cAMP mediated proteolysis of the catalytic subunit of cAMP-dependent protein kinase

Brian A. Hemmings

Friedrich Miescher-Institut, PO Box 2543, CH-4002 Basel, Switzerland

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The cAMP-dependent protein kinase from LLC-PK_1 cells can be activated in vivo by calcitonin and vasopressin, or forskolin. Continuous treatment of cells with these agents results in a decrease of total cAMP-PK activity. The loss of kinase activity was enhanced when either of these three agents was incubated in the presence of isobutylmethylxanthine. Results obtained using affinity purified antibodies to the catalytic subunit show that the loss of kinase was due to specific proteolysis of this subunit.

cyclic AMP *cyclic AMP dependence* *Protein kinase* *Hormone action* *Proteolysis*

1. INTRODUCTION

Hormonal regulation of cellular function through alterations of intracellular levels of cAMP is a basic control mechanism of all mammalian cells [1]. All the data so far available suggest that the cAMP-dependent protein kinase (cAMP-PK) mediates all the known effects of cAMP [2]. The holoenzyme of the cAMP-PK is a catalytically inactive tetramer composed of 2 regulatory (R) and 2 catalytic (C) subunits [3]. The R subunits bind 2 molecules of cAMP per monomer which results in the release of the catalytically active C subunits [4]. Two major classes (designated type I and II) of cAMP-PK are present in most mammalian cells, which have different R subunits but apparently identical C subunits [3].

Although the short-term effects of cAMP on the regulation of cAMP-PK have been intensively studied, very little information is known about the control mechanisms which modulate the total levels of either the type I or II kinase [5]. The amounts of type I and type II kinase synthesized in various tissues are different suggesting that the 2 isoenzymes have different biological functions. This hypothesis is supported by the fact that the ratio of type I and type II cAMP-PK changes in a number of cell lines during differentiation [5].

In this communication an additional in vivo mechanism for regulating the cAMP-PK is described. Long-term exposure to high levels of cAMP was found to result in a loss of kinase activity, which was apparently due to a decrease of C subunit protein.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Porcine epithelial cells, LLC-PK_1, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum at 37°C. Prior to hormone treatment or addition of drugs monolayer cultures were washed with DMEM (serum free) and then incubated in DMEM containing hormone or drug at 37°C.

2.2. *Enzyme assays*

The cAMP-PK was assayed using the synthetic peptide, Leu-Arg-Arg-Ala-Ser-Ala-Gly as substrate essentially as described in [6]. Protein kinase activity is expressed as units per mg protein (1 unit kinase activity is defined as the amount of enzyme

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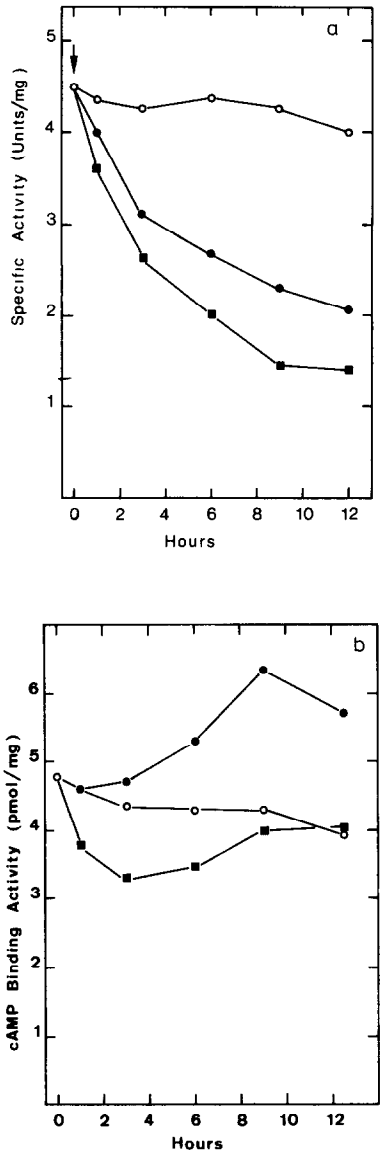
which catalyzes the transfer of 1 nmol phosphate from ATP to the peptide per min). cAMP binding activity was measured using the method of Gilman [7]. Protein concentrations were measured according to Bradford [8] with bovine serum albumin as standard.

Cell-free extracts were prepared in 20 mM Mes-NaOH, pH 6.8, containing 150 mM NaCl, 1 mM EDTA and 0.2% (v/v) Triton X-100. For the cAMP binding assay the Triton X-100 was omitted.



2.3. *Purification of cAMP-PK*

The C and R subunits of cAMP-PK were purified from bovine heart or skeletal muscle according to Beavo et al. [9].

2.4. *Preparation of antibodies specific for R_I, R_{II} and C subunits of cAMP-PK*

Purified subunits were cross linked to keyhole limpet hemocyanin according to Schwoch et al. [10] prior to injection into rabbits. Antisera were purified by $(NH_4)_2SO_4$ fractionation and chromatography on antigen-agarose columns.

2.5. *Immunoblot analysis*

Slab gel electrophoresis was performed on 10% separating gels in the presence of 0.1% SDS following the method of Laemmli [11]. The proteins were electrophoretically transferred to a nitrocellulose filter at 60 V for 3–4 h as described

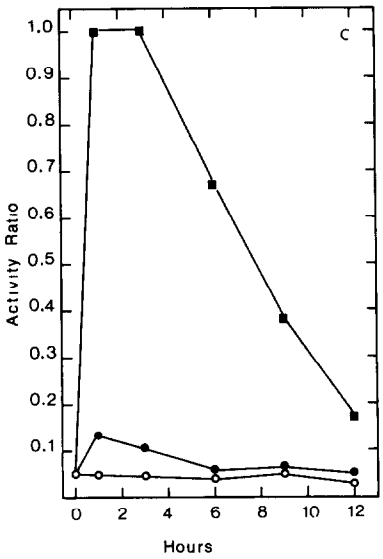


Fig.1. Effect of calcitonin in the presence or absence of IBMX on the total catalytic activity (a), activity ratio (b) and cAMP-binding (c), of the cAMP-PK from LLC-PK_1 cells. Cells were grown to 80% confluency in DMEM containing 10% (v/v) fetal calf serum. At the start of the experiment the cells were washed 3 times with DMEM and then incubated at 37°C in DMEM containing 10^{-8} M calcitonin (■) or 10^{-8} M calcitonin plus 0.5 mM IBMX (●). The control (○) was incubated in DMEM without any additions. At the times indicated cell free extracts were prepared and assayed for C and R subunits and the kinase activity ratio.

by Towbin et al. [12]. After transfer, nitrocellulose sheets were blocked using 3% (w/v) bovine serum albumin, 3% (w/v) ovalbumin and 1% (w/v) gelatin for 1 h. The proteins were visualized using the specific affinity purified antisera and ^{125}I-labeled protein A.

2.6. *Materials*

Dulbecco's modified Eagle's medium was from Gibco; fetal calf serum from Flow Laboratories; synthetic salmon calcitonin was a gift of Armour Pharmaceutical; dexamethasone was obtained from Sigma and [arginine]-vasopressin was purchased from Peninsula. Isobutylmethylxanthine, Kemptide, ATP, cAMP and Triton X-100 were from Sigma. [^{32}P]ATP and [^{3}H]cAMP and ^{125}I-protein A were obtained from Amersham. Phosphocellulose paper (P-81) was obtained from Whatman and nitrocellulose from Schleicher and Schull.

3. RESULTS

3.1. *Long treatment of LLC-PK$_1$ cells with agents that elevate cAMP levels*

Previously it was demonstrated that the addition of calcitonin, vasopressin or forskolin to monolayer cultures of LLC-PK$_1$ cells promoted a partial activation of the cAMP-PK which could be enhanced by the addition of IBMX to the culture medium ([13,14] also see fig.1b). These studies on the regulation of cAMP-PK in LLC-PK$_1$ cells have been extended to investigate the effect of long-term exposure of cells to hormones, forskolin and 8-bromo cAMP (BrcAMP). The data presented in table 1 show that treatment for 24 h of LLC-PK$_1$ cells with hormones, forskolin or BrcAMP resulted in a 20–40% decrease of total cAMP-PK activity. The simultaneous addition of IBMX (0.5 mM) with these compounds resulted in a 70% decrease of total cAMP-PK activity. The addition of dexamethasone, a steroid hormone which is known to modulate cAMP action in LLC-PK$_1$ cells [13], had no effect on the loss of C subunit activity (table 1).

The activity ratio of the cAMP-PK (the level of free C subunit relative to total cAMP-PK activity), the loss of total kinase and R subunit levels were investigated in more detail following calcitonin treatment in the presence or absence of IBMX

Table 1

Effect of various hormones and drugs on the in vivo stability of cAMP-dependent protein kinase

Treatment	cAMP-PK activity (units/mg protein)	% decrease
Expt I		
Control	4.55	0
Dexamethasone (10^{-7} M)	4.06	11
Calcitonin (3×10^{-8} M)	2.51	45
Dexamethasone (10^{-7} M) plus calcitonin	2.49	45
Calcitonin (3×10^{-8} M) plus IBMX (0.5 mM)	1.25	73
Dexamethasone (10^{-7} M), calcitonin (3×10^{-8} M) and IBMX (0.5 mM)	1.18	74
Expt II		
Control	4.38	0
IBMX (0.5 mM)	2.70	38
Arg^8-vasopressin (3×10^{-8} M)	2.91	34
Arg^8-vasopressin (3×10^{-8} M) plus IBMX (0.5 mM)	1.48	66
Forskolin (50 μM)	3.49	20
Forskolin (50 μM) plus IBMX (0.5 mM)	1.26	72
Expt III		
Control	4.63	0
IBMX (0.5 mM)	3.29	29
0.1 mM 8-BrcAMP	3.76	
0.1 mM 8-BrcAMP plus IBMX (0.5 mM)	2.22	52
0.5 mM 8-BrcAMP	2.93	36
0.5 mM 8-BrcAMP plus IBMX (0.5 mM)	1.43	69
1.0 mM 8-BrcAMP	2.14	54
1.0 mM 8-BrcAMP plus IBMX (0.5 mM)	1.25	73

Monolayer cultures of LLC-PK$_1$ cells were grown to ~80% of confluency, washed 3 × with prewarmed DMEM and then incubated at 37°C for 24 h in DMEM containing the indicated additions. Total cAMP-PK levels were determined as described in section 2. The above data are representative of several experiments that gave similar results

(fig.1a and b). These data show 2 important aspects of this regulatory mechanism: (i) the loss of kinase is relatively rapid ($t_{1/2}$ 5 h) and (ii) following the initial activation of the cAMP-PK, during the first 3 h of hormone stimulation, the levels of free C subunit decline. This latter result suggests that the synthesis of cAMP was down regulated. Determination of total R subunit levels by the [^{3}H]cAMP binding assay showed that the amount of cAMP binding activity remained relatively constant during the period when C subunit declined most rapidly (fig.1c). In fact, with the calcitonin treatment an increase of cAMP binding activity was observed.

3.2. *Investigations on the mechanism of loss of cAMP-PK activity*

Antibodies to purified bovine C, R_I and R_{II} subunits were prepared in rabbits and used to quantitate cAMP-PK subunit levels, by Western blotting, during prolonged hormone treatment. The 3 antibody preparations were affinity purified on antigen-agarose columns and found to cross react with purified porcine C, R_I and R_{II}.

Western blot analysis was used to quantitate C subunit levels following treatment with calcitonin plus or minus IBMX (fig.2). This analysis revealed that the levels of immunoreactive protein declined in parallel with the loss of kinase activity. After 12.5 h of treatment only ~25% of C subunit protein remained as judged by densitometric quantitation.

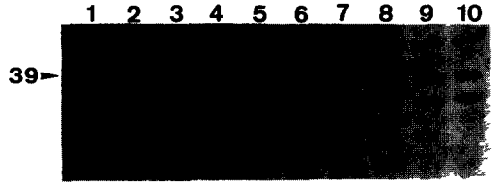


Fig.2. Determination of catalytic subunit levels in cell extracts following calcitonin treatment (in the presence or absence of IBMX). Cells were grown and treated as indicated in fig.1. Lane 1: 90 ng purified bovine skeletal muscle C subunit. All other lanes contain 225 μg cell extract. Lanes 2–4: control at 0, 6 and 12.5 h, respectively. Lanes 5–7: cells treated with calcitonin for 3, 6 and 12.5 h, respectively. Lanes 8–10: cells treated with calcitonin for 3, 6 and 12.5 h, respectively.

4. DISCUSSION

The data presented show that the cAMP-PK from LLC-PK_1 cells is regulated by 2 different mechanisms, (i) through changes in intracellular cAMP levels and (ii) by specific proteolysis.

In a previous publication [13] it was demonstrated that the activation (dissociation of the holoenzyme) and the inactivation (the reassociation of the R and C subunits) following hormone treatment are very rapid ($t_{1/2}$ for both processes of around 10 min), confirming that the activation state of cAMP-PK is rapidly responsive to fluctuations of cAMP levels caused by hormonal stimulation.

However, under conditions that lead to a long-term activation of cAMP-PK an additional control mechanism exists for regulating C subunit levels which involves proteolytic inactivation of this enzyme. Under normal growth conditions, prior to hormonal stimulation, the cAMP-PK was maintained predominantly in the holoenzyme form and not subject to proteolysis. Following the addition of hormones or compounds which elevate intracellular cAMP, free C subunit levels are increased to 100% of total C subunits, resulting in the loss of C subunit activity and immunoreactive protein. The R subunit did not appear to be rapidly degraded under these conditions.

Recently Shaltiel and co-workers [14,15] have described a C subunit specific protease which cleaves the native C subunit, when it is dissociated from R, to produce an inactive enzyme of 37 kDa. Whether a similar protease as that described above is present in LLC-PK_1 cells is currently being investigated. However, the immunoblot data suggest that a clipped form of the C subunit does not accumulate in vivo.

What is the physiological function of this control mechanism? It is possible that it represents a safety control mechanism which would normally operate during the down regulation phase of the cellular response to hormone stimulation. Such a mechanism would reduce free C subunit levels which might otherwise be inhibitory to normal cell growth. Recently mutant LLC-PK_1 cells have been isolated which are not growth inhibited by cAMP (unpublished). The level of C subunit activity in these cells is around 5% of that in the parent strain.

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