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Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes

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Certain amino acids, like glutamine and leucine, induce an anabolic response in liver. They activate p70 ribosomal protein S6 kinase (p70S6K) and acetyl-CoA carboxylase (ACC) involved in protein and fatty acids synthesis, respectively. In contrast, the AMP-activated protein kinase (AMPK), which senses the energy state of the cell and becomes activated under metabolic stress, inactivates by phosphorylation key enzymes in biosynthetic pathways thereby conserving ATP. In this paper, we studied the effect of AMPK activation and of protein phosphatase inhibitors, on the amino-acid-induced activation of p70S6K and ACC in hepatocytes in suspension. AMPK was activated under anoxic conditions or by incubation with 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAr) or oligomycin, an inhibitor of mitochondrial oxidative phosphorylation. Incubation of hepatocytes with amino acids activated p70S6K via multiple phosphorylation. It also activated ACC by a phosphatase-dependent mechanism but did not modify AMPK activation. Conversely, the amino-acid-induced activation of both ACC and p70S6K was blocked or reversed when AMPK was activated. This AMPK activation increased Ser79 phosphorylation in ACC but decreased Thr389 phosphorylation in p70S6K. Protein phosphatase inhibitors prevented p70S6K activation when added prior to the incubation with amino acids, whereas they enhanced p70S6K activation when added after the preincubation with amino acids. It is concluded that (a) AMPK blocks amino-acid-induced activation of ACC and p70S6K, directly by phosphorylating Ser79 in ACC, and indirectly by inhibiting p70S6K phosphorylation, and (b) both activation and inhibition of protein phosphatases are involved in the activation of p70S6K by amino acids. p70S6K adds to an increasing list of targets of AMPK in agreement with the inhibition of energy-consuming biosynthetic pathways.

Keywords: ACC; amino acid; AMPK; p70S6K; protein phosphatase.

p70 ribosomal protein S6 kinase (p70S6K) participates in the control of protein synthesis and is activated in response to hormones, mitogens and nutrients (reviewed in [1–3]). It phosphorylates the 40S ribosomal protein S6, which is involved in the translation of certain mRNAs, the so-called 5′-TOP mRNAs encoding ribosomal proteins and elongation factors. p70S6K is activated by insulin in muscle [1–3], but not in hepatocytes, according to our recent work [4]. In these cells, p70S6K is activated by amino acids like glutamine and leucine, which act synergistically [4]. However, a crosstalk between insulin and amino acids can be demonstrated with leucine, which enhances insulin signalling towards p70S6K in many cell types, including hepatocytes [3–6].

The mechanism of activation of p70S6K involves a complex sequence of multiple serine/threonine phosphorylations catalysed by several protein kinases. One of these is the mammalian target of rapamycin (mTOR), which phosphorylates p70S6K on Thr389 and is inhibited by the immunosuppressant rapamycin [7]. Phosphorylation of this site correlates with kinase activity [8]. mTOR may also phosphorylate and thereby inactivate a protein phosphatase that in turn inactivates p70S6K. Indeed, several studies suggest that the amino-acid signalling pathway leading to p70S6K activation comprises inhibition of a protein phosphatase [9,10]. Whatever the mechanism of activation of p70S6K by mTOR, the latter plays an essential role, because the activation of p70S6K caused by almost all stimuli so far tested is inhibited by rapamycin. Phosphorylation of Ser411, Thr421 and Ser424, which are within a Ser-Pro rich region located in the autoinhibitory domain, is also thought to modulate p70S6K activity [8,11]. In response to insulin, the 3-phosphoinositide-dependent protein kinase

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAr, 5-aminoimidazole-4-carboxyamide ribonucleoside; ZMP, AICA-ribotide; GAPP, glutamate-activated protein phosphatase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase; PKB, protein kinase B; PP2A, protein phosphatase 2A.

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(PDK1) is directly involved in p70S6K activation [11]. The target phosphorylation site for PDK1 is Thr229 in the catalytic domain of p70S6K. A role for protein kinase B (PKB) in the insulin-stimulated activation process of p70S6K has also been proposed [12], but has been ruled out for the amino-acid-induced activation of p70S6K in liver cells [4].

Acetyl-CoA carboxylase (ACC) is a regulatory enzyme in fatty acid synthesis (reviewed in [13–15]). We have shown that in liver cells ACC activation is correlated with cell swelling be it induced by amino acids that are cotransported with Na^+ or by hypotonic medium [16]. The activity of ACC is controlled by various mechanisms, including changes in the degree of polymerization, allosteric regulation by citrate and glutamate, and covalent modification by phosphorylation/dephosphorylation [13–18]. It is generally assumed that the active form is dephosphorylated, although phosphorylation has been invoked to explain ACC activation by insulin in adipocytes [19].

Under stress conditions, such as anoxia or inhibition of mitochondrial oxidative phosphorylation, the ATP balance becomes negative and, as a result, the AMP/ATP ratio increases. This leads to the activation of the AMP-activated protein kinase (AMPK), which functions as a metabolic master switch and inhibits anabolic processes, thereby preserving ATP (reviewed in [20–22]). ACC is phosphorylated *in vitro* by AMPK on Ser79, Ser1200 and Ser1250, the phosphorylation of Ser79 being responsible for inactivation [23]. AMPK-inactivated ACC can be reactivated by a glutamate-dependent type-2A protein phosphatase (GAPP), which dephosphorylates a synthetic peptide encompassing the Ser79 phosphorylation site for AMPK in ACC [24]. It is expected that in hepatocytes the activation state of ACC results from the balance between the activities of GAPP and AMPK, although the involvement of other protein kinase or phosphatases has not been ruled out.

Because ACC and p70S6K display a similar and parallel pattern of activation in hepatocytes incubated with glutamine [4], the question arises whether there is also a common mechanism for inactivation. It is indeed expected that ACC and p70S6K, which control energy-consuming biosynthetic pathways, are less active when ATP supply becomes limiting. Therefore, the effect of different activators of AMPK and the effect of inhibitors of protein phosphatases on the amino-acid-induced activation of ACC and p70S6K were examined in freshly prepared rat hepatocytes. Our results show that the activation of ACC and p70S6K depend on a protein phosphatase and that both enzymes may be inactivated under conditions leading to AMPK activation.

MATERIALS AND METHODS

Materials

5-Aminoimidazole-4-carboxyamide ribonucleoside (AICAr) and oligomycin were from Sigma. Okadaic acid and calyculin A were from Calbiochem. The peptides corresponding to the p70S6K substrate [4] and the AMPK substrate (SAMS) [25] were kindly provided by V. Stroobant (Ludwig Institute, Brussels, Belgium). Antibodies raised against a peptide containing phosphoSer79 of ACC was a generous gift from D. G. Hardie (Dundee, Scotland). Antibodies raised against synthetic phosphopeptides corresponding to p70S6K phosphorylation sites containing phosphoThr389 (anti-pThr389), phosphoSer-411 (anti-pSer411), or phosphoThr421 together with phosphoSer424 [anti-(ppThr421 + Ser424)] were purchased from Santa Cruz. The source of all other materials is given in [4,26].

Hepatocytes preparation and incubation

Hepatocytes from overnight-fasted male Wistar rats (170–200 g of body weight) were prepared as described previously [4] and incubated at 37 °C for the indicated periods of time following 15 min preincubation, at a concentration of about 50 $mg{\cdot}mL^{-1}$ in Krebs–Henseleit bicarbonate buffer in equilibrium with a 95% O_2/5% CO_2 gas phase in the presence of 20 mM glucose and other substances as indicated in the legends to the Figures. Anoxia was obtained by incubating the cells in a 95% N_2/5% CO_2 gas phase. At the end of the incubations, the cells were collected by centrifugation (2 s, microfuge) and the cell pellets were immediately stored in liquid nitrogen. The cell pellets were homogenized in 0.5 mL of the lysis buffer as described previously [4]. After centrifugation (20 000 ***g***, 15 min), the supernatants were stored at −80 °C.

Enzyme assays

Methods for the measurements of the activity of AMPK after precipitation with 6% (w/v) polyethylene glycol 6000, of ACC and p70S6K, and for immunoprecipitation of p70S6K from cell extracts have been described [4,27,28]. ACC was measured in the presence of 0.5 mM citrate-Mg [27]. One unit of enzyme activity corresponds to 1 nmol (protein kinases) or 1 μmol (ACC) of product formed per min under the assay conditions.

Other methods

The phosphorylation state of p70S6K in hepatocytes was evaluated by gel mobility shift assay [4] as well as by immunoblots with antiphosphopeptides. *In vitro* trials of p70S6K phosphorylation by purified AMPK were performed as follows. Immunoprecipitates of p70S6K from extracts of control and amino-acid-treated hepatocytes were incubated at 30 °C for 30 min in a total volume of 50 μL in the presence of 60 mU of purified liver AMPK [26] with or without 2 mM AMP and 100 μM [γ-^{32}P]ATP-Mg (3000 $c.p.m.{\cdot}pmol^{-1}$) for the measurement of the incorporation of radioactive phosphate after SDS/PAGE and autoradiography, or 1 mM ATP-Mg for the measurement of p70S6K activity [4].

RESULTS

AMPK is involved in the inactivation of ACC and p70S6K

In agreement with our previous studies [4], there was a similar pattern of activation of ACC and p70S6K in hepatocytes incubated with glutamine, suggesting a common point of control in their signalling pathways (Fig. 1). The effects of AMPK activation on p70S6K and ACC were compared. AMPK was activated by incubating the cells under stress conditions, namely anoxia (Fig. 1), AICAr or

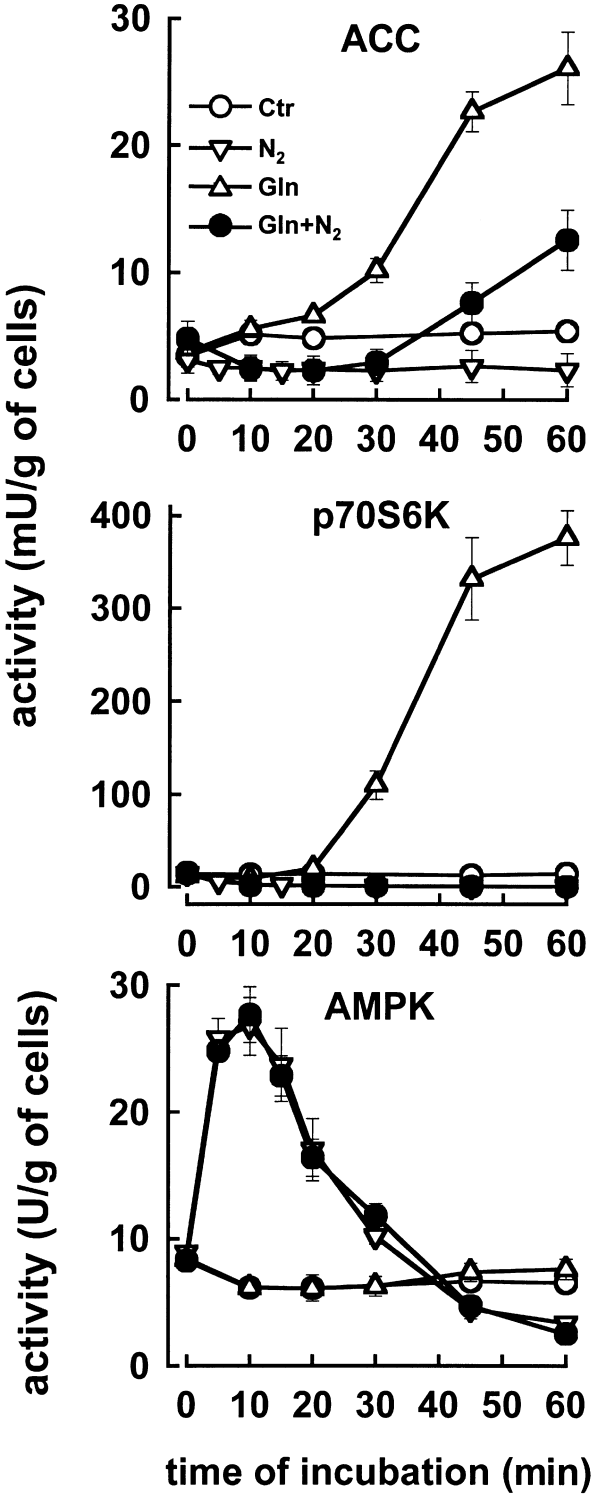

Fig. 1. Time-course of the effect of anoxia on ACC, p70S6K and AMPK activities. Hepatocytes were incubated for the indicated periods of time under control conditions (○, Ctr), in the presence of 10 mM glutamine (△, Gln), under a nitrogen atmosphere (▽, N_2) or in the presence of 10 mM glutamine under a nitrogen atmosphere (●, Gln + N_2). The values are the means ± SEM for three cell preparations.

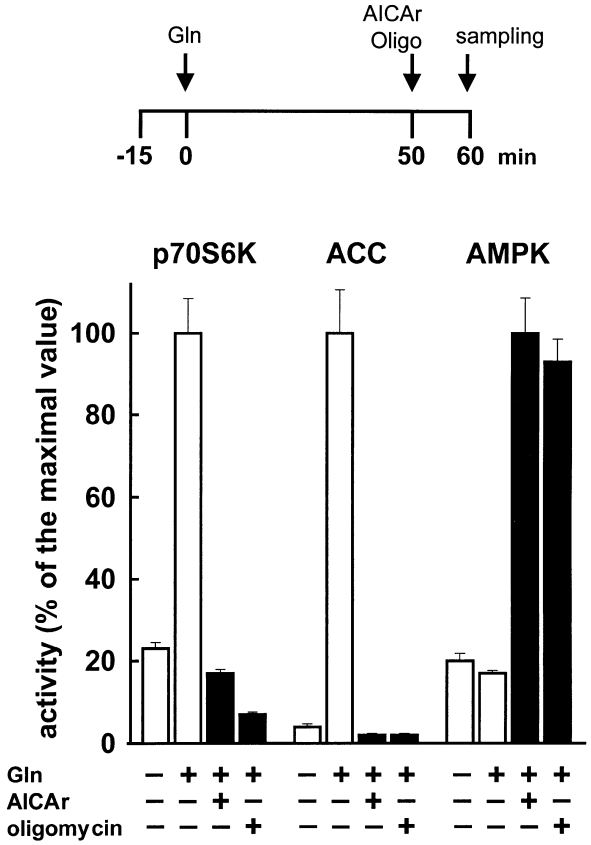

Fig. 2. Effect of AICAr and oligomycin on ACC, p70S6K and AMPK activities. The experimental protocol is shown schematically at the top of the figure. After an equilibrium period of 15 min, hepatocytes were incubated under control conditions or in the presence of 10 mM glutamine for 50 min (open bars). The cells were then further incubated with 0.5 mM AICAr or 1 μM oligomycin for 10 min (filled bars). Maximal activation was seen for ACC and p70S6K after a 60-min incubation with glutamine (32.8 ± 2.8 and 348 ± 34 $mU \cdot g^{-1}$ of cells, respectively; means ± SEM, $n = 3$) and for AMPK after a 10-min incubation with AICAr or oligomycin (24.5 ± 2.1 and 22.8 ± 1.3 $U \cdot g^{-1}$ of cells, respectively; means ± SEM, $n = 3$). The values are expressed as percent of maximal activity observed for each enzyme. Gln, glutamine; Oligo, oligomycin.

oligomycin, an inhibitor of mitochondrial oxidative phosphorylation (Fig. 2). Anoxia and oligomycin increase intracellular AMP concentration, whereas AICAr is phosphorylated into AICA-ribotide (ZMP), an AMP analogue. Both ZMP and AMP are activators of AMPK. As already observed in heart [26], AMPK activation by anoxia was transient, being maximal at 10 min before returning to basal or even lower values between 45 and 60 min of incubation (Fig. 1). Like anoxia, both AICAr and oligomycin activated AMPK (Fig. 2) and this activation was not changed in hepatocytes incubated with glutamine (Figs 1 and 2). Under these stress conditions, AMPK activation led to inactivation of both ACC and p70S6K, suggesting that AMPK activation overruled the control by amino acids. However, when AMPK activity returned towards basal levels at 45 and 60 min, ACC but not p70S6K started to reactivate. These

data suggest that AMPK plays a role in the inactivation process of ACC and p70S6K under metabolic stress conditions.

Rapamycin and AICAr exert different effects on the activation of ACC and p70S6K induced by glutamine

Rapamycin is a potent inhibitor of mTOR and of the insulin-dependent activation of p70S6K in skeletal muscle [29–31]. We compared the time-course of the effect of AICAr with that of rapamycin on ACC and p70S6K, both of which had been activated by a 50-min incubation with glutamine (Fig. 3). Rapamycin inactivated p70S6K but was without effect on ACC, in agreement with our previous findings [27]. This rules out a role for mTOR or p70S6K in the glutamine-mediated activation of ACC. The inactivation of p70S6K by rapamycin occurred within seconds (–27% after 20 s) and was complete between 5 and 10 min of incubation. In contrast, the inactivation of p70S6K by AICAr was slower and was half-maximal only at 7 min, whereas the inactivation of ACC by AICAr was half-maximal at about 1 min (Fig. 3). The velocity of the onset of ACC inactivation indicates that AICAr is quickly transported into the hepatocytes and indeed leads to an immediate activation of AMPK (Fig. 3), which in turn inactivates ACC by phosphorylating Ser79 (Fig. 4). The comparison of the sensitivity of ACC, p70S6K and AMPK towards AICAr showed that half-maximal effects were observed at about 30 μM for ACC and 110 μM for AMPK and p70S6K (Fig. 4).

ACC activity and Ser79 phosphorylation

Phosphorylation of Ser79 is known to inactivate ACC by decreasing the V_{max} [18,23]. *In vitro*, this site is phosphorylated by AMPK and dephosphorylated by GAPP. Immunoblotting hepatocyte extracts with an anti-phosphopeptide (anti-phosphoSer79 Ig) demonstrated that Ser79 was indeed phosphorylated (Fig. 4) when AMPK was activated. We confirmed that this ACC inactivation corresponded to a decrease in V_{max} (data not shown). In contrast, ACC activation by amino acids occurred without a change in Ser79 phosphorylation (Fig. 4), although it was blocked by protein phosphatase inhibitors (see below). This suggests that dephosphorylation occurs at other sites on ACC or that the process is indirect.

P70S6K is not a direct substrate of AMPK

We tested the possibility of a direct phosphorylation and inactivation of p70S6K by AMPK *in vitro*. p70S6K purified from control hepatocytes or from cells treated with amino acids could not be phosphorylated by purified AMPK (data not shown). In addition, attempts to inactivate p70S6K by AMPK *in vitro* also failed (without/with AMPK: control, 14/14; glutamine, 129/111; glutamine and leucine, 530/500 mU·g cells^{-1}, $n = 2$).

Inhibitors of protein phosphatases exert different effects on ACC and p70S6K activities

The effect of two inhibitors of protein phosphatases, namely okadaic acid and calyculin A, were investigated. Both inhibitors are cell permeable compounds with tumor

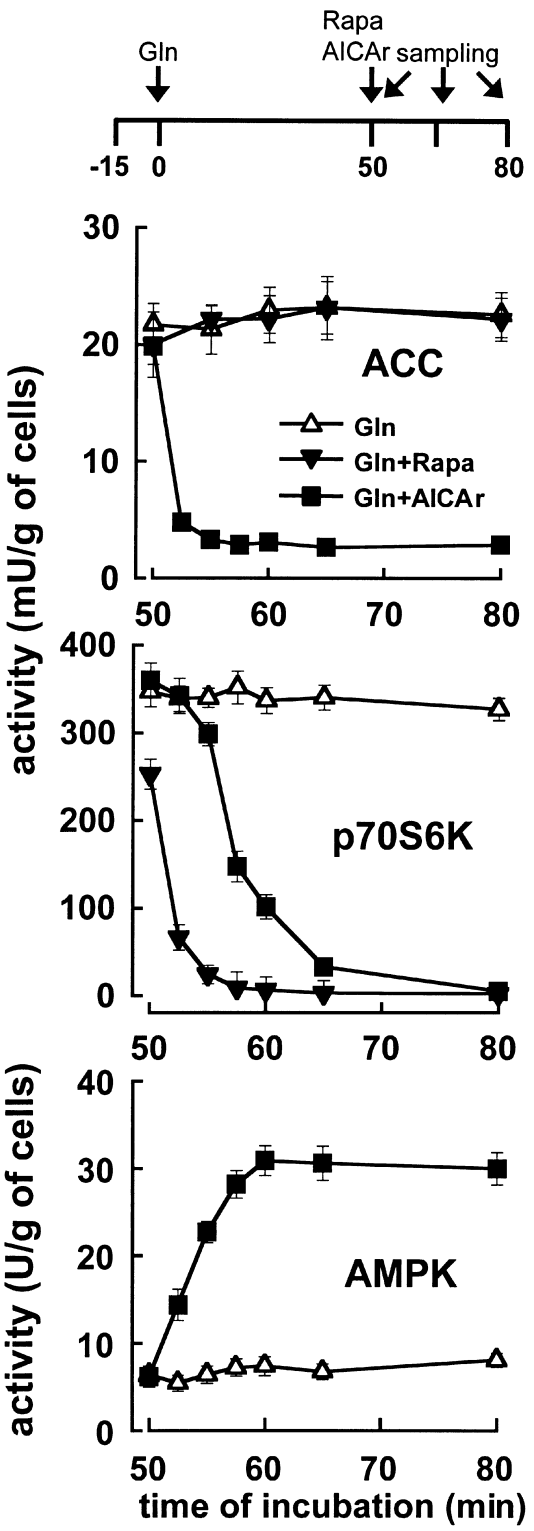

Fig. 3. Time-course of the effects of rapamycin or AICAr on ACC, p70S6K and AMPK activities. The experimental protocol is shown schematically at the top of the figure. Hepatocytes were incubated in the presence of 10 mM glutamine (Gln) to activate ACC and p70S6K. After 50 min, NaCl (0.9% final concentration, △), rapamycin (Rapa, 300 nM final concentration, ▼) or AICAr (0.5 mM final concentration, ■) were added and the cells were further incubated for up to 30 min. The values are the means ± SEM for three cell preparations.

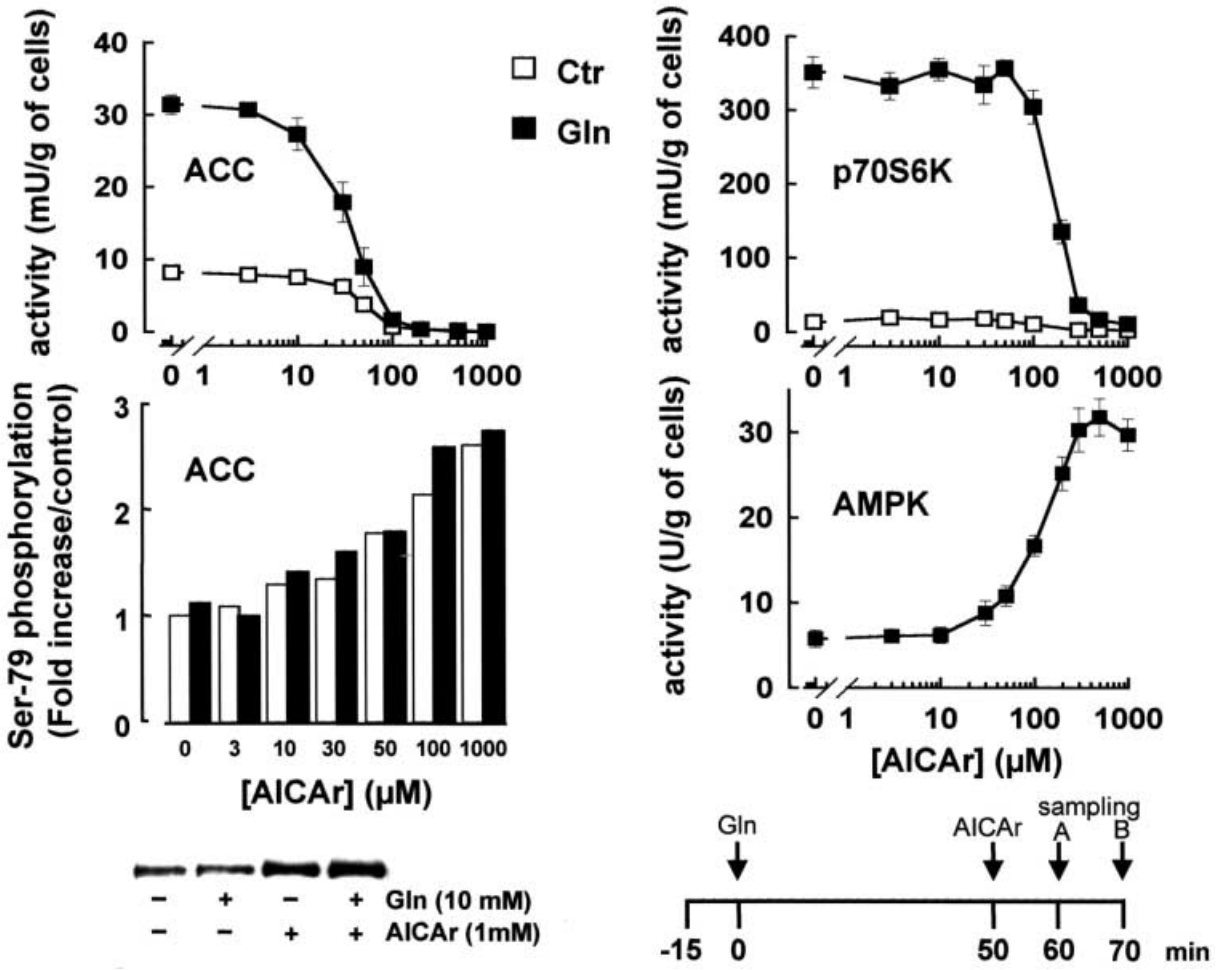

Fig. 4. Dose–response curve of the effect of AICAr on ACC, p70S6K and AMPK activities and Ser79 phosphorylation in ACC. The experimental protocol is shown schematically at the bottom right of the figure. Hepatocytes were incubated under control conditions or stimulated with 10 mM glutamine. After 50 min, AICAr was added and the cells were further incubated for 10 min (sampling A: ACC and AMPK) or 20 min (sampling B: p70S6K). AICAr (□, Ctr); glutamine plus AICAr (■, Gln). The values of enzyme activity are the means ± SEM for three cell preparations. Ser79 phosphorylation was detected by immunoblotting extracts (15 μg of proteins) with the anti-phosphoSer79 Ig (lower left panel) and was quantified by densitometry (middle left panel, mean of two experiments).

promoting properties that target on the serine/threonine protein phosphatases PP2A and PP1 [32]. Preincubation of hepatocytes with okadaic acid prevented the activation of ACC (100%) and of p70S6K (by ≈70%) in hepatocytes incubated with glutamine plus leucine (Fig. 5). These results suggest that a protein phosphatase is required for the activation of both ACC and p70S6K by amino acids. The effect of these inhibitors differed when they were added after a preincubation with amino acids to activate both enzymes. Under these conditions, okadaic acid inactivated ACC, whereas it enhanced p70S6K activation (Fig. 5). Similar results were obtained with calyculin A (data not shown).

The phosphorylation state of p70S6K

The activation of p70S6K involves multiple phosphorylations of the protein [1–3]. The phosphorylated forms can be detected by their reduced mobility during SDS/PAGE and by blotting with anti-phosphopeptides (Fig. 6). The proportion of slow electrophoretic, phosphorylated forms of p70S6K that appeared after stimulation of the cells with glutamine or glutamine plus leucine correlated with the increase in p70S6K activity brought about by these amino acids (Fig. 6, lanes 1–4). The data also show that increases in p70S6K activity correlate with increases in Thr389, Thr421 and Ser424 phosphorylation state, whereas Ser411 phosphorylation was unaffected (Fig. 6). AICAr did not change the phosphorylation state of p70S6K if compared with the controls (Fig. 6, compare lane 5 with lane 1), but it counteracted phosphorylation induced by amino acids and drastically decreased Thr389 phosphorylation (Fig. 6, compare lane 7 with lane 4). This suggests that AMPK did not directly target on p70S6K, thus confirming our lack of experimental evidence *in vitro* for a direct phosphorylation of p70S6K by AMPK. Addition of calyculin A after a preincubation with amino acids to activate p70S6K resulted in a further activation of p70S6K (Figs 5 and 6), which corresponded to the appearance of the slowest electrophoretic forms and maximal phosphorylation of Thr389, Thr421 and Ser424. (Fig. 6, compare lane 8 with lane 4).

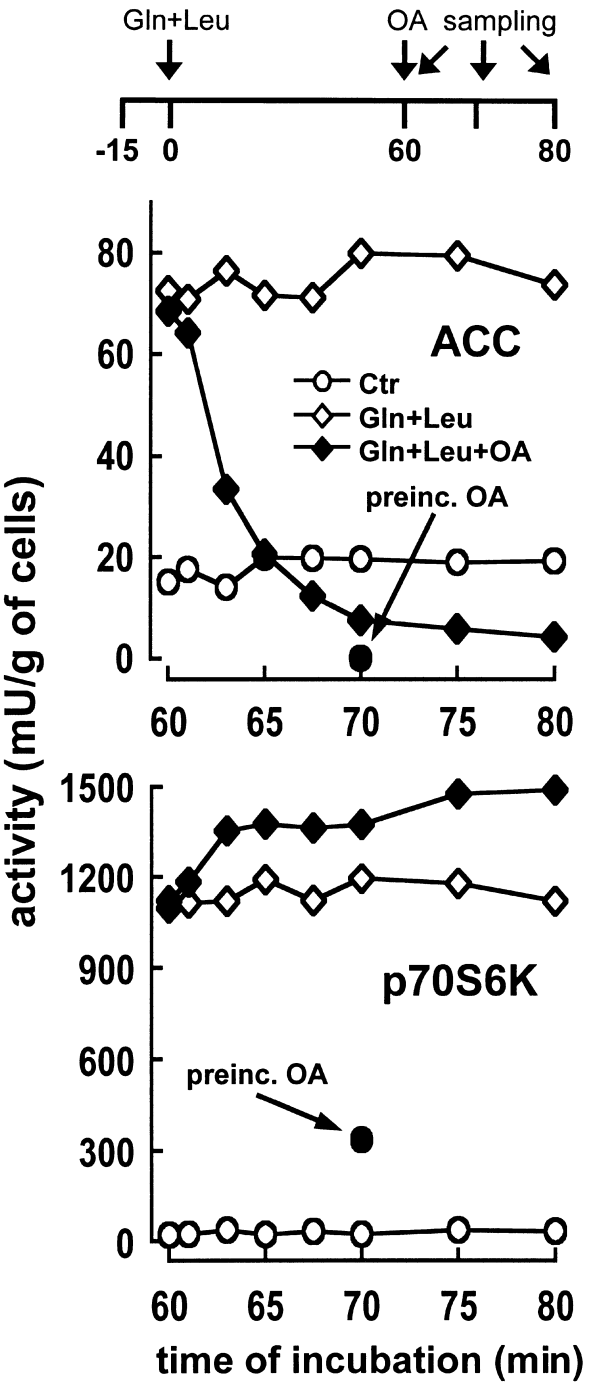

Fig. 5. Time-course of the effect of okadaic acid on ACC and p70S6K activity. The experimental protocol is shown schematically at the top of the figure. Hepatocytes were incubated under control conditions (○, Ctr) or were stimulated by 10 mM glutamine plus leucine (◇, Gln + Leu). After 60 min, okadaic acid (◆, OA, 100 nM final concentration) was added and the cells were further incubated for up to 20 min. In another experiment, the cells were preincubated with okadaic acid for 15 min prior to the stimulation by amino acids and samples were taken at the end of a 70-min incubation period (●, preinc. OA).

However, preincubation of the cells with calyculin A slightly activated p70S6K and increased phosphorylation of p70S6K mainly on Thr421 and Ser424 in control cells (Fig. 6, lane 6). In contrast, this preincubation with calyculin A decreased p70S6K activation and phosphorylation that resulted from further incubation with amino acids (Fig. 6, compare lane 9 with lane 4). Calyculin A was also found to antagonize, at least partially, the inactivation of p70S6K by rapamycin (Fig. 7, upper panel). This antagonism corresponded to an inhibition of p70S6K dephosphorylation by calyculin A. Indeed, the phosphorylation state of p70S6K from cells incubated with calyculin A was intermediate between the more (glutamine) and the less (glutamine plus rapamycin) phosphorylated forms. Taken together these data support the idea that p70S6K activity is finely tuned by different degrees in its phosphorylation state.

DISCUSSION

The results presented in this work demonstrate that the process of activation of ACC and p70S6K by amino acids is inhibited by okadaic acid and calyculin A, two inhibitors of type 1 and 2A protein phosphatase, and by incubation of liver cells with AICAr, which leads to AMPK activation. The interpretation of these results points to new mechanisms involved in the control of ACC and p70S6K by amino acids and AMPK. These mechanisms are shown schematically in Fig. 8, based on the available data in the literature and integrating the results obtained in the present study.

ACC is known to be activated by incubating hepatocytes with certain amino acids [16]. The activation process does not involve dephosphorylation of Ser79, as shown here, but is blocked by protein phosphatase inhibitors. We assume that the protein phosphatase involved is GAPP, the glutamate-dependent protein phosphatase type 2A, which is likely to be activated by the amino-acid-induced accumulation of glutamate and is inhibited by calyculin A [17]. Activation of AMPK inactivates ACC by direct phosphorylation of Ser79. An additional inactivation of the glutamate-sensitive protein phosphatase by AMPK cannot be ruled out. On the other hand, it is remarkable that ACC inactivation is very sensitive to AICAr and that partial activation of AMPK suffices to inactivate completely ACC.

In contrast with ACC, the active form of p70S6K is (multi)phosphorylated. Therefore, GAPP is unlikely to be directly involved. Nevertheless, it could participate in the activation cascade. p70S6K is activated by several protein kinases among which mTOR plays a crucial role. mTOR has a dual effect on p70S6K, it inhibits a downstream protein phosphatase, thereby relieving its inhibitory effect on p70S6K, and it phosphorylates p70S6K on Thr389 as part of the sequential phosphorylation/activation mechanism [10,33]. We have showed here that p70S6K activation by amino acids corresponds to an increased phosphorylation state of Thr389 and of other Ser/Thr residues that are located in the autoinhibitory domain. In agreement with the intervention of an inhibitory protein phosphatase downstream of mTOR [9,10] is our observation that the amino-acid-induced activation of p70S6K is reinforced by calyculin A if added after preincubation with amino acids. In this case, calyculin A would inhibit the mTOR-sensitive protein phosphatase and thereby reinforce the effect of

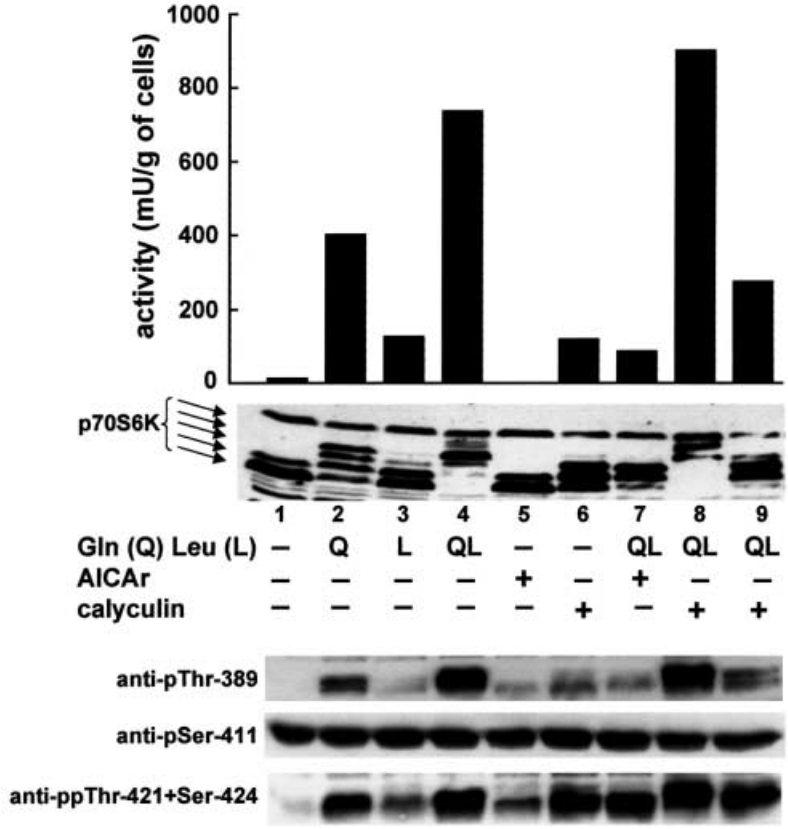

Fig. 6. Phosphorylation of p70S6K. The global phosphorylation state of p70S6K was evaluated by gel mobility shift as follows. Aliquots of extracts (150 μg of total protein) from hepatocytes were separated on a 7.5% acrylamide/0.05% bisacrylamide resolving gel and then blotted on poly(vinylidene difluoride) transfer membrane. p70S6K was immunodetected with a polyclonal antip70S6K antibody. The incubation times were the following: (1) 65 min control incubation; (2) 65 min incubation with Gln; (3) 15 min incubation with Leu; (4) 45 min incubation with Gln and Leu; (5) 45 min preincubation followed by 20 min incubation with AICAr; (6) 45 min preincubation followed by 20 min incubation with calyculin A; (7) 45 min preincubation with Gln and Leu followed by 20 min incubation with AICAr; (8) 45 min preincubation with Gln and Leu followed by 20 min incubation with calyculin A; (9) 20 min preincubation with calyculin A followed by 45 min incubation with Gln and Leu. The concentrations were 10 mM for Gln and Leu, 1 mM for AICAr and 100 nM for calyculin A. The values are from one cell preparation and are representative of three different experiments. The same extracts (200 μg of proteins) were also analysed by standard SDS/PAGE followed by immunodetection with anti-phosphopeptides [anti-pThr389 Ig, anti-pSer411 Ig and anti-(ppThr421 + Ser424) Ig].

mTOR, as shown by the increased Thr389 phosphorylation. Moreover, another protein phosphatase should also be involved in the activation of p70S6K by amino acids. Indeed, preincubation of hepatocytes with calyculin A prevents the activation and phosphorylation of p70S6K by amino acids, indicating that a protein phosphatase located upstream of mTOR is involved in the activation of p70S6K. We suggest that this upstream protein phosphatase is identical with GAPP, thereby causing the parallel activation of ACC and p70S6K after stimulation of hepatocytes with amino acids. To explain that the inhibition of this protein phosphatase blocks p70S6K activation, we speculate that this protein phosphatase leads to mTOR activation through an activation of mTOR kinase, the protein kinase responsible for mTOR activation, rather than via direct dephosphorylation of mTOR by GAPP. Indeed, there is no available evidence in the literature for an activation of mTOR by dephosphorylation. Taken together, these data support the hypothesis that two phosphatases are involved, one upstream of mTOR, the activation of which is required for the activation of both ACC and p70S6K, and one downstream of mTOR which inactivates p70S6K.

AICAr abrogates the amino-acid-induced activation of p70S6K indicating that AMPK is involved in the regulation of p70S6K. This observation may be related to the recent report of a sensitivity of mTOR to intracellular concentration of ATP [34]. The decrease in p70S6K activation by AMPK mainly results from a decrease in Thr389 phosphorylation, the site phosphorylated by mTOR. Moreover, our results show that the time-course of inactivation of p70S6K is slower than that of ACC, and that AMPK is not able to phosphorylate and inactivate p70S6K *in vitro*. This indicates that AMPK does not act directly on p70S6K *in vivo* but could inactivate mTOR by phosphorylation. However, the fact that p70S6K inactivation by AICAr is slower that the inactivation by rapamycin advocates against a direct action of AMPK on mTOR, suggesting that AMPK phosphorylates and inactivates a step upstream of mTOR, which could be GAPP or mTOR kinase.

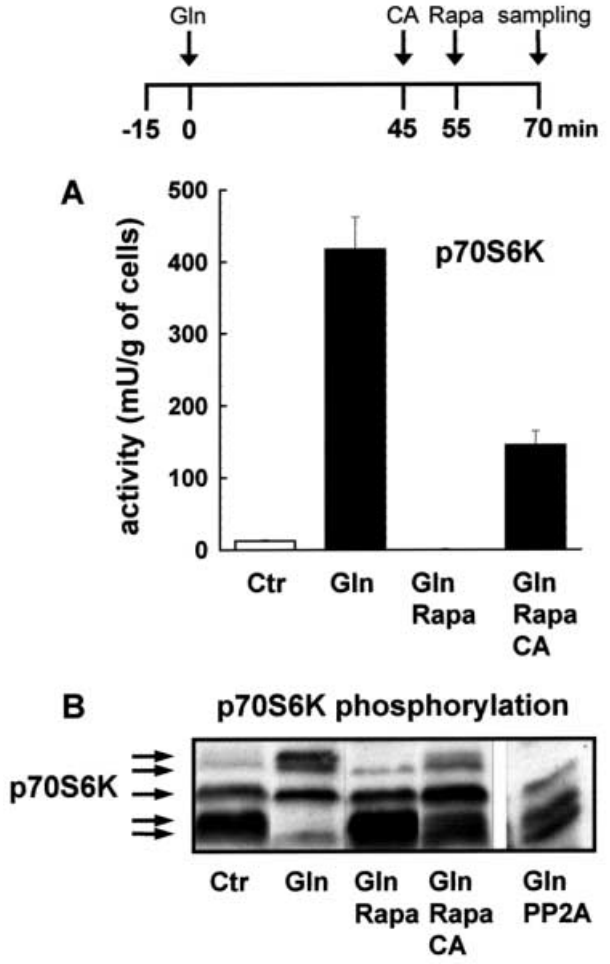

Fig. 7. Calyculin A partially inhibits rapamycin-induced dephosphorylation of p70S6K. The experimental protocol is shown schematically at the top of the figure. Hepatocytes were incubated for 45 min with 10 mM glutamine (Gln). The cells were then incubated with 100 nM calyculin A (CA) for 10 min before a further 15 min incubation with 300 nM rapamycin (Rapa). (A) p70S6K activity was measured after immunoprecipitation with an anti-p70S6K Ig. The values are the means ± SEM for three cell preparations. (B) The phosphorylation state of p70S6K was evaluated as described in the legend to Fig. 6. To verify that the mobility shift was due to protein phosphorylation, an extract prepared from cells stimulated with glutamine was incubated for 45 min in the presence of 40 $mU \cdot mL^{-1}$ of purified PP2A, 0.5 $mg \cdot mL^{-1}$ BSA, 1 mM $MnCl_2$, 50 mM Tris (pH 7.5), 0.03% Brij-35, 0.1 mM EGTA and 0.1% 2-mercaptoethanol. The reaction was stopped by boiling the incubation for 3 min in the presence of Laemli buffer. Ctr, control.

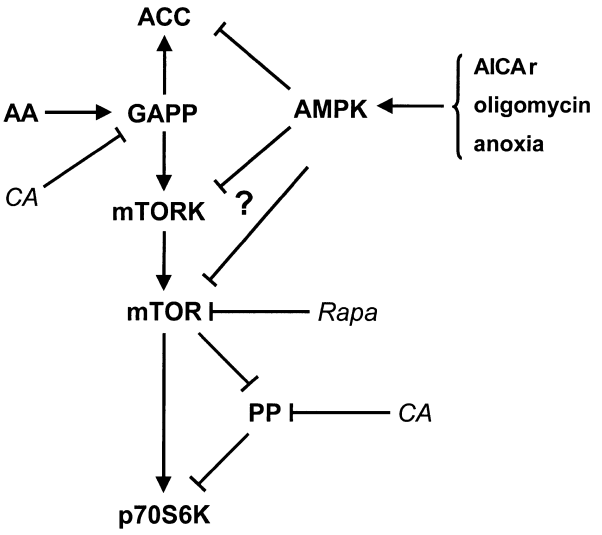

Fig. 8. Model for the control of ACC and p70S6K activity by amino acids and AMPK. AA, amino acids; CA, calyculin A; mTORK, mTOR kinase; PP, protein phosphatase; Rapa, rapamycin.

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