Differential Coupling of $\alpha_{1-}$, $\alpha_{2-}$, and $\beta$-Adrenergic Receptors to Mitogen-activated Protein Kinase Pathways and Differentiation in Transfected PC12 Cells*

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Three adrenergic receptor families that selectively activate three different G proteins (G$\alpha_{11}$, G$\alpha_{12}$, and G$\alpha_{14}$) were used to study mitogen-activated protein kinase (MAPK) activation and differentiation in PC12 cells. PC12 cells were stably transfected with $\alpha_{1A}$-, $\alpha_{2A}$-, or $\beta_1$-adrenergic receptors (ARs) in an inducible expression vector, and subclones were characterized. Norepinephrine stimulated inositol phosphate formation in $\alpha_{1A}$-transfected cells, inhibited cyclic adenosine 3′,5′-monophosphate (cAMP) formation in $\alpha_{2A}$-transfected cells, and stimulated cAMP formation in $\beta_1$-transfected cells. Nerve growth factor activated extracellular signal-regulated kinases (ERKs) in all cell lines; however, norepinephrine activated ERKs only in $\alpha_{1A}$- and $\beta_1$-transfected cells but not in $\alpha_{2A}$-transfected cells. Norepinephrine also activated c-Jun NH$\_2$-terminal kinase and p38 MAPK in $\alpha_{1A}$-transfected cells but not in $\beta_1$- or $\alpha_{2A}$-transfected cells. Norepinephrine caused differentiation of PC12 cells expressing $\alpha_{1A}$-ARs but not those expressing $\beta_1$- or $\alpha_{2A}$-ARs. However, norepinephrine acted synergistically with nerve growth factor in promoting differentiation of cells expressing $\beta_1$-ARs. Whereas ERKs are activated by G$\alpha_i$- but not G$\alpha_s$-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$\alpha_s$ signaling pathways activating all three major MAPK pathways.

Although mitogen-activated protein kinase (MAPK) 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 $\alpha_{1A}$-, $\alpha_{2A}$-, or $\beta_1$-adrenergic receptors (ARs) in an inducible expression vector, and subclones were characterized. Norepinephrine stimulated inositol phosphate formation in $\alpha_{1A}$-transfected cells, inhibited cyclic adenosine 3′,5′-monophosphate (cAMP) formation in $\alpha_{2A}$-transfected cells, and stimulated cAMP formation in $\beta_1$-transfected cells. Nerve growth factor activated extracellular signal-regulated kinases (ERKs) in all cell lines; however, norepinephrine activated ERKs only in $\alpha_{1A}$- and $\beta_1$-transfected cells but not in $\alpha_{2A}$-transfected cells. Norepinephrine also activated c-Jun NH$\_2$-terminal kinase and p38 MAPK in $\alpha_{1A}$-transfected cells but not in $\beta_1$- or $\alpha_{2A}$-transfected cells. Norepinephrine caused differentiation of PC12 cells expressing $\alpha_{1A}$-ARs but not those expressing $\beta_1$- or $\alpha_{2A}$-ARs. However, norepinephrine acted synergistically with nerve growth factor in promoting differentiation of cells expressing $\beta_1$-ARs. Whereas ERKs are activated by G$\alpha_i$- but not G$\alpha_s$-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$\alpha_s$ signaling pathways activating all three major MAPK pathways.

Although mitogen-activated protein kinase (MAPK) 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$\alpha_i$ (1, 2, 4, 5), and G$\alpha_q$ (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, Ga$\alpha_1$- 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$\alpha_q$-linked receptors are thought to involve both the $\alpha$- and $\beta$-subunits of G proteins, although the $\alpha_q$-dependent activation of protein kinase C is thought to play the predominant role (3, 22). Response to both G$\alpha_q$-linked (22, 23) and G$\alpha_i$-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 G$\alpha_i$, as well as $\beta$-$\gamma$-subunits (26). $\beta$-Dependent activation of p38 MAPK is inhibited by coexpression of G$\alpha_i$ in HEK 293 cells (26).

PC12 cells, derived from a rat pheochromocytoma, have been a primary model for studies of the 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$\alpha_{11}$-coupled) and lysophosphatidic acid (G$\alpha_i$-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$\alpha_{11}$-, G$\alpha_i$-, and G$\alpha_s$-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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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; [125I]BE, [125I]BE (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.
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 ($\alpha_1$A, $\alpha_2$, and $\beta$) of adrenergic receptor (AR) subtypes (30, 31) to assess whether $\alpha_1$, $\alpha_2$, and $\beta$-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. $\alpha_1$-ARs increase PI hydrolysis and intracellular Ca$^{2+}$ through $\alpha_1$-ARs inhibit adenylate cyclase through $G_i$, and $\beta$-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), phenolamine meylate from Ciba-Geigy (Summit, NJ), hygromycin B from Boehringer Mannheim, BE 2254 (2β-(4-hydroxyphethylaminomethyl)tetralone) from Beiersdorf AG (Hamburg, Germany), cyanopindolol from Sandoz (Basel, Switzerland), $[^{125}\text{I}]$rauwolscine (60 Ci/mmol) and carrier-free Na$^{125}$I from Amersham Pharmacia Biotech, $[^{3}\text{H}]$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 $\alpha_2$AR (32) was generously provided by Dr. G. Tasjimoto (National Children’s Hospital, Tokyo, Japan), the cDNA for the rat $\beta_1$-AR (33) was provided by Dr. Curtis A. Machida (Oregon Regional Primate Research Center, Beaverton, OR), and the cDNA for the human $\alpha_2$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 NotI fragment of pOPRSVICAT containing the chloramphenicol acetyltransferase reporter gene was ligated with the multiple cloning site of pBluescript KS+ (where an additional NotI site had been inserted 5’ to the XhoI 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 cultures were washed twice with ice-cold phosphate-buffered saline and lysed in Nonidet P-40 lysis buffer containing 135 mM NaCl, 20 mM Tris (pH 7.5), 1 mM MgCl$_2$, 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 determined by blotting the membrane with phosphospecific ERK, JNK/SAPK, or p38 MAPK antibodies (New England Biolabs) that specifically recognized 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 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 $\alpha_2$-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 $[^{125}\text{I}]$BE (1-AR), $[^{3}\text{H}]$rauwolscine ($\alpha_2$-AR), or $[^{3}\text{H}]$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 $\alpha_2$-AR agonist UK 14,304 (see also below), and no stimulation of $[^{3}\text{H}]$InsP formation by the $\alpha_1$-agonist 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 $\alpha_1$A, human $\alpha_1$A, or rat $\beta_1$-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 $\alpha_1$A or $\alpha_2$ARs, however, we were unable to obtain subclones showing inducible expression of $\beta_1$-ARs. Several subclones with constitutive expression of $\beta_1$-ARs were isolated and used for further studies. Constitutive and IPTG-induced receptor density in selected subclones is summarized in Table I.

**$\alpha_1$AR Expression and Induction**—Three subclones of PC12 cells expressing $\alpha_1$A-ARs were extensively characterized (Fig. 1). Saturation analysis of $[^{125}\text{I}]$BE binding showed that each subclone exhibited different levels of constitutive and IPTG-induced (1 mM, 48 h) receptor expression, with $\alpha_1$A-3 showing the highest degree of induction. The effect of NE on $[^{3}\text{H}]$InsP formation was studied to ensure that the expressed receptors were functional. Basal $[^{3}\text{H}]$InsP formation was similar in each subclone, and was not affected by treatment with IPTG (Fig. 1). NGF caused small increases in $[^{3}\text{H}]$InsP formation in each subclone, and this response was unaffected by treatment with IPTG. On the other hand, NE increased $[^{3}\text{H}]$InsP formation in each subclone, and this response was substantially increased by induction of receptor expression with IPTG. NE-stimulated $[^{3}\text{H}]$InsP formation was highly correlated with receptor den-
Adrenergic Receptor Subtypes and PC12 Cell Differentiation

Fig. 1. Radioligand binding and NE-stimulated inositol phosphate formation in subclones of α<sub>1A</sub>-transfected PC12 cells. Cells were transfected with the human α<sub>1A</sub>-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 [<sup>125</sup>I]IBE binding. [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.

| 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 β3/SS as described in the text. Individual subclones were isolated and propagated as described. Receptor density (B<sub>max</sub>) and affinity (K<sub>d</sub>) was quantitated by saturation analysis of radioligand (α<sub>1A</sub>, [H]rauwolscine; β<sub>2</sub>, [H]iodocyanopindolol) binding to membranes from cells treated with (+IPTG) or without (−IPTG) 1 mM IPTG for 48 h.

| Receptor Type | B<sub>max</sub> (fmol/mg of protein) | K<sub>d</sub> (μM) |
|---------------|----------------------------------|--------------|
| α<sub>1A</sub>-3 | 49 ± 16                          | 50 ± 5       |
| α<sub>2A</sub>-5 | 706 ± 158                        | 29 ± 5       |
| β<sub>1</sub>-3 | 50 ± 25                          | 37 ± 21      |

Values are means ± S.E. of three experiments performed in duplicate. *p < 0.001 compared to −IPTG.

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<sub>2A</sub>-ARs in uninduced cells is sufficient for maximal inhibition. α<sub>2A</sub>-AR activation did not affect InsP formation (data not shown), showing an absence of cross-talk with G<sub>q/11</sub>.

β<sub>1</sub>-AR Expression—All β<sub>1</sub>-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<sub>1A</sub>-AR and α<sub>2A</sub>-AR-expressing subclones, we used the constitutive expression of subclone β<sub>1</sub>-3 to study β<sub>1</sub>-AR responses in PC12 cells. Because the β<sub>1</sub>-AR is not activated in the absence of ligand, we did not make further attempts to isolate an inducible β<sub>1</sub>-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<sub>1</sub>-3 subclone (Fig. 3). Stimulation with the β<sub>1</sub>-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<sub>1</sub>-3 subclone. UK 14,304 had no effect on forskolin-stimulated cAMP accumulation in either parental PC12 cells or in the β<sub>1</sub>-3 subclone (Fig. 3), confirming the absence of endogenous α<sub>2A</sub>-ARs. β<sub>1</sub>-AR activation also did not affect InsP formation (data not shown), showing an absence of cross-talk with G<sub>q/11</sub>.

Activation of ERKs—We studied the effect of NE on ERK
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 (α1A-3, α2A-5, and β1-3). Exposure to NE (100 μM) had no effect in parental PC12 cells but caused activation of ERKs in the α1A-3 PC12 cells (Fig. 4). As expected, the degree of activation of ERKs by NE was increased by increasing α1A-AR expression with IPTG. NE also caused ERK activation in β1-3 PC12 cells, although this effect was not increased by IPTG, which does not increase β1-AR expression in these cells. Surprisingly, NE had no effect on ERK activation in α2A-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 α2A-AR-mediated ERK activation in PC12 cells, the effect of NE was also tested on the α2A-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 α1A-3, α2A-2, and β1-3 PC12 cells (Figs. 5 and 6). Exposure to NE (100 μM) caused a significant activation of JNK in the α1A-3 PC12 cells, and the effect of NE was markedly increased by increasing α1A-AR expression with IPTG (Fig. 5). NE had no significant effect on JNK activation in α2A-2 or β1-3 PC12 cells, irrespective of IPTG pretreatment (Fig. 6). Blotting for total JNK protein showed equivalent protein loading in all samples.

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 α1A-3, α2A-2, and β1-3 PC12 cells (Figs. 5 and 6). Exposure to NE (100 μM) caused a significant activation of p38 MAPK in the α1A-3 PC12 cells, and the effect of NE was again markedly increased by increasing α1A-AR expression with IPTG (Fig. 5). NE had no significant effect on p38 MAPK activation in α2A-2 or β1-3 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
the $\alpha_{1A}$-3, $\alpha_{2A}$-2, $\alpha_{2A}$-5, and $\beta_{1}$-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 $\alpha_{1A}$-3 PC12 cells. NE caused no detectable increase in activation of ERKs, JNK/SAPK, or p38 MAPK in either of the $\alpha_{2A}$ 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 $\beta_{1}$-3 PC12 cells.

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

**Differentiation of AR-expressing PC12 Cells**—Exposure of $\alpha_{1A}$-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 $\alpha_{1A}$-3 PC12 cells was dependent on the level of receptor expression. Cells expressing high levels of $\alpha_{1A}$-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 $\alpha_{1A}$-ARs (~40 fmol/mg of protein, not induced with IPTG) showed NE-induced differentiation only slightly higher than untreated cells. Exposure of IPTG-induced $\alpha_{1A}$-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 $\alpha_{1A}$-3 cells to both...
Control

vation of ERKs by expression with IPTG (1 mM, 48 h), in the presence or absence clones.

activation of Gq/11, Gi, and Gs on MAPK and differentiation in many tissues. We wanted to directly compare AR-mediated specificity, we used an IPTG-inducible vector system to cells. Because receptor density is critically important in signal-specificity with which these receptors activate MAPK in PC12

families to promote MAPK activation and differentiation of PC12 cells. Stimulation of both a2A- and b1-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 (8). We were surprised to find that stimulation of a2ARs did not activate ERKs in PC12 cells, even at high expression levels. Studies on inhibition of forskolin-stimulated cAMP accumulation showed that the expressed a2ARs were functional, and receptor density in the presence of IPTG was higher for a2ARs than either a1A- or b2-ARs (Table I). Although lysophosphatidic acid often acts via a2, it has also been reported to activate a1 in PC12 cells (41, 42), and it could be causing at least some of its effects via a1 in these cells.

PC2 differentiation in the absence of other stimuli. Most surprisingly, activation of a2ARs 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 a1ARs 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 a1ARs corresponded with a NE-induced differentiation of these cells into a neuronal-like phenotype. In cells expressing high levels of a1ARs, NE caused differentiation indistinguishable from that caused by NGF. Differentiation of a1AR-expressing 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 a1A-transfected PC12 cells, and these responses were also increased by IPTG exposure. a1AR-mediated activation of JNK/SAPK has been reported in cardiomyocytes (40); however, activation of p38 MAPK by a1-ARs has not been reported previously. p38 MAPK has been shown to be activated by Gq2-coupled m1 muscarinic receptors (26) and by activated forms of a1 (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 a1A-transfected PC12 cells are unusual in activation of these pathways by a single stimulus.

GPCRs coupling through a1 (including a2ARs) were among the first GPCRs shown to activate ERKs (1, 2, 4). Other a2-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 a2ARs did not activate ERKs in PC12 cells, even at high expression levels. Studies on inhibition of forskolin-stimulated cAMP accumulation showed that the expressed a2ARs were functional, and receptor density in the presence of IPTG was higher for a2ARs than either a1A- or b2-ARs (Table I). Although lysophosphatidic acid often acts via a2, it has also been reported to activate a1 in PC12 cells (41, 42), and it could be causing at least some of its effects via a1 in these cells.

The fact that a2ARs 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 G1-linked receptors appears to be mediated by bY-subunits (3, 22, 23, 43), and recent work suggests that bY signaling is impaired in the presence of either a1- or a-subunits (3, 44, 45). a1 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 a2 and a2 but not a1 (39). Because a1 is expressed selectively in PC12 cells, it may play a similar suppressive role in bY-mediated MAPK activation by a2ARs. Regardless, our results show that ERK activation is not a universal response to activation of G1-coupled receptors in PC12 cells.

Release of bY-subunits is also proposed to be important for ERK activation by a0-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 α₁A-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.
in PC12 cells caused by stimulation of an α-linked GPCR (rather than direct increases in cAMP), which would involve signaling from both α2- and βγ-subunits. We observed sustained activation of ERKs upon stimulation of β2-ARs in PC12 cells without observable differentiation in the absence of NGF. However, β1-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 Ca2⁺ 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 αq alone, but not of αi or αo, were capable of differentiating PC12 cells (14). Differentiation by αq coincided with activation of JNK, but ERK activation was not seen (14). The results using activated αq are less clear. One group reported that expression of activated αq caused proliferation of PC12 cells and constitutive activation of cAMP dependent pathways (54), whereas another group found that expression of activated αq caused differentiation of PC12 cells (55). Transfection of G protein subunits into Cos7 cells showed that ERK activation may be due to signaling from βγ rather than either αq, αi, or αo (3). These results have some similarities to studies in cardiac and smooth muscle, in which both α1- and β-ARs are involved in growth and differentiation (56). In both cases, α2-ARs dominate, causing rapid and divergent activation of MAPK pathways and transcription (40, 56–60). Because stimulation of α1A-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 α1-, α2-, 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 α1A-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.
