no effect on basal or insulin-stimulated GS activity [7]; and (iii) the phosphorylation state of GS is (in addition) regulated by the insulin-stimulated protein phosphatase 1 (for a review see [20]). In any case the insulin-induced inactivation of GSK-3 in adipocytes and Chinese hamster ovary cells has been described [21]. Recent experiments point to a possible role for phosphorylation of Ser-9 located near the N-terminus in inactivation of GSK-3, presumably catalysed by PKB [13,19]. Thus GSK-3 is clearly a target for insulin signalling, possibly primarily involved in the insulin regulation of protein synthesis and cytoskeletal reorganization, rather than of glycogen synthesis.

In addition to the identification of the signal-transduction pathways leading directly from the insulin receptor to downstream targets, several cross-talks have been delineated between signalling transmission by insulin and other hormones/growth factors or diverse exogenous stimuli, which either mimic (to a certain degree) or modulate in a positive or negative fashion metabolic and/or mitogenic insulin action in various cellular systems (see also the Discussion). Since none of these ligands activates the insulin receptor kinase directly, their signalling pathways may converge with that of insulin at a more distal signalling step. This property seems to be shared by phosphoinositideglycan-peptide (PIG-P) molecules prepared from the glycosylphosphatidylinositol anchor of yeast Gce1p which mimic metabolic insulin action to a significant degree without concomitant induction of insulin receptor kinase activity ([22,23]; see also for a discussion of the rationale for the use of PIG-P molecules). For elucidation of the site of interference of PIG structures and insulin signalling, we investigated in the present study the effects of five synthetic PIG compounds, which have recently been demonstrated to exert markedly different insulin-mimetic metabolic activity [24], on key elements of the insulin signalling pathway in isolated rat adipocytes. We found that structurally different PIG compounds affect the activity of PI 3-K and its downstream elements PKB and GSK-3 to a significant extent, with a relative potency which is correlated to their effects on glucose and lipid metabolism.

MATERIALS AND METHODS

Materials

PD98059 was bought from BioMol (Hamburg, Germany) and LY249002, wortmannin, genistein and rottlerin were supplied by Calbiochem (Bad Soden, Germany). The Rab4A-(191-219) peptide and Rab3C-(196-215) peptide, derived from the C-terminal domains of rat Rab4A and bovine Rab3C respectively were

synthesized and purified by HPLC to 85–90% homogeneity. Polyclonal antibodies against rat Rab4A were raised by immunizing a guinea pig with peptide QLRSPRRTQAPSAQE conjugated to keyhole limpet haemocyanin. In immunoblots using total homogenate of rat adipocytes, the anti-Rab4A antibodies recognized a single band with an apparent molecular mass of 25 kDa compatible with the size of Rab4A. Mouse monoclonal antibodies raised against the regulatory region of the human insulin receptor from human placenta, including Tyr-1146/1150/1151 (anti-IRa, 25B3; anti-IRb, 4B8), were bought from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Immunoprecipitation of metabolically labelled lysates from rat hepatoma cells and adipocytes with the two antibodies confirmed that they recognize the β -subunit of the rat receptor and, furthermore, demonstrated that these antibodies did not cross-react with other cellular proteins. Recombinant human insulin and PIG compounds 41, 37, 45, 7 and 1 were provided by the chemical synthesis and research departments of Hoechst Marion Roussel Deutschland GmbH (Frankfurt, Germany) as described elsewhere [24]. The sources of most of the other materials and radiochemicals used have been described previously [22,23,25,26] or are indicated for special reagents (e.g. antibodies) in the corresponding Materials and methods sections. All other materials were from Merck (Darmstadt, Germany) and of the highest purity available.

Preparation of rat adipocytes and incubation with PIG compounds, insulin and inhibitors

Rat adipocytes were isolated by collagenase digestion of epididymal fat pads from male Wistar rats (140–160 g) as described previously [25,26] and incubated at 0.7×10^5 cells/ml with PIG compounds (dissolved in doubly distilled water and stored at -70°C) or human insulin (dissolved as described [26]) in Krebs–Ringer phosphate (KRP)–Hepes buffer (130 mM NaCl/2 mM CaCl_2 /1.4 mM MgSO_4 /5.5 mM KCl/10 mM KH_2PO_4 /25 mM Hepes, pH 7.4) containing 5 mM glucose, 1% (w/v) BSA for 20 min at 37°C under mild shaking in an atmosphere of 95% O_2 /5% CO_2 , as described [23]. Wortmannin, LY249002, PD98059, genistein and rottlerin were dissolved in DMSO (final conc. during incubation 0.25%) and stored at -20°C . For adipocytes treated with these inhibitors, the appropriate control incubations contained 0.25% DMSO.

Tyrosine phosphorylation of the insulin receptor and IRS-1/2/3

Adipocytes (2.8×10^5) incubated with insulin or PIG compound (see above) were separated from the medium by flotation and lysed in 1 ml of chilled buffer containing 20 mM Tris/HCl (pH 7.4)/150 mM NaCl/10% (v/v) glycerol/2 mM EDTA/1 mM Na_3VO_4 /50 mM $\text{Na}_2\text{P}_2\text{O}_7$ /10 mM NaF/10 mM glycerol-3-phosphate/0.2 mM PMSF/20 $\mu\text{g/ml}$ leupeptin/10 $\mu\text{g/ml}$ pepstatin/25 $\mu\text{g/ml}$ aprotinin by 10 strokes in a loose-fitting Teflon-in-glass homogenizer on ice. The fat-free homogenate, prepared by centrifugation (1500 g, 3 min, 15°C), was supplemented with Triton X-100 (TX-100) (1% final conc.) and sodium deoxycholate (0.5% final conc.), incubated for 30 min at 4°C and finally centrifuged (13000 g, 10 min). The soluble fraction was pre-cleared by addition of 100 μl of Protein A/G–Sepharose (50 mg/ml of the same buffer; Pharmacia/LKB, Freiburg, Germany) and centrifugation as above. The supernatant was incubated with anti-human insulin receptor β -subunit (from guinea pig; Transduction Laboratories, Lexington, KY, U.S.A., 1:200), anti-rat C-terminal IRS-1, anti-rat IRS-2 (from rabbit; Upstate Biotechnology, Lake Placid, NY, U.S.A., 1:200)

or anti-phosphotyrosine (from rabbit; Biomol, 1:400) or non-immune rabbit IgG (Sigma, Deisenhofen, Germany, 1:100) for 4 h at 4 °C and then with Protein A/G-Sepharose (5 mg) overnight at 4 °C. The precipitates, collected by centrifugation, were washed three times with 1 ml each of lysis buffer containing 1 % TX-100 and lacking glycerol, and finally twice with buffer lacking TX-100 and NaCl. The anti-insulin receptor and IgG, as well as portions of the anti-IRS-1/2 and anti-phosphotyrosine immunoprecipitates, were subjected to SDS/PAGE and immunoblotted using mouse monoclonal anti-phosphotyrosine primary antibodies (1:2000 dilution; Biomol; clone 3B12) or anti-rat PI 3-K antibodies raised against a peptide corresponding to residues 500–519 of p85 protein (from rabbit; 1:500, Upstate Biotechnology) and with horseradish peroxidase-coupled secondary antibodies (Sigma), followed by chemiluminescence detection with the enhanced chemiluminescence (ECL) kit (performed according to the instructions of Amersham-Buchler (Braunschweig, Germany), or, alternatively, ¹²⁵I-labelled Protein A (Amersham-Buchler) as indicated in the Figure legends. Quantitative evaluation was performed by phosphorimaging (PhosphorImager; Molecular Dynamics, Storm 840 and ImageQuant Software, Krefeld, Germany).

For immunodepletion of IRS-1/2 from the anti-phosphotyrosine immunoprecipitates, the pelleted Protein A/G-Sepharose immunobeads were suspended in Laemmli sample buffer, heated (95 °C, 5 min) and centrifuged (13000 g, 2 min). The supernatant (25 µl) was supplemented with 1 ml of lysis buffer and then with 5 µl each of anti-IRS-1/2 antibodies. After incubation (4 h, 4 °C), 50 µl of Protein A/G-Sepharose (50 mg/ml) was added and the incubation was continued (overnight, 4 °C). The supernatant obtained by centrifugation (13000 g, 5 min, 4 °C) was precipitated by the addition of 50 µl of 100 % trichloroacetic acid, incubation (1 h on ice) and subsequent centrifugation (13000 g, 2 min). The pellet was washed twice with acetone, dried and then dissolved in Laemmli sample buffer for immunoblotting with anti-PI 3-K antibodies.

PI 3-K activity

Portions of the anti-IRS-1/2 immunoprecipitates (see above) were assayed for PI 3-K activity by incubating in 100 µl of 20 mM Tris/HCl (pH 7.0)/50 µM [γ -³²P]ATP (5 µCi)/10 mM MgCl₂/2 mM MnCl₂/100 mM NaCl/2 mM EDTA/0.5 µM wortmannin (for control incubations only) containing 10 µg of phosphatidylinositol and 1 µg of phosphatidylserine for 15 min at 22 °C. After addition of 20 µl of 8 M HCl and 160 µl of a 1:1 mixture of methanol/chloroform, the extracted phospholipids were resolved by TLC on plates (Silica Gel 60; Merck) coated with 1 % oxalate and developed in chloroform/methanol/water/ammonia (60:47:11.3:3.2, by vol.). Radiolabelled phosphatidylinositol 3-phosphate (PI3P) (average *R_f* value of 0.41 under these conditions) was visualized by autoradiography and quantified using phosphorimaging (PhosphorImager; Molecular Dynamics). For calculation of wortmannin-sensitive PI 3-K, all values were corrected for PI3P radiolabelled in the presence of wortmannin.

PKB activity

Adipocytes (5×10^5) incubated with insulin or PIG compound (see above) were washed with KRP-Hepes by flotation and then extracted in 1 ml of 50 mM Hepes/KOH, pH 7.6/0.2 mM EDTA/2.2 mM EGTA/2 mM dithiothreitol (DTT)/100 mM KCl/100 mM NaF/10 mM Na₂P₂O₇/2 mM Na₃VO₄/15 % (v/v)

glycerol/1 % Nonidet P-40/0.5 % deoxycholate (v/v)/1 µM microcystin/10 µg/ml pepstatin/25 µg/ml leupeptin/1 µg/ml antipain. Cell extract (900 µl) (prepared as described under tyrosine phosphorylation) was incubated with 10 µl of PKB antiserum (raised in rabbits by immunization with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence) precoupled to 10 mg of Protein A-Sepharose in 100 µl of extraction buffer for 2 h at 4 °C. The immunoprecipitates were collected by centrifugation (12000 g, 2 min, 4 °C), washed twice with 1 ml each of PKB assay buffer [20 mM Mops, pH 7.0/1 mM EDTA/1 mM EGTA/10 mM Na₂P₂O₇/50 mM NaF/1 mM Na₃VO₄/10 µg/ml aprotinin/0.1 mM PMSF/0.01 % Brij35/5 % (v/v) glycerol] containing 1 % Nonidet P-40/0.5 M NaCl and three times with 1 ml each of assay buffer lacking salt and detergent. The immunoprecipitates were finally resuspended in 45 µl of PKB assay buffer containing 10 mM MgCl₂/1 mM DTT/2.5 µM cAMP-dependent protein kinase inhibitor peptide (IP₂₀) and either the synthetic peptide, Crosstide (100 µM final conc.), which is based on the sequence surrounding the serine phosphorylation site of GSK-3 (Ser-21 of GSK-3 α and Ser-9 of GSK-3 β) [27] or alternatively, histone 2B (150 µg/ml). The assay was initiated by the addition of 5 µl of [γ -³²P]ATP (500 µM, 4 µCi) and terminated after incubation for 15 min at 30 °C by placing the test tubes on ice. For determination of Crosstide phosphorylation, the 10 µl portions of the samples were adsorbed on p81 phosphocellulose paper and washed extensively, as described below for the myelin basic protein kinase (MBPK) assay. The radioactivity associated with the paper was counted by liquid-scintillation counting. For determination of histone 2B phosphorylation, the samples were separated by 12 % SDS/PAGE. The autoradiogram was quantified by densitometry. Exposure times were chosen which guaranteed that the intensity of the bands was linearly related to the quantity of phosphoprotein contained in the bands.

PKB phosphorylation

For demonstration of phosphorylation by a size shift in SDS/PAGE analysis or immunoblotting, a fat-free homogenate was prepared from adipocytes treated with compound or insulin (see above), supplemented with TX-100 (1 % final conc.) and sodium deoxycholate (0.5 % final conc.), incubated for 30 min at 4 °C and finally centrifuged (13000 g, 10 min). The supernatant (100 µg of protein) was immunoprecipitated with 5 µg of rabbit anti-PKB antibodies (see above) coupled to Protein A-Sepharose beads. Immune pellets were washed three times with 1 ml each of immunoprecipitation buffer (20 mM Tris/HCl, pH 7.4/5 mM EDTA/100 mM NaF/10 mM Na₂P₂O₇/2 mM Na₃VO₄/1 % TX-100) and three times with 1 ml each of 50 mM Tris/HCl, pH 7.4/10 mM MgCl₂/1 mM DTT. The final immune pellets were suspended in Laemmli sample buffer, heated (95 °C, 2 min) and centrifuged (12000 g, 2 min), and proteins contained in the supernatant were separated by SDS/PAGE. For demonstration of phosphorylation by gel mobility-shift analysis, PKB was visualized by immunoblotting using polyclonal anti-rat PKB antibodies from sheep directed against the N-terminal part of the protein (Santa Cruz Biotechnology). For demonstration of phosphorylation by immunoblotting, phosphorylated PKB was detected using monoclonal antibodies raised against a phosphopeptide corresponding to residues 465–480 of human PKB α with Ser-473 in the phosphorylated state. (The antibodies were purified by batch adsorption to the corresponding non-phosphorylated peptide coupled to Sepharose beads. In control experiments using extracts from insulin-treated adipocytes, the antibodies not bound to these beads specifically recognized the

phosphorylated version of PKB, exclusively, as identified by its shift in gel mobility of the immunoblotted PKB or ^{32}P -labelling of the immunoprecipitated PKB.) For both analyses of PKB phosphorylation, the immunoblots were treated with ^{125}I -labelled Protein A and then subjected to autoradiography.

For demonstration of phosphorylation by $^{32}\text{P}_i$ incorporation, isolated rat adipocytes were incubated with [^{32}P]phosphate and then with PIG compounds, as described below for the evaluation of GS phosphorylation. A fat-free homogenate was prepared (see above) and centrifuged (15000 *g*, 60 min, 4 °C). Portions (1 ml) of the supernatant were immunoprecipitated with 10 μg of anti-PKB antibodies coupled to Protein A–Sepharose beads. The immunoprecipitates were washed and finally suspended in 50 μl of Laemmli sample buffer (2 % SDS), heated (95 °C, 2 min) and centrifuged (12000 *g*, 2 min). One half of each supernatant was separated by SDS/PAGE and radiolabelled PKB was visualized by phosphorimaging. The other half was diluted with 1 ml of immunoprecipitation buffer containing 10 $\mu\text{g}/\text{ml}$ aprotinin/20 $\mu\text{g}/\text{ml}$ pepstatin/10 $\mu\text{g}/\text{ml}$ leupeptin/1 mM PMSF and centrifuged again (12000 *g*, 2 min). The supernatant was supplemented with 20 μg of a kit containing four different monoclonal anti-phosphoserine antibodies (Biomol; clones 1C8, 4A3, 4A9, 4H4) and the monoclonal anti-phosphothreonine antibodies (clone 1E11). After incubation (4 h, 4 °C), the antibody complexes were precipitated during a 2 h incubation with 50 μl of Protein A–Sepharose [50 mg/ml of 50 mM Hepes/KOH (pH 7.4)/0.1 % TX-100]. The collected immunoprecipitates (12000 *g*, 2 min) were washed three times with 1 ml each of 50 mM Hepes/KOH (pH 7.4)/150 mM NaCl/100 mM NaF/0.2 mM Na_3VO_4 /0.1 % SDS/1 % TX-100 and finally twice with buffer lacking TX-100. Phosphoproteins were eluted from the washed precipitates by incubation with 500 μl of 50 mM Hepes/KOH (pH 7.4) containing 50 mM *p*-nitrophenylphosphate for 30 min at 4 °C. The supernatants obtained by centrifugation were precipitated with trichloroacetic acid. The acetone-washed pellets were suspended in 50 μl of Laemmli sample buffer and analysed by SDS/PAGE and phosphorimaging of the gel.

GSK-3 activity and autophosphorylation

Briefly, adipocytes (5×10^5) incubated with insulin or PIG compound (see above) were washed with KRP–Hepes by flotation and homogenized in 2 ml of 10 mM Tris/HCl, pH 7.4/50 mM NaCl/30 mM $\text{Na}_2\text{P}_2\text{O}_7$ /10 mM glycerol-3-phosphate/50 mM NaF/0.1 μM microcystin/200 μM Na_3VO_4 /0.5 $\mu\text{g}/\text{ml}$ leupeptin/2 $\mu\text{g}/\text{ml}$ aprotinin/1 $\mu\text{g}/\text{ml}$ pepstatin/1 mM PMSF by 10 strokes in a Teflon-in-glass homogenizer. A clear and fat-free cytosol was prepared by three sequential centrifugations at 1000 *g* for 2 min and twice at 20000 *g* for 15 min at 4 °C. Cytosol (100 μl) was incubated with 5 μl of GSK-3 α antiserum (raised in sheep by immunization with a synthetic peptide corresponding to amino acids 471–483 of rat GSK-3 α coupled to BSA) for 2 h at 4 °C. The immunocomplexes were precipitated with 2 mg of anti-sheep IgG coupled to agarose in 50 μl of homogenization buffer (under rotation for 2 h at 4 °C) after which the beads were collected by centrifugation (12000 *g*, 2 min), washed once with 1 ml of 100 mM Tris/HCl (pH 7.4) containing 0.2 % Nonidet P-40/0.5 M NaCl/0.5 M LiCl, once with 10 mM Tris/HCl and twice with GSK-3 assay buffer (20 mM Hepes/KOH, pH 7.4/10 mM MgCl_2 /1 mM DTT/10 mM NaF/10 mM $\text{Na}_2\text{P}_2\text{O}_7$). The kinase reaction was started by the addition of 50 μl of assay buffer containing 125 μM [γ - ^{32}P]ATP (15 μCi), 300 $\mu\text{g}/\text{ml}$ P-GS1 peptide (GS peptide with phosphorylated priming site), 10 mM MgCl_2 , 1 mM DTT and 10 $\mu\text{g}/\text{ml}$ heparin to the washed beads. After incubation for 15 min at 30 °C, 10 μl

aliquots of the supernatant (obtained by centrifugation as above) were spotted on p81 phosphocellulose papers (1 $\times$ 1 cm), which were then washed four times in 75 mM orthophosphoric acid. The radioactivity associated with the papers was measured by liquid-scintillation counting. In some experiments recombinant PP2AI (1 μg ; Calbiochem) was used as substrate instead of P-GS1, or autophosphorylation of GSK-3 was assayed in the absence of exogenous substrate. In these cases the kinase reaction (conditions as above) was terminated by rapid centrifugation (12000 *g*, 1 min, 4 °C) and separation of the supernatant from the immune pellet. The supernatant was precipitated in 10 % final conc. trichloroacetic acid for 1 h on ice and centrifuged at 15000 *g* for 15 min at 4 °C (for phosphorylated PP2AI). The immune pellet was washed twice with 1 ml of 100 mM Tris/HCl, pH 7.5/1 % TX-100/150 mM NaCl (for autophosphorylated GSK-3). The pellets were suspended in 50 μl of 2 $\times$ Laemmli sample buffer, heated (95 °C, 5 min) and centrifuged (12000 *g*, 2 min). The supernatants were run on SDS/PAGE (12 % resolving gel) and phosphorylated PP2AI and GSK-3 were visualized by autoradiography and quantified by phosphorimaging (PhosphorImager; Molecular Dynamics). For phosphoamino acid analysis of autophosphorylated GSK-3, the proteins resolved by SDS/PAGE were transferred to PVDF membranes. Pieces of the membrane containing ^{32}P -labelled GSK-3 (as identified by autoradiography) were excised and hydrolysed in 5.7 M HCl at 110 °C for 90 min. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis and detected by autoradiography in comparison with stained phosphoserine, phosphothreonine and phosphotyrosine markers run in parallel, as described previously [28]. For quantification, the material containing the ^{32}P -labelled amino acids was scraped off the plate and the Cerenkov radiation was counted.

GS phosphorylation

GS phosphorylation was carried out as described [29], but with the following modifications. Adipocytes were washed twice by flotation in low phosphate medium composed of KRP–Hepes modified to contain 50 μM KH_2PO_4 /2 mM glucose/2 % (v/v) BSA and then suspended in the same medium (7.5×10^5 cells/ml). Portions (5 ml) were incubated with [^{32}P]phosphate (0.2 mCi/ml) for 2 h before the addition of insulin or PIG compound and of 0.5 ml of 50 mM glucose. After incubation for 20 min at 37 °C, the cells were floated by centrifugation (1000 *g*, 1 min) and the infranatant was aspirated. The cells were suspended in 1.5 ml of cold 50 mM Tris/HCl, pH 7.6/100 mM KF/20 mM glycerol-3-phosphate/10 mM $\text{K}_4\text{P}_2\text{O}_7$ /10 mM EDTA/1 mM benzamidine/0.2 mM PMSF and homogenized by 10 strokes using a tight-fitting Teflon homogenizer in the same Eppendorf cup. The homogenate was centrifuged (18000 *g*, 30 min, 4 °C). A 1 ml volume of the post-mitochondrial supernatant was incubated with 10 μl of anti-GS antiserum raised in guinea pigs by immunization with purified GS from rabbit skeletal muscle. After incubation for 2 h at 4 °C, 50 μl of Protein A–Sepharose (10 % w/v in TES, see below) was added and the incubation continued overnight. The immunoprecipitates were collected by centrifugation (12000 *g*, 2 min, 4 °C) and washed twice with 1 ml each of TES (50 mM Tris/HCl, pH 7.4/1 mM EDTA/150 mM NaCl) containing 1 % TX-100, twice with TES and finally once with 50 mM Tris/HCl (pH 7.4). The beads were suspended in 25 μl of 2 $\times$ Laemmli sample buffer, heated (95 °C, 5 min) and centrifuged. The supernatant was subjected to SDS/PAGE (8 % resolving gel). The amount of ^{32}P contained in GS was determined by excising the corresponding gel piece and measuring its radioactivity by liquid-scintillation counting.

MBPK activity

Adipocytes (2.5×10^5) incubated with PIG compound or insulin (see above) were quickly washed with ice-cold KRP-Hepes by flotation and then homogenized in 200 μ l of 20 mM Hepes/KOH (pH 7.2)/20 mM EGTA/15 mM MgCl_2 /1 mM DTT/40 mM *p*-nitrophenylphosphate/50 mM glycerol-3-phosphate/5 mM $\text{Na}_4\text{P}_2\text{O}_7$ /20 mM NaF/0.2 mM PMSF/25 μ g/ml leupeptin/25 μ g/ml aprotinin by 10 strokes of a rotating tight-fitting Teflon pestle in the same Eppendorf cup at 4 °C. The homogenate was centrifuged (2000 *g*, 5 min, 4 °C). The infranatant below the fat cake was removed and centrifuged (30000 *g*, 5 min, 4 °C). After careful removal of the residual fat layer, the supernatant was used for measurement of MBPK by combining 5 μ l portions (about 10 μ g of protein) with 45 μ l of kinase buffer (50 mM glycerol-3-phosphate, pH 7.3/5 mM $\text{Na}_4\text{P}_2\text{O}_7$ /10 mM NaF/0.5 mM EDTA/15 mM MgCl_2 /2 mM DTT/4.4 mM protein kinase inhibitor peptide) containing 0.5 mg/ml MBP. The kinase reaction was initiated by the addition of 5 μ l of 1 mM [γ - ^{32}P]ATP (700 mCi/mmol). After a 10 min incubation at 30 °C, reactions were terminated by spotting 10 μ l of the reaction mixture onto 1 cm² sheets of p81 phosphocellulose paper (Whatman), which were immediately immersed in 0.85 % orthophosphoric acid under stirring and washed once in 95 % ethanol (10 ml per sheet) for 5 min. The papers were dried and ^{32}P was quantified by liquid-scintillation counting.

Mitogen-activated protein kinase (MAPK) activity

Adipocytes (5×10^5) incubated with insulin or PIG compound (see above) were washed with KRP-Hepes by flotation and then lysed in 1 ml of 50 mM Hepes/KOH, pH 7.2/100 mM NaCl/2 mM EDTA/1 % Nonidet P-40/0.5 mM Na_3VO_4 /40 mM *p*-nitrophenylphosphate/10 mM glycerol-3-phosphate/10 mM NaF/0.2 mM PMSF/25 μ g/ml leupeptin/25 μ g/ml aprotinin by incubation for 30 min on ice and vigorous vortexing three times for 10 s each at 10 min intervals. Lysates were cleared by centrifugation (12000 *g*, 10 min, 4 °C). The infranatant below the fat cake was removed using a syringe, re-centrifuged and used for measurement of MAPK by addition of 250 μ l portions to 250 μ l of Protein A-Sepharose (50 mg/ml of 50 mM Hepes/KOH, pH 7.2/100 mM NaCl), which had been pre-coupled with 10 μ l of rabbit anti-p42MAPK antiserum (raised against rat p42MAPK) and 5 μ l of rabbit anti-p44MAPK antiserum (raised against the C-terminal 14 amino acids of rat p44MAPK), and incubated for 2 h at 4 °C. The beads were collected by centrifugation (12000 *g*, 2 min, 4 °C), washed three times with 1 ml each of lysis buffer and three times with 1 ml each of 10 mM Hepes/KOH, pH 7.4/10 mM MgCl_2 and then resuspended in 20 μ l of the same buffer containing 2 mg of MBP per ml. The kinase assay was initiated by the addition of 20 μ l of 40 mM Hepes/KOH, pH 7.4/200 μ M [γ - ^{32}P]ATP (25 mCi/mmol)/40 mM MgCl_2 /20 mM NaF/0.2 mM Na_3VO_4 . After incubation for 30 min at 30 °C, the reaction was terminated by the addition of 20 μ l of 4 $\times$ Laemmli sample buffer. Samples were subjected to SDS/PAGE using a 15 % resolving gel. The gel was dried and the radiolabelled MBP was quantified by phosphorimaging (PhosphorImager; Molecular Dynamics).

Transfer of peptides or antibodies into rat adipocytes by electroporation

Rat adipocytes were washed and resuspended in high K^+ /low Ca^{2+} buffer (118 mM KCl/4.8 mM NaCl/0.4 mM CaCl_2 /1 mM EGTA/1.2 mM MgSO_4 /1.2 mM KH_2PO_4 /25 mM Hepes/KOH, pH 7.4/20 mg/ml BSA/3 mM sodium pyruvate

and then incubated for 30 min at 37 °C according to [30]. Subsequently, 0.5 ml aliquots (5×10^5 cells) of the cell suspension were mixed with 250 μ l of peptide or antibody solution to obtain the final peptide/antibody concentration as indicated in the Figure legends, and the electroporation was carried out three times in a Gene-Pulser (Bio-Rad, Munich, Germany) set at 25 μ F and 2.5 kV/cm.

Calculation of insulin-mimetic activity

Data from signalling assays were given as primary data (dpm or arbitrary units). Concentration–response curves were fitted to the equation $y = a + b[x/(x+k)]$ using a Marquardt–Levenberg non-linear least-squares algorithm. When plotted on linear–log axes, this equation gives a sigmoidal curve where the parameters are associated with the following properties: a = basal response; $a+b$ = maximal response; k = half-maximal concentration (EC_{50}); x = concentration of insulin. For all signalling assays at least four different adipocyte preparations with 2–5 independent incubations with insulin/PIG compounds for each preparation were performed. Adipocytes from each incubation were assayed at least in duplicate. Each point represents the mean value for the different cell preparations with $\pm$ S.D. indicated as bars.

Miscellaneous

Assays for insulin-mimetic metabolic activity (glucose transport and lipogenesis) using isolated rat adipocytes, SDS/PAGE, transfer of proteins onto PVDF membrane by semi-dry-blotting and trichloroacetic acid precipitation were performed according to [23,25,26].

RESULTS

Metabolic activity of the PIG compounds is not based on insulin receptor kinase stimulation

For identification of the putative site of convergence of the signalling pathways to the terminal effector systems for metabolic insulin actions (see Introduction) used by synthetic PIG compounds and insulin, we studied whether a correlation exists between the metabolic activity of the PIG compounds and their putative ability to induce tyrosine phosphorylation of the insulin receptor β -subunit or IRS-1/2/3 and regulate PI 3-K, PKB and GSK-3 activities in insulin-like fashion. The synthetic PIG compounds 41, 37, 45, 7 and 1 represent five different classes of potency. Compound 41, HO-SO₂-O-6Man α 1(Man α 1-2)-2Man α 1(6-HSO₃)-6Man α 1-4GluN β 1-6(D)inositol-1,2-(cyclic)-phosphate, compound 37, HO-PO(H)O-6Man α 1(Man α 1-2)-2Man α 1-6Man α 1-4GluN β 1-6(D)inositol-1,2-(cyclic)-phosphate, and compound 45, Man α 1-2Man α 1-2Man α 1(6-HSO₃)-6Man α 1-3GluN β 1-3(D/L)inositol-6-sulphate stimulated lipogenesis up to 90 % (at 20 μ M) of MIR but with differing EC_{50} values (2.5 ± 1 versus 5 ± 2 versus 11.5 μ M) as well as increased GS ($\text{EC}_{50} = 5$ versus 10 versus 18 μ M) and GPAT ($\text{EC}_{50} = 8$ versus 15 versus 35 μ M) activities, whereas compound 7, HO-PO(H)O-6Man α 1-4GluN α 1-6(L)inositol-1,2-(cyclic)-phosphate, was clearly less potent ($\text{EC}_{50} = 100 \mu$ M) and compound 1, HO-PO(H)O-6Man α 1-4GluN α 1-6(L)inositol was almost inactive. The same relative ranking between these five PIG compounds was also observed for other key pathways and enzymes of glucose and lipid metabolism in isolated rat adipocytes [24]. Immunoprecipitation of the insulin receptor with anti-insulin receptor antibodies from total homogenate of treated adipocytes, and subsequent immunoblotting with anti-phosphotyrosine antibodies (Figure 1), failed to detect a significant increase in the amount of tyrosine-phosphorylated insulin receptor β -subunit

Figure 1 Effects of compound 41 on tyrosine phosphorylation of the insulin receptor

Isolated rat adipocytes were incubated in the absence (lanes 1 and 5) or presence of increasing concentrations of human insulin (lanes 2–4) or compound 41 (lanes 6–8). The insulin receptor was immunoprecipitated from total fat-free homogenate with anti-insulin receptor antibodies (see the Materials and methods section) and then immunoblotted using anti-phosphotyrosine antibodies and ^{125}I -labelled Protein A. An autoradiogram of a typical experiment that was repeated four times with similar results is shown. Probing of parallel blots with anti-insulin receptor antibodies demonstrated (i) the identity of the phosphotyrosine-containing band with the insulin receptor β -subunit (IR β), and (ii) the comparable amounts of insulin receptor immunoprecipitated from the different samples.

Figure 2 Effects of compound 41 on tyrosine phosphorylation of IRS-1/2 and its association with the p85 subunit of PI 3-K in adipocytes

Isolated rat adipocytes were incubated in the absence (lanes 1, 5, 9, 13, 17, 21, 25 and 29) or presence of increasing concentrations of human insulin (lanes 2–4, 10–12, 18–20, 26–28) or compound 41 (lanes 6–8, 14–16, 22–24, 30–32). IRS-1 (lanes 1–16) or IRS-2 (lanes 17–32) was immunoprecipitated from total de-fatted homogenate using anti-IRS-1 or anti-IRS-2 antibodies respectively, and then immunoblotted with either anti-phosphotyrosine (lanes 1–8, 17–24) or anti-PI 3-K antibodies (lanes 9–16, 25–32) followed by treatment with ^{125}I -labelled Protein A. An autoradiogram of a typical experiment repeated three times with similar results is shown. Probing of parallel blots with anti-IRS-1/2 antibodies demonstrated (i) the identity of the phosphotyrosine-containing bands with IRS-1/2, and (ii) the comparable amounts of IRS-1/2 immunoprecipitated from the different samples. In control incubations, non-immune IgG did not immunoprecipitate tyrosine-phosphorylated or PI 3-K-associated IRS-1/2.

Figure 3 Effects of compounds 41, 37, 7 and 1 on various components of the metabolic signalling pathway of insulin in adipocytes

Isolated rat adipocytes were incubated with increasing concentrations of human insulin (▲) or compounds 41 (○), 37 (●), 7 (■) and 1 (□) and then assayed for tyrosine phosphorylation of IRS-1 (A), PI 3-K activity (B) and PKB activity (C). Tyrosine phosphorylation of IRS-1 and PI 3-K/PKB activities in basal cells were set at 1000 arbitrary units (arb. u.) and 100% respectively. The maximal insulin stimulation factors (at 10 nM) are as follows: IRS-1 tyrosine phosphorylation, 6.7 ± 1.2 ; PI 3-K activity, 4.4 ± 1.7 ; PKB activity, 3.9 ± 1.4 .

by compounds 41 (2452 ± 376 arbitrary units at $3 \mu\text{M}$ compared with 1634 ± 404 basal) and 37 (results not shown) under conditions which led to an about 14-fold elevation of insulin receptor tyrosine kinase activity by insulin (16845 ± 856 arbitrary units at 1 nM compared with 1132 ± 289 basal). Thus the synthetic PIG compounds apparently do not activate the insulin receptor kinase, which is compatible with their insulin-mimetic activity

Figure 4 Effects of compounds 41, 37, 45, 7 and 1 on the activity of PI 3-K in adipocytes

IRS-1 was immunoprecipitated using anti-IRS-1 antibodies from fat-free homogenate of rat adipocytes which had been incubated in the absence or presence of increasing concentrations of compounds 41, 37, 45, 7 and 1 or human insulin. The immunoprecipitates were incubated with [32 P]ATP and phosphatidylinositol as described in the Materials and methods section in the absence or presence of 0.5 μ M wortmannin (W) as indicated. After extraction with methanol/chloroform, the radiolabelled products were analysed by TLC and autoradiography. A typical experiment repeated twice with similar results is shown. M, marker lane containing 32 P-labelled PI3P and phosphatidylinositol-4,5-bisphosphate (PIP $_2$). In control incubations, immunoprecipitates obtained with non-immune IgG did not result in significant amounts of PI3P. The almost complete absence of radiolabelled phosphatidylinositol in the presence of wortmannin (W) demonstrated the specificity of the assay conditions for PI 3-K. For quantitative evaluation (see Figure 3B), the residual amount of PI3P generated in the presence of wortmannin (not visible in the autoradiogram of Figure 4) was subtracted from each value. The maximal insulin stimulation factor is 4.1 ± 0.5 .

in receptor-depleted adipocytes [24], and in this regard they behave like the peptide-containing PIG derivative, PIG-P, isolated from yeast [22].

Stimulation of the IRS-1/3-PI 3-K pathway correlates with the insulin-mimetic metabolic activity of PIG compounds

Next we analysed the effects of the synthetic PIG compounds on steps downstream of the insulin receptor. For detection of IRS-1/2 tyrosine phosphorylation, IRS-1 or -2 was immunoprecipitated with anti-IRS-1/2 antisera from the fat-free extract of adipocytes incubated with PIG/insulin and then immunoblotted using monoclonal anti-phosphotyrosine antiserum and 125 I-labelled Protein A, followed by autoradiography (Figure 2, lanes 1–8, 17–24) and quantitative evaluation by phosphorimaging (Figure 3A). For determination of the amount and activity of PI 3-K associated with (tyrosine-phosphorylated) IRS-1/2, portions of the immunoprecipitated IRS-1 or 2 were then immunoblotted with antibodies directed against the p85 subunit of PI 3-K and 125 I-labelled Protein A, followed by autoradiography (Figure 2, lanes 9–16, 25–32) and phosphorimaging. Other portions were incubated with [γ - 32 P]ATP and phosphatidylinositol in the absence or presence of wortmannin. Radiolabelled PI3P was identified by TLC analysis and autoradiography (Figure 4) and quantified by phosphorimaging (Figure 3B).

Compounds 41, 37, 45 (only part of the data are shown) and 7 induced a concentration-dependent increase in tyrosine phosphorylation of IRS-1 (Figure 2, lanes 5–8; Figure 3A), IRS-1-associated PI 3-K protein (Figure 2, lanes 13–16) and PI 3-K activity (Figures 4 and 3B). Compound 41 was more potent than compounds 37, 45 and 7, approaching at maximum (at 30 μ M) 48, 42, 31, 14% (IRS-1 tyrosine phosphorylation), 68, 55, 46,

Figure 5 Effects of compounds 41, 37, 45 and 7 on tyrosine phosphorylation of IRS-3 and its association with the p85 subunit of PI 3-K in adipocytes

Isolated rat adipocytes were incubated in the absence (lanes 1, 5, 9, 13, 17, 21 and 25) or presence of increasing concentrations of human insulin (lanes 2–4) or compound 45 (lanes 6–8), 7 (lanes 10–12), 41 (lanes 14–16, 22–24) and 37 (lanes 18–20, 26–28). Total tyrosine-phosphorylated proteins were immunoprecipitated from total de-fatted homogenate using polyclonal anti-phosphotyrosine antibodies. Portions of the immunoprecipitates were then immunoblotted with monoclonal anti-phosphotyrosine antibodies (lanes 1–20). Other portions were immunodepleted for IRS-1/2 (as described in the Materials and methods section) and then immunoblotted with anti-PI 3-K antibodies (lanes 21–28). The blots were treated with 125 I-labelled Protein A. An autoradiogram of a typical experiment repeated four times with similar results is shown. In control incubations, non-immune IgG did not immunoprecipitate tyrosine-phosphorylated p60 or PI 3-K. Furthermore, control immunoblotting of a parallel blot with anti-IRS-1/2 antibodies demonstrated the almost complete absence of IRS-1/2 in the immunodepleted samples (used in lanes 21–28). The apparent size of the p60 polypeptide was derived from molecular-mass markers run in parallel on the same gel.

32% (IRS-1-associated p85) and 63, 35, 30, 16% (PI 3-K activity) respectively of MIR (compare with Figure 2, lanes 1–4, 9–12; Figures 3A and 3B). The marginal effects elicited by compound 1 were not statistically significant. Interestingly, tyrosine phosphorylation of IRS-2 in response to compound 41 was much less pronounced compared with that of IRS-1 (Figure 2, lanes 21–24; 1274 arbitrary units at 30 μ M versus 566 arbitrary units basal) accompanied by a correspondingly reduced amount of p85 associated with IRS-2 (lanes 29–32; 4872 arbitrary units at 30 μ M compared with 2587 arbitrary units basal). In contrast, insulin caused considerable tyrosine phosphorylation of IRS-2 (lanes 17–20; 11548 arbitrary units at 2 nM compared with 2133 basal) and p85 association with IRS-2 (lanes 25–28; 14893 arbitrary units at 2 nM compared with 3510 basal), which is not drastically lower than that observed for IRS-1.

For detection of IRS-3 tyrosine phosphorylation, total tyrosine-phosphorylated proteins were immunoprecipitated with rabbit anti-phosphotyrosine antibodies from the fat-free extract of adipocytes incubated with PIG/insulin and then immunoblotted using monoclonal anti-phosphotyrosine antiserum and 125 I-labelled Protein A, followed by autoradiography (Figure 5, lanes 1–20). For determination of the amount of PI 3-K associated with (tyrosine-phosphorylated) IRS-3, IRS-1/2 was removed from portions of the anti-phosphotyrosine immuno-

Figure 6 Effects of compound 41 on tyrosine phosphorylation of IRS-1 in the adipocytes with inactivated insulin receptor

Isolated rat adipocytes in high K^+ /low Ca^{2+} buffer were incubated for 30 min at 37 °C to stabilize the basal kinase activity. The cells were then electroporated in the absence or presence of anti-insulin receptor antibodies (anti-IRa; 80 μ g/ml) as indicated. Subsequently the cells were incubated for 20 min in the absence or presence of insulin or compound 41 at the concentrations indicated. Insulin receptor β -subunit (upper panels) and IRS-1 (lower panels) were immunoprecipitated from total de-fatted homogenate using anti-insulin receptor and anti-IRS-1 antibodies respectively, and then immunoblotted with anti-phosphotyrosine antibodies followed by treatment with ^{125}I -labelled Protein A. An autoradiogram of a typical experiment repeated twice with similar results is shown. Probing of parallel blots with anti-insulin receptor β -subunit or anti-IRS-1 antibodies demonstrated (i) the identity of the phosphotyrosine-containing bands, and (ii) that comparable amounts of receptor and IRS-1 had been immunoprecipitated from the different samples.

precipitates by immunoprecipitation with anti-IRS-1/2 antibodies. The immunodepleted supernatants were then immunoblotted with antibodies directed against the p85 subunit of PI 3-K and ^{125}I -labelled Protein A, followed by autoradiography (Figure 5, lanes 21–28). Compounds 41, 37, 45 and 7 increased in a concentration-dependent fashion the tyrosine phosphorylation of a 60 kDa polypeptide (p60), which is presumably identical with IRS-3 (lanes 5–20), and the amount of PI 3-K associated with this protein (since the anti-phosphotyrosine immunoprecipitates have been immunodepleted for IRS-1/2; lanes 21–28). Compounds 41, 37, 45 and 7 approached at maximum (at 30 μ M) 89, 75, 39, 11 % of MIR for IRS-3 tyrosine phosphorylation and 95, 82, 44 and 15 % of MIR for IRS-3 association with p85 respectively, according to phosphorimage analysis of the corresponding blots. Compound 1 was inactive (results not shown). Thus the PIG compounds 41, 37, 45 and 7 stimulate PI 3-K in rat adipocytes predominantly via induction of tyrosine phosphorylation of IRS-1 and 3 with the same ranking order between the five compounds as manifested in their insulin-mimetic metabolic activity with respect to both MIR and effective concentrations. Taking into account the relative efficiencies of immunoprecipitation using the anti-IRS-1/2 and anti-phosphotyrosine antibodies (70, 85 and 95 % respectively, according to [31]), we estimated that IRS-1/3 may mediate approx. 50–60 % of the insulin-induced, but 85 to 95 % of compound 41-induced, PI 3-K activation, whereas the remaining portions are due to IRS-2. Comparable relationships in the signalling competence between the three IRS proteins were also found for compounds 37, 45 and 7 (results not shown).

Since the PIG compounds do not activate the insulin receptor kinase (see Figure 1), IRS1/3 must be phosphorylated by a different tyrosine kinase in response to PIG. This was confirmed by analysis of IRS-1 tyrosine phosphorylation in rat adipocytes

Table 1 Effects of anti-insulin receptor antibodies on glucose transport activation by compounds 41 and 37

Isolated rat adipocytes in high K^+ /low Ca^{2+} buffer were incubated for 30 min at 37 °C to stabilize the basal transport activity. The cells were then electroporated in the presence of two different anti-insulin receptor antibodies (anti-IRa; 80 μ g/ml; anti-IRb, 150 μ g/ml) or non-immune IgG (200 μ g/ml) as described in the Materials and methods section. Subsequently the cells were incubated in the absence or presence of insulin or compounds 41 and 37 at the concentrations indicated for 20 min and then assayed for glucose transport. Glucose transport activity is given as pmol 2-deoxyglucose/min per mg of protein. Each value represents the mean $\pm$ S.E.M. of two independent adipocyte preparations and electroporations with incubations/glucose transport determinations performed in quadruplicate.

	Anti-IRa	Anti-IRb	IgG
Basal	7.9 $\pm$ 1.1	7.2 $\pm$ 1.7	8.9 $\pm$ 2.0
Insulin			
0.2 nM	7.1 $\pm$ 1.2	8.0 $\pm$ 1.9	12.9 $\pm$ 3.1
1 nM	8.4 $\pm$ 1.5	11.1 $\pm$ 2.3	28.8 $\pm$ 4.8
10 nM	9.6 $\pm$ 1.9	14.2 $\pm$ 3.1	66.7 $\pm$ 7.1
100 nM	19.8 $\pm$ 3.3	27.5 $\pm$ 4.8	81.6 $\pm$ 9.9
Compound 37			
0.5 μ M	12.5 $\pm$ 1.4	14.9 $\pm$ 2.6	14.0 $\pm$ 1.8
2 μ M	24.6 $\pm$ 2.0	26.7 $\pm$ 5.1	29.4 $\pm$ 4.4
20 μ M	47.9 $\pm$ 5.3	50.4 $\pm$ 6.9	53.7 $\pm$ 8.1
50 μ M	60.2 $\pm$ 6.9	58.2 $\pm$ 5.4	64.9 $\pm$ 7.7
Compound 41			
0.5 μ M	26.8 $\pm$ 3.9	25.7 $\pm$ 2.4	23.7 $\pm$ 2.5
2 μ M	49.7 $\pm$ 5.5	48.8 $\pm$ 3.9	53.6 $\pm$ 4.1
20 μ M	60.4 $\pm$ 6.1	59.3 $\pm$ 5.1	64.2 $\pm$ 7.3
50 μ M	62.6 $\pm$ 5.9	60.1 $\pm$ 6.5	67.1 $\pm$ 6.8

with inactivated insulin receptor tyrosine kinase. For this, inhibitory anti-insulin receptor β -subunit antibodies were introduced into the cells by electroporation before stimulation with insulin or compound 41 and subsequent immunoprecipitation of the β -subunit and IRS-1 from the de-fatted adipocyte homogenate followed by immunoblotting with anti-phosphotyrosine antibodies (Figure 6). The ability of two different insulin receptor antibodies (anti-IRa/b) to interfere with the receptor kinase was first examined *in vitro* with insulin receptor partially purified from rat liver by wheat germ agglutinin–Sepharose chromatography. The insulin-stimulated (100 nM) increase (set at 100 %) in autophosphorylation and phosphorylation of exogenous substrates [histone, poly(Glu/Tyr) 4:1] was inhibited by 99 and 95 % respectively by anti-IRa and 94 and 90 % by anti-IRb (results not shown). Basal phosphorylation (set at 100 %) was repressed by 65 % by anti-IRa and 45 % by anti-IRb. Both autophosphorylation and exogenous substrate phosphorylation by the rat liver receptor was inhibited half maximally by these antibodies at 1–2 μ g/ml, with maximal inhibition at 15–20 μ g/ml. In agreement with these *in vitro* data, anti-IRa antibodies (80 μ g/ml) blocked autophosphorylation of the insulin receptor in isolated rat adipocytes electroporated with these antibodies (Figure 6) in the basal state (set at 100 %) to 20–30 % and in the insulin-stimulated (10 nM) state (set at 100 %) to 0.5–2.5 %. Despite the potent inhibition of insulin receptor tyrosine kinase by the anti-IRa antibodies, there was no significant difference in induction of tyrosine phosphorylation of IRS-1 by compound 41 between the absence (4.4-fold at 3 μ M; 8.2-fold at 30 μ M) or presence (5.3-fold at 3 μ M; 9.7-fold at 30 μ M) of the antibodies in the cells. Compatible with this finding, in electroporated adipocytes, tyrosine phosphorylation of the insulin receptor was not increased in response to compound 41 (Figure 6). In addition, glucose transport activation by compounds 41 and 37 was not significantly impaired in the presence, compared with the absence,

Figure 7 Effects of compounds 41, 37, 45, 7 and 1 on the activity of PKB in adipocytes

PKB was immunoprecipitated using anti-PKB antibodies from total fat-free extracts of rat adipocytes which had been incubated in the absence (lane 1) or presence of increasing concentrations of compounds 41 (lanes 13–16), 37 (lanes 5–8), 45 (lanes 9–12), 7 (lanes 17–20) and 1 (lanes 21–24) or human insulin (lanes 2–4). The immunoprecipitates were incubated with [32 P]ATP and histone 2B (H2B) as described in the Materials and methods section. The reaction mixtures were separated by SDS/PAGE and phosphorylated histone 2B was visualized by autoradiography. A typical experiment repeated twice with comparable results is shown. In control incubations using non-immune IgG, only marginal amounts of histone 2B were phosphorylated. The maximal stimulation factors are 5.9 ± 0.7 for insulin, 4.3 ± 0.6 for compound 41, 3.1 ± 0.4 for compound 37, 2.6 ± 0.4 for compound 45, 1.6 ± 0.2 for compound 7 and 1.1 ± 0.2 for compound 1.

of anti-IRa/b antibodies (Table 1), in contrast to the almost complete abrogation of the insulin-stimulated glucose transport by these antibodies (Table 1). Taken together, it appears very unlikely that a small increment in tyrosine phosphorylation of the insulin receptor (less than 3% of MIR), not inhibitable by the anti-insulin receptor antibodies and escaping detection by our analytical methods, can account for the almost full insulin-mimetic effects of compounds 41 and 37 on tyrosine phosphorylation of IRS-1/3 and glucose transport in rat adipocytes. In agreement with this view are our preliminary experiments with cells transfected with dominant-negative mutant insulin receptor harbouring an altered ATP binding site or tyrosine kinase regulatory domain which fail to respond to insulin, as has been shown previously [32], but are sensitive towards PIG compounds with regard to stimulation of glycogen synthesis and amino acid transport.

Stimulation of PKB and inhibition of GSK-3 correlates with the insulin-mimetic metabolic activity of PIG compounds

PKB is located downstream of PI 3-K within the insulin signalling cascade and is either directly or indirectly activated by the latter (see Introduction). We studied whether stimulation of PI 3-K by the synthetic PIG compounds of varying potency leads to corresponding increases in PKB activity. For determination of PKB activity, PKB was immunoprecipitated with anti-PKB antiserum from fat-free cell extracts of adipocytes treated with PIG/insulin and then incubated with Crosstide or histone 2B in the presence of [γ - 32 P]ATP and cAMP-dependent protein kinase inhibitor peptide. Samples containing Crosstide were adsorbed on phosphocellulose papers which were counted for radioactivity (Figure 3C). Samples containing histone 2B were analysed by SDS/PAGE and autoradiography (Figure 7). Compounds 41, 37, 45 and 7 increased the activity of immunoprecipitated PKB

Figure 8 Effects of compounds 41 and 37 on the phosphorylation of PKB in adipocytes

PKB was immunoprecipitated using anti-PKB antibodies from total fat-free extracts of rat adipocytes which had been incubated in the absence (lanes 1, 3, 10, 16) or presence of increasing concentrations of compounds 41 (lanes 4–9), 37 (lanes 11–15) and 1 (lanes 17–20) or human insulin (lane 2). The immunoprecipitates were separated by SDS/PAGE and immunoblotted using anti-PKB antibodies and [125 I]-labelled Protein A. The autoradiogram of a typical experiment repeated twice with identical results is shown. Probing of parallel blots with anti-serine/threonine antibodies as well as incorporation of 32 P into PKB (see Table 2) demonstrated the exclusive phosphorylation of the upper polypeptide band which had been immunoprecipitated with the anti-PKB antibodies (PKB-p). Quantitative evaluation of the autoradiogram by phosphorimaging revealed that the total recovery of PKB (the sum of the two lower non-phosphorylated and the upper phosphorylated PKB polypeptides) from the different immunoprecipitates was comparable. Control incubations using non-immune IgG for the immunoprecipitation or immunoblot did not lead to bands migrating at the size range of non-phosphorylated and phosphorylated PKB.

using either Crosstide (Figure 3C) or histone 2B (Figure 7) as substrate to up to 70–75 (lanes 13–16), 40–45 (lanes 4–8), 35–40 (lanes 9–12) and 10–15% (lanes 17–20) respectively of MIR. The effective concentrations closely matched those for PI 3-K activation. Compound 1 was completely ineffective (lanes 21–24). Thus, the ranking orders between the five compounds for PKB stimulation, PI 3-K activation and metabolic activity, with respect to MIR and effective concentrations, are identical.

The basis for PKB activation by insulin is assumed to rely on binding of phosphorylated phosphatidylinositol to its pleckstrin homology domain as well as on serine/threonine phosphorylation of its regulatory domain (see Introduction). We studied whether (part of) the increase in PKB activity provoked by the PIG compounds can be explained by phosphorylation of the enzyme. For this, PKB was immunoprecipitated from adipocytes incubated with compounds 41, 37 and 1 and the non-phosphorylated and phosphorylated forms of PKB were visualized by immunoblotting with anti-PKB antibodies (Figure 8). Under basal conditions (lanes 1, 3, 10, 16) a triplet of polypeptides of about 60 kDa was observed, the two upper ones corresponding to the different phosphorylation states of the protein [33–35], the lower one presumably representing a processed or degraded non-phosphorylated version (see legends to Figure 8 and Table 2). Treatment with insulin (lane 2) or compounds 41 (lanes 4–9) and 37 (lanes 11–15) caused the slowest migrating phosphorylated form to increase in intensity in a concentration-dependent fashion, and in parallel the intensity of the non-phosphorylated form migrating between the phosphorylated and the processed/

Table 2 Effects of compounds 41, 37, 7 and 1 on ^{32}P incorporation into PKB

Isolated rat adipocytes were labelled with [^{32}P]phosphate and then incubated with increasing concentrations of PIG compounds as indicated. PKB was immunoprecipitated using anti-PKB antibodies. One half of each immunoprecipitate was analysed directly by SDS/PAGE, the other half was analysed after another immunoprecipitation using anti-phosphoserine/threonine antibodies (anti-pS/T) as described in the Materials and methods section. The amount of radiolabelled PKB was determined by phosphorimaging of the dried gel (arbitrary units $\pm$ S.D., four experiments). Immunoblotting of a parallel gel with anti-PKB antibodies revealed that the amount of PKB immunoprecipitated did not vary significantly between the different incubations. The amount of radiolabelled PKB in response to 5 nM insulin was determined as 9835 ± 1034 arbitrary units in total immunoprecipitates and 7035 ± 802 in anti-phospho-serine/threonine immunoprecipitates, compared with 852 ± 123 and 575 ± 150 respectively in the basal state.

Compound ...	41		37		7		1	
	Direct	Anti-pS/T	Direct	Anti-pS/T	Direct	Anti-pS/T	Direct	Anti-pS/T
Basal	931 $\pm$ 89	535 $\pm$ 72	835 $\pm$ 94	602 $\pm$ 56	867 $\pm$ 97	539 $\pm$ 60	902 $\pm$ 81	644 $\pm$ 66
PIG compound (μM)								
0.5	1044 $\pm$ 121	599 $\pm$ 86	877 $\pm$ 97	659 $\pm$ 75	823 $\pm$ 105	550 $\pm$ 63	871 $\pm$ 75	668 $\pm$ 88
1	1355 $\pm$ 144	976 $\pm$ 103	1081 $\pm$ 176	860 $\pm$ 95	914 $\pm$ 90	637 $\pm$ 82	845 $\pm$ 59	612 $\pm$ 68
2	1763 $\pm$ 132	1362 $\pm$ 140	1377 $\pm$ 102	1035 $\pm$ 118	1074 $\pm$ 99	699 $\pm$ 77	885 $\pm$ 96	571 $\pm$ 74
5	3657 $\pm$ 403	2978 $\pm$ 351	3245 $\pm$ 377	2576 $\pm$ 288	1832 $\pm$ 215	1316 $\pm$ 174	934 $\pm$ 145	670 $\pm$ 88
10	6597 $\pm$ 759	4976 $\pm$ 512	5672 $\pm$ 503	4150 $\pm$ 587	2556 $\pm$ 341	1831 $\pm$ 214	854 $\pm$ 106	612 $\pm$ 71
20	8476 $\pm$ 934	6587 $\pm$ 781	6412 $\pm$ 832	4877 $\pm$ 612	2942 $\pm$ 351	2205 $\pm$ 289	843 $\pm$ 120	645 $\pm$ 88

Figure 9 Time course for the effects of compound 41 on the phosphorylation of PKB in adipocytes

PKB was immunoprecipitated using anti-PKB antibodies from total fat-free extracts of rat adipocytes which had been incubated in the absence (lanes 1 and 7) or presence of $10 \mu\text{M}$ compound 41 (lanes 8–12) or 5 nM human insulin (lanes 2–6) for increasing periods of time as indicated. Portions of the immunoprecipitates were separated by SDS/PAGE and immunoblotted using anti-PKB antibodies (lanes 1–6; lanes 7–12, right-hand lower panel) or antibodies raised against phosphorylated PKB (lanes 7–12, right-hand upper panel) and [^{125}I]-labelled Protein A (see Materials and methods section). The autoradiogram of a typical experiment repeated once with similar results is shown. Probing of parallel blots with anti-serine antibodies confirmed phosphorylation of the polypeptide band, exclusively, which was recognized by the anti-phospho-PKB antibodies (lanes 7–12, right-hand upper panel). Control incubations using non-immune IgG for the immunoblots instead of anti-PKB or anti-phospho-PKB antisera did not yield specific signals.

degraded forms decreased, suggesting that all these agents increased the phosphorylation state of PKB. Compound 1 was inactive (lanes 17–20).

This was confirmed by direct demonstration of phosphate incorporation into PKB immunoprecipitated from [^{32}P]phosphate-labelled and compound-treated adipocytes (Table 2). SDS/PAGE analysis and phosphorimaging revealed that compounds 41, 37 and 7 led to considerable elevations in the amount of ^{32}P -labelled PKB in a concentration-dependent fashion to up to about 80, 65 and 25% of MIR respectively. Compound 1 did not show any effect on radiolabelling of PKB. The nature of the phosphorylation was elucidated by using anti-phosphoserine/threonine antibodies for immunoprecipitation of the anti-PKB immunoprecipitates before the SDS/PAGE (Table 2). Previously we found that these antibodies are specific for phosphoserine/threonine compared with phosphotyrosine, but

under our experimental conditions they immunoprecipitate phosphoproteins with 70–80% efficiency only [28]. From 60 to 70% of the ^{32}P -labelled PKB in the basal state or generated in response to each concentration of compounds 41, 37 and 7 was recognized by the anti-phosphoserine/threonine antibodies. Taking into account the efficiency of these antibodies, we conclude that PKB becomes phosphorylated on serine/threonine residues, predominantly or even exclusively, upon challenge of adipocytes with PIG compounds. The serine/threonine phosphorylation of PKB, as investigated by the two independent methods (see Figure 8 and Table 2), seems to reflect its activation by compounds 41, 37 and 7 with respect to their maximal responses relative to MIR (80–90, 55–65 and 25–30% respectively), effective concentrations and relative ranking in potency ($41 > 37 \gg 7$). This argues strongly for serine/threonine phosphorylation as the major (or even only) mechanism for regulation of PKB by PIG compounds, as has been proposed for insulin according to current experimental evidence.

To corroborate this view, the time course of PKB phosphorylation upon challenge by PIG compounds was compared with that of the insulin response. For this, PKB was immunoprecipitated from adipocytes stimulated for various periods with compound 41 or insulin (Figure 9). The phosphorylated and non-phosphorylated versions of PKB were separated and identified by either gel mobility-shift during SDS/PAGE and immunoblotting with anti-PKB antibodies, as described above (for insulin, lanes 1–6), or by direct immunoblotting with antibodies specifically recognizing PKB phosphorylated at a C-terminal serine residue (Ser-473 in the human PKB α) (for compound 41, lanes 7–12, Figure 9, upper panel), and with anti-PKB antibodies not discriminating between the phosphorylated and non-phosphorylated versions (for compound 41, lanes 7–12, lower panel). The kinetics of the induction of PKB phosphorylation as manifested by both methods in a time-dependent increase in the amount of phosphorylated PKB and a concomitant decrease in the amount of the two non-phosphorylated PKB polypeptides was rather slow for compound 41. The maximum, 85–95% of the MIR, was reached between 90 and 120 min (lanes 11, 12) in comparison with that of insulin with the maximal response (7.5–9.5-fold above the zero time point) being obtained after 10–15 min (lanes 5, 6). Interestingly, the generation of a non-phosphorylated polypeptide of smaller apparent molecular mass than that of authentic PKB, which cross-reacts with

Figure 10 Effects of compounds 41, 37, 45, 7 and 1 on the activity of GSK-3 in adipocytes

GSK-3 was immunoprecipitated using anti-GSK-3 antibodies from fat-free cytosol of rat adipocytes (as described in the Materials and methods section) which had been incubated in the absence (lanes 1, 3, 6, 9, 12, 15) or presence of increasing concentrations of compounds 41 (lanes 4, 5), 37 (lanes 7, 8), 45 (lanes 10, 11), 7 (lanes 13, 14) and 1 (lanes 16, 17) or human insulin (lane 2). The immunoprecipitates were incubated with [32 P]ATP and PP2AI. After trichloroacetic acid precipitation, the proteins were analysed by SDS/PAGE and autoradiography. A typical experiment repeated three times with comparable results is shown. In control incubations, non-immune IgG failed to induce any phosphorylation of PP2AI. The amounts of GSK-3 immunoprecipitated from different samples were comparable, as revealed by immunoblotting of parallel blots with anti-GSK-3 antibodies.

the anti-PKB antibodies, is not restricted to compound 41 (lanes 7–12, Figure 9, lower panel; see also Figure 8) but also occurs in response to insulin (Figure 9, lanes 1–6). However, the insulin kinetics of both appearance (maximum at 2 min, lane 3) and disappearance (80–85% loss after 15 min, lane 6) of this PKB-related protein was drastically faster than the corresponding kinetics induced by compound 41 (maximum at 10–15 min, lanes 7, 8; 95% loss after 120 min, lane 12, lower panel). Thus, the mechanisms for activation by phosphorylation and inactivation, presumably by dephosphorylation/degradation of PKB, in isolated rat adipocytes are apparently similar for PIG compounds and insulin. The molecular basis for the kinetic differences remains to be clarified, but may be related to differential translocation of the PKB isoforms to different intracellular compartments in response to PIG compounds and insulin where phosphorylation, dephosphorylation and proteolysis occur.

One of the few physiological substrates identified so far for PKB is GSK-3. Consequently, we studied whether activation of PKB by the PIG compounds leads to changes in the activity and phosphorylation state of GSK-3. For some substrates of GSK-3, prior phosphorylation of the substrate to form the motif -S-X-X-X-S(P)- is a strict requirement. A synthetic peptide, P-GS1, derived from skeletal muscle GS and containing the four-residue spaced phosphorylation sites for GSK-3, 3b (Ser-644), 3c (Ser-648) and 4 (Ser-652), including the C-terminal priming phosphoserine residue corresponding to site 5 (Ser-656), proved to be efficient in mimicking the successive phosphorylation of GSK-3 *in vitro* [36,37]. For determination of the effect of the PIG compounds on GSK-3, its activity toward three different substrates (P-GS1, PP2AI and GS) and autophosphorylation were assayed. For this, GSK-3 was immunoprecipitated with anti-GSK-3 α antiserum from the fat-free cytosol of adipocytes treated with PIG/insulin and incubated with [γ - 32 P]ATP in the absence

(autophosphorylation) or presence of P-GS1 or PP2AI. Phosphorylation of GS was detected by immunoprecipitation of GS from the de-fatted post-mitochondrial supernatant of adipocytes which had been labelled with [32 P]phosphate before incubation with PIG/insulin. The phosphorylated substrates were separated from the incubation mixture by phosphocellulose binding for P-GS1 or SDS/PAGE for PP2AI (Figure 10), GSK-3 and GS and in all cases evaluated for radioactivity (Figure 11).

Compounds 41, 37, 45 (shown only for PP2AI) and 7 caused a concentration-dependent reduction in the amount of substrates, P-GS1 (Figure 11A) and PP2AI (Figures 10 and 11B), phosphorylated by GSK-3, as did insulin. Compound 41 (Figure 10, lanes 3–5) was most efficient with maximal inhibition of GSK-3 (at 10 μ M) approaching 70–85% of MIR (see lanes 1, 2) compared with compounds 37 (lanes 6–8; 55–75%), 45 (lanes 9–11; 30–45%) and 7 (lanes 12–14; 15–20%). Compound 1 did not exhibit significant activity (lanes 15–17). The same ranking order between the PIG compounds and comparable effective concentrations were obtained when inhibition of GSK-3 was assayed as its autophosphorylation (Figure 11C) or phosphorylation of GS (Figure 11D), with essentially no difference in the maximal response between insulin and compound 41 (at 50 μ M), lower potency of compounds 37 and 7 (65–75% and 40–50% of MIR respectively at 50 μ M) and inactivity of compound 1.

GSK-3 from rabbit skeletal muscle has been demonstrated to undergo autophosphorylation on Ser, Thr and Tyr residues in the case of the α -isoform and on Ser and Tyr residues only in the case of the β -isoform, resulting in a considerable reduction and stabilization respectively of the enzyme activity [37]. We studied the effect of PIG compounds on differential Ser/Thr/Tyr phosphorylation of GSK-3. For this, GSK-3 was immunoprecipitated from adipocytes which had been treated with PIG compounds or insulin, subsequently subjected to phosphorylation *in vitro* and then analysed for phosphoamino acids (Table 3). Compounds 41, 37 and 7, as well as insulin, reduced both the serine and tyrosine phosphorylation state of GSK-3 to up to 50% of the basal value. The ranking order between the PIG compounds in their potency in inhibiting GSK-3 serine and tyrosine phosphorylation (41 > 37 > 7) with compound 41 approaching almost full MIR was identical with that for impairment of GSK-3 phosphorylation activity towards exogenous substrates (see Figures 10 and 11). Compound 1 was completely ineffective. Threonine phosphorylation of GSK-3 was not significantly affected by PIG or by insulin (Table 3). These results demonstrate that inactivation of the dual-specificity kinase GSK-3 provoked by the PIG compounds comprises both phosphorylation of exogenous substrates and autophosphorylation on Ser and Tyr residues.

The following points argue strongly that the reduction of the net GSK-3 phosphorylation state on Ser and Tyr residues in response to PIG compounds is most likely to be based on interference with GSK-3 autokinase activity rather than on inhibition/stimulation of separate kinases/phosphatases present in the anti-GSK-3 immunoprecipitates. (i) Immunodepletion of PKB from the adipocyte cytosol used for immunoprecipitation of GSK-3 did not result in a significant reduction of the serine phosphorylation state of GSK-3 from basal and PIG compound-treated adipocytes compared with GSK-3 immunoprecipitated from cytosol containing PKB (Table 3). (ii) Immunoblotting of the GSK-3 immunoprecipitates from total or anti-PKB immunodepleted adipocyte cytosol with anti-PKB antibodies did not reveal any cross-reacting polypeptide in the size range of the phosphorylated/non-phosphorylated PKB versions (results not shown). (iii) The decrease in net serine phosphorylation of GSK-

Figure 11 Quantitative evaluation of the effects of compounds 41, 37, 7 and 1 on the activity of GSK-3 and phosphorylation of GS in adipocytes

Isolated rat adipocytes were incubated with increasing concentrations of insulin (▲) or compounds 41 (○), 37 (●), 7 (■) and 1 (□) and then assayed for GSK-3 activity with the substrates P-GS1 (A) or PP2AI (B), autophosphorylation of GSK-3 (C) and phosphorylation of GS (D). GSK-3 activity and autophosphorylation in basal cells were set at 100%. For phosphorylation of GS, the amount of ³²P-labelled GS is given in dpm. The maximal inhibition factors (percent of control divided by 100) provoked by insulin (at 10 nM) are as follows: P-GS1 phosphorylation, 2.6 ± 0.8; PP2AI phosphorylation, 2.3 ± 0.5; GSK-3 autophosphorylation, 1.8 ± 0.3; GS phosphorylation, 1.5 ± 0.2.

Table 3 Effects of compounds 41, 37, 7 and 1 on autophosphorylation of GSK-3

GSK-3 was immunoprecipitated with anti-GSK-3 antibodies from fat-free cytosol of isolated rat adipocytes which had been incubated with 10 μM compound 41, 37, 7 or 1 or 5 nM human insulin. The immunoprecipitates were incubated with [³²P]ATP and then separated by SDS/PAGE. After membrane transfer of the proteins and visualization of ³²P-labelled GSK-3 by autoradiography, the corresponding polypeptide band was excised, and then subjected to acid hydrolysis and phosphoamino acid analysis as described in the Materials and methods section. In a control experiment, the fat-free cytosol was subjected to two successive rounds of immunoprecipitation with anti-PKB antibodies and Protein A-Sepharose. After removal of the immune complexes, the cytosol immunodepleted of PKB was immunoprecipitated using anti-GSK-3 antibodies and the immuno-precipitate was further processed as described above (—PKB). Each value (dpm derived from Cerenkov counting of scraped TLC material) represents the mean ± S.E.M. of three independent adipocyte incubations with hydrolysis and amino acid analysis in duplicate each. The amount of phosphoserine and phosphotyrosine separately or the amount of the three phosphoamino acids together (total) in the basal state was set at 100%. The amount of phosphoserine in the '—PKB' samples was calculated as a percentage before the immunodepletion.

	Basal	Human insulin	Compound			
			41	37	7	1
Ser-P (dpm)	2785 ± 491	1504 ± 377	1647 ± 502	1982 ± 455	2180 ± 302	2904 ± 384
Percentage	100 ± 13	54 ± 8	59 ± 7	71 ± 9	78 ± 8	104 ± 9
—PKB (dpm)	2366 ± 513	1252 ± 412	1404 ± 355	1557 ± 522	1872 ± 414	2608 ± 460
Percentage	85 ± 7	83 ± 9	85 ± 7	79 ± 11	86 ± 6	90 ± 7
Thr-P (dpm)	309 ± 104	425 ± 85	519 ± 110	488 ± 152	407 ± 116	310 ± 82
Tyr-P (dpm)	1244 ± 292	601 ± 101	677 ± 123	805 ± 210	951 ± 188	1137 ± 235
Percentage	100 ± 11	48 ± 13	54 ± 16	65 ± 11	76 ± 14	91 ± 8
Total (%)	100 ± 8	58 ± 11	65 ± 9	75 ± 12	82 ± 12	99 ± 8

Figure 12 Time course for the effects of compounds 41, 37 and 7 on IRS-1 tyrosine phosphorylation and PI 3-K, GSK-3 and GS activities in adipocytes

Isolated rat adipocytes were incubated with 10 nM insulin ($\blacktriangle$) or 10 μ M compounds 41 ($\circ$) and 37 ($\bullet$) or 50 μ M compound 7 ($\blacksquare$) for increasing periods of time and then assayed for GSK-3 activity (A; assay period 10 min), IRS-1 tyrosine phosphorylation (B), PI 3-K activity (C; assay period 3 min) and GS activity (D; assay period 5 min). The basal (zero time point) and maximal insulin-induced values were set at 0 and 100% respectively in each case.

3 in response to PIG compounds as a result of inhibition of PKB putatively associated with the immunoprecipitated GSK-3 is not compatible with the observed stimulation of PKB upon those stimuli (see above). (iv) So far, kinases or phosphatases specifically associated with GSK-3 in the active or inactive state could not be identified by coimmunoprecipitation or yeast two-hybrid screens. Thus it seems likely that the net reduction in the PIG compound-induced serine and tyrosine phosphorylation of GSK-3 is based on PKB-mediated inhibition of GSK-3 autophosphorylation activity, although the participation of additional modulatory (yet unknown) extrinsic factors cannot be excluded. Taken together, in rat adipocytes, the inhibition of GSK-3 by the synthetic PIG compounds, consistently demonstrated as phosphorylation of different exogenous substrates as well as autophosphorylation, correlates well with the activation of PKB and PI 3-K, as reflected in the comparable maximal responses and effective concentrations as well as in the similar ranking order of the compounds with regard to their effects on these signalling components.

Kinetics of the effects of PIG compounds on insulin signalling components

To exclude the possibility that different PIG compounds exert their insulin-mimetic signalling and activity with different kinetics, which might affect their relative order of potency, we studied the time course for tyrosine phosphorylation of IRS-1 and PI 3-K, as well as inhibition of GSK-3 and activation of GS by compounds 41, 37 and 7 in comparison with insulin. The compounds were used at concentrations slightly below the maximal effective ones and the assay periods were reduced (as

indicated in the Figure legend) to avoid changes in the activity state of the immunoprecipitated enzymes during prolonged incubation *in vitro*. The insulin-mimetic effects of all three PIG compounds on GSK-3 (Figure 12A), tyrosine phosphorylation of IRS-1 (Figure 12B), PI 3-K (Figure 12C) and GS (Figure 12D) reached their maxima between 10 and 20 min after the start of incubation. The time-response curves exhibited very similar shapes for compounds 41, 37 and 7 and for the four effects studied, in general. This is compatible with a causal relationship between tyrosine phosphorylation of IRS-1, activation of PI 3-K, inhibition of GSK-3 and stimulation of GS in rat adipocytes. Thus, the incubation periods used for determination of PIG effects in rat adipocytes were apparently appropriate for comparison and differentiation between these compounds. Interestingly, insulin stimulation of GS, IRS-1 tyrosine phosphorylation and PI 3-K, as well as insulin inhibition of GSK-3, were significantly more rapid, as were their subsequent declines, compared with PIG action. These differences in the time-response curves were most striking for the increase in GSK-3 activity (Figure 12A) and the amount of tyrosine-phosphorylated IRS-1 (Figure 12B), which for insulin reached its maximum at 10 min and then declined to about 30% of the maximal level at 60 min. This may be explained by distinct tyrosine kinases triggered by insulin and compounds 41, 37 and 7 which phosphorylate IRS-1 and in consequence initiate downstream signalling with different kinetics.

PIG compounds are weak activators of the MAPK pathway

It has been reported that, in addition to PKB, GSK-3 activity is regulated by pp90^{Rsk} [38]. This Ser/Thr-specific protein kinase is

Figure 13 Effects of compounds 41, 37, 7 and 1 on the MAPK pathway and protein synthesis in adipocytes

Isolated rat adipocytes were incubated with increasing concentrations of insulin ($\blacktriangle$) or compounds 41 ($\circ$), 37 ($\bullet$), 7 ($\blacksquare$) and 1 ($\square$) and then assayed for MBPK (A), MAPKp44 (B), MAPKp42 (C) and protein synthesis (D). The phosphorylation of MBP and protein synthesis induced by 10 nM insulin were set at 100 arbitrary units (arb. u.). The maximal insulin stimulation factors (at 10 nM) are as follows: MBPK activity, 4.1 ± 0.7 ; MAPKp44 activity, 2.7 ± 0.9 ; MAPKp42 activity, 5.4 ± 1.0 ; protein synthesis, 2.4 ± 0.7 .

activated by insulin and other growth factors *in vivo* and phosphorylates GSK-3 stoichiometrically at an N-terminal serine residue, decreasing its activity dramatically. These observations suggested that pp90^{Rsk}, which is under the control of the MAPK cascade (for a review see [39]), may be involved in the regulation of GSK-3 by extracellular stimuli. Therefore, we studied the possibility that the synthetic PIG compounds provoke the observed inhibition of GSK-3 via activation of the MAPK pathway. In addition, this analysis should elucidate the specificity of PIG action. At present there is broad consensus that the PI 3-K pathway is more strictly coupled to metabolic insulin signalling compared with the MAPK pathway which seems to participate in the control of gene expression by insulin (for reviews see [3,40,41]). MAPK activity was determined using MBP as substrate and total MBPK contained in a fat-free post-mitochondrial supernatant from adipocytes incubated with PIG/insulin (Figure 13A). Alternatively, p42 and p44 MAPK isoforms were immunoprecipitated from a post-nuclear supernatant of pretreated adipocytes (Figures 13B and 13C). The MBP was separated from the incubation mixture by binding to phosphocellulose (for assaying total MAPK activity) or SDS/PAGE analysis (for assaying p42/p44 MAPK activity) and evaluated for radioactivity.

Compounds 41 and 37 increased total MAPK activity (measured as MBPK activity) in rat adipocytes to only about 40% of MIR (at 50 μ M), whereas the effects of compounds 7

and 1 were not significant (Figure 13A). These changes in total MAPK activity corresponded well with the elevations in the activity of the individual MAPK isoforms, in that both compounds 41 and 37 activated, in a concentration-dependent manner, p44 to up to 52% (Figure 13B) and p42 to up to 30% (Figure 13C) of MIR at 50 μ M. The stimulation by compounds 7 and 1 was low and below the limit of detection respectively.

Recently, a missing link within the insulin signalling pathway has been identified between insulin's ability to activate the MAPK pathway and to stimulate protein synthesis (for a review see [42]). eIF-4E, the mRNA cap-binding protein which controls the rate-limiting initiation step of translation, is inhibited by association with the binding protein, 4E-BP1, sometimes also called PHAS-1 protein [43,44]. When phosphorylated at the appropriate sites in response to insulin and growth factors, 4E-BP1 does not bind to eIF-4E, thus releasing it from inhibition and increasing its initiating activity. 4E-BP1 is an excellent substrate for MAPK *in vitro* and in response to insulin in adipocytes, although other kinases seem also to be involved in phosphorylating 4E-BP1 [43,45]. Consequently, in rat adipocytes the (modest) activation of MAPK by the synthetic PIG compounds should be reflected in stimulation of protein synthesis with a similar ranking order between the compounds with respect to both effects. We studied this prediction by determination of protein synthesis as incorporation of [³⁵S]methionine

Table 4 Effects of various inhibitors of cellular signalling on the metabolic activity of compounds 41, 37 and 7

Isolated rat adipocytes were incubated for 10 min with various concentrations of the inhibitors, as indicated, before the addition of insulin (10 nM final conc.), compounds 41 and 37 (final conc. 10 μ M) or compound 7 (50 μ M). After further incubation for 20 min, the adipocytes were assayed for lipogenesis (at high glucose; Lipo.) and GS activity which are expressed as a percentage $\pm$ S.D. of the maximal activation induced in the absence of inhibitor (set at 100%) for each stimulus. Basal lipogenesis and GS activity in the absence of both insulin and inhibitor are set at 0%.

	Insulin		Compound 41		Compound 37		Compound 7		Basal	
	Lipo.	GS	Lipo.	GS	Lipo.	GS	Lipo.	GS	Lipo.	GS
Genistein										
10 μ M	32 $\pm$ 7	48 $\pm$ 11	59 $\pm$ 13	71 $\pm$ 10	61 $\pm$ 9	77 $\pm$ 10	49 $\pm$ 7	63 $\pm$ 10	—4	—3
300 μ M	6 $\pm$ 4	9 $\pm$ 5	14 $\pm$ 6	20 $\pm$ 9	14 $\pm$ 6	21 $\pm$ 9	21 $\pm$ 10	25 $\pm$ 8	—6	—8
LY249002										
10 μ M	40 $\pm$ 9	33 $\pm$ 10	30 $\pm$ 9	23 $\pm$ 6	27 $\pm$ 7	22 $\pm$ 9	31 $\pm$ 8	45 $\pm$ 14	—9	—13
100 μ M	7 $\pm$ 3	5 $\pm$ 3	2 $\pm$ 2	4 $\pm$ 3	9 $\pm$ 3	10 $\pm$ 2	15 $\pm$ 6	12 $\pm$ 7	—26	—29
Wortmannin										
20 nM	53 $\pm$ 9	44 $\pm$ 7	41 $\pm$ 8	34 $\pm$ 7	30 $\pm$ 6	24 $\pm$ 5	48 $\pm$ 10	39 $\pm$ 7	—14	—19
100 nM	5 $\pm$ 3	0	4 $\pm$ 2	3 $\pm$ 2	7 $\pm$ 2	2 $\pm$ 2	10 $\pm$ 4	4 $\pm$ 1	—25	—30
PD98059										
10 μ M	95 $\pm$ 9	98 $\pm$ 8	97 $\pm$ 8	92 $\pm$ 5	90 $\pm$ 12	93 $\pm$ 8	89 $\pm$ 7	86 $\pm$ 10	—4	—2
100 μ M	88 $\pm$ 8	82 $\pm$ 10	92 $\pm$ 11	85 $\pm$ 8	81 $\pm$ 9	85 $\pm$ 6	93 $\pm$ 8	90 $\pm$ 7	—5	—7
Rottlerin										
20 μ M	93 $\pm$ 6	90 $\pm$ 8	94 $\pm$ 5	98 $\pm$ 7	88 $\pm$ 10	91 $\pm$ 12	102 $\pm$ 8	104 $\pm$ 6	—16	—9
200 μ M	65 $\pm$ 8	61 $\pm$ 7	96 $\pm$ 6	92 $\pm$ 4	95 $\pm$ 6	96 $\pm$ 7	92 $\pm$ 9	89 $\pm$ 10	—23	—13

into trichloroacetic acid-precipitable protein in adipocytes incubated with PIG/insulin.

Compounds 41 and 37 stimulated protein synthesis in rat adipocytes in a concentration-dependent manner up to 33 % of MIR (at 20 μ M), whereas the increase in protein synthesis detected with compounds 7 and 1 was very modest (Figure 13D). Thus the ability of the PIG compounds to activate protein synthesis corresponded well to their stimulatory potency on MAPK (see Figures 13A–13C). This argues for involvement of the MAPK pathway (and presumably phosphorylation of 4E-BP1), rather than of the PI 3-K pathway, in the regulation of translation by PIG compounds in rat adipocytes. More importantly, the modest signalling of the PIG compounds via the MAPK pathway relative to the PI 3-K pathway (25–40 %), compared with insulin signalling (set at 100 % for both pathways), suggests a certain selectivity of PIG compounds for inducing metabolic signalling. This view is supported by the present finding that compounds 41 and 37 preferentially activate glucose and lipid metabolism in rat adipocytes (80–90 % of MIR) rather than protein synthesis (30 % of MIR).

Characterization of the insulin-mimetic metabolic signalling by PIG compounds using inhibitors of kinases

The observed drastic stimulation of tyrosine phosphorylation of IRS-1/3 and activation of PI 3-K by synthetic PIG compounds, accompanied by only a modest increase in p44 and p42 MAPK activity, suggests involvement of certain tyrosine kinases and the PI 3-K pathway, but not of the MAPK pathway, in the insulin-mimetic action of PIG molecules. To strengthen this view, we studied the effects of a general inhibitor of tyrosine kinases, genistein [46], and the specific inhibitors of PI 3-K, LY249002 [47] and wortmannin [11,12], as well as of MAPK kinase, PD98059 [48], on the stimulation of lipogenesis and GS by compounds 41, 37 and 7 in comparison to that by insulin (Table 4). Inhibition of PI 3-K by either LY249002 or wortmannin concentration-dependently reduced stimulation of lipogenesis and GS by both insulin and all three PIG compounds to almost basal values at 100 μ M and 100 nM respectively, which reduced

Table 5 Effects of Rab4A peptide, anti-Rab4A antibodies and anti-phosphotyrosine antibodies on glucose transport activation by compounds 41 and 37

Isolated rat adipocytes in high K^+ /low Ca^{2+} buffer were incubated for 30 min at 37 °C to stabilize the basal transport activity. The cells were then electroporated in the presence of 0.5 mM Rab4A-(191–210) peptide or Rab3C-(196–215) peptide or anti-phosphotyrosine antibodies (anti-pY; 100 μ g/ml) or anti-Rab4A antibodies (500 μ g/ml) or non-immune IgG (500 μ g/ml). Subsequently the cells were incubated in the absence or presence of insulin or compounds 41 and 37, at the concentrations indicated, for 20 min and then assayed for glucose transport. Glucose transport activity is given as pmol 2-deoxyglucose/min per mg of protein. Each value represents the mean $\pm$ S.E.M. of three independent adipocyte preparations and electroporations with incubations in the presence of stimuli and glucose transport determinations in quadruplicate each.

	Rab4A-(191-210)	Rab3C-(196-215)	Anti-Rab4A	Anti-pY	IgG
Basal	6.8 $\pm$ 1.2	7.7 $\pm$ 2.0	7.3 $\pm$ 1.5	6.4 $\pm$ 1.4	8.0 $\pm$ 2.2
Insulin					
1 nM	12.3 $\pm$ 3.9	22.7 $\pm$ 5.9	9.6 $\pm$ 3.4	7.0 $\pm$ 2.5	25.6 $\pm$ 5.3
10 nM	28.5 $\pm$ 4.7	49.6 $\pm$ 4.8	23.6 $\pm$ 5.1	11.3 $\pm$ 3.8	58.9 $\pm$ 9.1
100 nM	39.5 $\pm$ 5.4	67.4 $\pm$ 7.0	31.2 $\pm$ 4.2	15.2 $\pm$ 3.4	75.3 $\pm$ 9.4
Compound 37					
0.5 μ M	9.9 $\pm$ 2.6	15.2 $\pm$ 2.7	8.8 $\pm$ 2.4	7.1 $\pm$ 2.0	17.4 $\pm$ 2.9
2 μ M	15.7 $\pm$ 2.9	29.6 $\pm$ 3.3	11.3 $\pm$ 2.2	10.3 $\pm$ 2.9	33.6 $\pm$ 5.3
20 μ M	22.9 $\pm$ 4.1	42.5 $\pm$ 5.9	19.8 $\pm$ 4.8	13.2 $\pm$ 2.6	48.3 $\pm$ 7.5
50 μ M	27.3 $\pm$ 5.6	49.3 $\pm$ 5.0	23.0 $\pm$ 4.5	14.1 $\pm$ 2.3	55.3 $\pm$ 8.1
Compound 41					
0.5 μ M	13.8 $\pm$ 1.9	24.0 $\pm$ 2.9	10.2 $\pm$ 1.7	6.2 $\pm$ 1.8	26.2 $\pm$ 3.0
2 μ M	19.0 $\pm$ 2.5	38.7 $\pm$ 3.1	17.2 $\pm$ 2.4	12.3 $\pm$ 2.1	43.5 $\pm$ 5.2
20 μ M	28.4 $\pm$ 3.9	46.3 $\pm$ 5.9	26.4 $\pm$ 3.7	17.5 $\pm$ 2.0	51.4 $\pm$ 6.5
50 μ M	31.5 $\pm$ 3.2	52.9 $\pm$ 6.1	29.6 $\pm$ 3.3	19.3 $\pm$ 2.8	59.6 $\pm$ 7.9

PI 3-K activity (in the absence as well as presence of insulin/PIG) to slightly below basal values. At inhibitor concentrations that provoked about 30–70 % inhibition of PIG-induced lipogenesis and GS activity, PIG-induced PI 3-K activity was blunted by 80–90 % compared with the control (results not shown). In contrast, blockade of the MAPK pathway by PD98059 or

simultaneous inhibition of protein kinase C (in particular the isoforms α , β , γ , δ , ϵ , η and ζ) and calmodulin-dependent kinase II by the (general protein kinase C) inhibitor, rottlerin, did not significantly affect the insulin-mimetic activity of the PIG compounds. Inhibition of tyrosine phosphorylation by genistein caused a concentration-dependent blockade of activation of both lipogenesis and GS by insulin as well as compounds 41, 37 and 7. However, the insulin effects tended to be more sensitive towards genistein compared with PIG action which is compatible with distinct tyrosine kinases being involved in insulin and PIG signalling (e.g. for phosphorylation of IRS-1). All the inhibitors used led to no significant reduction or only minor reduction of the basal lipogenesis or GS activities (Table 4).

Although genistein is a relatively specific inhibitor of tyrosine kinase activity, it is conceivable that the prevention of lipogenesis stimulation by the PIG compounds was caused by some other non-specific effect of this inhibitor. To address this issue, monoclonal anti-phosphotyrosine antibodies were introduced into isolated rat adipocytes by electroporation [49]. A number of studies have demonstrated that electroporation can be employed to introduce antibodies into mammalian cells without affecting the physiological integrity of the cells [30]. Our data confirm that this procedure *per se* was compatible with maintenance of the signalling pathways used by either insulin or the PIG compounds, although the fold-stimulation of glucose transport by compounds 37/41 and insulin was reduced by about 20 and 50 % respectively compared with untreated control cells (Table 5). This was due to both increased basal and diminished activatable transport activity of the electroporated adipocytes. Nevertheless, the relative difference in potency between the two PIG compounds was also observed with the electroporated cells, in that the maximal response was higher and the concentration-response shifted to the left for compound 41 compared with compound 37 (see Table 5, last column). The efficiency of the transfer of the antibodies was demonstrated by the about 85 % reduction of the insulin-induced glucose transport in cells loaded with anti-phosphotyrosine antibodies compared with those loaded with control IgG. The stimulation of glucose by compounds 41 and 37 was blocked in the presence of the anti-phosphotyrosine antibodies by about 70 and 80 % respectively compared with control IgG. Obviously the blockade of tyrosine phosphorylation in response to either insulin or PIG compounds by introduction of the anti-phosphotyrosine antibodies was somewhat less efficient than using the inhibitor genistein. Taken together, the effects of the cell-permeable tyrosine kinase and PI 3-K inhibitors and of the introduced anti-phosphotyrosine antibodies confirm the requirement of tyrosine phosphorylation and PI 3-K activation for the metabolic activity of the synthetic PIG compounds.

Rab4 is involved in stimulation of glucose transport by PIG compounds

Finally we wanted to evaluate whether the synthetic PIG compounds and insulin also share the same signalling components downstream of GSK-3 for regulation of terminal effector systems, such as activation of glucose transport and the underlying mechanism of GLUT4 translocation. The Rab family proteins of Ras-related small GTP-binding proteins have been implicated in regulation of intracellular vesicular traffic [50]. It has recently been demonstrated that Rab4 is associated with GLUT4-containing vesicles in rat adipocytes and is redistributed from the vesicles to the cytosol upon insulin stimulation [51]. In contrast, Rab4A was barely detectable in GLUT4-containing vesicles in rat cardiac myocytes, although insulin induced an

extensive shift of Rab4A from the cytosol and the microsomal fraction to the plasma membrane [52]. A recent study showed that a synthetic peptide corresponding to the Rab4 hypervariable C-terminal domain, Rab4A-(191–210), and anti-Rab4A antibodies transferred into rat adipocytes by electroporation, inhibited insulin-stimulated glucose transport and GLUT4 translocation by about 50 %, without affecting the basal transport activity [53]. Thus Rab4A seems to play a crucial role in the terminal steps of insulin signalling to the glucose transport system. Consequently, we studied the effects of the synthetic Rab4A-(191–210) peptide and of polyclonal anti-Rab4A antibodies on glucose transport stimulation by compounds 37 and 41 upon transfer into rat adipocytes by electroporation in comparison to the synthetic Rab3C-(196–215) peptide and control IgG (Table 5).

As expected, insulin stimulation of glucose transport was reduced by about 60 % in adipocytes electroporated in the presence of the Rab4A-(191–210) peptide. In contrast, the Rab3C-(196–215) peptide corresponding to the C-terminal domain of Rab3C, which has been shown to inhibit prolactin release in anterior pituitary cells [54], but not insulin-induced GLUT4 translocation in rat adipocytes [53], had only a very modest effect. Basal transport activity was not affected by the two peptides. Incubation of the adipocytes with either peptide in the absence of electroporation did not result in any impairment of glucose transport activation by insulin or PIG compounds (results not shown). Loading (by electroporation) of the adipocytes with the Rab4A-(191–210) peptide led to inhibition of glucose transport stimulation by compounds 37 and 41 by 40–50 % and 50–60 % when compared with cells electroporated in the presence of control IgG or Rab3C-(196–215) peptide respectively. At submaximal concentrations of both PIG compounds, the percentage inhibition was even more pronounced. The relative efficiency of the Rab4A-(191–210) peptide in blocking insulin and PIG action was comparable. The almost negligible effects of the Rab3C-(196–215) peptide on glucose transport activation by compounds 37 and 41 suggest that PIG action was abolished by the Rab4A C-terminal domain peptide in a specific fashion.

To confirm the results obtained with the Rab4A-(191–210) peptide, we examined the effect of polyclonal anti-Rab4 antibodies on insulin- and PIG-stimulated glucose transport activity. As shown in Table 5, in adipocytes electroporated in the presence of anti-Rab4A antibodies, the insulin effect on glucose transport was diminished by about 70 %. In contrast, non-immune IgG did not show any inhibitory effect. The anti-Rab4A antibodies reduced glucose transport activation by compounds 41 and 37 by about 60 and 70 % respectively compared with control IgG at the highest compound concentration (50 μ M), but with even greater potency at submaximal concentration. Control experiments showed that the anti-Rab4A antibodies were ineffective on insulin and PIG action in intact cells (results not shown). In general, blockade of Rab4A function in mediating both insulin and PIG action was more efficient upon transfer into adipocytes of anti-Rab4A antibodies compared with Rab4A-(191–210) peptide. Taken together, these results strengthen the aforementioned view that Rab4A protein plays an essential role in PIG action on glucose transport in rat adipocytes.

DISCUSSION

Not only insulin and IGF-1 receptors, but also several cytokine and interferon receptors can induce tyrosine phosphorylation of IRS proteins. In response to ligand binding, these receptors recruit and activate cytosolic tyrosine kinases of the Janus kinase

family, which in turn associate with and phosphorylate IRS-1 and IRS-2. More recently, IRS-1 has also been shown to be phosphorylated on tyrosine residues in response to angiotensin II and gastrin, ligands that bind to specific seven-transmembrane domain receptors. Ligand-dependent tyrosyl phosphorylation of proteins in the size range (160–170 kDa) appropriate for IRS-1 has also been observed for growth hormone and a number of other ligands that bind to members of the haematopoietin receptor family. This suggests that these ligands might utilize IRS-1 as a signalling molecule. In addition, PIG-P from yeast has recently been demonstrated to cause tyrosine phosphorylation of IRS-1 in isolated rat cardiomyocytes and adipocytes [22]. The present results are compatible with the view that different signalling pathways can use the IRS protein, in general, possibly as the site for cross-talk. The apparent correlation between the relative increment in IRS-1/3 tyrosine phosphorylation and PI 3-K activation for the synthetic PIG compounds studied here, as well as the identical ranking between five different molecules for both processes, strongly argues that PIG molecules exert (part of) their insulin-mimetic metabolic signalling via both IRS-1 and IRS-3.

Interestingly, adipocytes isolated from IRS-1 null mice revealed that IRS-1 plays a role in mediating 30–50 % of metabolic insulin action in adipocytes from wild-type mice [55], suggesting that other substrates exist that mediate the residual insulin action in adipocytes from IRS-1 null mice. A major tyrosine-phosphorylated 60 kDa protein, called pp60 or IRS-3, was found to coimmunoprecipitate with the p85 subunit of PI 3-K and this protein was detected in adipocytes but not in livers or muscles from wild-type and IRS-1 null mice [56]. The predicted amino acid sequence of IRS-3 contains an N-terminal pleckstrin homology domain followed by a phosphotyrosine-binding domain and a group of potential tyrosine phosphorylation sites, four of which are in the YXXM motif that binds to the SH2 domains of PI 3-K (for a review see [57]). This structure indicates that IRS-3 may interact with the activated insulin receptor and associate via its phosphorylated tyrosine residues with SH2-containing proteins such as PI 3-K. Interestingly, unlike muscles and livers in which IRS-2 was the alternative substrate in the absence of IRS-1, the amount of tyrosine-phosphorylated IRS-2 in adipocytes is very low. Thus it was concluded that pp60 but not IRS-2 is one major substrate that principally associates with PI 3-K in adipocytes and regulates the insulin-induced GLUT4 translocation in an additive fashion together with IRS-1. Our present findings that PIG compounds with potent insulin-mimetic activity (e.g. on glucose transport) are strong activators of tyrosine phosphorylation of IRS-1 and IRS-3, but not of IRS-2, support this view. In this regard, the comparative analysis of the signalling pathways used by synthetic PIG compounds and insulin may represent a valuable tool for evaluation of the role of a specific signalling step (e.g. phosphorylation of IRS-1/3 versus IRS-2) for a given biological activity of insulin.

Although previous results from other laboratories and the results presented here indicate that multiple ligands and compounds can utilize IRS-1 as a signalling molecule, the signalling response does not need to be identical for every ligand. IRS-1 contains at least 20 potential tyrosyl phosphorylation sites, at least eight of which are phosphorylated after insulin stimulation (for a review see [40]). Those in IRS-3 have not been identified so far. Consistent with the ability of SH2 domains in cellular proteins to bind with high affinity to phosphorylated tyrosyl residues in specific sequence motifs (for review see [57]), it has been demonstrated that SH2 domains from the 85 kDa regulatory subunit of PI 3-K, Grb2, Nck and the protein tyrosine phosphatase SHP2 bind to unique residues within IRS-1 (for reviews

see [40,41]). Differences in specificity of the kinase(s) activated in response to PIG compound compared with insulin could result in phosphorylation of unique subsets of IRS-1/3 tyrosyl residues and thereby alter the downstream signalling pathways activated. One would expect differences in the magnitude of the signalling response due to the differences in the ability of each compound to activate the relevant tyrosine kinase(s). There might also be differences in the specific response if the number of tyrosine residues phosphorylated differs as well. IRS-1/3-mediated responses unique to a specific PIG compound could also arise if signalling pathways unique to that compound acted in concert with pathways originating from IRS-1/3. Determination of the tyrosyl residues phosphorylated in IRS-1/3 and identification of the particular proteins that bind to IRS-1/3 and become activated in response to PIG compounds will deepen our understanding of the similarities and differences in cellular signalling originating from IRS-1/3 in response to stimuli that cause their tyrosine phosphorylation. Such differences may be reflected in the significantly lower activation of the MAPK pathway (both p42 and p44 isoforms) and protein synthesis by the PIG compounds compared with insulin, which cannot be explained solely by the rather small differences in the efficiency of tyrosine phosphorylation of IRS-1/3.

The molecular mechanism through which the PIG compounds provoke tyrosine phosphorylation of IRS-1/3 remains to be elucidated. Recently we observed tyrosine phosphorylation of the caveolar marker protein, caveolin, in isolated rat adipocytes and diaphragms upon challenge with PIG-P from yeast [58]. Tyrosine phosphorylation of caveolin has also been described in response to insulin in intact 3T3-L1 adipocytes and *in vitro* with caveolae isolated from insulin-treated cells [59]. The kinase responsible was suggested to be identical with the caveolae-associated acylated non-receptor tyrosine kinase p59^{lyn}, rather than the insulin receptor, which was barely detectable in the caveolae preparation [59]. Interestingly, recent findings from our laboratory suggest binding of PIG-P to a trypsin/salt- and *N*-ethylmaleimide-sensitive protein located in adipocyte caveolae, which seems to be involved in mediating the insulin-mimetic metabolic effects [58]. Taken together, these results open the possibility that the PIG compounds use a non-receptor tyrosine kinase residing in certain areas of the plasma membrane, the caveolae, for induction of tyrosine phosphorylation of IRS-1/3. Obviously, IRS-3 represents a better substrate for this putative tyrosine kinase compared with IRS-1 in adipocytes, which may be based on proper substrate presentation and localization. Alternative or supplementary modes for an increase in the tyrosine phosphorylation state of IRS-1/3, such as stimulation of growth factor or interleukin receptors as well as inhibition of certain (IRS-1/3-specific) tyrosine phosphatases, cannot be excluded, so far, as the molecular basis of PIG action on IRS-1/3.

In a study using adipocytes from IRS-1 null mice, glucose transport activation by insulin did not appear to simply parallel PI 3-K stimulation, although PI 3-K was found to be required for this process [55]. At 1 nM insulin, glucose transport was increased in adipocytes from IRS-1 null mice to only about 50 % of that in cells from wild-type mice, although PI 3-K activity was comparable between adipocytes from wild-type and IRS-1-deficient mice. In contrast, we observed an almost perfect correlation between the PIG-induced increase of glucose transport/GLUT4 translocation and PI 3-K in that: (i) the relative ranking of compounds 41, 37, 7 and 1 was identical for activation of these processes, and (ii) the percentage increase or fold stimulation above basal activity was comparable between glucose transport and PI 3-K.

Stimulation of glucose transport and GLUT4 translocation, in the absence of concomitant activation of PI 3-K, has been observed in several systems. Exercise or contraction of skeletal muscle cells or cardiomyocytes is a potent activator of glucose transport and GLUT4 translocation, which occurs in a wortmannin-insensitive manner (for a review see [1]). Furthermore, GLUT4 translocation induced by other stimuli, such as the phosphatase inhibitor okadaic acid, or uncouplers of oxidative phosphorylation, is also independent of PI 3-K activation. The ability of osmotic shock to stimulate glucose transport and GLUT4 translocation in 3T3-L1 adipocytes does not require PI 3-K and PKB [60]. In contrast, the results presented in this study reveal that the potent stimulation of glucose transport and GLUT4 translocation (up to 50 % of MIR) by PIG compounds in isolated rat adipocytes is apparently correlated with activation of PI 3-K and PKB and inhibition of GSK-3. Whereas the involvement of PI 3-K in metabolic PIG action could be demonstrated by using wortmannin or LY249002, the definitive proof for requirement of PKB and GSK-3 necessitates the development of specific inhibitors for these enzymes.

The present finding that Rab4A function is required for glucose transport activation by compounds 41 and 37 strongly argues for similarities in the molecular mechanism of GLUT4 translocation used by insulin and the synthetic PIG compounds in rat adipocytes. At present, the mechanism of the inhibition of PIG or insulin action on glucose transport by the Rab4A-(191–210) peptide is not clear. Because the C-terminal domain of Rab proteins contains structural elements necessary for the association with target membranes, the Rab4A-(191–210) peptide probably competes with endogenous Rab4A at the target(s). Recent studies indicate that one of the steps affected by this peptide is the insulin-induced exocytotic recruitment from the intracellular retention pool to the plasma membrane, rather than the insulin-induced blockade of the internalization of GLUT4 molecules, and that Rab4A may be involved in the former crucial step of insulin action [53]. Thus, the marked reduction of glucose transport stimulation by compounds 37 and 41 in adipocytes loaded with either the Rab4A-(191–210) peptide or anti-Rab4A antibodies would be compatible with involvement of Rab4A in promoting PIG-induced exocytotic fusion of GLUT4 vesicles with the plasma membrane in insulin-sensitive cells.

Taken together, the experimental evidence so far available suggests that the synthetic PIG compounds and insulin use the same signalling pathway between IRS-1/3 and terminal metabolic effector systems. Thus, the ability of PIG compounds to specifically control the activity of the corresponding signalling components, i.e. IRS-1/3, PI 3-K, PKB, GSK-3 and Rab4A, independent of the insulin receptor kinase, accompanied by only mild stimulation of the MAPK cascade, makes their site of action potentially interesting as a target amenable to therapeutic intervention in the management of insulin-resistant states, such as Type II diabetes and metabolic syndrome.

We thank Mrs. A. Unkelbach (Hoechst Marion Roussel Deutschland GmbH, Frankfurt, Germany) for expert assistance in preparing the Figures, and Professor J. Eckel (Diabetes Research Institute Düsseldorf, Germany) for helpful discussions.

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Received 18 June 1998/10 August 1998; accepted 28 August 1998