Insulin activates protein kinase B, inhibits glycogen synthase kinase-3 and activates glycogen synthase by rapamycin-insensitive pathways in skeletal muscle and adipose tissue

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Abstract Insulin stimulated protein kinase Bα (PKBα) more than 10-fold and decreased glycogen synthase kinase-3 (GSK3) activity by 50 ± 10% in skeletal muscle and adipocytes. Rapamycin did not prevent the activation of PKB, inhibition of GSK3 or stimulation of glycogen synthase up to 5 min. Thus rapamycin-insensitive pathways mediate the acute effect of insulin on glycogen synthase in the major insulin-responsive tissues. The small and very transient effects of EGF on phosphatidylinositol (3,4,5)P_3 PKBα and GSK3 in adipocytes, compared to the strong and sustained effects of insulin, explains why EGF does not stimulate glucose uptake or glycogen synthesis in adipocytes.

Key words: Insulin; EGF; Protein kinase B; Glycogen synthase kinase 3; Glycogen synthase; Rapamycin

1. Introduction

Insulin triggers the dephosphorylation and activation of glycogen synthase within minutes in skeletal muscle and adipose tissue, an important event in the stimulation of glycogen synthesis by this hormone. In skeletal muscle, most of the phosphate is released from a region of glycogen synthase that is phosphorylated in vitro by glycogen synthase kinase-3 (GSK3) [1,2] and dephosphorylated by a glycogen-associated form of protein phosphatase-1 (PP1.G_M) [3,4]. These findings suggest that insulin activates glycogen synthase by inhibiting GSK3 and/or activating PP1.G_M, and there is evidence that both of these mechanisms may operate in vivo. In skeletal muscle, insulin increases the phosphorylation of the glycogen-binding (G_M) subunit at Ser-48, which should increase the rate at which PP1.G_M dephosphorylates glycogen synthase [5], while in several cell lines insulin has been shown to inhibit GSK3 [6,7] by inducing its phosphorylation at serine/threonine residues [7–9].

In rat L6 myotubes, a skeletal muscle cell line, the residues in GSK3 that become phosphorylated in response to insulin are Ser-21 in GSK3α and Ser-9 in GSK3β [10], raising the question of which insulin-stimulated protein kinase phosphorylates these sites in vivo. This problem is complex because three distinct insulin-stimulated protein kinases catalyse the phosphorylation of Ser-21 and Ser-9 in vitro, namely p70 S6 kinase [11,12], MAP kinase-activated protein kinase-1 (MAPKAP-K1, also known as Rsk) [11,12] and protein kinase B (PKB, also known as c-Akt) [10]. In L6 myotubes [10] human myoblasts [13] or primary adipocytes [14], neither rapamycin (a drug which prevents the activation of p70 S6 kinase) nor PD 98059 (which inhibits activation of the MAP kinase pathway and hence the activation of MAPKAP-K1 [15]) have any effect on the inhibition of GSK3 by insulin. Moreover, the half-time for activation of p70 S6 kinase or MAPKAP-K1 by insulin (5–7 min) is much slower than the half-time for inhibition of GSK3 (2 min) [10,13]. Thus neither p70 S6 kinase nor MAPKAP-K1 are rate limiting for GSK3 inhibition by insulin in L6 myotubes, human myoblasts or primary adipocytes.

The activation of PKB by insulin, like the inhibition of GSK3, is unaffected by rapamycin and/or PD 98059 in L6 myotubes [10] or human myoblasts [13], and several further pieces of evidence suggest that PKB may mediate the inhibition of GSK3 in these cells. First, the half-time for activation of PKB (1 min) is consistent with it being an upstream activator of GSK3 [10]. Second, the activation of PKB by insulin, like the inhibition of GSK3, is prevented by inhibitors of phosphoinositide (PI) 3-kinase [10,15,16]. Third, GSK3 becomes inhibited when it is cotransfected with PKB in 293 cells (D. Alessi and P. Cohen, unpublished work).

A role for MAPKAP-K1 in the activation of glycogen synthase by insulin has been excluded by several findings. First, MAPKAP-K1β (Rsk-2) is not activated by insulin in the skeletal muscles of transgenic mice expressing a mutated insulin receptor, whereas the activation of glycogen synthase by insulin is unimpaired [18]. Secondly, PD 98059 has no effect on the stimulation of glycogen synthase or glycogen synthesis by insulin in human myotubes [13] or primary adipocytes [19]. Thirdly, epidermal growth factor (EGF) induces a stronger activation of MAP kinase than insulin in adipocytes, but does not stimulate glycogen synthase or glycogen synthesis [20].

Although, rapamycin does not inhibit the acute activation of glycogen synthase by insulin in L6 myotubes [10], or primary adipocytes [20], and has little effect in human myoblasts [13], the insulin-induced stimulation of glycogen synthase in 3T3-L1 adipocytes [21] and isolated rat hemidiaphragms [22] were reported to be inhibited by rapamycin. In addition, ra-

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[1]These investigators have made equally important contributions to this study.

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pamycin was reported to strongly suppress the insulin induced stimulation of glycogen synthesis in human myoblasts when measured after a long time period (2 h) [13] These findings suggested that the signal transduction pathway utilised by insulin to activate glycogen synthase may vary with cell type and with the length of stimulation. They also raised the question of whether, in some cells, p70 S6 kinase might mediate the inhibition of GSK3 by insulin and whether the activation of PKB might be prevented by rapamycin. For these reasons, we have investigated the effect of rapamycin on the activation of PKB, the inhibition of GSK3 and the activation of glycogen synthase in the major insulin-responsive tissues, namely skeletal muscle and primary adipocytes. Our results demonstrate that rapamycin has no effect on the insulin-induced activation of PKB or the inhibition of GSK3 in these tissues. Moreover, the activation of glycogen synthase induced by insulin in skeletal muscle is not affected by rapamycin after 5 min, although it is partially suppressed at later times. We conclude that rapamycin-insensitive pathways are largely responsible for mediating the acute inhibition of GSK3 and activation of glycogen synthase in the major insulin-responsive tissues. We also show that EGF does not mimic the effect of insulin on glycogen synthase in adipocytes because the activation of PI 3-kinase and PKB induced by this agonist are too small and transient for significant inhibition of GSK3 to take place.

2. Materials and methods

2.1. Materials

Rapamycin, a generous gift from Carol Germain (Warner-Lambert Co., Michigan, USA), was dissolved in dimethylsulphoxide (DMSO) at a concentration of 10 mM. Wortmannin was purchased from Sigma Chemical Co. (Poole, UK), insulin from Novo-Nordisk (Bagsvaerd, Denmark), sodium pentobarbitione (Sagatal) from May and Baker (Dagenham, UK) and epidermal growth factor from Boehringer (Lewes, UK). The 'complete' proteinase inhibitor cocktail was also purchased from Boehringer and used as recommended by the supplier.

2.2. Insulin stimulation of rat skeletal muscle in vivo

Male Wistar rats (200 g) were deprived of food overnight and anaesthetised by intraperitoneal injection with sodium pentobarbitione (5 mg/100 g). Once fully anaesthetized one of the lower limbs was partially skinned on the medial aspect to expose the saphenous vein. Fifteen microlitres of DMSO or rapamycin in DMSO (10 mM) were diluted with 0.5 ml of 150 mM NaCl and injected into the saphenous vein 5 min prior to injection of propranolol (3.0 mg/kg) or propranolol plus insulin (1.0 U/kg). When 30 min time points were being analysed, an additional injection of rapamycin was made 15 min after the injection of insulin. At various times, muscle from the contralateral leg was taken by freeze-clamping with Wollenberger Tongs which had been cooled to the temperature of liquid nitrogen. The frozen muscle was powdered finely under liquid nitrogen using a pestle and mortar precooled in liquid nitrogen and stored at −80°C until required. Each 1 g of powdered muscle was homogenised at 4°C using a Polytron in 3.0 ml of 4.0 mM EDTA, 50 mM NaF, pH 8.0, containing 1.0 mM sodium orthovanadate, 1.0 μM microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and the proteinase inhibitor cocktail. The homogenate was centrifuged at 13 000×*g* for 10 min at 4°C and the supernatant removed and used immediately for enzyme assays or snap-frozen in liquid nitrogen and stored in 0.5 ml aliquots at −80°C until required

2.3. Stimulation of rat adipocytes with insulin or EGF

Epididymal fat pads from eight male Wistar rats (170–180 g) were placed in a plastic beaker and each 1 g of tissue incubated at 37°C with 3.0 ml of 120 mM NaCl, 10 mM $NaHCO_3$, 30 mM HEPES, 1.2 mM $MgSO_4$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 40 mg/ml BSA, 1.0 mg/ml glucose and 0.2 μM adenosine, pH 7.4, and 3.0 mg of collagenase. The pads were finely chopped with scissors and incubated for 1 h at 37°C on a rocking platform. The adipocytes were strained twice through a 0.5 mm^2 plastic sieve, then washed 4 times at 37°C with fresh buffer without collagenase, resuspended in 20 ml of buffer and incubated for 20 min at 37°C. Aliquots (1.0 ml) of adipocyte suspension were then incubated for a further 20 min in a 37°C air incubator, before treatment for 10 min with DMSO carrier, rapamycin (100 nM) or wortmannin (100 nM) and then stimulated for various times without or with EGF (200 ng/ml) or insulin (200 nM) on a low-speed shaking platform (ensuring adipocytes were in a uniform suspension) at 37°C. Stimulations were stopped by addition of 10 μl of 0.2 M EDTA, 5.0 μl of 0.2 M EGTA, pH 8.0, containing 10 μl of 0.1 M sodium orthovanadate, pH 1.0, μl of 2-mercaptoethanol and 0.5 μl of 2.0 mM microcystin-LR, immediately snap-frozen in liquid nitrogen and stored at −80°C until required. Samples were then thawed, centrifuged at 13 000×*g* for 5 min at 4°C and the infranatants transferred into fresh tubes, recentrifuged and the supernatants assayed for the enzymatic activities as described below.

2.4. Immunoprecipitation and assay of protein kinases

PKBα [10] GSK3 [8], p42 MAP kinase [8], MAPKAP-K1β [8] and p70 S6 kinase [23] were immunoprecipitated from skeletal muscle and adipocyte extracts as described previously. PKBα and MAPKAP-K1β were both assayed using Crosstide [10], GSK3 using phospho-GS peptide-1 [8], p42 MAP kinase using myelin basic protein [24] and p70 S6 kinase using the specific peptide substrate KKRNRTLTV [25]. One unit of activity (*U*) was that amount which catalysed the phosphorylation of 1 nmol of substrate in 1 min.

2.5. Other analytical procedures

PI-(3,4,5)-trisphosphate was measured as described previously [26]. Glycogen synthase was assayed at pH 6.8 by measuring the incorporation of [^{14}C]glucose (G) from uridine diphospho-[^{14}C]glucose (UDP[^{14}C]G) into glycogen [2,27]. The activity was expressed as a ratio of the activity measured in the absence of glucose-6P divided by the activity in the presence of this allosteric activator (10 mM). Protein was measured according to Bradford [28] using bovine serum albumin (A1%/280 nm=6.5) as a standard.

3. Results

3.1. Regulation of PKB and GSK3 by insulin in rat skeletal muscle in vivo

Insulin stimulated PKBα activity about 10-fold (Fig. 1A) and decreased GSK3 activity by 40% (Fig. 1B) in skeletal muscle in vivo. As found in other cells, the activation of PKBα (data not shown) and the inhibition of GSK3 (Fig. 1B) were reversed by incubation with PP2A, a serine/threonine-specific phosphatase. Half-maximal activation of PKBα by insulin occurred within 2 min and maximal activation occurred after 5 min (Fig. 1A). Neither the activation of PKBα (Fig. 1A) nor the inhibition of GSK3 (Fig. 1B) were prevented by rapamycin under conditions where the activation of p70 S6 kinase was blocked completely (Fig. 2A). Moreover, rapamycin had no effect on the insulin-induced activation of glycogen synthase by insulin after 5 min (Fig. 2B), consistent with the lack of activation of p70 S6 kinase at this time (Fig. 2A). However, the small additional activation of glycogen synthase by insulin occurring between 5 and 30 min was partially suppressed by rapamycin. Little or no activation of MAPKAP-K1 by insulin was detected under these conditions (data not shown).

3.2. Regulation of PKB, GSK3 and MAP kinase by insulin and EGF in primary rat adipocytes

Insulin stimulated PKBα activity 14-fold (Fig. 3A) and decreased GSK3 activity by 60% (Fig. 3B) in adipocytes, and these effects were reversed by treatment with PP2A (data not

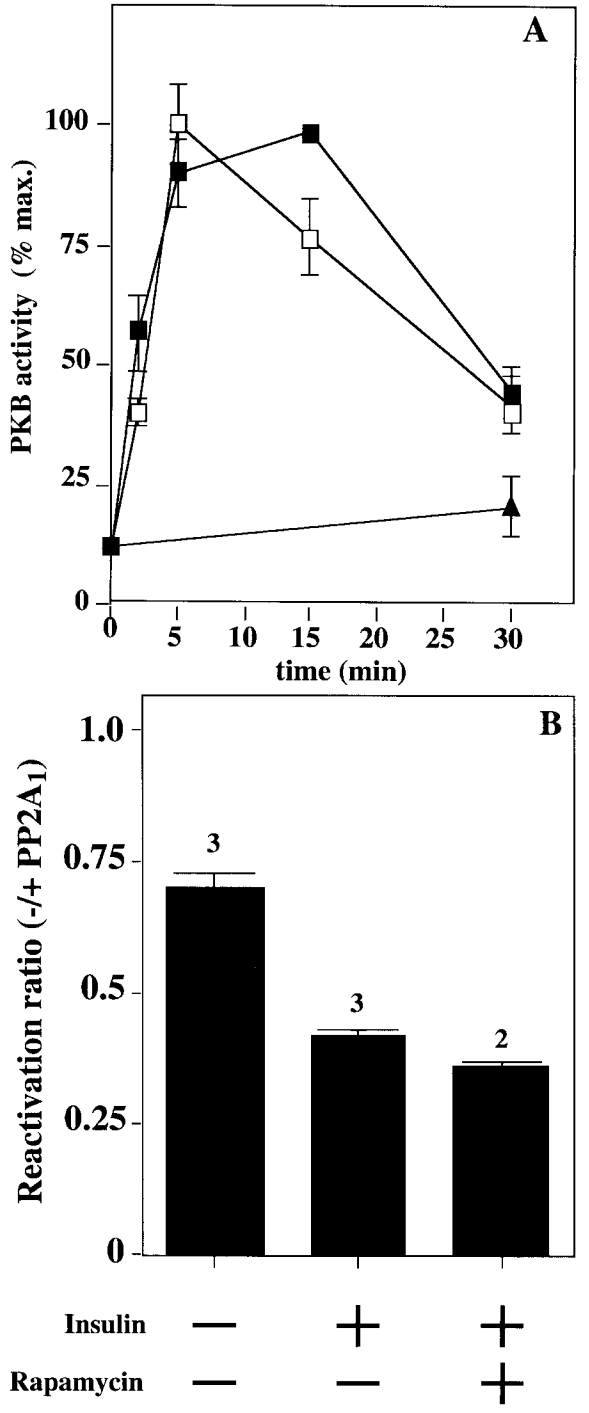

Fig. 1. Activation of PKB and inhibition of GSK3 by insulin in rat skeletal muscle in vivo. Skeletal muscles were exposed to rapamycin (■, ▲) or buffer (□) before stimulation with (□,■) or without (▲) insulin. A: PKBα was immunoprecipitated from the extracts and assayed in triplicate as described under Section 2. PKBα activities are presented as a percentage of the maximal specific activity attained after stimulation with insulin for 5 min in the absence of rapamycin (1.2 mU/mg) and are given ±SEM for three separate experiments. B: Skeletal muscles were freeze clamped 10 min after stimulation with insulin and both GSK3 isoforms were co-immunoprecipitated from the extracts. The immunoprecipitates were incubated without or with $PP2A_1$ prior to assay, and GSK3 activity is expressed as a reactivation ratio; i.e. GSK3 activity measured without $PP2A_1$ treatment divided by GSK3 activity after $PP2A_1$ treatment. The data are presented as the mean ± SEM for the number of different experiments shown in parentheses.

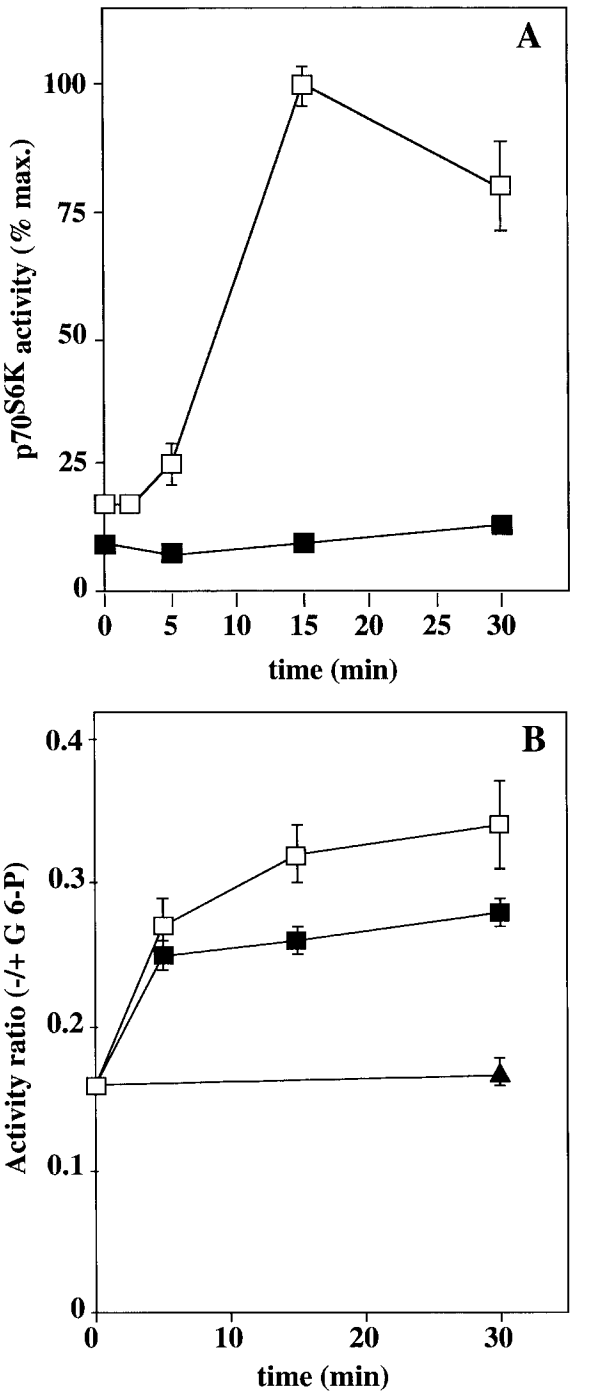

Fig. 2. Effect of rapamycin on the activation of p70 S6 kinase and glycogen synthase by insulin in skeletal muscle in vivo. Skeletal muscle in vivo was exposed to rapamycin (■, ▲) or buffer (□) and then stimulated with (□, ■) or without (▲) insulin for the times indicated. p70 S6 kinase was immunoprecipitated from the extracts and assayed as described under Section 2, while glycogen synthase activity was assayed directly in the cell extracts in the absence and presence of glucose-6P. p70S6 kinase activities are presented as a percentage of the maximal specific activity attained after stimulation with insulin for 15 min in the absence of rapamycin (2.2 mU/mg). Glycogen synthase activity is presented as an activity ratio−/+glucose-6P. Assays were performed in duplicate and the data are given as ±SEM for three separate experiments.

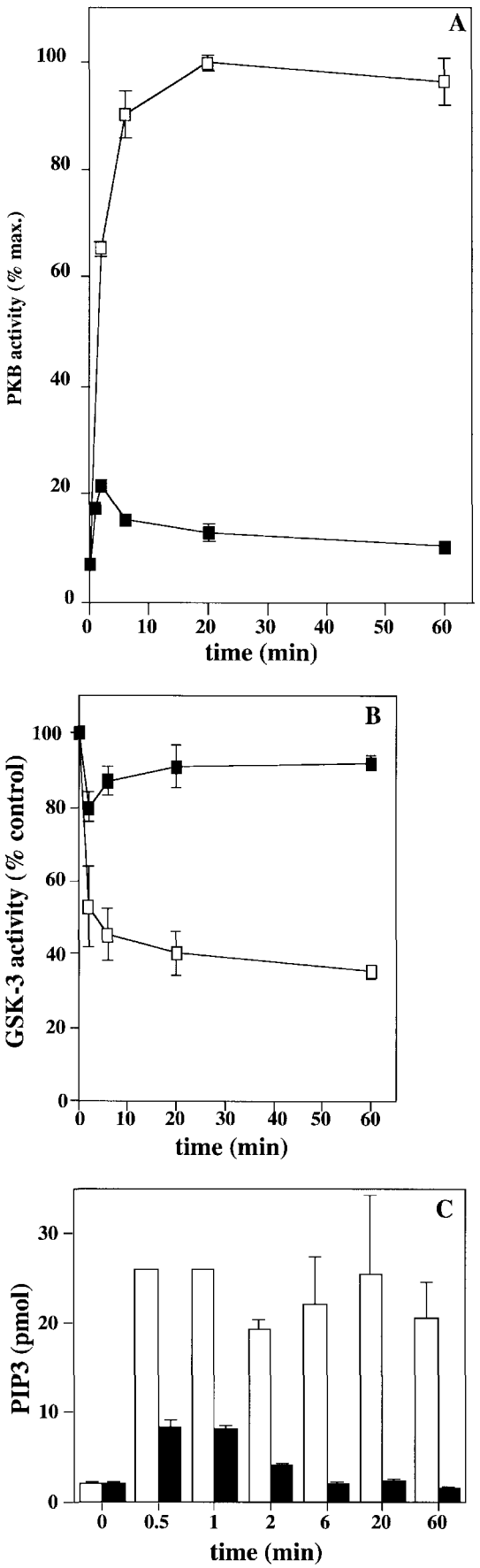

Fig. 3. Stimulation of PIP3 and PKB and inhibition of GSK3 in primary rat adipocytes. Adipocytes were stimulated with 200 nM insulin (open symbols or bars) or 200 ng/ml EGF (closed symbols or bars) for the times indicated and, after cell lysis, PKBα and both GSK3 isoforms were immunoprecipitated and assayed as described under Section 2. A: PKBα activity is presented as a percentage of the maximal activity attained after stimulation for 20 min with insulin (±SEM for three separate experiments). B: GSK3 activity is presented as a percentage of the activity measured in extracts from unstimulated adipocytes (±SEM for three separate experiments). C: The formation of PIP3 was measured in triplicate at each time point and the data show the results of at least two independent experiments.
←

shown). Half-maximal activation of PKBα and inhibition of GSK3 by insulin occurred within 2 min with a maximal effect after 5 min and, like the stimulation of glucose uptake and glycogen synthesis, the activation of PKBα and inhibition of GSK3 by insulin were sustained for at least 1 h.

In contrast to insulin, EGF induced only a 3-fold activation of PKBα, which peaked after 2 min and returned to near basal levels after 10–20 min (Fig. 3A). Consistent with this finding, EGF induced only a small (20%) inhibition of GSK3 after 2 min which returned to control levels after 10–20 min (Fig. 3B). EGF induced a stronger activation of p42 MAP kinase than insulin (data not shown) as reported in [20].

3.3. *Effect of insulin and EGF on the level of phosphatidylinositide-3,4,5-trisphosphate in primary rat adipocytes*

The insulin and EGF induced activation of PKBα and the insulin induced inhibition of GSK3 in adipocytes were unaffected by rapamycin but prevented by wortmannin (data not shown), indicating that, as in other cells, the regulation of these enzymes by insulin is mediated by the activation of PI 3-kinase. Consistent with the kinetics of activation of PKBα and inhibition of GSK3, insulin induced a greater than 10-fold increase in the level of phosphatidylinositol-3,4,5-trisphosphate (PIP3), the product of the PI 3-kinase reaction, which peaked within 1 min and was sustained for 1 h (Fig. 3C). In contrast, EGF induced a 3–4-fold activation of PIP3 which also peaked within 1 min but declined to near control levels after 6 min (Fig. 3C).

4. Discussion

To our knowledge, this is the first report that insulin activates PKB in the major insulin-responsive tissues of the body (skeletal muscle and adipocytes), and that the rate (half-time: 1–2 min) and extent (>10-fold) of activation (Fig. 1AFig. 3A) are similar to those observed in mammalian cell lines [10,16,17]. We also establish that insulin inhibits GSK3 in skeletal muscle in vivo by inducing its phosphorylation at a serine/threonine residue(s) (Fig. 1B). Moreover, the rate of inhibition of GSK3 in skeletal muscle and adipocytes is consistent with it lying downstream of PKB and upstream of glycogen synthase in the signal transduction pathway by which insulin activates glycogen synthase.

A strong correlation between the extent and duration of activation of PKBα and the extent and duration of inhibition of GSK3 also emerged when the effects of insulin were compared to those of EGF in adipocytes. Whereas insulin induced a greater than 10-fold activation of PKBα that was sustained for at least 1 h, EGF only induced a 3-fold activation of

PKBα which peaked after 2 min and returned to near basal levels after 10–20 min (Fig. 3A). This may explain why insulin induced a 60% decrease in GSK3 activity in adipocytes that was sustained for at least 1 h, whereas EGF only induced a maximum 20% decrease in GSK3 activity after 2 min which returned to control levels after 10–20 min. The stimulation of glucose transport and activation of glycogen synthase by insulin is also sustained for at least 1 h [20], and the small and very transient rise in PIP3 (Fig. 3C) and PKBα (Fig. 3A) and decrease in GSK3 activity (Fig. 3B) may explain why, in contrast to insulin, EGF fails to stimulate glucose transport and glycogen synthesis significantly in adipocytes. This would be analogous to the situation in pheochromocytoma (PC12) cells where NGF induces neuronal differentiation by triggering a sustained activation of the MAP kinase cascade, whereas EGF [29] and insulin [30] do not because the activation of MAP kinase by these agonists is too transient. The transient activation of PKB may also underlie the failure of platelet-derived growth factor (PDGF) to stimulate glucose transport in 3T3-L1 adipocytes [31], without the need to invoke more complex mechanisms such as the specific targetting of activated PI 3-kinase to GLUT-4 vesicles [31]. In primary adipocytes we failed to observe any effect of PDGF on the level of PIP3, or on the activities of PKBα and p42 MAP kinase (J. van der Kaay and M. Shaw and unpublished work), presumably because these cells lack PDGF receptors.

Rapamycin had no effect on the activation of PKBα (Fig. 1A) or on the inhibition of GSK3 (Fig. 1B) by insulin in skeletal muscle, or on the inhibition of GSK3, the activation of glycogen synthase or the stimulation of glycogen synthesis by insulin in primary adipocytes [14,20]. We also failed to find any effect of rapamycin on the activation of glycogen synthase in skeletal muscle up to 5 min after stimulation by insulin (Fig. 2B), a time point at which the increase in glycogen synthase activity had reached 70% of its maximal level. These findings were consistent with the observation that p70 S6 kinase, an enzyme whose activation is prevented by rapamycin, was not stimulated by insulin after 5 min but only at later times (Fig. 2A). Consistent with this finding, Chang et al. [18] have reported that rapamycin does not inhibit the activation of glycogen synthase in normal mouse skeletal muscle 20 min after an intravenous injection of insulin. We therefore conclude that rapamycin-insensitive pathways are mainly responsible for mediating the acute inhibition of GSK3 and activation of glycogen synthase in the major insulin-responsive tissues of the body.

Rapamycin has been reported to suppress the activation of glycogen synthase and glycogen synthesis by insulin in 3T3-L1 adipocytes [21] and incubated rat hemidiaphragm [22]. However, as found in primary adipocytes (Fig. 3) and in rat skeletal muscle in vivo (Fig. 1), the insulin-induced activation of PKBα and the inhibition of GSK3 are unaffected by rapamycin in the incubated rat hemidiaphragm (D. Cross and K. Walker, unpublished work) raising the question of how rapamycin inhibits the activation of glycogen synthase by insulin in 3T3-L1 adipocytes and hemidiaphragms, and in rat skeletal muscle after 5 min (Fig. 2B). One of many possible explanations is that PP1.G_M activity (see Section 1) is stimulated by a rapamycin-sensitive kinase, and that the relative contributions made by the inhibition of GSK3 and the activation of PP1G_M to the activation of glycogen synthase by insulin vary from tissue to tissue. However, p70 S6 kinase is unlikely to be the protein kinase which activates PP1.G_M in vivo because it does not phosphorylate the G_M subunit in vitro [32]. More detailed analysis of the effect of insulin on the phosphorylation of G_M in vivo will be needed to resolve this problem.

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