RESEARCH COMMUNICATION
Regulation of protein-synthesis elongation-factor-2 kinase by cAMP in adipocytes

Tricia A. DIGGLE*, Nicholas T. REDPATH*, Kate J. HEESOM† and Richard M. DENTON†

*Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K., and †Department of Biochemistry, School of Medical Sciences, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

Treatment of primary rat epididymal adipocytes or 3T3-L1 adipocytes with various agents which increase cAMP led to the phosphorylation of eukaryotic translation elongation factor-2 (eEF-2). The increase in eEF-2 phosphorylation was a consequence of the activation of eEF-2 kinase (eEF-2K), which is a Ca²⁺/calmodulin-dependent kinase. eEF-2K was shown to be essentially inactive at less than 0.1 μM free Ca²⁺ when measured in cell-free extracts. Treatment of adipocytes with isoprotrenol induced Ca²⁺-independent eEF-2K activity, and an 8–10-fold activation of eEF-2K was observed at Ca²⁺ concentrations of less than 0.1 μM. Increased cAMP in 3T3-L1 adipocytes led to the inhibition of total protein synthesis and decreased the rate of polypeptide-chain elongation. We also show that the phosphorylation of eEF-2 and the activity of eEF-2K are insulin-regulated in adipocytes. These results demonstrate a novel mechanism for the control of protein synthesis by hormones which act by increasing cytoplasmic cAMP.

Key words: insulin, protein kinase A, protein phosphorylation, translation.

INTRODUCTION
Eukaryotic translation elongation factor-2 (eEF-2) mediates the translocation step in peptide-chain elongation by promoting transfer of peptidyl-tRNA from the A- to the P-site of the ribosome and thereby moving the mRNA relative to the ribosome, bringing the next codon into alignment with the A-site (see [1]). eEF-2 is a monomeric 100 kDa protein that is inactivated by phosphorylation on Thr²⁴⁴[2–7]. eEF-2 is phosphorylated by a highly specific kinase, eEF-2 kinase (eEF-2K). eEF-2K is a Ca²⁺/calmodulin (CaM)-dependent kinase (originally termed ‘CaM kinase III’) that has recently been cloned [8–9].

The acute regulation of mRNA translation in animal cells in response to growth factors is most commonly mediated via changes in the phosphorylation of a number of initiation and/or elongation factors. Several reviews dealing with the role of protein phosphorylation in the control of translation initiation have been published [10–14]. More recently, a role for the regulation of protein synthesis at the elongation phase via eEF-2 phosphorylation has evolved, and we have shown that the phosphorylation of eEF-2 is regulated by insulin [15]. Insulin treatment of quiescent Chinese-hamster ovary cells expressing human insulin receptor (CHO.T cells) led to a 50% decrease in eEF-2K activity and the dephosphorylation of eEF-2 at 0.1 μM free Ca²⁺.

Major questions exist regarding the role of cAMP in the control of protein synthesis. A number of reports have implicated cAMP/cAMP-dependent protein kinase (PKA) as a negative regulator of cell proliferation and protein synthesis in various tissues [16–18]. The mechanisms involved in the short-term regulation of protein synthesis by cAMP are not fully understood. Some attention has been focused on the regulation of components involved in the initiation of mRNA translation, namely p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor-4E (eIF-4E) binding proteins (eIF-4E-BPs). The relevance of these components to the control of translation by cAMP is, however, still far from clear (see below for further discussion).

Here we describe a novel mechanism for the control of mRNA translation in response to elevated cAMP in adipocytes. This involves the inhibition of mRNA translation at the elongation stage through the increased phosphorylation of eEF-2. This is brought about by the induction of Ca²⁺-independent eEF-2K activity, which is likely to be mediated by the phosphorylation of eEF-2K by PKA. In confirmation of previous findings with CHO.T cells, we also show that, in adipocytes, insulin inhibits eEF-2K, leading to the dephosphorylation of eEF-2.

EXPERIMENTAL
All chemicals and biochemicals were obtained as previously described [8,19]. eEF-2 was purified from rabbit reticulocytes as described in [20]. Male Wistar rats were fed ad libitum up until the time of killing on a stock laboratory diet (CRM; Bioshore, Lavender Hill, Cambridge, Cambs., U.K.).

Cell culture
3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and were induced to differentiate by incubation with DMEM containing 10% (v/v) foetal-calf serum (Gibco), 0.2 μM insulin, 0.25 μM dexamethasone and 0.11 mg/ml isobutylmethylxanthine for 2 days. The cells were then...
incubated for 2 days with DMEM containing 10% foetal-calf serum and 0.2 \( \mu \)M insulin. Cells were then incubated for another 4 days with medium containing 10%, foetal-calf serum, which was replenished after 2 days. Adipocytes were then serum-starved overnight prior to experimentation.

**Preparation of cell extracts**

Fat cells were prepared from rat epididymal fat pads with the modifications described in [23]. Cells (250–300 mg dry cell weight) were incubated in gassed Krebs–Henseleit buffer (2 ml) containing 10 mM Heps, 2 mg/ml glucose and 1%, BSA with further additions as indicated in the Figure legends. Cells were extracted by vortex-mixing in glass in 50 mM \( \beta \)-glycerophosphate, pH 7.4, containing 1.5 mM EGTA, 0.5 mM Na\(_2\)VO\(_4\), 1 mM dithiothreitol (DTT), 1 \( \mu \)M microcystin and the proteinase inhibitors pepstatin, antipain and leupeptin (1 \( \mu \)g/ml each), 2 mM benzamidine and 0.1 mM PMSF. 3T3-L1 adipocytes were extracted with buffer containing 50 mM sodium \( \beta \)-glycerophosphate, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM DTT, 2 \( \mu \)g/ml pepstatin, leupeptin and antipain, 1 mM PMSF, 0.5 mM Na\(_2\)VO\(_4\), 1 \( \mu \)M microcystin, 1% Triton X-100 and 10% (v/v) glycerol.

**Assay of eEF-2 kinase**

eEF-2 kinase activity in 5 \( \mu l \) of primary-adipocyte-cell extract or 5 \( \mu g \) of 3T3-L1 adipocyte lysate was measured in a final volume of 30 \( \mu l \) in buffer [15] containing 50 pmol of eEF-2 in the absence or presence of 0.5 \( \mu g/ml \) CaM and 0.67 mM CaCl\(_2\). The assay buffer contained 2 mM N-hydroxyethylhydroxylethylaminetriacetic acid (HEDTA) and 0.4 mM EGTA to act as a Ca\(^{2+}\) buffer [22]. Addition of 0.67 mM CaCl\(_2\) to this buffer results in a free Ca\(^{2+}\) concentration of 5.6 \( \mu M \). To measure Ca\(^{2+}\)-independent activity, Ca\(^{2+}\) was omitted from the incubations. After 10 min at 30\(^\circ\)C, incubations were stopped by boiling in the presence of SDS/PAGE sample buffer. Samples were then separated by separation on a 10%-(w/v)-polyacrylamide gel followed by autoradiography. Incorporation of \(^{32}P\) into eEF-2 was assessed using a Molecular Dynamics computing densitometer equipped with ImageQuant software.

**Determination of changes in eEF-2 phosphorylation**

Adipocyte-cell extract (10 \( \mu l \)) was separated by isoelectric focusing [23] and eEF-2 was revealed by Western blotting using enhanced chemiluminescence (ECL\(^{\text{TM}}\); Amersham International). The relative levels of the phosphorylated and unphosphorylated forms of eEF-2 were assessed by densitometry.

**Protein synthesis and ribosomal transit times**

Rates of protein synthesis were measured by incubation of serum-starved 3T3-L1 adipocytes with insulin or agents, as indicated in the Figure legends, for 15 min prior to the addition of \(^{35}S\) methionine (5 \( \mu Ci/ml \)) for 30 min. Cells were washed with PBS, then lysed with 0.5% SDS, 0.1 M NaOH for 30 min at 4\(^\circ\)C. Protein was then precipitated with 10% (v/v) trichloroacetic acid before collection of the precipitates on GF filters and liquid-scintillation counting of radioactivity.

Transit times were carried out in 3T3-L1 adipocytes grown on 3.5 cm plates as described for CHO.T cells [15]. Serum-starved cells were incubated as indicated in the Figure legends. \(^{35}S\) Methionine (25 \( \mu Ci/ml \)) was then added and six plates were sampled between 10 and 30 min thereafter. Plates were washed with PBS and lysed in 1 ml of buffer as described in [15] except that the buffer contained 2 mM methionine and 1% detergents. Extracts were then processed as described in [15], except that extracts were not layered on to sucrose-containing buffer for ribosome sedimentation and the centrifugation was carried out for 1.25 h at 120000 \( g \).

**RESULTS**

**eAMP activates eEF-2K in adipocytes**

We have used two adipocytic cell types, namely primary rat epididymal adipocytes and 3T3-L1 adipocytes, to investigate the role of eEF-2 phosphorylation in the control of mRNA translation in response to agents which increase cytoplasmic cAMP. Treatment of rat adipocytes with the \( \beta \)-adrenergic agonist isoproterenol or 8-(4-chlorophenylthio)adenosine 3':5'-cyclic monophosphate (CPT-cAMP) led to a modest 1.2–1.4-fold increase in eEF-2K activity when it was measured in the presence of a saturating concentration of Ca\(^{2+}\) (5.6 \( \mu M \)) (Figure 1A). When measured in the absence of free Ca\(^{2+}\), eEF-2K from control adipocytes displayed some Ca\(^{2+}\)-independent activity, amounting to 5.4 \pm 1.6\% (mean \( \pm \) S.E.M., \( n = 5 \)) of the activity in the presence of Ca\(^{2+}\). This Ca\(^{2+}\)-independent activity was stimulated approx. 10-fold in adipocytes incubated with isoproterenol or CPT-cAMP (Figure 1B). Thus the Ca\(^{2+}\)-independent activity in fat cells stimulated with isoproterenol or CPT-cAMP represented 40–50% of the maximal activity. This is in agreement with the degree of Ca\(^{2+}\)-independence observed when eEF-2K was phosphorylated by PKA in vitro [24]. The effect of isoproterenol was rapid, maximal stimulation of eEF-2K being achieved within 1 min (results not shown). In 3T3-L1 adipocytes, similar results were observed using isoproterenol and CPT-cAMP as well as the adenylyl cyclase activator forskolin and the phosphodiesterase inhibitor theophylline (Figures 1C and 1D). The stimulation of Ca\(^{2+}\)-independent eEF-2K activity by these agents in 3T3-L1 adipocytes was lower, however, being approx. 3-fold. Treatment of adipocytes with insulin led to a 50% decrease in Ca\(^{2+}\)-dependent eEF-2K activity (Figures 1A and 1C), in agreement with a previous study using CHO.T cells [15]. Insulin also decreased the Ca\(^{2+}\)-independent eEF-2K activity by about 50% in rat adipocytes (Figure 1B).

**Regulation of eEF-2K by Ca\(^{2+}\)**

The Ca\(^{2+}\)-dependency of eEF-2K in adipocytes treated with insulin and isoproterenol was studied in more detail by assaying eEF-2K across a range of Ca\(^{2+}\) concentrations (Figure 2). The use of a buffer system containing HEDTA, EGTA and various concentrations of added Ca\(^{2+}\) allows for the tight control of free Ca\(^{2+}\) concentrations. Knowing the dissociation constants for the various metal-binding ligands in the assay, free Ca\(^{2+}\) concentrations can be accurately determined using an appropriate computer program (METLIG) [22]. Adipocytes were treated with insulin or isoproterenol as described above, and the extracts assayed for eEF-2K activity in the presence of the indicated concentrations of free Ca\(^{2+}\). eEF-2K from untreated adipocytes was inactive below about 0.1 \( \mu M \) free Ca\(^{2+}\) and was optimally activated at 1 \( \mu M \). Treatment of the adipocytes with isoproterenol, as seen in Figure 1, caused the activation of eEF-2K above the control level at all Ca\(^{2+}\) concentrations, although it did not significantly alter the half-maximal concentration (\( K_{\text{app}} \)) of Ca\(^{2+}\), which was 0.2 \( \mu M \) in untreated cells and 0.16 \( \mu M \) in isoproterenol-treated cells. Insulin appears to cause inhibition at all Ca\(^{2+}\) concentrations tested, again without altering the \( K_{\text{app}} \) for Ca\(^{2+}\) (0.25 \( \mu M \) in insulin-treated cells).
Control of elongation-factor-2 kinase by cAMP

Figure 1 Modulation of eEF-2K activity in fat cells by insulin and cAMP

Rat adipocytes (A, B) were left untreated (control) or treated with insulin (83 nM), isoproterenol (1 μM) or CPT-cAMP (2.5 mM). 3T3-L1 adipocytes (C, D) were incubated with insulin (20 nM), isoproterenol (1 μM), CPT-cAMP (1 mM), forskolin (25 μM) or theophylline (5 mM). eEF-2K activity in cell extracts was measured for 10 min in the presence (A, C) or 30 min in the absence (B, D) of Ca²⁺/CaM as described in the Experimental section. The control values in (A) and (C) are the activities of eEF-2K from untreated cells measured in the presence of Ca²⁺/CaM. In (B) and (D) the control eEF-2K activities are those from untreated cells measured in the absence of Ca²⁺/CaM. The Ca²⁺-independent activity of eEF-2K in control primary adipocytes (B) was about 5% of the maximal activity in control cells (A). Therefore, since the Ca²⁺-independent activity seen in the presence of isoproterenol or CPT-cAMP was increased 8–10-fold (B), this represents 40–50% of the maximal activity in control cells (A). The bars indicate the S.E.M. Assays were performed at least four times.

Figure 2 Ca²⁺-dependence of eEF-2K in adipocytes

Rat adipocytes were treated with insulin (●), isoproterenol (■) or vehicle (▲) as described in the legend to Figure 1. eEF-2K activity in cell extracts was measured across a range of free Ca²⁺ concentrations as indicated, in the presence of 0.5 μg/ml CaM. The control was taken as the maximum (100%) eEF-2K activity in cells treated with vehicle and measured at the highest free Ca²⁺ concentration.

Effects of insulin and cAMP on eEF-2 phosphorylation

Changes in the phosphorylation of eEF-2 in response to insulin, isoproterenol and CPT-cAMP were determined by isoelectric focusing of 3T3-L1 adipocyte extracts (Figure 3). In the experiment shown, insulin treatment of 3T3-L1 adipocytes brought about a 3-fold decrease in eEF-2 phosphorylation, while treatment with various agents which raised cAMP brought about a 2–3-fold increase. The experiment shown was typical of around

Figure 3 Effect of insulin and cAMP on eEF-2 phosphorylation in rat adipocytes

The phosphorylation state of eEF-2 in 3T3-L1 adipocytes was determined by polyacrylamide-gel isoelectric focusing of adipocyte extracts (5 μg) followed by transfer on to PVDF membrane, immunodetection of eEF-2 and revelation by ECL. Lane 1, control; lane 2, insulin; lane 3, isoproterenol, lane 4, CPT-cAMP; lane 5, theophylline; lane 6, forskolin. The concentrations of these were as given in the legend to Figure 1. The labelled arrows indicate the positions of migration of phosphorylated eEF-2 (eEF-2-P) and unphosphorylated eEF-2. The values indicate the percentage phosphorylation of eEF-2.
3T3-L1 adipocytes, although the effects of forskolin, CPT-cAMP largely similar to those obtained by Lin and Lawrence [25] in protein synthesis was linear for at least 30 min after the addition and theophylline all inhibited total protein synthesis by 50–60%.

Figure 4(A) illustrates that isoproterenol, CPT-cAMP, forskolin and agents which raise cAMP on eEF-2 phosphorylation and eEF-2K activity correlated with their effects on protein synthesis. In serum-starved 3T3-L1 adipocytes the average transit time was 3.5 ± 0.6 min (n = 4). The average transit times were increased by isoproterenol, CPT-cAMP, forskolin and theophylline by 300–400% (Figure 4B), indicating a corresponding decrease in the rate of peptide-chain elongation.

DISCUSSION

We were prompted to carry out the above study by the finding that PKA could phosphorylate purified eEF-2K in vitro and induce Ca<sup>2+</sup>-independent activity [24]. This suggested that the elevation of cAMP in cells could inhibit translation via increased phosphorylation of eEF-2. The above results indicate that this is indeed the case. This therefore represents a mechanism for the activation of eEF-2K in resting cells wherein the Ca<sup>2+</sup> concentration would only support a low level of eEF-2K activity (see Figure 2). The cytoplasmic Ca<sup>2+</sup> concentration in primary adipocytes has been reported to about 0.16 μM [32]. This would be consistent with the results presented in Figure 2 since, at this concentration, eEF-2K would be partially active (20%, of maximal activity). This would account for the basal level of eEF-2 phosphorylation observed in control cells (Figure 3) and would still allow for substantial activation (3-fold) by isoproterenol. This closely correlates with the increase in eEF-2 phosphorylation in response to isoproterenol (Figure 3).

Although this study does not directly demonstrate that PKA phosphorylates eEF-2K in vivo in response to elevated cAMP, the results strongly imply this, given the similarity of the effects of in vitro PKA phosphorylation on eEF-2K activity, to those observed in cells. Thus the phosphorylation and activation of eEF-2K by PKA is the most completely characterized mechanism, which accounts for the inhibition of translation in response to elevated cAMP, to be demonstrated. Since the agents which raised cAMP, brought about similar decreases in both the rate of elongation and the rate of total mRNA translation (Figure 4), it seems likely that the major effect of these agents on protein synthesis is via peptide-chain elongation. Other mechanisms have, however, been implicated in the regulation of peptide-chain initiation in response to cAMP (see below for further discussion).

Work by Ayuso-Parrilla and co-workers substantiated a link between elevated cAMP and the inhibition of protein synthesis by glucagon in liver cells and whole liver in vivo [17,18]. Significantly, it was found that glucagon increased the ribosomal transit time in hepatocytes, suggesting that glucagon may inhibit translation in hepatocytes in a manner analogous to that described above for adipocytes.

The mechanisms involved in the control of translation by cAMP have received little consideration. Although it is possible that cAMP may have multiple effects leading to alterations in translation rates, for example alterations in amino acid availability, or changes in the redox state, attention has focused on the phosphorylation of translation factors as a mechanism of control. Despite initial experiments which seemed to suggest that p70<sup>68k</sup> could be inhibited in response to elevated cAMP [26], this would appear now not to be the case, since cAMP analogues do not inhibit p70<sup>68k</sup> or block the activation of p70<sup>68k</sup> by growth
It has been shown that cAMP slightly attenuates the activation of p70\textsuperscript{S6K} by insulin in 3T3-L1 adipocytes [25]. However, our results indicate that, in serum-starved 3T3-L1 adipocytes, the phosphorylation of p70\textsuperscript{S6K}, as judged by mobility shift on SDS/PAGE, is not affected by isoproterenol or cAMP analogues (results not shown). Also in rat adipocytes, isoproterenol was shown not to effect p70\textsuperscript{S6K} activity in the presence of insulin, while it produced a small activation in the absence of insulin [29]. These results show that the inhibition of protein synthesis by cAMP in serum-starved 3T3-L1 adipocytes cannot be due to a decrease in p70\textsuperscript{S6K} activity.

It has been shown that the phosphorylation of eIF-4E-BP-2 is decreased in response to elevated cAMP in 3T3-L1 adipocytes [25,30] and aortic smooth-muscle cells [31]. This suggests that cAMP could regulate eIF-4E-BP-1 in 3T3-L1 adipocytes and in aortic smooth muscle cells, but this issue is complicated somewhat, since in primary rat epididymal adipocytes the phosphorylation of eIF-4E-BP-1 can be increased by cAMP in 3T3-L1 cells (results not shown). However, this issue is complicated somewhat, since in primary rat epididymal adipocytes the phosphorylation of eIF-4E-BP-1 can be increased by various agents which increase cAMP (results not shown) [19,21]. Since the phosphorylation of eIF-4E-BP-1 is associated with an increase in peptide chain initiation, the effect of cAMP on eIF-4E activity is necessary to determine the effects of cAMP on protein synthesis. Furthermore, since the effects of insulin on both eEF-2K activity and cAMP-2 phosphorylation in adipocytes (Figures 1 and 3) were similar to its effects in CHO.T cells [15], it seems very likely that the control of translation by insulin via eEF-2 dephosphorylation is a general mechanism in various insulin-responsive cells.

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