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Regulation of Protein Kinase B and Glycogen Synthase Kinase-3 by Insulin and β-Adrenergic Agonists in Rat Epididymal Fat Cells

ACTIVATION OF PROTEIN KINASE B BY WORTMANNIN-SENSITIVE AND -INSENSITIVE MECHANISMS*

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S. Kelly Moule‡§, Gavin I. Welsh¶, Nigel J. Edgell‡, Emily J. Foulstone‡‖, Christopher G. Proud¶, and Richard M. Denton‡

From the ‡Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol, BS8 1TD and the ¶Department of Biosciences, University of Kent, Canterbury, CT2 7NJ, United Kingdom

Previous studies using L6 myotubes have suggested that glycogen synthase kinase-3 (GSK-3) is phosphorylated and inactivated in response to insulin by protein kinase B (PKB, also known as Akt or RAC) (Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovic, M., and Hemmings, B. A. (1995) *Nature* 378, 785–789). In the present study, marked increases in the activity of PKB have been shown to occur in insulin-treated rat epididymal fat cells with a time course compatible with the observed decrease in GSK-3 activity. Isoproterenol, acting primarily through β_3-adrenoreceptors, was found to decrease GSK-3 activity to a similar extent (approximately 50%) to insulin. However, unlike the effect of insulin, the inhibition of GSK by isoproterenol was not found to be sensitive to inhibition by the phosphatidylinositol 3′-kinase inhibitors, wortmannin or LY 294002. The change in GSK-3 activity brought about by isoproterenol could not be mimicked by the addition of permeant cyclic AMP analogues or forskolin to the cells, although at the concentrations used, these agents were able to stimulate lipolysis. Isoproterenol, but again not the cyclic AMP analogues, was found to increase the activity of PKB, although to a lesser extent than insulin. While wortmannin abolished the stimulation of PKB activity by insulin, it was without effect on the activation seen in response to isoproterenol. The activation of PKB by isoproterenol was not accompanied by any detectable change in the electrophoretic mobility of the protein on SDS-polyacrylamide gel electrophoresis. It would therefore appear that distinct mechanisms exist for the stimulation of PKB by insulin and isoproterenol in rat fat cells.

Glycogen synthase kinase-3 (GSK-3)[1] was first discovered by virtue of its ability to phosphorylate and inactivate the regulatory enzyme of glycogen synthesis in mammals, glycogen synthase (GS) (reviewed in Refs. 1–3). In a number of tissues GS is activated by insulin, and in skeletal muscle this has been shown to involve the dephosphorylation of the sites phosphorylated by GSK-3 (4–7). This suggested that GSK-3 might be inactivated by insulin, although more attention was initially focused on the regulation (activation) of the relevant phosphatase, a glycogen-bound form of protein phosphatase-1 (PP-1G) (8). Recently, however, it has become clear that GSK-3 itself is indeed subject to acute regulation, being inactivated in response to insulin or growth factors (9–16) and also following T-cell stimulation (17). Where tested, this inactivation was reversed by treatment with serine/threonine phosphatases indicating that it was due to increased phosphorylation of GSK-3 on one or more Ser/Thr residues (11).

It has since been shown that the inactivation of GSK-3 is associated with increased phosphorylation of a serine residue near the N terminus (Ser-9 of the β-isoform of mammalian GSK-3 which corresponds to Ser-21 of the α-isoenzyme) (11, 14, 16). This is of special interest since several insulin/growth factor-activated protein kinases have been shown to phosphorylate these residues in GSK-3 *in vitro*. These include the p90 ribosomal S6 protein kinase ($p90^{rsk}$), which is downstream of the mitogen-activated protein kinase (MAP kinase) cascade, and the p70 ribosomal S6 kinase ($p70^{S6k}$) (18, 19). Studies employing rapamycin, which selectively blocks activation of $p70^{S6k}$, appear to rule out a role for this enzyme in regulating GSK-3 in the cell types thus far studied (10, 12, 13). The evidence for a role for the MAP kinase pathway is mixed, with transfection approaches (either using $p90^{rsk}$ itself (14) or dominant negative mutants of an upstream kinase (15)) providing evidence for a role for this pathway and recent data from fat cells (13) and L6 myotubes (16) giving, respectively, correlative and strong evidence against a role for the MAP kinase pathway in regulating GSK-3 activity in response to insulin. The latter studies (16) provided evidence that protein kinase B (PKB, also known as RAC or Akt) might be responsible for the control of GSK-3 in L6 myotubes. PKB phosphorylates Ser-9 and Ser-21 in the β- and α-isoforms of GSK-3, respectively, is activated by insulin (16, 20, 21), and may lie downstream of phosphatidylinositol 3′-kinase (PI 3′-kinase) (16, 22). PI 3′-kinase is implicated in the control of GSK-3 in several cell types by the observation that wortmannin, a selective but not absolutely specific inhibitor of PI 3′-kinase, blocks the inactivation of GSK-3 by insulin and other agents (10, 12, 13, 16). LY 294002, a structurally unrelated inhibitor of PI 3′-kinase (23), has recently been shown to block the insulin-induced inactivation of GSK-3 in L6 myotubes (16).

In this study we demonstrate that insulin activates PKB in rat fat cells and that this may underlie the effects of insulin on GSK-3 activity in these cells. In addition, we report that the

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§ To whom correspondence should be addressed. Tel.: +44 117 9287432; Fax: +44 117 9288274.

‖ Recipient of an MRC Postgraduate Studentship.

[1] The abbreviations used are: GSK-3, glycogen synthase kinase-3; GS, glycogen synthase; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; PKB, protein kinase B; $p70^{S6k}$, p70 ribosomal S6 protein kinase; $p90^{rsk}$, p90 ribosomal S6 protein kinase; PI 3′-kinase, phosphatidylinositol 3′-kinase; cpt cAMP, chlorophenylthio-cAMP; db-cAMP, dibutyryl cAMP; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

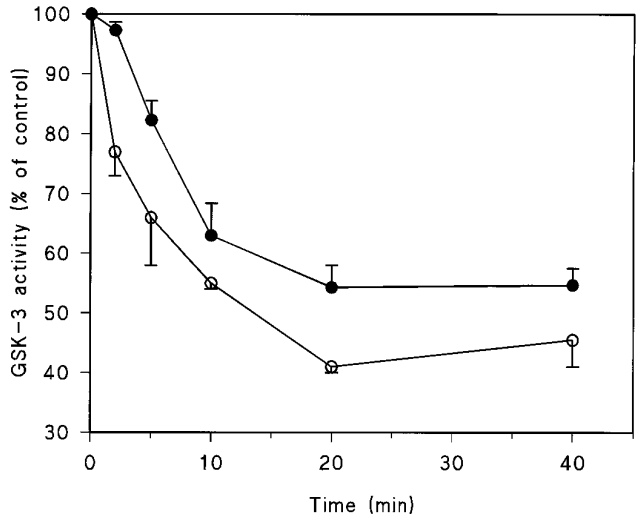

FIG. 1. **Time course for the effect of insulin and isoproterenol of GSK-3.** Adipocytes were incubated with insulin (83 nM, ○) or isoproterenol (1 μM, ●) for the times indicated prior to extraction. Results are expressed as a percentage of the control value at time 0 (39.5 ± 4.9 pmol of phosphate incorporated into substrate peptide/min/g dry cells, $n = 3$) and are means ± S.E. for three separate cell preparations. In the absence of hormones the activity of GSK-3 did not alter significantly with time and was 39.3 ± 3.2 pmol of phosphate/min/g dry cells ($n = 3$) after the 40-min incubation.

β-adrenergic agonist, isoproterenol, also causes the stimulation of PKB and inactivation of GSK-3 in fat cells. The effects of isoproterenol on these two kinases do not appear to be mediated by increases in the intracellular level of cAMP. A number of lines of evidence suggest that distinct mechanisms are involved in the activation of PKB by insulin and isoproterenol.

EXPERIMENTAL PROCEDURES

Materials—Male Wistar rats were fed *ad libitum* up to the time of killing on a stock laboratory diet (CRM; Bioshore, Manea, Cambs, UK). Collagenase was purchased from Worthington Diagnostic Systems (Freehold, NJ). Enhanced chemiluminescence Western blotting detection kits and [γ-^{32}P]ATP were from Amersham Int. (Amersham, Bucks, UK), and pepstatin, leupeptin, and antipain were from Cambridge Research Biochemicals (Harston, Cambs, UK). Microcystin, dithiothreitol, and rapamycin were obtained from Calbiochem (Nottingham, UK). LY 294002 was purchased from the Alexis Corp. (Nottingham, UK). All other chemicals and biochemicals were from Sigma or BDH (both of Poole, Dorset, UK). The β_3-agonist, BRL 37344, was a gift from Dr. P. Young of SmithKline Beecham Pharmaceuticals (Welwyn, Herts, UK). The PKB antiserum was raised in rabbits immunized with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence and was used without further purification. Anti-GSK-3 serum was a gift from Dr. J. Vandenheede (Katholieke Universiteit, Leuven, Belgium). All synthetic peptides, including those used for the assay of GSK-3, p70^{S6k}, and PKB were synthesized by Dr. G. Bloomberg (Dept. of Biochemistry, University of Bristol, UK). Wortmannin and rapamycin were dissolved in dimethyl sulfoxide and stored at −20 °C.

Preparation and Incubation of Epididymal Fat Cells—Adipocytes were isolated from epididymal fat pads, preincubated, and then incubated (at 150–250 mg cell dry weight/ml) as described previously (13, 24). For fat cells treated with wortmannin, LY 294002, or rapamycin, the appropriate control incubations contained 0.1% dimethyl sulfoxide.

Assays of GSK-3, p70^{S6k}, and PKB—The activities of GSK-3 and p70^{S6k} were measured in immunoprecipitates using peptide substrates as described previously (13). For the measurement of PKB activity, adipocytes (150–200 mg dry cell weight) were extracted in 1 ml of 50 mM Hepes (pH 7.6), 0.2 mM EDTA, 2.2 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, 10% glycerol, 1% Triton X-100, 1 μM microcystin, and 1 μg/ml each of pepstatin, leupeptin, and antipain. PKB was then immunoprecipitated from 750 μl of cell extracts with 5 mg of protein A-Sepharose and 5 μl of PKB antiserum for 2 h at 4 °C. Immunoprecipitates were washed twice with 1 ml of PKB assay buffer (20 mM MOPS (pH 7.0), 1 mM EDTA, 1 mM EGTA, 0.01% Brij 35, 5% glycerol) containing 0.5 M NaCl and twice with 1 ml of the same buffer without added

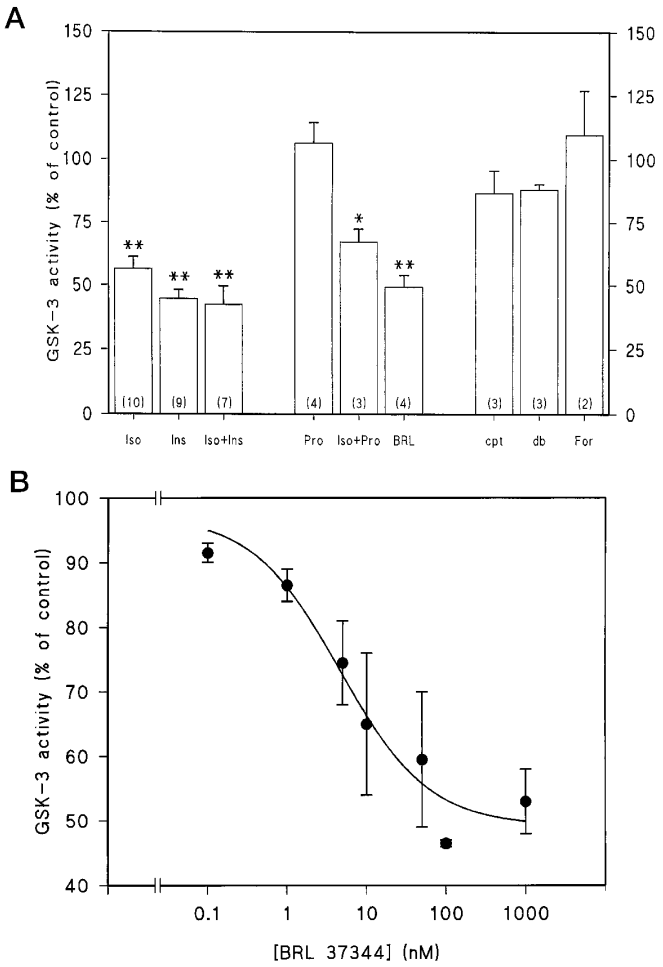

FIG. 2. **The effect of β-adrenergic agonists and agents that increase intracellular cAMP levels on GSK-3 activity.** *A*, adipocytes were incubated for 10 min with 83 nM insulin (*Ins*), 1 μM isoproterenol (*Iso*), 5 nM BRL37344 (*BRL*), 2 mM cpt-cAMP (*cpt*), 5 mM db-cAMP (*db*), or 5 nM forskolin (*For*) prior to extraction. Where indicated 10 μM propranolol (*Pro*) was added 10 min before addition of isoproterenol. Results are expressed as a percentage of the control value (no additions) for GSK-3 activity (45.6 ± 3.0 pmol of phosphate incorporated into substrate peptide/min/g dry cells, n = nine separate cell preparations) and are means ± S.E. for the number of separate cell preparations shown in *parentheses*. Significance, as assessed by the Student's t test, is indicated as follows: **, $p < 0.0001$, *, $p < 0.001$ *versus* appropriate control. *B,* dose response for the effect of BRL37344 on GSK-3 activity. Adipocytes were incubated for 10 min with varying concentrations of BRL 37344. Results are expressed as a percentage of the control value in the absence of BRL37344 (see *A*) and are means ± S.E. for two separate cell preparations.

salt. The protein A beads were finally resuspended in 40 μl of PKB assay buffer containing 0.1% mercaptoethanol and 2.5 μM cAMP-dependent protein kinase inhibitor peptide (IP$_{20}$). The activity of PKB in these immunoprecipitates was measured using either myelin basic protein (MBP, 0.5 mg/ml final concentration) or the synthetic peptide based on the sequence surrounding the serine phosphorylation site of GSK-3 (Ser-21 of GSK-3α and Ser-9 of GSK-3β), "cross-tide" (16) (100 μM), for 15 min at 30 °C.

Anion Exchange Chromatography, Gel Electrophoresis, and Immunoblotting of PKB—To separate proteins by anion exchange chromatography, fat cells were extracted as above and applied to a Pharmacia SMART system Mono Q column equilibrated with Buffer A (50 mM Tris (pH 7.3), 2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.03% Brij 35 (w/v), 0.1% mercaptoethanol, and 1 μg/ml pepstatin, leupeptin, and antipain). The column was developed with a 0–50% gradient of Buffer B (Buffer A plus 1 M NaCl) at a flow rate of 50 μl/min. Fractions (100 μl) were assayed for cross-tide kinase activity (10-μl fraction in a final reaction volume of 25 μl).

Western blotting was performed using immunoprecipitates run on 16-cm SDS-10% polyacrylamide gels (25) and blotted onto Immobilon-P

TABLE I

The effect of various agents on lipolysis and glycogen synthase activity ratio

Adipocytes were incubated with effectors as shown for 10 min prior to extraction. Extracts were assayed for GS activity in the presence or absence of glucose 6-phosphate, while the cell incubation media were retained for glycerol assays. Results are shown as the average measurement determined from the number of separate cell preparations shown in parentheses. Where appropriate (for $n > 2$), values are ± S.E., and significance ($p < 0.01$ *versus* control) is assessed by Student's t test and is denoted by *.

Condition	Rate of lipolysis (nmol glycerol released/10 min/g dry cells)	GS activity ratio ((−/+)-glucose 6-phosphate)
Control	207 ± 39 (6)	0.048 ± 0.009 (6)
Insulin (83 nM)	225 ± 47 (6)	0.238 ± 0.048 (6)*
Isoproterenol (1 μM)	2495 ± 340 (6)*	0.059 ± 0.008 (6)
Insulin + isoproterenol	2833 (2)	0.113 (2)
BRL37344 (5 nM)	1393 (2)	0.030 (2)
cpt-cAMP (2 mM)	1648 (2)	0.063 (2)
db-cAMP$^{\alpha}$ (5 mM)	2512 (2)	0.055 (2)
Forskolin (5 nM)	2425 (2)	0.057 (2)

membrane (Millipore, Watford, Herts, UK). Blots were incubated with PKB antiserum at a 1:400 dilution and immunoreacting proteins were visualized using the enhanced chemiluminescence detection system.

Assays of Glycogen Synthase and Lipolysis—The activity of glycogen synthase in fat cell extracts was determined as described elsewhere (13). Lipolysis was assayed by measuring the appearance of glycerol in the incubation medium (26).

RESULTS

Inactivation of GSK-3 in Fat Cells in Response to Isoproterenol—Incubation of adipocytes with isoproterenol led to a rapid decrease in the activity of GSK-3 (Fig. 1). Maximum inactivation of GSK-3 in response to isoproterenol was seen after approximately 20 min and was sustained for at least 40 min. This time course and the extent of inactivation (approximately 50%) were broadly similar to the changes in GSK-3 activity seen in response to insulin (see Fig. 1 and Ref. 13), although the effect of insulin was more rapid in onset. The effects of insulin and isoproterenol on GSK-3 activity were not additive (Fig. 2*A*). GSK-3 activity in all experiments was measured by following phosphate incorporation into a peptide substrate corresponding to the GSK-3 phosphorylation site in eukaryotic initiation factor-2B.[2] A similar inactivation of GSK-3 in response to isoproterenol was also observed when the kinase was assayed using a peptide based on its phosphorylation site in GS (see Ref. 13) (data not shown).

In order to investigate the mechanism by which isoproterenol inactivates GSK-3 in fat cells, a number of agents designed to either mimic or inhibit the effects of β-adrenergic agents was tested. The effects of isoproterenol on fat cells are mediated through its actions on β-adrenergic receptors. However, the effects of isoproterenol on GSK-3 in this system were not blocked by the classical β_1-/β_2-antagonist propranolol (Fig. 2*A*). This finding suggested that isoproterenol may be acting primarily via the β_3-receptor which is known to be insensitive to propranolol (see Ref. 28). The β_3-receptor is thought to be the primary adrenergic receptor mediating the effects of β-adrenergic agents on lipolysis in rat white adipose tissue (29). Like isoproterenol, the β_3-receptor-specific agonist, BRL 37344 (30, 31), caused a significant decrease in the activity of GSK-3 (Fig. 2*A*). The effects of BRL 37344 on GSK-3 activity were half-maximal at approximately 5 nM (Fig. 2*B*). This is consistent with reported EC_{50} values for the actions of BRL 37344 on the stimulation of lipolysis in this cell type (32–34).

The binding of β-adrenergic agonists to their receptors activates adenylate cyclase and thereby increases cytoplasmic cAMP levels. In order to address the possibility that the inactivation of GSK-3 was a consequence of elevated cAMP, we adopted two approaches. In the first, cells were treated with the cell-permeant cAMP analogues, db-cAMP and cpt-cAMP. This had no effect on the activity of GSK-3 (Fig. 2*A*) although they did stimulate lipolysis over 8-fold (see Table I). In the second, we made use of forskolin, which activates adenylate cyclase. Treatment of cells with forskolin also failed to cause inactivation of GSK-3 (Fig. 2*A*) but did increase the rate of lipolysis in these cells to an extent comparable with isoproterenol (Table I). It therefore seems unlikely that isoproterenol acts via cAMP to inactivate GSK-3 in these cells. Table I also shows the effects of various agents on the activity ratio ((−/+)-glucose 6-phosphate) of GS in fat cells. Despite decreasing the activity GSK-3, neither isoproterenol nor BRL 37344 on their own had any effect on the activity ratio of GS. The pronounced stimulation of GS seen with insulin was approximately halved when isoproterenol was included in the incubation.

The Isoproterenol-induced Inactivation of GSK-3 Is Not Blocked by Inhibitors of PI 3′-Kinase or by Rapamycin—Since the inactivation of GSK-3 by isoproterenol could not be mimicked by raising intracellular cAMP levels, we sought to examine other possible mechanisms by which this effect might occur. In rat fat cells, the inactivation of GSK-3 by insulin is blocked by wortmannin, an inhibitor of PI 3′-kinase (13). In contrast, wortmannin had no effect on the inactivation of GSK-3 by isoproterenol or by BRL 37344. (Fig. 3). Since wortmannin does block the regulation of GSK-3 by other stimuli such as epidermal growth factor and insulin-like growth factor-1 in other cell types (10, 12, 16), the mechanism by which adrenergic agonists decrease GSK-3 activity appears to be quite distinct from that employed by agents acting through receptor tyrosine kinases. To further test for the possible involvement of PI 3′-kinase in the effect of isoproterenol on GSK-3, the fat cells were pretreated with the unrelated PI 3′-kinase inhibitor LY 294002. As is the case for wortmannin, although LY 294002 abolished the effect of insulin on GSK-3, it was unable to prevent the inhibition seen in response to isoproterenol (Fig. 3).

As the inactivation of GSK-3 induced by isoproterenol was not affected by wortmannin, which completely blocks activation of $p70^{S6k}$ by insulin in fat cells (13), it is very unlikely that the inactivation of GSK-3 is mediated by this signaling pathway. This is supported by the observation that rapamycin (which specifically blocks the activation of $p70^{S6k}$ without affecting MAP kinase) had no effect upon the inactivation of GSK-3 induced by isoproterenol or BRL 37344 (Fig. 3). Rapamycin also has no effect upon the control of GSK-3 in other cell types tested (10, 12, 13, 16). At the dose used here (20 nM), rapamycin completely blocked the activation of $p70^{S6k}$ induced by insulin (see Ref. 13).

Insulin and Isoproterenol Activate PKB in Rat Epididymal Fat Cells—Cross *et al.* (16) have recently shown that PKB can phosphorylate GSK-3 *in vitro* and cause its inactivation. PKB phosphorylates the same N-terminal serine residue phosphorylated by $p70^{S6k}$ or $p90^{rsk}$ (18, 19). Since PKB is thought to be

[2] G. I. Welsh, J. C. Patel, and C. G. Proud, submitted for publication.

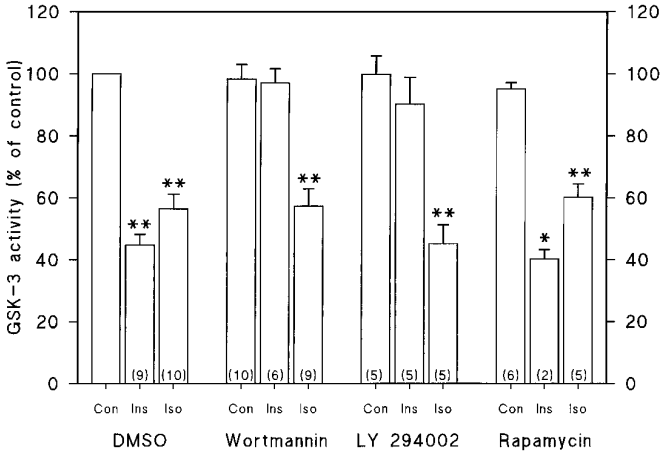

FIG. 3. **The effects of wortmannin, LY 294002, and rapamycin on the stimulation of GSK-3 activity by insulin and β-adrenergic agonists.** Adipocytes were pretreated with wortmannin (100 nM) or rapamycin (20 nM) for 30 min or with LY 294002 (50 μM) for 15 min prior to the addition of hormones for a further 10 min. Abbreviations for the effectors are as outlined in Fig. 2*A*. Results are expressed as a percentage of the control value (see Fig. 2*A*) and are means ± S.E. for the number of separate cell preparations shown in *parentheses*. Where appropriate significance (assessed as in Fig. 2*A*) is indicated as follows: **, $p < 0.001$; *, $p < 0.05$.

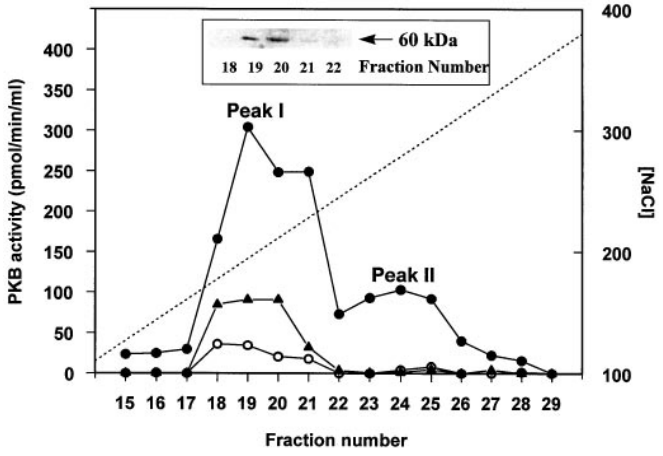

FIG. 4. **Identification of PKB by Mono Q chromatography.** Extracts were prepared from fat cells treated with insulin (●), isoproterenol (▲), or no additions (○) and fractionated by Mono Q chromatography. PKB activity is expressed as pmol of phosphate incorporated into cross-tide/min/ml fraction. The salt gradient is indicated by the *dotted line*. Similar results were obtained with four separate cell preparations. *Inset,* Western blot showing elution position of PKB from insulin-treated extracts. PKB was not detected in any other fractions. The elution position of PKB did not change under control or isoproterenol-treated conditions.

downstream of PI 3′-kinase (20, 22), this would provide an explanation of the ability of wortmannin to block completely the regulation of GSK-3 by insulin and other stimuli. Thus, insulin by, for example, activating PI 3′-kinase would also bring about the activation of PKB and therefore the phosphorylation and inactivation of GSK-3.

We have therefore examined the regulation of PKB activity by insulin and isoproterenol in fat cells. Extracts from adipocytes treated with insulin or isoproterenol were separated by Mono Q chromatography essentially as described by Cross *et al.* (16). Insulin increased the kinase activity toward cross-tide in two broad peaks (I and II) (Fig. 4). Peak I (eluting between 150–250 mM NaCl) was found to coincide with the elution position of PKB as shown by immunoblotting (Fig. 4, *inset*). The cross-tide kinase activity eluting at this position was thus due, at least in part, to the activity of PKB. Immunoreactive PKB could not be detected in peak II, and thus the activity toward cross-tide in these fractions is likely to be due to some other unidentified kinase. Isoproterenol increased the kinase activity toward cross-tide in peak I but not peak II. The effect of isoproterenol on the kinase activity in this peak was less than the stimulation seen in response to insulin.

The stimulation of PKB by both insulin and isoproterenol in whole cell extracts was confirmed by measuring kinase activity in anti-PKB immunoprecipitates using either cross-tide or myelin basic protein as a substrate. Table II shows that the both hormones increase the activity of PKB, with the effects of insulin being greater than those of isoproterenol. The fold effects observed were similar whether cross-tide or MBP was used to measure kinase activity. To further verify that the kinase activity measured in immunoprecipitates was actually PKB, we preincubated the protein A-Sepharose beads with anti-PKB antibody plus the peptide to which the antiserum was raised (see "Experimental Procedures") prior to the addition of beads to fat cell extracts. This peptide was able to compete with PKB for binding to the antiserum, as shown by its ability to abolish kinase activity toward MBP in immunoprecipitates from control, insulin-, or isoproterenol-treated cell extracts (data not shown).

The time courses for activation of PKB by insulin and isoproterenol are shown in Fig. 5. Insulin caused an extremely

TABLE II

Measurement of PKB activity using myelin basic protein or cross-tide

Adipocytes were incubated with insulin (83 nM) or isoproterenol (1 μM) for 10 min prior to extraction. The MBP or cross-tide kinase activity was measured in anti-PKB immunoprecipitates as described under "Experimental Procedures." Results are expressed as pmol of ^{32}P incorporated into substrate/min/g dry ± S.E. for the number of separate cell preparations shown in parentheses. Significance of the hormone effects (*versus* control) is assessed by the Student's *t* test, and is denoted by * ($p < 0.001$).

Condition	MBP kinase activity	Cross-tide kinase activity
Control	0.56 ± 0.04 (10)	0.46 ± 0.03 (3)
Insulin	1.85 ± 0.13 (17)*	2.20 ± 0.32 (3)*
Isoproterenol	1.15 ± 0.05 (15)*	1.24 ± 0.13 (3)*

rapid stimulation of PKB activity which declined slowly over 40 min. In contrast, the activation in response to isoproterenol was slower and more transient. A comparison of the time courses for GSK-3 inactivation (Fig. 1) and PKB stimulation (Fig. 5) shows a number of discrepancies between the two. First, complete activation of PKB, even with isoproterenol, was seen after 5 min, while maximal inhibition of GSK-3 occurred only after 20 min. Second, inactivation of GSK-3 was sustained even after PKB activities had returned to basal levels.

The stimulation of PKB by insulin has also been reported in a number of other cell types (16, 20, 21) and is accompanied by a change in the mobility of the protein on SDS-PAGE. This band-shift in response to insulin is thought to be a result of changes in the phosphorylation of PKB (21, 22, 35) and may be seen on stimulation of fat cells with the hormone (see Fig. 6 *inset* and Ref. 21). Interestingly the increase in PKB activity on treatment with isoproterenol is not associated with any detectable change in the electrophoretic mobility of the protein. The effects of insulin and isoproterenol on PKB activity were not additive (Fig. 6). The primary effect of isoproterenol again appears to be via the β_3-adrenergic receptor because BRL 37344 is also able to stimulate PKB. The lack of effect of either cpt-cAMP or db-cAMP indicates that the increase of PKB activity in response to β-adrenergic agonists does not occur as a result of increased cytoplasmic cAMP levels (Fig. 6).

The data in Fig. 7 show that while the effect of insulin was

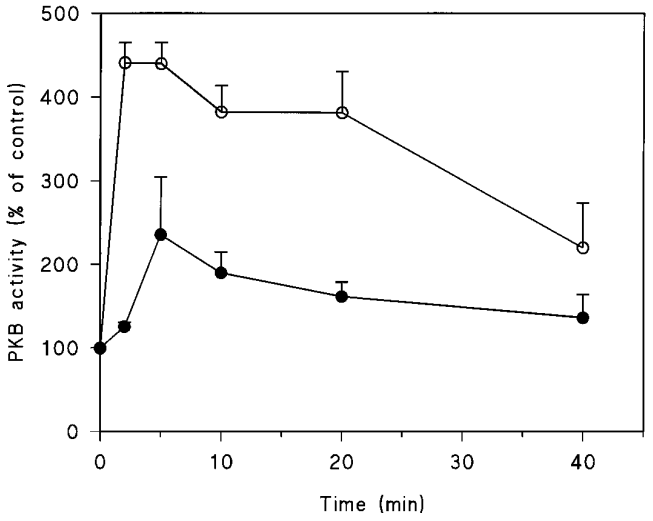

FIG. 5. **Time course for activation of PKB by insulin and isoproterenol.** Adipocytes were incubated with insulin (○) or isoproterenol (●) for the times indicated. The results are expressed as percentage increase from the control PKB activity at time 0 (0.53 ± 0.11 pmol of phosphate incorporated into MBP/min/g dry cells, $n = 3$ separate cell preparations) and are the average ± S.E. from three separate cell preparations. PKB activity in the absence of hormones did not alter significantly over the incubation period and was 0.49 ± 0.05 pmol of phosphate/min/g dry cells ($n = 3$) at 40 min.

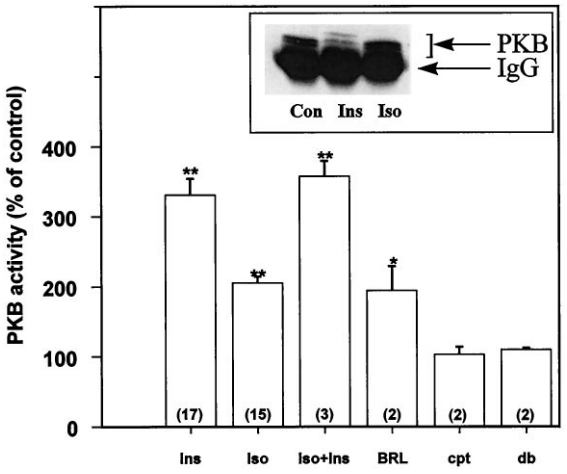

FIG. 6. **The effect of insulin, β-adrenergic agonists, and cAMP analogues on PKB activity.** Adipocytes were incubated with 83 nM insulin (*Ins*), 1 μM isoproterenol (*Iso*), 5 nM BRL 37344 (*BRL*), 2 mM cpt-cAMP (*cpt*), or 5 mM db-cAMP (*db*) for 10 min prior to extraction. Results are expressed as a percentage of the control value (no additions) for PKB activity (0.56 ± 0.04 pmol of phosphate incorporated into MBP/min/g dry cells, $n = 10$ separate cell preparations) and are means ± S.E. for the number of separate cell preparations shown in *parentheses*. Where appropriate significance (assessed as in Fig. 2*A*) is indicated as follows: **, $p < 0.001$; *, $p < 0.05$. *Inset* shows a Western blot of anti-PKB immunoprecipitates from control (*Con*), insulin (*Ins*), or isoproterenol (*Iso*)-treated fat cell extracts. *IgG* indicates the position of the immunoglobulin heavy chains.

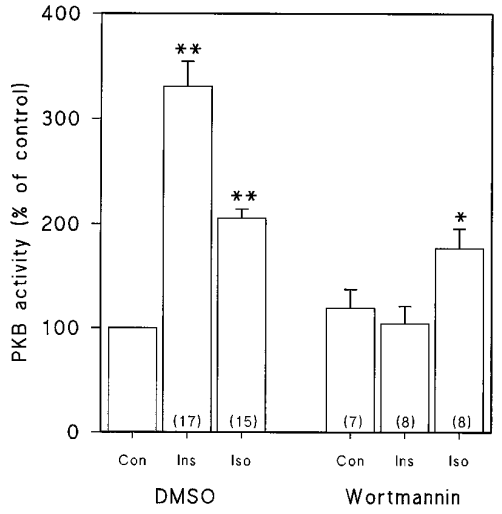

FIG. 7. **The effect of wortmannin on the activation of PKB by insulin and isoproterenol.** Adipocytes were preincubated with wortmannin (100 nM) for 30 min. Cells were then incubated for a further 10 min with no further additions (*Con*), 83 nM insulin (*Ins*), or 1 μM isoproterenol (*Iso*). Results are expressed as a percentage of the control value for PKB activity (see Fig. 6) and are means ± S.E. for the number of separate cell preparations shown in *parentheses*. Where appropriate significance (assessed as in Fig. 2*A*) is indicated as follows: **, $p < 0.001$; *, $p < 0.05$. *DMSO*, dimethyl sulfoxide.

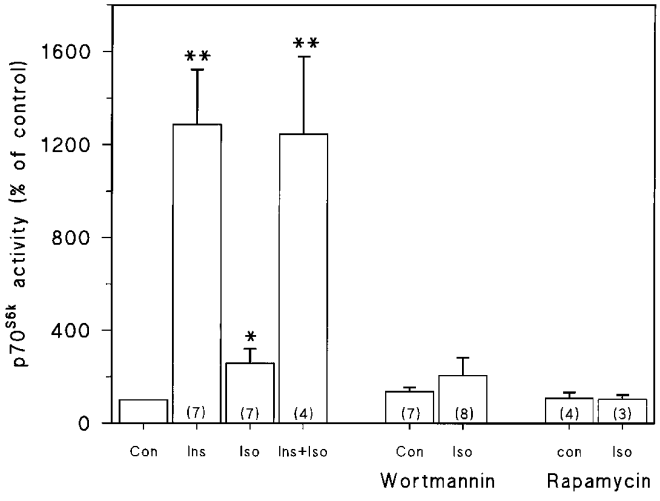

FIG. 8. **Effect of isoproterenol on p70^{S6k} activity.** Adipocytes were preincubated with wortmannin (100 nM) or rapamycin (20 nM) for 30 min as indicated prior to the addition of insulin (*Ins*) or isoproterenol (*Iso*). Results are expressed as a percentage of the control (*Con*) value (4.0 ± 0.5 pmol of phosphate incorporated into substrate peptide/min/g dry cells, $n = 9$) and are means ± S.E. for the number of separate cell preparations shown in *parentheses*. Where appropriate significance (assessed as in Fig. 2*A*) is indicated as follows: **, $p < 0.001$; *, $p < 0.05$.

abolished by preincubation of the adipocytes with wortmannin, the inhibitor had no significant effect on the stimulation of PKB by isoproterenol. In agreement with others (21), wortmannin also reversed the effect of insulin on the band-shift seen with PKB on SDS-PAGE (data not shown). Rapamycin was without effect on changes in the activity of PKB in response to either hormone (data not shown).

Given that PKB is reported to lie upstream of p70^{S6k} (22), we determined whether isoproterenol activated this kinase. Isoproterenol gave a significant activation, which was blocked by rapamycin but was modest in comparison to that induced by insulin (Fig. 8). In contrast to the effect of isoproterenol on PKB activation and GSK-3 inhibition, the increase in p70^{S6k} activity was largely eliminated by wortmannin and rapamycin.

DISCUSSION

GSK-3 in Rat Epididymal Fat Cells Is Activated by Isoproterenol as Well as Insulin—The data presented in this paper show that in freshly isolated rat adipocytes treated with isoproterenol, there is a marked inhibition of GSK-3 activity. Isoproterenol was also found to increase the activity of the kinase proposed to be upstream of GSK-3, namely PKB. We have been unable to find any change in the activity ratio of GS

((−/+)-glucose 6-phosphate) in response to isoproterenol; indeed, isoproterenol partially reverses the activation of GS seen in response to insulin (see also Ref. 36). Since decreases in GSK-3 activity have been proposed to be responsible, at least in part, for activation of GS, these findings were of interest. Our results seem to indicate that GSK-3 is not the primary regulator of GS activity in rat fat cells and suggest that PP1-G probably plays a more important role in the regulation of glycogen synthesis *in vivo*. It has been suggested that PP1-G plays the dominant role in the regulation of rat muscle GS (see Ref. 37). The mechanisms involved in the regulation of glycogen metabolism in different tissues may be distinct. In rat muscle (37) and in 3T3-L1 adipocytes (38) the stimulation of GS by insulin is partially sensitive to inhibition by rapamycin, whereas in primary rat adipocytes it is not (13).

PKB in Rat Epididymal Fat Cells Is Activated by Insulin and Isoproterenol by Apparently Different Mechanisms—As PKB has been implicated as the direct upstream regulator of GSK-3 in L6 myotubes (16), it was important to determine its activity in adipocytes. Here we report the first direct measurement of PKB activity in rat fat cells and have shown substantial and rapid stimulation of the kinase in response to insulin. Isoproterenol was also found to activate PKB in these cells. Overall, the congruence between the actions of all effectors on GSK-3 inactivation and PKB activation supports the view that PKB is the upstream regulator of GSK-3 in fat cells, as concluded by Cross *et al.* (16) from their studies in L6 myotubes. In further agreement with this view, the activation of PKB in the present study is considerably more rapid than the inactivation of GSK-3 (Figs. 1 and 5). However, in the absence of a specific inhibitor of PKB it is not possible to obtain conclusive proof that PKB is indeed upstream of GSK-3.

The finding that isoproterenol also causes a small stimulation of $p70^{S6k}$ is interesting in light of the proposal that this kinase is downstream of PKB (22). The physiological relevance for the activation of this S6 kinase under these conditions remains to be explained.

Our results also have important implications for understanding the mechanisms by which PKB is regulated in fat cells. The exact means by which PKB activity is controlled remains obscure, with a number of different factors being implicated, acting either independently or in conjunction with each other. In other cell types there is strong evidence that the activation of PKB is mediated via PI 3′-kinase (20, 22), although the direct involvement of PI 3′-kinase products remains controversial (20, 39). In fat cells, the differing effects of wortmannin on PKB activation by insulin and isoproterenol, along with the failure of isoproterenol to produce a band-shift in PKB, would seem to indicate that different mechanisms are involved in the stimulation of the kinase by these hormones. A role for PI 3′-kinase in the activation of PKB by insulin in fat cells seems likely as the stimulation is abolished by preincubation with wortmannin. Changes in PKB activity in response to isoproterenol do not appear to involve PI 3′-kinase activation as this inhibitor was without effect. Isoproterenol has no effect on adipocyte PI 3′-kinase activity when measured in anti-PI 3′-kinase p85 immunoprecipitates (40). It is, however, possible that β-adrenergic agonists increase the amount of PI 3′-kinase products in fat cells via activation of another, as yet uncharacterized, isoform of PI 3′-kinase. Indeed, a $G_{\beta\gamma}$-sensitive isoform of PI 3′-kinase has been described in myeloid-derived cells and in platelets (41, 42).

In agreement with Kohn *et al.* (21), the activation of PKB in fat cells in response to insulin was accompanied by a change in the mobility of the kinase on SDS-PAGE. In this respect, the mechanism by which isoproterenol activates PKB again clearly differs from that involved in insulin action, as it is not associated with a change in the migration of the protein on gel electrophoresis (Fig. 6, *inset*). The activation of the kinase and SDS-PAGE band-shift in response to platelet-derived growth factor in Rat-1 fibroblasts is thought to be a direct result of changes in the phosphorylation of PKB, as both are reversed by treatment with phosphatase (22). It is likely that activation of PKB by insulin in fat cells involves a similar phosphorylation step. Activation in response to isoproterenol, however, appears to occur independently of the phosphorylation event responsible for band-shift of the protein. It is possible that isoproterenol induces changes in the phosphorylation of PKB which cause changes in kinase activity but are not manifested as changes in its electrophoretic mobility.

Finally, it has been proposed that activation of PKB can occur as a result of protein-protein interactions mediated by its pleckstrin homology domain (43, 44). The pleckstrin homology domains from the three known PKB isoforms (α, β, and γ) have recently been shown to interact with PKC-α, -δ, and -ζ subtypes and with G protein βγ subunits ($G_{\beta\gamma}$) *in vitro* (43), but the effects of these interactions on PKB activity have yet to be determined. It is possible that the effect of isoproterenol is mediated by the binding of $G_{\beta\gamma}$ subunits to the pleckstrin homology domain, in a manner analogous to the activation of β-adrenergic receptor kinase on binding of $G_{\beta\gamma}$ (see Ref. 27). Further work clearly needs to be done to clarify the question of how β-adrenergic agonists stimulate PKB.

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