Involvement of Both Phosphatidylinositol 3-Kinase and p44/p42 Mitogen-Activated Protein Kinase Pathways in the Short-Term Regulation of Pyruvate Kinase L by Insulin*

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ABSTRACT

Pyruvate kinase L (PK-L) is a key regulatory enzyme of the hepatic glycolytic/gluconeogenic pathway that can be dephosphorylated and activated in response to insulin. However, the signaling cascades involved in this insulin effect have not been established. In this work we have investigated the potential involvement of phosphatidylinositol 3-kinase (PI 3-K) and p44/p42 mitogen-activated protein kinase (MAPK) pathways in the short-term modulation of PK-L by insulin in primary cultures of rat hepatocytes. Wortmannin, at a concentration of 100 nM, caused a marked inhibition of the PI 3-K/protein kinase B (PKB) signaling pathway. This occurs by rapid recruitment and activation of PI 3-K in the proximity of the plasma membrane, followed by an intracellular increment in phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] concentration and activation of PK-L, respectively. However, this PI 3-K inhibitor also reduced insulin-mediated phosphorylation of p44/p42 MAPK in cultured rat hepatocytes, indicating that both the PI 3-K and MAPK pathways could be involved in PK-L activation by insulin. Three facts appear to reinforce this hypothesis: 1) the selective and complete inhibition of the PI 3-K/protein kinase B pathway by LY294002 (50 μM) was accompanied by a partial blockade of insulin-induced PK-L activation; 2) when signaling through the MAPK cascade was selectively suppressed by the presence of PD98059 (50 μM), a 50% reduction of insulin-induced activation of PK-L was observed; and 3) the effect of PD98059 (50 μM) on PK-L activation was reinforced by the additional presence of 100 nM wortmannin. We also observed that the blockade of p70 S6-kinase by rapamycin did not affect the activation of PK-L by insulin. From these findings it can be concluded that both PI 3-K and MAPK pathways, but not p70 S6-kinase, are involved in the short-term activation of PK-L by insulin in rat hepatocytes. (Endocrinology 142: 1057–1064, 2001)

Insulin plays a key role in regulating a wide range of cellular processes, including carbohydrate and lipid metabolism, protein synthesis, and cell growth (1). In recent years, the molecular mechanisms involved in insulin signaling have been extensively investigated. Thus, insulin receptor undergoes autophosphorylation on tyrosine residues upon binding of the hormone, which enhances its tyrosine kinase activity toward different cytosolic proteins, such as insulin receptor substrates (IRSs) and Shc (1, 2). Tyrosine phosphorylation of both IRS-1 and IRS-2 causes the activation of the phosphatidylinositol 3-kinase (PI 3-K)/protein kinase B (PKB) signaling pathway. This occurs by rapid recruitment and activation of PI 3-K in the proximity of the cell membrane, followed by an intracellular increment in phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] concentration (3, 4). This metabolite is required for the activation of PKB (5–7) and may also bind guanine nucleotide exchange factors, phospholipase Cγ, and other cytosolic proteins (4). There is also evidence that stimulation of PI 3-K is involved in the activation of p70 S6-kinase (p70 S6-K) by insulin (8). On the other hand, tyrosine-phosphorylated IRSs and Shc also interact with SH-2 domains of growth factor receptor-binding protein 2 (1, 2). This protein binds proline-rich motifs on SOS through its SH-3 domains, which results in the activation of Ras. This is followed by translocation of Raf-1 to the plasma membrane and activation of the mitogen-activated protein kinase (MAPK) cascade (9, 10).

The involvement of the above-mentioned signaling cascades in the long- and short-term metabolic effects elicited by insulin, such as stimulation of glucose transport (11), acceleration of both glycogen (12, 13) and protein synthesis (14), inhibition of lipolysis (15), and repression of both phosphoenolpyruvate carboxykinase and glucose 6-phosphatase gene transcription (16, 17) has been recently established. However, the signaling pathways involved in the short-term control of key regulatory steps of hepatic gluconeogenesis by insulin have not been elucidated. In the liver, the glycolytic/gluconeogenic pathway is tightly regulated by hormones such as insulin, glucagon, and epinephrine, acting at both the fructose 6-P/fructose 1,6-P2 and the phosphoenolpyruvate/ pyruvate substrate cycles (18, 19). In the latter substrate cycle, the short-term hormonal control is exerted by modulating PK-L activity. This enzyme is regulated by allosteric effectors and phosphorylation/dephosphorylation mechanisms. In the liver, glucagon can elicit the phosphorylation and inactivation of PK-L through the stimulation of a CAMP-depen-
dent protein kinase (18, 19). Dephosphorylation and re-activation of PK-L appear to be mediated by protein phosphatases 2A and/or 2C (20–22). It is known that insulin may antagonize the effects of glucagon on PK-L by decreasing the hepatic concentration of cAMP (18, 19). Furthermore, insulin may induce the activation of PK-L without significant changes in the hepatic content of cAMP (19, 23, 24) by a process in which the dephosphorylation of the enzyme has been implicated (23–25). In connection with this, it has been demonstrated that the activation of PK-L by insulin in isolated rat hepatocytes is accompanied by a transient increase in pyruvate kinase phosphatase activity (22, 25).

In this work we have investigated the potential involvement of PI 3-K/PKB and p44/p42 MAPK [extracellular signal-regulated kinase 1/2 (ERK-1/ERK-2)] signaling pathways as well as p70 S6-K in the insulin-induced activation of PK-L in primary cultures of rat hepatocytes. Our results show that the presence of PI 3-K inhibitors, such as wortmannin or LY294002, in the incubation medium significantly reduced the activation of PK-L by insulin. Furthermore, PD98059 at a concentration that completely blocked insulin signaling through the MAPK cascade caused a 50% reduction in insulin-induced activation of PK-L, an effect that was further reinforced by the additional presence of a suboptimal concentration of wortmannin. It was also observed that the blockade of p70 S6-K by rapamycin did not significantly affect the activation of PK-L by insulin. These results indicate that both p44/p42 MAPK and PI 3-K signaling pathways, but not p70 S6-K, are implicated in the short-term regulation of PK-L by insulin.

Materials and Methods

Reagents

Antibodies against IRS-1 and p85 were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Mouse antiphosphotyrosine and anti-IRS2 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against the phosphorylated forms of p44/p42 MAPK (Thr182/Tyr202), PKB (Ser473 and Thr383), and p70 S6-K (Thr389) as well as the p44/p42 MAPK antibody were obtained from New England Biolabs, Inc. (Taunus, Germany). Polyclinilinene difluoride (PVDF) membranes were purchased from Millipore Corp. (Bedford, MA). Collagenase A, from Novo Industri (Copenhagen, Denmark). Earle’s medium 199 was obtained from ICN Iberica, SA (Madrid, Spain). Wortmannin and tert-phosphatidylinositol were purchased from Sigma (St. Louis, MO). Rapamycin, PD98059, and LY294002 were purchased from Calbiochem (San Diego, CA). Insulin was from Novo Industri (Copenhagen, Denmark). Earle’s medium 199 was obtained from ICN Iberica, S.A. (Barcelona, Spain). Collagenase A, substrates, auxiliary enzymes, and coenzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany). [γ-32P]ATP (3 Ci/μmol) and enhanced chemiluminescence reagents were provided by Amersham Pharmacia Biotech (Barcelona, Spain). The remaining reagents, all of analytical grade, were obtained from Roche, Sigma, or Merck & Co., Inc. (Darmstadt, Germany).

Animals

Fed male Wistar rats from our inbred colony were used. The animals were maintained on standard chow (A.04 Panlab SL, Barcelona, Spain) and water ad libitum and were housed in animal quarters at constant temperature (23 C) with a fixed light cycle (12 h). The animal experiment described has been conducted in accordance with the highest standards of humane animal care. All animals were anesthetized with sodium pentobarbital (5 mg/100 g BW) immediately before the experiments.

Culture conditions and cell incubations

Hepatocytes were isolated by perfusion of the liver with collagenase (26) and suspended in medium 199 supplemented with 10% FBS, 0.1% BSA, 1 μM T3, 1 μM dexamethasone, and glucose up to 10 mM. Hepatocytes were plated to attain a cell density of 1.2 × 10^5 cells/cm² and cultured in a cell incubator at 37 C in an atmosphere of 5% CO2. Four hours after plating, the medium was replaced by fresh supplemented medium 199. After 19 h of culture, hepatocyte monolayers were washed three times with PBS and incubated for an additional 2 h in medium 199 supplemented with 20 mM HEPES (pH 7.4), 0.1% BSA, and 10 mM glucose. Then, cells were treated with insulin for 5 or 10 min, as indicated. Wortmannin, LY294002, and rapamycin were added to the incubation medium 10 min before the addition of insulin, whereas PD98059 was incorporated into the medium 30 min before the addition of hormone. Appropriate vehicle controls were included in every experiment.

Analytical procedures

For the measurement of pyruvate kinase activity, hepatocyte monolayers were washed once with ice-cold 0.9% NaCl and immediately frozen in liquid N2 at the indicated times. Then, cell monolayers were thawed in a medium containing 50 mM glycyglycine, 15 mM EGTA, and 100 mM potassium fluoride, pH 7.4, and homogenized with a Polytron PT 1200C (Kinematica AG, Lucerne, Switzerland). After centrifugation (10,000 × g for 5 min), pyruvate kinase activity was determined in the supernatants using a spectrophotometric assay as described by Felıú et al. (27). The incubation mixture contained 50 mM glycyglycine (pH 7.4), 0.1 M KCl, 10 mM MgCl₂, 1.25 mM Mg-ADP, 0.15 mM NADH, and 1 U lactate dehydrogenase in a total volume of 1 ml. Aliquots of the supernatant were added to the mixture and allowed to stand for 10 min at 30 C; the reaction was started by the addition of either 0.15 mM (v0.15) or 5 mM phosphoenolpyruvate (Vmax).

For immunoprecipitations, hepatocytes were washed once with ice-cold 0.9% NaCl and immediately homogenized in a lysis medium containing 50 mM HEPES (pH 7.4), 10 mM NaPO₄, 100 mM NaF, 10 mM EDTA, 1 mM NaN₃VO₄, 1% Triton X-100, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM benzamidine, and 2 mM phenylmethylsulfonyl fluoride. Cell lysates were centrifuged (10,000 × g for 30 min), and the supernatants were incubated for 3 h with the corresponding antibody. Then, the immunocomplexes were captured with protein A-agarose (Sigma), and the immunoprecipitates by32P incorporation into phosphatidylinositol (28).

For immunoblotting, cell lysates or immunoprecipitates were subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and blocked with 5% nonfat dried milk dissolved in a solution (TBS) containing 20 mM Tris (pH 7.4), 0.05% Tween-20, and 150 mM NaCl. Then, PVDF membranes were probed with the corresponding antibody and washed four times (5 min each) with TBSB. Afterward, membranes were incubated with horseradish peroxidase conjugated to antirabbit or antimouse IgG (1:2000). Finally, membranes were washed four times with TBSB and developed by enhanced chemiluminescence. All immunoprecipitations, immunoblots, and PI 3-K activity assays were performed in at least two separate experiments.

Statistical analysis

Where applicable, data are expressed as the mean ± SEM, with n equal to the number of different experiments. Paired Student’s t test was performed when two groups were compared. One-way ANOVA with repeated measures was used, followed by Tukey-Kramer’s test, when multiple groups were compared (Instat, GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant when P < 0.05.

Results

Activation of PI 3-K by insulin

As expected, insulin elicited a dose-dependent increase in tyrosine phosphorylation of IRS-1 and IRS-2 in primary cul-

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tured rat hepatocytes (Fig. 1). This was accompanied by a dose-dependent association of p85 with phosphorylated IRSs and an activation of PI 3-K. It is of note that clear increases in tyrosine phosphorylation of both IRS-1 and IRS-2, in p85 association, and in PI 3-K activity were observed at the lowest insulin concentration tested (0.1 nm).

**Effect of wortmannin on insulin-induced PK-L activation**

When cultured hepatocytes were incubated for 10 min with increasing concentrations of insulin, a significant dose-dependent increment in the PK-L activity ratio ($v_{0.15}/V_{max}$) was observed (by ANOVA, $P < 0.0001$; Fig. 2). It is well established that the PK-L activity ratio is inversely related to the degree of phosphorylation of the enzyme (18, 29). Moreover, in good agreement with other reports (23, 25), the activation of PK-L by insulin was still evident after ammonium sulfate precipitation of the enzyme (data not shown), suggesting that a covalent change in the PK-L molecule (possibly dephosphorylation) occurs in response to insulin (22–25).

To examine the role of PI 3-K in the activation of PK-L by insulin, we used wortmannin, a well known inhibitor of PI 3-K activity (30). Thus, treatment of cultured hepatocytes with 100 nm wortmannin completely abolished the activation of PK-L caused by 0.1 nm insulin and decreased the hormonal effects elicited by 1 and 10 nm insulin (reductions of 38% and 32%, respectively). When wortmannin was used at a concentration of 500 nm, the activation of PK-L was completely blocked, even in the presence of saturating concentrations of insulin (Fig. 2).

The differences in insulin-induced PK-L activation observed in the presence of 100 and 500 nm wortmannin could be related to distinct degrees of PI 3-K inhibition. To check this possibility, PI 3-K activity was measured in anti-IRS-1 and anti-IRS-2 immunoprecipitates obtained from hepatocytes incubated with increasing concentrations of insulin in the absence and presence of the inhibitor. As shown in Fig. 3, both 100 and 500 nm wortmannin strongly inhibited insulin-dependent activation of PI 3-K associated with IRS-1 and IRS-2, at all of the assayed insulin concentrations. However, slight differences appeared to exist between residual PI 3-K activities found in hepatocytes incubated in the presence of 100 and 500 nm wortmannin. To assess the consequence of these slight differences in PI 3-K activity in steps of the insulin signaling cascade downstream of this enzyme, we examined the degree of phosphorylation of PKB on both Thr$^{308}$ and Ser$^{473}$ residues, which is known to be dependent on PI 3-K activation (5–7). As shown in Fig. 4A, cultured hepatocytes incubated with different concentrations of insulin for 5 min showed a dose-dependent increase in the phosphorylation status of both Thr$^{308}$ and Ser$^{473}$ residues. The presence of 100 nm wortmannin completely blocked the phosphorylation of PKB elicited by 0.1 nm insulin. However,
some residual PKB phosphorylation was detected in hepatocytes treated with 1 and 10 nM insulin. When 500 nM wortmannin was used, a complete suppression of both Thr308 and Ser473 phosphorylation was observed at all the assayed insulin concentrations (Fig. 4B). A good direct correlation appeared to exist between insulin-mediated PK-L activation and the extent of PKB phosphorylation in the presence of 100 and 500 nM wortmannin.

Effect of wortmannin on insulin-induced MAPK activation

Although wortmannin has been widely considered as a specific inhibitor of the PI 3-K signaling pathway (30), it has been demonstrated that this agent can also reduce activation of the MAPK pathway in different cellular systems (31, 32). To examine whether this inhibitor does or does not affect the MAPK pathway in cultured rat hepatocytes, we analyzed the effect of wortmannin on the activation of this cascade by insulin. For this purpose, cultured hepatocytes were incubated with different concentrations of insulin for 5 min, and MAPK activation was assessed using an antibody that only recognizes the phosphorylated forms of ERK-1 and ERK-2. As shown in Fig. 4, insulin increased the extent of phosphorylation of both ERK-1 and ERK-2 in a dose-dependent manner. The effect on ERK-1 was far lower than that observed on ERK-2; this is in agreement with the data reported by Peak et al. (13), who found a barely detectable phosphorylation and activation of ERK-1 in response to either insulin or epidermal growth factor in rat hepatocytes. When cultured hepatocytes were preincubated with 100 nM wortmannin, insulin-induced phosphorylation of ERK-1/ERK-2 was partially inhibited (Fig. 4A), whereas it was completely abolished, at all the assayed insulin concentrations, in hepatocytes treated with 500 nM wortmannin (Fig. 4B). All of these findings indicate that in cultured rat hepatocytes wortmannin is a powerful inhibitor of both PI 3-K- and MAPK-dependent pathways.

Effect of LY294002 on insulin-induced PK-L activation

To further support the involvement of PI 3-K in the activation of PK-L by insulin, LY294002, a PI 3-K inhibitor not structurally related to wortmannin (3), was used. As shown in Fig. 4C, treatment of cultured hepatocytes with LY294002 (50 μM) completely blocked the insulin-dependent activation of the PI 3-K/PKB pathway at all of the assayed hormone concentrations. When the influence of LY294002 on the MAPK signaling pathway was tested (Fig. 4C), it was observed that, in contrast to what occurred with wortmannin, insulin-induced phosphorylation of ERK-1/ERK-2 was not reduced, but even appeared to be increased. These results were in good agreement with those reported by Zimmerman and Moelling (33), who showed that the blockade of PKB activation by LY294002 increased insulin-like growth factor I-mediated activation of the MAPK cascade in HEK-293 and MCF-7 cells.

When the influence of LY294002 on the insulin-dependent activation of PK-L was studied, it was observed that 50 μM
LY294002 significantly reduced the PK-L activity ratio in hepatocytes incubated under basal conditions (0.168 ± 0.012 vs. 0.105 ± 0.014, for control and LY294002-treated hepatocytes, respectively; by paired Student’s t test, P < 0.001; n = 4 experiments). This effect was not due to any interference of LY294002 with the assay of PK-L activity (data not shown). Furthermore, LY294002 clearly blocked the activation of PK-L by saturating concentrations of insulin (0.1 nm). At higher concentrations of hormone (1 and 10 nm), the PK-L activity ratio increased, but to levels far below those reached in hepatocytes incubated in the absence of LY294002 (Fig. 5).

Effects of PD98059 on insulin-induced MAPK phosphorylation and PK-L activation

To evaluate the possible involvement of the MAPK pathway in the insulin-induced activation of PK-L, PD98059 was used. This organic compound is a specific inhibitor of the MAPK pathway, which has been shown not to affect the activation of PI 3-K by insulin in 3T3-L1 adipocytes (34). Thus, cultured hepatocytes treated with PD98059 (50 μM) showed a complete blockade of insulin-induced MAPK phosphorylation at all of the assayed hormone concentrations. Under these conditions, the activation of the PI 3-K/PKB pathway by insulin was not affected (Fig. 4D).

When hepatocytes were incubated with 50 μM PD98059 in the absence of insulin, a small, but statistically significant, increase was observed in the basal PK-L activity ratio (0.154 ± 0.08 vs. 0.173 ± 0.08 for control and PD98059-treated hepatocytes, respectively; by paired Student’s t test, P < 0.05; n = 6 experiments; Fig. 6). We have no clear explanation for this effect, but the presence of this inhibitor in the reaction mixture did not interfere with the assay of PK-L activity (data not shown). Nevertheless, as also shown in Fig. 6, PD98059, at a concentration that completely blocked the MAPK cascade (Fig. 4D), significantly reduced the activation of PK-L elicited by saturating concentrations of insulin (1 and 10 nm).

Moreover, when insulin-induced PK-L activation was quantified as a percentage of the respective basal value, a partial, but statistically significant, reduction of the insulin effect was observed at all of the assayed hormone concentrations in hepatocytes treated with PD98059 (50%, 47%, and 45% reduction vs. the corresponding control incubations for 0.1, 1, and 10 nm insulin, respectively; P < 0.001). The additional presence of 100 nM wortmannin in the incubation medium enhanced the inhibitory effect elicited by PD98059 on the insulin-mediated activation of PK-L. This enhancement was statistically significant at 0.1 and 1 nm insulin. All of these findings suggest that both PI 3-K and MAPK pathways are involved in the short-term activation of PK-L by insulin.

Effects of rapamycin on insulin-induced p70 S6-K phosphorylation and PK-L activation

It has been established that stimulation of p70 S6-K activity is dependent on PI 3-K activation (8). In connection with this, previous reports have shown that the activation of p70 S6-K by insulin in human myoblasts (35) and rat skeletal muscle (12) takes several minutes and is preceded by PKB activation. To study the possible involvement of p70 S6-K in the insulin-induced activation of PK-L, we tested the influence of rapamycin on this process. This macrolide is the most potent inhibitor of p70 S6-K described to date, blocking the activation of this kinase by all known agents (36). First, we carried out a time-course study of the insulin action on both PKB and p70 S6 kinase activation in primary cultures of rat hepatocytes. As shown in Fig. 7A, maximal PKB phosphorylation in response to 10 nm insulin was achieved as early as 2 min after hormone treatment, and this kinase remained phosphorylated for at least 20 min. Nevertheless, phosphorylation of p70 S6-K on Thr368 residue, which is essential to bring about full kinase activation (37), was barely detected 4 min after insulin stimulation and increased gradually until min
Materials and Methods

A, Rat hepatocytes were cultured for 21 h as indicated in Materials and Methods and were deprived of serum and hormones for the last 2 h. Then, cells were incubated with 10 nM insulin and homogenized at the indicated times. Proteins from cell extracts were resolved by SDS-PAGE (50 µg protein/lane) and immunoblotted with both anti-phospho-PKB (Ser473) and anti-phospho-p70 S6-K (Thr389) polyclonal antibodies. B, Rat hepatocytes were cultured for 21 h as indicated in Materials and Methods and were deprived of serum and hormones for the last 2 h. Then cells were preincubated for 10 min with 100 nM rapamycin or vehicle. Afterward, insulin was added, and 10 min later, cell monolayers were immediately homogenized. Proteins from cell extracts were resolved by SDS-PAGE (50 µg protein/lane) and immunoblotted with both anti-phospho-PKB (Ser473) and anti-phospho-p70 S6-K (Thr389) polyclonal antibodies. Data are representative of at least two independent experiments.

20 (Fig. 7A). As shown in Fig. 7B, the phosphorylation of p70 S6-K by insulin was also dose dependent and was completely suppressed at all of the assayed hormone concentrations when cultured hepatocytes were treated with 100 nM rapamycin. Under these conditions, the phosphorylation of PKB at Ser473 was not inhibited. As shown in Fig. 8, this same concentration of rapamycin did not significantly affect the activation of PK-L by insulin. These findings indicate that the activation of p70 S6-K is not required for the insulin-dependent activation of PK-L.

Discussion

It is well known that PK-L activity is modulated by phosphorylation/dephosphorylation of the enzyme molecule (18, 19). In liver, the phosphorylation and subsequent inactivation of PK-L can be mediated by cAMP- and Ca2+-calmodulin-dependent protein kinases (18, 19). However, there is no agreement about the protein phosphatase(s) responsible for the dephosphorylation and reactivation of PK-L (20–22). With respect to the short-term modulation of PK-L activity by insulin, it has been reported that this hormone may cause the activation of this enzyme, without significant changes in either cAMP levels or protein kinase A activity (23, 24). There is experimental evidence supporting the hypothesis that the stimulation of a protein phosphatase activity is implicated in the regulation of PK-L by insulin (22, 23, 25). However, the cellular signaling pathway leading to the short-term activation of PK-L by this hormone has not been established.

The PI 3-K/PKB signaling pathway has been shown to play a central role in the modulation of glucose metabolism by insulin. Thus, activation of this pathway has been shown to mediate insulin effects on GLUT-4 translocation to the plasma membrane in L6 myoblasts (11) as well as on the activation of glycogen synthase and the stimulation of glycogen synthesis in different cell types, including cultured hepatocytes (12, 13). To investigate the possible involvement of the PI 3-K-dependent signaling pathway in the insulin-mediated activation of PK-L, wortmannin, a fungal metabolite that is a known inhibitor of PI 3-K activity (3, 30), was used. Thus, 100 nM wortmannin totally suppressed the activation of PK-L elicited by a subsaturating concentration of insulin (0.1 nM), whereas the inhibitory effect of this agent was only partial at 1 and 10 nM insulin. These findings were paralleled by an inhibition of insulin-stimulated PI 3-K activity and PKB phosphorylation, elicited by this concentration of wortmannin in cultured rat hepatocytes. When a higher concentration of wortmannin (500 nM) was used, the activation of PK-L was completely blocked at all of the assayed insulin concentrations. Under these conditions, insulin signaling through the PI 3-K/PKB pathway was totally suppressed in cultured hepatocytes, as indicated by the complete blockade of PKB phosphorylation. It is of note that PKB phosphorylation on both Thr308 and Ser473 is a well known PI 3-K-dependent process (5–7). All of these findings suggest that the PI 3-K pathway is involved in the short-term activation of PK-L by insulin.

However, it must be mentioned that wortmannin has been reported to reduce the activation of the MAPK cascade in response to insulin in L6 cells (31), but not in human skeletal muscle (38). Considering these controversial data, we also investigated the possible influence of wortmannin on the activation of the MAPK signaling pathway by insulin in...
cultured rat hepatocytes. We observed that at a concentration of 100 nm, this inhibitor partially antagonized insulin effects on the phosphorylation of ERK-1/ERK-2, whereas 500 nm wortmannin completely suppressed these insulin effects. Taking into account the fact that wortmannin was able to inhibit both PI 3-K and MAPK pathways in cultured rat hepatocytes, the wortmannin effect on insulin-mediated PK-L activation could be due to the blockade of either of these two pathways, or both. The mechanism by which wortmannin inhibits the MAPK pathway is not well understood; it has been suggested that this agent may block the activation of this pathway between Ras and Raf in L6 cells (31), whereas a decrease in GTP loading of Ras has been observed in 3T3L1 adipocytes (39).

To further investigate the involvement of PI 3-K in insulin-induced PK-L activation, LY294002, a selective PI 3-K inhibitor, was used. In our cell system, this inhibitor blocked insulin signaling through the PI 3-K/PKB pathway, and in contrast to the results obtained with wortmannin, LY294002 did not reduce but, rather, increased the insulin effect on the MAPK cascade. In this respect it has been demonstrated in different types of cells that the activation of PKB results in Raf phosphorylation on Ser239, which reduces its kinase activity and consequently the activation of the p44/p42 MAPK pathway (33, 40). Furthermore, inhibition of PKB activation by LY294002 has been shown to increase Raf and ERK activities in HEK 293 and MCF-7 cells (33). Our results suggest that this cross-regulation between PI 3-K/PKB and MAPK pathways may also occur in cultured hepatocytes.

When the influence of LY294002 on insulin-induced PK-L activation was investigated, it was observed that this inhibitor, in contrast to what occurred with wortmannin, caused a significant reduction in the PK-L activity ratio in hepatocytes incubated in the absence of insulin, although we have no clear explanation for this phenomenon. In hepatocytes treated with a subsaturating concentration of insulin (0.1 nm), LY294002 almost completely blocked the activation of PK-L, whereas at higher concentrations of insulin (1 and 10 nm), the effect of LY294002 was markedly reduced. However, in these conditions the values for the PK-L activity ratio attained in response to insulin were far lower than those reached in control hepatocytes. From these data and from the studies carried out with wortmannin, it can be concluded that the PI 3-K signaling pathway is implicated in the short-term activation of PK-L by insulin. In addition, it must be pointed out that the difference between the degrees of inhibition of insulin-mediated PK-L activation elicited by 500 nm wortmannin and 50 mM LY294002 may have been due to the fact that wortmannin at this concentration blocked the activation of the MAPK cascade, whereas LY294002 did not.

p70 S6-K is an enzyme that lies downstream of PI 3-K in the insulin signaling pathway (9). In previous studies (12, 13), rapamycin, a potent inhibitor of this kinase, has been used to study the possible involvement of p70 S6-K in the regulation of glycogen synthase activity by insulin. In our work we observed that 100 mM rapamycin, a concentration that completely blocked the phosphorylation of p70 S6-K in Thr389, did not significantly affect insulin-mediated activation of PK-L in cultured rat hepatocytes. This finding allows us to rule out the implication of this kinase in the signaling cascade that leads to the short-term activation of PK-L by insulin.

To analyze the possible involvement of the p44/p42 MAPK cascade in the activation of PK-L by insulin, PD98059 was used. Thus, we observed that 50 mM PD98059, which completely blocked ERK-1/ERK-2 phosphorylation without affecting PI 3-K/PKB activation, led to a 50% inhibition of the insulin-induced activation of PK-L. This finding strongly suggests that the activation of the p44/p42 MAPK pathway plays a relevant role in the short-term activation of PK-L by insulin. The additional presence of 100 mM wortmannin in the incubation medium significantly reinforced the inhibitory effect of PD98059 on insulin-induced activation of PK-L. As the signal transduction through the MAPK pathway was completely suppressed by the presence of PD98059, this wortmannin action must be attributed to the partial inhibition of PI 3-K elicited by this agent. In good agreement with this, when both the PI 3-K and MAPK pathways were completely blocked by the presence of 500 mM wortmannin, activation of PK-L by insulin was totally suppressed.

The activation of the PI 3-K/PKB pathway has been shown to be essential for most of the metabolic actions of insulin (3), whereas the MAPK cascade has been considered to be implicated mainly in mitogenic signals (10). Nevertheless, recent reports have affirmed that both pathways are involved in different actions of insulin and insulin-like growth factor I, such as protection of cells from apoptosis (41), stimulation of protein synthesis (42), and regulation of insulin receptor trafficking (43). Moreover, cross-talk between the PI 3-K and MAPK pathways has been reported at different levels in several cell systems (33, 39, 40, 44), suggesting that integration of signals through different pathways is crucial for the coordinated responses of cells to hormonal stimuli.

In summary, our findings provide evidence that both p44/p42 MAPK and PI 3-K pathways, but not p70 S6-kinase, are involved in the short-term activation of PK-L by insulin.

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References

9. Leevers SJ, Paterson HF, Marshall CJ 1994 Requirement for Ras in Raf acti-
viation is overcome by targeting Raf to the plasma membrane. Nature 369:411–414


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