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Regulation of polypeptide-chain initiation in rat skeletal muscle

Starvation does not alter the activity or phosphorylation state of initiation factor eIF-2

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In rats, 48-h starvation causes a decrease in the rate of protein synthesis in skeletal (e.g. gastrocnemius) muscle, due largely to impairment of peptide-chain initiation. In other cell types inhibition of initiation is associated with decreased activity and recycling of initiation factor eIF-2, and increased phosphorylation of its α-subunit. However, 48-h starvation has no effect on the activity or recycling of eIF-2 measured in extracts of gastrocnemius muscle, or on the level of α-subunit phosphorylation. The effects of starvation on peptide-chain initiation in skeletal muscle must therefore involve alterations in other components of the translational machinery.

Skeletal muscle; Protein synthesis; Initiation; Initiation factor-2 (eIF-2); Protein phosphorylation

1. INTRODUCTION

The rate of protein synthesis is decreased in skeletal muscle of starved or experimentally-diabetic animals. Over the short to medium term (up to 3 days) this is due to inhibition of translation at the level of peptide-chain initiation. Similar effects are observed in perfused muscle, and inhibition of initiation is quickly reversed after administration of insulin [1].

This inhibition of initiation in skeletal muscle is accompanied by a fall in the ability of muscle extracts to form 40 S-initiation complexes containing Met-tRNA$_i$ (the initiator tRNA) bound to the 40 S ribosomal subunit [2,3]. Formation of these complexes is mediated by initiation factor-2 (eIF-2). Added, purified eIF-2 stimulates formation of 40 S-initiation complexes to a greater extent in extracts of muscle from starved or diabetic rats than in corresponding control samples [2]. Taken together, these data suggest that changes in eIF-2 activity may be important in the regulation of peptide-chain initiation in skeletal muscle. The activity of eIF-2 can be regulated by phosphorylation of its α-subunit (eIF-2α) which inhibits its recycling (mediated by another initiation factor, GEF = guanine nucleotide-exchange factor), and thus also inhibits eIF-2 activity. This mechanism is important in the control of translation by haem in reticulocytes [4] and in virus-infected cells [5,6]. The level of eIF-2α phosphorylation increases in HeLa cells under conditions which inhibit initiation [7–9] and, conversely, insulin may decrease the level of eIF-2α phosphorylation in chondrocytes [10].

We have therefore studied the possible involvement of changes in the activity and phosphorylation state of eIF-2α in the control of peptide-chain initiation in skeletal muscle.

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2. MATERIALS AND METHODS

2.1. *Materials*

Materials were obtained as described [11,12], with the following additions. Ampholytes were from LKB (Selsdon, Surrey, England). eIF-2 and GEF were prepared from rat liver as described ([11] and [12], respectively). Nylon membranes (Biodyne A) were from Pall Ultrafine Filtration Corp., Glen Cove, NY, USA. The eIF-2α kinase HCR and eIF-2 were purified from reticulocyte lysates as described [13].

2.2. *Buffers*

The following buffers were used: buffer A = 50 mM Tris-HCl, pH 7.8, 250 mM KCl, 5 mM $MgCl_2$; buffer B = 20 mM Tris-HCl, pH 7.6, 250 mM KCl, 2 mM EDTA, 4 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol, 2.3% (v/v) glycerol; buffer C = 20 mM Tris-HCl, pH 7.6, 0.1% (v/v) β-mercaptoethanol, 10% (v/v) glycerol; buffer D = 25 mM Tris-HCl, pH 7.6, 450 mM KCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 4 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol, 5% (v/v) glycerol; buffer E = 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.05% (v/v) Tween 20.

2.3. *Animals*

Male Sprague-Dawley rats (150–200 g) were bred and kept in the departmental animal unit on an 07.00/19.00 light/dark cycle, and allowed water and food ad libitum (except for starved animals for which food withdrawal began at 09.00). Rats were killed by cervical dislocation between 08.30 and 09.30.

2.4. *Preparation and analysis of polyribosomes*

Gastrocnemius muscles were rapidly removed, frozen in liquid nitrogen and pulverised in a mortar and pestle. Weighed amounts were homogenised in five volumes of buffer A containing 0.1 mM EGTA, 1 mg/ml heparin and 4 mM sodium pyrophosphate using a Polytron homogeniser (position 4, for three 5 s bursts at 0–4°C). After centrifugation at $10000 \times g_{av}$ for 10 min, 0.4 ml of the resulting supernatant was layered over a 0.4 ml cushion of 0.8 M sucrose in buffer A containing 0.25 mg/ml heparin and centrifuged at $150000 \times g_{av}$ for 2.8 h. Ribosome pellets were resuspended in 0.5 ml of the same buffer and stored at −80°C. For analysis, samples were layered over 20–50% (w/v) linear sucrose gradients in the same buffer and centrifuged (2 h at $150000 \times g_{av}$) in a Beckman SW50.1 rotor.

2.5. *Preparation of samples for assay of eIF-2 activity*

Muscle homogenates, and postmitochondrial and postribosomal supernatants were prepared as described above but using buffer B. Samples were stored at −80°C prior to assay. Tissue RNA and protein levels were determined as described [2,14].

2.6. *Assessment of eIF-2(αP) phosphatase activity in muscle extracts*

Purified eIF-2 was phosphorylated in incubations (25 μl) containing 12.5 μg eIF-2, 10 U of HCR, 20 mM Tris-HCl, pH 7.6, 0.05 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 100 mM KCl, 2 mM $MgCl_2$, 5% (v/v) glycerol and 0.2 mM [γ-^{32}P]ATP (4 mCi μmol^{-1}). After 45 min at 30°C, an equal volume of buffer C containing 0.1 mM EDTA was added. The sample was divided into two equal fractions. To one was added 115 μl of muscle extract prepared as described in section 2.7 (i.e. in the presence of phosphatase inhibitors, NaF and pyrophosphate), and to the other, 115 μl of extract prepared without these compounds (NaF was replaced by extra KCl). The presence of excess EDTA in these assays chelates the Mg^{2+} ions from the phosphorylation incubations (calculated residual free $Mg^{2+} \simeq$ 2 pM) and eliminate further HCR activity. Mixtures were incubated either at 4 or 30°C as shown, and samples were removed for assessment of eIF-2α phosphorylation by SDS-polyacrylamide gel electrophoresis and autoradiography.

2.7. *Initiation factor assays*

eIF-2 was assayed by the formation of ternary complexes as previously described [15,16], and modified as described in the text. GEF was essentially as described by Matts and London [17] but using muscle postmitochondrial supernatants. The preincubation period was only 1 min.

2.8. *Isoelectric focusing and immunoblotting*

Muscle postmitochondrial supernatants were made by the procedure described above (section 2.3) using buffer D. Extracts were then diluted with the same buffer without KCl to adjust the salt concentration. Postmitochondrial supernatants were applied to phosphocellulose (Whatman P-11) columns in buffer C containing 250 mM KCl, 50 mM NaF and 1 mM EDTA. The eIF-2 was eluted with this buffer containing 0.7 M KCl.

Isoelectric focusing was performed essentially as described [18] with gels containing 6% (w/v) acrylamide, 3% (v/v) ampholytes mixed in the ratio 4:1 pH 5–7:pH 3.5–10 and 9 M urea, but using the Bio-Rad mini-slab gel apparatus. Gels were run for 2 h at 2 W followed by 30 min at 1000 V. The separated proteins were transferred to nylon membranes at 250 mA for 3 h in 0.7% acetic acid. After transfer, membranes were fixed for 20 min in 0.5% (v/v) glutaraldehyde in phosphate-buffered saline [19], and then washed twice in Tris-buffered saline. Membranes were then blocked overnight in buffer E containing 25% (w/v) low-fat milk powder. All subsequent steps were carried out in buffer E. The membrane was incubated successively with anti-eIF-2α antibody, biotin-conjugated anti-mouse IgG and avidin-peroxidase, in each case for 1 h with extensive washing with TTN between stages. Finally, the blot was visualised using 4-chloro-1-naphthol as substrate for peroxidase.

3. RESULTS AND DISCUSSION

Gastrocnemius muscle was used here because starvation causes marked inhibition of protein synthesis in this tissue [20,21]. Fasting of rats for 48 h resulted in a substantial fall in the proportion of ribosomes in polysomes (fed 65 ± 3%; 48-h starved 28 ± 3%; fig.1) in line with previous work [2,21], and indicating impairment of peptide chain-initiation. Each experimental determination represents a different individual animal for these and subsequent assay procedures. Statistical data therefore reflect inter-assay and inter-animal variations.

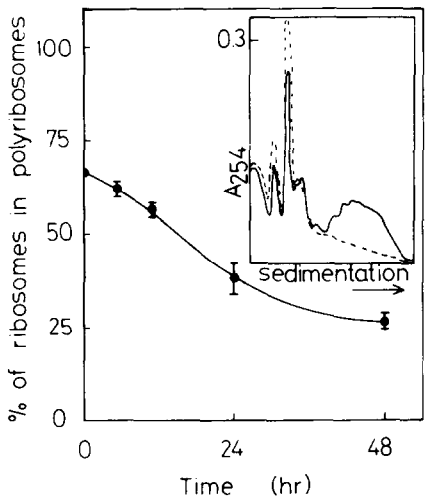

Fig.1. Assessment of proportion of ribosomes in polysomes in samples from rat gastrocnemius muscle. This was assessed as described in section 2; the period of food deprivation is indicated on the abscissa. Inset: Sucrose density gradient profiles for ribosome/polyribosome samples from fed (——) or 48-h starved rats (– – –).

3.1. *Isolation and assay of eIF-2 from gastrocnemius muscle*

To assess the importance of changes of eIF-2 activity in the control of peptide-chain initiation, it was first necessary to find suitable conditions for the isolation and assay of eIF-2. The inclusion of 0.25 M KCl in the extraction buffer was necessary to achieve maximal recoveries of eIF-2 activity as previously reported [22]. The extraction medium contained EDTA to chelate Mg^{2+} ions and thus inhibit protein kinases and sodium pyrophosphate to inhibit protein phosphatases [23]. Fluoride ions were not used because MgF_2 is only sparingly soluble and eIF-2 assays contain Mg^{2+} ions.

Postribosomal supernatant samples were used here for the measurement of eIF-2 activity because they (unlike postmitochondrial supernatants) gave time courses which were linear up to 20 min (fig.2A) and up to 40% (v/v) of sample in the assay (fig.2B). The eIF-2 in muscle fractions prepared under these conditions retained its sensitivity both to HCR and to GEF (fig.2C,D). It thus exhibited the properties expected for this factor and changes in its phosphorylation state should be detected as alterations in eIF-2 activity in the ternary complex assay.

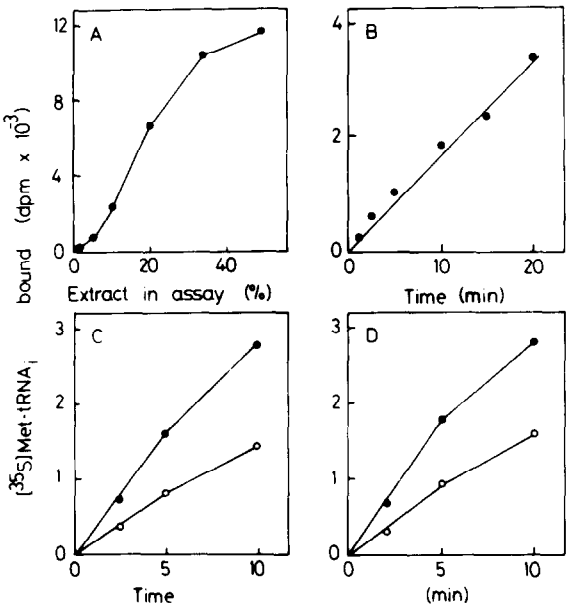

Fig.2. Characterisation of ternary complex [eIF-2·GTP·Met-tRNA$_i$] formation assay in postribosomal supernatants from gastrocnemius muscles. A, Standard incubations (50 μl) contained the proportion shown (% v/v) of postribosomal supernatant from fed rats. Samples (45 μl) were taken for determination of complex formation after 5 min. B, Standard 100 μl incubations contained 5 μl of postribosomal supernatant and samples (15 μl) were withdrawn at the times indicated. C, Incubations (50 μl) contained the equivalent of 5 μl of postribosomal supernatant pretreated by incubation for 5 min at 30°C in the presence of 2 mM Mg(acetate)$_2$ and 0.5 mM ATP with (○) or without (●) 0.5 U of HCR. Mg^{2+} concentrations were then adjusted to 0.5 mM by adding EDTA as appropriate. Samples (15 μl) were removed as indicated. D, Incubations (50 μl) contained 2.5 μl of postribosomal supernatant with (●) or without (○) added purified rat liver GEF (0.3 μg) and 15 μl samples were taken as shown.

Similar recoveries of eIF-2 in postribosomal supernatants were obtained from fed or starved animals (table 1) and eIF-2 activity, measured both as the rate (table 1) and the extent (not shown) of ternary complex formation and expressed either relative to tissue RNA or protein, was not significantly different for the two groups. The activities were also the same when eIF-2 was assayed in with or without Mg^{2+} ions, which should inhibit ternary complex formation more strongly in samples where eIF-2 is more highly phosphorylated. Our work extends earlier observations that eIF-2 activity (measured under conditions where changes in its phosphorylation level would pro-

Table 1

eIF-2 activity in extracts of gastrocnemius muscles from fed or starved rats

	Fed	Starved
eIF-2 activity in postmitochondrial supernatant (pmol [^{35}S]Met-tRNA$_i$ bound mg protein^{-1})		
Assayed in the presence of 0.5 mM Mg^{2+} (12)	0.26 ± 0.01	0.27 ± 0.01
% recovery in postribosomal supernatant[a] (4)	77 ± 3	81 ± 5
Rate of [^{35}S]Met-tRNA$_i$ binding in postribosomal supernatant (pmol [^{35}S]Met-tRNA$_f$ × 10^3 bound min^{-1} μg^b RNA^{-1})		
Assayed in the presence of 0.5 mM Mg^{2+} (12)	1.80 ± 0.18	2.14 ± 0.20
Assayed in the presence of 2.5 mM Mg^{2+} (12)	0.71 ± 0.08	0.84 ± 0.04
(pmol [^{35}S]Met-tRNA$_f$ bound min^{-1} mg protein^{-1})		
Assayed in the presence of 0.5 mM Mg^{2+} (12)	0.306 ± 0.010	0.318 ± 0.008
Assayed in the presence of 2.5 mM Mg^{2+} (12)	0.130 ± 0.006	0.136 ± 0.004

[a] % recovery calculated as

$$\frac{\text{activity in postribosomal supernatant}}{\text{(activity in postribosomal supernatant plus activity in ribosomal pellets)}} \times 100$$

Ternary complexes [eIF-2·GTP·[^{35}S]Met-tRNA$_f$] were determined under standard conditions in the presence or absence of Mg^{2+} as indicated

[b] Where normalised to RNA, this is total tissue RNA

The number of experiments in each case is indicated in parentheses, and the standard errors are also given

bably not be detected, i.e. without Mg^{2+} ions [22]) in psoas muscle was not altered by starvation. It suggests that the overall amount and the intrinsic activity of eIF-2 in muscle is unchanged following a 48 h fast.

3.2. *Guanine nucleotide exchange activity in muscle*

This was assayed as the ability of muscle postmitochondrial supernatant samples to mediate exchange of labelled GDP bound to eIF-2 for unlabelled GTP. The displacement of labelled GDP was linear with respect to the amount of extract in the assay up to 40% (v/v) (fig.3A) and with respect to time up to 3 min (fig.3B). As expected, displacement of GDP was strongly inhibited by prior treatment of the muscle postmitochondrial supernatant sample with HCR (typically, pretreatment for 5 min with 5 U ml^{-1} led to an 80% inhibition of the rate of nucleotide exchange (not illustrated)). The rate at which muscle postmitochondrial supernatants mediated this displacement was not significantly different for samples derived from gastrocnemius muscle of fed or starved animals, whether expressed relative to tissue RNA or protein (fig.3B,C). Impairment of the recycling of eIF-2 does, therefore, appear to be the cause of the inhibition of peptide-chain initiation in gastrocnemius muscles from starved rats.

3.3. *The level of phosphorylation of eIF-2α in gastrocnemius muscle*

The level of phosphorylation of eIF-2α was very low (<5%) in muscle extracts and was not significantly altered by starvation for 48 h (fig.4). These measurements were performed with five different sets of animals and gave essentially identical results in each case. Therefore the inhibition of chain initiation which occurs in starvation is not associated with increased phosphorylation of eIF-2α and this finding is in line with our inability to detect changes in the activities of eIF-2 or GEF under these conditions.

The levels of phosphorylation of eIF-2α found here are lower than those reported for some other types of cells – e.g. haem-supplemented reticulocyte lysates (10% [25]) and Ehrlich ascites tumour cells (about 50% [18]), but are similar to

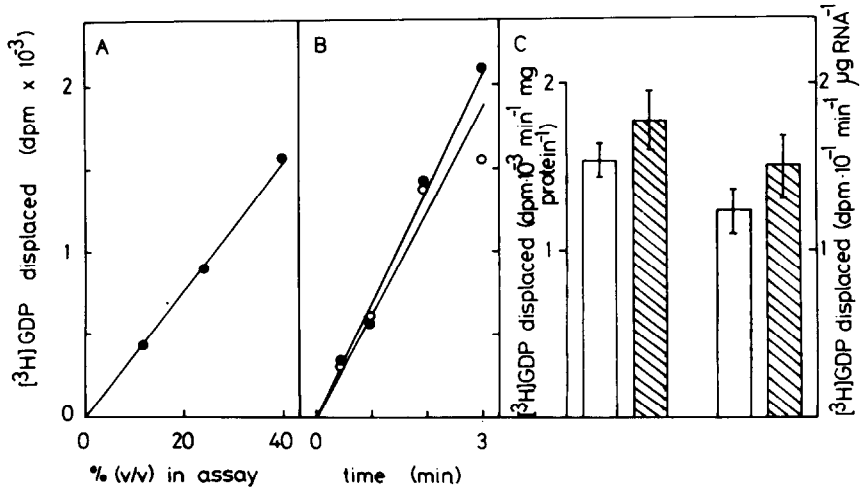

Fig.3. GDP-exchange assays containing postmitochondrial supernatants from rat gastrocnemius muscle. A, Standard assays (100 µl) contained the indicated percentage (v/v) of muscle postmitochondrial supernatant and preformed {eIF-2·[^{3}H]GDP} complexes (about 15000 dpm). Samples (30 µl) were removed at 3 min for the assay of remaining bound [^{3}H]GDP. B, Standard incubations (100 µl) contained 25 µl of muscle postmitochondrial supernatant from fed (•) or starved (○) rats and preformed {eIF-2·[^{3}H]GDP} complexes (about 37000 dpm). Samples (20 µl) were removed and processed as indicated. C, Results from experiment in B expressed as rates of displacement of [^{3}H]GDP/min per unit of tissue RNA or protein for 48-h starved animals (shaded columns) and fed rats (open columns) (mean of 8 determinations with standard errors shown by the vertical bars).

those seen in serum-stimulated HeLa cells [8]. The buffers used here for the preparation of eIF-2 contained 50 mM NaF, 1 mM EDTA, 1 mM EGTA and 4 mM pyrophosphate, which are established inhibitors of protein phosphatases. Indeed, 25 mM NaF and 4 mM EDTA have been shown to prevent dephosphorylation of a range of phosphoproteins in muscle extracts [26–29]. To verify that under our extraction conditions there was no eIF-2(αP) phosphatase activity in muscle extracts, samples of ^{32}P-labelled eIF-2(αP) were incubated with muscle extracts prepared under standard conditions with or without the main phosphatase inhibitors, NaF and sodium pyrophosphate. In the presence or absence of phosphatase inhibitors, at 4°C (the temperature at which all the extraction steps were performed), there was no significant dephosphorylation of eIF-2α (fig.5) up to 90 min. Only at 30°C, in the absence of phosphatase inhibitors, was there detectable phosphatase activity. It is therefore very unlikely that complete dephosphorylation of eIF-2 occurs during the isolation procedure and eIF-2α seems not to be significantly phosphorylated in gastrocnemius muscles of fed or starved rats.

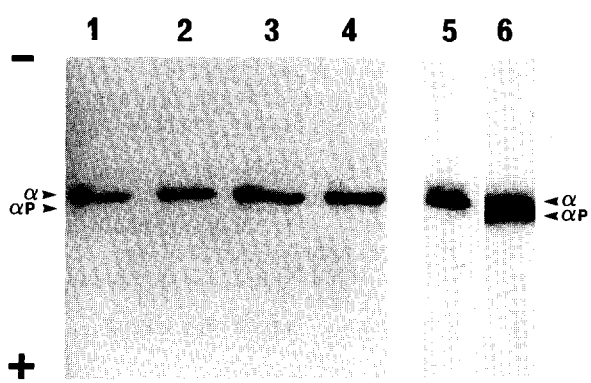

Fig.4. Phosphorylation state of eIF-2α. eIF-2 was analysed by isoelectric focusing and immunoblotting. Tracks 1 and 2: eIF-2 from gastrocnemius muscle of two fed rats, tracks 3 and 4: eIF-2 from gastrocnemius muscle of two rats starved for 48 h, tracks 5 and 6: reticulocyte lysates incubated in the presence of haem (5) or dsRNA (6), and thus indicating the focusing positions of eIF-2α and eIF-2α(P) (also shown by arrows). Rat and rabbit eIF-2α and eIF-2α(P) cofocus exactly. The polarity is indicated.

3.4. *Concluding remarks*

The data presented here strongly indicate that the impairment of peptide-chain initiation in skeletal muscle following 48-h starvation in skeletal muscle is not associated with any detectable change in the activity of eIF-2 or in the

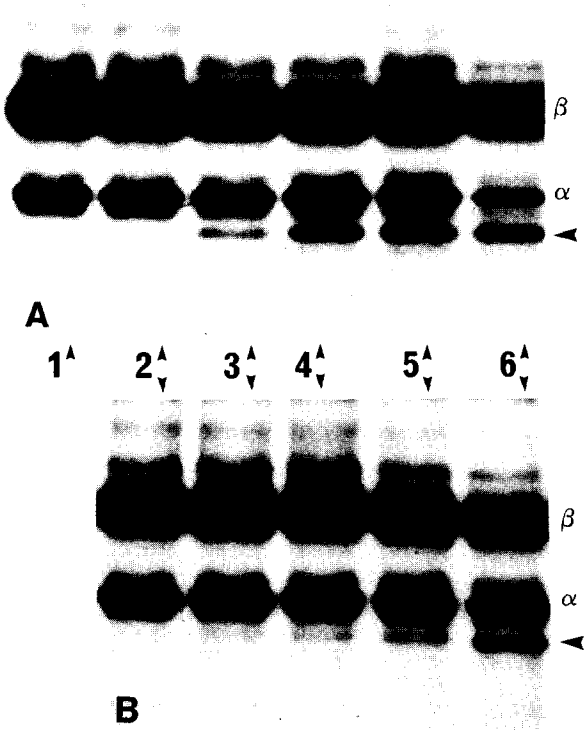

Fig.5. Protein phosphatase activity against eIF-2(αP) in muscle extracts. eIF-2(α[^{32}P]) was incubated with muscle extracts prepared in buffers without (A) or with (B) phosphatase inhibitors (NaF and sodium pyrophosphate) at 4°C (tracks 1–5) or 30°C (track 6). Samples were taken at 0 (track 1), 20 (track 2), 40 (track 3), 60 (track 4) and 90 (tracks 5,6) min for analysis by SDS-PAGE and subsequent autoradiography. The figure is an autoradiograph and the direction of migration was from top to bottom. The positions of eIF-2α and eIF-2β are indicated; phosphorylation of eIF-2β is due to casein kinase-2 present in the HCR used here. The band marked with an arrow, which appears during the incubation, seems to be a degradation product of eIF-2β, which is very sensitive to proteolysis [11].

phosphorylation state of eIF-2α. It must therefore result from alteration(s) in another component(s) of the translational machinery.

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REFERENCES

[1] Jefferson, L.S. (1980) Diabetes 29, 487–498.
[2] Harmon, C.S., Proud, C.G. and Pain, V.M. (1984) Biochem. J. 223, 687–696.
[3] Kelly, F.J. and Jefferson, L.S. (1985) J. Biol. Chem. 260, 6677–6683.
[4] Proud, C.G. (1986) Trends Biochem. Sci. 11, 73–77.
[5] Samuel, C.E., Duncan, R., Knutson, G.S. and Hershey, J.W.B. (1984) J. Biol. Chem. 259, 13451–13457.
[6] Siekierka, J., Mariano, T.M., Reichel, P.A. and Mathews, M.B. (1985) Proc. Natl. Acad. Sci. USA 82, 1959–1963.
[7] Duncan, R. and Hershey, J.W.B. (1984) J. Biol. Chem. 259, 11882–11889.
[8] Duncan, R. and Hershey, J.W.B. (1985) J. Biol. Chem. 260, 5493–5497.
[9] Duncan, R. and Hershey, J.W.B. (1987) Mol. Cell. Biol. 7, 1293–1295.
[10] Towle, C.A., Mankin, H.J., Avruch, J. and Treadwell, B.V. (1984) Biochem. Biophys. Res. Commun. 121, 134–140.
[11] Colthurst, D.R. and Proud, C.G. (1986) Biochim. Biophys. Acta 868, 77–86.
[12] Proud, C.G. (1987) Biochim. Biophys. Acta 914, 64–73.
[13] Colthurst, D.R., Campbell, D.G. and Proud, C.G. (1987) Eur. J. Biochem. 166, 357–363.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Proud, C.G. and Pain, V.M. (1982) FEBS Lett. 143, 55–59.
[16] Proud, C.G., Clemens, M.J. and Pain, V.M. (1982) FEBS Lett. 148, 214–220.
[17] Matts, R.L. and London, I.M. (1984) J. Biol. Chem. 259, 6708–6711.
[18] Wong, S.-T., Mastropaolo, W. and Henshaw, E.C. (1983) J. Biol. Chem. 257, 5231–5238.
[19] Scorsone, K.A., Panniers, P., Rowlands, A.G. and Henshaw, E.C. (1987) J. Biol. Chem. 262, 14536–14543.
[20] Garlick, P.J., Millward, D.J., James, W.P.T. and Waterlow, J.C. (1975) Biochim. Biophys. Acta 414, 71–84.
[21] Li, J.B., Higgins, J.E. and Jefferson, L.S. (1979) Am. J. Physiol. 236, E222–E228.
[22] Rannels, S.R., Rannels, D.E., Pegg, A.E. and Jefferson, L.S. (1978) Am. J. Physiol. 235, E134–E139.
[23] Khandelwal, R.L. (1978) Arch. Biochem. Biophys. 191, 764–773.
[24] Pain, V.M. and Clemens, M.J. (1983) Biochemistry 22, 726–733.
[25] Leroux, A. and London, I.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2147–2151.
[26] Yeaman, S.J. and Cohen, P. (1975) Eur. J. Biochem. 51, 93–104.
[27] Foulkes, J.G. and Cohen, P. (1979) Eur. J. Biochem. 97, 251–256.
[28] Parker, P.J., Embi, N., Caudwell, F.B. and Cohen, P. (1982) Eur. J. Biochem. 124, 47–55.
[29] Parker, P.J., Caudwell, F.B. and Cohen, P. (1983) Eur. J. Biochem. 130, 227–234.