Effects of Bone Morphogenetic Protein (BMP) on Adrenocorticotropin Production by Pituitary Corticotrope Cells: Involvement of Up-Regulation of BMP Receptor Signaling by Somatostatin Analogs

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The mechanism by which somatostatin analogs suppress ACTH production by corticotropinomas has yet to be fully elucidated. We here studied the effects of somatostatin analogs on ACTH secretion using mouse corticotrope AtT20 cells focusing on the biological activity of bone morphogenetic proteins (BMPs). BMP ligands, receptors and Smads, and somatostatin receptors (SSTRs)-2, -3, and -5 were expressed in AtT20 cells. BMP-2, -4, -6, and -7 decreased basal ACTH production with BMP-4 effects being the most prominent. BMP-4 also inhibited CRH-induced ACTH production and proopiomelanocortin (POMC) transcription. However, the decrease in CRH-induced cAMP accumulation caused by BMP-4 was not sufficient to completely account for BMP-4 actions, indicating that ACTH suppression by BMPs was not directly linked to cAMP inhibition. CRH-activated ERK1/ERK2, p38-MAPK, stress-activated protein kinase/c-Jun NH_2-terminal kinase, protein kinase C, and Akt pathways and CRH-induced ACTH synthesis was significantly decreased in the presence of U0126 or SB203580. Because BMPs attenuated CRH-induced ERK and p38 phosphorylation, it was suggested that BMP-4 suppresses ACTH production by inhibiting CRH-induced ERK and p38 phosphorylation. Somatostatin analogs octreotide and pasireotide (SOM230) significantly suppressed CRH-induced ACTH and cAMP production in AtT20 cells and reduced ERK and p38 phosphorylation. Notably, CRH-induced ACTH production was enhanced in the presence of noggin, a BMP-binding protein. The inhibitory effects of octreotide and SOM230 on CRH-induced ACTH production were also attenuated by noggin, implying that the endogenous BMP system plays a key role in inhibiting CRH-induced ACTH production by AtT20 cells. The findings that OCT and SOM230 up-regulated BMP-Smad1/Smad5/Smad8 signaling and ALK-3 and BMPRII and down-regulated inhibitory Smad6/7 establish that the activation of endogenous BMP system is functionally involved in the mechanism by which somatostatin analogs suppress CRH-induced ACTH production. (*Endocrinology* 151: 1129–1141, 2010)

Abbreviations: ActRII, Activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPR, BMP receptor; BtcAMP, N^6,O^2-dibutyryl cAMP monosodium salt; CRHR1, CRH receptor 1; DAPI, 4′,6′-diamidino-2-phenylindole; dNTP, deoxynucleotide triphosphate; D2R, dopamine D2 receptor; FCS, fetal calf serum; FSK, forskolin; JNK, c-Jun NH_2-terminal kinase; pCMV-β-gal, cytomegalovirus-β-galactosidase plasmid; PKA, protein kinase A; POMC, proopiomelanocortin; PPAR, peroxisome proliferator-activated receptor; RPL19, ribosomal L19; SAPK, stress-activated protein kinase; SSTR, somatostatin receptor.

ACTH secretion is physiologically inhibited by somatostatin, a 14- or 28-amino-acid cyclopeptide that is also involved in the regulation of GH secretion from the anterior pituitary (1, 2). Somatostatin effects are mediated through G protein-coupled somatostatin receptors (SSTR)-1 to -5 and can be activated by various somatostatin analogs having different affinities for the five receptors. For instance, octreotide is a SSTR-2-prefering agonist, and pasireotide (also called SOM230) is a multireceptor agonist that binds to SSTR-1, -2, -3, and -5 with high affinity, having a 30- and 40-fold higher and prolonged affinity to SSTR-1 and SSTR-5, respectively, as compared with the effects of octreotide (3, 4).

Human corticotrope adenomas also express multiple SSTRs, with the expression of SSTR-5 predominating over the expression of the other SSTRs (5–7). Studies using the murine corticotrope AtT20 cell line demonstrated that the inhibition of cAMP accumulation and regulation of ACTH secretion are predominantly mediated by SSTR-2 and -5 (8), suggesting that SOM230 could be effective for inhibiting ACTH release in human corticotrope tumors. SOM230 has also been shown to inhibit basal ACTH release from human ACTH-secreting pituitary adenoma cells (7). In addition, SOM230 also suppresses CRH- and adrenalectomy-induced plasma ACTH levels in rats *in vivo* (9, 10). These data strongly argue for the usefulness of somatostatin analogs with high affinity for SSTR-2 and -5, such as SOM230, to regulate plasma ACTH and cortisol levels in Cushing's patients. However, the detailed mechanism underlying ACTH suppression by somatostatin analogs has yet to be elucidated.

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily member. A variety of physiological BMP actions in many endocrine tissues including the ovary (11–13), pituitary (14–18), hypothalamus (19), thyroid (20), and adrenal (21–25) has been recently identified. There has been increasing evidence that locally produced BMPs play critical roles in the pituitary differentiation. For instance, BMP-4, -6, -7, and -15 activate FSH production by pituitary gonadotropes (14, 26, 27). Human gonadotropinomas were found to have reduced expression of the activin/BMP-binding protein, follistatin, as compared with nonfunctioning tumors (15). In addition, peroxisome proliferator-activated receptor (PPAR)-γ activation is functionally linked to the inhibition of BMP receptor (BMPR) signaling in gonadotorope LβT2 cells (28). It is also interesting that BMP-4 is overexpressed in various lactotrope tumor models including dopamine D2-receptor-null mice, estrogen-induced rat prolactinomas, and human prolactinomas (17), in which molecular interaction of BMP-4, Smad4, and estrogen receptor is functionally involved to regulate prolactin-promoter activity

(29). Notably, Giacomini and colleagues (18) have reported that BMP-4 expressed in the pituitary inhibits corticotrope cell proliferation and ACTH production by pituitary corticotrope tumor cells, in which BMP-4 expression is augmented by retinoic acid. Thus, the pituitary BMP system is likely to act as a regulator not only for pituitary differentiation but also for the transformation of differentiated pituitary cells (14–18, 28–30).

In the present study, we examined the effects of somatostatin analogs on ACTH suppression using mouse corticotrope AtT20 cells and focusing on the biological activity of BMPs. Arzt's laboratory (31, 32) has demonstrated that CRH stimulates a MAPK, ERK, which is activated downstream of cAMP-protein kinase A (PKA) through CRH receptor 1 (CRHR1) on corticotropes. We here have found that BMP-4 suppresses ACTH production by inhibiting CRH-induced ERK and p38 pathways in corticotrope cells. In this regulatory system, somatostatin analogs act to facilitate the BMP-Smad signaling. This new cross-communication among BMP-4, CRH, and somatostatin actions may play a key role in controlling ACTH production in the pituitary corticotrope.

Materials and Methods

Reagents and supplies

Human and rat CRH, forskolin (FSK), N^6,O^2-dibutyryl cAMP monosodium salt (BtcAMP), and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human BMP-2, -4, -6, and -7 and mouse noggin were purchased from R&D Systems Inc. (Minneapolis, MN); an ERK inhibitor, U0126, and a p38-MAPK inhibitor, SB203580, were from Promega Corp. (Madison, WI); and a stress-activated protein kinase (SAPK)/c-Jun NH$_2$-terminal kinase (JNK) inhibitor, SP600125, was from Biomol Laboratories Inc. (Plymouth Meeting, PA). Normal rat pituitary tissues were collected from 8-wk female Wister rats. Mouse ovary total RNA was purchased from Ambion (Austin, TX). Pasireotide (SOM230) and octreotide acetate were provided from Novartis International Pharmaceutical Ltd. (Basel, Switzerland).

Measurement of ACTH and cAMP

AtT20/D16v (AtT20) cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin at 37 C in a 5% CO$_2$ humidified atmosphere. To assess effects of BMPs on ACTH synthesis, AtT20 cells (1×10^4 viable cells per well) were precultured in 24-well plates with 10% FCS for 24 h. The medium was then changed to serum-free DMEM and subsequently treated with octreotide, SOM230, BMPs, noggin, and various inhibitors in the presence or absence of CRH, FSK or BtcAMP. After 24 to 72 h culture, the supernatant of the culture media was collected and stored at −80 C until assay. ACTH concentrations in the media were measured by a radioimmunometric assay that enables to specifically detect ACTH 1-39 peptide (ACTH IRMA kit; Mitsubishi Chemical, Tokyo,

Japan). To assess cellular cAMP synthesis, AtT20 cells (1×10^4 viable cells per well) were cultured in serum-free DMEM containing 0.1 mM IBMX. After 24 to 48 h culture, the conditioned medium was collected and the extracellular contents of cAMP were determined by a RIA with assay sensitivity of 0.6 nM (Yamasa Shoyu, Tokyo, Japan).

RNA extraction, RT-PCR, and quantitative real-time PCR analysis

AtT20 cells (1×10^5 viable cells per well) were precultured in serum-free DMEM in 12-well plates, and cells were treated with indicated concentrations of BMPs, octreotide, and SOM230 in combination with CRH or FSK. After 24 h culture, total cellular RNAs were extracted using TRIzol (Invitrogen Corp., Carlsbad, CA). The expression of SSTRs, dopamine D2 receptor (D2R), proopiomelanocortin (POMC), PPARα, PPARγ, D2R, BMP ligands and receptors, Smads, BMP-binding protein follistatin, and housekeeping gene ribosomal L19 (RPL19) was detected by RT-PCR. The extracted RNA (1 μg) was subjected to RT reaction using first-strand cDNA synthesis system (Invitrogen) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42 C for 50 min and 70 C for 10 min. Hot-start PCR was performed using $MgCl_2$ (1.5 mM), dNTP (0.2 mM), and *Taq* DNA polymerase (2.5 U) (Invitrogen). PCR primer pairs were selected from different exons of the corresponding genes as follows: SSTR-1, 526-546 and 869-889 (from GenBank accession no. X62314); SSTR-2, 240-260 and 559-579 (M93273); SSTR-3, 501-521 and 782-802 (X63574); SSTR-4, 400-420 and 781-801 (M96544); SSTR-5, 98-118 and 368-388 (L04535); D2R, 542-562 and 851-871 (X56065); PPARα, 601-622 and 932-953 (NM_011144); PPARγ, 350-371 and 721-742 (NM_011146); and POMC, 155-175 and 374-394 (BC061215). Primer pairs for mouse BMP-2, -4, -6, and -7; activin receptor-like kinase (ALK)-2, -3, -4, and -6; and activin type II receptor (ActRII), BMPRII, Smad1-8, follistatin, and RPL19 were selected as we reported (16, 19, 28, 33). The aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of POMC, BMPRs, Smad6/Smad7, and RPL19 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA master SYBR Green I system (Roche Diagnostic Co., Tokyo, Japan). Accumulated levels of fluorescence for each product were analyzed by the second-derivative method after the melting-curve analysis (Roche), and then, after the assay validation by calculating each amplification efficiency, the expression levels of target genes were quantified based on standard curve analysis for each product and normalized by RPL19 level in each target.

Thymidine incorporation assay

AtT20 cells (1×10^5 viable cells per well) were precultured in 12-well plates with DMEM containing 10% FCS for 24 h. After preculture, medium was replaced with fresh serum-free medium and cells were treated with indicated concentrations of CRH, BMPs, octreotide, and SOM230. After 24 h culture, 0.5 μCi/ml [*methyl-*³H]thymidine (Amersham Pharmacia, Piscataway, NJ) was added and incubated for 3 h at 37 C, and the incorporated thymidine was detected as we reported (34). Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 60 min at 4 C, and solubilized in 0.5 M NaOH, and radioactivity was determined with a liquid scintillation counter.

Western immunoblot analysis

AtT20 cells (1×10^5 viable cells per well) were precultured in serum-free DMEM. After 24 h preculture, BMPs, octreotide, SOM230, CRH, and BtcAMP were added either alone or in the indicated combinations. After 15 to 60 min stimulation with hormones and/or growth factors, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM NaF, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis (23, 33), using anti-phospho- and anti-total-ERK1/2 MAPK antibody, anti-phospho- and anti-total-p38 MAPK antibody, anti-phospho- and anti-total-SAPK/JNK MAPK antibody, anti-phospho- and anti-total-Akt antibody, anti-phospho-PKC (pan) antibody, anti-Smad5 and anti-phospho-Smad1/Smad5/Smad8 (pSmad1/5/8) antibody (Cell Signaling Technology, Inc., Beverly, MA), and anti-actin antibody (Sigma-Aldrich). The relative integrated density of each protein band was digitized by NIH Image J 1.34s.

Immunofluorescence microscopy

For immunofluorescence study, AtT20 cells were precultured in serum-free DMEM using chamber slides (Nalge Nunc Int., Naperville, IL) and treated with BMP-4 (100 ng/ml) for 1 h. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature, and washed three times with PBS. The cells were then incubated with anti-phospho-Smad1/5/8 antibody (Cell Signaling Technology) for 1 h and washed three times with PBS and then with Alexa Fluor 488 antirabbit IgG (Invitrogen Corp.) in a humidified chamber for 1 h. After washing with PBS, cells were also incubated with phalloidin-conjugated Alexa Fluor (Invitrogen), followed by application of the counter medium containing 4′,6′-diamidino-2-phenylindole (DAPI), and then stained cells were visualized under a fluorescent microscope.

Transient transfection and luciferase assay

AtT20 cells (1×10^5 viable cells per well) were precultured with 10% FCS for 24 h. The cells were transiently transfected with 500 ng POMC-luc or BRE-luc reporter plasmid with 50 ng cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal) using FuGENE6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h as we reported (16, 28). Cells were then treated with indicated concentrations of BMP-4, octreotide, and SOM230 in combination with CRH in serum-free medium. After 24 h culture, the cells were washed with PBS and lysed with cell culture lysis reagent (Toyobo, Osaka, Japan). Luciferase activity and β-galactosidase (β-gal) activity of the cell lysate were measured by a luminometer. The data were shown as the ratio of luciferase to β-gal activity.

Statistical analysis

All results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Fisher's protected least significant difference

test (StatView 5.0 software; Abacus Concepts, Inc., Berkeley, CA). *P* values < 0.05 were accepted as statistically significant.

Results

We first examined expression profile of the SSTRs and key components of the BMP system in AtT20 cells and rat normal pituitary tissues by RT-PCR (Fig. 1A). Ovarian tissue RNA was used as positive expression control for the BMP system molecules. AtT20 cells expressed three subtypes of SSTRs including SSTR-2, -3, and -5 but lacked detectable expression of SSTR-1 and -4 subtypes. Rat normal pituitary tissues expressed all five SSTR subtypes as well as POMC, PPARα, PPARγ, and D2R. In AtT20 cells, POMC and PPARγ expression was clearly detected, but PPARα or D2R expression was not detected. In addition, AtT20 cells expressed BMP ligands (BMP-2, -4, -6, and -7), BMP/activin type I (ALK-2, -3, and -4), type II (ActRII, ActRIIB, and BMPRII) receptors, binding protein follistatin, and Smads (Smad1, 2, 3, 4, 5, 6, 7, and 8) (Fig. 1A). The expression patterns of BMP ligands, receptors, and Smads in AtT20 cells were similar to that of normal pituitary tissues. BMP type IB receptor ALK-6 was not expressed in AtT20 cells or whole pituitary tissues.

We also investigated the ACTH secretory profile of AtT20 cells in response to CRH. As shown in Fig. 1B, basal ACTH production increased time dependently during 72 h culture. CRH (10–300 nM) increased ACTH production in the medium approximately 2-fold in a concentration-dependent manner during 24 h culture in serum-free conditions (Fig. 1C). FSK (10 μM) and BtcAMP (1 mM) also increased ACTH production by AtT20 cells; however, the maximal effects of cAMP-PKA activation by cAMP compounds were 25% less potent than that induced by CRH (Fig. 1C).

The effects of somatostatin analogs on ACTH production were next examined in AtT20 cells. As shown in Fig. 1D, basal ACTH production was not significantly changed by pasireotide (SOM230) during 72 h exposure. Dose-dependent experiments for 24 h with octreotide and SOM230 marginally reduced basal ACTH; however, the effects were not statistically significant (Fig. 1D). The effects of somatostatin analogs on CRH-induced ACTH (Fig. 1E) and cAMP (Fig. 1F) production were then examined in AtT20 cells. SOM230 suppressed CRH-induced ACTH secretion for 72 h, in which a marked difference was detected in 24-h culture experiments (Fig. 1E). SOM230 also suppressed CRH-induced cAMP secretion for 48 h (Fig. 1F). SOM230 and octreotide reduced CRH-induced ACTH (Fig. 1E) and cAMP (Fig. 1F) production concentration dependently for 24 h.

To elucidate the involvement of endogenous BMP actions on CRH-induced ACTH production, cells were treated with a BMP-binding protein noggin, which antagonizes BMP signaling, and CRH-induced ACTH secretion was examined. As shown in Fig. 2A, noggin (30 ng/ml) increased basal levels of ACTH accumulated in the medium over 24–72 h culture. ACTH levels induced by CRH (3–100 nM) were also up-regulated in the presence of noggin. Notably, the ACTH reduction caused by SOM230 (30 μM) and octreotide (30 μM) was restored by cotreatment with 10–100 ng/ml noggin (Fig. 2A), suggesting that endogenous BMPs are likely to play inhibitory roles in ACTH production by AtT20 cells.

To characterize the BMP signaling pathway in AtT20 cells, cells were treated with various BMP ligands in the presence or absence of CRH. Immunofluorescence demonstrated the nuclear localization of phosphorylated Smad1/Smad5/Smad8 in AtT20 cells stimulated by BMP-4 (Fig. 2B). Western immunoblots also showed Smad1/Smad5/Smad8 phosphorylation by BMP-2, -4, -6, and -7, which was maintained regardless of the presence of CRH (100 nM) in AtT20 cells. Next, the effects of BMPs on basal and CRH-induced ACTH production were examined. For 24- to 72-h experiments, BMP-4 potently suppressed basal and CRH-induced ACTH production (Fig. 2C). As shown in Fig. 2D, BMP-2, -4, -6, and -7 significantly decreased basal and CRH-induced ACTH production concentration dependently for 24 h culture. Each of the tested BMPs suppressed ACTH secreted into the medium (both in the presence and absence of CRH), with BMP-4 being the most efficacious (Fig. 2D). The IC_{50} values for suppressing basal and CRH-induced ACTH were as follows: BMP-4, 3 ng/ml; BMP-2, 10 ng/ml; BMP-6, 30 ng/ml; and BMP-7, 30 ng/ml.

We next examined whether the BMP effects on ACTH reduction were accompanied by transcriptional regulation of POMC gene. POMC transcription was examined by AtT20 cells transiently transfected with POMC-luc plasmid including 0.7 kb 5′-promoter region of rat POMC gene (35). As shown in Fig. 3A, POMC-promoter activity was significantly increased by CRH (100 nM) and, less effectively, by FSK (10 μM). BMP-2 and BMP-4 were potent in inhibiting CRH-induced POMC-promoter activity, although BMP-2 and BMP-4 did not affect POMC-luc activity in AtT20 cells in the absence of CRH. Steady-state POMC mRNA levels were assessed by quantitative PCR. Basal and CRH-induced POMC mRNA levels were significantly reduced by BMP-2 and BMP-4 (Fig. 3B). The inhibitory effects of BMP-4 on POMC transcription were more potent than the effects of BMP-2. Thus, BMP-2 and -4 suppress POMC transcription regardless of the presence of CRH actions, leading to the inhibition of ACTH production by AtT20 cells. We further
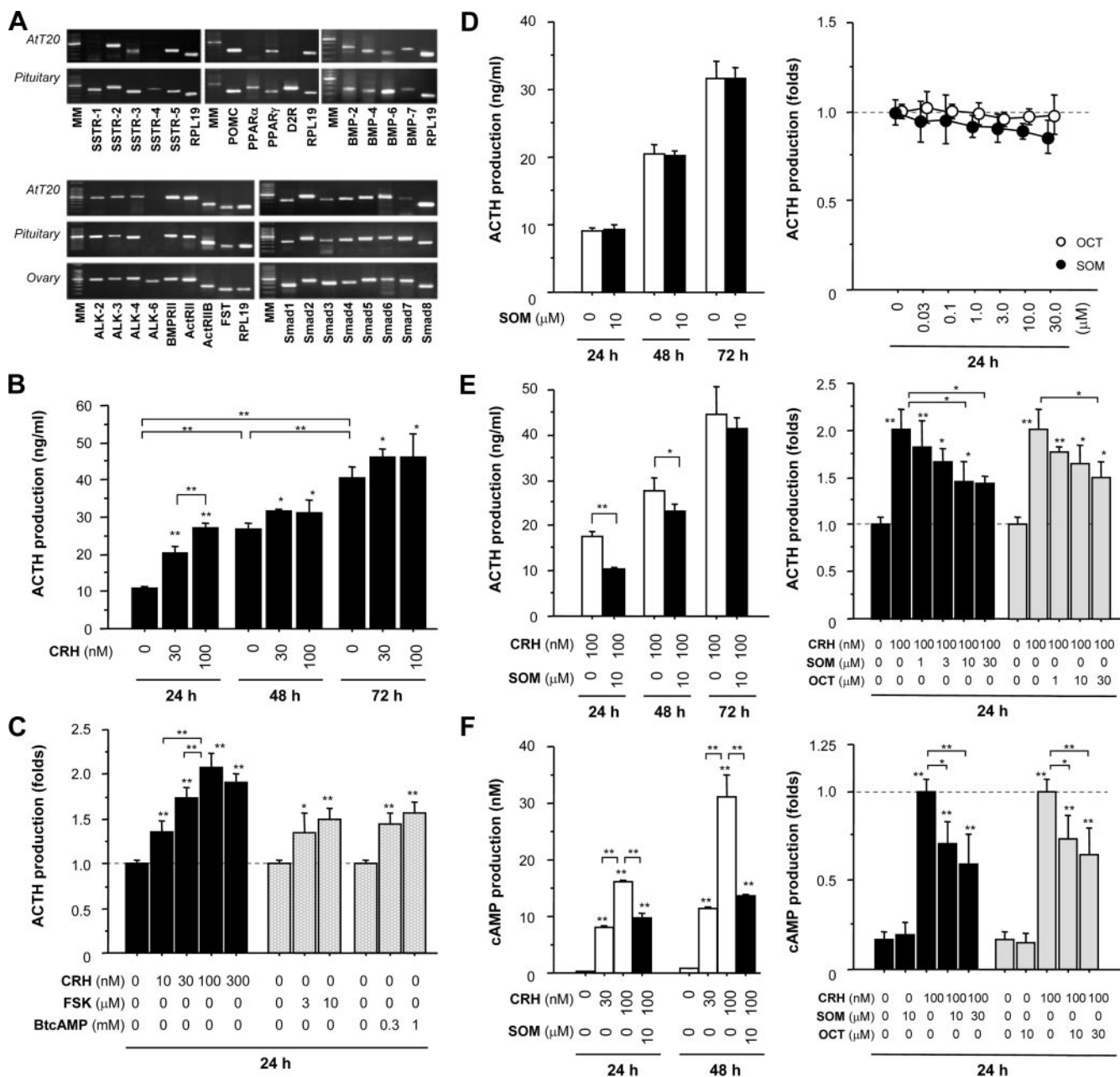
FIG. 1. Characterization of AtT20 cells regarding the BMP system and effects of somatostatin analogs on basal and CRH-induced ACTH and cAMP production. A, Total cellular RNAs were extracted from AtT20 cells and quantified by measuring the absorbance of the sample at 260 nm. The expression of mRNAs encoding SSTRs, D2R, POMC, PPARs, BMP ligands and receptors, follistatin (FST), Smads, and housekeeping gene RPL19 was examined by RT-PCR analysis in AtT20 cells compared with control samples extracted from rat pituitary and mouse ovary tissues. Aliquots of PCR products were electrophoresed on 1.5% agarose gel, visualized by ethidium bromide staining, and shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker. B and C, AtT20 cells (1×10^4 viable cells per well) were cultured in 24-well plates with DMEM containing 10% FCS for 24 h. The medium was then changed to serum-free DMEM and subsequently treated with the indicated concentrations of CRH, FSK, and BtcAMP. After 24–72 h culture, the culture medium was collected and ACTH levels were determined by radioimmunometric assay. D and E, AtT20 cells (1×10^4 viable cells per well) were treated with the indicated concentrations of SOM230 (SOM) or octreotide (OCT) in the absence or presence of CRH (100 nm) in serum-free conditions. After 24–72 h culture, the supernatant of the culture media was collected and ACTH concentrations in the culture media were measured. F, To assess cellular cAMP synthesis, cells (1×10^4 viable cells per well) were cultured in 24-well plates with serum-free DMEM containing 0.1 mm IBMX. After 24–48 h culture with indicated treatments, the conditioned medium was collected and the extracellular content of cAMP was determined by RIA. Results are shown as mean $\pm$ SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ vs. control groups or between the indicated groups.

examined the effects of BMPs on CRH-induced cAMP accumulation. BMP-2, -6, and -7 did not affect CRH-induced cAMP accumulation in the medium after a 24-h incubation (Fig. 3C). However, 100 ng/ml BMP-4 had a weak but significant inhibitory effect on CRH-induced cAMP in AtT20 cells (Fig. 3C).
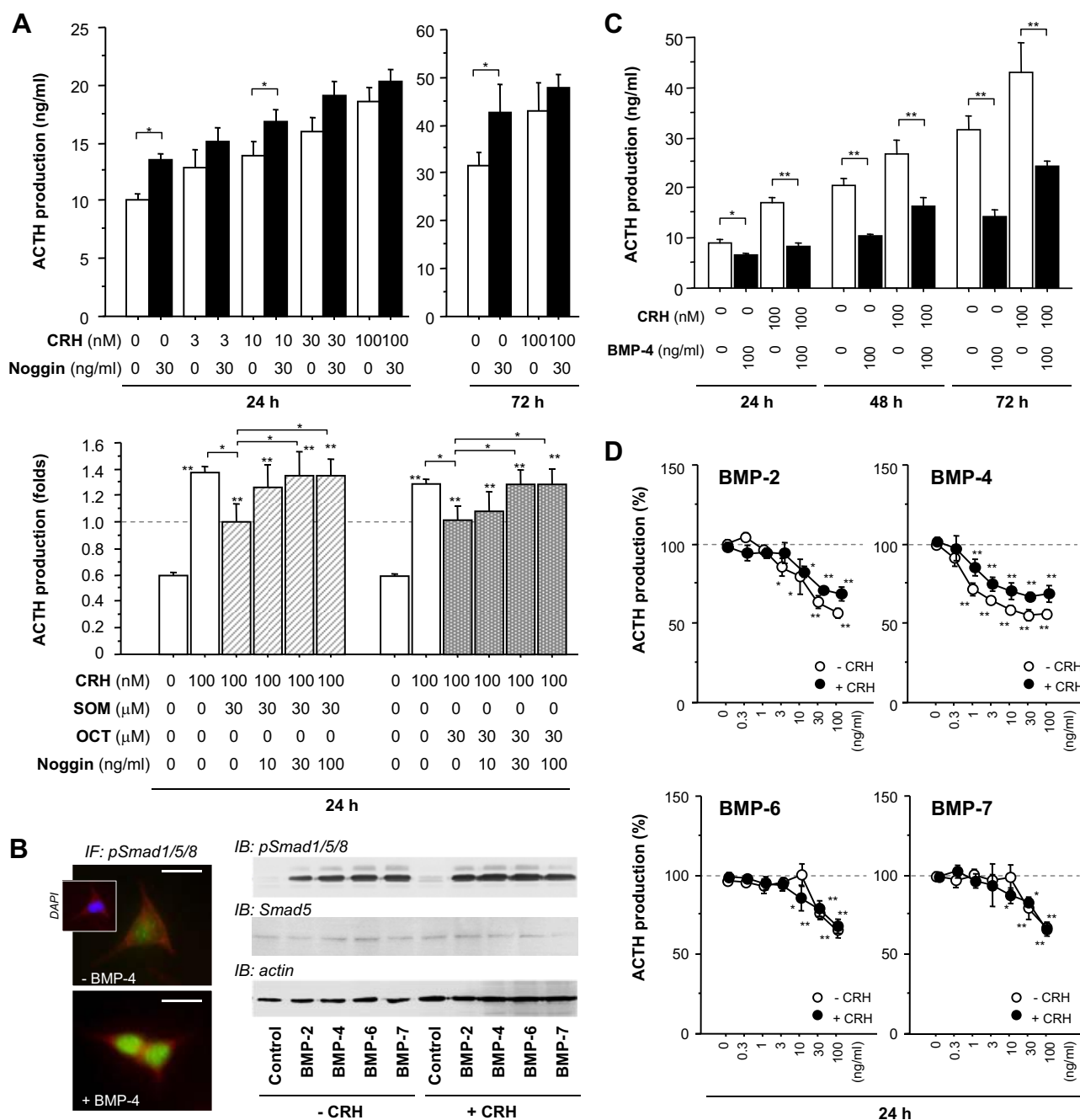
FIG. 2. Effects of noggin and BMPs on basal and CRH-induced ACTH production by AtT20 cells. A, AtT20 cells (1 × 10⁴ viable cells per well) were cultured in 24-well plates with DMEM containing 10% FCS for 24 h. The medium was then changed to serum-free DMEM and subsequently treated with the indicated concentrations of noggin and CRH (*upper panel*), in combination with SOM230 (SOM) and octreotide (OCT) (*lower panel*). After 24–72 h culture, the supernatant of the culture media was collected and ACTH concentrations in the culture media were measured by radioimmunometric assay. B, Cells were precultured for 24 h and stimulated with BMP-4 (100 ng/ml) for 1 h. Immunofluorescence (IF) studies were performed using anti-phospho-Smad1/Smad5/Smad8 (pSmad1/5/8) antibody and phalloidin staining for actin on BMP-4-treated and untreated cells (*left panel*). DAPI indicates counterstaining with DAPI. *Scale bars*, 20 μm. Cells (1 × 10⁵ viable cells per well) were precultured for 24 h and stimulated with BMP-2, -4, and -7 (100 ng/ml) in the absence or presence of CRH (100 nM). After 60 min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-pSmad1/5/8, Smad5, and actin antibody (*right panel*). The results shown are representative of those obtained from three independent experiments. C and D, AtT20 cells (1 × 10⁴ viable cells per well) were treated with the indicated concentrations of BMP-2, -4, -6, and -7 in the absence or presence of CRH in serum-free conditions. After 24–72 h culture, the supernatant of the culture media was collected and ACTH concentrations in the media were measured. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P <$ 0.05; **, $P <$ 0.01 *vs.* control groups or between the indicated groups.

Cell lysates of AtT20 cells stimulated with CRH (100 nM) for 15 and 60 min were applied for SDS/PAGE-immunoblot analysis. As shown in Fig. 4A, CRH (100 nM)

activated phosphorylation of MAPKs including ERK1/ERK2, p38-MAPK, and SAPK/JNK, PKC, and Akt although BMP-2, -4, -6, and -7 alone did not affect the
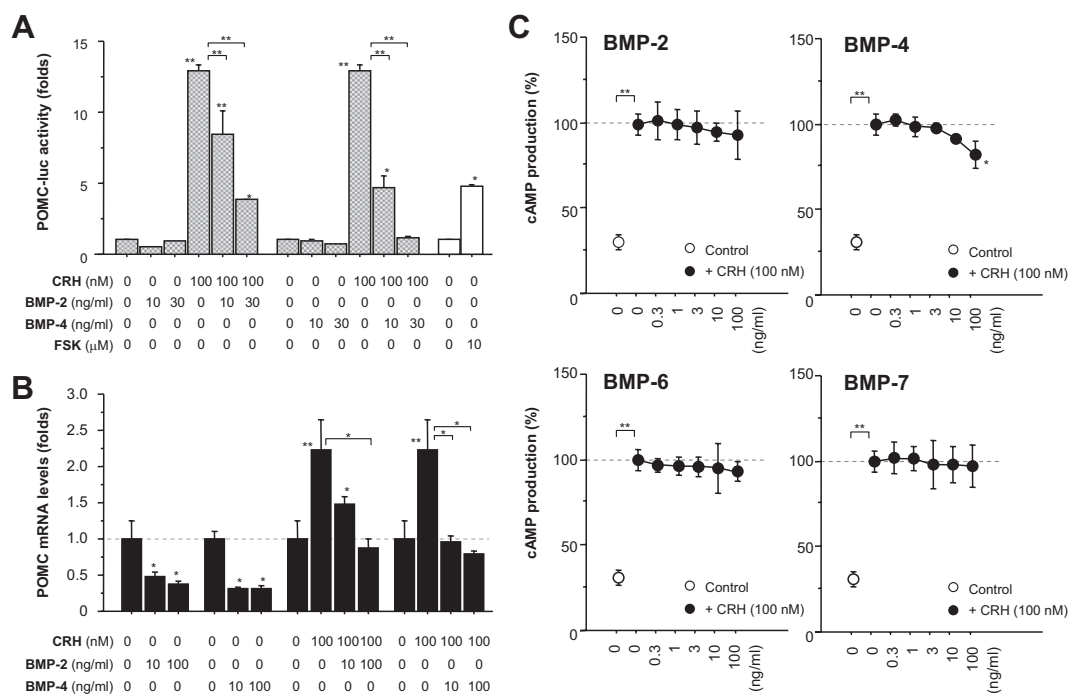
FIG. 3. Effects of BMPs on POMC transcription and CRH-induced cAMP synthesis by AtT20 cells. A, AtT20 cells (1×10^5 viable cells per well) were transiently transfected with POMC-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24 h treatment with indicated concentrations of CRH, BMP-2 and -4, and FSK, cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-gal activity. B, AtT20 cells (1×10^5 viable cells per well) were treated with indicated concentrations of CRH amd BMP-2 and -4 in serum-free DMEM for 24 h. Total cellular RNA was extracted, and POMC mRNA levels were examined by quantitative real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. C, AtT20 cells (1×10^4 viable cells per well) were treated with the indicated concentrations of BMP-2, -4, -6, and -7 in the presence of CRH in serum-free DMEM containing 0.1 mM IBMX. After 24 h culture, the supernatant of the culture media was collected and the extracellular contents of cAMP were determined by RIA. Results are shown as mean $\pm$ SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ vs. control groups, between the indicated groups or CRH-treated basal levels (C).

phosphorylation of this signaling. CRH-induced phosphorylation of SAPK/JNK, PKC, and Akt by CRH was not affected by BMPs. However, it was of note that BMP-2, -4, -6, and -7 inhibited CRH-induced phosphorylation of ERK1/ERK2 and p38-MAPK (Fig. 4B). In addition, cAMP also activated ERK phosphorylation with similar potent to CRH (Fig. 4C). The cAMP effect was much less potent on p38 phosphorylation than that of CRH (Fig. 4C). Moreover, CRH-induced phosphorylation of ERK and p38 was significantly suppressed by SOM230 and octreotide, with SOM230 more potent than octreotide (Fig. 4D). We next examined functional roles of ERK and p38 pathways in CRH-induced ACTH production using chemical inhibitors for each pathway. U0126 is an established selective inhibitor of ERK1/ERK2 that works by inhibiting the kinase activity of the upstream ERK1/ERK2 kinases, MEK1/MEK2 (36). SB203580 is an ATP-competitive inhibitor for p38-MAPK but not for the upstream p38-MAPK kinases such as MKK3 or -6 (37). Among the isoforms including p38α, -β, -γ, and -δ, SB203580 specifically inhibits the phosphorylation of p38α/p38β (37). SP600125 is a selective and reversible ATP-competitive inhibitor of JNK1, -2, and -3 (38). CRH-

induced ACTH synthesis was significantly reduced by U0126, SB203580, and a PKA inhibitor H89 but not by SP600125 (Fig. 4E).

We also studied the involvement of BMP-4 in AtT20 cell proliferation. As seen in Fig. 5A, 24 h exposure of BMP-4 (100 ng/ml), CRH (100 nM), octreotide (10 μM), or SOM230 (10 μM) did not suppress basal AtT20 cell mitosis significantly. In addition, octreotide or SOM230 did not affect thymidine uptake by CRH-treated AtT20 cells. Notably, in the presence of BMP-4, octreotide and SOM230 elicited significant suppression of AtT20 cell mitosis. To elucidate the interaction between BMP signaling and the effects of octreotide and SOM230, changes of BMP-responsive promoter BRE-luc activity were evaluated in AtT20 cells. As shown in Fig. 5B, BMP-4 readily stimulated BRE-luc activity. Octreotide and SOM230 alone had no specific effects on basal BRE-promoter activity. However, BMP-4-induced BRE-luc activity was further up-regulated in AtT20 cells treated with octreotide or SOM230. Interestingly, cotreatment with octreotide and SOM230 additively enhanced BMP-4-induced BRE-luc activation in AtT20 cells (Fig. 5B). In the presence of CRH, the BMP-4 responsiveness
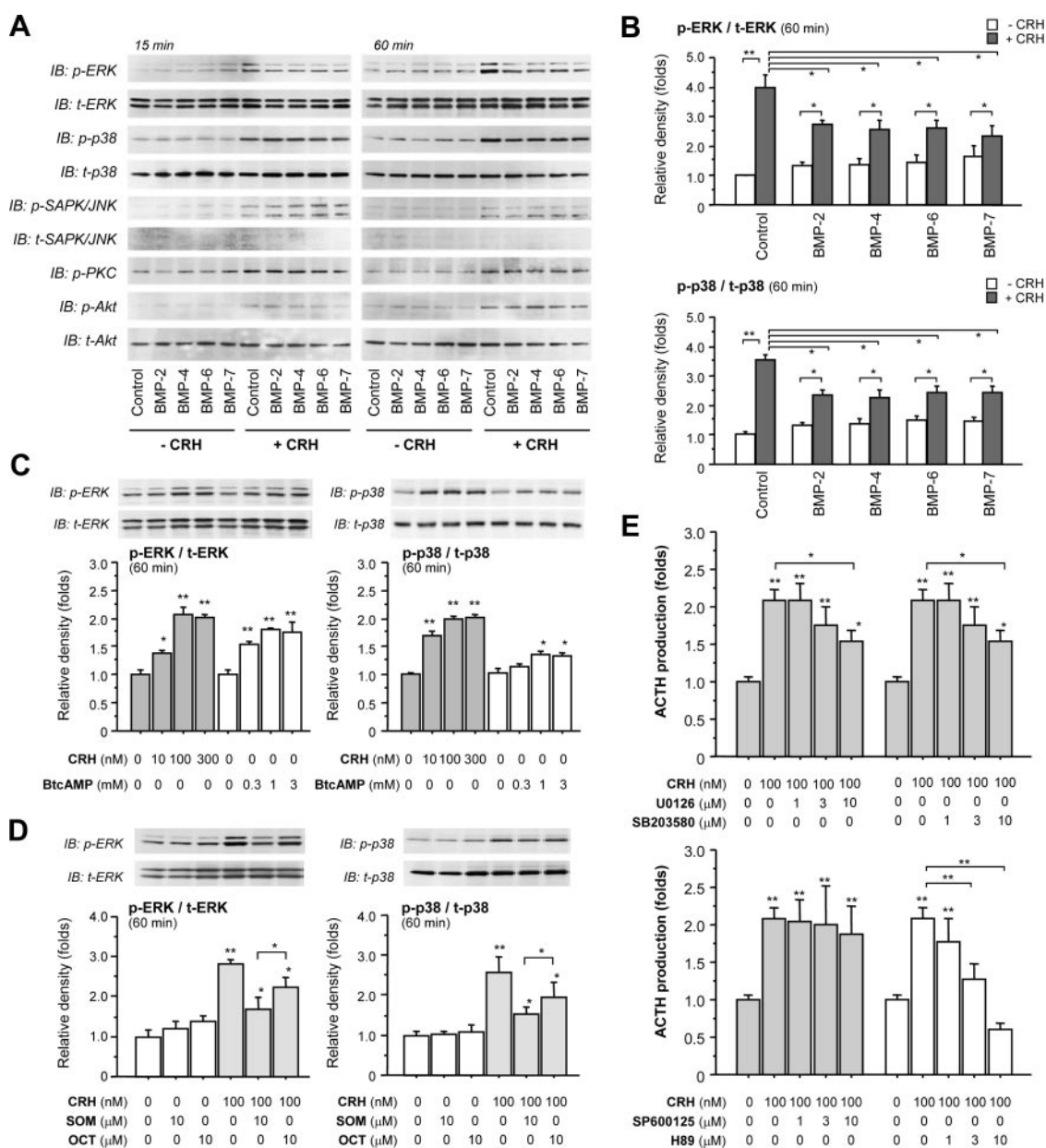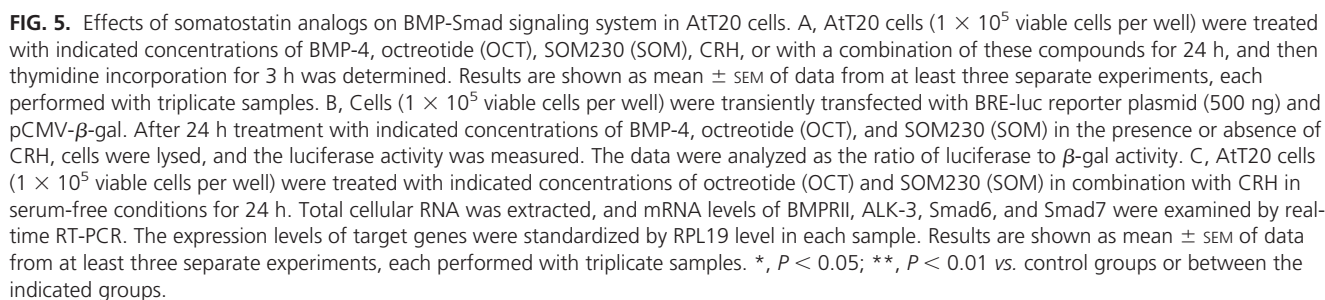
FIG. 4. Effects of BMPs and somatostatin analogs on CRH-induced MAPK activation and effects of MAPK inhibition on CRH-induced ACTH production by AtT20 cells. A, AtT20 cells (1×10^5 viable cells per well) were precultured for 24 h and treated with BMP-2, -4, -6, and -7 (100 ng/ml) in the absence or presence of CRH (100 nM). After 15 and 60 min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-phospho-ERK1/2 (p-ERK1/2) and anti-total-ERK1/2 (t-ERK1/2), anti-phospho-p38 (p-p38) and anti-total-p38 (t-p38), anti-phospho-SAPK/JNK (p-SAPK/JNK) and anti-total-SAPK/JNK (t-SAPK/JNK), anti-phospho-pan PKC (p-PKC), and anti-phospho-Akt (p-Akt) and anti-total-Akt (t-Akt) antibodies. The results shown are representative of those obtained from three independent experiments. B, The bands on the x-ray film were scanned and digitized, and the signal intensities were numerically converted. The relative integrated density of each protein band was digitized by NIH image J 1.34s. C and D, AtT20 cells (1×10^5 viable cells per well) were precultured for 24 h and treated with indicated concentrations of CRH, BtcAMP, SOM230 (SOM), or octreotide (OCT) or with a combination of these compounds. After 60 min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-p-ERK1/2 and anti-t-ERK1/2 and anti- p-p38 and anti-t-p38 antibodies. The results shown are representative of those obtained from three independent experiments. The relative integrated density of each protein band was digitized by NIH image J 1.34s. E, AtT20 cells (1×10^4 viable cells per well) were treated with the indicated concentrations of U0126, SB203580, SP600125, and H89 in the presence of CRH in serum-free conditions. After 24 h culture, the supernatant of the culture media was collected and ACTH concentrations in the media were measured by radioimmunometric assay. Results are shown as mean ± SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ *vs.* control groups or between the indicated groups.

and the effects of octreotide and SOM230 were further augmented (Fig. 5B).

To pursue the mechanism by which octreotide and SOM230 increase BMPR signaling, the expression levels of BMPRs and Smads were examined (Fig. 5C). As a result, mRNA levels of a key pair of BMP-4 receptors, BMPRII and ALK-3, were increased by octreotide and SOM230 regardless of CRH actions. In contrast, mRNA levels of

FIG. 5. Effects of somatostatin analogs on BMP-Smad signaling system in AtT20 cells. A, AtT20 cells (1×10^5 viable cells per well) were treated with indicated concentrations of BMP-4, octreotide (OCT), SOM230 (SOM), CRH, or with a combination of these compounds for 24 h, and then thymidine incorporation for 3 h was determined. Results are shown as mean $\pm$ SEM of data from at least three separate experiments, each performed with triplicate samples. B, Cells (1×10^5 viable cells per well) were transiently transfected with BRE-luc reporter plasmid (500 ng) and pCMV-β-gal. After 24 h treatment with indicated concentrations of BMP-4, octreotide (OCT), and SOM230 (SOM) in the presence or absence of CRH, cells were lysed, and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β-gal activity. C, AtT20 cells (1×10^5 viable cells per well) were treated with indicated concentrations of octreotide (OCT) and SOM230 (SOM) in combination with CRH in serum-free conditions for 24 h. Total cellular RNA was extracted, and mRNA levels of BMPRII, ALK-3, Smad6, and Smad7 were examined by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ SEM of data from at least three separate experiments, each performed with triplicate samples. *, $P < 0.05$; **, $P < 0.01$ vs. control groups or between the indicated groups.

inhibitory Smad6 and Smad7 were down-regulated by octreotide and SOM230, which was not affected by treatment with CRH. The SOM230 effects increasing BMPR expression and decreasing inhibitory Smads expression were higher than the effects of octreotide (Fig. 5C).

Discussion

The BMP system has been known to play important roles in the initial development of the anterior pituitary (39). BMP-4 is required during the first stage of pituitary organogenesis for the proliferation of the Rathke's pouch, which gives rise to Pit-1 lineage cells including lactotrope cells. During the subsequent stages of pituitary organogenesis, inhibition of BMP-2 by fibroblast growth factor (FGF)-8 leads to differentiation of corticotrope cells (40, 41). BMP-4 not only governs the pituitary organogenesis but also plays a key role in the pathogenesis of differentiated pituitary lineages. For instance, BMP-4 promotes cell proliferation in lactotropes in conjunction with Smad-estrogen receptor interaction under the influence of noggin (17).

BMP-4 is also involved in antiproliferative action induced by retinoic acid on corticotropinoma cells (30). Based on Giacomini's data, antiproliferative effects of BMP-4 were observed after 3- to 5-d treatments in AtT20 cells (although 1–2 d culture with BMP-4 failed to suppress AtT20 cell proliferation) (30). Here we focused on earlier periods of 24 h culture when BMP-4 and somatostatin analogs efficaciously suppressed ACTH production by AtT20 cells. In our experiments, neither BMP-4 nor somatostatin analog elicited significant suppression of AtT20 cell proliferation for 24 h, regardless of the presence or absence of CRH. Nevertheless, it was notable that BMP-4 showed a significant inhibition of cell mitosis in the presence of somatostatin analogs, SOM230, and octreotide. These findings provide insight suggesting that somatostatin analogs facilitate BMP-4 actions and/or signal transduction.

The receptors for TGF-β superfamily members consist of type I and type II receptors, each of which exhibits serine/threonine kinase activity. Several preferential combinations of BMP ligands and receptors have been recognized to date; e.g. BMP-2 and BMP-4 preferentially bind

to ALK-3 and/or ALK-6, BMP-6 and BMP-7 most readily bind to ALK-2 and/or ALK-6 (42–45), and BMP-15 efficiently binds to ALK-6 with much lower affinity for ALK-3 (46). Regarding type II receptors, ActRII, which was originally identified as an activin receptor, also acts as a receptor for BMP-6 and BMP-7 (43, 44). BMPRII binds exclusively to BMP ligands including BMP-2, -4, -6, -7, and -15 (46–49). Because ALK-6 is not expressed in AtT20 cells, the receptor pairs of ALK-3 and BMPRII are likely to be the major functional complex for BMP-4 for regulating cAMP and ACTH production. Our data show that somatostatin analogs up-regulate BMP-Smad1/Smad5/Smad8 signaling by augmenting BMPRII and ALK-3 expression as well as by reducing the expression of inhibitory Smad6 and Smad7 in AtT20 cells. This new interaction of BMP-Smad pathway and CRHR signaling may be involved in controlling ACTH production and secretion from corticotrope cells in an autocrine/paracrine manner.

After stress-induced stimulus, CRH is synthesized in the hypothalamus and released into the portal system in the pituitary. Interaction of CRH with its receptor in corticotrope cells leads to increased transcription of POMC mRNA and secretion of ACTH into the circulatory system (50). CRH induces POMC expression in pituitary primary cultures and in AtT20 cells (51, 52). BMP-2 and BMP-4 were potent in inhibiting CRH-induced POMC-promoter activity. Basal and CRH-induced POMC mRNA levels were also reduced by BMP-2 and -4. It is therefore possible that BMP-2 and -4 may also facilitate the degradation of POMC mRNA in addition to the inhibitory effects on POMC transcriptional activity induced by CRH. In corticotrope cells, CRH also leads to an increase in intracellular cAMP levels and activation of PKA (53, 54). The actions of the CRH family peptides are mediated through seven-transmembrane domain G protein-coupled receptors, CRHR1 and CRHR2 (55). CRHR1 and CRHR2 exist in splice-variant forms that are differentially expressed in the brain and periphery. In AtT20 cells, CRHR1 is predominantly expressed (56). CRHR activation by their cognate ligands results in GTP/GDP exchange on the G protein subunit, which participates in the activation of a number of signaling pathways. CRHR activation has been shown to stimulate the MAPK pathway, in particular, the ERKs (31, 32, 56–60).

In our study, cAMP accumulation induced by CRH was not significantly suppressed by low concentrations (1–10 ng/ml) of BMP-4, suggesting that BMP-4 effects on ACTH suppression were not directly linked to cAMP inhibition in corticotrope cells. Arzt and colleagues (31, 32) have previously shown that CRH stimulates ERK1/ERK2 signaling, and the ERK activation occurs at the downstream of

cAMP-PKA through CRHR1 on corticotropes. We here further found that CRH also activated ERK, p38, SAPK/JNK, PKC, and Akt pathways, and BMPs reduced the CRH-induced ERK and p38 phosphorylation. CRH-induced ACTH synthesis was significantly reduced in the presence of U0126 or SB203580, suggesting that ERK and p38 pathways are linked to CRH-induced ACTH production. Stimulation with cAMP analogs also directly activated ERK1/ERK2 phosphorylation; however, the cAMP effect on p38 was much less potent as compared with the CRH action. We therefore concluded that cAMP-PKA and ERK pathways were functionally connected, leading to ACTH production by AtT20 cells as previously described (31, 32). Ben-Shlomo *et al.* (61) have demonstrated that SSTR signaling plays critical roles in ACTH production via the MAPK pathway. In that study, selective silencing of SSTR-2, -3, and -5 causes intracellular signaling changes including increased cAMP as well as enhanced ERK1/2 phosphorylation, in which the knockdown of SSTR-5 preferentially induced ERK1/2 phosphorylation higher than that of SSTR-2 and -3 (61). It is unclear whether this action is due to ligand-independent receptor dimerization and/or receptor interaction with downstream proteins. However, it appeared that the endogenous SSTR-2, -3, and -5 signal constitutively restrains adenylate cyclase activity and MAPK signaling, leading to suppression of ACTH production.

We here demonstrated that CRH induces cAMP and MAPK pathways, in which BMP-4 suppresses ACTH production by inhibiting CRH-induced ERK and p38-MAPK pathways in AtT20 cells. Differences in the efficacy of ACTH suppression between SOM230 and octreotide were also observed. SOM230 and octreotide up-regulated BMP-Smad1/Smad5/Smad8 signaling by augmenting BMPRII and ALK-3 expression as well as by reducing the expression of inhibitory Smad6 and Smad7. However, the effects of SOM230 on BMP-4 signaling were significantly higher than the effects of octreotide, indicating the differences of SSTR affinities between two somatostatin analogs. In an *in vitro* study using human corticotrope adenoma cells, the incubation with SOM230 resulted in inhibition of ACTH release in 30–40%, whereas octreotide inhibited ACTH release in only one culture (7). In another study using primary corticotrope adenoma cells, SOM230 inhibited ACTH secretion in five of six tumors and also inhibited cell proliferation in all tumors (5). Moreover, the suppression of CRH-induced ACTH release seen with SOM230 in AtT20 cells was not affected by dexamethasone pretreatment, whereas the suppressive effects of octreotide were almost completely blocked by exogenously added dexamethasone (6, 7). Given that SSTR-2 is down-regulated by excess glucocorticoids, and

that SSTR-5 is more resistant to glucocorticoids, the inhibition of SSTR-2 expression by cortisol may explain the lack of efficacy of octreotide in patients with Cushing's disease who have elevated levels of cortisol.

The efficacy of somatostatin analogs is linked to the SSTR selectivity profile. The binding capability to SSTR-2 and -5 appears critical (62) and is negatively coupled to adenylate cyclase, leading to a reduction of intracellular cAMP levels (63). Therefore, the presence of SSTR-2/-5 on corticotrope adenomas and the numerous pathways of somatostatin action (including tyrosine phosphatases, potassium and calcium channel modulation, phospholipase C, and adenylate cyclase pathways) suggests that somatostatin analogs with modified receptor subtype affinities with prolonged binding capacity would have the potential to be developed as new treatment modalities for corticotrope adenomas.

Collectively, functional interaction between the effects of somatostatin analogs and BMP-4 signaling for CRH-induced ACTH suppression was here uncovered. Considering that somatostatin analogs up-regulated BMP-Smad1/Smad5/Smad8 signaling in corticotrope cells, the activation of the endogenous BMP system may be crucial in the mechanism by which SOM230 and/or octreotide suppress CRH-induced ACTH production in corticotrope tumors.

Acknowledgments

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