Insulin stimulation of pyruvate dehydrogenase in adipocytes involves two distinct signalling pathways

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In isolated rat adipocytes, the insulin stimulation of pyruvate dehydrogenase can be partially inhibited by inhibitors of PI3K (phosphoinositide 3-kinase) and MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase). In combination, U0126 and wortmannin completely block the insulin stimulation of pyruvate dehydrogenase. It is concluded that the effect of insulin on pyruvate dehydrogenase in rat adipocytes involves two distinct signalling pathways: one is sensitive to wortmannin and the other to U0126. The synthetic phosphoinositolglycan PIG41 can activate pyruvate dehydrogenase but the activation is only approx. 30 % of the maximal effect of insulin. This modest activation can be completely blocked by wortmannin alone, suggesting that PIG41 acts through only one of the pathways leading to the activation of pyruvate dehydrogenase.

Key words: fatty acid synthesis, mitochondria, mitogen-activated protein kinase, phosphoinositide 3-kinase, phosphoinositolglycan.

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex is an intramitochondrial multi-enzyme complex that catalyses the oxidative decarboxylation of pyruvate and the concomitant formation of acetyl-CoA, CO_2 and NADH [1]. The complex is regulated by reversible phosphorylation and, in mammalian tissues, is essentially inactive in the phosphorylated state and active in the non-phosphorylated state [2,3]. Interconversion is brought about by highly specific kinases and phosphatases. Currently, four isoforms of the PDH kinase have been identified in mammalian tissues [4–8]. The kinases are expressed in a tissue-specific manner and differ substantially in kinetics and allosteric modulation [9]. PDH phosphatase (PDP) appears to be a heterodimer of catalytic and regulatory subunits [10,11]. To date, two isoforms of the catalytic subunit have been identified with different sensitivities to Mg^{2+}, Ca^{2+} and spermine [12].

Insulin activates PDH in adipocytes [13] and other lipogenic tissues [14–16] by increasing the proportion of the complex in the active, dephosphorylated form. This activation is of importance because, together with the activation of glucose transport and acetyl-CoA carboxylase, it represents the means by which insulin increases the conversion of glucose to fatty acids [17]. Extensive studies on mitochondria prepared from control and insulin-treated adipocytes have indicated that the effect of insulin on the activity of PDH within adipocytes is largely brought about through the stimulation of PDP involving a decrease in $K_{0.5}$ for Mg^{2+} [18,19].

The signalling system whereby insulin acting through the insulin receptor located at the plasma membrane brings about the activation of PDP within adipocyte mitochondria is poorly understood. It is well established that many of the metabolic activations of insulin are brought about through the activation of phosphoinositide 3-kinase (PI3K) and the subsequent activation of protein kinase B (PKB) [20]. However, recent studies from this laboratory using intact rat epididymal fat pads concluded that this signalling pathway may not be involved in the activation of PDH by insulin, since this activation was essentially unaffected by concentrations of the inhibitor of PI3K, wortmannin, that blocked other actions of insulin including the activation of PKB [21]. In this study we report results using adipocytes isolated from rat epididymal fat tissue.

Many studies have implicated a soluble mediator in the metabolic actions of insulin, including the activation of PDH [22–25]. Several putative mediators have been described [26,27], some apparently containing a phosphorylated inositol group and a disaccharide moiety. However, in early work, preparations were not pure and had variable insulin mimetic effects. It was concluded that insulin might act through a phosphoinositolglycan (PIG) released from the cell surface following actions of phospholipase D or phospholipase C on the glycosylphosphatidylinositol anchor of an unknown cell-surface protein. A substantial advance in this area was made by the production of synthetic PIG molecules that have insulin-mimetic properties [28,29]. Studies by the group of Müller showed that the most potent compound, PIG41, had a wide range of insulin-mimetic effects on rat adipocytes including the activation of glucose transport and PKB. The maximal effect of PIG41 was typically 70–90 % of that of insulin but the effect on PDH was not measured. The authors conclude that PIG41 acts through a yet-to-be-fully-defined cell-surface receptor resulting in the activation of focal adhesion kinase and Lyn tyrosine kinase, and hence an increase in the tyrosine phosphorylation of insulin receptor substrate-1 and thus the activation of PI3K and PKB [28,30–32].

In the present study, rather surprisingly, PIG41 was found to have only a modest effect on PDH compared with other metabolic effects of insulin. This observation, taken together with studies on the effects of wortmannin and the specific mitogen-activated

Abbreviations used: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/ERK kinase; PDH, pyruvate dehydrogenase; PDP, PDH phosphatase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PIG, phosphoinositolglycan.

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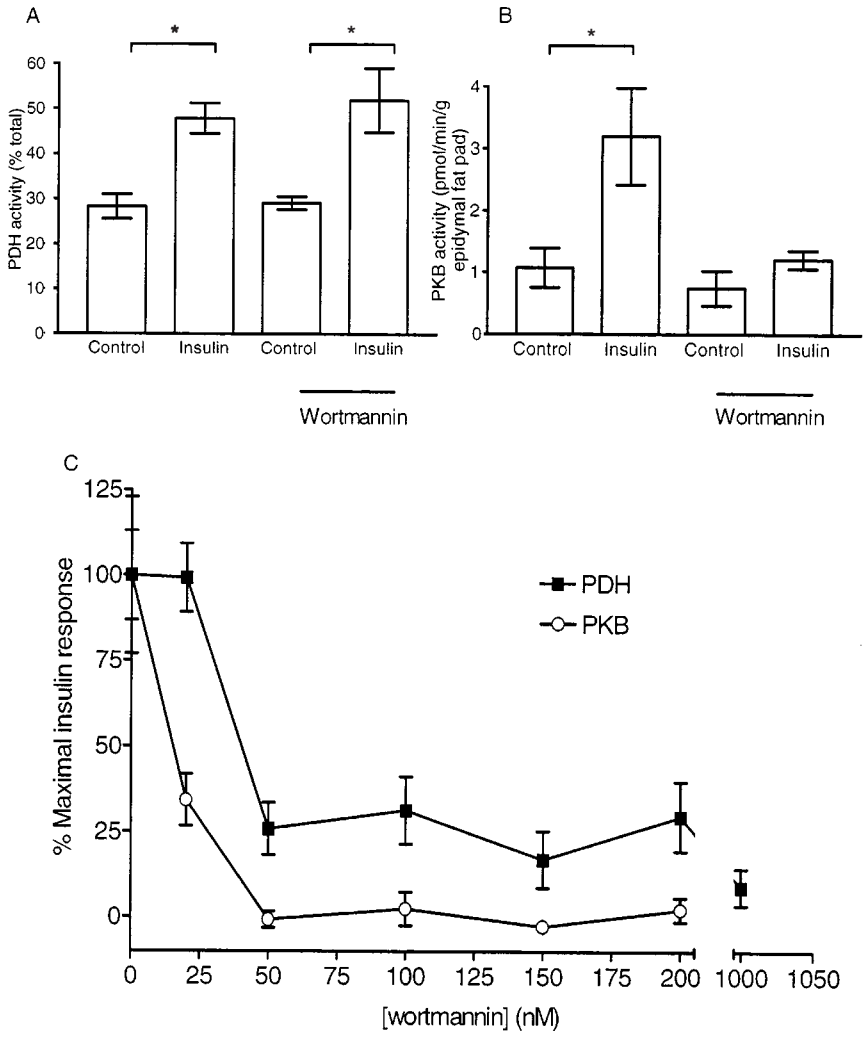

Figure 1 Effect of wortmannin on the activation of PDH and PKB by insulin in intact epididymal fat pads and isolated adipocytes

Intact epididymal fat pads were preincubated with wortmannin (100 nM) or DMSO control for 30 min before stimulation with insulin (80 nM, 10 min) and extraction for assay of (**A**) PDH and (**B**) PKB. * Indicates that the effects of insulin are significant to $P < 0.05$; means ± S.E.M. from four independent observations. (**C**) Isolated adipocytes were preincubated with a range of concentrations of wortmannin for 30 min before stimulation with insulin (80 nM, 10 min) and extraction and assay of PDH and PKB. Data are expressed as a percentage of maximal insulin response, seen in the absence of wortmannin (insulin-stimulated samples, 170 ± 16% of control; basal activity, 56 ± 5% of total PDH activity in the absence of wortmannin).

protein kinase/extracellular signal-regulated kinase (ERK) kinase 1/2 (MEK1/2) inhibitor U0126, indicates that insulin activates PDH in rat adipocytes through two distinct signalling pathways, of which only one is stimulated by PIG41.

EXPERIMENTAL

Materials

All chemicals and biochemicals were from BDH or Sigma (both of Poole, Dorset, U.K.) except acetyl-CoA (Roche, Lewes, E. Sussex, U.K.), γ-^{32}P-labelled ATP and enhanced chemiluminescence reagents (Amersham Biosciences, Little Chalfont, Bucks., U.K.), collagenase (Worthington Biochemical Corporation, Freehold, NJ, U.S.A.), anti-active-ERK antibody and U0126 (Promega, Southampton, U.K.), and PIG41 and PIG1 (gifts from Dr Günter Müller, Aventis, Frankfurt, Germany). Wortmannin (10 mM stock solution) and U0126 (10 mM stock solution) were dissolved in DMSO and stored in aliquots at −20 °C.

Preparation, incubation and extraction of rat epididymal fat pads

Animals were killed and their epididymal fat pads removed and placed in gassed Krebs–Ringer buffer [33] containing 5 mM glucose equilibrated with 95% O_2/5% CO_2. After 30 min of equilibration in the presence of inhibitor or DMSO control, half the pads were stimulated with 80 nM insulin for 10 min. The pads were blotted briefly and then frozen in liquid nitrogen. Polytron extraction was performed on the frozen pads with 2 ml of extraction buffer/g of tissue [extraction buffer: 100 mM KH_2PO_4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 5% (v/v) rat serum and 0.1% Triton X-100, pH 7.2] followed by freeze–thawing to ensure complete mitochondrial breakage. The samples were centrifuged for 5 min in a bench-top centrifuge and the infranatant taken for assay.

Preparation, incubation and extraction of primary rat adipocytes

Primary rat adipocytes were prepared from the epididymal fat pads of male Wistar rats (180–220 g) as in [34]. Adipocytes were pre-incubated in 2 ml samples (approx. 20 % cytocrit) for 30 min in a gassed Krebs–Ringer buffer plus 1 % BSA and 5 mM glucose and then incubated in the same media with additions of insulin and synthetic PIG as given in the Figure legends and text. Additions of inhibitors were made 30 min before additions of insulin or PIG. The cells were rapidly extracted by removing the infranatant below the floating fat cells and snap-freezing the cells in 200 μl of extraction buffer (see above). The samples were freeze–thawed three times to ensure complete cell and mitochondrial breakage and centrifuged at 14000 ***g*** for 5 min at 4 °C and the infranatant taken for assay.

Assay of PDH and PKB activities

These enzymes were assayed as described previously [35].

Detection of active ERK using Western blots

Fat cell extract prepared as above (15 μl) was mixed with 5 μl of sample buffer [50 mM Tris/HCl, pH 6.8, 20 % (v/v) glycerol, 4 % SDS, 10 % (v/v) 2-mercaptoethanol and 0.1 % (w/v) Bromophenol Blue] and boiled for 5 min. Samples were separated by SDS/PAGE (10 % gel) as in [36] and transferred to Immobilon P membrane using semi-dry blotting. The membrane was then immersed in anti-active-ERK antibody (1:2000 dilution) for 1 h followed by extensive washing in TBS/Tween (50 mM Tris/HCl, pH 7.6, 150 mM NaCl and 0.05 % Tween 20) and a 1 h incubation in anti-rabbit horseradish peroxidase conjugated secondary antibody (1:5000 dilution). Binding of the antibody was detected by enhanced chemiluminescence. Blots were stripped of bound antibody by three 1 h washings in 1 M glycine at pH 2.5. This was then reblocked and probed as above but with an anti-ERK antibody to detect the total amount of ERK present. Multiple exposures of each immunoblot were produced to ensure that the quantification of intensity using ImageQuant was in the linear range.

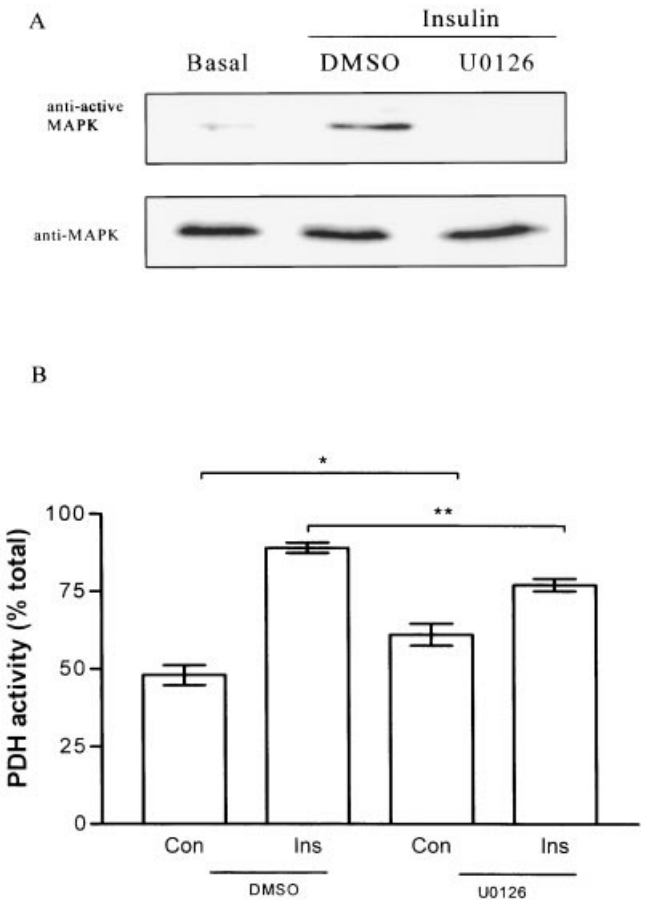

Figure 2 Effect of U0126 on the insulin stimulation of ERK/mitogen-activated protein kinase and PDH

(**A**) Effect of U0126 on ERK/mitogen-activated protein kinase activity. Immunoblot of total cell lysate from cells treated in parallel with U0126 (10 μM) or DMSO control for 30 min. The upper blot was probed with anti-active-ERK antibody that detects double-phosphorylated ERK1/2. The lower blot shows a reprobe with anti-ERK1/2 antibody which detects total ERK1/2. Results are representative of five experiments. (**B**) Effect of pre-incubation with U0126 (10 μM) for 30 min on the activation of PDH by insulin in isolated adipocytes. Data shown are means $\pm$ S.E.M. from five observations. The significance of the effects is indicated by * ($P < 0.05$) and ** ($P < 0.01$).

RESULTS AND DISCUSSION

In agreement with our earlier study [21], 100 nM wortmannin had no discernable effect on the stimulation of PDH by insulin in intact epididymal fat pads, whereas the effect of insulin on PKB activity was inhibited by about 90 % (Figures 1A and 1B). However, when dose–response studies were carried out with isolated adipocytes prepared from rat epididymal fat pads it was clear that wortmannin in this system was able to cause a partial inhibition of the insulin stimulation of PDH when wortmannin was present at a concentration above 20 nM (Figure 1C). At concentrations of wortmannin between 50 and 200 nM, approx. 25 % of the maximal insulin stimulation remained. In contrast, the effects of insulin on PKB activity and glucose uptake were completely blocked under these conditions (Figure 1C and results not shown). It also appears that the insulin stimulation of PDH requires a higher concentration of wortmannin than the insulin stimulation of PKB to produce the same amount of inhibition: at 20 nM wortmannin there was no detectable effect on the insulin activation of PDH, whereas the insulin activation of PKB was reduced by around 70 %. The likely explanation for the earlier observations with intact epididymal fat pads [21] is that wortmannin is less able to permeate the pads than isolated adipocytes and observations at 100 nM wortmannin with the pads are comparable with 20 nM wortmannin in isolated adipocytes. Differences in the effects of wortmannin between intact pads and isolated adipocytes may only be apparent in the measurement of the insulin stimulation of PDH due to its reduced and partial sensitivity to wortmannin when compared with other actions of insulin.

The MEK1/2 inhibitor U0126, at a concentration of 10 μM, was found to completely inhibit the increased phosphorylation of ERK1/2 in insulin-treated isolated rat adipocytes (Figures 2A and 3C). Associated with this inhibition was a 75 % decrease in the effect of insulin on PDH activity. However, it should be noted that this was partially due to an increase in PDH activity in the absence of insulin. However, U0126 also had a significant effect on the activity of PDH in the presence of insulin. U0126 (10 μM) had no detectable effect on glucose uptake in the presence or absence of insulin (results not shown). In the presence of both U0126 (10 μM) and wortmannin (100 nM) the effect of insulin on PDH activity was completely blocked (Figure 3). As

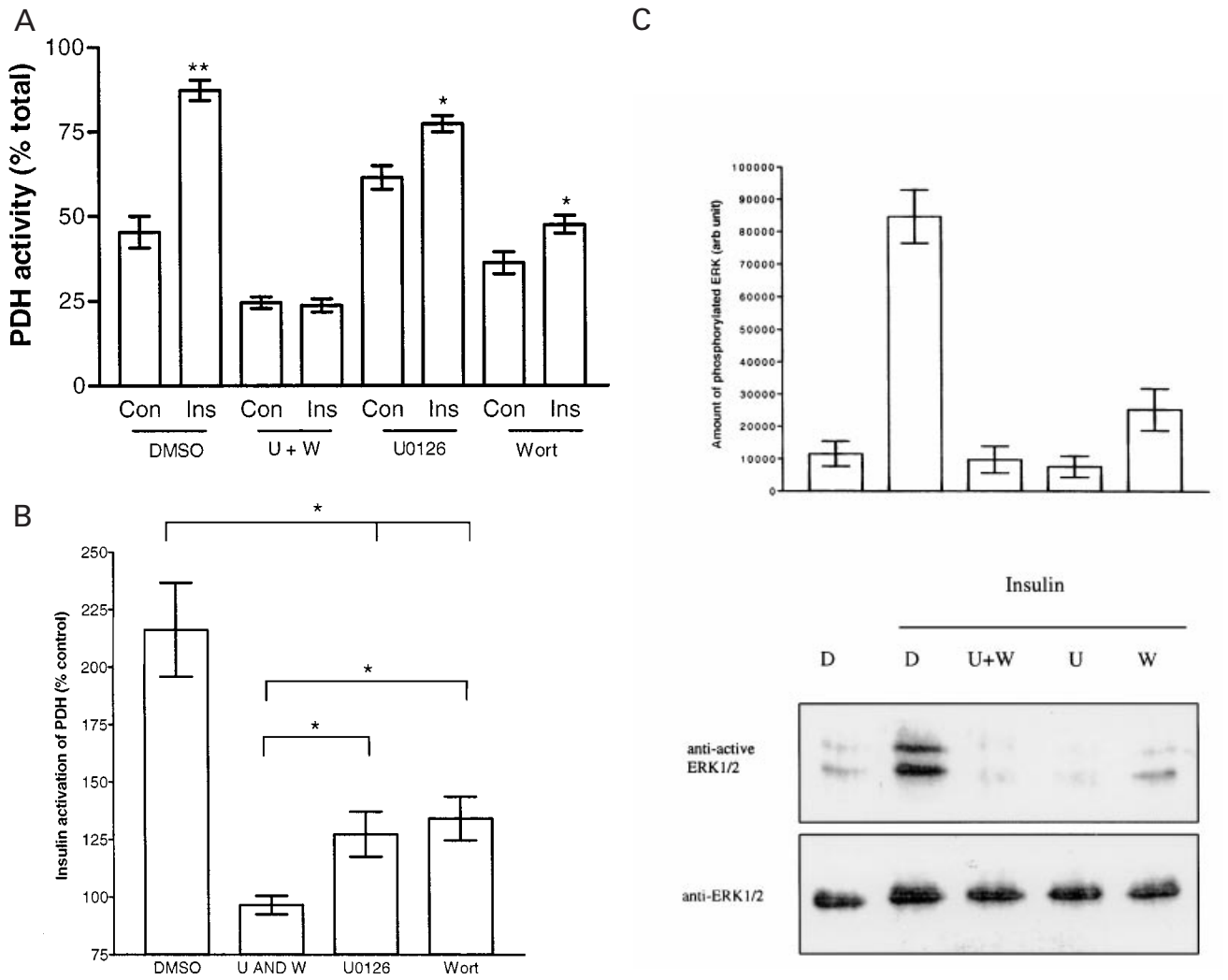

Figure 3 Synergistic effects of U0126 (10 μM) and wortmannin (100 nM) on the activation of PDH by insulin in isolated adipocytes

(**A**) PDH activity as percentage of total PDH activity in control and insulin-treated adipocytes in the presence of DMSO, or U0126 (U), wortmannin (Wort) or both. Data shown are means $\pm$ S.E.M. from five observations. The significance of the effects of insulin is indicated by * ($P < 0.05$) and ** ($P < 0.01$). (**B**) Data re-expressed as insulin activation of PDH as a percentage of the control. (**C**) The effect of wortmannin and U0126 on the activation of ERK1/2 by insulin. D, DMSO; U, U0126; W, wortmannin. The histogram is a quantification of five immunoblots detecting dual-phosphorylated active ERK1/2. A representative immunoblot is shown probed with anti-active-ERK1/2 antibody (upper panel) and reprobed with anti-ERK1/2 antibody (lower panel).

found previously [37], wortmannin alone resulted in a decrease in the phosphorylation and activation of ERK1/2 in these cells. A small activation of ERK remained in the presence of wortmannin, which may be sufficient for activation of PDH via the putative ERK-dependent pathway. A more detailed measurement of the insulin-activation of ERK in the presence of 100 nM wortmannin showed a fold activation of 1.8 ± 0.3 ($P < 0.05$) compared with 10.5 ± 2.3 fold in the absence of wortmannin (seven observations).

These observations are compatible with insulin activating PDH in rat adipocytes through two signalling pathways. One pathway is inhibited by wortmannin, whereas the other is inhibited by U0126. It is necessary to completely block both pathways to see a complete inhibition of the insulin stimulation of PDH.

Studies with the synthetic PIG41 fit into this proposed model (Figure 4). Earlier studies [29,38] indicated that PIG41 is able to stimulate PKB in isolated rat adipocytes to approx. 80% of the maximal response to insulin, with a half-maximal effect at about 5 μM. PIG1, a related PIG, had no insulin-mimetic effects. Comparable effects were found in the present study (Figure 4A). However, when the effects of PIG41 on PDH were studied the maximal effect was only 30 ± 15% of that of insulin. PIG1 was without any appreciable effect. Wortmannin caused complete abolition of the effects of PIG41 on PDH (Figure 4B).

In conclusion, the means whereby insulin is able to influence the activity of PDH appears to involve a combination of two distinct signalling pathways. The wortmannin-sensitive pathway, which is also activated by PIG41, may involve PI3K since both insulin and PIG41 stimulate this enzyme. However, other members of the PI3K superfamily are inhibited by wortmannin [39] and the sensitivity of the insulin stimulation of PDH appeared to be somewhat lower than the insulin stimulation of PKB. The second pathway is likely to involve ERK1/2 because of the inhibitory effects of the MEK 1/2 inhibitor U0126. PIG41 would not be expected to stimulate this pathway, as earlier studies have shown that PIG41 has only a small stimulatory effect on ERK1/2 compared with that of insulin [38].

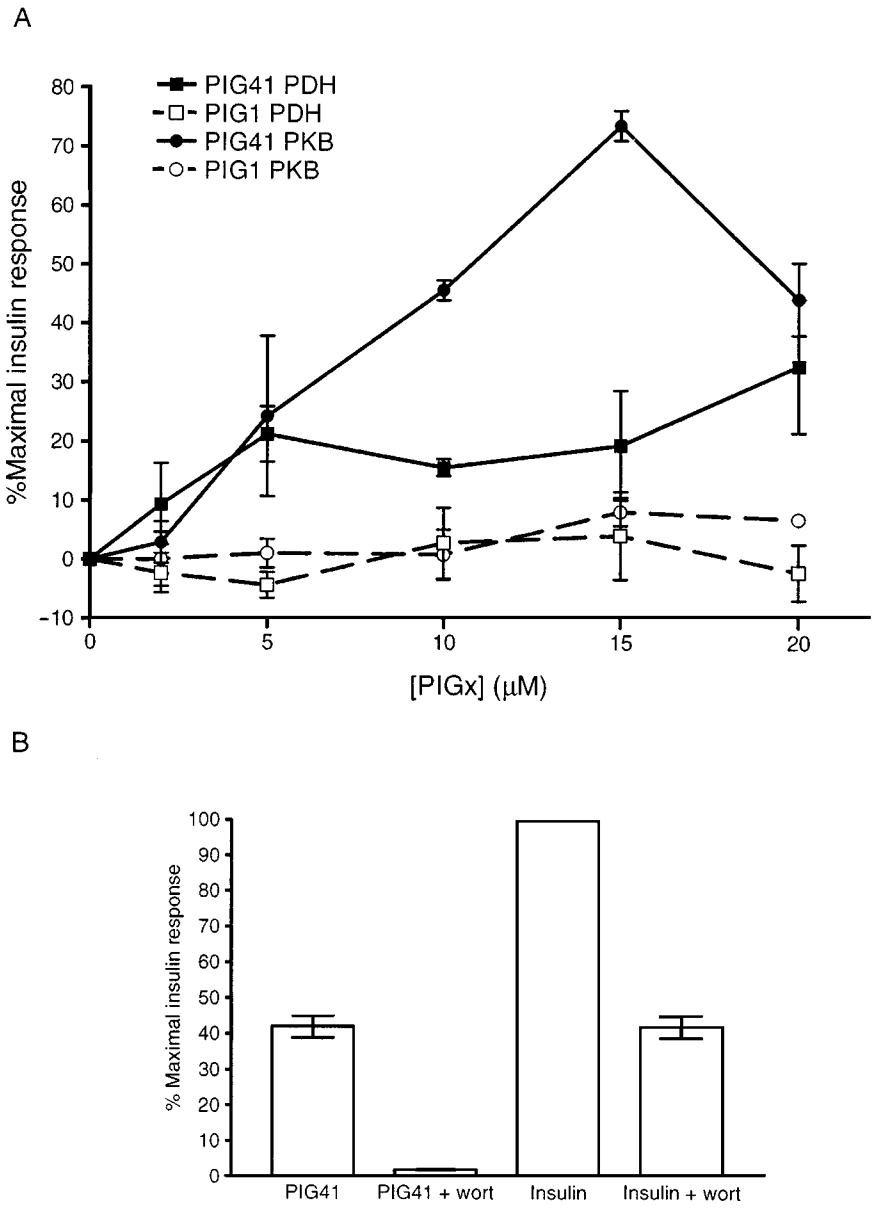

Figure 4 Effect of PIG41 and PIG1 on the activity of PKB and PDH in isolated adipocytes

(**A**) Data are expressed as the means ± S.E.M. of the percentage maximum insulin response ($n = 5$ observations). The insulin effect on PDH activity was 217 ± 30% of basal. Insulin response of PKB activation was 10.5 ± 5.1-fold. (**B**) Effect of PIG41 or insulin, with or without inhibition by wortmannin (100 nM), on PDH activity in isolated adipocytes. The activation of PDH is expressed as a percentage of maximum insulin response; data are expressed as means ± S.E.M. from three (PIG41) and six (insulin) observations. Insulin-stimulated value was 149 ± 1% of the control.

While the present paper was in preparation, Caruso et al. [40] reported evidence suggesting that protein kinase Cδ was involved in the stimulation of PDH activity in L6 skeletal muscle cells and immortalized mouse hepatocytes (Hep cells). However, insulin may activate PDH by different mechanisms in these cells to those in primary rat adipocytes, as no inhibition of the effect of insulin on PDH was observed with either wortmannin or the MEK1/2 inhibitor PD98059. Nevertheless, a surprising aspect of the studies of Caruso et al. [40] was the near-complete inhibition of the activation of PDH by insulin in the presence of Rottlerin (which was assumed to be a specific inhibitor of protein kinase Cδ). Other studies have shown that in fact Rottlerin has no effect on this protein kinase, although it does inhibit a number of other protein kinases some of which are downstream of PI3K, including 3-phosphoinositide-dependent protein kinase 1, PKB and glycogen synthase kinase 3β [39]. These authors also concluded that insulin caused protein kinase Cδ to translocate into mitochondria and to phosphorylate and activate the intramitochondrial PDP. This would require the rapid transfer of protein kinase Cδ across the mitochondrial membranes but the authors do not comment on how this might be achieved.

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