Regulation of metabolism by amino acid dependant signal transduction
Dubbelhuis, P.F.

Citation for published version (APA):
Dubbelhuis, P. F. (2004). Regulation of metabolism by amino acid dependant signal transduction

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Chapter 6

Studies on leucine-dependent activation of the mTOR/p70S6 kinase pathway
Involvement of chloride as second messenger?

Abstract

In this chapter the effects and underlying mechanisms of amino acid-induced cell swelling on leucine-dependent activation of the mTOR/p70S6 kinase pathways were studied. In rat hepatocytes, the ability of a complete mixture of amino acids to stimulate the mTOR/p70S6 kinase pathway was strictly dependent on the presence of small amounts of leucine. Cell swelling, either induced by hypotonicity of the medium or by concentrative Na+-dependent amino acid transport, strongly potentiated the ability of leucine alone to activate the mTOR/p70S6 kinase pathway. Cell swelling induced by glutamine, proline or asparagine resulted in part from accumulation of intracellular glutamate and aspartate. Leucine-dependent phosphorylation of p70S6 kinase was greatly stimulated when hepatocytes were exposed to lactate and NH₃. The combination of lactate and NH₃ promoted the synthesis and accumulation of intracellular glutamate and aspartate. Inhibition of intracellular conversion of glutamate into aspartate (and vice versa) did not affect leucine-dependent phosphorylation of p70S6 kinase.

Because cell swelling is accompanied by the release of KCl, resulting in a decrease in the intracellular chloride concentration, we tested the hypothesis that chloride may influence amino acid-mediated p70S6 kinase phosphorylation. Intracellular chloride was increased by blocking the Na⁺/K⁺ ATP pump with ouabain, which caused inhibition of p70S6 kinase phosphorylation and increased AMPK phosphorylation. In permeabilized hepatocytes, chloride, but not glutamate, stimulated de-phosphorylation of p70S6 kinase.

Furthermore, the hypothesis was tested that uncharged leucyl-tRNA acts as a starvation signal. For this purpose, hepatocytes were incubated with leucinol, an inhibitor of leucyl-tRNA synthetase. In the presence of a complete mixture of amino acids, leucinol inhibited activation of the mTOR/p70S6 kinase pathway. Histidinol and other amino alcohols were less or not effective. Further analysis revealed that the strong inhibitory effect of leucinol on amino acid-dependent activation of the mTOR/p70S6 kinase pathway may be explained by its ability to activate AMPK.

We conclude that in rat hepatocytes the mTOR/p70S6 kinase pathway may be controlled by uncharged leucyl-tRNA the level of which could be influenced by amino acid availability and/or intracellular chloride. In addition, chloride may affect the mTOR/p70S6 kinase pathway by stimulating de-phosphorylation of p70S6 kinase and/or decreasing mTOR activity by activation of AMPK.
1. Introduction

In many cell types leucine is the most effective amino acid in stimulating the mTOR/p70S6 kinase pathway, resulting in stimulation of protein synthesis and inhibition of autophagic protein degradation ([1] see for references and see also chapter 1). However, for the full activation of the mTOR/p70S6 kinase pathway leucine alone is not sufficient and other amino acids are also needed. The ability of some of these other amino acids to promote cell swelling may be the explanation for their synergy with leucine, as was suggested by van Sluijters et al. [1]. In line with this suggestion is the finding in rat hepatocytes that leucine-induced phosphorylation of ribosomal protein S6 by p70S6 kinase is strongly potentiated by hypotonicity-induce dd cell swelling [2,3]. So far, however, the mechanism underlying the potentiation of leucine-dependent signalling by cell swelling is not known.

Cell swelling, whether induced by hypotonicity of the extracellular medium or by concentrative Na⁺-dependent amino acid transport, causes many cell types to respond by releasing KCl in an attempt to restore their original volume, a process known as "regulatory volume decrease" (RVD) [4]. This can result in significant decreases in intracellular chloride concentration. For example, in rat hepatocytes the decrease in intracellular chloride after RVD activates glycogen synthase phosphatase, and thus increases glycogen synthesis, because chloride is an inhibitor of glycogen synthase phosphatase [5]. In certain bacteria amino acid-tRNA synthetases are inhibited by chloride ions [6]. If this also occurs in mammalian cells, protein synthesis may be accelerated when intracellular chloride falls. Indeed, in mammalian cells (T-lymphoblastoid Jurkat cells) it was shown that inhibition of amino acid-tRNA synthetases by amino alcohols interfered with amino acid-mediated p70S6 kinase activation [7]. This led to the suggestion that amino acid availability is sensed by mTOR via the degree of aminoacylated tRNA [7]. However, these data could not be confirmed in studies with freshly isolated adipocytes [8] or with CHO cells [9].

In this chapter we show that in rat hepatocytes leucine proved to be indispensable for activation of the mTOR/p70S6 kinase pathway. For full activation, however, cell swelling, was also required, regardless of whether it was caused by hypotonicity of the incubation medium, by concentrative Na⁺-dependent amino acid transport or by the intracellular generation of impermeant glutamate and/or aspartate. Among various amino alcohols, leucinol inhibits amino acid-mediated phosphorylation of p70S6 kinase most potently. Leucinol also activates AMPK, which we previously showed to antagonize mTOR-dependent signalling (chapter 4, [10]). In the present experiments evidence was obtained that chloride ions promote de-phosphorylation of p70S6 kinase. It is concluded that in rat hepatocytes free, uncharged, leucyl-tRNA and intracellular chloride ions may both act as starvation signals.
2. Materials and methods

2.1. Materials

Insulin, rapamycin, okadaic acid, AICAriboside, ouabain, amino alcohols, aminooxyacetate and the chemicals for enhanced chemi-luminescence (ECL) were from Sigma (St. Louis, MO, USA). Digitonin was from Merck (Darmstadt, Germany). LY294002 (2-(4-morpholinyl)-8-phenylchromone) was obtained from Biomol (Plymouth Meeting, PA, USA). Calpain inhibitor I (N-Acetyl-Leu-Leu-norleucinal) was from Boehringer (Mannheim, Germany). Phosphospecific anti-PKB (Thr\textsuperscript{308}), phosphospecific anti-4E-BP1(Thr\textsuperscript{37/46}), phosphospecific anti-S6 (Ser\textsuperscript{235/236}) and phosphospecific anti-AMPK(Thr\textsuperscript{172}) were from Cell Signaling Technology Inc. (Leusden, The Netherlands). Rabbit anti-p70S6 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit-HRP was from Biorad (Hercules, CA, USA). All other chemicals and enzymes were obtained from either Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, USA). Rapamycin, LY294002, calpain inhibitor I, and okadaic acid were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 0.25% (v/v). This concentration of DMSO did not affect the processes that were studied.

Male Wistar rats (about 250 g) were obtained from T.N.O., Zeist, The Netherlands, and were maintained on standard laboratory chow and water \textit{ad libitum}, until initiation of the fasting period.

2.2. Preparation of hepatocytes

Hepatocytes from male Wistar rats, fasted for 16-20 h, were isolated by collagenase perfusion as described in[11].

2.3. Hepatocyte incubation and sample preparation

Hepatocytes (5-10 mg dry weight/ml) were pre-incubated for 20 min at 37° C in minimal medium (Krebs-Henseleit bicarbonate buffer plus 10 mM Na-Hepes, pH 7.4, and 20 mM glucose) followed by incubations with components as indicated in the legends to the figures. Final incubation volume was 2 ml. The gas atmosphere was \textit{O}_2 / \textit{CO}_2 (19:1, v/v). When hepatocytes were incubated in a hypo-osmotic medium, the NaCl concentration in the Krebs-Henseleit bicarbonate buffer was decreased from 120 to 70 mM.

At the end of incubations, samples of hepatocytes were taken and prepared for metabolite analyses. For the determination of intracellular aspartate and glutamate samples were obtained by centrifugation of the cells through a layer of silicone oil (AR 200, Wacker Chemie) into a layer of HClO\textsubscript{4} (14% m/v). The acid cell extracts were neutralized to pH 7 with 2 M KOH plus 0.3 M Mops.
When hepatocytes were permeabilized with digitonin, cells were first pre-incubated for 45 min at 37°C in minimal medium supplemented with amino acids (4AA; see below) to achieve full p70S6 kinase phosphorylation. After two wash steps with sucrose medium (0.3 M sucrose, 2.5 mM K-phosphate (pH 7.4), 1 mM EGTA, 20 mM Na-HEPES (pH 7.4) en 2 mM MgCl₂) hepatocytes were incubated in the sucrose medium supplemented with components as indicated in the legend to the figure together with 30 μg/ml digitonin and p70S6 kinase de-phosphorylation was analysed.

Protein synthesis was measured with [³H]valine (0.5 μCi/ml), as in [3]. In order to ensure uniformity of the labelling of the intracellular valine pool, a concentration of 5 mM valine was used.

For amino acid determination, incubations were terminated by the addition of ice-cold sulfosalicylic acid (final concentration 6.7%). The precipitated protein was removed and the supernatant was adjusted to pH 2.2 with LiOH (3M). Amino acids were determined with an amino acid analyser (LKB, alpha plus), using a Li-citrate buffer system.

The composition of the complete mixture of all amino acids (4AA) used in the experiments was such that the concentration of each of the amino acids was present at a concentration 4 times that found in the portal vein of a fasted rat. The composition of the 1AA mixture was exactly as described in [12] except that the leucine concentration was 250 μM.

2.4. Metabolite assays

Aspartate and glutamate were measured fluorometrically as described [13].

2.5. Immunoblotting

At the end of the incubations, hepatocytes were diluted with 3 volumes of an ice-cold medium containing 145 mM NaCl and 10 mM Na⁺-Hepes, pH 7.4; cells were collected by rapid centrifugation in a microcentrifuge for 1 s. The cell pellet was lysed with Laemmli sample buffer and incubated at 95°C for 5 min.

Cell protein was determined by the method of Lowry [14] and 30 μg of protein was separated by 12.5% (w/v) SDS-PAGE. After separation, a standard Western Blotting procedure was performed and the polyvinylidene fluoride (PVDF) blots were incubated with appropriate antibodies. The proteins were visualised by enhanced chemiluminescence (ECL).

2.6. Gel retardation assay

Equivalents of 60 μg of cell protein per sample was separated by SDS-PAGE. The different phosphorylated forms of p70S6 kinase were separated by 7.5% (w/v) SDS-PAGE. After separation, a standard Western Blotting procedure was performed and the blots were incubated with an antibody against p70S6 kinase. The proteins were visualised by ECL.
3. Results

3.1. Leucine depletion inhibited p70S6 kinase phosphorylation

As indicated in the introduction to this chapter, leucine is the most potent amino acid in stimulating signalling in hepatocytes. We wanted to know whether deletion of leucine from a complete mixture of all amino acids would affect signalling (Fig. 1A). It had previously been demonstrated that omission of valine from such a mixture did not affect signalling [3]. In the experiment of Fig. 1A we used histidine depletion as another control because the degree of charging of histidine-tRNA with histidine had been reported to be extremely important in the control of protein turnover in CHO cells [15]. In Fig. 1A, hepatocytes were incubated with 4AA, in the presence or absence of leucine and/or histidine. In one series of incubations we also added calpain inhibitor I (CI) in order to prevent the production of leucine and histidine by proteolysis [12] (cf. also chapter 3).

Phosphorylation of p70 S6 kinase in the presence of 4AA was not affected by CI (Fig. 1A). Depletion of histidine did not affect 4AA-dependent p70 S6 kinase phosphorylation, neither in the absence or presence of CI. By contrast, depletion of leucine, whether or not combined with histidine depletion, prevented 4AA-induced p70 S6 kinase phosphorylation although it took 90 min of incubation before the effect could be observed (Fig. 1A). Apparently, the depletion of leucine by ongoing protein synthesis is slow under these conditions. Addition of 50 μM leucine to the leucine-depleted amino acid mixture was sufficient for full restoration of p70 S6 kinase phosphorylation (Fig. 1B).

By contrast, in the presence of CI, leucine depletion inhibited p70 S6 kinase after 45 min but the inhibition, unexpectedly, disappeared after 90 min of incubation (Fig. 1A). In order to find an explanation for this surprising result we considered the possibility that CI (N-acetyl-leu-leu-norleucinal) might have been degraded to leucine in the course of the incubations. If true, the data are the result of two opposing effects: inhibition by CI of leucine production from proteins, but production of leucine from degradation of CI. This, indeed, turned out to be the case (Fig. 1C). Production of valine and isoleucine from breakdown of intracellular proteins was 90% inhibited by CI, but production of leucine strongly increased. The relative occurrence of valine, isoleucine and leucine in protein from hepatocytes is exactly known [16,17] and was used to calculate the production of leucine from protein degradation assuming that CI was not degraded. Degradation of CI resulted in an increase in leucine production (15.27 ± 1.66 μmol/gdw/h) whereas it should have been much lower when CI would have been stable during the incubation (2.43 μmol/gdw/h, calculated).
Fig. 1. Leucine depletion inhibited 4AA-induced p70S6 kinase phosphorylation. A, hepatocytes were pre-incubated for 20 min in minimal medium followed by 45 and 90 min of incubation with the indicated addition or omission. B, hepatocytes were pre-incubated for 20 min in minimal medium followed by 90 min of incubation with the indicated addition or omission. Lysates were separated by SDS-PAGE, immunoblotted with anti-p70S6 kinase antibody, and visualised by ECL as described under Materials and Methods. The data are from experiments carried out with two different hepatocyte preparations. C, hepatocytes were incubated for 90 min in minimal medium in the presence or absence of CI. At the end of the incubation period, the amount of branched-chain amino acids was determined as described under Materials and Methods. The data in C are the means (± S.E.) of experiments carried out with seven different hepatocyte preparations. Abbreviations: Ctl, control (minimal medium); CI, calpain inhibitor I at 150 μM; -His, without histidine; -Leu, without leucine; -Leu-His, without leucine and histidine; +Leu, leucine at the concentration indicated in μM; Rapa, rapamycin at 100 nM; Val, valine; Ileu, isoleucine; Leu, leucine; Exp, expected leucine levels without CI degradation.

3.2 Leucine-dependent activation of the mTOR pathway and the effect of amino acid induced cell swelling

Hypo-osmotically induced cell swelling potentiates the ability of leucine to stimulate signalling [3]. In order to test the possibility that amino acid-induced cell swelling can also promote leucine-dependent signalling, hepatocytes were incubated in the presence of either
Leucine-dependent signalling

glutamine, proline or asparagine or of a combination of lactate and ammonia, in the absence and presence of leucine. In the presence of glutamine, proline or asparagine, cell swelling not only occurs because of $\text{Na}^\text{+}$-dependent concentrative transport across the plasma membrane [18] but also because of the generation of (impermeant) intracellular glutamate and aspartate in the course of their catabolism [19]. In the presence of lactate and ammonia, glutamate and aspartate are synthesized via glutamate dehydrogenase and aspartate aminotransferase, respectively [20].

In the experiment of Fig. 2A, phosphorylation was measured of both p70S6 kinase and of 4E-BP1, another downstream target of mTOR. Incubations with rapamycin as a negative control were also carried out. Interestingly, leucine could only stimulate the phosphorylation of p70S6 kinase when glutamine, proline or asparagine were present (Fig. 2A). Cell swelling, induced by decreasing the $\text{NaCl}$ concentration of the medium, also stimulated leucine-mediated activation of the mTOR pathway (cf. [3]). Hepatocytes incubated with glutamine, proline or asparagine had increased levels of intracellular glutamate and aspartate (Fig. 2B). The same phenomenon was observed when hepatocytes were incubated with lactate combined with ammonium chloride (Fig. 2B), under which condition leucine-dependent activation of the mTOR pathway was also highly stimulated (Fig. 2A). Incubation of hepatocytes with aminooxyacetate, a transaminase inhibitor, did not influence the phosphorylation of p70S6 kinase induced by leucine in the presence of glutamine, proline or asparagine, although it did influence the intracellular concentrations of glutamate and aspartate (Fig. 2A,B). In the presence of lactate and ammonia, aminooxyacetate prevented intracellular production of glutamate and aspartate (in agreement with Meijer et al. [20]) and stimulation of p70S6 kinase by leucine was also lost under these conditions (Fig. 2A,B). The reason for the inhibition of lactate metabolism by aminooxyacetate is that oxaloacetate efflux from the mitochondrion occurs as aspartate [20].

3.3. Effect of ouabain on p70S6 kinase and AMPK

To assess whether a decrease in intracellular chloride concentrations, due to amino acid-induced cell swelling (cf. introduction) could be responsible for the activation of leucine-mediated phosphorylation of p70S6 kinase, hepatocytes were incubated with ouabain. Ouabain is an inhibitor of the $\text{Na}^+$/K+ ATPase and causes a rise in the intracellular chloride concentration [21]. Ouabain inhibited amino acid-induced phosphorylation of p70S6 kinase and of S6 (Fig. 3A,B). In hepatocytes incubated with ouabain, the phosphorylation of AMPK at Thr$^{172}$ was stimulated. Ouabain-induced phosphorylation of AMPK at Thr$^{172}$ was attenuated in the presence of 4AA but was unaffected by the amino acid combination leucine, tyrosine and phenylalanine (Fig. 3C). Because AMPK activation strongly inhibits protein synthesis [10] (chapter 4), the effect of ouabain on protein synthesis was also studied. Ouabain strongly inhibited this process (Fig. 3D).
Fig. 2. Conditions that result in cell swelling enhance leucine-dependent activation of the mTOR/p70S6 kinase pathway. A, hepatocytes were pre-incubated for 20 min in minimal medium followed by 30 min of incubation with the indicated additions. Lysates were separated by SDS-PAGE, immunoblotted with anti-p70S6 kinase and anti-phospho-4E-BP1(Thr^{37/46})-antibody, and visualised by ECL as described under Materials and Methods. B, hepatocytes were pre-incubated for 20 min in minimal medium followed by 30 min of incubation with the indicated additions. Intracellular glutamate and aspartate levels were measured as described under Materials and Methods. The data are from experiments carried out with two different hepatocyte preparations. Abbreviations: Ctl, control (minimal medium); Leu, leucine at 2 mM; Gln, glutamine at 10 mM; Hypo, hypotonicity 70 mM NaCl; Pro, proline at 10 mM; Lac, lactate at 15 mM; NH_{4}Cl, ammonium chloride at 15 mM; Rapa, rapamycin at 100 nM; AOA, aminooxyacetate at 1 mM; Asn, asparagine at 10 mM; gdw, gram dry weight.
Fig. 3. Ouabain inhibited amino acid-dependent activation of the mTOR/p70S6 kinase pathway and activated AMPK. A, B and C, hepatocytes were pre-incubated for 20 min in minimal medium followed by 60 min of incubation with the indicated additions. Lysates were separated by SDS-PAGE, immunoblotted with anti-p70S6 kinase, anti-phospho-S6(Ser^{235/236}) and anti-phospho-AMPK(Thr^{172})-antibodies, and visualised by ECL as described under Materials and Methods. D, hepatocytes were incubated for 1 h in the presence of a complete amino acid mixture plus [H]valine; the concentrations of valine was 5 mM under all conditions. Where present, ouabain was added at the indicated concentrations in mM. Values in D represent the amount of [H]valine incorporated into protein. Abbreviations: Ctl, control (minimal medium); Oua, ouabain at the concentration indicated in mM; PTL, a mixture of 200 μM phenylalanine, 300 μM tyrosine and 1 mM leucine; Aicar, AICArriboside at 250 μM; Rapa, rapamycin at 100 nM; gdw, gram dry weight.
3.4. Chloride enhanced de-phosphorylation of p70S6 kinase

Because the intracellular chloride concentration can only be manipulated in an indirect manner we decided to study its effect in permeabilized hepatocytes and to test whether or not chloride could affect de-phosphorylation of phosphorylated p70S6 kinase. After prior incubation of the cells with 4AA to allow phosphorylation of p70S6 kinase to occur, the cells were permeabilized with digitonin and further incubated with various concentrations of KCl or K-glutamate. It was found that KCl enhanced de-phosphorylation of p70S6 kinase in a concentration dependent manner, while K-glutamate had no effect (Fig. 4A). K-aspartate was also ineffective (data not shown). The acceleration by KCl of p70S6 kinase de-phosphorylation could be inhibited by the PP2A inhibitor okadaic acid (Fig. 4B).

![Image of experiment showing chloride stimulated p70S6 kinase de-phosphorylation in permeabilized hepatocytes.](image)

**Fig. 4. Chloride stimulated p70S6 kinase de-phosphorylation in permeabilized hepatocytes.** A and B, hepatocytes were pre-incubated for 60 min in minimal medium supplemented with 4AA, followed by 5 min of incubation in a sucrose medium supplemented with digitonin (30 μg/ml), as described in Material and Methods, and with the indicated addition. Lysates were separated by SDS-PAGE, immunoblotted with anti-p70S6 kinase-antibody, and visualised by ECL as described under Materials and Methods. Abbreviations: KCl, potassium chloride at the concentration indicated in mM; Glu, potassium glutamate at the concentration indicated in mM; Oka, okadaic acid at 20 nM.

3.5. Amino alcohols interfere with amino acid induced p70S6 kinase phosphorylation.

It has been proposed that amino acid depletion may be sensed by mTOR via the levels of uncharged, free tRNA [7]. Since amino alcohols are competitive inhibitors of tRNA synthetases and decrease the levels of aminoacylated tRNA [22,23], rat hepatocytes were incubated with various amino alcohols and their effect on amino acid/insulin-mediated p70S6 kinase phosphorylation was studied. As shown in Fig 5A, addition of 5 and 10 mM leucinol or of 10 mM histidinol inhibited amino acid/insulin-mediated p70S6 kinase phosphorylation. Alaninol and serinol did not inhibit amino acid/insulin-mediated p70S6 kinase phosphorylation. Leucinol was the only amino alcohol found to stimulate phosphorylation of AMPK at Thr172 (Fig. 5B). Phosphorylation of p70S6 kinase in the presence of amino acids and insulin was completed in 30 min and remained constant thereafter (Fig. 5C). At all time points measured, p70S6 kinase
phosphorylation was inhibited by 10 mM leucinol. By contrast 10 mM histidinol only retarded the onset of p70S6 kinase phosphorylation (Fig. 5C). Incubation with leucinol, but not with histidinol, stimulated phosphorylation of AMPK at Thr\(^{172}\) (Fig. 5D). The AMP analogue AICAR stimulated the phosphorylation of AMPK at Thr\(^{172}\) and also inhibited amino acid/insulin mediated p70S6 kinase phosphorylation but not as strong as leucinol (Fig. 5C,D).

**Fig. 5. Effect of amino alcohols on p70S6 kinase phosphorylation.** A and B, hepatocytes were pre-incubated for 20 min in minimal medium followed by 60 min of incubation with the indicated additions. C and D, hepatocytes were pre-incubated for 20 min in minimal medium followed by 15, 30 and 45 min of incubation with the indicated additions. Lysates were separated by SDS-PAGE, immunoblotted with anti-p70S6 kinase and anti-phospho-AMPK(Thr\(^{172}\))-antibodies and visualised by ECL as described under Materials and Methods. E, hepatocytes were incubated for 1 h in the presence of a complete amino acid mixture plus [H]valine; the concentration of valine was 5 mM under all conditions. Amino alcohols, if present, were added at the indicated concentrations in mM. The basal rate of protein synthesis under control conditions (100%) was equal to 0.98 \(\mu\)mol valine incorporated into protein per g of dry mass of cells after 60 min of incubation. Abbreviations: Ctl, control (minimal medium); Ins, insulin at 10\(^{-7}\) M; Sol, serine at the concentration indicated in mM; Hol, histidinol at the concentration indicated in mM; Lol, leucinol at the concentration indicated in mM; Aol, alaninol at the concentration indicated in mM; Aicar, AICAriboside at 250 \(\mu\)M; Rapa, rapamycin at 100 nM.
Activation of the mTOR/p70S6 kinase pathway by amino acids results in the stimulation of protein synthesis. We wanted to know if inhibition of p70S6 kinase phosphorylation by amino alcohols also results in inhibition of protein synthesis. In hepatocytes incubated with a complete amino acid mixture, leucinol or histidinol strongly inhibited protein synthesis, in agreement with [24], whereas alaninol or serinol only had a mild effect on protein synthesis (Fig. 5E).

4. Discussion

Amino acid-dependent activation of the mTOR/p70S6 kinase pathway has been studied in many different cell types (see, e.g., Ref. [1]) and much information is now available about the mechanism of mTOR-dependent activation of p70S6 kinase by amino acids [1,25]. In most of these studies, including our own, leucine was the most effective amino acid. The fact that leucine is able to stimulate signalling in the presence of the transaminase inhibitor AOA (Fig. 2A) strongly suggests that it is leucine itself, rather than a catabolite, is responsible for the stimulation of signalling.

How leucine levels are sensed by the cell and activate the mTOR/p70S6 kinase pathway is still unclear, however, one possibility, suggested by Liboshi et al. [7] is that levels of tRNA-charging control the mTOR/p70S6 kinase pathway (Fig. 6). This mechanism is based on observations in yeast showing that upon amino acid starvation uncharged tRNA binds with high affinity to the protein kinase GCN2, because the carboxyterminus of GCN2 structurally resembles histidinyl-tRNA synthetase and other aminoacyl-tRNA synthetases. Activated GCN2 phosphorylates eIF2α and inhibits protein synthesis [26]. Although mammalian homologues of GCN2 exist [27], the mechanism regulating their activity is not yet known. The specific stimulation of the mTOR/p70S6 kinase pathway by leucine may be explained by the high frequency of leucine in proteins and by the existence of multiple leucyl-tRNA synthetases due to the 6-fold codon degeneration [28]. Studies performed with purified aminoacyl-tRNA synthetases from certain bacteria showed that chloride can inhibit these enzymes by weakening the interaction between the aminoacyl-tRNA synthetase and the tRNA [6]. Whether or not chloride can also inhibit aminoacylation of tRNA in mammalian cells is not known. If true, it would satisfactorily explain the ability of cell swelling to potentiate leucine-dependent signalling.

In rat hepatocytes, leucine-induced activation of the mTOR/p70S6 kinase pathway depends on the presence of specific amino acids such as glutamine, proline and asparagine that induce cell swelling and increase accumulation of intracellular glutamate and aspartate [2,3] (Fig. 2A,B). Control experiments carried out in our laboratory (data not shown) indicated that cell swelling does not affect leucine transport across the plasma membrane. According to Krause et al. [29], amino acid-induced accumulation of glutamate can stimulate a glutamate-dependent protein phosphatase (GAPP) located upstream of mTOR (Fig. 6). If, indeed, activation of GAPP is needed for mTOR-mediated activation of p70S6 kinase by leucine, GAPP is probably not only stimulated by glutamate but also by aspartate because leucine-dependent phosphorylation of
p70S6 kinase was enhanced when intracellular levels of aspartate were high and intracellular levels of glutamate were low (Fig. 2A,B). Because the effect of leucine on p70S6 kinase phosphorylation is also potentiated by hypo-osmotic cell swelling (Fig. 2A), it cannot be ruled out, however, that GAPPP is activated by the fall in chloride due to RVD.

Fig. 6 Regulation of the mTOR/p70S6 kinase pathway by amino acids and the possible involvement of chloride. Leucine-dependent activation of the mTOR/p70S6 kinase pathway is potentiated by cell swelling. This can be explained by assuming that chloride ions inhibit GAPPP and/or AA-tRNA synthetase and that they stimulate AMPK, resembling starvation conditions. These effects of chloride are relieved when chloride levels fall due to RVD. Abbreviations: Na\(^+\)-AA, specific amino acids, such as glutamine, proline and asparagine, which are transported into the cell in a Na\(^+\)-dependent manner; RVD, regulatory volume decrease; Cl\(^-\), chloride; Leu, leucine; GAPPP, glutamate-activated protein phosphatase; Glu/Asp, glutamate and aspartate; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase; p70S6K, p70S6 kinase; S6-P, phosphorylated ribosomal protein S6; AA-tRNA, aminoacyl-tRNA; AA, amino acids; PP2A, protein phosphatase 2A.

In addition to GAPPP, there may be other protein phosphatases downstream of mTOR that are involved in p70S6 kinase regulation [1]. In permeabilized hepatocytes chloride, but not glutamate, enhanced de-phosphorylation of amino acid-dependent phosphorylated p70S6 kinase (Fig. 4A), possibly by activation of the protein phosphatase 2A (PP2A) because the process was sensitive to okadaic acid (Fig. 4B). It must be stressed, however, that the permeabilized hepatocyte is an artificial model in which protein kinases are no longer active. In intact
hepatocytes okadaic acid had no effect on p70S6 kinase phosphorylation (see chapter 5). Perhaps the local concentration of okadaic acid in intact hepatocytes is lower than in permeabilized cells.

The involvement of chloride in amino acid signalling was further emphasized by the experiment with ouabain (Fig. 3), the inhibitor of the Na$^+$/K$^+$ ATP pump which increases the intracellular levels of chloride [21]. Ouabain inhibited amino acid-mediated activation of the mTOR/p70S6 kinase pathway and amino acid-induced stimulation of protein synthesis; ouabain also stimulated the phosphorylation of AMPK on Thr$^{172}$ (Fig. 3), which parallels its activity [30]. The activation of AMPK phosphorylation by ouabain was not influenced by the combination of phenylalanine, tyrosine and leucine (PTL), a mixture that does not induce cell swelling, whereas it was attenuated by increasing concentrations of the complete mixture of all amino acids. This suggests that chloride levels are important in the regulation of AMPK activity. The mechanism responsible for the inhibition of p70S6 kinase phosphorylation and inhibition of protein synthesis by ouabain is at least partly related to the activation of AMPK [10] (see also chapter 4). It is also possible that ouabain interferes with amino acid transport across the plasma membrane and by this means inhibits p70S6 kinase phosphorylation and protein synthesis.

Amino acid-dependent activation and phosphorylation of p70S6 kinase was also inhibited by amino alcohols [7] (Fig 5A,C). Because AMPK negatively controls the mTOR/p70S6 kinase pathway [10,29,31,32], the activation of AMPK by leucinol, but not histidinol, may explain the strong inhibitory effect of leucinol on amino acid-dependent phosphorylation of p70S6 kinase compared to that of histidinol. The inhibition of p70S6 kinase phosphorylation by histidinol is probably due to an increase in uncharged histidinyl-tRNA levels, as suggested by liboshi et al. [7]. Depletion of uncharged leucyl-tRNA is probably also involved in leucinol-dependent inhibition of p70S6 kinase phosphorylation. The simultaneous stimulation of AMPK by leucinol may account for the stronger inhibitory effect of leucinol than that of histidinol and also of AICAR (Fig. 5).

In conclusion, on the basis of these data conclude that the minimal requirement for activation of the mTOR/p70S6 kinase pathway is the presence of leucine and an increase in cell volume, either induced by hypo-osmolarity of the extracellular environment or by intracellular accumulation of glutamate and aspartate. The mechanism responsible for the potentiation of the leucine-dependent activation of the mTOR/p70S6 kinase pathway by cell swelling may involve a decrease in intracellular chloride due to RVD, which subsequently stimulates the activity of GAPP and/or aminoacyl tRNA synthetases and keeps the activity of AMPK low (Fig. 6).
References


Chapter 6


