Kinetics of calcium and calmodulin-dependent protein kinase III from embryonic chicken leg muscle cells

Bent Riis*, Odd Nygård

Department of Zoological Cell Biology, Wenner-Grens Institute, Arrhenius Laboratories for Natural Sciences E5, Stockholm University, S-106 91, Stockholm, Sweden

Received 14 February 1997

Abstract Embryonic chicken muscle cells (CELM) contain the calmodulin-dependent protein kinase that specifically phosphorylates eukaryotic elongation factor 2. The kinase requires Ca^{2+} and maximum activity in CELM was observed at 10 μM Ca^{2+}. The ATP concentration required for half the maximum activity of CaM PKIII in CELM was calculated to be 0.15 mM. In CELM, dephosphorylation of eEF-2 was catalyzed by phosphoprotein phosphatase PP2A alone. The activity of PP2A was relatively low and the half-life of added phosphorylated eEF-2 was more than 15 min. Due to the low phosphoprotein phosphatase activity, inhibition of the PP2A activity by addition of okadaic acid had little effect on the eEF-2 phosphorylation kinetics.

Key words: Protein synthesis regulation; Calcium and calmodulin-dependent protein kinase III; eEF-2 kinase; Elongation factor-2

1. Introduction

In higher eukaryotes, the protein synthesis elongation cycle is catalyzed by two soluble elongation factors, eEF-1α and eEF-2 [1]. The enzyme eEF-1α is responsible for bringing cognate aminoacyl tRNAs to the ribosomal A site while eEF-2 promotes translocation of peptidyl tRNA from the A to the P site on the ribosome. The efficiency of the translocation step is regulated by reversible phosphorylation of eEF-2 [1–3]. Phosphorylated eEF-2 (P-eEF-2) has decreased affinity for the pre-translocation type of ribosome and is thus unable to promote translocation [2]. This results in a reduced rate of protein synthesis and an accumulation of polysomes [3]. P-eEF-2 is dephosphorylated by phosphoprotein phosphatases 2A and 2C, PP2A and PP2C, respectively [4].

Phosphorylation of eEF-2 is catalyzed by a Ca^{2+} and calmodulin-dependent protein kinase (CaM PKIII), also known as the eEF-2 kinase [3,5]. Recently, the elusive CaM PKIII kinase has been cloned. Deduced from cDNA analysis, it is an enzyme with an apparent M_r of 95 kDA and it shows low homology to other known kinases [6]. The activity of CaM PKIII is influenced by hormones [7] and depends on the phosphorylation status of the kinase itself [8], indicating that the kinase activity and thus the rate of translation could be regulated via a kinase cascade. The well-known second messenger Ca^{2+} activates CaM PKIII via calmodulin [9,10]. The concentration of free Ca^{2+} ions in various compartments of the cell fluctuates in response to various stimuli. Two different pathways exist for supplying free Ca^{2+} ions [10]: influx of Ca^{2+} ions through membrane channels and release of Ca^{2+} from Ca^{2+}-sequestering proteins, mainly various glycoproteins located in the endoplasmic reticulum. Total depletion as well as relatively high concentrations of calcium inhibit translation, but the underlying mechanisms remain unclear in both cases [9,11].

It has been shown that proliferating undifferentiated cells, i.e. cells that have an extensive protein synthesis, show high CaM PKIII activity [12]. This unexpected observation made us investigate the balance between the CaM PKIII-dependent phosphorylation of eEF-2 and the PP2A/PP2C dephosphorylation of P-eEF-2 in developing chicken embryonic muscle cells (CELM), a tissue known to have substantial CaM PKIII activity.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (specific activity 110 TBq/mmol) was obtained from Amersham, UK. Nitrocellulose filters, type HA 0.45, were from Millipore, Bedford, MA, USA. Calmodulin was from Boehringer, Mannheim, Germany. OA was a generous gift from Dr. Y. Tsukitani. Rat liver eEF-2 was purified as described [12]. The specific eEF-2 kinase, CaM PKIII, was partially purified as described [13].

2.2. Preparation of cell-free extracts from CELM cells

The major leg muscles from 15-day-old chicken embryos were homogenized in ice-cold buffer containing 0.25 M sucrose, 30 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$ and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using a Dounce homogenizer. The homogenates were centrifuged at 10000×*g* for 10 min to remove organelles, membranes and cellular debris. The supernatant was frozen and stored at −80°C.

2.3. Determination of CaM PKIII activity

The activity of CaM PKIII was determined by measuring the [^{32}P]phosphate incorporation into eEF-2. The assays contained, in final volumes of 50 μl, Tris-HCl (pH 7.6) 20 mM, glycerol 10% (v/v), KCl 0.1 M, 2-mercaptoethanol 15 mM, $MgCl_2$ 10 mM, $CaCl_2$ 1.5 mM, EGTA 1 mM, and EDTA 0.1 mM (buffer A) and 50 μg protein from the CELM cells. In the experiments where eEF-2 was used as the variable substrate, [γ-^{32}P]ATP was added to a final concentration of 0.5 mM. The samples were incubated at 30°C as indicated. When ATP was used as the variable substrate the reaction mixture contained 13.5 pmol of purified eEF-2. In some experiments, okadaic acid (OA) was added to a final concentration 0.2 μM. Incubation was stopped by addition of 1 ml ice-cold 10% trichloric acid (TCA). The samples were applied to nitrocellulose filters and extensively washed with 5% TCA before liquid scintillation counting. The background was rou-

*Corresponding author. Present address: Department of Product Quality, Danish Institute of Agricultural Science, P.O. Box 39, DK-8830 Tjele, Denmark. Fax: (45) 89 99 15 64.
E-mail: Bent.Riis@sh.dk

Abbreviations: ATP, adenosine triphosphate; CELM, chicken embryonic leg muscle cells; CaM PKIII, calcium and calmodulin-dependent protein kinase III; eEF-1α and eEF-2, eukaryotic elongation factors 1α and 2, respectively; OA, okadaic acid; P-eEF-2, phosphorylated eEF-2; PP2A and PP2C, phosphoprotein phosphatases 2A and 2C, respectively; PMSF, phenylmethylsulfonyl fluoride; RRL, rabbit reticulocyte lysate

PII S0014-5793(97)00277-9

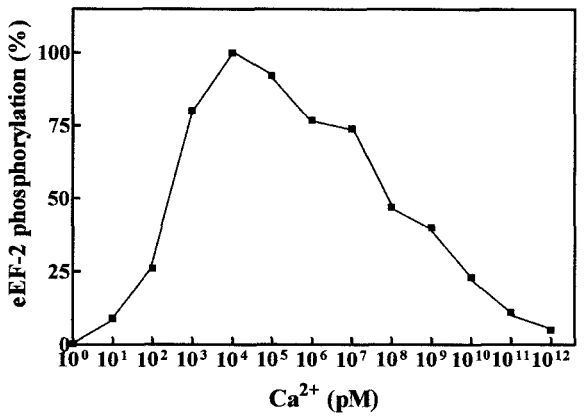

Fig. 1. Effect of Ca^{2+} on the CaM PKIII activity in homogenates from CELM. The homogenates were depleted of endogenous Ca^{2+}, and the kinase activity was determined by incubation for 8 min at 30°C in the presence of added Ca^{2+} as described in Section 2. The extent of eEF-2 phosphorylation was analyzed by gel electrophoresis and autoradiography. The autoradiographs were quantified using a computer-assisted image analysis system.

tinely subtracted. Alternatively, the reaction was stopped by boiling for 2 min in the presence of 10 μl sample buffer [14]. The material was analyzed by SDS gel electrophoresis using 10–15% (weight by volume) polyacrylamide gradient gels followed by autoradiography. The autoradiographs were quantified using a microcomputer assisted image analysis system.

2.4. Dephosphorylation of eEF-2

Purified eEF-2 was labelled with [^{32}P]phosphate and dialysed extensively against buffer A. For dephosphorylation, 13.5 pmol of [^{32}P]PO_4-labelled eEF-2 was incubated with 50 μg of CELM protein at 30°C. Samples were withdrawn as indicated and spotted onto filter paper disks. The filter paper was allowed to dry and put in ice-cold 10% TCA containing 1% phosphoric acid for 30 min. The filters were rinsed in 5% TCA for 10 min at room temperature, dehydrated in 99% alcohol for 10 min and dried. The radioactivity bound to the filters was determined by scintillation counting.

2.5. Determination of eEF-2 and total protein content

The content of endogenous eEF-2 in the samples was estimated using the diphtheria toxin-dependent ADP-ribosylation assay as previously described [15]. The total content of protein was determined according to Bradford [16].

2.6. Determination of Ca^{2+} influence on CaM PKIII

The Ca^{2+} requirement for CaM PKIII-dependent activity was determined by dialyzing the CELM homogenates against a buffer containing 1.0 mM Ca^{2+} and 1.0 mM EDTA for 1 h. Thereafter, the kinase activity was determined in the presence of added Ca^{2+} as indicated.

3. Results and discussion

Before studying the ability of CELM homogenates and rabbit reticulocyte lysate, RRL, to phosphorylate eEF-2, we wanted to determine the effect of varying Ca^{2+} concentrations on the activity of CaM PKIII in the CELM extract. The reactions were performed in a Ca^{2+}/EGTA buffered solution in which the final concentration of Ca^{2+} ions was adjusted by addition of free Ca^{2+}. As seen in Fig. 1, concentrations between 1 nM and 1 mM stimulated eEF-2 phosphorylation, whereas concentrations above 1 mM were found to be strongly inhibitory. We also wanted to ensure that the concentration of ATP used in our assays was not rate limiting. For this purpose the kinase activity was determined using increasing concentrations of ATP (Fig. 2). The K_m and V_{max} for the phosphorylation reaction were calculated to 145 ± 10 μM and 4.5 ± 0.2 pmol/min, respectively. The K_m is comparable to that observed for the partially purified kinase from RRL [17]. Based on these results, the ATP concentration used in the following experiments was selected to allow maximum CaM PKIII activity.

The maximum rate of eEF-2 phosphorylation in the CELM system was determined in experiments using eEF-2 as the variable substrate. Two different experimental conditions were employed. In one series of experiments, the CaM PKIII activity was determined in the absence of the PP2A inhibitor, OA (Fig. 3). These experiments allowed determination of the rate of eEF-2 phosphorylation at steady state and thereby the calculation of the apparent kinetic constants $K_{m(app)}$ and

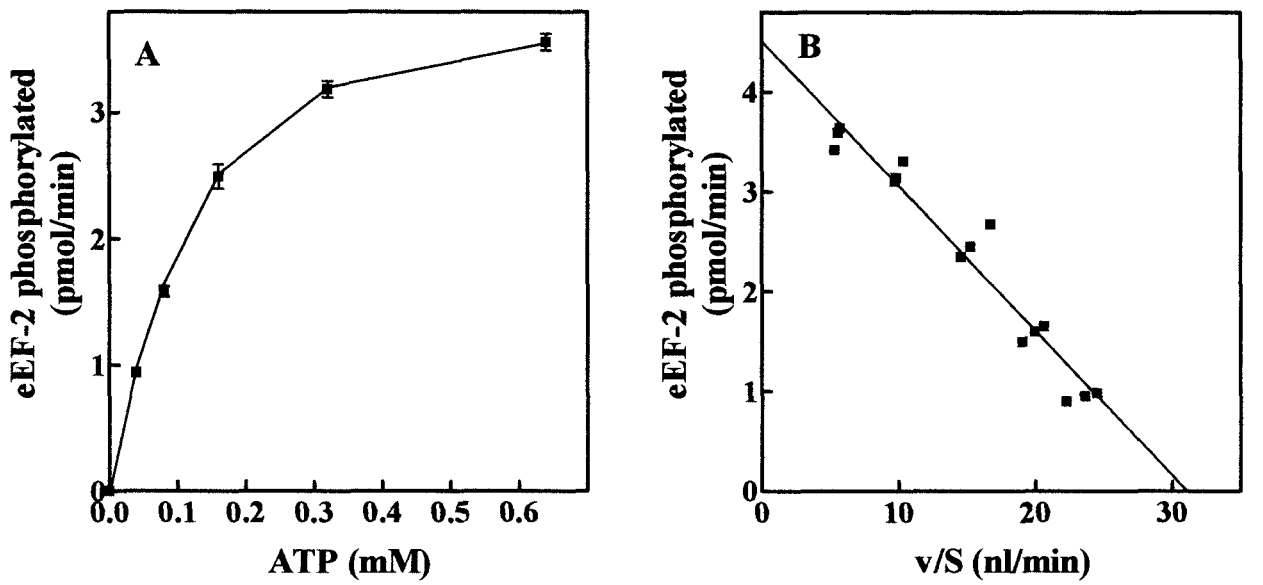

Fig. 2. Phosphorylation of eEF-2 in CELM in the presence of increasing concentrations of ATP (A). The incubation was as described in Section 2. Linearization of the data was done according to Eadie and Hofstee [19,20] (r=0.97) (B).

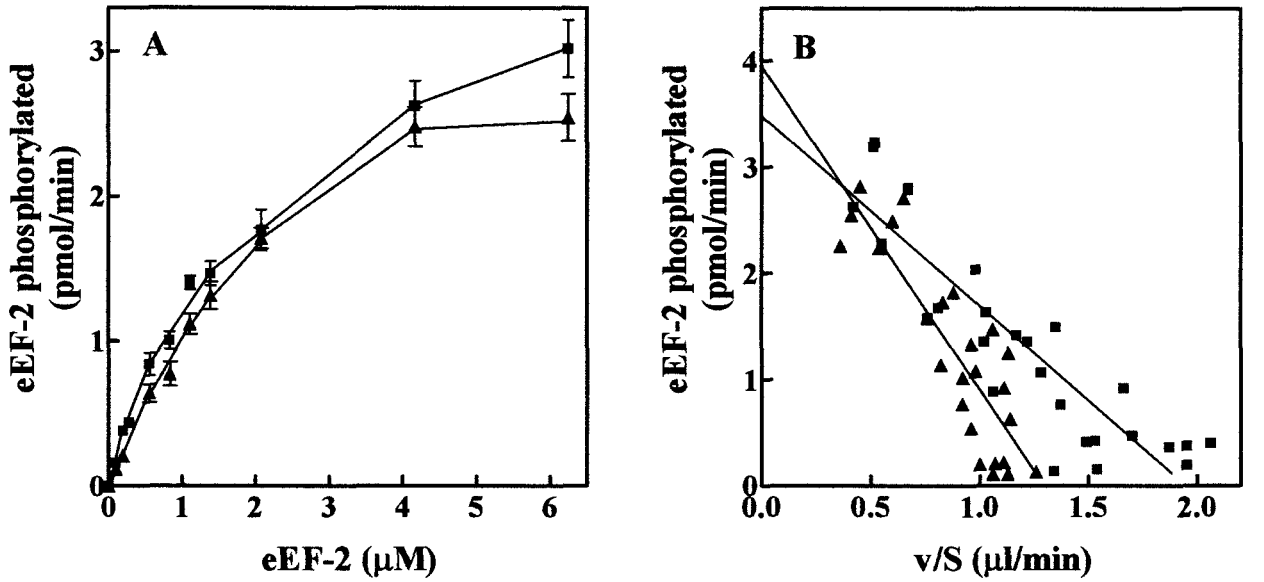

Fig. 3. Phosphorylation of eEF-2 in CELM in the presence of increasing concentrations of eEF-2 (A). The reaction mixtures containing 0.5 mM ATP were incubated in the absence (▲) or presence (■) of okadaic acid as described in Section 2. Linearization of the data was done according to Eadie and Hofstee [19,20] ($r = 0.89$ (▲) and 0.86 (■)) (B).

$V_{max(app)}$ for the eEF-2 phosphorylation under conditions where phosphorylation was counterbalanced by dephosphorylation. Under these conditions the $V_{max(app)}$ and $K_{m(app)}$ were calculated to 4.0 ± 0.4 pmol/min and 3.1 ± 0.4 μM, respectively. An analysis of the kinase activity in RRL using identical experimental conditions (not illustrated) showed that the $V_{max(app)}$ and $K_{m(app)}$ were 4.3 ± 0.7 pmol/min and 11.3 ± 2.9 μM, respectively. As seen the maximum rate of phosphorylation was approximately similar in both CELM and RRL, while the concentration of eEF-2 needed for obtaining half maximum rate of phosphorylation was more than three times higher in RRL than in CELM.

In the second series of experiments, the PP2A activity was inhibited by addition of OA. This series of experiments allowed calculation of the V_{max} and K_m for the phosphorylation reaction without interference from the dephosphorylation reaction. As seen in Fig. 3, addition of OA had little effect on the phosphorylation kinetics of the CELM; and the V_{max}, 3.5 ± 0.2 pmol/min, was almost unaffected by the addition of OA. However, the K_m, 1.8 ± 0.2 μM, was approximately half of that observed in the absence of OA. Identical experiments performed in RRL gave a V_{max} of 5.3 ± 1.9 pmol/min and a K_m of 5.1 ± 0.9 μM (not illustrated). These results indicate that the rate of PP2A catalyzed dephosphorylation of P-eEF-2 in the CELM was low in comparison to the maximum capacity of the eEF-2 kinase to phosphorylate eEF-2.

The concentration of eEF-2 in CELM was determined to 88 ± 4.2 nM using the specific diphtheria toxin catalyzed ADP-ribosylation of eEF-2 [15]. As the amount of P-eEF-2 is directly proportional to the concentration eEF-2 at low substrate concentrations, the capacity of CELM to phosphorylate eEF-2 under optimal Ca^{2+} concentration could be calculated to 0.11 pmol eEF-2/min in the absence of OA and 0.16 pmol/min in the presence of OA.

The small effect of the phosphoprotein phosphatase inhibitor OA prompted us to directly determine the phosphatase activity in CELM. As seen in Fig. 4, incubation of added ^{32}P-labelled eEF-2 in CELM resulted in a dephosphorylation of the factor. No dephosphorylation was observed in the presence of OA (not illustrated). As OA is a potent inhibitor of PP2A activity, the data show that PP2A is the main protein phosphatase responsible for reactivating P-eEF-2 in CELM. The data presented in Fig. 4 allowed calculation of the time needed to dephosphorylate 50% of the added P-eEF-2. The $t_{1/2}$ was calculated to be 16.3 min for the CELM homogenate and 4.5 min for RRL. The reason for the difference in phosphatase activity between the two systems is not known, but may depend on low amounts of protein phosphatases in the CELM tissue in analogy with the situation in rat corpus luteum [7]. Alternatively, PP2A is inactivated in the CELM system due to the presence of inhibitors as is the case in some muscle cells [18].

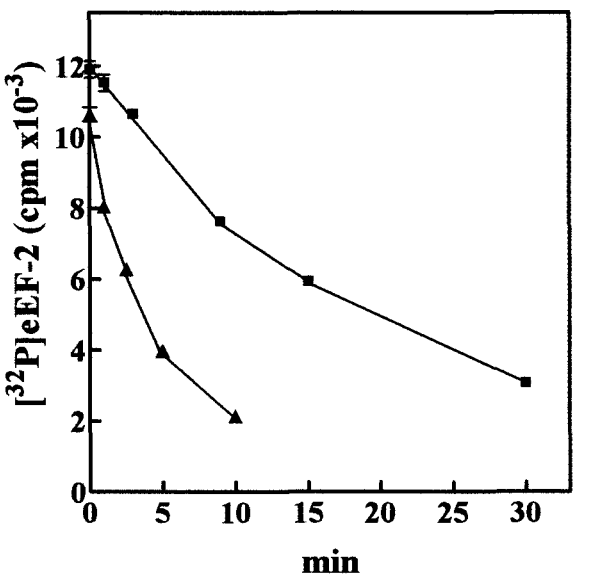

Fig. 4. Dephosphorylation of [^{32}P]eEF-2 in homogenates from CELM (■) and in RRL (▲). Dephosphorylation was determined as described in Section 2.

Acknowledgements: We thank Mrs. B. Lundberg for technical assistance. This work was supported by a grant from the Wenner-Grens Institute, Stockholm, to B.R. and from the Swedish Natural Science Research Council (B-Bu-8463-3039) to O.N.

References

[1] W.C. Merrick, J.W.B. Hershey, in: J.W.B. Hershey, M.B. Mathews, N. Sonenberg (Eds.), Translational Control. Monograph 30, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA, 1996, pp. 31–69.
[2] U. Carlberg, A. Nilsson, O. Nygård, Eur J Biochem 191 (1990) 639–645.
[3] A.G. Ryazanov, E.A. Shestakova, P.G. Natapov, Nature 334 (1988) 170–173.
[4] M. Gschwendt, W. Kittstein, G. Mieskes, F. Marks, FEBS Lett 257 (1989) 357–360.
[5] C.A. Nairn, B. Bhagat, C.H. Palfrey, Proc Natl Acad Sci USA 82 (1985) 7939–7943.
[6] N.T. Redpath, N.T. Price, C.G. Proud, J Biol Chem 271 (1996) 17547–17554.
[7] C.T. Albarracin, H.C. Palfrey, W.R. Duan, M.C. Rao, G. Gibori, J Biol Chem 269 (1994) 7772–7776.
[8] O. Nygård, A. Nilsson, U. Carlberg, L. Nilsson, R. Amons, J Biol Chem 266 (1991) 16425–16430.
[9] A.C. Nairn, H.C. Palfrey, in: J.W.B. Hershey, M.B. Mathews, N. Sonenberg (Eds.), Translational Control. Monograph 30, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, USA, 1996, pp. 295–318.
[10] R.W. Tsien, R.Y. Tsien, Annu Rev Cell Biol 6 (1990) 715–760.
[11] C.O. Brostrom, M.A. Brostrom, Annu Rev Physiol 52 (1990) 577–590.
[12] A. Nilsson, O. Nygård, Biochim Biophys Acta 1268 (1995) 263–268.
[13] L. Nilsson, O. Nygård, Biochim Biophys Acta 782 (1984) 49–54.
[14] U.K. Laemmli, Nature 227 (1970) 680–685.
[15] B. Riis, S.I.S. Rattan, B.F.C. Clark, J Biochem Biophys Methods 19 (1989) 319–326.
[16] M.M. Bradford, Anal Biochem 72 (1976) 248–254.
[17] A. Nilsson, U. Carlberg, O. Nygård, Eur J Biochem 195 (1991) 377–383.
[18] S. Pelech, P. Cohen, M.J. Fischer, C.I. Pogson, M.R. El-Maghrabi, S.J. Pilkins, Eur J Biochem 145 (1984) 39–49.
[19] G.S. Eadie, J Biol Chem 146 (1942) 85–93.
[20] B.H.J. Hofstee, Nature 184 (1959) 1296–1298.