Involvement of cAMP response element-binding protein in the regulation of cell proliferation and the prolactin promoter of lactotrophs in primary culture

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Involvement of cAMP response element-binding protein in the regulation of cell proliferation and the prolactin promoter of lactotrophs in primary culture. Am J Physiol Endocrinol Metab 293: E1529–E1537, 2007. First published October 9, 2007; doi:10.1152/ajpendo.00028.2007.—Hypothalamic hormones, including dopamine, regulate critical functions of pituitary cells via the cAMP-protein kinase A (PKA) pathway. The PKA-downstream transcription factor cAMP response element (CRE)-binding protein (CREB) is an integrating molecule that is also activated by many other protein kinase pathways. We investigated the involvement of CREB in the regulation of cell proliferation and the PRL promoter of rat lactotrophs in primary cell culture. Recombinant adenosinoviruses were used for efficient gene delivery into pituitary cells. Bromocriptine, a dopaminergic agonist known to decrease intracellular cAMP concentrations, caused inhibition of PRL promoter activity and lactotroph proliferation, which was accompanied by decreases in CRE-mediated transcription and CREB phosphorylation in lactotrophs. Expression of a dominant-negative form of CREB (MCREB), which was effective in suppressing CRE-mediated transcription induced by the adenylate cyclase activator forskolin, inhibited basal and forskolin-induced PRL promoter activity and PRL mRNA expression. MCREB expression lowered basal proliferative levels and blocked forskolin-induced proliferation of lactotrophs. Insulin-like growth factor I (IGF-I), a potent mitogen in lactotrophs, did not affect forskolin-induced proliferation of lactotrophs. Insulin-like growth factor I (IGF-I), a potent mitogen in lactotrophs, did not affect forskolin-induced proliferation of lactotrophs. Insulin-like growth factor I (IGF-I) stimulates proliferation in thyroid and ovarian granulosa cells (2, 5, 32). Thus, cAMP utilizes multiple signaling pathways to regulate cell proliferation in a cooperative manner.

Adenosine 3',5'-cyclic monophosphate; dopamine; insulin-like growth factor I

ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (cAMP) stimulates or inhibits cell proliferation, depending on the cell type. cAMP action is mediated mostly by its downstream effector, protein kinase A (PKA). PKA-dependent cAMP signaling for proliferation involves activation of the transcription factor cAMP response element (CRE)-binding protein (CREB) (29) or the Rap1-mediated kinase cascade (36). PKA acts directly on CREB and causes phosphorylation of the serine residue at position 133 (Ser133), which is an essential step for CREB activation and dimerization (14). The activation by PKA endows CREB with the ability to bind to a CRE sequence in the promoters of numerous target genes and stimulate their transcription (46). The CREB target genes related to cell proliferation include c-fos, cyclins A and D, and proliferating cellular nuclear antigen (29, 47). CREB, originally identified as a transcription factor activated by the cAMP-PKA pathway, is also phosphorylated at the same Ser133 by many other protein kinases, including ribosomal S6 kinase 2 (44), mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (40), calcium/calcmodulin-dependent kinases (26), and protein kinase B/Akt (10). The fact that CREB is phosphorylated by a variety of protein kinase-mediated signaling pathways suggests that CREB is a convergent molecule for regulating essential cell functions such as cell proliferation, differentiation, and survival (35). Involvement of CREB in the regulation of cell proliferation has been shown in vivo and in vitro in somatotrophs (37), vascular smooth muscle cells (41), developing thymocytes (15), and cell lines from thyroid follicles (43) and myeloid leukemia (34).

However, the PKA-CREB pathway is not the sole pathway for mediating the mitogenic signal of cAMP (36). PKA activates Rap1, a member of the Ras superfamily of small G proteins that, in turn, stimulates and inhibits the activity of MAPK kinase kinases such as B-Raf and Raf-1, respectively, leading to altered activity of the MAPK extracellular signal-regulated kinase (ERK) (33, 42) that is critical for growth factor-induced proliferation. Importantly, Rap1 is also activated by cAMP through the cAMP-guanine nucleotide exchange factor (GEF) in a PKA-independent manner (9, 19). Activation of Rap1 by the cAMP-GEF pathway activates the MAPK pathway (22) and has been implicated in cell proliferation in several cell types (13, 17). Furthermore, cAMP also activates phosphoinositide 3-kinase and its downstream molecule Akt in PKA-dependent and -independent manners to stimulate proliferation in thyroid and ovarian granulosa cells (2, 5, 32). Thus, cAMP utilizes multiple signaling pathways to regulate cell proliferation in a cooperative manner.

There are several lines of evidence indicating that cAMP is a positive regulator of the proliferation of pituitary lactotrophs and mediates the action of hypothalamic hormones on proliferation. First, we have previously shown that increased intracellular cAMP concentrations caused by treatment with a membrane-permeable cAMP analog or forskolin, an adenylate cyclase activator, stimulate proliferation of lactotrophs in primary culture in a PKA-dependent manner (38, 45), although an inhibitory action of cAMP has been reported in the somato-
mammotroph cell line GH3 (18). Second, the hypothalamic hormone dopamine and its agonists, which inhibit markedly lactotroph functions, including the secretion and synthesis of prolactin (PRL) and cell proliferation (1), lower intracellular cAMP concentrations (28, 39). Third, forskolin-induced lactotroph proliferation is suppressed by treatment with bromocriptine, a dopaminergic agonist, suggesting that the anti-mitogenic action of dopamine is, at least in part, mediated by inhibition of cAMP production (38). In addition to regulation by the cAMP-PKA pathway, pituitary-intrinsic growth factors and their signaling kinase cascade, the MAPK, are other major regulators of lactotroph proliferation. Interestingly, we (38, 45) have previously shown that insulin-induced lactotroph proliferation is blocked by the PKA inhibitors H89 and KT5720, suggesting that there is a functional interaction between the cAMP-PKA and growth factor-MAPK pathways.

The role of CREB in the regulation of lactotroph functions is poorly understood. All studies published to date have been restricted to investigating the involvement of CREB in the regulation of PRL gene expression using GH3 cells (6, 24). Therefore, we used normal lactotrophs in primary culture and investigated 1) whether bromocriptine inhibits CRE-mediated transcription and CREB phosphorylation in lactotrophs, 2) whether expression of a dominant-negative CREB mutant suppresses forskolin- or insulin-like growth factor I (IGF-I)-induced stimulation of PRL promoter activity and lactotroph proliferation, and 3) whether IGF-I increases intracellular cAMP concentrations and CREB phosphorylation in lactotrophs.

MATERIALS AND METHODS

Plasmids and adenovirus vectors. Adenovirus vectors were produced using the Adeno-X Expression and Adeno-X Tet-On Expression systems according to the manufacturer’s protocol (Clontech Laboratories, Mountain View, CA). The adenovirus vector Ad-TRE/MCREB.Tag expresses a dominant-negative CREB mutant (MCREB) (14) and a 6xHis tag at its NH2 terminus in a tetracycline-regulated manner. A 1.3-kb fragment of the MCREB cDNA was excised by NheI and Sdel digestion of pcDNA-MCREB, a plasmid originating from RSV-MICREB, and subcloned into the pTRE-Shuttle2 vector (Clontech Laboratories) at the NheI/EcoRV sites. A 6xHis tag was then added at the NH2 terminus of MCREB of the pTRE-Shuttle2 using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and primers of 5’-caccgtgctagatgctcaccacccatcataaatggag-3’ and its antisense sequence. A fragment containing the cDNA for 6xHis-tagged MCREB was isolated from the pTRE-Shuttle2 by digestion with L-Ceu and PI-Scel and inserted into a site created by the same restriction enzymes in Adeno-X viral DNA, a replication-incompetent adenoviral genome. The Adeno-X virus carrying 6xHis-tagged MCREB was transfected into HEK-293 cells using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) for virus production, and the recombinant adenoviruses were further propagated by serial infection in HEK-293 cells. The adenoviruses obtained were purified with the Adeno-X Virus Purification kit (Clontech Laboratories) and concentrated with the Centricon Centrifugal Filter Unit (Millipore, Bedford, MA). The titer of adenovirus vectors (infectious units) was determined using the Adeno-X Rapid Titer kit (Clontech Laboratories). Ad-TRE/wtCREB, an adenovirus vector expressing wild-type CREB, was produced from pcDNA-CREB by a procedure similar to that for Ad-TRE/MCREB.Tag production. Ad-TRE/β-gal and Ad-Tet.On, adenoviruses expressing β-galactosidase in a tetracycline-regulated manner and rTetR-VP16, respectively, were produced from Clontech Laboratories.

To produce an adenovirus vector expressing the luciferase reporter gene driven by a promoter containing the consensus CRE (Ad-CRE/Luc) or serum response element (SRE) sequence (Ad-SRE/Luc), the pCRE-Luc and pSRE-Luc plasmids (Mercury Pathway Profiling System; Clontech Laboratories) were digested with BamHI and NotI, and the resultant fragment containing the CRE- or SRE-driven luciferase gene was subcloned into a BamHI- and NotI-digested fragment of a pTRE-Shuttle2 vector lacking its tetracycline-responsive expression cassette (positions 34–1,582), named pTRE-Shuttle2-NruLMCS.Smald. pTRE-Shuttle2-NruLMCS.Smald was constructed by excising the multiple cloning region from the pSL1180 vector (Amersham Biosciences, Arlington Heights, IL) using NruI and Smal and then inserting it into a BsgII-digested and end-blunted pTRE-Shuttle2 vector. To produce an adenovirus vector carrying a PRL promoter activity reporter gene (Ad-Prl/Luc), a 3.2-kb fragment of the 5’-flanking region of the rat PRL gene (positions −3,221 to +33) was flanked from pSV2A-Prl/Luc (a generous gift from Dr. Harry Elsholtz, University of Toronto, Toronto, ON, Canada) by digestion with BamHI and HindIII and subcloned into the pGL3-Basic vector (Promega, Madison, WI) at the BgIII/HindIII sites. A NotI- and NotI-digested fragment of the pGL3 vector containing the reporter gene was inserted into the pTRE-Shuttle2-NruLMCS.Smald vector at a site created by the same restriction enzymes. The pTRE-Shuttle2-NruLMCS.Smald vector containing the CRE-, SRE-, or PRL promoter-driven luciferase gene was digested with L-Ceu and PI-Scel, and the reporter gene was inserted into Adeno-X viral DNA. The lactotroph-specific expression of luciferase by infection with Ad-Prl/Luc was confirmed by selective localization of luciferase-immunoreactivity in some PRL-immunoreactive pituitary cells, as demonstrated by double immunostaining for luciferase and PRL.

Cell culture and virus infection. Experiments were conducted in accordance with the guidelines of the Ethics Committee of Animal Experiments of the University of Yamashiro and were approved by the committee. Seven-week-old female Wistar rats purchased from Japan SLC (Shizuoka, Japan) were used to obtain anterior pituitary cells for primary culture. Anterior pituitary cells were dispersed as described previously (20). A 100-μl aliquot of pituitary cell suspension containing 2.0 × 10⁶ cells in a 1:1 mixture of DMEM and Ham’s nutrient mix F-12 without phenol red and containing 15 mM HEPES, penicillin, and streptomycin (DMEM-F-12) was plated in poly-D-lysine-coated culture dishes. For infection with adenovirus vectors, Ad-Tet.On in combination with either Ad-TRE/MCREB.Tag or Ad-TRE/β-gal and, in reporter assay experiments, Ad-Prl/Luc or Ad-CRE/Luc was added into the cell suspension at appropriate multiplicities of infection (MOIs). The cells were subsequently allowed to attach to the surface of the dishes in a humidified CO₂ incubator for 1 h. The pituitary cells were then flooded with 2 ml of DMEM-F-12 containing 500 ng/ml insulin and precultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 1 day. After 1 day of preculture, the pituitary cells were washed with DMEM-F-12 to remove adenoviruses, and cultures were initiated with a serum-free, chemically defined medium (20) in which doxycycline (Clontech Laboratories) was added at a concentration of 10 μg/ml to induce the reverse Tet repressor protein and thereby either MCREB or β-galactosidase protein. Cultured pituitary cells were treated with mitogens such as forskolin, IGF-I, and bromocriptine (all from Sigma Chemical, St. Louis, MO). For labeling proliferating pituitary cells, the cultured cells were treated with 200 μM 5-bromo-2’-deoxyuridine (BrdU; Sigma Chemical) for 3 h prior to the end of culture.

Immunostaining. To determine the BrdU-labeling index, pituitary cells labeled with BrdU were detached from culture dishes at the end of culture by trypsin treatment. The cells were attached to poly-D-lysine-coated glass slides by centrifugation, using a cytocentrifuge (SC-2; Tomy, Tokyo, Japan), and fixed with ice-cold methanol for 30 min. The pituitary cells attached to glass slides were double immunostained for BrdU and PRL as described previously (20). Immunostained slides were covered with PermaFluor (Immuron, Pittsburgh, PA).

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PA) and examined with a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan) equipped with a dual-hand mirror unit for FITC and Texas Red (U-DM-FI/TX). A total of 1,000 PRL-immunoreactive cells were examined in randomly chosen fields for each slide to determine the BrdU-labeling index, which was the percentage of cells immunoreactive for both PRL and BrdU of the total PRL-immunoreactive cells counted.

For double immunostaining for phosphorylated CREB (pCREB) and PRL, pituitary cells cultured on dishes were fixed with 1.8% paraformaldehyde in 0.1 M phosphate buffer for 10 min and rinsed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T). The pituitary cells were permeabilized with TBS containing 1% Tween 20 for 30 min, followed by blocking of endogenous peroxidase with 3% hydrogen peroxide in phosphate-buffered saline for 10 min. After blocking nonspecific binding by using the 0.5% blocking reagent supplied in the TSA Fluorescence System (NEL701A; Dako, Tokyo, Japan) at a 1:100 dilution for 1 h, 1) a FITC-labeled anti-rabbit IgG antibody (Cell Signaling Technology; Beverly, MA) at a 1:100 dilution for 4 h at 4°C; 2) an anti-CREB antibody (Cell Signaling Technology) at a 1:100 dilution for 1 h, and 3) a peroxidase-labeled sheep anti-fluorescein antibody (Roche Diagnostics) at a 1:200 dilution for 1 h, 4) FITC-labeled tyramide (PerkinElmer) at a 1:50 dilution for 10 min, 5) a monoclonal mouse anti-rat PRL antibody (QED Biosciences, San Diego, CA) at a 1:10,000 dilution for 1 h, and 6) an Alexa fluor 594-labeled anti-mouse IgG antibody (Molecular Probes, Eugene, OR) at a 1:100 dilution for 1 h. Fluorescence images of the immunostained pituitary cells were captured with a digital charge-coupled device camera (DP50; Olympus) and stored in a personal computer. pCREB immunoreactivity was quantified by measuring the fluorescence intensity of nuclear FITC with Photoshop imaging software (Adobe Systems, San Jose, CA), and a cell with a fluorescence intensity higher than a level of 60 was defined as a pCREB-immunoreactive cell. Pituitary cells that were immunoreactive for both pCREB and PRL were counted in a total of 200 PRL-immunoreactive cells.

To validate the adenovirus-mediated expression of MCREB protein, paraformaldehyde-fixed pituitary cells were immunostained for MCREB or the 6×His tag with 1) a rabbit anti-pCREB antibody (Cell Signaling Technology) at a 1:2,000 dilution for 4 h at 4°C; 2) an anti-CREB antibody (Cell Signaling Technology) at 1:2,000 dilution or a rabbit anti-His-Tag antibody (Cell Signaling Technology) at 1:2,000 dilution for 15 min, 2) a peroxidase-labeled polymer reagent (Envision System, Dako Japan, Tokyo, Japan) for 1 h, and 3) cyanine 5-labeled tyramide (PerkinElmer) at a 1:50 dilution for 10 min. To verify the lactotroph-specific expression of the reporter luciferase gene, pituitary cells infected with Ad-Prl/Luc were attached to glass slides after desiccation and fixed with ice-cold methanol. The pituitary cells were permeabilized, blocked for endogenous peroxidase and nonspecific binding, and then subjected to double immunostaining for luciferase and PRL with the following reagents: 1) a rabbit anti-luciferase antibody (Sigma Chemicals) at a 1:12,000 dilution overnight at 4°C; 2) a peroxidase-labeled polymer reagent (Envision System, Dako Japan, Tokyo, Japan) for 1 h, and 3) cyanine 5-labeled tyramide (PerkinElmer) at a 1:50 dilution for 10 min. The primary antibodies were labeled with a horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody (1:10,000 dilution in TBS-T; Amersham Biosciences). The membrane was blocked for 1 h with 5% nonfat dry milk in TBS-T and then subjected to incubation with a rabbit anti-CREB antibody (no. 238461; Calbiochem, Darmstadt, Germany) at 1:2,000 dilution, at which overexpressed MCREB and wild-type (wt)CREB but not endogenous CREB were detected, or a mouse anti-β-actin antibody (A-5441; Sigma Chemical) at a 1:10,000 dilution in TBS-T containing 5% BSA at 4°C overnight. The primary antibodies were labeled with a horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody (1:10,000 dilution in TBS-T; Amersham Biosciences).

Intracellular cAMP concentrations were determined using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI). Pituitary cells were treated with 300 µl of 0.1 N HCl for 15 min, and cell lysates were centrifuged at 15,000 g for 5 min. The supernates were vortexed for 10 s and centrifuged at 18,000 g for 2 min. Forty-microliter aliquots of the supernatants were assayed for cAMP activity using the Luciferase Assay System (Promega). Light intensity was measured with a luminometer (BLR-201; Aloka, Tokyo, Japan) and is expressed in terms of relative luciferase units.


cAMP assay. Intracellular cAMP concentrations were determined using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI). Pituitary cells were treated with 300 µl of 0.1 N HCl for 15 min, and cell lysates were centrifuged at 15,000 g for 5 min. The supernates were acetylated with acetic anhydride and triethylamine and assayed for cAMP. The minimum detectable concentration for the cAMP was 0.016 pmol/assay tube (90% B/B0). The intra-assay coefficient of variation was 6.4%.

Statistical analysis. Experiments were replicated at least three times with separate batches of cell preparations. Differences between groups were statistically analyzed using one-way ANOVA followed by Fisher’s protected least significant difference test.

RESULTS

Bromocriptine-induced inhibition of PRL promoter activity and lactotroph proliferation is accompanied by inhibition of CRE-luciferase activity and lactotroph CREB phosphorylation. PRL promoter activity was measured by infection with Ad-Prl/Luc, an adenovirus carrying a PRL promoter-driven luciferase reporter gene. Treatment of pituitary primary cultures with 1
μM forskolin increased PRL promoter-regulated luciferase reporter activity and lactotroph proliferation, as determined by BrdU labeling (Fig. 1A and B). Pretreatment with 10 nM bromocriptine 1 h earlier significantly inhibited basal and forskolin-induced PRL promoter-luciferase activity by 59 and 58%, respectively, compared with vehicle pretreatment (P < 0.05). Bromocriptine was also effective in inhibiting basal and forskolin-induced lactotroph proliferation by 78 and 76%, respectively (P < 0.05), as shown previously (38). To investigate whether bromocriptine alters CRE-mediated transcription, CRE-luciferase activity was determined in vehicle- and bromocriptine-treated cells by infection with Ad-CRE/Luc, an adenovirus carrying a luciferase reporter gene driven by two tandem repeats of the consensus CRE sequence. CRE-luciferase activity was markedly increased by forskolin treatment (Fig. 1C). Basal and forskolin-induced CRE-luciferase activity was significantly inhibited by bromocriptine pretreatment by 22 and 79%, respectively (P < 0.05). Because the CRE-luciferase activity measured was attributed not only to lactotrophs but also in part to other pituitary cell types, we further investigated whether bromocriptine alters lactotroph-specific activation of CREB upstream of CRE regulation. We determined the proportion of pCREB-immunoreactive (pCREB-ir) lactotrophs with double-immunocytochemical staining for PRL and pCREB using an antibody that specifically recognizes phosphorylation of Ser133 of CREB. The double-immunocytochemical staining revealed nuclear pCREB immunoreactivity in a proportion of pituitary cells (Fig. 2A). In parallel to the changes in CRE-luciferase activity, the percentage of pCREB-ir lactotrophs was increased by forskolin treatment, and the forskolin-induced increase was significantly inhibited by bromocriptine pretreatment by >80% compared with vehicle pretreatment (P < 0.05; Figs. 1D and 2B–D).

Adenovirus-mediated and tetracycline-regulated expression of MCREB in pituitary cells. We used a dominant-negative form of CREB, MCREB, to inhibit CREB function. MCREB, in which Ser133 is substituted with Ala (14), can bind to the CRE sequence but does not transactivate CREB-responsive genes and thereby acts as a CREB inhibitor via direct competition with endogenous CREB or other CREB family members. To verify the appropriate expression of MCREB in a tetracycline-regulated manner by an adenovirus vector, pituitary cells coinfected with Ad-TRE/MCREB.Tag and Ad-Tet.On were treated with or without doxycycline, a tetracycline analog, for 2 days and subjected to immunocytochemical studies. Because no antibodies available can immunologically distinguish MCREB from endogenous CREB, MCREB expression was verified by two indirect methods. The first method involved immunostaining with a CREB antibody at a dilution at which no antibodies available can immunologically distinguish MCREB from endogenous CREB, MCREB expression was confirmed by two indirect methods. The first method involved immunostaining with a CREB antibody at a dilution at which no antibodies available can immunologically distinguish MCREB from endogenous CREB, MCREB expression was verified by two indirect methods. The first method involved immunostaining with a CREB antibody at a dilution at which no antibodies available can immunologically distinguish MCREB from endogenous CREB, MCREB expression was verified by two indirect methods. The first method involved immunostaining with a CREB antibody at a dilution at which no antibodies available can immunologically distinguish MCREB from endogenous CREB, MCREB expression was verified by two indirect methods. 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of virus-infected pituitary cells (Fig. 3, B and C). Second, identical results were obtained by immunostaining with an antibody against the 6×His tag, which was added at the NH₂ terminus of MCREB (Fig. 3, D and E). Furthermore, MCREB expression was functionally verified by examining the effect of adenovirus infection on forskolin-induced CRE-luciferase activity. In addition to Ad-Tet.On and Ad-TRE/MCREB.Tag at varying MOIs, pituitary cells were infected with Ad-CRE/Luc. Reporter assays revealed that forskolin-induced CRE-luciferase activity was decreased in an Ad-TRE/MCREB.Tag dose-dependent manner only in the presence of doxycycline (Fig. 4A). MCREB expression also inhibited the luciferase activity of the cytomegalovirus promoter known to contain CREs (data not shown) but did not alter fetal bovine serum-induced SRE activity, indicating CRE specificity of the effect of Ad-TRE/MCREB.Tag (Fig. 4B). On the basis of these results, an MOI of 2 was chosen as an optimum dose of Ad-TRE/MCREB.Tag and Ad-Tet.On for blocking CREB activation in the subsequent experiments.

Effects of MCREB expression on PRL promoter activity. MCREB was expressed to mimic the suppression of CRE-luciferase activity and CREB activation observed in bromocriptine-treated lactotrophs. Treatment of Ad-TRE/β-gal- and Ad-Tet.On-coinfected pituitary cells with forskolin increased PRL promoter activity approximately fourfold above the basal level (Fig. 5A). Forskolin-induced PRL promoter activity was not affected by expression of β-galactosidase induced by doxycycline treatment. MCREB expression induced by Ad-TRE/MCREB.Tag and Ad-Tet.On coinfection and doxycycline treatment significantly inhibited basal and forskolin-induced PRL promoter activity by 85 and 92%, respectively, compared with Ad-TRE/MCREB.Tag-infected controls without doxycycline (P < 0.05). Similar inhibitory effect of MCREB expression was also found in PRL mRNA expression (Fig. 5B). To determine the specificity of the MCREB inhibition of PRL promoter activity, the antagonistic effect of the expression of wild-type CREB was examined in
pituitary cells infected with both Ad-TRE/MCREB.Tag and Ad-TRE/wtCREB. Western blot analysis verified that infection with Ad-TRE/MCREB.Tag at 2 MOIs or Ad-wtCREB at MOI 4 MOIs alone and their combination induced CREB-immuno
detectable in infected cells (Fig. 5C).}

**Effects of MCREB expression on lactotroph proliferation.** Forskolin treatment of pituitary cells coinfectected with Ad-TRE/β-gal and Ad-Tet.On significantly increased lactotroph proliferation 4.2-fold (P < 0.05; Fig. 6A). Neither expression of β-galactosidase induced by doxycycline treatment of these infected cells nor infection with Ad-TRE/MCREB.Tag and Ad-Tet.On had a significant effect on forskolin-induced prolifera
tion. MCREB expression induced by doxycycline treatment of the Ad-TRE/MCREB.Tag-infected cells had a marked inhibitory effect on basal proliferation (P < 0.05). In addition, MCREB expression also blocked forskolin-induced prolifera
tion (P < 0.05).

Because IGF-I is another major regulator of lactotroph proliferation and the IGF-I-MAPK pathway has been sug
gested to interact with the cAMP-PKA pathway (38, 45), the effects of MCREB expression on IGF-I-induced lactotroph proliferation were also studied. Treatment with 20 ng/ml IGF-I increased lactotroph proliferation threefold in pituitary cells coinfectected with Ad-TRE/β-gal and Ad-Tet.On regardless of doxycycline treatment (Fig. 6B). Coinfection of pituitary cells with Ad-TRE/MCREB.Tag and Ad-Tet.On alone did not affect IGF-I-induced proliferation, whereas MCREB expression induced by treatment of infected cells with doxycycline attenuated the fold increase in IGF-I-induced proliferation by 54% (P < 0.05).

**Lactotroph CREB phosphorylation in IGF-I-treated pituitary cells.** The finding that MCREB inhibited IGF-I-induced lactotroph proliferation raised the possibility that IGF-I-induced lactotroph proliferation is mediated by activation of the cAMP-PKA-CREB pathway. Therefore, we determined whether treatment of pituitary cells with IGF-I increases intra
cellular cAMP concentrations. Treatment with forskolin, as a positive control, markedly increased intracellular cAMP concentrations at both 20 min and 1 h (P < 0.05), whereas IGF-I treatment did not alter them at either time (Fig. 7A). Next, we tested whether IGF-I activates lactotroph CREB without changes in cAMP concentration, because CREB has been shown (29) to be activated in response to growth factors. The percentage of pCREB-ir lactotrophs significantly increased 20 min after forskolin treatment and reached a peak with a 10-fold increase at 3 h (P < 0.05) that lasted ≤9 h (Fig. 7B). IGF-I treatment also increased the percentage of pCREB-ir lactotrophs but was not effective as forskolin treatment; a 2.4-fold increase was observed only at 20 min and 1 h (P < 0.05).

**DISCUSSION**

For physiological studies, primary cultures of normal cells are superior to cell lines, but gene transfer using conventional methods is difficult to achieve for these cells. To express a dominant-negative form of CREB and reporter genes we used adenovirus vectors, which have proved efficient for expressing proteins in normal pituitary cells in primary culture (3, 30). MCREB expression was effective in inhibiting PRL promoter activity and lactotroph proliferation, suggesting that CREB plays a critical role in regulating the function of pituitary lactotrophs. However, it should be noted that the effects of MCREB found in the present study could be partly due to blockade of transcriptional activities of other CREB family members, such as CRE modulator and activating transcription factor 1, because the consensus CRE sequence to which...
MCREB binds is also a site targeted by these CREB family members (12, 16).

In previous studies using GH3 cells, there have been conflicting results regarding the effect of expression of dominant-negative forms of CREB on cAMP-activated PRL promoter activity. A dominant-negative form of CREB, in which residues 1–243 of the transactivation domain were deleted, did not affect cAMP-induced activation of a reporter gene directed by a 0.6-kb PRL promoter region (24), whereas KCREB, a mutant form of CREB with Arg287 substitution with Leu, inhibited cAMP-induced activation of a shorter PRL promoter (6). The results of the present study demonstrate that MCREB expression in lactotrophs in primary culture markedly suppresses basal activity and forskolin-induced activation of a 3.2-kb PRL promoter. These results, which are consistent with the results of MCREB inhibition of endogenous PRL mRNA expression, suggest that CREB is closely involved in maintenance of basal activity and cAMP-activation of the PRL promoter, at least in normal lactotrophs. Most functional CREs are located within 200 nucleotides of the transcription start site (47). There is a half-CRE sequence located at positions −99 to −92 in the proximal region of the PRL promoter. However, no significant CRE binding has been detected in the proximal PRL promoter containing this half-CRE sequence (21, 24). Given these findings, CREB or a related protein may bind to a CRE-like sequence in the distal region of the PRL promoter used in the present study or may be involved in PRL promoter regulation in an indirect manner.

In addition to PRL promoter activity, basal and forskolin-induced proliferation of lactotrophs was greatly inhibited by MCREB expression, suggesting requirement of CREB for both basal and cAMP-induced lactotroph proliferation. These results are the first to imply involvement of CREB in the regulation of lactotroph proliferation. CREB activated by the cAMP-PKA pathway may upregulate expression of its target genes, including c-fos, cyclins, and proliferating cellular nuclear antigen to stimulate lactotroph proliferation. However, Struthers et al. (37) reported that transgenic mice expressing

![Fig. 6. Effects of MCREB expression on forskolin- and IGF-I-induced lactotroph proliferation. Cultured pituitary cells were coinfected for 1 day with Ad-Tet.On (MOI 2) and either Ad-MCREB (MOI 2) or Ad-β-gal (MOI 2). The pituitary cells were treated with 10 μg/ml doxycycline (Dox) or vehicle for 40 h, and 1 μM forskolin (A) or 20 ng/ml IGF-I (B) or vehicle was added 24 h before the end of culture. The pituitary cells were treated with 200 μM BrdU for 3 h and fixed with ice-cold methanol for 30 min for double immunostaining for PRL and BrdU. BrdU labeling indexes are expressed as a fold increase relative to the group coinfected with Ad-Tet.On and Ad-β-gal and not treated with Dox and forskolin (A) or IGF-I (B). Data are means ± SE of triplicate determinations from a representative experiment. *Significantly different from the Ad-β-gal-infected and Dox-treated groups.](http://ajpendo.physiology.org/)
MCREB under the control of the growth hormone promoter had a dwarf phenotype with a marked deficiency in somatotrophs and that, although lactotrophs in the transgenic mice also expressed the mutant CREB, lactotroph cell densities in the anterior pituitary were apparently normal. These findings in transgenic mice seem inconsistent with those in the present study in which the same dominant-negative CREB mutant was expressed in cultured rat pituitary cells. Presently, the reason for this discrepancy remains unknown. Lower expression of MCREB in lactotrophs than in somatotrophs, due to expression regulation by the growth hormone promoter, may be insufficient to suppress lactotroph proliferation. The development of transgenic mice that express MCREB under the direction of the PRL promoter may be helpful for testing this view.

We showed that MCREB expression markedly inhibited IGF-I-induced lactotroph proliferation. This finding raised the possibility that IGF-I may activate the cAMP-PKA-CREB pathway to stimulate lactotroph proliferation. We addressed this possibility and found that IGF-I did not alter intracellular cAMP concentrations but stimulated CREB phosphorylation in lactotrophs to a limited degree. This is in agreement with the findings of other studies that growth factors cause Ser\(^{133}\) phosphorylation of CREB via ribosomal S6 kinase 2 (8, 44), MAPK-activated protein kinase 2 (31, 40), or mitogen- and stress-activated protein kinase 1 (7). The cAMP-independent activation of CREB by IGF-I in lactotrophs suggests that CREB partly mediates the mitogenic signal of IGF-I in lactotrophs. However, considering our previous findings that even expression of PKA, which is upstream of CREB activated by IGF-I and is not affected by IGF-I, suppressed insulin-induced lactotroph proliferation, it seems likely that, in addition to CREB activation, the mitogenic action of IGF-I or insulin on lactotrophs requires basal activity of the cAMP-PKA pathway at an unidentified step of growth factor signaling.

Hypothalamic dopamine exerts a profound effect on many lactotroph functions, including cell proliferation, synthesis and secretion of PRL, apoptosis, cell morphology, intracellular Ca\(^{2+}\) concentration, and membrane potentials (1). The signal transduction pathways mediating many of the cellular responses of lactotrophs to dopamine have not been fully determined and are unlikely to involve a common mechanism. The primary mechanism by which dopamine inhibits PRL gene expression seems to be inhibition of adenylate cyclase activity and the subsequent cAMP-PKA pathway (11, 27, 28, 39), although other signaling pathways mediated by protein kinase C (4), Pit-1 (23), and ERK (25) have been proposed for the dopamine inhibition. In contrast, the mechanism of the antimitogenic action of dopamine is less well understood. Therefore, we tested the hypothesis that dopamine inhibits PRL promoter activity and lactotroph proliferation by inhibiting the cAMP-PKA-CREB pathway. First, we showed that bromocriptine inhibited basal and forskolin-induced CRE-luciferase activity and lactotroph CREB phosphorylation. Second, CREB expression, mimicking suppression of CRE-mediated transcription and CREB phosphorylation observed in the bromocriptine-treated lactotrophs, inhibited lactotroph proliferation and PRL promoter activity. These results support the idea that inhibition of CREB activity is responsible for the dopamine inhibition of both PRL promoter activity and cell proliferation in normal lactotrophs.

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