Regulation of Glycogen Synthase in Rat Hepatocytes

EVIDENCE FOR MULTIPLE SIGNALING PATHWAYS*

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Louis Lavoie§, Christian J. Band§, Mei Kong, John J. M. Bergeron¶, and Barry I. Posner∫
From the Polypeptide Hormone Laboratory, Faculty of Medicine, and ¶Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec H3A 2B2, Canada

We examined the signaling pathways regulating glycogen synthase (GS) in primary cultures of rat hepatocytes. The activation of GS by insulin and glucose was completely reversed by the phosphatidylinositol 3-kinase inhibitor wortmannin. Wortmannin also inhibited insulin-induced phosphorylation and activation of protein kinase B/Akt (PKB/Akt) as well as insulin-induced inactivation of GS kinase-3 (GSK-3), consistent with a role for the phosphatidylinositol 3-kinase/PKB-Akt/GSK-3 axis in insulin-induced GS activation. Although wortmannin completely inhibited the significantly greater level of GS activation produced by the insulin-mimetic bisperoxovanadium 1,10-phenanthroline (bpV(phen)), there was only minimal accompanying inhibition of bpV(phen)-induced phosphorylation and activation of PKB/Akt, and inactivation of GSK-3. Thus, PKB/Akt activation and GSK-3 inactivation may be necessary but are not sufficient to induce GS activation in rat hepatocytes. Rapamycin partially inhibited the GS activation induced by bpV(phen) but not that affected by insulin. Both insulin- and bpV(phen)-induced activation of the atypical protein kinase C (ζ) (PKC (ζ)) was reversed by wortmannin. Inhibition of PKC (ζ) with a pseudosubstrate peptide had no effect on GS activation by insulin, but substantially reversed GS activation by bpV(phen). The combination of this inhibitor with rapamycin produced an additive inhibitory effect on bpV-(phen)-mediated GS activation. Taken together, our results indicate that the signaling components mammalian target of rapamycin and PKC (ζ) as well as other yet to be defined effector(s) contribute to the modulation of GS in rat hepatocytes.

Much current work has focused on defining the nature of the downstream effectors mediating key effects of insulin. The regulation of glycogen synthase (GS), 1 the rate-limiting enzyme in glycogen synthesis, by insulin has received increasing attention. GS is regulated by a complex interplay of diurnal, nutritional, and hormonal factors (reviewed in Refs. 1–3) that ultimately modulate the phosphorylation state and hence the activity of the enzyme. Whereas there have been numerous studies of GS activation in skeletal muscle, the modulators of GS in liver have been less completely evaluated. Studies to date indicate that glucose and insulin are the major physiologic effectors of hepatic GS activation (1, 2). It appears that glucose and/or its metabolite glucose 6-phosphate activate hepatic GS by physically associating with critical regulatory enzymes upstream of GS or with GS itself (2, 4, 5), although other mechanisms have been suggested (6). The mechanisms by which insulin effects activation of GS and stimulation of glycogen synthesis in liver are poorly defined, although data have been obtained indicating that modulation of PP1-G (1, 7) as well as PKB/Akt (8) and GSK-3 (9) may be involved.

Insulin activation of the insulin receptor kinase (IRK) is followed by tyrosine phosphorylation of insulin receptor substrates (IRSs), the two major ones in liver being IRS-1 and -2 (10, 11). The IRSs recruit, on defined phosphotyrosine motifs, key molecules involved in signal transduction (reviewed in Ref. 12). In this fashion, PI 3-kinase associates with IRS-1/2 and is activated, resulting in the activation of downstream signaling molecules including PKB/Akt (13–15), p70s6k (16), and atypical PKC isoforms such as PKC (ζ/λ) (17–19). The central role of PI 3-kinase activation in realizing insulin metabolic effects has been established (reviewed in Ref. 20).

The peroxovanadium compounds, insulin-mimetic agents that activate the IRK by inhibiting an IRK-associated protein-tyrosine phosphatase (21, 22), mimic a range of insulin actions in target tissues including liver (23–27) and thus provide tools for amplifying our understanding of the control mechanisms operating on biologic processes influenced by insulin. In the present study, we sought to identify, in primary rat hepatocyte cultures, the molecular components and signal transduction pathways involved in the regulation of GS by both insulin and bpV(phen). Our results demonstrate that neither PKB/Akt activation nor GSK-3 inactivation is sufficient for GS activation and hence raise a question about the role suggested for this pathway in insulin-mediated GS activation. We demonstrate the involvement of two other pathways in the modulation of GS activation, accessed by bpV(phen) but not insulin, a rapamycin-sensitive component that is not p70s6k (28), and wortmannin-sensitive activation of PKC (ζ).

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¶ To whom correspondence should be addressed: Polypeptide Hormone Laboratory, Strathcona Anatomy Bldg., 3640 University St., Montreal, Quebec H3A 2B2, Canada. Tel.: 514-398-4101; Fax: 514-398-3923; E-mail: mc85@musica.mcgill.ca.
∫ The abbreviations used are: GS, glycogen synthase; PPI-G, type 1 protein phosphatase associated with glycogen; PKB/Akt, protein kinase B/Akt; GSK-3, glycogen synthase kinase-3; IRK, insulin receptor kinase; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; p70s6k, p70/p85 ribosomal S6 protein kinase; PKC, protein kinase C; bpV(phen), bisperoxovanadium 1,10-phenanthroline; MAP, mitogen-activated protein; mTOR, mammalian target of rapamycin; DMEM, Dulbecco’s modified Eagle’s medium; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; MOPS, 4-morpholinepropanesulfonic acid; ANOVA, analysis of variance; PTEN, phosphatase and tensin homolog deleted on chromosome 10.

2 Since currently available antibodies do not permit a distinction between the two atypical PKCs ζ and λ, we have used the terminology of Standaert et al. (71) and thus refer to the atypical PKC as PKC (ζ).
**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine insulin was from Lilly. Wortmannin was purchased from Calbiochem, and the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor PD98059 (28) was obtained from New England Biolabs Inc. (Beverly, MA). Peroxovanadium bpV(phen) was synthesized and purified as described previously (21). Collagenase was from Worthington. Cell culture media, antibiotics, and protein phosphatase assay system kit were from Life Technologies, Inc. (Life Technologies, Burlington, Ontario, Canada), and Vitrogen-100 was from Collagen Corp. (Toronto, Canada). [U-3H]UDP-glucose and [γ-32P]ATP were purchased from NEN Life Science Products, and okadaic acid was previously (27). The inhibitors wortmannin (100 nM), rapamycin (200 μM glucose (0.12%)), and caffeine (3 mg/ml) were combined and incubated for 45 min at 30 °C. Twenty microilters of supernatants (60 μg of protein) were incubated with 20 μl of okadaic acid (2 nm) in buffer A for 5 min at room temperature. The protein phosphatase reaction, initiated by adding 60 μg of [γ-32P]labeled phosphatase a, was carried out for 10 min at 30 °C and terminated by adding 200 μl of 20% ice cold trichloroacetic acid. The reaction tubes were kept on ice for 10 min and centrifuged at 12,000 × g for 5 min. After centrifugation, the supernatants was counted in scintillation fluid. Nonenzymatic hydrolysis of [32P] from substrate was accounted for less than 4% of the total amount released in the samples. The inhibitors were assayed as described previously (27).

**Cell Culture and Stimulations**—Hepatocytes, isolated from 120–140-g fed male Harlan Sprague Dawley rats (Charles River, St-Constant, Canada) by in situ liver perfusion with collagenase, were seeded onto six-well plates (Corning Costar Corp., Cambridge, MA) coated with collagen (Vitrogen-100) at a density of 5 × 10⁵ cells/cm². Primary cultures were incubated for 16 h in Dulbecco’s modified Eagle’s medium/Hams F-12 (DMEM/F-12) containing 10% fetal bovine serum, 10 mM Hepes-buffered saline. Beads were incubated in 20 μl of kinase assay buffer containing 2.5 mM Tris, pH 7.5, 25 mM magnesium chloride, 1 mM EDTA, and 1 μM (γ-32P)ATP (6 μCi/ml), and 1 μg of phosphorylase-BS peptide-2 substrate for 30 min at 30 °C. The kinase reaction was stopped by the addition of 6× concentrated Laemmli sample buffer. [32P]-Phosphorylated peptide (294 daltons) was resolved by Tricine SDS-polyacrylamide gel electrophoresis (34) and quantitated by autoradiography or scintillation counting of the phosphorylated peptide extracted from the gels.

**PKB/Akt Phosphorylation Assay**—Western blot analysis of Thr308 and Ser473 of PKB/Akt was carried out using the Phospho-specific® PKB antibody kit (New England Biolabs Inc.) according to the manufacturer’s protocol. Gels were reprobed with a rabbit anti-PKB/Akt antibody (provided in the kit), and data were corrected for total PKB/Akt levels after densitometric scanning with a Bio-Rad model GS-700 imaging densitometer.

**PKB/Akt Activity Assay**—Hepatocytes were lysed in 500 μl of buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1% β-mercaptoethanol, 1 mM microcystin-LR, 1 mg/ml glycogen, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A), lysates were centrifuged at 12,000 × g for 10 min, and 450 μl of supernatant (450 μg protein) was incubated with mild agitation for 90 min at 4 °C with 2 μg of anti-GSK-3β antibody preadsorbed to protein A-Sepharose beads. Immobilized immune complexes were recovered by centrifugation at 12,000 × g for 2 min and washed three times with HEPES-buffered saline containing 1% Triton X-100, 200 mM sodium orthovanadate, 4 mM sodium fluoride and once with Hepes-buffered saline. Beads were incubated in 20 μl of kinase assay buffer containing 2.5 mM Tris, pH 7.5, 25 mM magnesium chloride, 1 mM EDTA, and 1 μM (γ-32P)ATP (6 μCi/ml), and 1 μg of phosphorylase-BS peptide-2 substrate for 30 min at 30 °C. The kinase reaction was stopped by the addition of 6× concentrated Laemmli sample buffer. [32P]-Phosphorylated peptide (294 daltons) was resolved by Tricine SDS-polyacrylamide gel electrophoresis (34) and quantitated by autoradiography or scintillation counting of the phosphorylated peptide extracted from the gels.
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**RESULTS**

**Effect of Insulin, bpV(phen), and Glucose on Hepatocyte GS Activation**—Fig. 1A shows that insulin at a dose of 100 nM stimulates GS activity in our primary rat hepatocyte cultures by 170% of basal level. Whereas PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, the upstream activator of p44/42 MAP kinase (40, 41), inhibited glucose-mediated GS activation (data not shown) as previously reported by others (6). Fig. 1B shows that glucose-induced GS activation was also partially reversed by rapamycin. These data strongly suggest that in addition to allosteric regulation, glucose affects GS activation by a signal transduction-based mechanism. Glucose did not activate p70s6k (Fig. 2), suggesting that a mTOR-dependent event(s), other than p70s6k activation, is involved in mediating GS activation by glucose.

**The Role of Glycogen Phosphorylase Inactivation and Phosphorylase Phosphatase Activation**—We sought to evaluate the role of phosphorylase and its phosphatase as downstream signaling elements leading to GS activation by insulin and bpV(phen). Active glycogen phosphorylase exerts an inhibitory effect on hepatic GS phosphatase (PP1-G) activity and hence opposes GS activation (4). Inactivation of GS phosphatase by phosphorylase appears to be an important mechanism leading to GS activation by glucose in liver (4). Inactivation of liver phosphorylase by insulin in vivo has also been demonstrated and proposed to account for the activation of GS by the hormone (40, 41). As observed in Fig. 3, insulin treatment of hepatocytes did not significantly inhibit phosphorylase activity, whereas glucose was, as predicted, inhibitory. Interestingly, phosphorylase activity was significantly inhibited by bpV(phen). Whereas rapamycin substantially reversed the inhibitory effect of bpV(phen), no such reversal of the glucose inhibitory effect was observed (Fig. 3). Thus, phosphorylase inactivation can be mediated by mTOR and/or mTOR-dependent signaling events(s) and may contribute to bpV(phen)-induced GS activation. It is unlikely that p70s6k is involved, since both insulin and bpV(phen) activate p70s6k in a rapamycin-sensitive manner (27) but only bpV(phen)-induced GS activation showed sensitivity to rapamycin. Glucose-mediated phosphorylase inactivation is not sensitive to rapamycin; hence, its activation of GS involves a mTOR-dependent effector distinct from phosphorylase.

Since dephosphorylation and inactivation of phosphorylase is effected by phosphorylase phosphatase (4), we examined the effect of insulin and bpV(phen) on the activity of this enzyme.
While bpV(phen) augmented phosphorylase phosphatase activity, insulin did not (Table I). This is consistent with the effect of these agents on phosphorylase activity. Rapamycin blocked bpV(phen)-induced phosphorylase phosphatase activation (Table I). Taken together, these data indicate that 1) neither phosphorylase phosphatase nor phosphorylase are directly involved in the acute activation of GS by insulin in liver, and 2) mTOR is a critical signaling element for bpV(phen)-mediated phosphorylase phosphatase activation and phosphorylase inactivation.

Insulin and bpV(phen) Modulation of PKB/Akt and GSK-3 Activities—The serine/threonine protein kinase GSK-3 inhibits GS by phosphorylation (3) and is considered to be a major regulator of GS in several cell types including liver cells (1). We investigated the effect of insulin and bpV(phen) on GSK-3 activity and found that both agents were comparably inhibitory (Fig. 4). Rapamycin and PD98059 were without effect on both insulin- and bpV(phen)-mediated GSK-3 inactivation (Fig. 4). Although wortmannin treatment resulted in the complete reversal of insulin-induced GSK-3 inactivation, it only partially reversed bpV(phen)-mediated GSK-3 inactivation (Fig. 4).

GSK-3 is a direct substrate for PKB/Akt in vitro (42), and there is evidence that PKB/Akt phosphorylates and inactivates GSK-3 in vivo (43, 44). Phosphorylation of both Thr^308 and Ser^373 of PKB/Akt are required for its full activation (45). In primary rat hepatocytes, maximal PKB/Akt activation in response to insulin occurred at 2 min (8). In contrast, bpV(phen) induces maximal IRK-dependent signaling events at 20 min (21, 22). We thus assessed the phosphorylation of Thr^308 and Ser^373 of PKB/Akt by Western blotting using phosphospecific antibodies toward Thr^308 and Ser^373 as well as PKB/Akt immunoprecipitation activity at these respective times in insulin- and bpV(phen)-treated hepatocytes. We observed that insulin-induced phosphorylation of Thr^308 and Ser^373 (Fig. 5A) and activation (Fig. 5B) of PKB/Akt were completely abolished by wortmannin as reported in many cell types (reviewed in Ref. 46). bpV(phen)-mediated phosphorylation of Thr^308 and Ser^373 and activation of PKB/Akt were 3–4-fold greater than that observed for insulin. This ratio was maintained after correcting for total PKB/Akt levels measured by immunoblotting the gels with a PKB/Akt antibody (Fig. 5A, bottom). In contrast to insulin, wortmannin did not inhibit Thr^308 and Ser^373 phosphorylation and only minimally inhibited (30%) the activity of PKB/Akt following bpV(phen) treatment (Fig. 5, A and B). Because wortmannin has previously been shown to inhibit insulin- and bpV(phen)-activated PI 3-kinase to the same extent in primary rat hepatocytes (27), then bpV(phen)-induced PKB/Akt phosphorylation and activation must be largely independent of PI 3-kinase.

Role of PKC (ζ/λ) in the Regulation of GS by Insulin and bpV(phen)—Evidence suggests that atypical isoforms of PKC act downstream of PI 3-kinase (47, 48) and are involved in the regulation of insulin-stimulated glucose transport (17, 49, 50) and protein synthesis (18). We investigated whether one such isoform, PKC (ζ/λ), plays a role in signaling to GS. We measured PKC (ζ/λ) activity in response to insulin and bpV(phen) in cultured rat hepatocytes. Fig. 6 shows that insulin and bpV(phen) activated PKC (ζ/λ) by 1.5- and 2-fold, respectively, and that this activity was suppressed to below basal levels by the pseudosubstrate inhibitor of atypical PKC isoforms. Wortmannin reduced insulin- and bpV(phen)-induced PKC (ζ/λ) activity to basal levels, consistent with a requirement for PI 3-kinase for its activation.

Since PKC (ζ/λ) and GS activation are both wortmannin-
tocytes were incubated for 45 min with 100 nM wortmannin (W) and bpV(phen) in cultured rat hepatocytes. Serum-starved hepatocyte preparations. The phosphorylation of Thr\(^{308}\) and Ser\(^{473}\) residues of PKB/Akt (A, top and middle, respectively). Gels were probed with a rabbit anti-PKB/Akt antibody for total PKB/Akt levels (A, bottom). The phosphorylation of Thr\(^{308}\) and Ser\(^{473}\), corrected for total PKB/Akt, was 4.5- and 3.2-fold that with insulin and 2.8-fold those of insulin. bpV(phen)-stimulated PKB/Akt activity, over that with insulin. Following wortmannin, these levels were 2.9- and 2.8-fold those of insulin. bpV(phen)-stimulated PKB/Akt activity, corrected for total PKB/Akt, was 4.5- and 3.2-fold that with insulin alone in the absence and presence, respectively, of wortmannin. The data are representative of experiments on three independent hepatocyte preparations.

sensitive, we investigated whether PKC (\(\zeta/\lambda\)) plays a role in insulin- and bpV(phen)-mediated signaling to GS. Fig. 7 shows that a PKC (\(\zeta/\lambda\)) pseudosubstrate peptide did not inhibit the stimulation of GS by insulin. In contrast, a marked inhibitory effect was noted in bpV(phen)-treated cells. Furthermore, the combination of the PKC (\(\zeta/\lambda\)) pseudosubstrate and rapamycin resulted in an additive inhibitory effect on bpV(phen)-induced GS activation, thus suggesting that PKC (\(\zeta/\lambda\)) and mTOR are on distinct signaling pathways leading to GS activation by bpV(phen).

**DISCUSSION**

In the present study, we evaluated the modulation of GS in primary rat hepatocytes by glucose, insulin, and the insulin-mimetic compound bpV(phen). Previous work demonstrated the allosteric actions of glucose and/or its primary metabolite glucose 6-phosphate on the key enzymes associated with GS activation or with GS itself in liver (2, 4). The present study showed that a signaling-based mechanism contributes to glucose-mediated GS activation. Thus, as previously observed (6), wortmannin partially reversed glucose-induced GS activation (data not shown), implicating PI 3-kinase in glucose signaling to GS. However, glucose did not activate PI 3-kinase activity when assayed in phosphotyrosine immunoprecipitates (data not shown). Because the lipid products of PI 3-kinase are potent inhibitors of hepatic glucose-6-phosphatase (51), the inhibitory effect of wortmannin may be explained by a reduction in the levels of such lipid products, resulting in augmented glucose-6-phosphatase activity and hence reduced amounts of glucose 6-phosphate available for GS activation. A novel finding was the demonstration that glucose-induced GS activation is sensitive to rapamycin. A role for amino acids in the control of mTOR function has recently been proposed (52). Our data suggest a role for yet another nutrient, glucose, in modulating mTOR function. The rapamycin-sensitive glucose effector(s) remains to be identified, but it does not appear to be p70\(^{euk}\) (Fig. 2) or phosphorylase (Fig. 3).

We examined the roles of PI 3-kinase, MAP kinase, and p70\(^{euk}\) in effecting insulin-mediated GS activation in the cultured hepatocyte system. Wortmannin blocked insulin-induced GS activation, indicating a role for PI 3-kinase, as observed previously in various cell lines (38, 39, 53, 54), rat adipocytes (55), and rat hepatocytes (8, 56). PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, the upstream activator of p44/42 MAP kinases, had no effect on insulin-mediated GS activation, excluding a role for MAP kinases. This is consistent with earlier work on 3T3-L1 adipocytes, cultured muscle cells, and rat diaphragm (37, 39, 57, 58), and by Peak et al. (8) on primary rat hepatocytes. Previous studies have shown that rapamycin, an inhibitor of p70\(^{euk}\) activation (35, 36), partially or completely inhibits insulin-induced GS activation in rat diaphragm muscle in vitro (37), 3T3-L1 adipocytes (38), and human myoblasts (39). However, we failed to observe an inhibitory effect of rapamycin on insulin-induced GS activation in rat hepatocytes, consistent with the lack of effect of this inhibitor on insulin-mediated hepatic glycogen synthesis (8). Thus, the stimulation
GSK-3 may play a necessary role but is clearly insufficient for GS activation in hepatocytes. The observation that bpV(phen)-induced phosphorylation and activation of PKB/Akt were only minimally affected by wortmannin raises interesting mechanistic considerations. PKB/Akt activation appears to be effected when its pleckstrin homology domain interacts with phospholipid products of PI 3-kinase to induce a conformational change in PKB/Akt, rendering Thr308 and Ser473 accessible for phosphorylation and full activation of the enzyme (15). Because bpV(phen) is a powerful protein-tyrosine phosphatase inhibitor, our results point to a critical role for a protein-tyrosine phosphatase(s) in attenuating PKB/Akt phosphorylation and activation. In light of the recent demonstration that the protein-tyrosine phosphatase PTEN is a lipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (63, 64) and that PKB/Akt activity and phosphorylation are constitutively elevated in PTEN-deficient mouse embryonic fibroblasts (65), it is possible that bpV(phen) inhibits PTEN, leading to sustained phosphoinositide 3-phosphate levels and hence PKB/Akt activity. It is also possible that bpV(phen) inhibits a protein-tyrosine phosphatase(s) involved in negatively regulating the activities of 3-phosphoinositide-dependent protein kinases 1 and 2, which have been described as the enzymes responsible for phosphorylating PKB/Akt at Thr308 and Ser473, respectively (15). In keeping with the current model for PKB/Akt activation (reviewed in Ref. 66), this would mean that in the presence of wortmannin, sufficient PI 3-kinase lipid products accumulate due to PTEN inhibition so as to induce the conformational change in PKB/Akt necessary for Thr308 and Ser473 phosphorylation.

We sought to identify the PI 3-kinase-dependent downstream protein kinase(s) that were inactivated by wortmannin and hence could be implicated in modulating GS activation by bpV(phen). PKC (ζ/λ) is activated in vitro by phosphatidylinositol 3,4,5-triphosphate (47) and has been shown to lie downstream of PI 3-kinase in rat adipocytes (17) and L6 muscle cells (49). Our observation of a reversal by wortmannin of insulin- and bpV(phen)-induced PKC (ζ/λ) activation in rat hepatocytes is consistent with a role for PI 3-kinase lipid products in PKC (ζ/λ) activation in hepatocytes. Whereas the addition of the PKC (ζ/λ) pseudosubstrate inhibitor had no effect on insulin-induced GS activation, it significantly suppressed bpV(phen)-induced GS activation. Thus, PKC (ζ/λ) is not the downstream kinase activated by insulin. The pseudosubstrate studies implicate the involvement of PKCs in bpV(phen)-induced GS activation. We know that the pseudosubstrate inhibits both typical and atypical PKCs (see Ref. 17). However, activation of typical PKCs leads to GS inactivation (1), and their inhibition should hence augment GS activation by bpV(phen). This is clearly not the case, as seen in Fig. 7. Furthermore, since wortmannin fully inhibits bpV(phen)-induced GS activation, it must subsume that portion which is PKC-dependent and hence implicates atypical PKCs (i.e. PKC (ζ)) whose activation is inhibited by wortmannin.

Since the PKC (ζ/λ) pseudosubstrate only effected partial inhibition (~35%) of bpV(phen)-induced GS activation, we explored the role of other pathways in this process. Unlike the case with insulin, rapamycin significantly reduced the magnitude of bpV(phen)-stimulated GS activity, pointing to the involvement of mTOR-dependent event(s) in bpV(phen) action. Indeed, our data demonstrate that the activation of PKC (ζ/λ) combined with a rapamycin-sensitive step constitutes most of the stimulation of GS by bpV(phen). Prior in vivo studies implicated a role for phosphorylase inactivation in insulin-mediated hepatic GS activation (40, 41). However, the confounding effects of circulating glucose levels rendered interpretation difficult. In the present study, we show that in hepatocytes incu-
bated with insulin, GS was activated, while phosphorylase activity was not significantly diminished. This agrees with earlier studies performed on rat hepatocytes (67, 68) and indicates that phosphorylase inactivation is unlikely to be involved in the activation of hepatic GS by insulin. In contrast to these findings, we observed that bpV(phen) significantly modulated both phosphorylase phosphatase and phosphorylase activity, and that the changes effected by bpV(phen) were rapamycin-sensitive. Indeed, this might explain the greater ability of bpV(phen) compared with insulin to stimulate GS. How mTOR signals to these enzymes is at present unclear, but p70S6k is not involved, since its activation by insulin and bpV(phen) is both similar in magnitude and sensitive to rapamycin (Ref. 27 and data not shown). Effectors downstream of mTOR other than p70S6k have been described (69), and mTOR itself possesses serine kinase activity (70) such that it may directly regulate phosphorylase phosphatase and/or phosphorylase by phosphorylation.

In summary, we have presented data indicating that the activation of PKB/Akt and the inhibition of GSK-3 in a PI 3-kinase-dependent manner may be necessary for insulin-induced GS activation but cannot be sufficient. We suggest that an additional pathway needs to be activated to realize the effect of insulin. Our observations with bpV(phen) identified mTOR and PKC (ζα) as other PI 3-kinase-dependent signaling components that contribute to GS activation in hepatocytes. They do not appear to be the additional postulated component activated by insulin in the course of achieving GS activation. Further work is required to identify the downstream effector(s) involved.

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REFERENCES

1. Puga-Zahutendi, S., and Kandhelwa, R. L. (1995) Mol. Cell. Biochem. 149, 95–101
2. Villar-Palasi, C., and Guinovart, J. J. (1997) FASEB J. 11, 544–558
3. Lawrence, J. C., Jr., and Vlahos, C. J. (1997) J. Biol. Chem. 272, 1666–1673
4. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) J. Biol. Chem. 270, 13151–13156
5. Coffer, P. J., Jin, J., and Woodgett, J. R. (1998) Biochem. J. 335, 1–13
6. Aoki, K., Takahashi, R., Moriya, S., Nishikawa, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S.-I., Mizuno, K., Hirai, S.-I., Shizukuda, O., and Ohno, S. (1996) J. Cell. Physiol. 165, 788–798
7. Sawai, H., Kogo, T., and Yonezawa, K. (1997) J. Biol. Chem. 272, 17–22
8. Sato, H., Kano, M., and Mak, T. W. (1997) EMBO J. 16, 4360–4369
9. Hozumi, H., and Saffitz, J. E. (1998) J. Biol. Chem. 273, 10727–10733
10. Imai, Y., Caruso, M., Beguinot, F., Formisano, P., and Accili, D. (1998) J. Biol. Chem. 273, 1274–1280
11. Rother, K. I., Imai, Y., Caruso, M., Beguinot, F., Formisano, P., and Accili, D. (1997) J. Biol. Chem. 272, 27713–27719
12. Avruch, J. (1998) Mol. Cell. Biochem. 194, 54–62
13. Cohen, P., Klumpp, S., and Scheling, D. L. (1989) Biochem. J. 258, 193–194
14. Kadowaki, T. (1995) J. Biol. Chem. 270, 13151–13156
15. Bevan, P. A., Burgess, J. W., Yale, J. F., Drake, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) J. Biol. Chem. 270, 20425–20430
16. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7688
17. Thomas, J. A., Schliender, K. K., and Lerner, J. A. (1968) Anal. Biochem. 25, 486–499
18. Bevan, P. A., Burgess, J. W., Yale, J. F., Drake, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) J. Biol. Chem. 270, 4860–4865
19. Thomas, J. A., Schliender, K. K., and Lerner, J. A. (1968) Anal. Biochem. 25, 486–499
20. Band, C. J., Posner, B. I., Dumas, V., and Contreras, J. O. (1997) Mol. Endocrinol. 11, 1899–1907
21. Bevan, P. A., Drake, P. G., Yale, J. F., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 181–190
22. Bevan, P. A., Burgess, J. W., Yale, J. F., Drake, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Am. J. Physiol. 268, 60–66
23. Band, C. J., and Posner, B. I. (1997) J. Biol. Chem. 272, 138–145
24. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 49–58
25. Bevan, P. A., Burgess, J. W., Yale, J. F., Drake, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) J. Biol. Chem. 270, 9031–9035
26. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) J. Biol. Chem. 270, 9031–9035
27. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 181–190
28. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 181–190
29. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) J. Biol. Chem. 270, 9031–9035
30. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 181–190
31. Bevan, P. A., Burgess, J. W., Yale, J. F., Drakes, P. G., Lachance, D., Baquiram, G., Shaver, A., and Posner, B. I. (1995) Mol. Cell. Biochem. 153, 181–190