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Cyclic AMP Regulation of Eukaryotic Gene Transcription by Two Discrete Molecular Mechanisms

Abstract. *In experiments designed to study the mechanism by which peptide hormones binding to their plasma membrane receptors stimulate the expression of specific genes, the transcription of two neuroendocrine genes, prolactin and growth hormone, was analyzed in a rat pituitary cell line. The results showed that cyclic adenosine monophosphate (cyclic AMP) stimulates the transcription of discrete subsets of eukaryotic genes by at least two independent molecular mechanisms. Cyclic AMP stimulated growth hormone gene transcription and phosphorylation of a 19,000-dalton nuclear protein; this appears to reflect direct nuclear actions of the cyclic AMP–dependent protein kinase. In contrast, the stimulation by cyclic AMP of prolactin gene transcription appears to reflect activation of a discrete calcium-dependent event.*

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Evolutionary conservation of critical regulatory signal systems has been reported for organisms as distantly related as bacteria and mammals. An example of such a conserved system is 3′,5′-adenosine monophosphate (cyclic AMP). The discovery of the cyclic AMP receptor protein (CRP) and its identification as the regulatory subunit of cyclic AMP–dependent protein kinase in eukaryotes (*1*) has led to its characterization as an important regulator of prokaryotic gene expression. In the presence of cyclic AMP, bacterial cyclic AMP receptor protein binds to specific DNA sequences in the promoter region of catabolite-sensitive operons (about 10 percent of the bacterial genome), permitting the binding of RNA polymerase and subsequent transcription (*2*). In eukaryotic organisms, cyclic AMP or hormones that stimulate adenylate cyclase influence the synthesis of various proteins (*3*). A direct effect of cyclic AMP at the level of gene transcription has been shown for several genes, including prolactin (*4, 5*), phosphoenolpyruvate carboxykinase (*6, 7*), lactate dehydrogenase (*8*), and possibly the tyrosine hydroxylase (*9*) genes. The two mammalian forms of CRP appear to have lost the DNA-binding domain characteristic of bacterial CRP (*10*), which suggests the importance of the catalytic subunit in regulation of gene transcription. Analysis of mutations of cyclic AMP–dependent protein kinase in several cell lines has suggested that most, and perhaps all, of the intracellular effects of cyclic AMP are mediated by cyclic AMP–dependent protein kinase (*11*). The effects of cyclic AMP on tyrosine aminotransferase gene expression are mediated by an activation of cyclic AMP–dependent protein kinase (*12*).

We have previously used the clonal rat pituitary cell line GH_4 to characterize the regulation of prolactin gene transcription by peptide hormones—thyrotropin-releasing hormone (TRH) and epidermal growth factor (EGF)—and cyclic AMP and to describe the hormone-dependent phosphorylation in the nucleus of a 23-kilodalton (kD) basic protein, referred to as BRP (*5, 13*).

Although TRH produces only small stimulations of adenylate cyclase (*14*), various cyclic AMP analogs and forskolin rapidly stimulate prolactin gene transcription, inducing an increase in transcription by a factor of 3 to 5 in less than 10 minutes (*5*). In the present study, the maximum stimulation of prolactin gene transcription produced by cyclic AMP was no more than 50 to 60 percent of that produced by the peptide hormone TRH (Fig. 1) and was not augmented by simultaneous addition of the calcium ionophore A23187 to mimic the increase in intracellular free calcium produced by TRH (*15*). Simultaneous treatment of GH_4 cells with maximally stimulating concentrations of TRH and forskolin produced the same stimulation of prolactin gene transcription as treatment with TRH alone. This is consistent with the possibility that the effects of TRH and forskolin are mediated by the same mechanism.

One such mechanism may be the activation of cyclic AMP–dependent protein kinase by TRH. Cyclic AMP–dependent protein kinase phosphorylates two subtypes of histone H1 at a single site (Ser^{37}) both in vivo and in vitro (*16*). Tryptic peptide maps of the combined histone H1 fraction showed a marked phosphorylation of the peptide containing Ser^{37}

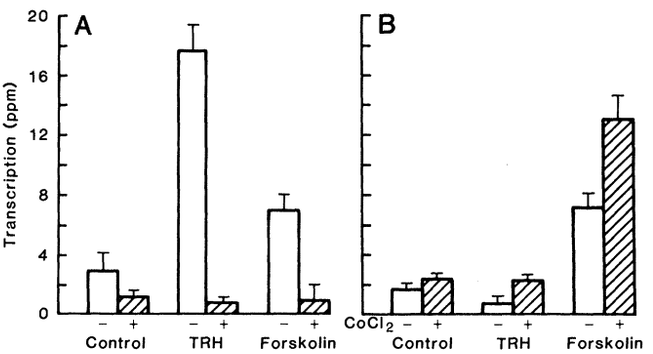


Fig. 1. Differential inhibition of forskolin-stimulated prolactin (A) and growth hormone (B) gene transcription. Rates of prolactin and growth hormone gene transcription were simultaneously measured in GH_4 cultures treated with TRH ($3 \times 10^{-7}M$ for 30 minutes) or forskolin ($10^{-6}M$ for 30 minutes). Where indicated (shaded bars), cobalt chloride ($CoCl_2$, 1 m*M*) was added 5 minutes before addition of TRH or forskolin. Prolactin gene transcription was measured by DNA excess hybridization of nascent RNA transcripts isotopically labeled by chain elongation in isolated nuclei as described (*5, 13*). Results are the average of triplicate determinations ± standard error of the mean. TRH, forskolin, and cobalt had no effect on the transcription rate of three constitutively expressed genes.

in forskolin-treated GH_4 cell cultures (Fig. 2A). Because TRH did not stimulate phosphorylation of histone H1 at Ser^{37} under conditions where prolactin gene transcription and BRP phosphorylation are maximally stimulated, it is unlikely that the nuclear effects of TRH are mediated by the activation of cyclic AMP–dependent protein kinase. However, treatment of GH_4 cells with forskolin stimulated phosphorylation of BRP (Fig. 3A) to an extent comparable to that reported for the response to TRH (*13*). Tryptic peptide maps of BRP isolated from cells treated with TRH or forskolin (Fig. 4) revealed an identical pattern of phosphopeptides, suggesting that cyclic AMP and TRH stimulate phosphorylation at the same sites; each site is a serine residue, as determined by phosphoamino acid analysis. The most plausible explanation of these data is that the binding of TRH to its plasma membrane receptor and the stimulation of cyclic AMP–dependent protein kinase produce an activation of a shared protein kinase that is distinct from the cyclic AMP–dependent protein kinase.

The effect of cyclic AMP on growth hormone gene transcription was evaluated by examining the response to forskolin and cyclic AMP analogs. Elevated amounts of intracellular cyclic AMP rapidly increased stimulation of growth hormone gene transcription in GH_4 cells by a factor of about 3, which is comparable to the increase seen in primary cultures of pituitary cells treated with forskolin (*17*) (Fig. 1). In contrast to the reciprocal effects of most hormonal agents on the biosynthesis of prolactin and growth hormone, increased amounts of intracellular cyclic AMP stimulated the synthesis of both prolactin and growth hormone (Fig. 1).

We have shown that the calcium antagonist cobalt chloride is a potent inhibitor of the stimulation of prolactin gene transcription by peptide hormones (*13*). These results are interpreted as defining

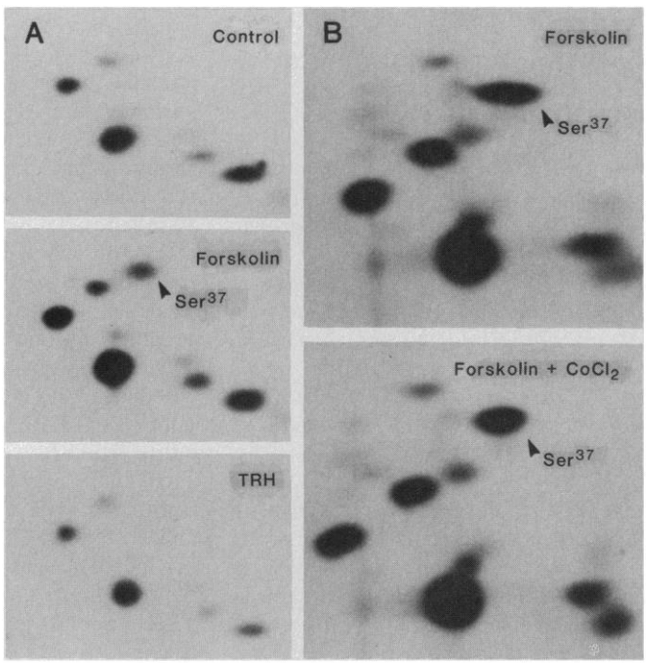
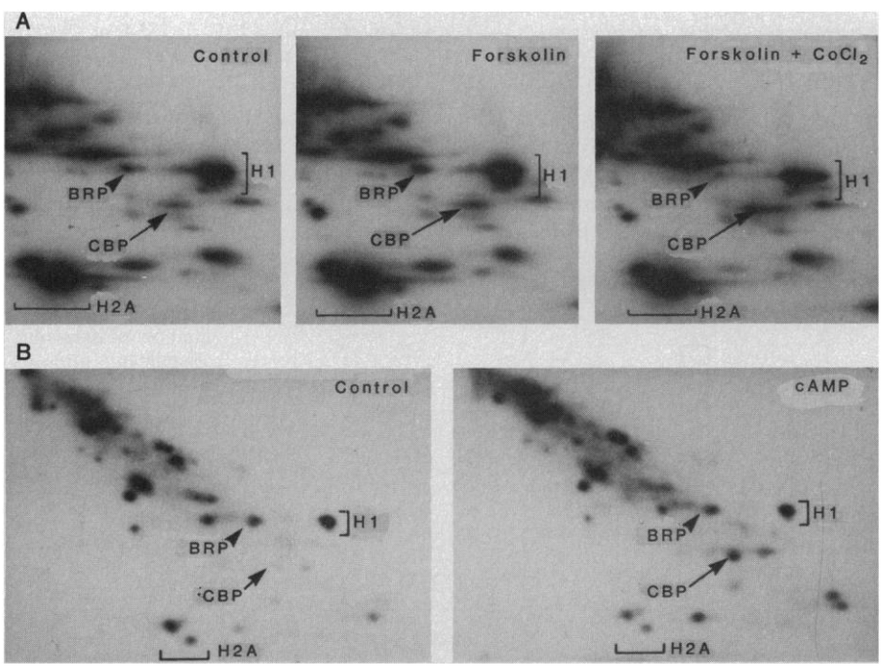


Fig. 2. Autoradiographs of tryptic phosphopeptide maps of histone H1. GH_4 cells were treated with ^{32}P-labeled phosphate before use, and proteins were prepared and subjected to electrophoresis as described (*13*). The complete set of H1 proteins was isolated from two-dimensional gels of acid-soluble nuclear proteins from (A) control, forskolin-treated ($10^{-6}M$ for 30 minutes), or TRH-treated cells ($3 \times 10^{-7}M$ for 30 minutes) or (B) cells treated for 30 minutes with forskolin alone or with forskolin after a 5-minute incubation with cobalt chloride (1 m*M*). Tryptic maps were prepared as described (*22*).



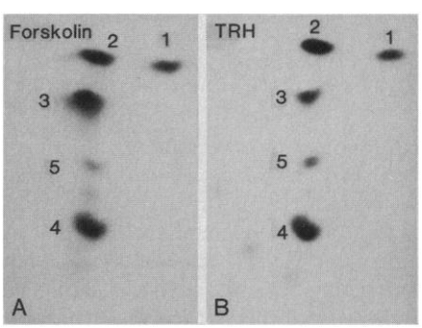


Fig. 3 (left). (A) Phosphorylation of BRP and CBP in forskolin-treated GH_4 cells. GH_4 cells were treated with ^{32}P-labeled phosphate before use, and proteins were prepared and subjected to electrophoresis as described (*13*). Autoradiographs are of two-dimensional gels of acid-soluble nuclear proteins from unstimulated cultures or from cells treated with forskolin alone ($10^{-6}M$ for 30 minutes) or with forskolin after a 5-minute incubation with cobalt chloride (1 m*M*). (B) Phosphorylation of CBP by cyclic AMP in broken cells. Homogenates of GH_4 cells were labeled [90 seconds at 20°C in 150 m*M* sucrose, 25 m*M* Hepes (*p*H 7.2), 5 m*M* magnesium acetate, and 5 m*M* dithiothreitol] with adenosine [α-^{32}P]triphosphate ($2 \times 10^{-6}M$, >400 Ci/mmol) in the presence or absence of cyclic AMP ($10^{-5}M$). Autoradiographs are of two-dimensional gels of acid-soluble chromatin proteins. Fig. 4 (right). Tryptic phosphopeptide maps of BRP isolated from GH_4 cells treated with forskolin (A) or TRH (B). BRP was isolated by punching the appropriate protein spot from two-dimensional gels prepared from cells treated with ^{32}P-labeled phosphate and with TRH ($3 \times 10^{-7}M$ for 30 minutes) or forskolin ($10^{-6}M$ for 30 minutes); the material was subjected to trypsin digestion and two-dimensional chromatography as described (*22*).

a calcium-dependent event that is essential for prolactin gene regulation by peptide hormones. We therefore investigated the possibility that cobalt ions might distinguish the effects of cyclic AMP on prolactin and growth hormone gene transcription. Cobalt inhibited the stimulation of prolactin gene transcription by forskolin (Fig. 1) or 8-bromo cyclic AMP, which is comparable to the effects of cobalt on TRH-stimulated prolactin gene transcription. In contrast to the effects on prolactin gene expression, the stimulation of growth hormone gene transcription by forskolin was potentiated by cobalt ions (Fig. 1). Cobalt also decreased the level of prolactin gene transcription in unstimulated cultures by 50 percent (Fig. 1). We (*13*) and others (*18*) have noted that, under usual conditions of culture with 10 percent serum, prolactin gene transcription is not basal but is approximately two times greater than the level observed in serum-free culture conditions, presumably because of low concentrations of hormones in the serum. Thus, prolactin gene transcription was 1.5 to 2 parts per million per kilobase in serum-free conditions, and no further decrease in transcription rate was observed when cobalt was added. The possibility that the regulatory mechanism for cyclic AMP stimulation of prolactin and growth hormone gene expression is the same, but that a calcium-dependent process operates selectively in prolactin but not in growth hormone gene transcription, is therefore unlikely.

Cobalt ions inhibited forskolin-stimulated BRP phosphorylation (Fig. 3A) to the levels observed in cells cultured in serum-free media; however, cobalt ions did not prevent forskolin stimulation of cyclic AMP accumulation (*19*) or the phosphorylation of histone H1 at Ser^{37} (Fig.2B), showing that cobalt does not inhibit cyclic AMP–dependent protein kinase activity. Cobalt ions also potentiated phosphorylation by cyclic AMP of a 19-kD basic nuclear protein (CBP) (Fig. 3A) that was soluble in dilute acid, could be precipitated by perchloric acid, and could be distinguished from previously described histone and high-mobility group proteins. The phosphorylation of CBP was greatly stimulated by addition of cyclic AMP to homogenates of GH_4 cells (Fig. 3B). Phosphorylation of BRP was unaffected by addition of cyclic AMP to identical homogenates (Fig. 3B). Therefore, CBP may be a direct substrate of the cyclic AMP–dependent protein kinase in vivo. A relation between the phosphorylation of BRP and CBP and the mediation of altered prolactin and growth hormone gene transcription is unlikely on the basis of their abundance. However, their analysis together with the transcriptional data shows that increased amounts of intracellular cyclic AMP modulate a second protein kinase that exhibits calcium-dependent activation or activity.

Cyclic AMP exerts various effects on cellular calcium metabolism (*20*) and can modulate many kinase systems either directly or by activation of a protein phosphatase inhibitor (*21*). The identification of discrete regulatory pathways mediating the effects of cyclic AMP on prolactin and growth hormone gene transcription suggests that a single intracellular mediator can simultaneously regulate the transcription of different sets of responsive genes by stimulating independent biochemical events. In this regard, it is important to establish the pathway by which cyclic AMP acts to regulate transcription of each responsive gene.

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Ca^{2+} and Ca^{2+}-Activated K^+ Currents in Mammalian Gastric Smooth Muscle Cells

Abstract. *Inward movement of calcium through voltage-dependent channels in muscle is thought to initiate the action potential and trigger contraction. Calcium-activated potassium channels carry large outward potassium currents that may be responsible for membrane repolarization. Calcium and calcium-activated potassium currents were identified in enzymatically isolated mammalian gastric myocytes. These currents were blocked by cadmium and nifedipine but were not substantially affected by diltiazem or D600. No evidence for a tetrodotoxin-sensitive sodium current or an inwardly rectifying potassium current was found.*

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Electrophysiological studies of mammalian gastric muscle strips have shown membrane activity consisting of slow wave depolarizations, spike discharges, plateau phases, or a combination of these activities (*1–3*). The presence of any particular type of activity depends on the species as well as on the anatomical location of the gastric muscle from which it is recorded. These studies have also examined the ionic and pharmacologic dependence of the electrophysiological properties. Voltage clamp studies, however, have been complicated by the syncytial nature of this tissue. Temporal and spatial clamp inhomogeneity and the accumulation and depletion of ions in restricted extracellular spaces have made it difficult to characterize the ionic currents. These problems are largely overcome by studying isolated cells, and such studies have already been performed on toad gastric cells (*4*) and mammalian jejunal cells (*5*). We now describe the electrophysiological properties of isolated single gastric myocytes studied by the whole-cell voltage clamp technique (*6*). A new method was used to isolate gastric cells from the corpus region of guinea pig and rabbit stomach (*7*). We found that gastric myocytes have