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Differential Coupling of α_1-, α_2-, and β-Adrenergic Receptors to Mitogen-activated Protein Kinase Pathways and Differentiation in Transfected PC12 Cells*

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Nidhi Gupta Williams, Hongying Zhong, and Kenneth P. Minneman‡

From the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Three adrenergic receptor families that selectively activate three different G proteins ($\alpha_1/G_{q/11}$, α_2/G_i, and β/G_s) were used to study mitogen-activated protein kinase (MAPK) activation and differentiation in PC12 cells. PC12 cells were stably transfected with α_{1A}-, α_{2A}-, or β_1-adrenergic receptors (ARs) in an inducible expression vector, and subclones were characterized. Norepinephrine stimulated inositol phosphate formation in α_{1A}-transfected cells, inhibited cyclic adenosine 3′5′-monophosphate (cAMP) formation in α_{2A}-transfected cells, and stimulated cAMP formation in β_1-transfected cells. Nerve growth factor activated extracellular signal-regulated kinases (ERKs) in all cell lines; however, norepinephrine activated ERKs only in α_{1A}- and β_1-transfected cells but not in α_{2A}-transfected cells. Norepinephrine also activated c-Jun NH_2-terminal kinase and p38 MAPK in α_{1A}-transfected cells but not in β_1- or α_{2A}-transfected cells. Norepinephrine caused differentiation of PC12 cells expressing α_{1A}-ARs but not those expressing β_1- or α_{2A}-ARs. However, norepinephrine acted synergistically with nerve growth factor in promoting differentiation of cells expressing β_1-ARs. Whereas ERKs are activated by G_i- but not G_s-linked receptors in many fibroblastic cell lines, we observed the opposite in PC12 cells. The results show that activation of the different G protein signaling pathways has different effects on MAPKs and differentiation in PC12 cells, with G_q signaling pathways activating all three major MAPK pathways.

Although mitogen-activated protein kinase (MAPK)[1] pathways were originally thought to be activated primarily by growth factor receptors with intrinsic tyrosine kinase activity, it is now clear that G protein-coupled receptors (GPCRs) can also activate MAPK pathways (1–11). GPCRs acting through G_s (12), G_i (1, 2, 4, 5), and $G_{q/11}$ (3, 6–8, 10, 14), have all been shown to activate MAPK, although the mechanisms involved appear to be dependent on cell phenotype. In some cases, MAPK activation is downstream of known second messengers such as cAMP (12), Ca^{2+} (6, 8), and/or protein kinase C (10). In other cells, Gα- and/or G$\beta\gamma$-subunits may directly or indirectly activate the Ras/Raf pathway (1–5, 11) through adapter proteins, tyrosine kinases, and/or phosphoinositide 3 kinase (9). MAPK pathways have been shown to be inhibited by increases in cAMP concentrations in some fibroblastic cell lines (15, 16).

MAPKs are subdivided into three major pathways (17). Extracellular signal regulated kinases 1 and 2 (ERKs) are stimulated by growth factors and cytokines and stimulate growth and differentiation. The proto-oncogene c-*ras* and the cytoplasmic kinases c-Raf and MEK are known to play important roles in the activation of ERKs (18–20). The other two MAPK pathways, c-Jun-NH_2-terminal kinase (JNK) (also known as stress-activated protein kinase) and p38 MAPK, are generally activated by stresses such as inflammatory cytokines, osmotic shock, or UV irradiation and may be involved in inhibition of cell growth and/or apoptosis. The balance between these pathways may be critical in determining cell fate (21).

The mechanisms of activation of ERKs by GPCRs remain controversial. Responses to G_q-linked receptors are thought to involve both the α- and $\beta\gamma$-subunits of G proteins, although the α_q-dependent activation of protein kinase C is thought to play the predominant role (3, 22). Response to both G_i-linked (22, 23) and G_s-linked (12) receptors are thought to be due primarily to release of $\beta\gamma$-subunits, although other mechanisms have also been proposed (12, 24). Similar mechanisms have been implicated in activation of JNK/SAPK by GPCRs (24, 25), and GPCR activation of p38 MAPK was recently suggested to involve Gα_q- as well as $\beta\gamma$-subunits (26). $\beta\gamma$-Dependent activation of p38 MAPK is inhibited by coexpression of Gα_o in HEK 293 cells (26).

PC12 cells, derived from a rat pheochromocytoma, have been a primary model for studying mechanisms underlying neuronal differentiation (27). Nerve growth factor (NGF) acts on receptors with tyrosine kinase activity to differentiate these cells into a neuronal phenotype, through a Ras-dependent activation of ERKs (28). Stimulation of both bradykinin ($G_{q/11}$-coupled) and lysophosphatidic acid (G_i-coupled) receptors also activates ERKs in PC12 cells, apparently through the tyrosine kinases Pyk2 (6) and Src (8) in a Ras-dependent manner. cAMP analogs also activate ERKs and potentiate NGF-induced neurite formation in PC12 cells (29). Thus, $G_{q/11}$-, G_i-, and G_s-linked receptors may all activate ERKs in this cell line.

This raises questions about signaling specificity. If all three types of G proteins can activate ERKs, albeit through different mechanisms, do they have similar functional consequences? To what extent are known second messengers involved in activation of the MAPK pathways? Are the functional consequences of G protein activation similar to those of tyrosine kinase receptor activation?

We wanted to directly address the specificity by which

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‡ To whom correspondence should be addressed. Tel.: 404-727-5985; Fax: 404-727-0365.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; AR, adrenergic receptor; cAMP, cyclic adenosine 3′5′-monophosphate; ERK, extracellular signal-regulated kinase (also known as p42 and p44 MAPKs); GPCR, G protein-coupled receptor; [^{125}I]BE, [^{125}I]BE 2254 ((2-β-(4-hydroxyphenyl)ethylaminomethyl)-tetralone); InsP, inositol phosphate; IPTG, isopropylthiogalactose; JNK, c-Jun NH_2-terminal kinase; NE, norepinephrine; NGF, nerve growth factor; SAPK, stress-activated protein kinase.

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GPCRs activate MAPK pathways in a single cell line and study their functional consequences. To do this, we transfected PC12 cells with inducible expression vectors coding for one of each of the three families (α_{1A}, α_{2A}, and β_1) of adrenergic receptor (AR) subtypes (30, 31) to assess whether $G_{q/11}$-, G_i-, and G_s-linked pathways all activate ERKs in this cell line. ARs affect growth and differentiation of a variety of cell types, although the mechanisms involved are not yet clear. All ARs are activated by norepinephrine (NE), but they initiate signals through different G proteins. α_1-ARs increase PI hydrolysis and intracellular Ca^{2+} through $G_{q/11}$, α_2-ARs inhibit adenylate cyclase through G_i, and β-ARs stimulate adenylate cyclase through G_s. We wanted to directly assess which signaling pathways were linked to which MAPK pathways and determine whether activation of any of these GPCRs would cause differentiation of PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: Lac-Switch vector system from Stratagene (La Jolla, CA), phentolamine mesylate from Ciba-Geigy (Summit, NJ), hygromycin B from Boehringer Mannheim, BE 2254 ((2-β-(4-hydroxyphenyl)ethylaminomethyl)-tetralone) from Beiersdorf AG (Hamburg, Germany), cyanopindolol from Sandoz (Basel, Switzerland), [^{3}H]rauwolscine (60 Ci/mmol) and carrier-free Na^{125}I from Amersham Pharmacia Biotech, [^{3}H]inositol (20–40 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO), fetal bovine serum, Geneticin, and trypsin/EDTA from Life Technologies, Inc.; and (−)norepinephrine bitartrate, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and other chemicals from Sigma. The cDNA for the human α_{1A}-AR (32) was generously provided by Dr. G. Tsujimoto (National Children's Hospital, Tokyo, Japan), the cDNA for the rat β_1-AR (33) was provided by Dr. Curtis A. Machida (Oregon Regional Primate Research Center, Beaverton, OR), and the cDNA for the human α_{2A}-AR (34) was obtained from ATCC (Manassas, VA). PC12 cells were obtained from Cindy Miranti and Michael Greenberg (Harvard Medical School, Boston, MA). NGF was generously provided by David Ginty (Johns Hopkins, Baltimore, MD).

Preparation of Expression Vectors—The full-length AR sequences were cloned into the multiple cloning site of the operator vector (pOPRSVICAT) of the inducible Lac-Switch system. The *Not*I fragment of pOPRSVICAT containing the chloramphenicol acetyltransferase reporter gene was replaced with the multiple cloning site of pBluescript KS+ (where an additional *Not*I site had been inserted 5′ to the *Xho*I site) to facilitate insertion of the gene of interest (35).

Cell Culture—Rat pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum, 5% fetal bovine serum, 10 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C in a humidified atmosphere with 5% CO_2. Confluent cells were subcultured in a 1:3 ratio. Where indicated, transfected cells were treated with 1 mM IPTG for various time periods to induce receptor expression.

Transfection—PC12 cells were co-transfected with the LacSwitch repressor (p3′SS) and operator vectors by calcium phosphate precipitation and propagated for several weeks in the presence of 250 μg/ml hygromycin and 500 μg/ml Geneticin to obtain resistant cells. Subclones expressing each of the different ARs were obtained by screening for cell lines that exhibited low constitutive and high inducible receptor levels by radioligand binding. Cells for radioligand binding and second messenger measurements were plated at lower (1/10) antibiotic concentrations.

Radioligand Binding—Confluent 100-mm plates of cells were washed in phosphate-buffered saline (20 mM $NaPO_4$, 154 mM NaCl, pH 7.6) and harvested by scraping. Cells were homogenized with a Polytron, and membranes collected by centrifugation at 30,000 × *g* for 10 min, washed, and resuspended by homogenization. Receptor density was determined by saturation analysis of specific antagonist radioligands. Binding of [^{125}I]BE 2254 (α_1; [^{125}I]BE), [^{125}I]-iodocyanopindolol (β) and [^{3}H]rauwolscine (α_2) to membrane preparations was performed as described previously (36–38). For saturation analysis, increasing concentrations of radioligand ([^{125}I]BE, 25–800 pM; [^{125}I]cyanopindolol, 10–300 pM; [^{3}H]rauwolscine, 200–4000 pM) were used. Nonspecific binding was defined as binding in the presence of 10 μM phentolamine (α_1 or α_2) or 50 μM isoproterenol (β).

InsP Formation—Accumulation of [^{3}H]InsPs was determined in confluent 35-mm dishes. Cells were treated with or without 1 mM IPTG for 48 h and labeled with *myo*-[^{3}H]inositol (2mCi/plate) for 1–2 days. Production of total [^{3}H]InsPs in the presence of 10 mM LiCl was determined as described previously (35). Results are expressed as percentage of hydrolysis of [^{3}H]inositol incorporated into lipid.

cAMP Accumulation—cAMP accumulation was measured by the [^{3}H]adenine prelabeling technique in confluent 35-mm dishes as described previously (37). Results are expressed as percent conversion of incorporated [^{3}H]ATP into [^{3}H]cAMP.

Western Blots—Confluent cells were serum-starved for 2 h at 37 °C before treatment. Agonists were generally added for 15 min, and cells were washed twice with ice-cold phosphate-buffered saline and lysed in Nonidet P-40 lysis buffer containing 137 mM NaCl, 20 mM Tris-Cl (pH 8), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 20 mM sodium orthovanadate, 20 mM leupeptin, and 10 μg/ml aprotinin. 20 μg of total protein was subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to a nitrocellulose membrane. Activation of ERK1 and 2, JNK/SAPK, or p38 MAPK was detected by blotting the membrane with phosphospecific ERK, JNK/SAPK, or p38 MAPK antibodies (New England Biolabs) that specifically recognize the activated, threonine, and tyrosine dually phosphorylated forms. Blots were stripped and probed with nonphosphospecific antibodies to the enzymes to control for protein loading. Proteins were visualized using a horseradish peroxidase-conjugated goat anti-rabbit IgG and by ECL (Amersham Pharmacia Biotech).

RESULTS

Lack of Endogenous ARs in Parental PC12 Cells—We used radioligand binding and functional approaches to determine whether any endogenous ARs are expressed in PC12 cells. Low levels of endogenous α_2-ARs have been reported in PC12 cells (39), but we found no detectable levels of any AR subtype in our PC12 cells. No specific binding was detected in membrane preparations using the antagonist radioligands [^{125}I]BE (α_1-AR), [^{3}H]rauwolscine (α_2 -AR), or [^{125}I]cyanopindolol (β-AR) (<5 fmol/mg of protein, data not shown). There was also no detectable stimulation of cAMP by the β-AR agonist isoproterenol, no inhibition of forskolin-stimulated cAMP by the α_2-AR agonist UK 14,304 (see also below), and no stimulation of [^{3}H]InsP formation by the α_1-AR agonists phenylephrine or NE in parental PC12 cells (data not shown). These data suggest that this is one of the few cell lines that do not express measurable levels of any AR subtype.

Characterization of Stably Transfected PC12 Subclones—PC12 cells were co-transfected with the lac repressor vector and the lac operator vector containing either human α_{1A}-, human α_{2A}-, or rat β_1-AR coding sequences. Subclones expressing each receptor were screened for low constitutive expression and inducibility by IPTG. Saturation analysis of specific radioligand binding was used to measure receptor density. Several subclones were isolated with inducible expression of α_{1A}- or α_{2A}-ARs; however, we were unable to obtain subclones showing inducible expression of β_1-ARs. Several subclones with constitutive expression of β_1-ARs were isolated and used for further studies. Constitutive and IPTG-induced receptor density in selected subclones is summarized in Table I.

α_{1A}-AR Expression and Induction—Three subclones of PC12 cells expressing α_{1A}-ARs were extensively characterized (Fig. 1). Saturation analysis of [^{125}I]BE binding showed that each subclone exhibited different levels of constitutive and IPTG-induced (1 mM, 48 h) receptor expression, with α_{1A}-3 showing the highest degree of induction. The effect of NE on [^{3}H]InsP formation was studied to ensure that the expressed receptors were functional. Basal [^{3}H]InsP formation was similar in each subclone, and was not affected by treatment with IPTG (Fig. 1). NGF caused small increases in [^{3}H]InsP formation in each subclone, and this response was unaffected by treatment with IPTG. On the other hand, NE increased [^{3}H]InsP formation in each subclone, and this response was substantially increased by induction of receptor expression with IPTG. NE-stimulated [^{3}H]InsP formation was highly correlated with receptor den-

sity, being highest in subclone 3, which expressed the highest density of α_{1A}-ARs and lowest in subclone 9, which expressed the lowest density of α_{1A}-ARs (Fig. 1). α_{1A}-AR activation had no effect on cAMP levels (data not shown), showing an absence of cross-talk with G_i or G_S.

α_{2A}-AR Expression and Induction—Two PC12 subclones showing inducible expression of α_{2A}-AR receptors (α_{2A}-2 and α_{2A}-5) were characterized extensively. Saturation analysis of [^{3}H]rauwolscine binding indicated similar levels of basal and IPTG-induced receptor expression in each cell line. The concentration-response curve for IPTG-stimulation of α_{2A}-AR expression in subclone 5 is shown in Fig. 2. IPTG caused about a 7-fold increase in receptor expression with an EC_{50} around 5–10 μM. Inhibition of forskolin-stimulated cAMP accumulation in these cells by the α_2-AR-selective agonist UK 14,304 was used to ensure that the expressed receptors were functional. As expected, there was no effect of IPTG-induced receptor expression on forskolin-stimulated cAMP accumulation (Fig. 2). However, the potency of UK 14,304 in inhibiting forskolin-stimulated cAMP accumulation was enhanced 5–7-fold by IPTG pretreatment (1 mM, 48 h). Interestingly, the maximal inhibition of the forskolin response by UK 14,304 was not affected by IPTG, suggesting that the density of α_{2A}-ARs in uninduced cells is sufficient for maximal inhibition. α_{2A}-AR activation did not affect InsP formation (data not shown), showing an absence of cross-talk with $G_{q/11}$.

TABLE I
Adrenergic receptor density and properties in subclones of transfected PC12 cells: effect of IPTG induction

PC12 cells were co-transfected with one of the three adrenergic receptor cDNAs subcloned into the LacSwitch operator vector and p3′SS as described in the text. Individual subclones were isolated and propagated as described. Receptor density (B_{max}) and affinity (K_D) was quantitated by saturation analysis of radioligand (α_{1A}, [^{125}I]BE 2254; α_{2A}, [^{3}H]rauwolscine; β_1, [^{125}I]iodocyanopindolol) binding to membranes from cells treated with (+IPTG) or without (−IPTG) 1 mM IPTG for 48 h.

	K_D		B_{max}	
	−IPTG	+IPTG	−IPTG	+IPTG
	pM		*fmol/mg of protein*	
α_{1A}-3	49 ± 16	29 ± 5	37 ± 16	293 ± 65[a]
α_{2A}-5	706 ± 158	925 ± 174	124 ± 37	902 ± 82[a]
β_1-3	50 ± 25	37 ± 21	209 ± 74	195 ± 78

[a] $p < 0.001$ compared to −IPTG.

β_1-AR Expression—All β_1-AR-expressing PC12 subclones that we isolated showed constitutive receptor expression. Each subclone showed a receptor density around 200 fmol/mg of protein, which was not significantly altered by treatment with IPTG (1 mM, 48 h). Because this receptor density is in the range of expression of the α_{1A}-AR- and α_{2A}-AR-expressing subclones, we used the constitutive expression of subclone β_1-3 to study β_1-AR responses in PC12 cells. Because the β-AR is not activated in the absence of ligand, we did not make further attempts to isolate an inducible β-AR PC12 cell line. Stimulation with forskolin (30 μM) caused about a 10-fold increase in cAMP accumulation in both parental PC12 cells and the β_1-3 subclone (Fig. 3). Stimulation with the β-AR agonist isoproterenol (10 μM) had no effect on cAMP accumulation in parental PC12 cells but caused a significant 50–100% increase in the β_1-3 subclone. UK 14,304 had no effect on forskolin-stimulated cAMP accumulation in either parental PC12 cells or in the β_1-3 subclone (Fig. 3), confirming the absence of endogenous α_2-ARs. β_1-AR activation also did not affect InsP formation (data not shown), showing an absence of cross-talk with $G_{q/11}$.

Activation of ERKs—We studied the effect of NE on ERK

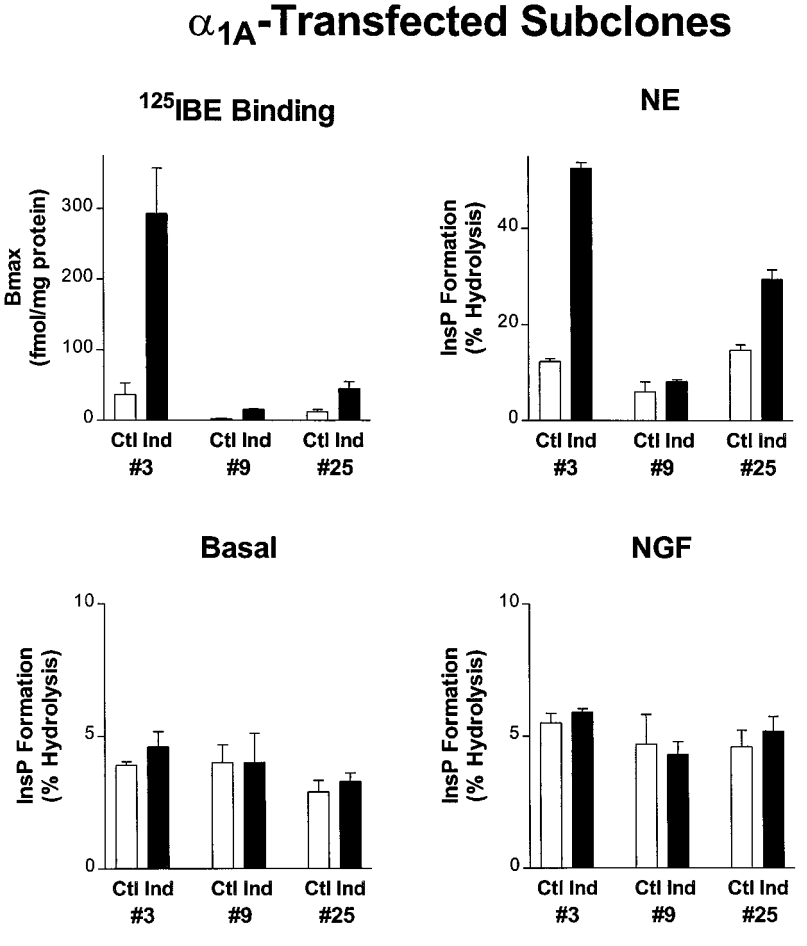

FIG. 1. **Radioligand binding and NE-stimulated inositol phosphate formation in subclones of α_{1A}-transfected PC12 cells.** Cells were transfected with the human α_{1A}-AR in the LacSwitch vector system, selected, and propagated as described under "Experimental Procedures." Receptor density (*top left*) was determined in subclones 3, 9, and 25 treated without (*Ctl*) or with (*Ind*) 1 mM IPTG for 48 h by saturation analysis of specific [^{125}I]BE binding. [^{3}H]Inositol phosphate formation was determined in each subclone with or without IPTG induction of receptor expression, in the presence of vehicle (*bottom left*), 100 μM NE (*top right*), or 100 ng/ml NGF (*bottom right*). Each value is the mean ± S.E. of three experiments performed in duplicate.

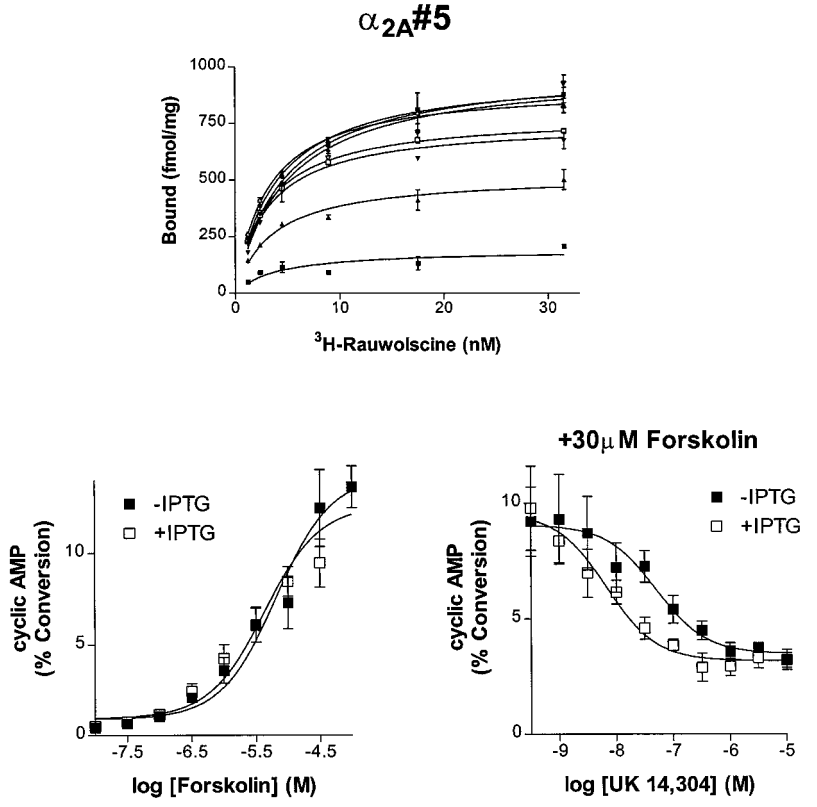

FIG. 2. **Radioligand binding and cAMP accumulation in α_{2A}-5 PC12 subclone.** *Top,* receptor density was determined by saturation analysis of specific [^{3}H]rauwolscine binding in cells treated for 48 h with IPTG at the following concentrations: none (■), 5 (▲), 10 (▼), 20 (♦), 50 (●), 100 (□), 1000 (△), or 5000 (▽) μM IPTG. *Bottom left,* concentration-response relationship for forskolin-stimulated [^{3}H]cAMP formation in cells treated without (*−IPTG*) or with (*+IPTG*) 1 mM IPTG for 48 h. *Bottom right,* concentration-dependent inhibition of 30 μM forskolin-stimulated [^{3}H]cAMP by UK 14,304 in the same cells. Each value is the mean ± S.E. of three experiments performed in duplicate.

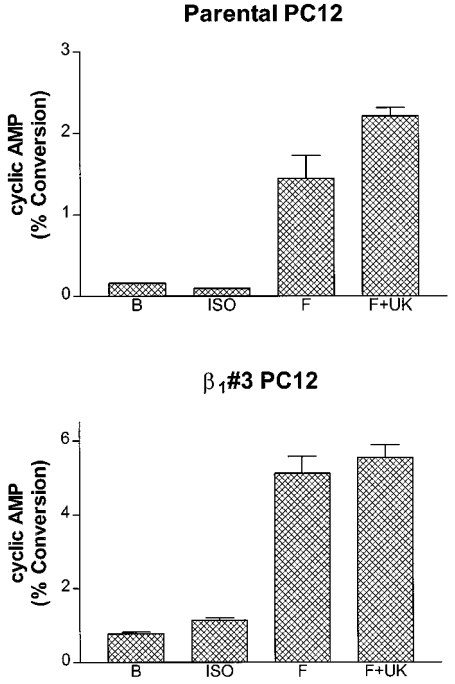

FIG. 3. **cAMP accumulation in untransfected PC12 cells and in a subclone of PC12 cells transfected with the rat β_1-AR.** Data from untransfected parental PC12 cells is shown in the *top panel*, and data from the β_1-3 subclone is shown in the *bottom panel*. [^{3}H]cAMP accumulation was determined under basal conditions (*B*) and after stimulation with isoproterenol (*ISO*, 10 μM), forskolin (*F*, 30 μM), or forskolin + 10 μM UK 14,304 (*F+UK*) as described in the text. Each value is the mean ± S.E. of three individual determinations.

activation in PC12 cells expressing the different AR subtypes. Exposure to NGF (100 ng/ml) caused activation of ERK 1 and 2 phosphorylation in parental PC12 cells (Fig. 4), as well as in PC12 cells expressing each of the AR subtypes (α_{1A}-3, α_{2A}-5, and β_1-3). Exposure to NE (100 μM) had no effect in parental PC12 cells but caused activation of ERKs in the α_{1A}-3 PC12 cells (Fig. 4). As expected, the degree of activation of ERKs by NE was increased by increasing α_{1A}-AR expression with IPTG. NE also caused ERK activation in β_1-3 PC12 cells, although this effect was not increased by IPTG, which does not increase β_1-AR expression in these cells. Surprisingly, NE had no effect on ERK activation in α_{2A}-5 PC12 cells, even after increasing receptor density by IPTG exposure (Fig. 4). Blotting for total ERK protein showed equivalent sample loading for each condition (Fig. 4). To confirm the lack of α_{2A}-AR-mediated ERK activation in PC12 cells, the effect of NE was also tested on the α_{2A}-2 subclone. Again, NGF increased ERK phosphorylation in this cell line, whereas NE had no effect either with or without exposure to IPTG (data not shown).

Activation of JNK/SAPK—Several GPCRs have also been shown to activate JNK/SAPK in various cells (7, 40). Exposure to NGF (100 ng/ml) caused a slight activation of JNK phosphorylation in α_{1A}-3, α_{2A}-2, and β_1-3 PC12 cells (Figs. 5 and 6). Exposure to NE (100 μM) caused a significant activation of JNK in the α_{1A}-3 PC12 cells, and the effect of NE was markedly increased by increasing α_{1A}-AR expression with IPTG (Fig. 5). NE had no significant effect on JNK activation in α_{2A}-2 or β_1-3 PC12 cells, irrespective of IPTG pretreatment (Fig. 6).

Activation of p38 MAPK—GPCRs have also been shown to activate p38 MAPK in certain cells (26). Exposure to NGF (100 ng/ml) caused only a small activation of p38 MAPK phosphorylation in α_{1A}-3, α_{2A}-2, and β_1-3 PC12 cells (Figs. 5 and 6). Exposure to NE (100 μM) caused a significant activation of p38 MAPK in the α_{1A}-3 PC12 cells, and the effect of NE was again markedly increased by increasing α_{1A}-AR expression with IPTG (Fig. 5). NE had no significant effect on p38 MAPK activation in α_{2A}-2 or β_1-3 PC12 cells, irrespective of IPTG pretreatment (Fig. 6). Blotting for total p38 MAPK protein showed equivalent protein loading in all samples.

Summary of AR-mediated Activation of MAPK Pathways in PC12 Subclones—Fig. 6 shows a summary of the effects of NE and NGF on activation of ERKs, JNK/SAPK, and p38 MAPK in

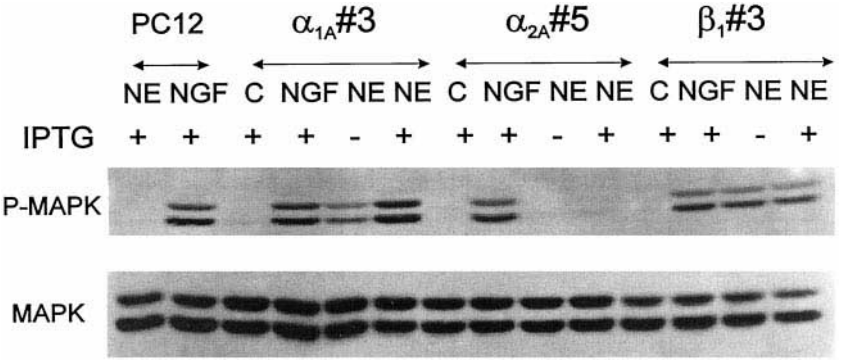

FIG. 4. **Activation of MAPK by NE and NGF in PC12 cell subclones.** Cells were treated with (+) or without (−) 1 mM IPTG for 48 h; serum-starved for 2 h; treated with vehicle (*C*), NE (100 μM), or NGF (100 ng/ml) for 15 min; lysed; and harvested as described. 20 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for activated phosphorylated MAPK (*P-MAPK*) or total MAPK (*MAPK*) as described. Data from parental PC12 cells and subclones transfected with each of the three AR subtypes are shown. Data are representative of seven or more separate experiments.

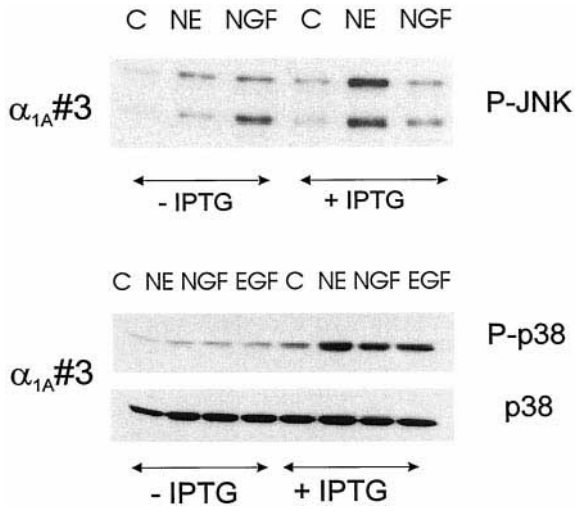

FIG. 5. **Activation of JNK/SAPK and p38 MAPK by NE and NGF in α_{1A}-3 PC12 cells.** Cells were treated with (*+IPTG*) or without (*−IPTG*) 1 mM IPTG for 48 h; serum-starved for 2 h; treated with vehicle (*C*), NE (100 μM), or NGF (100 ng/ml) for 15 min; lysed; and harvested as described. 20 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for activated phosphorylated JNK (*P-JNK*), activated phosphorylated p38 MAPK (*P-p38*), or total p38 MAPK (*p38*) as described. Data are representative of five or more separate experiments.

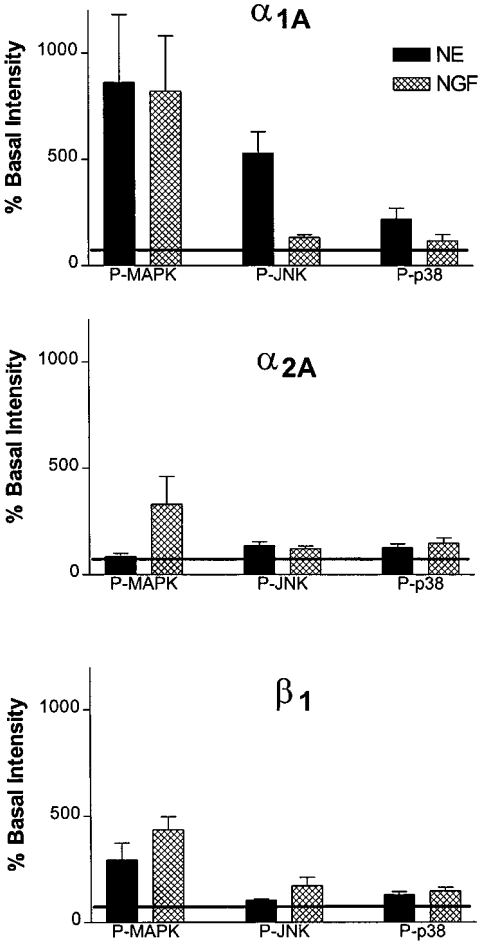

FIG. 6. **Summary of the effects of NE and NGF on activation of MAPK pathways in PC12 cells stably expressing different AR subtypes.** The degree of activation of MAPK (*P-MAPK*), JNK (*P-JNK*), and p38 MAPK (*P-p38*) by NE (100 μM) or NGF (100 ng/ml) was quantitated by densitometry of Western blots. Data from PC12 cell subclones stably transfected with α_{1A}-AR (*top*), α_{2A}-AR (*middle*), or β_1-AR (*bottom*) subtypes is shown. Each value is the mean ± S.E. of values from 3–7 experiments. *Bars* indicate basal intensity in the absence of agonist (100%).

the α_{1A}-3, α_{2A}-2, α_{2A}-5, and β_1-3 PC12 subclones. NGF caused substantial increases in ERK activation and small increases in JNK/SAPK and p38 MAPK activation in all cell lines studied. NE caused an 8.6-fold activation of ERKs, 5.3-fold activation of JNK/SAPK, and 2.2-fold increase in p38 MAPK activation in IPTG-induced α_{1A}-3 PC12 cells. NE caused no detectable increase in activation of ERKs, JNK/SAPK, or p38 MAPK in either of the α_{2A} PC12 subclones examined, either with or without IPTG treatment. NE caused a 3-fold increase in ERK activation, but no detectable increase in either JNK/SAPK or p38 MAPK in β_1-3 PC12 cells.

Time Course of NE-stimulated ERK Activation in α_{1A} and β_1-AR PC12 Cells—Because activation of either α_{1A}- or β_1-ARs activated ERKs in PC12 cells, we compared the time course of the two responses. Fig. 7 shows that NE activation of α_{1A}-ARs caused a large and sustained activation of ERKs, which was highly dependent on receptor induction by IPTG. NE activation of β_1-ARs also caused sustained ERK activation.

Differentiation of AR-expressing PC12 Cells—Exposure of α_{1A}-3 PC12 cells to either NE or NGF caused differentiation of the cells within 36–48 h after exposure (Fig. 8). The extent of NE-induced differentiation of α_{1A}-3 PC12 cells was dependent on the level of receptor expression. Cells expressing high levels of α_{1A}-ARs (~300 fmol/mg of protein following induction with IPTG) displayed NE-induced differentiation similar to that observed with NGF alone, whereas cells expressing lower levels of α_{1A}-ARs (~40 fmol/mg of protein, not induced with IPTG) showed NE-induced differentiation only slightly higher than untreated cells. Exposure of IPTG-induced α_{1A}-3 cells to both NE and NGF caused differentiation levels (size and number of neurites) greater than those caused by either agonist alone (Fig. 8), suggesting that differentiation in response to the two agonists is additive. Exposure of uninduced α_{1A}-3 cells to both

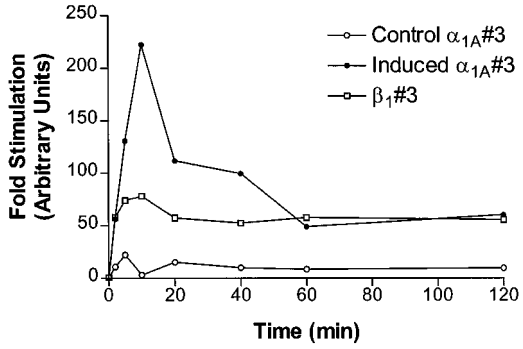

FIG. 7. **Time course for NE activation of ERKs in PC12 subclones.** α_{1A}-3 PC12 cells were treated with (●, *Induced*) or without (○, *Control*) 1 mM IPTG for 48 h, and β_1-3 cells (□) were cultured without IPTG. Cells were serum-starved for 2 h, treated with NE (100 μM) for 0–120 min, lysed, and harvested as described. 20 μg of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for activated phosphorylated MAPK as described. Data are representative of two separate experiments. The degree of stimulation is expressed in arbitrary units and is relative to basal intensity. The densitometer was adjusted for maximal sensitivity to emphasize responses.

NE and NGF caused differentiation similar to that of cells exposed to NGF alone, confirming that the effects of NE and NGF are additive, not synergistic.

NE treatment had no significant effect on the differentiation state of either of the α_{2A}-AR-expressing PC12 subclones (subclones 2 and 5), either with or without induction of receptor expression with IPTG (1 mM, 48 h), in the presence or absence of NGF, or at any time point examined (data not shown).

Exposure of β_1-3 PC12 cells to NE for 24–36 h had little visible effect on differentiation (Fig. 9). However, NE had a synergistic effect on differentiation caused by NGF. Exposure to both NGF and NE for just 24 h caused differentiation of cells to levels higher than that seen in cells exposed to either NGF or NE alone (Fig. 9).

DISCUSSION

In this report, we directly compare the effects of $G\alpha_{q/11}$, $G\alpha_i$, and $G\alpha_S$-coupled ARs on mitogenic responses and differentiation of PC12 cells. ARs affect growth and differentiation of many cells, although the subtypes and mechanisms involved are not yet clear. PC12 cells have been widely used to study the events involved in cellular differentiation (27). NGF causes differentiation of PC12 cells through a sustained activation of the Ras/Raf/ERK pathway (21, 28). We took advantage of the fact that the three AR families selectively couple to three major G protein families ($\alpha_1/G_{q/11}$, α_2/G_i, and β/G_S) to examine the specificity with which these receptors activate MAPK in PC12 cells. Because receptor density is critically important in signaling specificity, we used an IPTG-inducible vector system to control receptor expression within a range normally observed in many tissues. We wanted to directly compare AR-mediated activation of $G_{q/11}$, G_i, and G_S on MAPK and differentiation responses in the same cellular phenotype. PC12 cells also allow direct comparison of signals generated by GPCRs and tyrosine kinase receptors involved in growth, differentiation, and apoptosis.

We found dramatic differences in the ability of the three AR families to promote MAPK activation and differentiation of PC12 cells. Stimulation of both α_{1A}- and β_1-ARs caused ERK activation, whereas stimulation of α_{2A}-ARs did not. The activation of ERKs by α_{1A}- and β_1-ARs was sustained and elevated even 1 h after stimulation. Only in α_{1A}-AR transfected cells did NE cause significant activation of the JNK/SAPK and p38 MAPK pathways. In addition, only the α_{1A}-AR subtype caused PC12 differentiation in the absence of other stimuli. Most surprisingly, activation of α_{2A}-ARs at either low or high density had no effect on ERK activation in PC12 cells, despite previous studies showing activation of this pathway through the endogenous lysophosphatidic acid receptor in these cells (6, 8).

Activation of α_{1A}-ARs by NE caused a substantial activation of ERKs in PC12 cells, and this effect was increased by IPTG exposure, suggesting that it was proportional to receptor density. ERK activation by α_{1A}-ARs corresponded with a NE-induced differentiation of these cells into a neuronal-like phenotype. In cells expressing high levels of α_{1A}-ARs, NE caused differentiation indistinguishable from that caused by NGF. Differentiation of α_{1A}-AR cells exposed to both NGF and NE was no more than additive and occurred on the same time scale as that caused by NGF alone (36–48 h).

NE also caused a large activation of JNK/SAPK and a smaller activation of p38 MAPK in α_{1A}-transfected PC12 cells, and these responses were also increased by IPTG exposure. α_1-AR-mediated activation of JNK/SAPK has been reported in cardiomyocytes (40); however, activation of p38 MAPK by α_1-ARs has not been reported previously. p38 MAPK has been shown to be activated by $G_{q/11}$-coupled m1 muscarinic receptors (26) and by activated forms of α_q (14). Activation of JNK/SAPK and p38 MAPK has previously been associated with stress responses (17, 21); however, recent data in cardiomyocytes suggest a role for JNK in cell growth (40). In most cases, ERK, JNK/SAPK, and p38 MAPK pathways are activated by different stimuli (17), and α_{1A}-transfected PC12 cells are unusual in activation of these pathways by a single stimulus.

GPCRs coupling through α_i (including α_2-ARs) were among the first GPCRs shown to activate ERKs (1, 2, 4). Other α_i-coupled receptors, such as lysophosphatidic acid receptors, have also been reported to activate ERKs in PC12 cells (8). We were surprised to find that stimulation of α_{2A}-ARs did not activate ERKs in PC12 cells, even at high expression levels. Studies on inhibition of forskolin-stimulated cAMP accumulation showed that the expressed α_{2A}-ARs were functional, and receptor density in the presence of IPTG was higher for α_{2A}-ARs than either α_{1A}- or β_1-ARs (Table I). Although lysophosphatidic acid often acts via α_i, it has also been reported to activate α_q in PC12 cells (41, 42), and it could be causing at least some of its effects via α_q in these cells.

The fact that α_{2A}-ARs activate ERKs in fibroblastic cell lines such as Rat1a cells (2, 4) in a PTX-sensitive manner but do not activate ERKs in PC12 cells indicates that there are important mechanistic differences in signaling between the cell types. Activation of ERKs by G_i-linked receptors appears to be mediated by $\beta\gamma$-subunits (3, 22, 23, 43), and recent work suggests that $\beta\gamma$ signaling is impaired in the presence of either α_t- or α_o-subunits (3, 44, 45). α_o is selectively expressed in brain and many neuronal cell lines, including PC12 cells (39, 46, 47). Fibroblastic cell lines, such as NIH3T3 and Rat1a, express α_{i2} and α_{i3} but not α_o (39). Because α_o is expressed selectively in PC12 cells, it may play a similar suppressive role in $\beta\gamma$-mediated MAPK activation by α_{2A}-ARs. Regardless, our results show that ERK activation is not a universal response to activation of G_i-coupled receptors in PC12 cells.

Release of $\beta\gamma$-subunits is also proposed to be important for ERK activation by α_S-linked GPCRs, although the specificity of these interactions in PC12 cells is not yet clear (12, 43, 48). The effects of increased levels of cAMP on differentiation of PC12 cells and activation of ERKs have been well studied (29, 49, 50). In most other cell lines, increases in cAMP generally inhibit ERK activation (15, 16), and PC12 cells are relatively unusual in that forskolin-induced increases in cAMP activate ERKs (29, 49–51). This study is one of the few examples of ERK activation

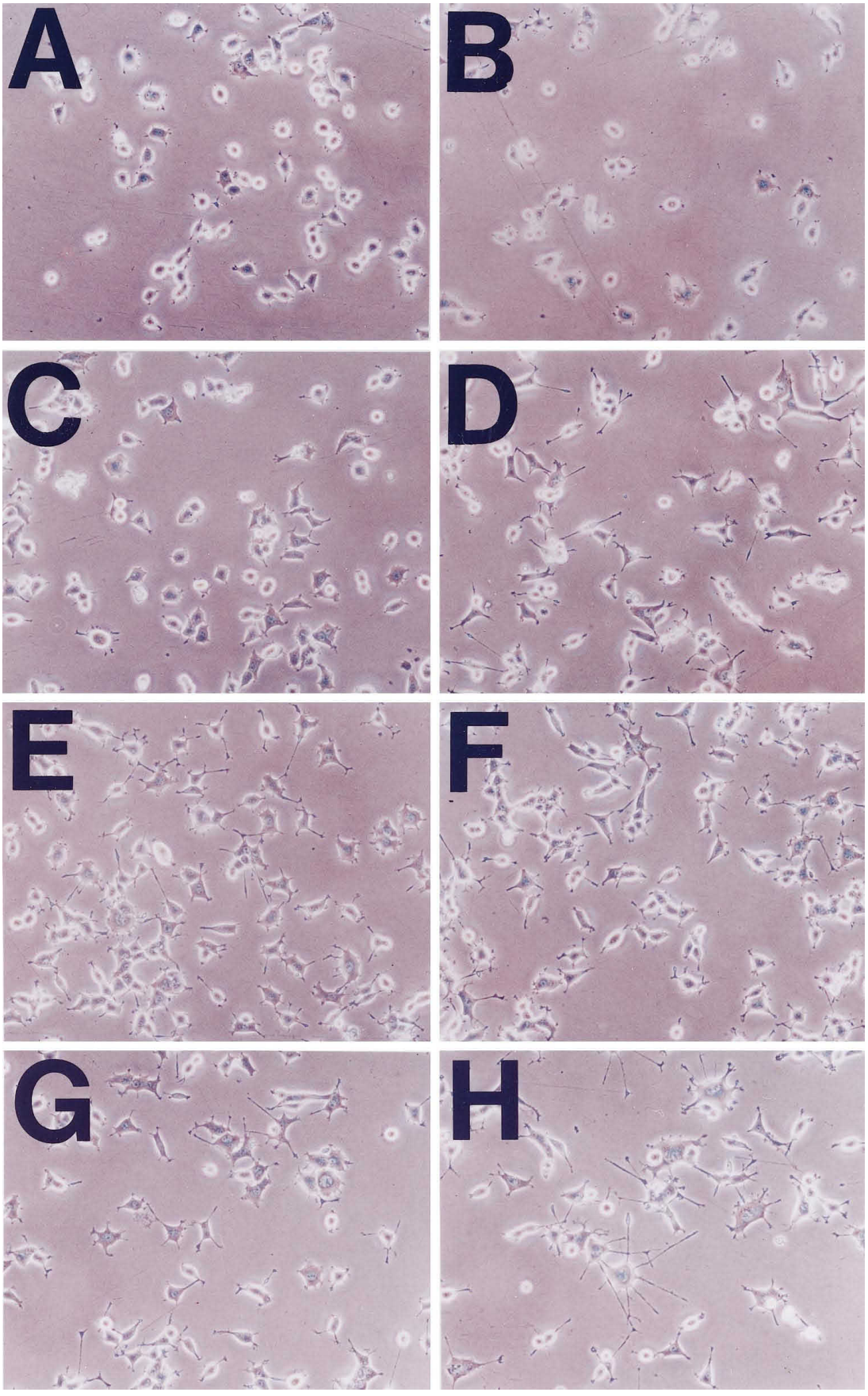

FIG. 8. **Effects of NE and NGF on differentiation of the α_{1A}-3 subclone of PC12 cells.** Cells were plated on collagen-coated plates and treated without (*A, C, E,* and *G*) or with (*B, D, F,* and *H*) 1 mM IPTG for 48 h to induce receptor expression. Before treatment with agonists, the medium was replaced with Dulbecco's modified Eagle's medium containing 1% horse serum. Cells were then treated for 48 h with vehicle (*A* and *B*), 10 μM NE (*C* and *D*), 100 ng/ml NGF (*E* and *F*), or NE + NGF (*G* and *H*). Fresh NE was added every 24 h. A representative field of cells is shown in each case.

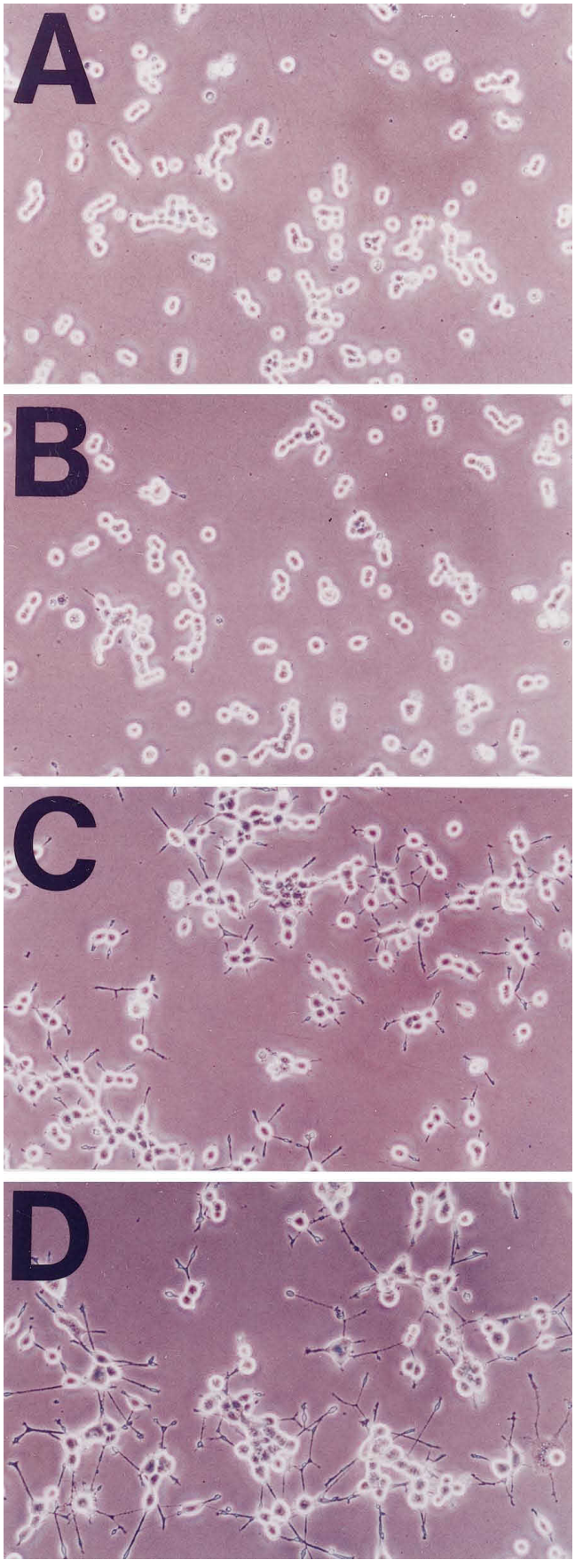

FIG. 9. **Effects of NE and NGF on differentiation of the β_1-3 subclone of PC12 cells.** Cells were plated on collagen-coated plates. Before treatment with agonists, the medium was replaced with Dulbecco's modified Eagle's medium containing 1% horse serum. Cells were the treated for 24–30 h with vehicle (*A*), 100 μM NE (*B*), 100 ng/ml NGF (*C*), or NE + NGF (*D*). Fresh NE was added every 24 h. A representative field of cells is shown in each case.

in PC12 cells caused by stimulation of an α_s-linked GPCR (rather than direct increases in cAMP), which would involve signaling from both α_S- and $\beta\gamma$-subunits. We observed sustained activation of ERKs upon stimulation of β_1-ARs in PC12 cells without observable differentiation in the absence of NGF. However, β_1-AR activation strongly potentiated NGF-induced differentiation, causing the appearance of neurites within 24 h after addition of both NE and NGF. This is consistent with previous studies suggesting that large increases in cAMP can alone cause differentiation of PC12 cells, but smaller increases in cAMP only potentiate growth factor-induced differentiation (52). Intracellular nonmitochondrial Ca^{2+} pools have been shown to be necessary for the synergistic effects of NGF and cAMP analogs on PC12 cell differentiation (53).

Previous studies in which PC12 cells were transfected with activated forms of G protein α-subunits showed that activated forms of α_q alone, but not of α_i or α_o, were capable of differentiating PC12 cells (14). Differentiation by α_q coincided with activation of JNK, but ERK activation was not seen (14). The results using activated α_S are less clear. One group reported that expression of activated α_S caused proliferation of PC12 cells and constitutive activation of cAMP dependent pathways (54), whereas another group found that expression of activated α_S caused differentiation of PC12 cells (55). Transfection of G protein subunits into Cos7 cells showed that ERK activation may be due to signaling from $\beta\gamma$ rather than either α_q, α_i, or α_S (3).

These results have some similarities to studies in cardiac and smooth muscle, in which both α_1- and β-ARs are involved in growth and differentiation (56). In both cases, α_1-ARs dominate, causing rapid and divergent activation of MAPK pathways and transcription (40, 56–60). Because stimulation of α_{1A}-ARs activates ERKs, JNK/SAPK, and p38 MAPK and promotes differentiation of PC12 cells, it will be interesting to compare the signaling pathways involved with those in myocytes (13, 60, 61). Studies in cardiac and smooth muscle cells are generally performed in primary cultures or *in vivo*, and PC12 cells may be a useful alternative in defining the transcriptional effects of receptor activation and how they relate to growth and differentiation.

This report shows that activations of α_1-, α_2-, and β-ARs in transfected PC12 cells have different effects on MAPK pathways and differentiation, suggesting a clear specificity in activation of MAPK pathways. The marked stimulation of all three MAPK pathways and differentiation by α_{1A}-AR activation may provide a useful system for studying the mechanisms by which GPCRs control cellular growth and differentiation and the relationship of these pathways to those activated by tyrosine kinase receptors.

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