β-Adrenergic agonists increase phosphorylation of elongation factor 2 in cardiomyocytes without eliciting calcium-independent eEF2 kinase activity

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Abstract The β-adrenergic agonist isoproterenol increased the phosphorylation of elongation factor eEF2 in ventricular cardiomyocytes from adult rats (ARVC). Phosphorylation of eEF2 inhibits its activity, and protein synthesis was inhibited in ARVC concomitantly with increased eEF2 phosphorylation. eEF2 kinase activity in ARVC extracts was completely dependent upon Ca^{2+}/calmodulin. In contrast to other cell types, however, treatments designed to raise intracellular cAMP failed to induce Ca^{2+}/calmodulin-independent activity. Instead, they increased maximal eEF2 kinase activity. Similar data were obtained when partially purified ARVC eEF2 kinase was treated with cAMP-dependent protein kinase in vitro. These data suggest that ARVC possess a distinct isoform of eEF2 kinase.

Key words: Protein synthesis; Elongation; Myocyte; Heart; Kinase; Calcium

1. Introduction

A number of agents and conditions modulate the rate of mRNA translation in isolated cardiac myocytes from adult animals. These include insulin (and related growth factors) and α-adrenergic agonists, such as phenylephrine, which activate protein synthesis acutely [1–6]. This is connected with the general anabolic effect of insulin and the ability of the latter two agents to promote hypertrophy of cardiac tissue. Conversely, β-adrenergic stimulation results in inhibition of protein synthesis [7]. Such agonists exert inotropic and chronotropic effects on cardiac contraction, a process which, like protein synthesis, consumes ATP. It is therefore physiologically reasonable that in parallel with promoting contraction, such treatment would also switch off processes such as protein synthesis, which compete with it for the available ATP. However, the mechanisms underlying the ability of β-agonists to inhibit cardiac protein synthesis remain obscure. Since one of the main actions of these agents is to raise cellular cAMP levels, it seems likely that cAMP may act to inhibit a component or components of the translational machinery.

Although a good deal of effort has been devoted to understanding the molecular mechanisms involved in regulating mRNA translation in animal cells, little attention has been directed to exploring possible links to cAMP. One possible link is the phosphorylation of elongation factor 2, eEF2, which inhibits the activity of this protein. eEF2 is phosphorylated at Thr56 by a protein kinase termed eEF2 kinase (reviewed in [8]), which is a member of a recently discovered and novel group of protein kinases [9]. eEF2 kinase is normally dependent on Ca ions and calmodulin (Ca/CaM) for activity [8,10,11]. An early study showed that eEF2 kinase was a substrate for cAMP-dependent protein kinase (PKA) in vitro and that this led to activation of eEF2 kinase, by rendering it less dependent on Ca/CaM for activity [12]. This therefore provided a potential mechanism for the regulation of elongation by agonists which raise cAMP. Consistent with this, two subsequent studies, in adipocytes [13] and myeloid cells [14], have shown that treatment of cells with agents which activate PKA leads to increased phosphorylation of eEF2 and inhibition of peptide-chain elongation.

Here we show that β-adrenergic agonists increase eEF2 phosphorylation in isolated ventricular cardiomyocytes and that this correlates with inhibition of protein synthesis. Surprisingly, these treatments do not alter the requirement of cardiac eEF2 kinase for Ca/CaM but instead increase its maximal activity.

2. Materials and methods

2.1. Chemicals and biochemicals

These items were obtained as described earlier or were purchased from Sigma (Poole, Dorset).

2.2. Isolation, culture and extraction of cardiac myocytes

Cardiomyocytes (ARVC, adult rat ventricular myocytes) were prepared and maintained in culture overnight (unless otherwise stated) as described earlier [3].

2.3. Gel electrophoresis and immunoblotting

These techniques were performed as described earlier [3]. The anti-(P)eEF2 antibody was prepared using the peptide described by Marin et al. [15] and purified by chromatography successively on columns to which the phospho and dephospho forms of the peptide had been immobilised. The final preparation of anti-(P)eEF2 antibody did not recognise non-phosphorylated eEF2.

2.4. Assays for eEF2 kinase activity

eEF2 kinase assays were performed using 15–20 μg cell protein per assay in the presence and absence of calcium ions and calmodulin, as described earlier [16,17] using purified eEF2 (0.3 μg/assay) as substrate. Assays were performed for 20 min at 30°C and reactions were stopped with the addition of 5×SDS sample buffer. Samples were analysed on 10% SDS–PAGE. The gels were Coomassie stained, destained, dried and then exposed to X-ray film (Konica).

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2.5. Measurement of protein synthesis

Cells were isolated and treated as before. Cells were incubated with methionine-free MEM for 2 h to reduce background incorporation of methionine into synthesised proteins. The experiment was performed in triplicate. [^{35}S]methionine (15–20 μCi/ml) was added to control cells for 15 min. Isoproterenol was added to experimental cells for designated times (2, 5 or 10 min) before the addition of [^{35}S]methionine for 15 min. Cells were lysed and protein extracted as before. Protein content was quantified and three equal amounts of each lysate were spotted onto squares of 3 mm filter paper. The paper was boiled twice for 2 min in 5% TCA with 0.5 mM cold methionine. The papers were washed in cold 5% TCA and then in ethanol and dried. [^{35}S]methionine incorporation was measured by scintillation counting.

2.6. Partial purification of eEF2 kinase

eEF2 kinase was purified from extracts of control or isoproterenol-treated heart cells by ion-exchange chromatography on Mono-Q using an Äkta FPLC system (Pharmacia). The column was washed with low salt buffer (0.1 M KCl in 20 mM HEPES–KOH, pH 7.5; 0.1 mM EDTA; 15 mM β-mercaptoethanol) before the samples were applied. The column was developed with an increasing gradient of salt up to 0.5 M KCl in the same buffer. Fractions were collected and assayed as before for eEF2 kinase activity.

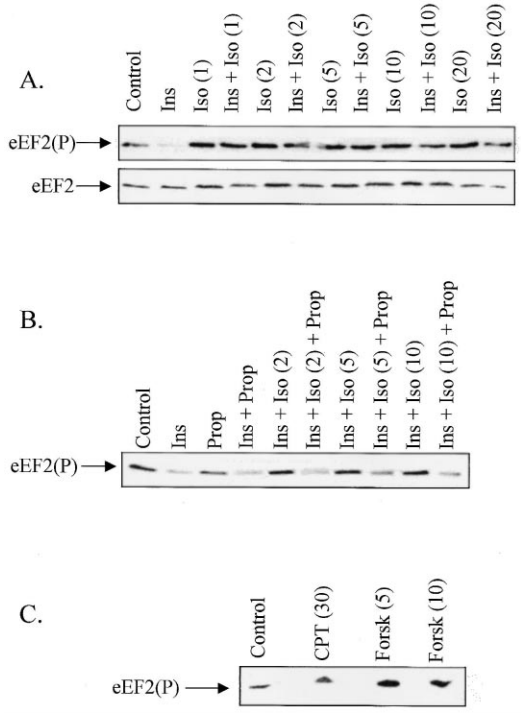

Fig. 1. Effect of insulin and isoproterenol on eEF2 phosphorylation in ARVC. Panel A: ARVC were cultured overnight and, in some cases (where shown) were then treated with insulin (Ins, 10 nM) for 20 min. Where indicated, isoproterenol (Iso, 1 μM) was added to the times shown. Cells were extracted and samples were applied to SDS–PA gels and subjected to immunoblotting with anti-phospho eEF2 antibody (upper blot) and, as a loading control, an anti-eEF2 antibody which does not distinguish the phosphorylated protein (lower blot). Panel B: Overnight cultures of ARVC were treated with insulin and isoproterenol, as for panel A. In some cases, cells were incubated with 20 μM propranolol (prop) for 20 min prior to the addition of isoproterenol. The blots were probed with an anti-phospho eEF2 antibody. Loading controls (as in panel A) established equal loading of samples in each lane (data not shown). Panel C: Overnight cultures of ARVC were treated with 0.5 mM CPT-cAMP or 1 μM forskolin for the times indicated (min). The blot was probed with an anti-phospho eEF2 antibody. Loading controls (as in panel A) established equal loading of samples in each lane (data not shown).

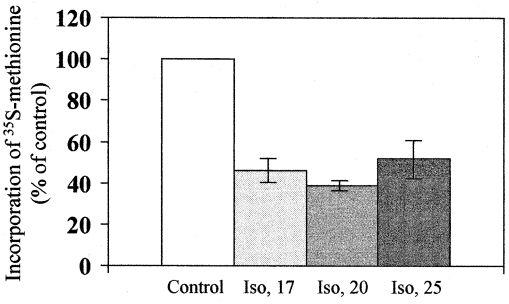

Fig. 2. Effect of isoproterenol on the rate of protein synthesis in ARVC. Overnight cultures of ARVC were incubated for 2 h in methionine-free medium before addition of isoproterenol for the specified times. The times indicate the end of the 15 min incubation with [^{35}S]methionine. Cells were extracted at this time point, and the protein concentration of each extract was assayed. The incorporated radioactivity was counted in a scintillation counter, and corrected for the protein concentration of the extract. Assays were performed in triplicate plates for each condition, and each plate was assayed in duplicate. The data shown are representatives of two further experiments performed.

3. Results

3.1. Isoproterenol elicits phosphorylation of eEF2 in ARVC via the β-adrenergic receptor

Isoproterenol is a potent β-adrenergic agonist that increases cAMP levels in the heart. In ARVC it brought about a rapid increase in phosphorylation of eEF2 as analysed using an anti-serum that specifically recognises eEF2 when it is phosphorylated as the main site of phosphorylation, Thr56. In 10 separate experiments, isoproterenol increased the phosphorylation state of eEF2 (Fig. 1A), the increase being evident as early as 1 min after treatment of the cells with isoproterenol. The level of eEF2 phosphorylation remained elevated for at least 20 min after isoproterenol stimulation. We have previously shown that insulin decreases the level of eEF2 phosphorylation in ARVC [3] (see also Fig. 1A). It was therefore of interest to study whether isoproterenol still increased eEF2 phosphorylation in cells pretreated with insulin. Addition of isoproterenol to insulin-treated ARVC led to a substantial rise in eEF2 phosphorylation which increased almost to the levels observed in cells which had not been pretreated with insulin, i.e. more markedly than in control cells where there is significant basal phosphorylation of eEF2. Thus, the effect of isoproterenol is clearly dominant over the effect of insulin.

To verify that the effects of isoproterenol were mediated through the β-adrenoreceptor, we employed the β-specific antagonist, propranolol. This compound completely blocked the ability of isoproterenol to induce phosphorylation of eEF2 in control or insulin-pretreated AVRC (Fig. 1B).

3.2. Artificially elevating cAMP increases eEF2 phosphorylation in ARVC

Since isoproterenol increases cAMP levels by activating adenyl cyclase, it was likely that effect of isoproterenol upon the phosphorylation of eEF2 was mediated by cAMP. To investigate this, we employed strategies designed to increase the intracellular cAMP level in ARVC. Treatment of ARVC with the cAMP analogue chlorophenylthio-cAMP (CPT-cAMP) also induced the phosphorylation of eEF2, albeit over a somewhat longer time-scale than isoproterenol (Fig. 1C). This indicates that artificial increases in cAMP can cause

this effect. As an alternative way to increase intracellular cAMP concentrations, we treated ARVC with forskolin, which activates adenyl cyclase. This results in increased phosphorylation of eEF2 (Fig. 1C), to a greater extent than CPT-cAMP. Taken together, these data strongly suggest that the effect of isoproterenol is mediated through cAMP rather than any other possible downstream consequence of activating β-adrenoreceptors.

3.3. Increased phosphorylation of eEF2 correlates with inhibition of protein synthesis in ARVC

To study whether the treatment of cells with isoproterenol affected the rate of protein synthesis, we measured the incorporation of [^{35}S]methionine into newly synthesised protein in ARVC. Treatment with isoproterenol markedly reduced the protein synthesis as compared to untreated controls (Fig. 2). Thus, the isoproterenol-induced increase in eEF2 phosphorylation is associated with inhibition of overall protein synthesis, consistent with the fact that phosphorylation of eEF2 inactivates it. The standard procedures to assess whether elongation is indeed inhibited – i.e. transit time measurements or polysome profiles – could not be carried out using ARVC due to the very small amounts of radiolabel incorporated and/or insufficient quantities of isolated cells available.

3.4. Isoproterenol and CPT-cAMP cause activation of eEF2 kinase in ARVC but do not increase its Ca/CaM-independent activity

In the case of eEF2 kinase from rabbit reticulocytes, phosphorylation by PKA increased its Ca/CaM-independent activity without increasing its activity measured in the presence of Ca/CaM [12]. Similarly, in both adipocytes and myeloid cells [13,14], treatments designed to increase intracellular cAMP led to a marked rise in the Ca/CaM-independent activity without discernibly altering the maximal activity of the kinase. In contrast, when eEF2 kinase activity was measured in extracts from ARVC treated with isoproterenol or CPT-cAMP, no Ca-independent activity was evident, as was also the case for untreated cells (Fig. 3).

3.5. Characteristics of eEF2 kinase in ARVC

It was clearly possible that isoproterenol activated a second isoform of eEF2 kinase which is distinct from the form(s) so far found in other tissues. To examine whether ARVC express more than one isoform of eEF2 kinase, extracts from control or isoproterenol-treated ARVC were fractionated by FPLC on Mono-Q and fractions were assayed for their ability to

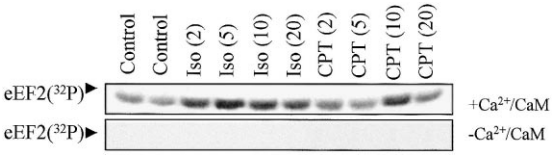

Fig. 3. Effect of isoproterenol or CPT-cAMP on eEF2K activity in ARVC. ARVC which had been cultured overnight were treated with 1 μM isoproterenol (Iso) or 0.5 mM CPT-cAMP (CPT) for the times indicated (in min). Extracts were prepared and assayed for eEF2K activity in the presence or absence of Ca^{2+}/CaM (as indicated). The intensity of the signals for the assays performed in the presence of Ca^{2+}/CaM was assessed by phosphorimage analysis (Fuji). The strength of each signal is expressed as a percentage of the control (numbers below each lane).

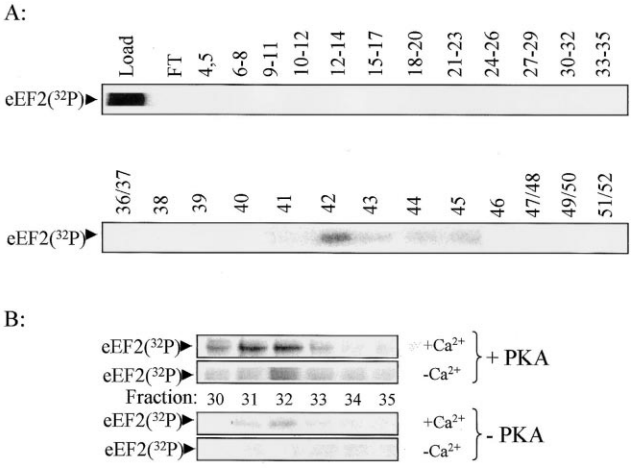

Fig. 4. Characteristics of eEF2K activity in ARVC. Panel A: ARVC which had been cultured overnight were treated for 5 min with isoproterenol (1 μM). The cell lysate was applied to a Mono-Q column which was developed with a gradient of KCl and fractions were collected. The fractions were assayed for eEF2K activity in the presence of Ca/CaM, along with the load (lysate) and the flow-through from the column. Certain fractions were pooled prior to assay, as indicated. Panel B: Freshly isolated ARVC were extracted and applied to a Mono-Q column which was developed with a gradient of KCl. Fractions were collected. Those containing the eEF2K activity were preincubated with cold ATP/Mg with or without PKA (as indicated, 20 mU in 20 μl total volume) for 20 min in the absence of Ca^{2+}/CaM. The fractions were assayed for eEF2 kinase activity using [γ-^{32}P]ATP in the presence or absence of Ca^{2+}/CaM, as indicated. Fraction numbers differ from those in panel A due to differences in the FPLC programme used, but peak eEF2 kinase activity eluted in the same position (KCl concentration) as in panel A.

phosphorylate eEF2. All the eEF2 kinase activity was retained on the column, which was then developed with a gradient of KCl up to 0.5 M. Only a single symmetrical peak of eEF2 kinase activity was observed in either case (Fig. 4A). The elution position of this peak is similar (in terms of ionic strength) to that of eEF2 kinase from other sources (e.g. reticulocyte lysates [18]). This indicates that it is unlikely that isoproterenol activates an additional form of eEF2 kinase or an alternative kinase that phosphorylates eEF2.

The peak fractions from the Mono-Q column from control cells were pretreated with PKA prior to assay for eEF2 kinase activity to examine whether, as for eEF2 kinase from other sources, this manipulation increased the Ca-independent activity of eEF2k. PKA treatment clearly markedly increased the maximal activity of cardiac eEF2 kinase (measured in the presence of Ca ions) but had no effect upon the Ca-independent activity (Fig. 4B). Thus, PKA does not elicit Ca-independent activity for eEF2 kinase from ARVC.

4. Discussion

The data presented here show for the first time that β-adrenergic stimulation leads to the phosphorylation of eEF2 in cardiomyocytes, an effect which is known to cause inactivation of eEF2. The phosphorylation of eEF2 correlates with inhibition of overall protein synthesis induced by β-adrenergic stimulation of the cells and it is likely that the inhibitory effect of β-adrenergic stimulation on protein synthesis is due (at least in part) to the increased phosphorylation of eEF2. Inhibition of protein synthesis under this condition will reduce the

consumption of ATP making more metabolic energy available to support cardiac contraction, which is activated by β-adrenergic agonists. Earlier work showed that glucagon inhibits protein synthesis in liver cells and that this involved reduced rates of peptide-chain elongation [19,20]. Similarly, the recent study of Diggle et al. [13] showed that β-adrenergic stimulation of adipocytes led to inhibition of both protein synthesis and rates of elongation. Thus, inhibition of elongation may be a general response to elevation of cAMP in mammalian cells, as a mechanism to divert either metabolic energy or metabolic precursors (e.g. amino acids in liver) away from protein synthesis and into other processes (contraction in heart or gluconeogenesis in liver). Elongation represents a more suitable point of inhibition here, since polysomes remain intact and translation could quickly be resumed once the requirement for energy or fuel has passed. Inhibition of initiation would, on the other hand, cause disaggregation of polysomes with likely differential effects upon the translation of different transcripts.

A striking difference between the findings of this study and those of earlier investigators is that here elevating cAMP does not alter the basal, Ca-independent, activity of eEF2k but rather increases its maximal activity measured in the presence of Ca/CaM. This effect was consistently observed in extracts of intact cells treated with β-adrenergic agonist, following treatment of cell extracts with purified PKA and when the partially purified eEF2 kinase from ARVC was treated with PKA. One plausible explanation for this difference from other cell types previously examined (reticulocytes, 3T3-L1 adipocytes, primary adipocytes, IPC-81 myeloid cells) is that ventricular myocytes express a different isoform of eEF2 kinase which has different regulatory properties. It could well be physiologically important that heart cells do not rely on changes in the Ca-sensitivity of eEF2 kinase in order to regulate elongation, since Ca levels fluctuate on a beat-to-beat basis in these cells, unlike many other cell types where they are only raised acutely in response to growth factors, hormones or other stimuli. Altering the maximal activity of eEF2 kinase would thus offer a more rational basis for affecting eEF2 phosphorylation in ARVC.

The idea that different cell types may possess different forms of eEF2 kinase is not entirely new, although this report provides the first indication that different isoforms may have distinct regulatory properties. Hait et al. [21] have previously provided immunological evidence that certain tissues express distinct forms of eEF2 kinase. For example, an antibody raised to eEF2 kinase failed to detect a protein of the expected size in several tissues (heart was not analysed) which possess high levels of eEF2 kinase activity. In this context, it is notable that we were unable to obtain any signal for eEF2 kinase on Western blots for the peak fractions from the FPLC purification experiments described here using any of three different antisera to eEF2 kinase from other tissues. These were raised either to the whole eEF2 kinase protein (expressed in *Escherichia coli* using the muscle cDNA described in [22]) or two peptides who were based on parts of the translated amino acid sequence of this cDNA. This observation is consistent with the existence in cardiomyocytes of a distinct isoform of eEF2 kinase, although further work will be required to clarify this.

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