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Effects of Adenine Nucleotides on Choriogonadotropin α and β Subunit Synthesis

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The α subunit of human chorionic gonadotropin (CG) contains a discrete cAMP response element in the 5' flanking region of the gene. Since cAMP also stimulates the synthesis of the CGβ subunit the presence of a cAMP *cis* element in the CGβ gene was examined. Deletion mutants bearing various lengths of CGβ 5' region in front of the chloramphenicol acetyl transferase (CAT) gene were transfected in placental tumor cells. No discrete cAMP response element could be identified. Unexpectedly we also observed that AMP and adenosine not only stimulated CAT activity driven by CGβ promoter sequences but also enhanced synthesis of CGα and β subunits in cultured choriocarcinoma cells. GMP, CMP, guanosine, and cytosine were inactive at comparable concentrations. These data suggest that the response of the CGα and β genes to the non-cyclic adenine derivatives occurs by a mechanism that differs from cAMP.

The placental hormone, human chorionic gonadotropin (hCG) is composed of two non-covalently-linked α and β subunits. The α subunit is encoded by a single gene (1), and the CGβ subunit is encoded by a multigene cluster of six homologous sequences (genes or pseudogenes) (2,3). Both subunits are developmentally regulated, i.e., α subunit production increases through the course of pregnancy, while β subunit synthesis is maximal at about the 11th week of gestation and declines thereafter (4). The factors regulating the CG subunit genes are unclear. There is evidence that activation of the CG subunit genes occurs at different steps in trophoblast differentiation (4). Cyclic AMP stimulates hCG production by enhancing the steady-state levels of the α and β subunit mRNAs. However, the increase of each subunit occurs with different kinetics (13). The α subunit gene contains an element in the 5' region which is conserved among a number of cyclic AMP responsive genes (5-8). This region contains a 18-base pair repeated sequence which is a binding site for one or more nuclear proteins (9-11). Previous identification of *cis* acting elements associated with the expression of the CGβ gene has been unsuccessful due to an inability to express the CGβ genes by transfection. Using constructs derived from a cosmid clone containing the entire CGβ cluster, we recently identified promotor sequences for one of the genes (12). Here, we report the effects of 8-Bromo cyclic AMP and related nucleosides on the expression of the CGβ gene. While the CGβ gene(s) were responsive to cyclic AMP, we were unable to detect a cAMP

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responsive element in the 5' flanking region of the gene. In addition, unexpectedly, we observed that several non-cyclic adenine nucleosides were also more potent in stimulating CGβ gene expression. These data suggest that the cAMP responsiveness of the CG subunit genes is through different mechanisms and other adenine nucleosides and nucleotides can stimulate CG synthesis.

Materials and Methods

Cell Culture

The choriocarcinoma cell line designated JAr was maintained in DME medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml) (12). Agonists were added on the day after replating (cell density 1 x 10^6 cells/75 cm^2) and incubated in 5% CO_2-Air incubator at 37° C for the indicated times.

Plasmids

Throughout this paper will refer to the CGβ genes according to the number assignments of Boorstein et al (14). CGβ gene 5 is expressed *in vivo* (15) and previous transfection experiments showed that it is the most actively expressed gene in the cluster (12,15).

Vectors containing the neomycin-resistance gene and the CGβ5 promoter region in front of CAT gene were used (see Fig. 1 in ref. 12). Successive deletion mutants bearing the CGβ5 5' region extending from the KpnI site (-3.5 Kb) or Acc 1 site (-634 bp) to the SpeI site (+103 bp) in the gene were created by exonuclease III digestion, as previously described (12). These vectors were transfected into JAr cells (8,12) and stable transformants were selected with the antibiotic G-418 (0.5 mg/ml).

Labeling and Immunoprecipitation

Cells cultured in 6 well plates were washed once with phosphate buffered saline (PBS) and incubated with cysteine-free media containing 25 μCi/ml [^{35}S]cysteine for 6 h. The media were transferred to new tubes and the cells were washed with PBS, and lysed in 0.4 ml of lysis buffer (50 mM Tris-HCl; pH 7.8, 150 mM NaCl, 5 mM EDTA and 0.6% SDS). The lysate was then diluted with 1.6 ml of lysis dilution buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA and 0.6% Triton X100) containing protease inhibitors (0.3 mg/ml phenylmethylsulfonyl fluoride and 0.3 mg/ml iodoacetamide). Intra- and extracellular material were immunoprecipitated and the proteins resolved on SDS gels as previously described (12).

CAT Assay

Chloramphenicol acetyltransferase activity in stably transfected JAr cells was determined as previously described (8,12). Twenty μg of cell extract was incubated with 0.1 μCi of ^{14}C Chloramphenicol for 6 h, extracted with ethyl acetate, and loaded on silica gel TLC plate.

Results

Effect of 8-Bromonucleot(s)ides on hCG Production by JAr cells

Synthesis of the α and β subunits of hCG was examined by labeling JAr cells with [^{35}S]cysteine. Aliquots of media were precipitated with antiserum against the native CGβ subunit. This antiserum precipitates hCG dimer which accounts for the appearance of the α subunit (Fig. 1, lane 8). Addition of 50 μM 8-Bromo cyclic AMP stimulated β subunit synthesis 7.5-fold, and the α subunit levels increased over 4-fold (lane 1). However, unexpectedly, we observed that 50 μM of 8-bromo 5'-AMP (lane 2) and 8-bromo adenosine (lane 3) were potent agonists for hCG synthesis. The effect is specific for adenine derivatives since 50 μM of 8-bromo cyclic GMP (not shown), 8-bromo GMP (lane 4), 8-

bromo guanosine (lane 5), 5-bromo CMP (lane 6) and 5-bromo cytidine (lane 7) were inactive. This stimulation was not a consequence of a general increase in protein synthesis, since no differences in the distribution of radioactivity in total protein derived from untreated and treated cells were seen (data not shown).

Effects of adenine derivatives on CGβ promoter-driven chloramphenicol acetyl transferase (CAT) constructs

Although the cAMP responsive regions in the α gene are well characterized (5-11), no such regulatory information is available for the CGβ gene. To assess if these agonists stimulate transcription of the CGβ subunit gene, the expression of the bacterial enzyme CAT was examined in JAr cells transfected with constructs containing the entire 5' flanking region of CGβ 5 linked to the CAT gene (see Materials and Methods). Since CAT is not synthesized in mammalian cells, this approach allows examination of the effects of the nucleosides on β transcription independent of their action on the endogenous β genes. CAT constructs bearing the CGβ5 promoter region (12) from KpnI site (-3.5 Kb) to SpeI site (+103 bp) (Fig. 3) were studied. The cells were exposed to adenosine derivatives for 24 hours and cellular extracts were incubated with [^{14}C]chloramphenicol and the acetylated derivatives quantitated by thin layer chromatography. As observed with [^{35}S]labeled subunits, adenosine (lane 4), 5' AMP (lane 3), cyclic AMP (lane 2), and forskolin (lane 1) enhanced CAT expression (Fig. 2). The minimal concentration of 8-bromo adenosine needed to stimulate CGβ5-CAT expression was 5μM; this approximates the concentration of the total adenine pool in the trophoblast which is 1 μM (16).

To address if cyclic AMP and the non-cylic derivatives act by the same mechanism, JAr cells were incubated in the presence of a 10 μM forskolin and 50 μM adenosine (Fig.

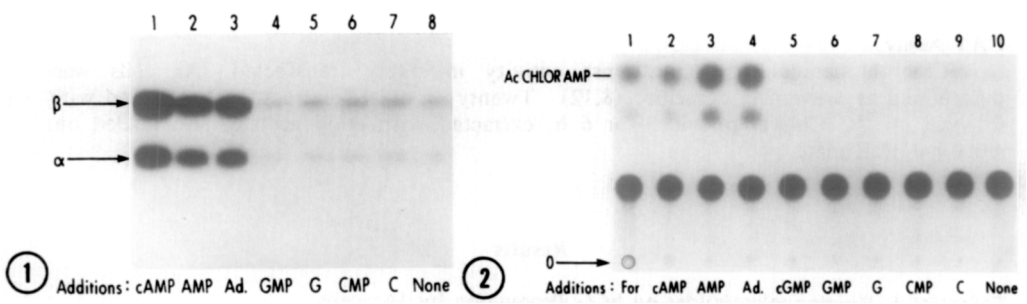

Fig. 1. CG subunits secreted by JAr cells treated with 50 μM of the indicated nucleotide. Cells were incubated with these compounds for 72 h before labeling with [^{35}S]cysteine. Protein was immunoprecipitated with antibody directed against the CGβ subunit. The agents used are: cAMP, 8-Bromo Cyclic AMP; AMP, 8-Bromo AMP; Ad, 8-Bromo Adenosine; GMP, 8-Bromo GMP; G, 8-Bromo Guanosine; CMP, 8-Bromo CMP; C, 8-Bromo cytosine.

Fig. 2. Expression of the chloramphenicol acetyl transferase (CAT) in JAr choriocarcinoma cells stably transfected with CAT gene linked to the CGβ gene 5 Kpn-Spe1 promoter fragment (See Materials and Methods). The cells were treated for 72h with 50 μM of nucleosides or 10 μM forskolin (FOR) and the extracts were assayed as described in methods section. Ad, adenosine; G, guanosine; C, cytosine. O corresponds to the origin of chromatography and AcChloramp corresponds to acetylated chloramphenicol, the product of CAT activity.

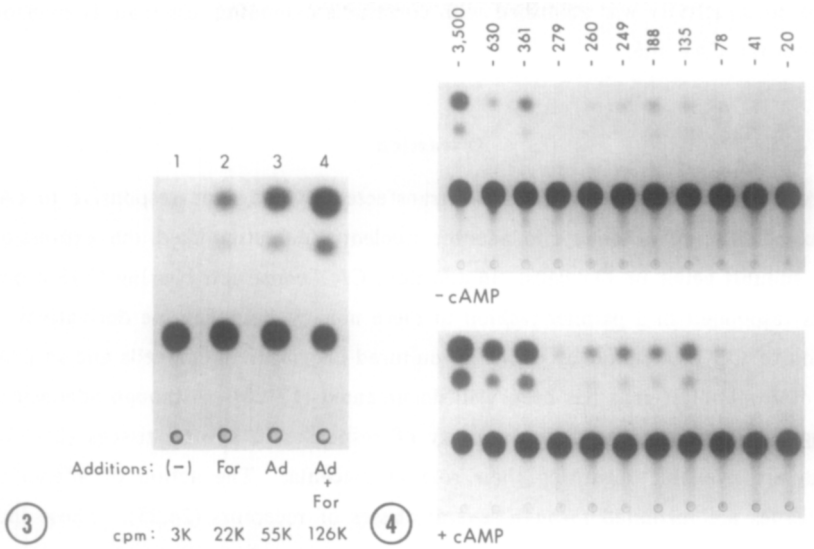

Fig. 3. CAT expression by JAr cells transfected with the CAT construct described in Fig. 2. The cells were incubated for 72 h with 50 μM of 8-bromo adenosine, 10 μM forskolin or both and extracts were prepared and assayed for CAT expression. Note that CAT activity is higher in cells treated with both compounds together than with either agent alone.

Fig. 4. Expression of the CAT gene in JAr cells in the absence or presence of cAMP. The cells were incubated as described in the legend to Fig. 3 stabile clones expressing exonuclease III-generated CGβ5 deletions were analyzed. The **numbers** on top of each lane indicate the number of nucleotides upstream from the cap site.

3). If they act through different mechanisms one might expect to see a synergistic effect when they are mixed together. Following electrophoresis the acetylated chloramphenicol derivatives were scraped from the plate and the radioactivity determined. Compared to the additive effects of each agent alone, there is 1.8 times greater enhancement when the two agonists are added together. These data suggest that cAMP and adenosine act by different mechanisms.

Expression of CAT driven by successive deletion mutants of CGβ5

It is well documented that the CGα gene contains cAMP response elements with the 200 bp 5' ward of the cap site. To determine if discrete sequences in the 5' flanking sequence of the CGβ5 gene are involved in the adenine stimulation, a series of deletion mutants at the 5' end of the β gene were produced. They were constructed so that each would have decreasing amounts of 5' flanking β DNA in an otherwise identical plasmid background. Thus, starting from the KpnI site (-3.5 Kb) to the cap site, constructs containing deleted 0.3-0.4 Kb segments, and ending at the SpeI site (+103 bp) were created by exonuclease digestion and transfected into JAr cells. Stable transformants were obtained (12) treated with cyclic AMP, forskolin or adenosine and the level of CAT expression determined. In contrast to the α gene, no discrete site corresponding to a cyclic AMP response element was identified in the β Kpn fragment. Using β constructs both the stimulated and unstimulated CAT activity parallel each other (Fig. 4) and under

both conditions, activity was abolished with constructs extending less than 41 nucleotides 5'ward from the CAP site.

Discussion

Here we show that, not only is the transfected CGβ 5 gene responsive to cAMP, but, unexpectedly, other noncyclic adenine nucleot(s)ides stimulated the expression of both CG subunit genes in JAr cells. In addition, CAT constructs bearing CGβ promoter sequences responded in a parallel fashion to these non-cyclic adenosine derivatives. The stimulation of CG production by cAMP in cultured choriocarcinoma cells and in primary cultures of trophoblast cells has been well documented (17-20). Although adenosine and its analogs are known to mediate a variety of responses in several tissues (21-23), no such data are available regarding their role in placenta. The action of adenosine in various tissues are mediated through several classes of receptors (24,25). These include (1) A1 and A2 receptors which are external receptors coupled to adenylate cyclase, (2) P2 receptors which are external and have an affinity for ATP, and (3) a P receptor, located intracellularly and also coupled to adenylate cyclase. The placenta apparently has at least two types of adenosine receptors, but they have yet to be classified (26,27). The effects of 8-bromo adenosine on trophoblast cells may be mediated through these receptors.

Both cyclic AMP and forskolin showed a distinct pattern in enhancing CGβ production compared to adenosine or AMP. Moreover, cells transfected with constructs bearing the CGβ promoter region in front of the CAT gene were more responsive to a combination of forskolin and adenosine than either compound used alone. Analysis of the CGβ gene deletion mutants showed that no discrete element is responsible for the response to cAMP. Thus, unlike the case for the α gene which contains a cAMP response element upstream from the promotor, cyclic AMP and adenosine stimulate CGβ production indirectly. Presumably, the CGβ gene(s) contain multiple *cis*-acting elements which may bind several overlapping regulatory proteins. Together, these data support previous evidence that the responses of the α and β genes to cAMP occurs via different mechanisms (13,20).

The physiological role of cAMP in regulating CG biosynthesis in placenta is still yet to be defined. Since 5' AMP is as effective an agonist as cAMP in stimulating hCG expression in JAr cells, the response is not restricted to cyclic derivatives. That exogenous adenosine derivatives (including ATP) change cell metabolism resulting in secondary effects cannot be excluded. For example, such agents may induce differentiation of progenitor trophoblastic cells into hCG producing cells. Since the concentration of adenosine nucleot(s)ides used here are at least 10-fold lower than in previous studies describing the effects of 8-bromo-cyclic AMP, our data suggest that adenosine may be an intracellular mediator associated with modulating synthesis of trophoblast proteins including CG during pregnancy.

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