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Catalytic subunit of cAMP-dependent protein kinase is essential for cAMP-mediated mammalian gene expression

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Cyclic AMP-stimulated mRNA levels in cultured rat hepatocytes were inhibited by three different inhibitors of cAMP-dependent protein kinase activity: (i) Rp-cAMPS, a cAMP analog with a sulfur substitution at the equatorial oxygen of the cyclic monophosphate; (ii) H8, an isoquinoline sulfonamide derivative; and (iii) PKI, a 20-amino acid synthetic peptide of the Walsh protein kinase inhibitor. These inhibitors specifically blocked the cAMP-stimulated increase in mRNA for tyrosine aminotransferase and phospho*enol*pyruvate carboxykinase; they had no effect on the level of albumin mRNA which is not cAMP regulated. These results provide functional evidence that kinase activity involving protein phosphorylation is required in cAMP-mediated gene expression in mammalian cells.

Protein kinase subunit inhibitor; cyclic AMP-stimulated transcription; Tyrosine aminotransferase; Phospho*enol*pyruvate carboxykinase; (Hepatocyte)

1. INTRODUCTION

cAMP-regulated gene expression has been demonstrated both in procaryotic and eucaryotic cells, however, the mediators of cAMP action in these cell types are strikingly different. In *E. coli* cAMP binds to a cAMP receptor protein (CRP or CAP) and activates the expression of a series of genes for enzymes of sugar catabolism [1]. Eucaryotic cells contain an analogous cAMP receptor protein which has amino acid homologies to CRP, that is, the regulatory subunit (R) of cAMP-dependent protein kinase (cAMP-PK) [2]. The protein kinase however also consists of a catalytic subunit (C) which is the prime mediator of most cAMP actions in eucaryotes [3]. Nevertheless, it has been recently reported that in the lower eucaryote *Dictyostelium discoideum* a cell surface receptor for cAMP, not protein kinase, mediates gene expression [4,5]. Cell surface R has been reported in mammalian cells, and R has been shown to be associated with both soluble and membrane fractions, as well as associated with intracellular structures including the cytoskeleton in the area of microtubules and Golgi [6-8]. In addition, there are at least three distinct types of R subunits which are products of different genes [9,10]. The greater complexity of the cAMP mediator in mammalian cells has necessitated the investigation of whether the regulatory or catalytic subunit of cAMP-PK is essential for the mechanism of cAMP-regulated gene expression in these cells. cAMP increases mRNA synthesis for a number of mammalian proteins [11,12] including two in liver, tyrosine aminotransferase (TAT) [13,14] and phospho*enol*pyruvate carboxykinase (PEPCK) [15], which were examined here. The results indicate that protein phosphorylation by the catalytic subunit is required for cAMP stimulation of mRNA for these enzymes. These data have been presented in abstract form [16].

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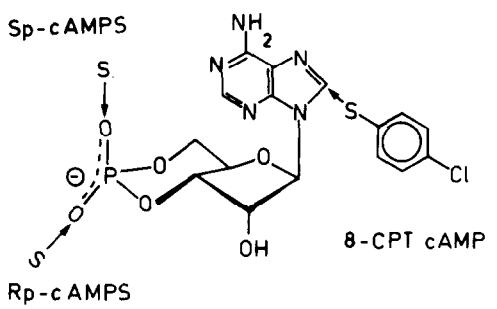
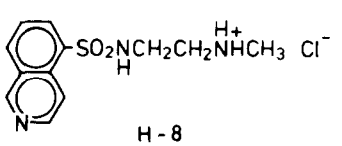
2. MATERIALS AND METHODS

2.1. *Kinase activators and inhibitors (structures in fig.1)*

Activators of cAMP-dependent protein kinase used were 8-chlorophenylthio-cAMP (8CPT-cAMP) and Sp-cAMPS, a cAMP analog with a sulfur substitution at the axial oxygen of the cyclic monophosphate. 8CPT-cAMP was used in most experiments since its properties are superior to cAMP for studies on intact cells, i.e., it is more lipophilic [17] and more resistant to hydrolysis by phosphodiesterase [18]. These properties also apply to the agonist Sp-cAMPS which was used in experiments in which the antagonist Rp-cAMPS was examined. Sp-cAMPS has a lower affinity than 8CPT-cAMP for cAMP-PK [19] and can be more readily competed against by Rp-cAMPS [20]. Rp-cAMPS is an inhibitor of both type I and type II cAMP-PK in vitro and in vivo [19], is slightly more lipophilic than cAMP [17], at high concentrations can inhibit cAMP and cGMP hydrolysis by phosphodiesterase [21] and is itself not hydrolyzed by any mammalian phosphodiesterase so far tested [18]. The effectiveness of Rp-cAMPS as a cAMP-PK antagonist in intact hepatocytes has been demonstrated [20].

Rp-cAMPS inhibits cAMP-PK by preventing holoenzyme dissociation, whereas two other inhibitors, used directly, inhibit kinase free C activity. H8, an isoquinoline derivative which mimics the adenine of ATP, inhibits cAMP-PK, as well as other protein kinases, to various extents [22]. Two other inhibitors, each 20-amino acid synthetic peptides representing the active domain of the Walsh protein kinase inhibitor (PKI) which acts as a competitive pseudosubstrate of cAMP-PK [23], were constructed and are referred to as PKI-1 and PKI-2. These inhibitors bind to and block the kinase substrate recognition site but do not themselves serve as substrates since they lack the essential serine to be phosphorylated, and rather contain alanine at that site. Other compounds used as control peptides unable to act as such strong inhibitors of cAMP-PK included Kemptide, a synthetic substrate, and the peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$.





L R R A S L G Kemptide
I A S G R T G R R N A I H D I L V S S A PKI 1
T T Y A D F I A S G R T G R R N A I H D PKI 2

Fig.1. Structures of kinase activators and inhibitors. The cAMP-PK substrate recognition site is underlined in Kemptide. PKI-1 and PKI-2 inhibitors lack serine at the obligatory position for phosphorylation.

2.2. *Hepatocyte isolation and culture*

Hepatocytes were prepared from adult male Sprague-Dawley rats (200–300 g) using a modification [24] of the collagenase perfusion method of Seglen [25]. Isolated cells (5×10^6) were cultured in 10 cm Falcon tissue culture dishes for 48 h at 37°C in Ham's F-12 medium (Biochrom) containing 10% fetal calf serum (FCS), insulin (1 μg/ml), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Four hours prior to experiments, cells were changed to medium without FCS and insulin. Thereafter, cells were treated as described for individual experiments in the figure legends. In all experiments, inhibitors (either H8, PKI or Rp-cAMPS) were added to cells 30 min before stimulants were added for an additional 3 h. 8CPT-cAMP was obtained from Boehringer, Kemptide and the peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ from Peninsula, and H8 from Dr Hugo De Jonge (Rotterdam). Sp-cAMPS and Rp-cAMPS were synthesized in our laboratory (Jastorff, B.). PKI-1 and PKI-2 were synthesized by Dr Dieter Palm (Würzburg).

2.3. *Measurement of mRNA levels by Northern blot analysis*

Total cellular RNA was extracted using a LiCl-urea method [26], applied to agarose gels and transferred to nitrocellulose for Northern blot hybridization analysis as described [27,28]. The mRNA levels for TAT, PEPCK and albumin were measured by hybridization of ^{32}P-labeled complementary RNA transcripts made from SP6 plasmids containing the respective cDNAs as described [27,28]. Radioactively labeled mRNA was visualized by exposure of nitrocellulose blots to Kodak XAR-5 film with intensifiers, and was quantitated by liquid scintillation counting of radioactive bands cut from the blots.

3. RESULTS

3.1. *Inhibition of cAMP-stimulated mRNA levels by H8*

In preliminary experiments (not shown), the stimulation of mRNA for TAT in hepatocytes was observed to be maximal at 3 h of incubation with 20 μM 8CPT-cAMP. 3 h incubations with 50 μM 8CPT-cAMP were used for examining inhibitors of mRNA levels in the following experiments. A 20-fold stimulation of $mRNA^{TAT}$ levels above control was observed in cells treated with 50 μM cAMP and this was half maximally inhibited by 40 μM H8 (fig.2). This inhibitor concentration observed in intact cells appears reasonable considering the reported in vitro K_i value of H8 for

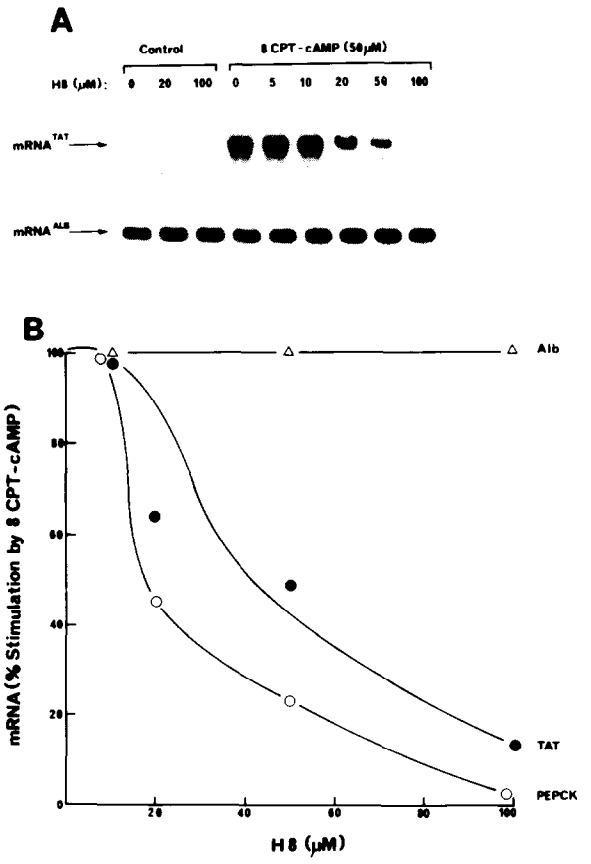


Fig.2. Northern blot (A), and its quantitation (B), of the mRNA level for TAT, PEPCK and albumin in hepatocytes in response to 8CPT-cAMP, in the absence or presence of increasing concentrations of H8.

cAMP-PK of 1.2 μM [22] and that which we observed (3.5 μM) using an in vitro protein kinase assay with purified C and Kemptide as substrate (not shown).

The mRNA level of PEPCK, another cAMP-regulated enzyme in rat liver, was also analyzed and a similar inhibition by H8 as that observed for the mRNA of TAT was found (fig.2B). In contrast, the level of mRNA for albumin, which is not regulated by cAMP, was unchanged by the treatment of hepatocytes with H8 (fig.2).

3.2. *Inhibition of cAMP-stimulated mRNA levels by PKI-1 and PKI-2*

PKI-1 corresponds to the 20-amino acid synthetic peptide described as having a K_i value of 0.8 μM [29], and which we determined in our experiments to be 0.7 μM using an in vitro protein kinase assay with purified C and Kemptide as substrate (not shown). PKI-2 also contained 20 amino acids, but compared to PKI-1, its sequence started 6 amino acids closer to the NH_2-terminal end of the Walsh protein, and it had a reported K_i value of 0.3 nM, or 5 nM in our assay, as analyzed by Henderson plot analysis of tight-binding inhibitors [23]. 1000-fold higher concentrations were required for equal inhibition of cGMP-PK compared to cAMP-PK (not shown), which is consistent with other published data [30]. Concentrations between 200 μM and 1 mM of both PKI-1 and PKI-2 inhibited the cAMP-stimulated mRNA levels for

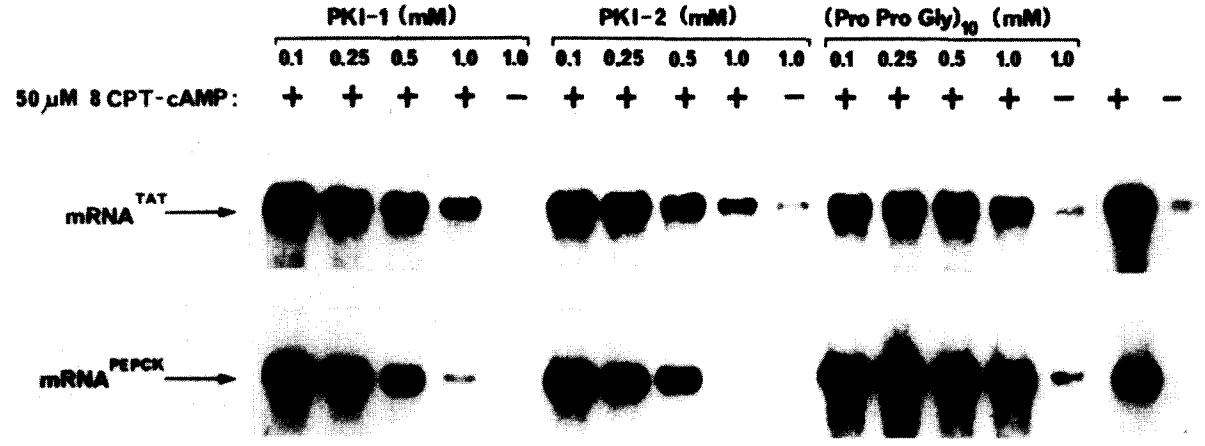


Fig.3. Northern blot showing stimulation of mRNA levels for TAT and PEPCK by 8CPT-cAMP and its dose-dependent inhibition by the active peptide fragments, PKI-1 and PKI-2 of the Walsh inhibitor. An equivalent concentration of another unspecific peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$ was an ineffective inhibitor. PKI-1 and PKI-2 did not reduce the mRNA levels of albumin (not shown).

TAT and PEPCK, although the inhibition by PKI-2 was somewhat greater (fig.3). As with H8, these peptides had no effect on the mRNA level of albumin which is not cAMP regulated. Other peptides were studied to determine the specificity of the PKI inhibition. Kemptide, for example, a 7-amino acid synthetic substrate of cAMP-PK ($K_m = 16\ \mu M$) was not a successful inhibitor of cAMP-stimulated mRNA levels for TAT or PEPCK (not shown). Another unrelated peptide of comparable molecular mass to PKI-1 and PKI-2, $(Pro\text{-}Pro\text{-}Gly)_{10}$ (fig.3) had little or no effect on these mRNA levels. The inhibition by PKI-1 and PKI-2 was dose dependent and was 80–90% complete at 1 mM PKI-2. The proposed mechanism of entry of PKI into hepatocytes, and the small fraction of the administered PKI which most likely reaches the intracellular protein kinase are considered in section 4.

3.3. *Inhibition of cAMP-stimulated mRNA levels by Rp-cAMPS*

Whereas H8 and PKI are inhibitors of kinase catalytic activity, Rp-cAMPS is a cell permeable, competitive antagonist for cAMP binding to R of cAMP-PK, and R occupation by Rp-cAMPS prevents the dissociation of cAMP-PK [31]. Using the Kemptide assay and Sp-cAMPS stimulation of cAMP-PK, Rp-cAMPS was determined to have a K_i value of $5\ \mu M$ in vitro (not shown). In intact hepatocytes in culture, $200\ \mu M$ Rp-cAMPS produced nearly complete inhibition of the increase in mRNA for TAT (fig.4) or PEPCK (not shown) in response to $10\ \mu M$ Sp-cAMPS. A residual level of

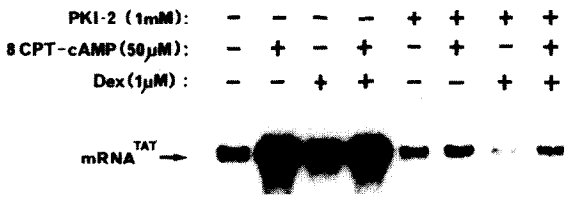


Fig.5. Inhibition of both 8CPT-cAMP and dexamethasone stimulated $mRNA^{TAT}$ levels by PKI-2. Stimulation by maximal doses of both 8CPT-cAMP and dexamethasone together (in the absence of PKI-2) were not additive.

mRNA remained, however, similar to the level observed when Rp-cAMPS was present alone. Also shown in fig.4 is the stimulation of mRNA for TAT by dexamethasone which was not inhibited by Rp-cAMPS. This lack of inhibition of dexamethasone's effect by Rp-cAMPS has also been observed by others [28], and was in contrast to the inhibition of dexamethasone by H8 (not shown) and by PKI (see section 3.4).

3.4. *Stimulation of $mRNA^{TAT}$ by dexamethasone and 8CPT-cAMP are not additive*

The question of whether dexamethasone and 8CPT-cAMP influence the mRNA levels for TAT by completely separate mechanisms was investigated. Maximal concentrations of both agents used together were not additive (fig.5), suggesting that some common element may be involved in both pathways of stimulation. PKI-2 inhibition of either agent alone, or in combination, was observed (fig.5).

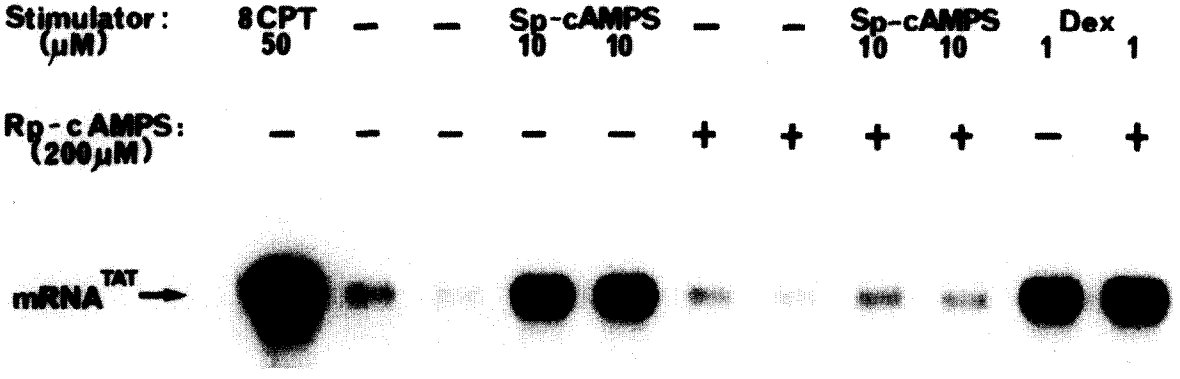


Fig.4. Northern blot analysis of the antagonism of Rp-cAMPS on the $mRNA^{TAT}$ level stimulated by Sp-cAMPS. Rp-cAMPS did not antagonize dexamethasone-stimulated levels.

4. DISCUSSION

Three inhibitors of cAMP-PK acting by different mechanisms decreased the level of cAMP-stimulated mRNA for TAT and for PEPCK. H8 and PKI inhibit the catalytic activity of C whereas Rp-cAMPS prevents the dissociation of the kinase holoenzyme and thereby the formation of the active free C. Results with all three inhibitors support the primary importance of cAMP-dependent protein kinase activation in cAMP-regulated gene expression. Furthermore the results obtained with H8 and PKI indicated that C-dependent phosphorylation is clearly required for cAMP stimulation of mRNA levels. Although H8 can inhibit other protein kinases besides cAMP-PK [22], in the experimental setting being used here, such kinases would not contribute to the effects observed since they would not be cAMP stimulated.

Additionally, PKI was tested since it is a specific peptide inhibitor containing an arginine cluster that mimics the well-established basic amino acid subsite that is the primary recognition site for substrates of cAMP-PK. Two lines of evidence indicated that the inhibition by PKI of mRNA levels for TAT and PEPCK was specific. First, mRNA levels for albumin were unchanged implying that PKI did not have a general deleterious effect on the cells. Second, other peptides of similar size did not inhibit the mRNA for TAT or PEPCK. High concentrations of PKI were needed since it is expected that this size molecular mass peptide does not efficiently enter cells. However, it has been possible to introduce molecules as large as a monoclonal antibody [32] and DNA [33] into cells that endocytose. Small amounts enter the cell, and a small fraction of that avoids digestion, and in the case of DNA can even eventually enter the nucleus and result in expression of new genes [33]. A recent preliminary report presented in abstract form [34] also indicated that intracellular expression of a minigene coding for a fragment of PKI could inhibit the activity of a fusion gene containing the cAMP-regulated promoter for enkephalin and the chloramphenicol acetyltransferase reporter gene.

Both H8 and PKI were able to inhibit the dexamethasone stimulation of TAT and PEPCK mRNA levels (not shown). It is possible that cAMP plays a permissive role in the effect of dexamethasone on gene expression and that there is some intermediate factor common to both pathways of stimulation. This was suggested by experiments in which the effects of 8CPT-cAMP and dexamethasone were shown not to be additive. Dexamethasone-stimulated mRNA levels were resistant to inhibition by Rp-cAMPS perhaps because this was not a strong enough inhibitor of cellular endogenous cAMP to totally block the permissive effect of cAMP. Also, synthetic Rp-cAMPS itself had a certain basal stimulatory activity since it appeared not to be completely free of contamination by cAMP (see also [27]). This was particularly obvious in in vitro protein kinase assays. Alternatively, in intact cell experiments Rp-cAMPS may also elevate cAMP levels due to its ability to inhibit phosphodiesterase [21].

The data presented indicate that unlike the mechanism of cAMP-mediated gene expression in procaryotes and in a lower eucaryote, *Dictyostelium discoideum*, the mechanism in mammals requires C activity and a potential phosphoprotein intermediary factor. The proposed cAMP regulatory element consensus sequence suggested for several genes [11,12,35] thus most likely binds a protein whose phosphorylation is regulated by C. One such protein has been postulated to be a 40 kDa nuclear protein [36]; functional studies will be needed to test this. Although R-II is also a prominent substrate for C subunit, our data (Büchler, W., Walter, U., Lohmann, S.M., Schmid, W. and Schütz, G., unpublished) do not support a direct role for R-II as a DNA regulatory protein; no specific binding of either phospho- or dephospho-R-II to TAT promoter regions was detected.

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