0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(12):5332–5340

doi: 10.1210/en.2005-0474

Regulatory Roles of Bone Morphogenetic Proteins and Glucocorticoids in Catecholamine Production by Rat Pheochromocytoma Cells

Yoshihiro Kano, Fumio Otsuka, Masaya Takeda, Jiro Suzuki, Kenichi Inagaki, Tomoko Miyoshi, Manabu Miyamoto, Hiroyuki Otani, Toshio Ogura, and Hirofumi Makino

Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama City 700-8558, Japan

We here report a new physiological system that governs catecholamine synthesis involving bone morphogenetic proteins (BMPs) and activin in the rat pheochromocytoma cell line, PC12. BMP type I receptors, including activin receptor-like kinase-2 (ALK-2) (also referred to as ActRIA) and ALK-3 (BMPRIA), both type II receptors, ActRII and BMPRII, as well as the ligands BMP-2, -4, and -7 and inhibin/activin subunits were expressed in PC12 cells. PC12 cells predominantly secrete dopamine, whereas noradrenaline and adrenaline production is negligible. BMP-2, -4, -6, and -7 and activin A each suppressed dopamine and cAMP synthesis in a dose-dependent fashion. The BMP ligands also decreased 3,4-dihydroxyphenylalanine decarboxylase mRNA expression, whereas activin suppressed tyrosine hydroxylase expression. BMPs induced both Smad1/5/8 phosphorylation and Tlx2-Luc activation, whereas activin stimulated 3TP-Luc activity and p38 MAPK phosphorylation. ERK signaling was not affected by BMPs or activin. Dexamethasone enhanced catecholamine synthesis, accompanying increases in tyrosine hydroxylase and 3,4-dihydroxyphenylalanine decarboxylase transcription without cAMP accumulation. In the presence of dexamethasone, BMPs and activin failed to reduce dopamine as well as cAMP production. In addition, dexamethasone modulated mitotic suppression of PC12 induced by BMPs in a ligand-dependent manner. Furthermore, intracellular BMP signaling was markedly suppressed by dexamethasone treatment and the expression of ALK-2, ALK-3, and BMPRII was significantly inhibited by dexamethasone. Collectively, the endogenous BMP/activin system plays a key role in the regulation of catecholamine production. Controlling activity of the BMP system may be critical for glucocorticoid-induced catecholamine synthesis by adrenomedullar cells. (*Endocrinology* 146: 5332–5340, 2005)

THE PC12 PHEOCHROMOCYTOMA cell line is a clone of cells derived from a rat adrenal medullary tumor (1). PC12 cells exhibit many properties of adrenal medullary chromaffin cells, including catecholamine synthesis, storage, and secretion (1). The catecholamine biosynthetic pathway in which tyrosine is converted to dopamine is initially catalyzed by tyrosine hydroxylase (TH) to produce 3,4-dihydroxyphenylalanine (DOPA). DOPA is then converted to dopamine by DOPA decarboxylase (DDC), also called aromatic L-amino acid decarboxylase. Dopamine-β-hydroxylase (DBH) converts dopamine to noradrenaline. Cofactors, such as molecular oxygen, ferrous iron, and tetrahydrobiopterin, are essential for the TH-catalyzed hydroxylation reaction (2).

In adrenomedullary cells, TH is considered to be the rate-limiting enzyme in catecholamine biosynthesis. TH activity can be governed by both acute and chronic regulatory mechanisms (3). Acute regulation of TH activity occurs at a post-transcriptional level, mainly through the phosphorylation of TH resulting in the activation of the enzymes present in the cells. Chronic activation, which can last from minutes to days, is exerted through the regulation of TH transcription. The activity of both TH and DBH is regulated by second messenger mechanisms involving the activation of cAMP, protein kinase A, and protein kinase C in PC12 cells (4).

There is accumulating evidence that several growth factors and cytokines act as local autocrine/paracrine regulators of catecholamine production in PC12 cells as well as proliferation and differentiation of PC12 cells. Dopamine-inducing factors include pituitary adenylyl cyclase-activating polypeptide (5), endothelin-1 (6), nerve growth factor (7, 8), high glucose (9), and glucocorticoids (10, 11). In contrast, IGFs (12), neuropeptide Y (13), and κ-opioid (14) have been reported to be dopamine suppressors. Recently, the bone morphogenetic protein (BMP) system has been implicated in the regulation of the differentiation process of PC12 cells. For instance, BMP ligands have been shown to potentiate the neurotrophic activity of PC12 cells induced by nerve growth factor or fibroblast growth factor (15, 16). However, the effects of BMPs on catecholamine secretion remain unclear.

In the present study we investigated roles of the BMP system in the regulation of catecholamine synthesis. BMP ligands belong to the TGF-β superfamily and were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. Recent studies have demonstrated diverse roles for BMPs in numerous physiological activities in endocrine tissues such as the ovary

First Published Online September 8, 2005

Abbreviations: ActRII, Activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, bone morphogenetic protein type II receptor; DBH, dopamine-β-hydroxylase; DDC, 3,4-dihydroxyphenylalanine decarboxylase; Dex, dexamethasone; DOPA, 3,4-dihydroxyphenylalanine; FCS, fetal calf serum; GDNF, glial cell line-derived neurotrophic factor; HS, horse serum; IBMX, 3-isobutyl-1-methylxanthine; TH, tyrosine hydroxylase.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

(17–19), pituitary (20, 21), thyroid (22), and adrenal cortex (23). We demonstrate novel roles of BMPs in controlling adrenomedullar function.

Materials and Methods

Reagents and supplies

Dexamethasone (Dex) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Human TGF-β1 was obtained from PeproTech EC Ltd. (London, UK). Recombinant human BMP-2, -4, -6, and -7 and activin A were purchased from R&D Systems, Inc. (Minneapolis, MN). Human adult ovary total RNA was purchased from Stratagene, Inc. (La Jolla, CA). PC12 cells were provided by Dr. Isao Date (Okayama University, Okayama, Japan). Human adrenal tumor tissues were obtained from patients who had been diagnosed by clinical and pathological criteria with written permission regarding the experimental use of the tissues in advance of the surgery. Tumor lesions of adrenal tissues were completely separated from normal portions and subjected to extraction of total cellular RNAs. Normal rat whole adrenals and adrenal medullar tissues were obtained from 7-wk-old male Sprague Dawley rats (Charles River Laboratories, Inc., Wilmington, MA). The protocol was conducted in accordance with an animal use protocol approved by our institutional committee. The tissues were immediately frozen in liquid nitrogen and stored at −80 C until RNA extraction.

Cell culture and catecholamine analysis

PC12 cells were maintained in DMEM (Sigma-Aldrich Corp.), supplemented with 10% fetal calf serum (FCS) and 10% horse serum (HS) with penicillin-streptomycin solution (Sigma-Aldrich Corp.) in humidified 5% CO_2 at 37 C. PC12 cells (1×10^5 viable cells) were cultured in DMEM containing the indicated concentrations (0–10%) of FCS and HS in 96-well plates. Unless otherwise indicated, the cells (1×10^5 viable cells) were cultured in DMEM containing 1% FCS and 1% HS for 24 h. After preculture, the cells were treated with the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; TGF-β1; and/or Dex (micromolar concentrations). The culture medium was collected after 24-h culture, and catecholamine levels, including dopamine, noradrenaline, and adrenaline, were determined by HPLC (Tosoh Analysis and Research Center Co., Shunan, Japan). For counting the cell number, PC12 cells were cultured in 24-well plates (1×10^5 viable cells) with DMEM containing the indicated serum concentrations (0–10%) for 24 h. The cells were then washed with PBS, trypsinized, and applied to a Coulter counter (Beckman Coulter, Inc., Fullerton, CA).

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

PC12 cells (1×10^6 viable cells) were cultured in DMEM containing 1% FCS and 1% HS in six-well plates. After preculture, the cells were treated with the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; and/or Dex (micromolar concentrations). After 24- or 48-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol (Invitrogen Life Technologies, Inc., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 C until assay. The expression of BMP/activin ligands, receptors, and follistatin mRNAs was detected by RT-PCR analysis. The extracted RNA (1 μg) was subjected to an RT reaction using the First-Strand cDNA Synthesis System (Invitrogen Life Technologies, Inc.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxy-NTP (0.5 mM) at 42 C for 50 min and at 70 C for 10 min. Subsequently, hot-start PCR was performed using $MgCl_2$ (1.5 mM), deoxy-NTP (0.2 mM), and 2.5 U *Taq* DNA polymerase (Invitrogen Life Technologies, Inc.) under the conditions we previously reported (20, 24). Oligonucleotides used for PCR were custom-ordered from Kurabo Biomedical Co. (Osaka, Japan). PCR primer pairs were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosome DNA contaminants. The primer pairs for BMP receptors, inhibin/activins, and ribosomal protein-L19 (L19) were selected as we have previously reported (20, 24). For the BMP ligand genes, the following sequences were selected for PCR primers: BMP-2, 198–218 and 468–488 from GenBank accession number Z25868; BMP-4, 711–730 and 1091–1110 from NM_012827; BMP-6, 820–838 and 1321–1340 from AY184240; BMP-7, 63–80 and 431–448 from AF100787; BMP-15, 250–269 and 624–643 from NM_021670; and follistatin, 361–385 and 525–550 from NM_012561. For the catecholamine synthase genes, the following sequences were used: TH, 502–522 and 926–946 from M10244; DDC, 405–425 and 771–791 from M27716; and DBH, 880–901 and 1222–1243 from L12407. The PCR product sizes are as follows: ALK-2, 706 bp; ALK-3, 510 bp; ALK-4, 529 bp; ALK-6, 456 bp; ActRII, 492 bp; BMPRII, 522 bp; BMP-2, 291 bp; BMP-4, 400 bp; BMP-6, 521 bp; BMP-7, 386 bp; BMP-15, 394 bp; inhibin-α, 201 bp; activin-βA, 180 bp; activin-βB, 227 bp; follistatin, 190 bp; and L19, 195 bp. The aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of ALK-2, ALK-3, ActRII, BMPRII, TH, DDC, and DBH mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system (Roche, Tokyo, Japan) under conditions of annealing at 60 C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by the second derivative method after the melting curve analysis (Roche), and then the expression levels of target genes were standardized by L19 level in each sample.

Thymidine incorporation assay

PC12 cells were precultured in 12-well plates (2×10^5 viable cells) with DMEM containing 1% FCS and 1% HS for 16 h, and the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; TGF-β1; and/or Dex (micromolar concentrations) were added. After 24-h culture, 0.5 μCi/well [*methyl*-^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) was added, and incubation was performed for 3 h at 37 C. The incorporated thymidine was detected as we previously reported (25). 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 (Tri-Carb 2300TR, Packard Co., Meriden, CT).

Measurement of cAMP production

To assess the effect of treatments on cAMP synthesis, PC12 cells (1×10^5 viable cells) were cultured with DMEM containing 1% FCS and 1% HS. After preculture, the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; TGF-β1; and/or Dex (micromolar concentrations) were added in the presence of 0.1 mM IBMX (specific inhibitor of phosphodiesterase activity). After 24-h culture, the conditioned medium (96-well plates) and cell lysates solubilized with 0.1 M HCl (24-well plates) were collected, and the extracellular cAMP concentrations and intracellular cAMP contents, respectively, were determined by cAMP enzyme immunoassay (Sigma-Aldrich Corp.) after the acetylation of each sample. Intracellular cAMP levels were standardized by the protein contents.

Immunoblot analysis of phosphoproteins

PC12 cells (5×10^5 viable cells) were precultured in a 12-well plates in DMEM containing 1% FCS and 1% HS. After 24-h preculture, BMP-2, -4, -6, and -7 and activin A (100 ng/ml) were added to the culture medium either alone or in combination with Dex (3 μM). After 20- and 60-min stimulation with growth factors, cells were solubilized in 100 μl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY) containing 1 mM Na_3VO_4, 1 mM sodium fluoride, 2% sodium dodecyl sulfate, and 4% β-mercaptoethanol. The cell lysates were then subjected to SDS-PAGE/immunoblotting analysis as we previously reported (22), using antiphospho- and antitotal p38 MAPK antibody (Cell Signaling Technology, Inc., Beverly, MA), antiphospho- and antitotal ERK1/2 MAPKs (Cell Signaling Technology, Inc.), and antiphospho-Smad1/5/8 (Cell Signaling Technology, Inc.).

Transient transfection and luciferase assay

PC12 cells were precultured in 12-well plates in DMEM for 24 h. Cells (at ~70% confluence) were then transiently transfected with 1.0 μg of each luciferase reporter plasmid [Tlx2-Luc, 3TP-Luc, and $(CAGA)^9$-Luc] and 0.1 μg of cytomegalovirus-β-galactosidase plasmid using FuGene 6

(Roche, Indianapolis, IN) for 24 h. The cells were then treated with BMP-2, -4, -6, and -7; activin A; and TGF-β1 (100 ng/ml) in the presence or absence of Dex (3 μM) in DMEM containing 1% FCS and 1% HS for 24 h. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-galactosidase activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan) as we previously reported (23). The data are shown as the ratio of luciferase to β-galactosidase activity.

Statistical analysis

All results are shown as the mean ± SEM of data from three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA). $P < 0.05$ was accepted as statistically significant.

Results

To characterize components of the BMP system present in PC12 cells, we first examined the expression of BMP receptor subunits by RT-PCR using total cellular RNA of PC12 cells, rat adrenal, and various human adrenal tumors. Normal ovary RNA was used as a positive control. As shown in Fig. 1, PC12 cells, rat whole adrenal glands, and medullar tissues expressed ALK-2 (ActRIA) and ALK-3 (BMPRIA), but not ALK-4 (ActRIB) or ALK-6 (BMPRIB). BMP type II receptors, including ActRII and BMPRII, were clearly expressed. The patterns of BMP receptor expression in rat adrenal tissues, human pheochromocytoma, adrenal adenoma, and myelolipoma were compared. The pattern of BMP receptor expression in rat adrenal glands resembles those in pheochromocytoma and adrenal adenoma, although myelolipoma tissue only expressed ALK-3. As for the BMP ligands (Fig. 2), BMP-2, -4, and -7 and inhibin/activin subunits (inhibin-α, activin-βA, and activin-βB) were expressed in PC12 cells. In comparison with the pattern expressed in the whole adrenal tissue, PC12 cells lacked expression of BMP-6 and the BMP/activin-binding protein, follistatin.

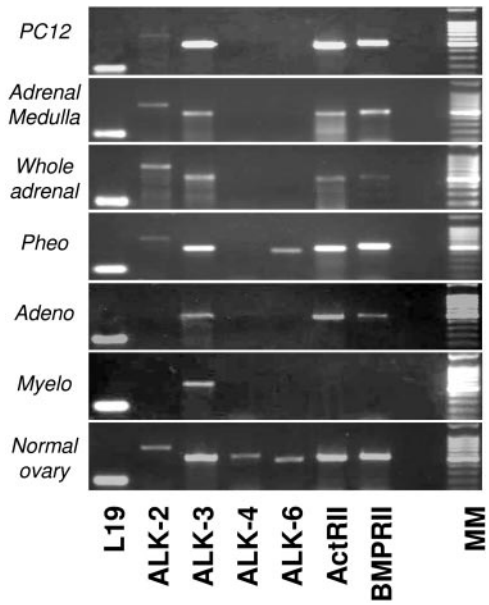

FIG. 1. Expression of BMP receptors in PC12 cells and adrenal tissues. Total cellular RNAs were extracted from PC12 cells, rat adrenal medulla and whole adrenal tissues, and human adrenal tumor tissues. The steady-state expression of mRNAs encoding BMP type I (ALK-2, -3, -4, and -6) and type II (ActRII and BMPRII) receptors and the housekeeping gene L19 was examined by RT-PCR analysis. Human ovary RNA was used for a control study. Aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. MM, Molecular weight marker; Pheo, pheochromocytoma; Adeno, adrenocortical adenoma; Myelo, myelolipoma.

As a first step in elucidating the biological roles of BMPs in PC12 cells, the cell culture conditions were optimized to examine catecholamine production in the conditioned medium. PC12 cells were cultured in medium containing 0%, 1%, 5%, and 10% serum (FCS and HS), and levels of dopamine secreted in the conditioned medium were determined by HPLC. As shown in Fig. 3A, culturing PC12 cells in medium containing 1% serum produced the highest dopamine secretion into the medium. In addition, exposing PC12 cells to medium with 1% serum arrested cell proliferation induced in cells during culture in 10% serum-containing medium. Furthermore, a time-course study during 72-h culture revealed that dopamine levels are saturated after 48-h culture and are the least variable at the 24 h point. Therefore, we cultured PC12 cells in medium containing 1% of FCS and HS for 24 h to determine dopamine levels in the conditioned medium.

Functional roles of BMPs and activin in PC12 cells were tested. Dopamine secretion reached approximately 200 pg/ml in the conditioned medium during 24-h culture (Fig. 3B), although the secreted levels of noradrenaline and adrenaline by PC12 cells did not reach detectable ranges. BMP-2, -4, -6, and -7 and activin A caused the suppression of PC12 dopamine secretion in a dose-dependent manner. In contrast TGF-β1 had no significant effect on dopamine secretion by PC12 cells. Levels of cAMP, a well-established second messenger in the transduction pathway regulating catechol-

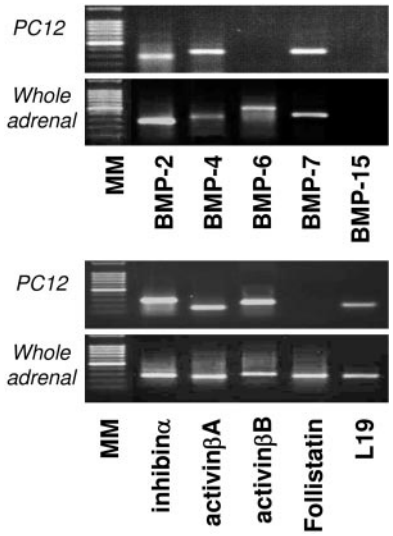

FIG. 2. Expression of BMP ligands in PC12 cells and rat whole adrenal. Total cellular RNAs were extracted from PC12 cells and rat whole adrenals. The steady-state expression of mRNAs encoding BMP-2, -4, -6, -7, and -15; inhibin/activin subunits; follistatin; and L19 was examined by RT-PCR analysis. Aliquots of PCR products were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. MM, Molecular weight marker.

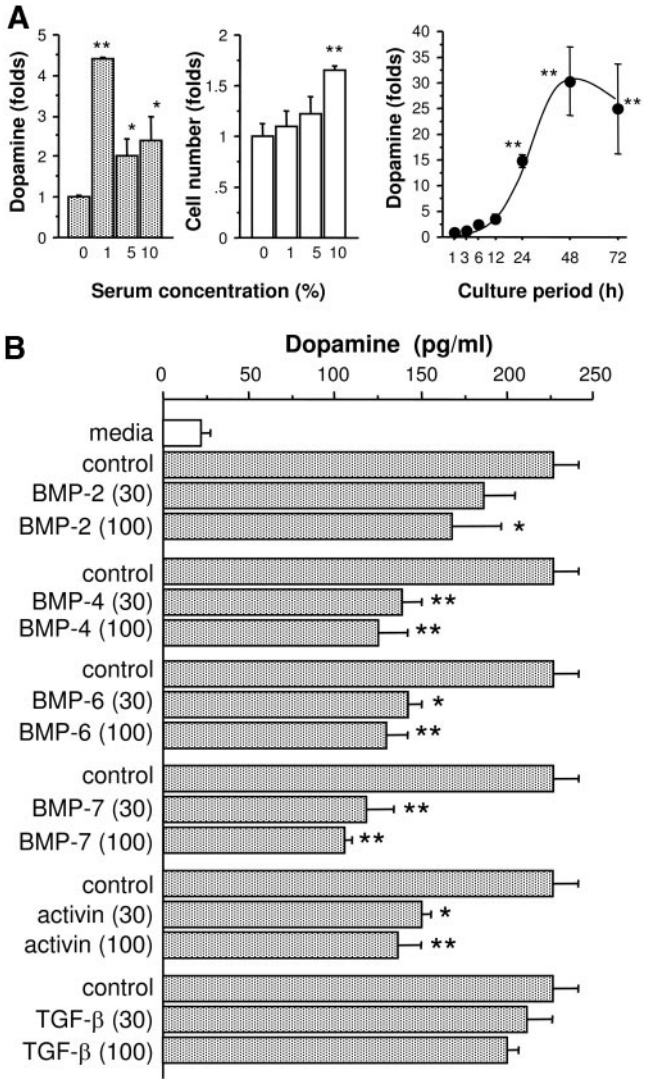

FIG. 3. Effects of BMPs and activin on dopamine production by PC12 cells. A, PC12 cells (1×10^5 viable cells) were cultured in DMEM containing the indicated concentrations (1–10%) of FCS and HS for 24 h (*left panel*). The culture medium was collected, and catecholamine levels were determined by HPLC. For counting the cell number, PC12 cells were cultured in 24-well plates (1×10^5 viable cells) with DMEM containing the indicated serum concentrations for 24 h (*middle panel*). The cells were then washed with PBS, trypsinized, and examined in a Coulter counter. The time-course study of dopamine production was performed in DMEM containing 1% FCS and HS during 72-h culture (*right panel*). Results show the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ [*vs.* control (0% serum or 1-h culture) group]. B, PC12 cells (1×10^5 viable cells) were cultured with the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; and TGF-β1 in DMEM containing 1% FCS and HS for 24 h. Dopamine levels in the conditioned medium were determined by HPLC. Results show the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* each control group).

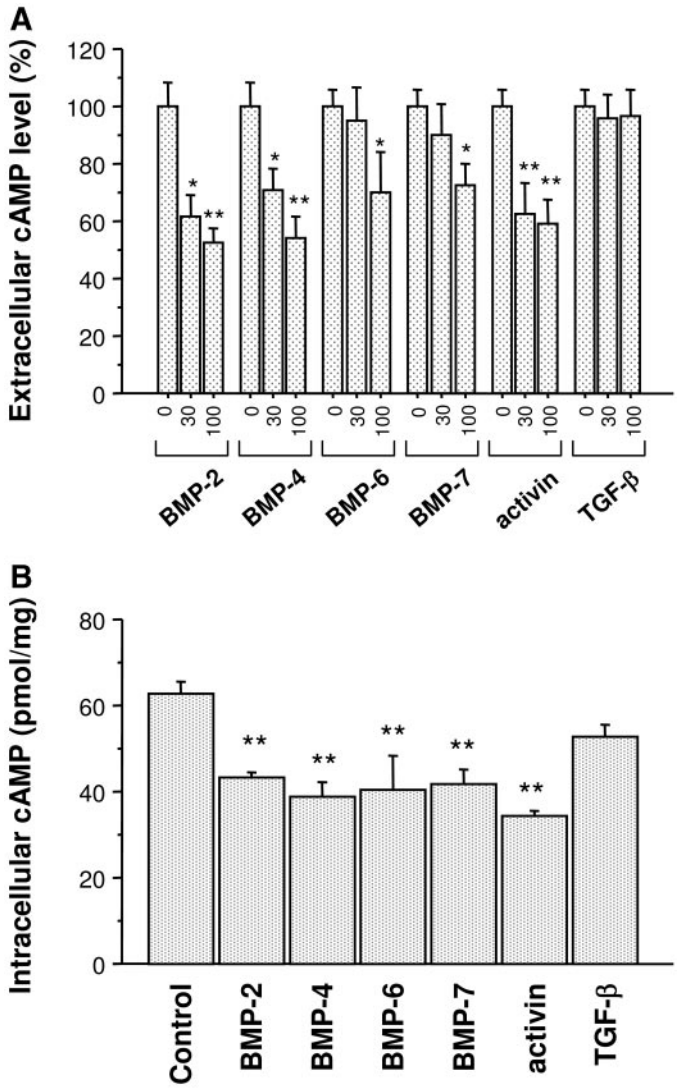

FIG. 4. Effects of BMPs and activin on cAMP production by PC12 cells. A, Changes of extracellular cAMP levels. Cells (1×10^5 viable cells in 96-well plates) were cultured in DMEM containing 1% FCS and 1% HS with the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; and TGF-β1 in the presence of IBMX (0.1 mM) for 24 h. The conditioned medium was collected, and the accumulated cAMP levels were determined by enzyme immunoassay after the acetylation of each sample. Results show the mean ± SEM of data obtained from quadruplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* each control group). B, Changes in intracellular cAMP levels. Cells (1×10^5 viable cells in 24-well plates) were cultured in DMEM containing 1% FCS and 1% HS with BMP-2, -4, -6, and -7; activin A; and TGF-β1 (100 ng/ml) in the presence of IBMX (0.1 mM) for 24 h. The cell lysates solubilized with 0.1 M HCl were collected, and the cAMP contents were determined by enzyme immunoassay after acetylation and were standardized by protein contents. Results show the mean ± SEM of data obtained from quadruplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* each control group).

amine synthesis and secretion, were also determined. Consistent with their effects on dopamine secretion, BMP-2, -4, -6, and -7 and activin A, but not TGF-β1, significantly suppressed cAMP production by PC12 cells (Fig. 4A). In addition, intracellular cAMP contents were examined in the cell lysates treated with BMPs. As shown in Fig. 4B, intracellular cAMP levels were significantly reduced by BMPs and activin, but not by TGF-β1, which was consistent with the changes in extracellullar cAMP levels in the medium.

Next, the levels of catecholamine synthase mRNAs, including TH, DDC, and DBH, were determined by quantitative real-time PCR analysis. As shown in Fig. 5, steady-state

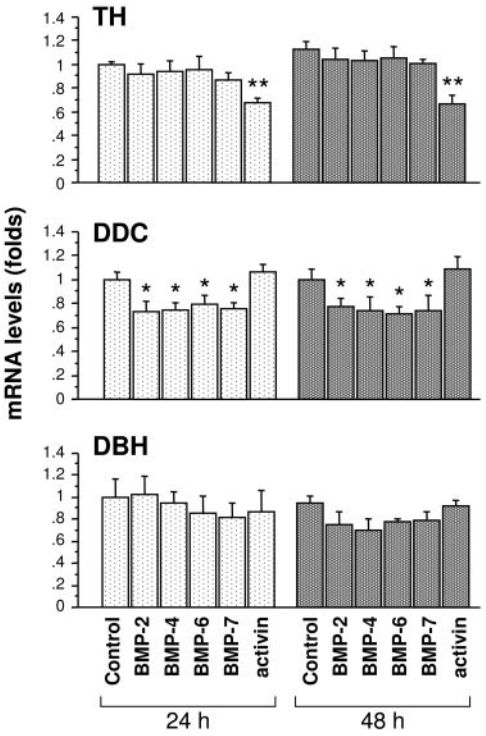

FIG. 5. Effects of BMPs and activin on catecholamine synthase expression by PC12 cells. Cells (1×10^6 viable cells) were cultured with indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7 and activin A for 24 and 48 h at 37 C. The total cellular RNAs were then extracted, and the steady-state mRNA levels of TH, DDC, DBH, and L19 were analyzed by quantitative real-time RT-PCR. The level of each target mRNA expression was standardized by the level of L19 in each sample. Results show the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* the control group).

mRNA levels of TH were preferentially reduced by activin A treatment, whereas DDC expression was specifically suppressed by BMP-2, -4, -6, and -7 treatment. Levels of DBH mRNA transcripts were not significantly altered by any ligand. The changes in the levels of TH, DDC, and DBH mRNA transcripts were the same at 24 and 48 h culture points.

To evaluate BMP signaling in PC12 cells, activation of the major BMP signaling pathway, Smad1/5/8, was examined by Western blotting analysis using antiphospho-Smad1/5/8 antibodies that detect phosphorylated (activated) Smad proteins. As shown in Fig. 6, BMP-2, -4, -6, and -7 activated Smad1/5/8 phosphorylation, whereas activin had no effect on Smad1/5/8 activation. Activation of MAPK signaling and p38 and ERK phosphorylation was also examined in the presence of BMP-2, -4, -6, and -7 and activin A. BMPs failed to activate p38 and ERK phosphorylation, whereas activin A stimulated phosphorylation of p38. These effects were also confirmed by luciferase assays using BMP/activin-responsive reporters, including Tlx2-Luc, 3TP-Luc, and $(CAGA)^9$-Luc. As shown in Fig. 7, BMP-2, -4, -6, and -7 potently increased Tlx2-Luc activity, whereas activin A specifically induced 3TP-Luc and $(CAGA)^9$-Luc activation.

To investigate the physiological effects of BMP ligands and activin in PC12 cells, the functional interaction with glucocorticoid, a major inducer of catecholamines (10), was investigated. We preliminarily examined the effects of Dex on catecholamine production. Dex potently induced dopamine production by PC12 cells, but failed to stimulate noradrenaline and adrenaline levels to detectable ranges. As shown in

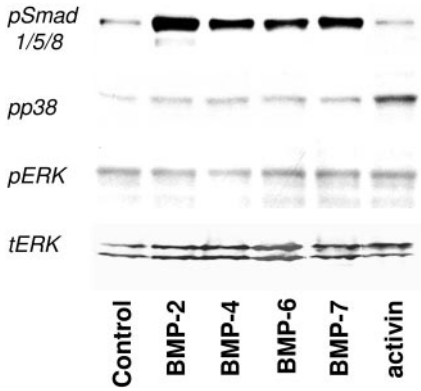

FIG. 6. Smad1/5/8 and MAPK phosphorylation by BMPs and activin in PC12 cells. Cells (5×10^5 viable cells) were precultured in 12-well plates for 24 h and then stimulated with BMP-2, -4, -6, and -7 and activin A (100 ng/ml). After 60-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting analysis using antibodies that detect phosphorylated Smad1/5/8 molecules (pSmad1/5/8). The cell lysates collected after 20-min incubation were subjected to immunoblot analysis for MAPK signaling using antiphospho-p38 (pp38), antiphospho-ERK1/2 (pERK), and anti-total-ERK1/2 (tERK) antibodies. The results shown are representative of those obtained from three independent experiments.

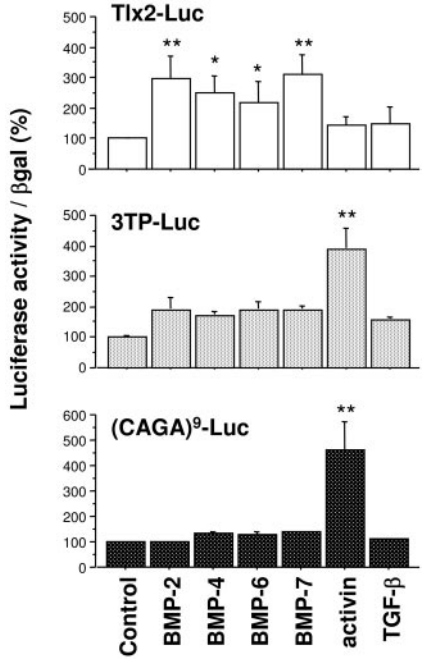

FIG. 7. Target gene transcription by BMPs and activin in PC12 cells. Cells (~70% confluence) were transiently transfected with Tlx2-Luc, 3TP-Luc, or $(CAGA)^9$-Luc reporter plasmid (1 μg) and cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal; 0.1 μg). Cells were then treated with BMP-2, -4, -6, and -7; activin A; and TGF-β1 (100 ng/ml) for 24 h. The cells were washed with PBS and lysed, and luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. The results shown are the ratio of luciferase to βgal activity and are graphed as the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control group).

Fig. 8A, Dex increased dopamine secretion in a dose-dependent manner, with the maximal effects at approximately 1 μM Dex. Dex (1–3 μM) also caused significant increases in TH, DDC, and DBH mRNA expression by PC12 cells (Fig. 8B); however, Dex did not have any effect on extracellular cAMP production (Fig. 8C). The synergistic interactions of Dex and BMPs in PC12 cells were investigated. Interestingly, BMP-2, -4, -6, and -7 and activin A failed to suppress dopamine production by PC12 cells in the presence of Dex (3 μM; Fig. 9A). BMPs also failed to suppress cAMP accumulation in

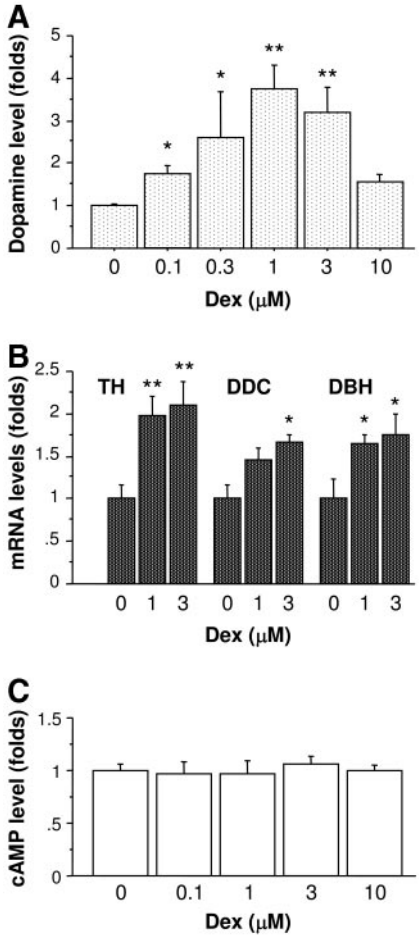

FIG. 8. Effects of Dex on catecholamine synthesis by PC12 cells. A, Changes in dopamine levels: PC12 cells (1×10^5 viable cells) were cultured with increasing concentrations (micromolar) of Dex for 24 h at 37 C, and dopamine levels in the conditioned medium were determined by HPLC. Results show the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ [*vs.* Dex (0) group]. B, Changes in catecholamine synthase expression. Cells (1×10^6 viable cells) were cultured with the indicated concentrations of Dex (micromolar) for 24 h at 37 C. Steady-state mRNA levels of TH, DDC, DBH, and L19 were analyzed by quantitative real-time RT-PCR in the extracted total cellular RNA. The level of each target mRNA expression was standardized by level of L19 in each sample. Results show the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ [*vs.* Dex (0) group]. C, Changes in cAMP production. Cells (1×10^5 viable cells) were cultured with the indicated concentrations of Dex (micromolar) in the presence of IBMX (0.1 mM) for 24 h, then the conditioned medium was collected, and the accumulated cAMP concentration was determined after acetylation of the samples. Results show the mean ± SEM of data obtained from triplicate treatments.

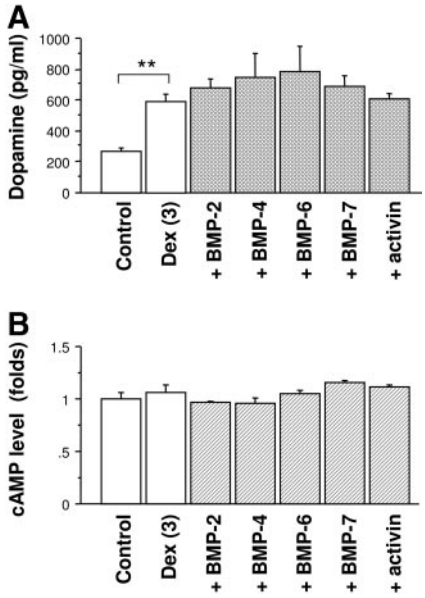

FIG. 9. Effects of Dex on BMP regulation of dopamine and cAMP in PC12 cells. A, Changes in dopamine levels. PC12 cells (1×10^5 viable cells) were cultured with BMP-2, -4, -6, and -7 and activin A (100 ng/ml) in combination with 3 μM Dex for 24 h at 37 C, then dopamine levels in the conditioned medium were determined by HPLC. Results show the mean ± SEM of data obtained from triplicate treatments. **, $P < 0.01$ *vs.* control group. B, Changes in cAMP production. Cells (1×10^5 viable cells) were cultured with BMP-2, -4, -6, and -7 and activin A (100 ng/ml) in combination with 3 μM Dex in the presence of IBMX (0.1 mM) for 24 h at 37 C, then the conditioned medium was collected, and the accumulated cAMP concentration was determined after acetylation of the samples. Results show the mean ± SEM of data obtained from triplicate treatments.

Dex-treated cells (Fig. 9B). Accordingly, Dex was found to be a functional repressor of BMP actions in PC12 cells.

The mitotic properties of PC12 cells were evaluated using [^{3}H]thymidine uptake assays (Fig. 10). BMP-2, -4, -6, and -7; activin A; and TGF-β1 exhibited dose-dependent suppression of thymidine uptake by PC12 cells (Fig. 10A). Notably, in the presence of 3 μM Dex, a ligand-dependent difference of thymidine uptake was demonstrated in PC12 cells treated with BMPs and activin (Fig. 10B). Namely, BMP-2 and BMP-4 caused dose-responsive reductions in DNA synthesis independently of Dex treatment, whereas BMP-7 and activin A efficaciously suppressed DNA synthesis in the presence of Dex.

To elucidate the mechanism by which Dex antagonizes BMP action, BMP/activin signaling was evaluated in cells cultured with Dex and BMPs. Phospho-Smad1/5/8 activation induced by BMP-2, -4, -6, and -7 was examined by immunoblotting analysis in the presence and absence of dexamethasone (3 μM). BMP-induced Smad1/5/8 phosphorylation was suppressed by Dex treatment (Fig. 11A). As shown in Fig. 11B, the BMP signaling detected by Tlx2-Luc was significantly impaired in the presence of Dex. Dex also potently decreased 3TP-Luc activities induced by BMP-2, -4, and -7 and activin A. To elucidate the underlying mechanism by which Dex elicits inhibitory actions on BMP signaling, we also examined changes in BMP receptor expression in the presence of Dex (1–3 μM). Quantitative real-time PCR anal-

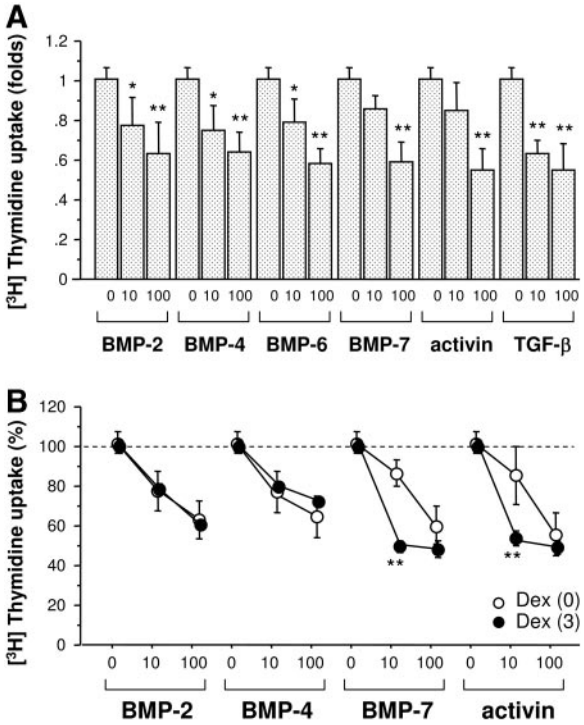

FIG. 10. Effects of BMPs and Dex on mitotic action by PC12 cells. After preculture, PC12 cells (2×10^5 viable cells) were cultured with the indicated concentrations (nanograms per milliliter) of BMP-2, -4, -6, and -7; activin A; and TGF-β1 in the absence (A) or presence (B) of Dex (3 μM) for 24 h. [*Methyl*-^{3}H]thymidine (0.5 μCi/well) was then added and incubated for 3 h at 37 C. The cells were washed, precipitated by trichloroacetic acid, and solubilized in NaOH, and radioactivity was counted. Results show the mean ± SEM of data obtained from triplicate incubations. *, $P < 0.05$; **, $P < 0.01$ [*vs.* the control of each group (A) or *vs.* Dex (0) group (B)].

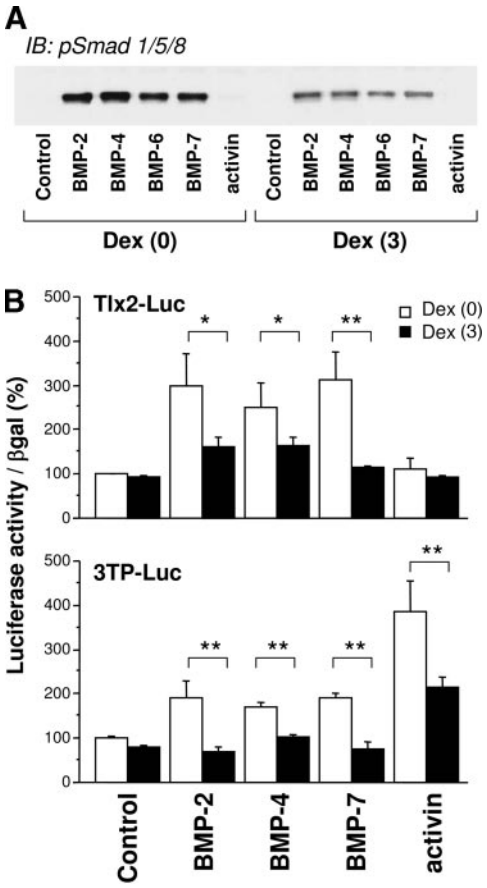

FIG. 11. Effects of Dex on BMP signaling in PC12 cells. A, After preculture, PC12 cells (5×10^5 viable cells) were treated with BMP-2, -4, -6, and -7 and activin A (100 ng/ml) in the absence or presence of Dex (3 μM). After 60-min stimulation with growth factors, cells were solubilized, and the cell lysates were subjected to SDS-PAGE/ immunoblotting (IB) analysis using antiphospho-Smad1/5/8 antibody (pSmad1/5/8). B, Cells (~70% confluence) were transiently transfected with Tlx2-Luc or 3TP-Luc reporter plasmid (1 μg) and cytomegalovirus-β-galactosidase plasmid (pCMV-β-gal; 0.1 μg). Cells were then treated with BMP-2, -4, and -7 and activin A (100 ng/ml) in the presence or absence of Dex (3 μM) for 24 h. The cells were washed with PBS and lysed, and luciferase activity and β-galactosidase (βgal) activity were measured by luminometer. Results are shown as the ratio of luciferase to βgal activity and are graphed as the mean ± SEM of data obtained from triplicate treatments. *, $P < 0.05$; **, $P < 0.01$ (between the indicated groups).

ysis revealed that Dex significantly decreased the levels of ALK-2, ALK-3, and BMPRII mRNA steadily expressed in PC12 cells, whereas Dex had no effect on L19 mRNA levels (Fig. 12). Thus, Dex inhibition of BMP/activin receptors is likely to be involved in the inhibition of BMP/activin signaling, which may lead to fine-tuned regulation of dopamine secretion as well as DNA synthesis by PC12 cells.

Discussion

In the present study we demonstrate the presence of a functional BMP and activin system in rat pheochromocytoma PC12 cells that includes BMP receptors (ALK-2, ALK-3, ActRII, and BMPRII) and BMP ligands (BMP-2, -4, and -7). Functional BMP signaling pathways were also demonstrated in undifferentiated PC12 cells by showing ligand-induced phosphorylation of BMP signaling molecules, Smad1/5/8, and activation of the BMP-responsive luciferase reporter constructs, Tlx2-Luc and 3TP-Luc.

The BMP/activin system was found to play a role in modulation of the endocrine functions of PC12 cells. BMP-2, -4, -6, and -7 and activin A produced a dose-dependent suppression of dopamine and cAMP synthesis by PC12 cells. Furthermore, BMP-2, -4, -6, and -7 suppressed DDC mRNA expression, and activin A mainly decreased TH mRNA levels. DBH levels were not affected by BMPs and activin. DDC and TH are involved in the synthesis of dopamine and other monoamine neurotransmitters, and their activities can be regulated by a number of physiological stimuli. TH, not DDC, has been recognized as a rate-limiting enzyme for the synthesis of catecholamines (26, 27). However, some recent reports suggest a significant role for DDC in the process of catecholamine synthesis. An irreversible blocker of monoamine B oxidase increases DDC gene expression, and these compounds can exert neuronal protective effects (28). In addition, DDC activity is up-regulated by dopamine receptor blockers (29, 30). Thus, DDC plays a dynamic role in controlling monoamine synthesis (31). Considering our present results showing that BMPs preferentially control DDC mRNA expression, but not TH expression, the independent

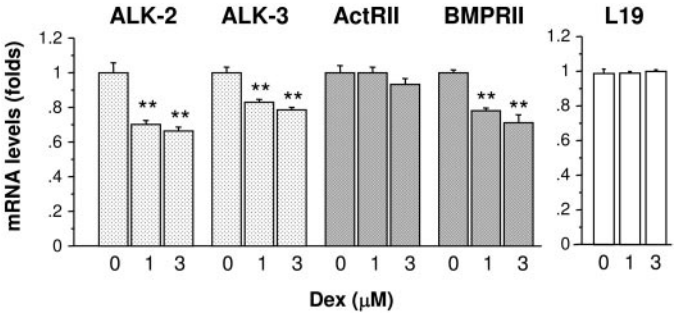

FIG. 12. Effects of Dex on BMP receptor expression in PC12 cells. PC12 cells (1×10^6 viable cells) were cultured with increasing doses of Dex (micromolar) for 24 h at 37 C. The total cellular RNAs were then extracted, and the levels of BMP type I (ALK-2 and -3), type II receptor (ActRII and BMPRII), and L19 mRNA were analyzed by quantitative real-time RT-PCR. Levels of BMP receptor mRNA expression were standardized by the level of L19 in each sample. Results show the mean ± SEM of data obtained from triplicate treatments. **, $P < 0.01$ *vs.* each control group.

roles and distinct regulation of DDC and TH are evident in the process of dopamine synthesis.

BMP signalings including Smad1/5/8 phosphorylation and Tlx2-Luc activation were clearly activated by BMP-2, -4, -6, and -7. In contrast, activin A specifically stimulated p38 phosphorylation in PC12 cells in addition to 3TP-Luc and $(CAGA)^9$-Luc activities. Many apoptotic signals have been shown to stimulate p38 MAPK activity that coincides with the induction of cellular apoptosis. The magnitude of p38 MAPK activation has been proposed as a factor determining the choice between cell growth and apoptosis (32). Activin signaling identified in the present study may induce the apoptotic change in PC12 cells by activating p38 phosphorylation, which leads to growth arrest and suppression of catecholamine production. In contrast, BMP signaling, which affects neither p38 nor ERK, appears to modulate catecholamine production and cellular growth in PC12 cells. It has recently been shown that the activation of MAPK is necessary and sufficient for PC12 cell differentiation (33), although the point of convergence of signaling pathways in PC12 cells is a complex of specific cellular responses.

Our present data show that Dex enhances TH, DDC, and DBH transcription without activating cAMP second messenger signaling pathways. In the presence of Dex, BMPs and activin completely failed to reduce dopamine synthesis or cAMP generation in PC12 cells. Dex decreased BMP signaling, including Smad1/5/8 phosphorylation and activation of Tlx2-Luc and 3TP-Luc reporters. This modulation of BMP signaling could be due at least in part to a reduction of ALK-2, ALK-3, and BMPRII expression by Dex. Dex treatment also modulated BMP actions on DNA synthesis in PC12 cells. In the present study it is notable that the effects of BMP-2 and BMP-4 on DNA synthesis were not influenced by Dex pretreatment; however, the effects of BMP-7 and activin were rather efficaciously elicited in the presence of Dex. This difference could be due to the differential suppression of BMP type II receptors by Dex. In this regard, BMP-2 and BMP-4 primarily bind to ALK-3 and/or ALK-6 type I receptors, whereas BMP-6 and BMP-7 bind ALK-2 and/or ALK-6 (19). Regarding the type II receptors, BMP-2 and BMP-4 require BMPRII binding, whereas BMP-6 and BMP-7 can bind either ActRII or BMPRII subunits (34). The sustained expression of ActRII in the presence of Dex may have caused additive effects of BMP-7 and activin on the mitotic inhibition.

Glucocorticoids and catecholamines play important roles in metabolic and cardiovascular responses. Many physiological stressors result in the release of both cortisol and catecholamines from the adrenal into the circulation, and both are critical for various stress responses. After release from the adrenal cortex, glucocorticoids first enter sinusoids that traverse the adrenal medulla before entering the systemic circulation. The present data demonstrate a functional link between glucocorticoids and catecholamine release through the BMP system in adrenomedullar cells. Endogenous glucocorticoids contribute to maintaining catecholamine secretion by the adrenal medulla (35, 36) in addition to its induction of catecholamine synthetic enzymes (37). In this machinery of catecholamine secretion, inhibition of the endogenous BMP system by glucocorticoid hormones may be pivotal for controlling circulating catecholamine levels. Additional investigation is necessary to clarify the molecular interaction between glucocorticoid and the endogenous BMP system in PC12 cells.

Germline mutations in the *ret* gene are responsible for the inheritance of multiple endocrine neoplasia characterized by familial pheochromocytomas. The mutations convert a transmembrane receptor, Ret, into constitutively active form of the intrinsic tyrosine kinase. The ligands for Ret have been recently identified as members of the glial cell line-derived neurotrophic factor (GDNF) family, which functions through conformation of the receptor complex consisting of a common Ret receptor and the homodimers of ligand-specific GDNF family receptor α (38). Interestingly, GDNF family ligands belong to the TGF-β superfamily member, and GDNF family ligands require TGF-β molecule for their neurotrophic actions through membrane translocation of GDNF family receptor α1 (39). Given the pronounced conformational similarity of GDNF to TGF-β and BMP-7 (40), one may consider the possibilities of cross-reaction among their receptor subunits and intracellular involvement of signal crosstalk between the GDNF-Ret system and the BMP system in the process of adrenomedullar tumorigenesis.

Collectively, the endogenous BMP/activin system plays a key role in the regulation of catecholamine secretion by rat pheochromocytoma cells. Controlling the activity of the BMP system could be a key process for glucocorticoids, leading to the enhancement of dopamine synthesis by PC12 cells.

Acknowledgments

We thank Dr. R. Kelly Moore for helpful discussion and critical reading of the manuscript. We also thank Drs. Isao Date, Jeff Wrana, Tetsuro Watabe, and Kohei Miyazono for providing PC12 cell, Tlx2-Luc, 3TP-Luc, and $(CAGA)^9$-Luc plasmids, respectively.

Received April 20, 2005. Accepted August 31, 2005.

Address all correspondence and requests for reprints to: Dr. Fumio Otsuka, Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama City 700-8558, Japan. E-mail: fumiotsu@md.okayama-u.ac.jp.

This work was supported by in part by Grant-in-Aid for Scientific Research 16790525, the Uehara Memorial Foundation, the Japan Research Foundation for Clinical Pharmacology, the Research Foundation for Pharmaceutical Sciences, the Tokyo Biochemical Research Foundation, and the Takeda Science Foundation.

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