Activation of Mitogen-Activated Protein Kinase in Freshly Isolated Rat Hepatocytes by Both a Calcium- and a Protein Kinase C–Dependent Pathway

Angela Romanelli and Gérald van de Werve

In the present study, we investigated the role of calcium and protein kinase C (PKC) in the activation of mitogen-activated protein kinase (MAPK) in isolated rat hepatocytes. We found that the glycogenolytic hormone norepinephrine (NE), acting through the α_1-adrenergic receptor and the G protein Gq, was able to induce a dose- and time-dependent activation of MAPK in hepatocytes. Vasopressin, which acts through a different receptor but also through stimulation of the Gq-dependent pathway, also caused a twofold activation of MAPK. Activation of MAPK by both agonists required the presence of free extracellular calcium and was blocked by the specific PKC inhibitor, Ro 31-8220. MAPK activation was also induced by phorbol myristate acetate (PMA), confirming that a PKC-dependent pathway exists for MAPK activation in liver. Furthermore, calcium-mobilizing agents such as thapsigargin and ionomycin were able to induce an activation of MAPK by a PKC-independent pathway that was totally abolished by preincubation of cells with EGTA. A second pathway for MAPK activation that relies solely on calcium may therefore exist. Ro 31-8220 did not affect phosphorylase activation by NE, vasopressin, thapsigargin, and ionomycin, indicating that PKC inhibition did not interfere with the signaling pathway leading to inositol-1,4,5-triphosphate (IP_3)-induced calcium mobilization or with changes in calcium fluxes. The role of MAPK activation by NE and vasopressin in the regulation of hepatic carbohydrate metabolism is discussed.

MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) was originally isolated as an insulin-stimulated microtubule-associated protein-2 kinase in 3T3-L1 cells.[1] Since then, MAPK, also known as ERK (extracellular signal–regulated kinase), has been cloned[2] and several isoforms have been identified. The best characterized are the 44-kd and 42-kd MAPKs, also known as ERK1 and ERK2, respectively.[3] In different cell types, several stimuli have been reported to lead to an increase in MAPK activity, including activation of receptors coupled to heterotrimeric G proteins such as Gq or Gi,[4-8] as well as tyrosine kinase receptors.[9] Gi-coupled receptors can activate the small guanosine triphosphate–binding protein *Ras,* which triggers sequential stimulation of the MAPK cascade: *Raf,* MAPK kinase (MEK), and MAPK. Gq and tyrosine kinases can stimulate phospholipase C-β and -γ, respectively, leading to an increase in intracellular calcium via inositol-1,4,5-triphosphate (IP_3) generation and activation of protein kinase C (PKC) via production of diacylglycerol. Most reports suggest that growth factors activate MAPK by a *Ras*-dependent and calcium-independent pathway,[10-13] whereas MAPK activation by Gq-coupled receptors such as the α_{1B}-adrenergic receptor has been shown to be mediated by a phospholipase C-β– and PKC-dependent pathway.[4,5] Phorbol esters that directly activate PKC have been reported to activate MAPK in intact cells,[14-16] and in vitro studies demonstrate that PKC is able to phosphorylate *Raf*[17-19] and activate MAPK in a cell-free system.[20] Calcium-mobilizing agents can also induce MAPK activation in fibroblasts.[13]

The mechanisms thought to be involved in growth factor–induced MAPK activation in liver following tyrosine kinase activation include both PKC-dependent and pertussis toxin–sensitive pathways.[16,21] In this study we show that norepinephrine ([NE] in the presence of the β-adrenergic antagonist propranolol) and vasopressin, which act via Gq-linked receptors, can activate MAPK in hepatocytes by both a calcium- and a PKC-dependent pathway. Furthermore, calcium-mobilizing agents such as thapsigargin or ionomycin can also activate MAPK even in the presence of PKC inhibition by Ro 31-8220, suggesting that calcium alone can also be sufficient for MAPK activation. This is an interesting finding, since calcium is reputed to be a glycogenolytic signal in liver. We performed parallel assays of phosphorylase activation as an indicator of calcium mobilization and also to evaluate the role of PKC in hormone-, thapsigargin-, and ionomycin-induced increases in intracellular calcium. The potential role of MAPK in regulating hepatic glucose metabolism is discussed.

MATERIALS AND METHODS

Materials

Collagenase, dithiothreitol, phenylmethylsulfonyl fluoride, vasopressin, NE, propranolol, staurosporine, *p*-nitrophenylphosphate, aprotinin, leupeptin, β-glycerophosphate, sodium orthovanadate, phorbol myristate acetate (PMA), and analytical-grade chemicals were purchased from Sigma (St Louis, MO). Glucagon was obtained from Lilly (Indianapolis, IN), and thapsigargin and ionomycin were purchased from Calbiochem (San Diego, CA). [γ-^{32}P]adenosine triphosphate was obtained from (Dupont New England Nuclear, Boston, MA). Myelin basic protein–derived peptide was purchased from Upstate Biotechnology (Lake Placid, NY). Ro 31-8220 was a generous gift from Roche (Welwyn Garden City, Hertfordshire, UK).

Animals and Cells

Male Wistar rats (200 to 250 g) were housed for 1 week in the Department's animal quarters before experimentation and were fed ad libitum with Purina Rat Chow (Charles River Laboratories, Wilmington, MA). Hepatocytes were prepared as previously described,[22] and cell viability was assessed by hormone responsiveness and by Trypan

From the Laboratoire d'Endocrinologie Métabolique, Departments of Nutrition and Biochemistry, Université de Montréal, Montréal, Québec, Canada.

Submitted August 26, 1996; accepted December 3, 1996.

Supported by Grant No. MT-10804 from the Medical Research Council of Canada.

Present address: A.R., Department of Cell Biology, Harvard Medical School, 240 Longwood Ave, Boston, MA 02115.

Address reprint requests to Gérald van de Werve, PhD, Laboratoire d'Endocrinologie Métabolique, Université de Montréal, Department of Nutrition. CP 6128 Succ. Centre-Ville (Qué). H3C 3J7. Canada.

0026-0495/97/4605-0015$03.00/0

blue exclusion (85% to 95%). The cells (40 mg/mL) were preincubated for 30 minutes at 37°C in a Krebs-Henseleit medium containing 10 mmol/L glucose and in the presence or absence of Ro 31-8220, dimethylsulfoxide (DMSO), EGTA, or propranolol as indicated. The hepatocytes were then further incubated in the presence of different agonists, and the reaction was stopped at different times by freezing 50 µL of the cell suspension in liquid nitrogen. The samples were kept at −80°C pending phosphorylase *a* and MAPK assays, with the latter performed the next day.

Sample Preparation and MAPK Assay

MAPK was assayed as previously described.[23] Briefly, cell extracts were prepared by thawing cell suspensions in homogenization buffer containing 50 mmol/L Tris hydrochloride, pH 7.4, 100 mmol/L sodium chloride, 50 mmol/L sodium fluoride, 5 mmol/L ethylenediamine tetraacetic acid, 40 mmol/L β-glycerophosphate, 200 µmol/L sodium orthovanadate, 100 µmol/L phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1 mmol/L dithiothreitol, and 1 µmol/L leupeptin. The homogenates were then sonicated for 2 to 3 seconds at 60% output and centrifuged at 15,000 × *g* for 10 minutes. The MAPK activity assay was started by addition of 30 µL kinase assay buffer (812 µg/mL myelin basic protein–derived peptide, APRTPGGRR, 1.3 mmol/L dithiothreitol, 13 mmol/L $MgCl_2$, 1.3 µmol/L staurosporine, 26 mmol/L HEPES, pH 7.4, 65 µmol/L ATP, and 2,000 Ci/mmol [γ-^{32}P]ATP) to 10 µL supernatant (10 to 15 µg cellular protein). The mixture was incubated for up to 10 minutes at 30°C and stopped at 0, 5, and 10 minutes by spotting 10 µL onto phosphocellulose P81 paper (Whatman, Maidstone, UK). The papers were washed three times for 30 minutes with 4 L 1% phosphoric acid and once in acetone, dried, and counted in a liquid scintillation counter.

Phosphorylase a Assay

Phosphorylase *a* was assayed at 30°C in homogenates of thawed cell suspensions as previously described.[24]

Expression of Results and Statistical Analyses

MAPK activity was measured as picomoles of phosphate incorporated into the substrate per minute per milligram of cell extract protein as calculated by linear regression of the time course of incubation. MAPK activity is expressed as fold-activation relative to activities determined at time 0 (shown in text) of hepatocyte incubation following the 30-minute preincubation. Phosphorylase *a* activity is expressed as units per minute per gram wet weight of liver, where 1 U is defined as 1 µmol substrate used/min. Data are representative of the indicated number of hepatocyte preparations and are expressed as the mean ± SEM. Significant differences between groups of data were determined using ANOVA and unpaired Bonferroni tests as indicated.

RESULTS

NE and Vasopressin Stimulate MAPK Activity in Isolated Rat Hepatocytes

Incubation of hepatocytes with increasing concentrations of NE (in the presence of 10 µmol/L propranolol, which blocks the β-adrenergic component of NE) led to a dose-dependent increase in MAPK activity up to a concentration of 1 µmol/L (Fig 1). With this concentration of NE, a transient, almost twofold activation of MAPK was observed (Fig 2a). For all concentrations of NE, maximal activation was reached after 2.5 minutes of incubation following hormone addition. Vasopressin (100 nmol/L), acting via a different receptor (V1) that is also coupled to the heterotrimeric G protein Gq and thus also leads to stimulation of phospholipase C-β, was found to induce a twofold increase in MAPK activity (Fig 3a).

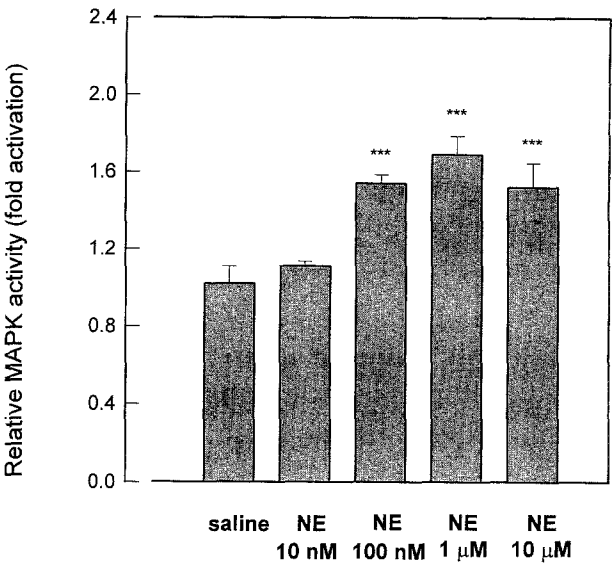

Fig 1. Effect of increasing concentrations of NE on MAPK activity. Cells were preincubated for 10 minutes in the presence of 10 µmol/L propranolol and then challenged with increasing concentrations of NE as indicated for 2.5 minutes, at which time aliquots were frozen pending MAPK assay. Results are expressed as the mean value only or as the mean ± SEM (n = 3 to 11). Statistical significance is indicated for differences between control and NE (*$P < .001$).**

The Role of PKC in MAPK and Phosphorylase Activation

Figure 4a shows that the time course of MAPK activation by NE was completely abolished by the specific PKC inhibitor, Ro 31-8220.[25] Vasopressin-induced MAPK activation was also inhibited by pretreatment of cells with Ro 31-8220 (Fig 5a). DMSO, which is the vehicle for Ro 31-8220, slightly attenuated MAPK activation by NE and vasopressin compared with preincubation in the absence of the solvent (Figs 2a, 3a, 4a, and 5a). Ro 31-8220 did not modify phosphorylase activation by NE (Fig 4b) or vasopressin (Fig 6a). This suggests that the hormone-induced calcium signal that leads to phosphorylase activation was not affected by PKC inhibition by Ro 31-8220. Preincubation of cells with the PKC inhibitor for 30 minutes did not significantly affect basal MAPK activity ($t = 0$ minutes) with respect to the condition in which cells are preincubated with 0.1% DMSO (5.92 ± 0.61 *v* 5.64 ± 0.49 pmol/min/mg protein, respectively; n = 6 to 7).

In keeping with a requirement for PKC in the action of NE and vasopressin on MAPK, the addition of PMA to isolated rat hepatocytes also led to a substantial activation of MAPK that was greater than twofold after 2.5 minutes and was sustained for up to 15 minutes (not shown). Furthermore, this activation was completely blocked by Ro 31-8220, suggesting that the inhibitor is indeed acting on phorbol ester–sensitive isoforms of PKC.

The Role of Calcium in MAPK and Phosphorylase Activation

Since calcium influx has been reported to be an important component in the signaling pathway mediating responses originating from Gq-coupled receptors,[26] we tested whether

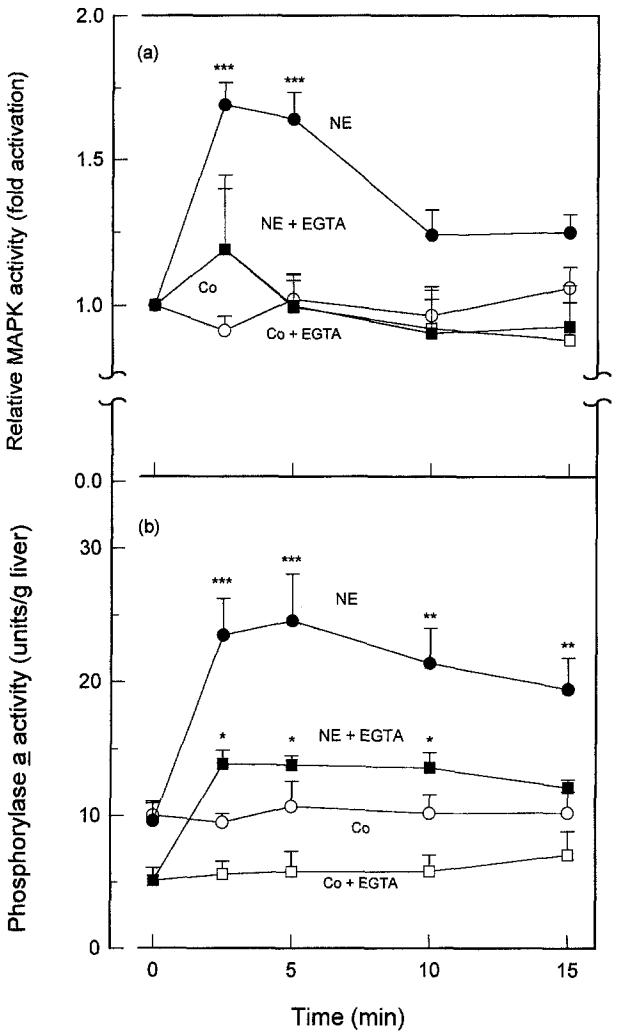

Fig 2. Effect of EGTA on the time course of MAPK and phosphorylase activation by NE. Hepatocytes were preincubated in a Krebs-Henseleit medium containing 10 mmol/L glucose with (□,■), or without (○,●) 2.5 mmol/L EGTA for 15 minutes. Propranolol (10 μmol/L) was added 10 minutes before addition of 1 μmol/L NE (●,■) or saline (○,□). MAPK (a) or phosphorylase *a* (b) activities were assayed at the indicated times. Results are expressed as the mean ± SEM (n = 3 to 11). Statistical significance is indicated for differences between control and NE and between control + EGTA and NE + EGTA (*$P < .05$, **$P < .01$, ***$P < .001$).

removal of free calcium from the incubation medium affected MAPK activation. For this purpose, we preincubated the hepatocytes in the presence of 2.5 mmol/L EGTA for 15 minutes before addition of the hormone. EGTA blunts the activation of MAPK by NE (Fig 2a). Consistent with this, vasopressin-induced MAPK activation is also inhibited in these conditions (Fig 3a). In the presence of EGTA, whereas calcium influx is prevented, it can still be mobilized from IP_3-sensitive intracellular pools. To verify this, we tested phosphorylase activation in the same conditions, which is an indication of calcium-stimulated phosphorylase kinase. Figure 2b demonstrates that activation of phosphorylase by NE is significantly attenuated by EGTA preincubation. Activation of phosphorylase by 100 nmol/L vasopressin was completely blunted (Fig 7a). This is not so surprising, since the regulation of calcium fluxes by these two agonists has been shown to differ.[27] The activation of phosphorylase induced by NE measured in the presence of EGTA, albeit attenuated, is probably due to mobilization of calcium from internal stores. Figure 2b also shows that basal phosphorylase *a* is decreased by preincubation of the cells in the presence of EGTA. However, pretreatment of cells with EGTA for 15 minutes did not significantly alter basal MAPK activities (6.12 ± 0.869 *v* 5.30 ± 0.310, n = 3 to 14).

We also tested the effects of thapsigargin on MAPK and phosphorylase activation. This compound is a sesquiterpene lactone that inhibits endoplasmic reticulum Ca^{2+}-ATPase[28] and thus increases intracellular calcium by allowing it to leak out of the endoplasmic reticulum. Calcium influx is then triggered following internal store depletion.[29] Figure 3b shows that thapsigargin-induced MAPK activation is completely blunted by EGTA, as is phosphorylase activation (Fig 7b). EGTA also blocked MAPK and phosphorylase activation by ionomycin, a

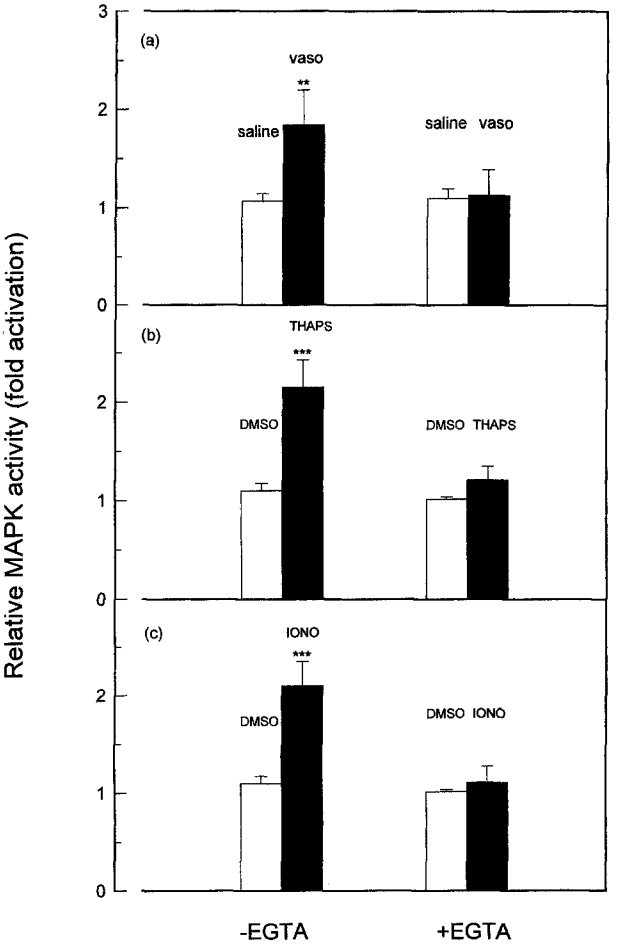

Fig 3. Effect of EGTA on MAPK activation by vasopressin, thapsigargin, and ionomycin. Hepatocytes were preincubated in the presence of EGTA as described in Fig 2 and challenged as described in Fig 5. MAPK activity was assayed in aliquots frozen 5 minutes following the addition of each agent. Results are expressed as the mean ± SEM (n = 3 to 5). Statistical significance is indicated for differences between the appropriate control and each agonist and for differences between control + EGTA and agonist + EGTA ($P < .01$, ***$P < .001$). Abbreviations are as in Fig 5.**

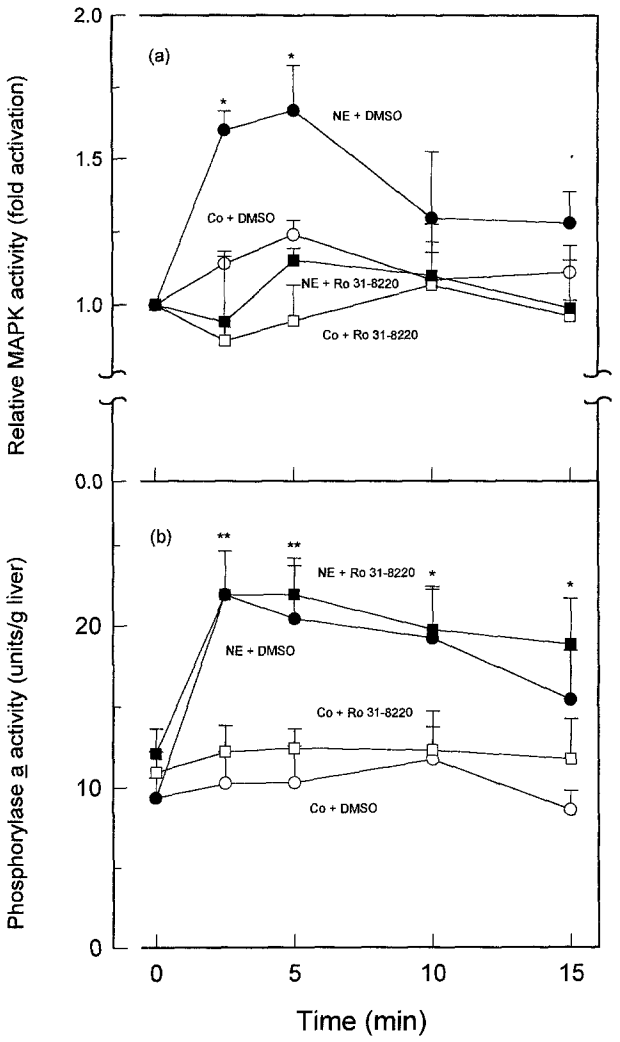

Fig 4. Effect of Ro 31-8220 on the time course of MAPK and phosphorylase activation by NE. Hepatocytes were preincubated for 30 minutes in a Krebs-Henseleit medium containing 10 mmol/L glucose with the vehicle 0.1% DMSO (○,●) or with (□,■) 10 μmol/L Ro 31-8220. Propranolol (10 μmol/L) was added 10 minutes before addition of 1 μmol/L NE (●,■) or saline (○,□). MAPK (a) or phosphorylase *a* (b) activities were assayed at the indicated times. Results are expressed as the mean ± SEM (n = 3 to 6). Statistical significance is indicated for differences between control + DMSO and NE + DMSO and for differences between control + Ro 31-8220 and NE + Ro 31-8220 (*$P < .05$, **$P < .01$).

calcium ionophore with an effect that relies primarily on calcium influx (Figs 3c and 7c). We next tested whether the effects of the calcium-mobilizing agents were mediated by an activation of PKC, since the results with NE, vasopressin, and PMA suggest that this kinase is implicated in the regulation of MAPK activity. Figure 5b and c shows that MAPK activation by thapsigargin and ionomycin is unaffected by the presence of Ro 31-8220. To verify that Ro 31-8220 was not affecting calcium mobilization, we measured the effect of this inhibitor on phosphorylase activation by these same agents. Figure 6b shows that phosphorylase activation by thapsigargin is not modulated by Ro 31-8220, whereas the effect of ionomycin is slightly attenuated (Fig 6c).

We also wanted to verify that EGTA did not affect MAPK per se. We therefore tested the effect of PMA on MAPK activity in cells preincubated with EGTA. Phorbol esters mimic the action of diacylglycerol; however, the former usually do not depend on calcium for inducing activation of PKC.[30] PMA was still able to activate MAPK in the presence of EGTA (not shown). We also tested whether glucagon was able to activate phosphorylase in cells preincubated with EGTA, to ensure that EGTA did not affect cellular viability. Indeed, glucagon (10 nmol/L) was still able to significantly activate phosphorylase in these cells (glucagon alone, 23.78 ± 4.41 U/g liver, n = 3; glucagon + 2.5 mmol/L EGTA, 19.31 ± 3.30, n = 4; measured 5 minutes following hormone addition). From this, we can conclude that EGTA did not alter signaling pathways in a nonspecific fashion, including the cyclic adenosine monophosphate (cAMP)-dependent pathway induced by glucagon.

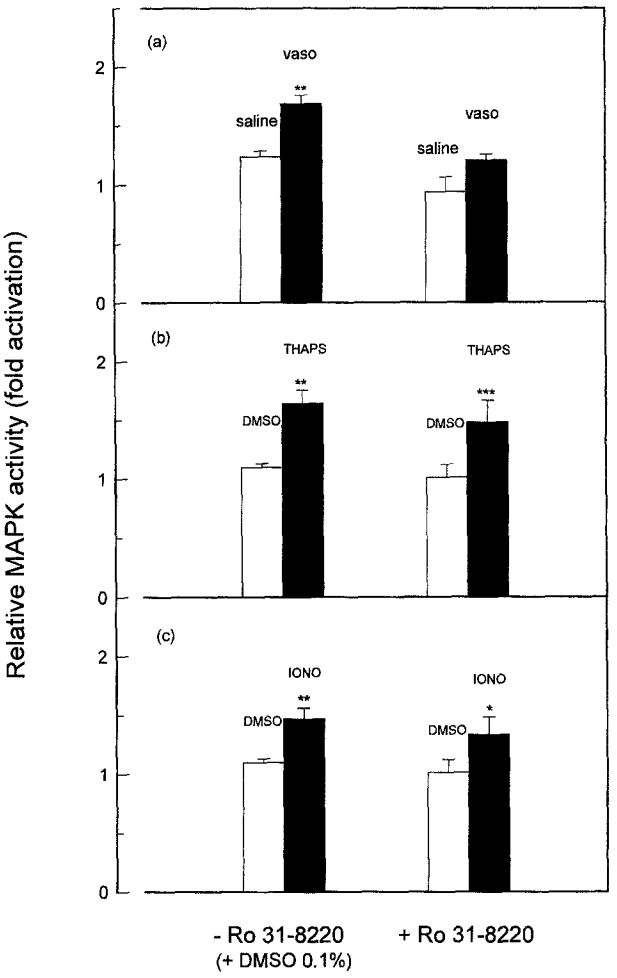

Fig 5. Effect of Ro 31-8220 on MAPK activation by vasopressin, thapsigargin, and ionomycin. Hepatocytes were preincubated in the presence of Ro 31-8220 or 0.1% DMSO as described in Fig 4. MAPK activity was assayed in aliquots frozen 5 minutes following the addition of (a) 100 nmol/L vasopressin (vaso), (b) 1.5 μmol/L thapsigargin (THAPS), and (c) 10 μmol/L ionomycin (IONO). Results are expressed as the mean ± SEM (n = 3 to 4). Statistical significance is indicated for differences between the appropriate control (saline or 0.5% DMSO) + DMSO and each agonist + DMSO and for differences between control + Ro 31-8220 and agonist + Ro 31-8220 (*$P < .05$, **$P < .01$, ***$P < .001$).

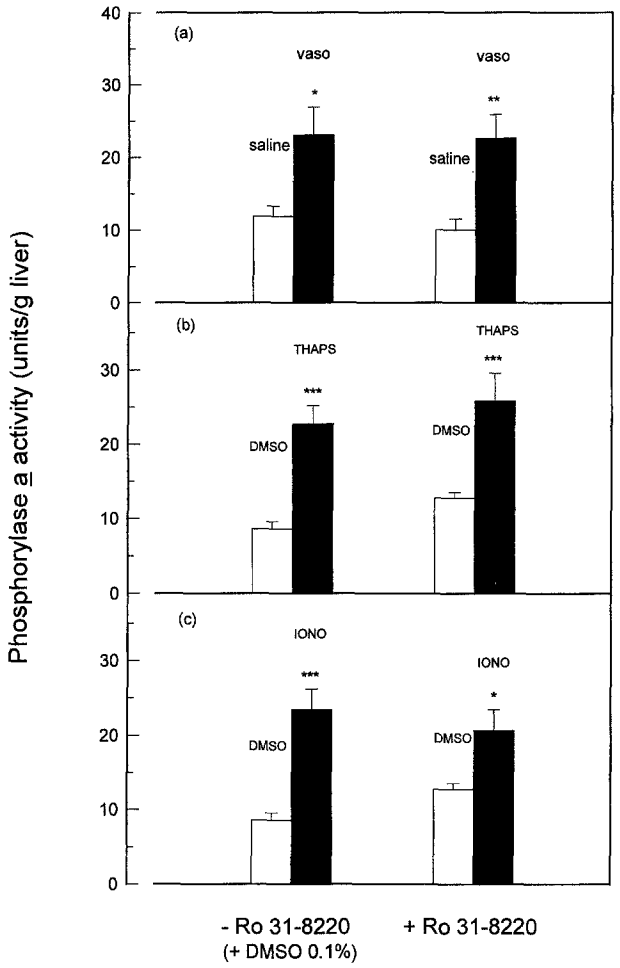

Fig 6. Effect of Ro 31-8220 on phosphorylase activation by vasopressin, thapsigargin, and ionomycin. Hepatocytes were preincubated and challenged as described in Fig 5. Phosphorylase *a* activity was assayed in aliquots frozen 5 minutes following the addition of each agent. Results are expressed as the mean ± SEM (n = 3 to 6). Statistical significance is indicated for differences between the appropriate control + DMSO and each agonist + DMSO and for differences between control + Ro 31-8220 and agonist + Ro 31-8220 (*$P < .05$, **$P < .01$, ***$P < .001$). Abbreviations are as in Fig. 5.

DISCUSSION

Regulation of MAPK Activity in Liver by Calcium and PKC

Although the MAPK pathway has been extensively studied, few reports have focused on the regulation of this enzyme in the liver, which is a key tissue for glucose homeostasis and thus a major target for hormones and catecholamines such as insulin, glucagon, and (nor)epinephrine that regulate hepatic glucose storage and output. To our knowledge, this study is the first to show that seven membrane-spanning receptors coupled to Gq (eg, α_1-adrenergic and vasopressin receptors) are able to mediate MAPK activation in nondividing, differentiated cells such as freshly isolated hepatocytes. Furthermore, we show that this activation is both calcium- and PKC-dependent. A recent study[12] has reported that vasopressin, ionomycin, and PMA are unable to elicit MAPK activation in cultured hepatocytes. However, our results corroborate those of a different study, also performed in cultured hepatocytes, showing that PMA is able to stimulate MAPK activation in these cells and that hepatocyte growth factor–induced MAPK activation is at least partially mediated by PKC.[16]

Although we observe a significant activation of MAPK in the presence of either calcium-mobilizing agent, thapsigargin or ionomycin, both calcium and PKC seem to be required for the activation of MAPK by NE and vasopressin. In the presence of Ro 31-8220, NE and vasopressin are unable to activate MAPK. However, the PKC inhibitor does not interfere with phosphorylase activation by these agonists. Thus, it seems that the calcium signal generated under these conditions by the stimulation of phospholipase C-β is sufficient for activation of phosphorylase but not for activation of MAPK, and that PKC is necessary for activation of the latter by NE and vasopressin. Conversely, only a small transient activation of MAPK by NE can still be detected when hepatocytes are preincubated in the presence of EGTA. This can be explained as follows. Although PMA can activate PKC in a calcium-independent manner, receptor-induced PKC activation requires the presence of calcium.[31] It is known that in the liver, among other cells, the sustained phase

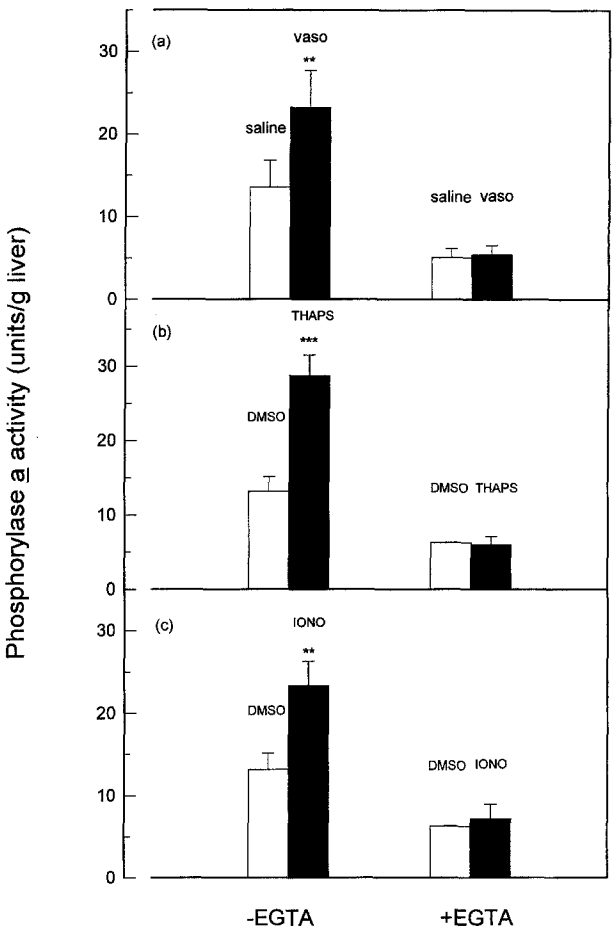

Fig 7. Effect of EGTA on phosphorylase activation by vasopressin, thapsigargin, and ionomycin. Hepatocytes were preincubated and challenged as described in Fig 3. Phosphorylase *a* activity was assayed in aliquots frozen 5 minutes following the addition of each agent. Results are expressed as the mean ± SEM (n = 3). Statistical significance is indicated for differences between the appropriate control and each agonist and for differences between control + EGTA and agonist + EGTA ($P < .01$, ***$P < .001$). Abbreviations are as in Fig. 5.**

of diacylglycerol production is due to the hydrolysis of phosphatidylcholine by phospholipase D.[32] Stimulation of this phospholipase can be both calcium- and PKC-dependent and follows the initial stimulation of phospholipase C-β.[33,34] The transient increase in intracellular calcium elicited by NE in the presence of EGTA therefore may not be sufficient for receptor-induced PKC activation. So it is highly likely that PKC and calcium act in a synergistic fashion to activate MAPK. This would explain why EGTA prevents activation of MAPK by NE and vasopressin despite the fact that PMA alone can activate MAPK through PKC.

Interestingly, MAPK activation induced by thapsigargin and ionomycin is not modified by Ro 31-8220, indicating that their action is PKC-independent. Furthermore, Ro 31-8220 does not abolish activation of phosphorylase by these agents, suggesting that PKC inhibition does not interfere with calcium fluxes, since thapsigargin and ionomycin increase intracellular calcium directly, bypassing the Gq-dependent signaling system. Consequently, calcium increases induced by these agents are different from those induced by hormones. For the latter, mechanisms such as receptor desensitization and calcium oscillations induced by IP_3-mediated release of calcium from the endoplasmic reticulum are associated with their responses.[29] Calcium fluxes induced by thapsigargin, for example, exhibit different rates of release from intracellular stores and more sustained increases, probably due to a greater rate of calcium influx.[28] Such a sustained increase in intracellular calcium may trigger a different pathway for MAPK activation that is PKC-independent. A calcium-dependent activation of MAPK has been previously reported in human foreskin fibroblasts.[13] Several studies have shown that in different cell types, an increase in intracellular calcium can lead to tyrosine kinase activation and thus activation of the MAPK pathway.[35-37] Thus, it is possible that two distinct pathways of MAPK activation exist in liver: one that is dependent on calcium only and seems to require sustained calcium influx, and another that is mediated by Gq and is both calcium- and PKC-dependent. The PKC-mediated pathway could involve phosphorylation and activation of *Raf* or MEKK, of which the latter is thought to be most likely involved in the activation of MAPK by heterotrimeric G proteins.[38]

Role of MAPK in the Regulation of Hepatic Metabolism

Although MAPK has clearly been shown to be essential for mitogenic signaling,[3] its role as a modulator of metabolic processes is not well understood, since a direct link between MAPK and metabolic functions has yet to be established. Stimulation of glycogen synthesis by insulin is achieved by dephosphorylation and activation of glycogen synthase. This results from activation of a phosphatase associated with glycogen (PP1G) and inhibition of glycogen synthase kinase-3 (GSK-3).[39] MAPK is thought to play a role in the activation of PP1G through the activation of p90RSK.[40-42] Tobe et al[43] observed the activation of MAPK kinase, ERK1 and ERK2, and p90RSK in rat liver extracts following injection of insulin via the hepatic portal vein. Other studies have reported the stimulation of MAPK activity by insulin and/or growth factors in cultured and freshly isolated rat hepatocytes.[12,16,21,44,45] A role for MAPK in the activation of glycogen synthesis has therefore been postulated. However, studies using PD98059, an inhibitor of MEK activation, suggest that the MAPK cascade is not required for stimulation of glucose uptake, lipogenesis, and glycogen synthesis in 3T3-L1 adipocytes and L6 myotubes.[46] Furthermore, insulin and epidermal and platelet-derived growth factors all significantly increase MAPK activity, but only insulin induced an increase in the aforementioned parameters.[47-50] Therefore, the importance of MAPK in the regulation of intermediary metabolism remains undefined.

In this study, we find that calcium, which is known to be a glycogenolytic signal leading to phosphorylase activation in liver, can also activate MAPK and is required for Gq-mediated MAPK activation. Vasopressin and NE are not known to activate glycogen synthase or stimulate glycogen synthesis, but instead induce glycogen degradation. We find that these hormones activate MAPK with a time course similar to that of phosphorylase activation. In these conditions, net glycogen synthesis could not take place. Although a role for MAPK in the stimulation of glycogen synthesis should not be dismissed, our results suggest that MAPK activation under different conditions probably leads to different metabolic responses. Indeed, inhibition of GSK-3 has been shown to be wortmannin-sensitive.[51] Thus, it is possible that the activation of two parallel pathways, one mediated by MAPK and the other involving phosphatidylinositol-3-kinase, is required for glycogen synthesis in liver. These two pathways could be simultaneously activated by insulin.

What role could MAPK activation play in the regulation of hepatic carbohydrate metabolism under these conditions in which phosphorylase is activated simultaneously? In the liver of fed animals, activation of phosphorylase by calcium-mobilizing agonists does not lead to a substantial increase in glucose production. This has been attributed to the increase in fructose-2,6-bisphosphate (F-2,6-BP) levels. Because cAMP is not increased, 6-phosphofructo-2-kinase is not inhibited and will use the accumulated fructose 6-phosphate from increased glycogenolysis to synthesize F-2,6-BP, which is a potent stimulator of glycolysis.[52,53] Some reports have also suggested that PKC may play a role in the stimulation of glycolysis.[54,55] Therefore, it is possible that hormones such as NE and vasopressin stimulate glycolysis via activation MAPK through PKC. The potential target(s) of MAPK would need to be defined. MAPK could either directly or via another kinase and/or phosphatase affect the activity of a glycolytic/gluconeogenic enzyme.

This study suggests that in liver, activation of MAPK can be induced by calcium-mobilizing agonists and cannot lead to a stimulation of glycogen synthesis under these conditions. This enzyme may therefore be implicated in several metabolic effects. It has been shown that insulin can increase 6-phosphofructo-2-kinase and pyruvate kinase activities in perfused rat liver, possibly via the action of a phosphatase. MAPK may play a role in the stimulation of glycolysis by insulin and calcium-mobilizing agents. Further studies are needed to fully characterize the role of MAPK in the regulation of hepatic glucose metabolism.

ACKNOWLEDGMENT

We thank Dr Pierre Haddad and Dr Sylvain Meloche for helpful discussions. We are also grateful to Guylaine Gévry for technical assistance.

REFERENCES

1. Ray LB, Sturgill TW: Characterization of insulin-stimulated microtubule-associated protein kinase. J Biol Chem 263:12721-12727, 1988a

2. Boulton TG, Yancopoulos GD, Gregory JS, et al: An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64-67, 1990

3. Seger R, Krebs EG: The MAPK cascade. FASEB J 9:726-735, 1995

4. Faure M, Voynoyasenetskaya TA, Bourne HR: cAMP and β-γ subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. J Biol Chem 269:7851-7854, 1994

5. Hawes BE, van Biesen T, Koch WJ, et al: Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. J Biol Chem 270:17148-17153, 1995

6. Koch WJ, Hawes BE, Allen LF, et al: Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by Gβγ activation of $p21^{ras}$. Proc Natl Acad Sci USA 91:12706-12710, 1994

7. Winitz S, Russell M, Qian NX, et al: Involvement of *ras* and *Raf* in the Gi-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. J Biol Chem 268:19196-19199, 1993

8. Johnson GL, Gardner AM, Lange-carter C, et al: How does the G protein, Gi2, transduce mitogenic signals? J Cell Biochem 54:415-422, 1994

9. Malarkey K, Belham CM, Paul A, et al: The regulation of tyrosine kinase signaling pathways by growth factor and G-protein-coupled receptors. Biochem J 309:361-375, 1995

10. Thomas SM, De Marco M, D'Arcangelo S, et al: *Ras* is essential for nerve growth factor– and phorbol ester–induced tyrosine phosphorylation of MAP kinases. Cell 68:1031-1040, 1992

11. Wood KW, Sarecki C, Roberts TM, et al: *ras* mediates nerve growth factor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell 68:1041-1050, 1992

12. Ginès P, Li X, Zamarripa JL, et al: Tyrosine kinase growth factor receptors but not seven-membrane–spanning receptors or phorbol esters activate mitogen-activated protein kinase in rat hepatocytes. Hepatology 22:1296-1303, 1995

13. Chao T-SO, Byron KL, Lee K-M, et al: Activation of MAP kinases by calcium-dependent and calcium-independent pathways. J Biol Chem 267:19876-19883, 1992

14. Kribben A, Wieder ED, Li X, et al: AVP-induced activation of MAP kinase in vascular smooth muscle cells is mediated through protein kinase C. Am J Physiol 265:C939-C945, 1993

15. Chao T-SO, Foster DA, Rapp UR, et al: Differential *Raf* requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters and calcium. J Biol Chem 269:7337-7341, 1994

16. Adachi T, Nakashima S, Saji S, et al: Mitogen-activated protein kinase activation in hepatocyte growth factor stimulated rat hepatocytes: Involvement of protein tyrosine kinase and protein kinase C. Hepatology 23:1244-1253, 1996

17. Sozeri O, Vollmer K, Liyanage M, et al: Activation of the c-*Raf* protein kinase by protein kinase C phosphorylation. Oncogene 7:2259-2262, 1992

18. Kolch W, Heidecker G, Kochs G, et al: Protein kinase Cα activates *RAF*-1 by direct phosphorylation. Nature 364:249-252, 1993

19. Carroll MP, May WS: Protein kinase C–mediated serine phosphorylation directly activates *Raf*-1 in murine hematopoietic cells. J Biol Chem 269:1249-1256, 1994

20. VanRenterghem B, Browning MD, Maller JL: Regulation of mitogen-activated protein kinase activation by protein kinases A and C in a cell-free system. J Biol Chem 269:24666-24672, 1994

21. Gines P, Li X, Brown SES, et al: Inhibitory actions of cyclic adenosine monophosphate and pertussis toxin define two distinct epidermal growth factor–regulated pathways leading to activation of mitogen-activated protein kinase in rat hepatocytes. Hepatology 23:1167-1173, 1996

22. van de Werve G: Isolation and characteristics of hepatocytes. Toxicology 18:179-185, 1980

23. Meloche S: Cell cycle reentry of mammalian fibroblasts is accompanied by the sustained activation of $p44^{mapk}$ and $p42^{mapk}$ isoforms in the G1 phase and their inactivation at the G1/S transition. J Cell Physiol 168:577-588, 1995

24. Hue L, Bontemps F, Hers HG: The effect of glucose and of potassium ions on the interconversion of the two forms of glycogen phosphorylase and of glycogen synthase in isolated rat liver preparations. Biochem J 152:105-114, 1975

25. Budworth J, Gescher A: Differential inhibition of cytosolic and membrane-derived protein kinase C activity by staurosporine and other kinase inhibitors. FEBS Lett 362:139-142, 1995

26. Bygrave FL, Benedetti A: Calcium: Its modulation in liver by cross-talk between the actions of glucagon and calcium-mobilizing agonists. Biochem J 296:1-14, 1993

27. Altin JG, Bygrave FL: The Ca^{2+}-mobilizing actions of vasopressin and angiotensin differ from those of the α-adrenergic agonist phenylephrine in the perfused liver. Biochem J 232:911-917, 1985

28. Thastrup O, Cullen PJ, Drøbak BK, et al: Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA 87:2466-2470, 1990

29. Berridge MJ: Inositol trisphosphate and calcium signalling. Nature 361:315-325, 1993

30. Gopalakrishna R, Barsky SH, Thomas TP, et al: Factors influencing chelator-stable, detergent-extractable, phorbol diester–induced membrane association of protein kinase C. Differences between Ca^{2+}-induced and phorbol ester–stabilized membrane bindings of protein kinase C. J Biol Chem 261:16438-16445, 1986

31. Rasmussen H, Isales CM, Calle R, et al: Diacylglycerol production, Ca^{2+} influx, and protein kinase C activation in sustained cellular responses. Endoc Rev 16:649-681, 1995

32. Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607-614, 1992

33. Siddiqui RA, Exton JH: Oleate stimulation of diacylglycerol formation from phosphatidylcholine through effects on phospholipase D and phosphatidate phosphohydrolase. Eur J Biochem 210:601-607, 1992

34. Yeo E-J, Exton JH: Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells. J Biol Chem 270:3980-3988, 1995

35. Sadoshima J, Qiu Z, Morgan JP, et al: Angiotensin II and other hypertrophic stimuli mediated by G protein–coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. Circ Res 76:1-15, 1995

36. Earp HS, Huckle WR, Dawson TL, et al: Angiotensin II activates at least two tyrosine kinases in rat liver epithelial cells. J Biol Chem 270:28440-28447, 1995

37. Rosen LB, Greenberg ME: Stimulation of growth factor receptor signal transduction by activation of voltage-sensitive calcium channels. Proc Natl Acad Sci USA 93:1113-1118, 1996

38. Lange-Carter CA, Pleiman CM, Gardner AM, et al: A divergence in the MAP kinase regulatory network defined by MEK kinase and *Raf*. Science 260:315-319, 1993

39. Cohen P: Dissection of the protein phosphorylation cascades

involved in insulin and growth factor action. Biochem Soc Trans 21:555-567, 1993

40. Sturgill TW, Ray LB, Erikson E, et al: Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature 334:715-718, 1988

41. Dent P, Lavoinne A, Nakielny S, et al: The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature 348:302-308, 1990

42. Lavoinne A, Erikson E, Maller JL, et al: Purification and characterisation of the insulin-stimulated protein kinase from rabbit skeletal muscle; close similarity to S6 kinase II. Eur J Biochem 199:723-728, 1991

43. Tobe K, Kadowaki T, Hara K, et al: Sequential activation of MAP kinase activator, MAP kinases, and S6 peptide kinase in intact rat liver following insulin injection. J Biol Chem 267:21089-21097, 1992

44. Ray LB, Sturgill TW: Insulin-stimulated microtubule associated protein kinase is detectable by analytical gel chromatography as a 35-kDa protein in myocytes, adipocytes and hepatocytes. Arch Biochem Biophys 262:307-313, 1988b

45. Peak M, Yeaman SJ, Agius L: Epidermal growth factor and insulin stimulate MAP kinase activity in cultured hepatocytes. Biochem Soc Trans 21:494S, 1993 (suppl, abstr)

46. Lazar DF, Wiese RJ, Brady MJ, et al: Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. J Biol Chem 270:20801-20807, 1995

47. Robinson LJ, Razzack ZF, Lawrence JC, et al: Mitogen-activated protein kinase activation is not sufficient for stimulation of glucose transport or glycogen synthase in 3T3-L1 adipocytes. J Biol Chem 268:26422-26427, 1993

48. Fingar DC, Birnbaum MJ: Characterization of the mitogen-activated protein kinase/90-kilodalton ribosomal protein S6 kinase signaling pathway in 3T3-L1 adipocytes and its role in insulin-stimulated glucose transport. Endocrinology 134:728-735, 1994

49. Lin TA, Lawrence JC: Activation of ribosomal protein S6 kinases does not increase glycogen synthesis or glucose transport in rat adipocytes. J Biol Chem 269:21255-21261, 1994

50. Wiese RJ, Corley Mastick C, Lazar DF, et al: Activation of mitogen-activated protein kinase and phosphatidylinositol 3′-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. J Biol Chem 270:3442-3446, 1995

51. Shepherd PR, Navé BT, Siddle K: Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: Evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase. Biochem J 305:25-28, 1995

52. Hue L, van Schaftingen E, Blackmore PF: Stimulation of glycolysis and accumulation of a stimulator of phosphofructokinase in hepatocytes incubated with vasopressin. Biochem J 194:1023-1026, 1981

53. van de Werve G, Jeanrenaud B: Fructose-2,6-bisphosphate: A third messenger in endocrine regulation of liver metabolism?, in Bianchi L, Gerok W, Landmann L, et al (eds): Liver in Metabolic Diseases. Boston, MA, MTP, 1983, pp 263-275

54. Conricode KM, Ochs RS: Epidermal growth factor and 12-*O*-tetradecanoylphorbol 13-acetate stimulate lactate production and the pentose phosphate pathway in freshly isolated rat hepatocytes. J Biol Chem 265:20931-20937, 1990

55. Conricode KM, Ochs RS: Vasopressin stimulates pyruvate utilization through a Ca^{2+}-dependent mechanism and lactate formation by a protein kinase C–dependent mechanism in isolated rat hepatocytes. Biochim Biophys Acta 1095:161-168, 1991