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Transcriptional Responses to Growth Factor and G Protein-Coupled Receptors in PC12 Cells: Comparison of α_1-Adrenergic Receptor Subtypes

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Abstract: Transcriptional responses to growth factor and G protein-coupled receptors were compared in PC12 cells using retroviral luciferase reporters. In cells stably expressing α_{1A}-adrenergic receptors, norepinephrine activated all five reporters [AP1 (activator protein-1), SRE (serum response element), CRE (cyclic AMP response element), NFκB (nuclear factor-κB), and NFAT (nuclear factor of activated T cells)], whereas nerve growth factor (NGF) and epidermal growth factor activated only AP1 and SRE. Activation of P2Y2 receptors by UTP did not activate any reporters. Protein kinase C inhibition blocked NFκB activation by norepinephrine, but potentiated CRE. Mitogen-activated protein kinase kinase inhibition blocked AP1 activation by norepinephrine, but also potentiated CRE. p38 mitogen-activated protein kinase inhibition reduced most norepinephrine responses, but not NGF responses. Inhibition of Src eliminated SRE responses to norepinephrine and NGF, and reduced all responses except CRE. Phosphatidylinositol 3-kinase inhibitors markedly potentiated CRE activation by norepinephrine, with only small effects on the other responses. Comparison of the three human subtypes showed that the α_{1A} activated all five reporters, the α_{1B} showed smaller effects, and the α_{1D} was ineffective. Cell differentiation caused by norepinephrine, but not NGF, was reduced by all inhibitors studied. These experiments suggest that α_{1A}-adrenergic receptors activate a wider array of transcriptional responses than do growth factors in PC12 cells. These responses are not linearly related to second messenger production, and different subtypes show different patterns of activation. **Key Words:** G protein—Adrenergic receptors—Growth factor—Transcription—Reporters—Gene expression.
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α_1-Adrenergic receptors (ARs), like many G protein-coupled receptors (GPCRs), activate mitogenic responses and regulate growth and proliferation of many cells (Zhong and Minneman, 1999*a*). There are three α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D}), all of which signal through the $G_{q/11}$ family of G proteins (Zhong and Minneman, 1999*a*). In rat PC12 cells transfected with α_{1A}-ARs, norepinephrine (NE) activates all three mitogen-activated protein kinase (MAPK) pathways and causes the cells to differentiate into a neuronal-like phenotype similar to that caused by nerve growth factor (NGF) (Williams et al., 1998; Zhong and Minneman, 1999*b*). These mitogenic responses appear to be independent of known second messenger pathways (Berts et al., 1999), but involve activation of a number of tyrosine kinases, particularly Pyk2 and Src (Zhong and Minneman, 1999*c*).

As activation of either the α_{1A}-GPCR or the NGF tyrosine kinase receptor causes PC12 cells to differentiate into a similar neuronal-like phenotype, this provides an opportunity to compare and contrast the signaling pathways involved. GPCR and growth factor receptors were traditionally thought to activate totally separate signaling pathways. Growth factors were known to control nuclear transcriptional activity through MAPK pathways, whereas GPCRs were thought to control metabolic activity and second messenger levels near the plasma membrane. With the realization that GPCRs also activate MAPK pathways, there has been a growing interest in the transcriptional pathways activated by these receptors and the mechanisms by which such pathways are activated.

In this study, we use novel retroviral luciferase reporter constructs (Abbott et al., 2000) to compare di-

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Abbreviations used: AP1, activator protein-1; AR, adrenergic receptor; CRE, cyclic AMP response element; CREB, CRE binding protein; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase (also known as p42 and p44 mitogen-activated protein kinases); GPCR, G protein-coupled receptor; IL-2, interleukin-2; IPTG, isopropylthiogalactose; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; NE, norepinephrine; NFAT, nuclear factor of activated T cells; NFκB, nuclear factor-κB; NGF, nerve growth factor; PI-3-kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; SRE, serum response element.

rectly the transcriptional responses to α_1-AR and NGF receptor activation in PC12 cells. We find that α_{1A}-ARs activate a much wider variety of transcriptional responses than does NGF in these cells, but that the other two α_1-AR subtypes show smaller responses. We also use a variety of specific inhibitors to begin to dissect the mechanisms involved in activation of these transcriptional responses, and to study their role in cell differentiation in response to NE or NGF.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: SB202190, GFX203290, PP2, PD98059, and BAPTA-AM, Calbiochem (La Jolla, CA, U.S.A.); (−)-NE bitartrate, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and LY294002, Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

PC12 cells were propagated in 100-cm^2 plates at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 1.4% glutamine, 20 m*M* HEPES, 100 mg/L streptomycin, 10^5 units/L penicillin, 10% donor horse serum, and 5% fetal bovine serum (Williams et al., 1998). Cells were detached by trypsinization and gentle trituration, and subcultured at a ratio of 1:3 upon reaching confluency.

Construction of retroviral luciferase reporter vectors

Multimers of synthetic oligonucleotide pairs bearing well characterized enhancer sequences were cloned upstream of a minimal interleukin-2 (IL-2) gene promoter to drive luciferase expression. Concatenation was achieved by a brief (<5 min) preligation of the oligonucleotides, followed by adding the minimal IL-2 promoter/luciferase shuttle vector pVB1 (Boss et al., 1998), which was first prepared by *Bam*HI digestion with or without partial fill-in with dGTP, depending on the reporter. Shuttle clones with two or more copies of enhancer were identified by sequencing. Digestions with *Sal*I and *Hin*dIII released these enhancer/promoter inserts, which were then cloned into the promoterless retroviral luciferase plasmid pKA9 (Boss et al., 1998). The upper strand enhancer sequence (in bold), copy number in vector, and references are: nuclear factor-κB (NFκB), 5′-ATC AGT TGA GG**G GAC TTT CC**C AGG C (five copies) (Sen and Baltimore, 1986); nuclear factor of activated T cells (NFAT), 5′-GAT CAG GA**G GAA AAA CTG TTT CA**T ACA GAA GGC (three copies) (Durand et al., 1988); cyclic AMP response element binding protein (CREB), 5′-GAT CAG GCT GG**T GAC GTC A**CT G**TG ATG TCA** GTG CTC (eight copies) (Molina et al., 1993); activator protein-1 (AP1), 5′-TCG AA**T GAG TCA** GCT CTA **TGA GTC A**GA TCC A**TG AGT CA**G CTC TA**T GAG TCA** G (eight copies) (Angel et al., 1987); and serum response element (SRE), 5′-GAT C**AC ACA GGA TGT CCA TAT TAG GAC ATC** T (two copies) (Price et al., 1995).

Preparation of cell lines

PC12 cells stably expressing the human α_{1A}-, α_{1B}, or α_{1D}-AR cDNAs in an isopropylthiogalactose (IPTG)-inducible expression vector (Hirasawa et al., 1993; Esbenshade et al., 1995) were used for all experiments (Williams et al., 1998; Zhong and Minneman, 1999*a–c*). Except where noted, cells were treated for 48 h with 1 m*M* IPTG to induce receptor expression. Cells were transfected further with retrovirus coding for reporters. Phoenix-producer cells (ATCC) were purchased with the permission of Dr. G. P. Nolan (Stanford University) for transient production of nonreplicating recombinant retrovirus. Infectious retroviral supernatants were generated by a helper virus-free protocol, and PC12 cells were infected as described elsewhere (Abbott et al., 2000).

Positive controls

Expression of reporters was verified by screening for responses to positive controls, using 100 n*M* phorbol 12-myristate 13-acetate (PMA) for AP1, NFκB, and SRE reporters, 100 n*M* PMA + 100 n*M* ionomycin for NFAT reporter, and 10 μM forskolin for cyclic AMP response element (CRE) reporter. Final vehicle concentrations were generally 1% dimethyl sulfoxide for PMA and 0.1% ethanol for forskolin, except for the SRE where 1% dimethyl sulfoxide was found to alter the response and the dimethyl sulfoxide concentration was reduced to 0.01%. Because of the large variability between reporters and transfections, most data were normalized as a percentage of the response caused by the appropriate positive control in that particular experiment.

Luciferase measurements

Confluent cells were incubated in serum-free medium with various stimuli for 4 h at 37°C. When inhibitors were used, they were added 30 min before addition of stimulus. Cells were then washed twice with phosphate-buffered saline and lysed in buffer containing 25 m*M* Tris (pH 7.8), 4 m*M* EGTA, 1% Triton X-100, 10% glycerol, and 2 m*M* dithiothreitol. Lysates were centrifuged briefly in a microfuge, and the supernatant containing ~50 μg of protein was used for activity measurements. Luciferase activity was determined by mixing the lysate with 0.35 ml of assay buffer containing 25 m*M* Tris (pH 7.8), 40 m*M* $MgSO_4$, 4 m*M* EGTA, 2 m*M* ATP, 1 m*M* dithiothreitol, and 100 μl of 0.75 m*M* luciferin (GibcoBRL).

$[Ca^{2+}]_i$ determinations

$[Ca^{2+}]_i$ transients were determined using fura-2 as described previously (Esbenshade et al., 1995). Confluent 100-mm plates were washed with balanced salt solution (130 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1.5 m*M* $CaCl_2$, 20 m*M* HEPES, 10 m*M* glucose, 0.1% bovine serum albumin), and cells were detached by mild trypsinization (0.25%). Cells were rinsed three or four times with balanced salt solution and stored on ice. One milliliter of cell suspension (1×10^6 cells/ml) was incubated with 1 μM fura-2/acetoxymethyl ester for 10 min at 37°C, rinsed 10 min with balanced salt solution, and diluted to 3 ml before the experiment. The cell suspension was transferred to a cuvette and placed in a Perkin–Elmer (Beaconsfield, Buckinghamshire, U.K.) LS 50B luminescence spectrofluorometer with a thermostatted (37°C) stirred cell holder. The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. Calibration of the fluorescence signals for calculation of $[Ca^{2+}]_i$ was performed by equilibrating intracellular and extracellular Ca^{2+} with 30 μM digitonin (R_{max}), followed by addition of 300 m*M* EGTA, 1 *M* Tris (pH 9.0) (R_{min}), and using a K_D of 225 n*M* for fura-2 (Grynkiewicz et al., 1985).

Differentiation

PC12 cells were grown in collagen-coated six-well plates at a starting density of 60,000 cells/ml. After induction with 1 m*M* IPTG for 2 days, agonists were added to the medium and pictures taken 48 h after agonist addition. The length of neurites

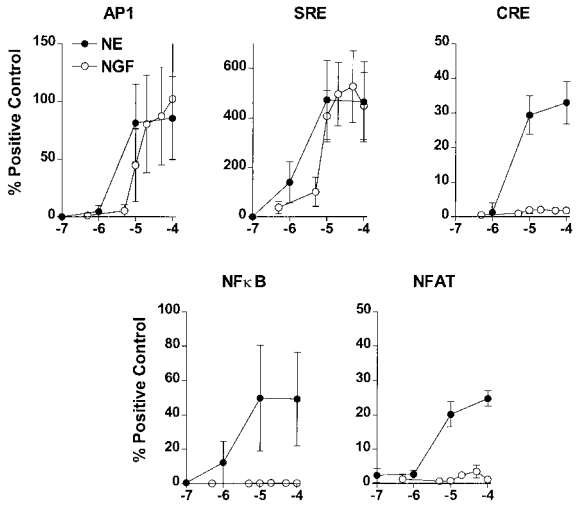

FIG. 1. Transcriptional responses to NE and NGF in transfected PC12 cells. α_{1A}-PC12 cells (subclone 3) were transfected with retroviral luciferase reporter constructs for AP1, SRE, CRE, NFκB, or NFAT as described in the text. Transfections were verified by screening for responses to positive controls using 100 n*M* PMA for AP1, NFκB, and SRE, 100 n*M* PMA + 100 n*M* ionomycin for NFAT, and 10 μM forskolin for CRE. Cells were then treated with the indicated concentrations of NE (log *M*) or NGF (log mg/ml) for 4 h and harvested, and luciferase activity was measured as described. Values are expressed as a percentage of the positive control response and are the means ± SEM of three to seven individual experiments. Fold stimulation by the positive control was 32 ± 8 (AP1), 6 ± 2 (SRE), 93 ± 46 (CRE), 113 ± 30 (NFκB), and 111 ± 27 (NFAT).

relative to the width of cell body was quantified as follows: 0, no neurite longer than 50% of cell body width; 1, longest neurite extended 50–100% of cell body width; 2, longest neurite extended 100–150% of cell body width; 3, longest neurite extended 150–200% of cell body width; 4, longest neurite extended >200% of cell body width.

RESULTS

Transfection and characterization of luciferase reporters in α_{1A}-PC12 cells

PC12 cells stably transfected with human α_{1A}-ARs under the control of an IPTG-inducible promoter (Zhong and Minneman, 1999*b*) were transfected with retroviral luciferase reporters for AP1, SRE, CRE, NFκB, or NFAT (Abbott et al., 2000). Following IPTG induction, these cells (subclone 3) express α_{1A}-ARs at a high normal density of 268 ± 10 fmol/mg of protein, and differentiate in response to either NE or NGF (Zhong and Minneman, 1999*b*). Successful reporter expression was verified by screening for luciferase activity following treatment with an appropriate positive control (100 n*M* PMA for AP1, NFκB, and SRE, 100 n*M* PMA plus 100 n*M* ionomycin for NFAT, and 10 μM forskolin for CRE). The positive controls typically caused 10–200-fold increases in luciferase activity for each of the five reporter constructs.

Transcriptional responses to NE and NGF in α_{1A}-PC12 cells

The effect of NE and NGF on each of the five transcriptional reporters expressed in α_{1A}-PC12 cells is shown in Fig. 1. Both NE and NGF strongly activated the reporters for AP1 and SRE, increasing activity to an extent equal to or substantially greater than that caused by the positive control PMA. NE also activated the reporters for CRE, NFκB, and NFAT, although this response was smaller (25–50%) than that caused by the positive controls (forskolin, CRE; PMA, NFκB; PMA plus ionomycin, NFAT). NGF did not cause measurable activation of the reporters for CRE, NFκB, or NFAT (Fig. 1).

Activation by other agonists

To rule out phenotypic variation among subclones, responses to NE, NGF, and other agonists were also examined in a different α_{1A}-PC12 cell subclone expressing a similar density of receptors (subclone 28 expressing 373 ± 10 fmol/mg of protein). Although there were quantitative differences between the magnitude of stimulation caused by NE in this subclone, Fig. 2 shows that qualitatively similar patterns of transcriptional activation were observed in both subclones. Both NE and NGF caused a large activation of the AP1 and SRE reporters in the α_{1A}-28 cells, and NE (but not NGF) activated the CRE, NFκB, and NFAT reporters. Pertussis toxin pretreatment had no effect on any of the reporter responses to NE (data not shown).

PC12 cells also contain endogenous $G_{q/11}$-coupled P2Y2 receptors, which can be activated by UTP (Soltoff et al., 1998). Activation of these receptors causes increases in intracellular Ca^{2+} that are essentially identical

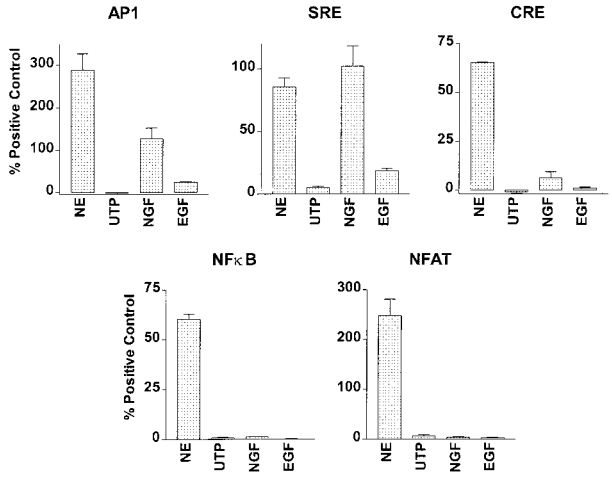

FIG. 2. Comparison of agonist-activated transcriptional responses in transfected PC12 cells. α_{1A}-PC12 cells (subclone 28) were transfected with the specified reporter constructs and screened, and luciferase responses to NE (100 μM), UTP (100 μM), NGF (100 ng/ml), or EGF (100 ng/ml) were determined. Values are expressed as a percentage of the positive control response and are the means ± SEM of three or four experiments.

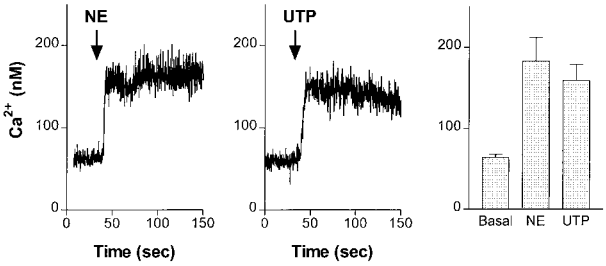

FIG. 3. Comparison of Ca^{2+} responses to NE and UTP in α_{1A}-PC12 cells. Cells were induced with IPTG for 48 h and loaded with fura-2, and intracellular Ca^{2+} responses were determined as described. Representative tracings show the effect of 100 μM NE (**left**) and 100 μM UTP (**center**). Average results from five or six experiments are plotted on the **right.**

to those caused by NE in α_{1A}-PC12 cells (Fig. 3), and causes a slightly smaller activation of MAPK (Berts et al., 1999). It is interesting that UTP did not significantly increase transcriptional activity of any of the five reporter constructs, even in cases where NE caused very large responses. PC12 cells also contain endogenous epidermal growth factor (EGF) receptors, which activate MAPK pathways in a manner similar to that caused by NGF. EGF treatment of these cells caused a pattern of responses similar to, but smaller than, that caused by NGF. EGF caused small, but significant, increases in AP1 and SRE reporter activity, but not CRE, NFκB, or NFAT reporter activity (Fig. 2).

Effect of a protein kinase C inhibitor and BAPTA

The role of protein kinase C activation and release of stored Ca^{2+} in transcriptional responses to NE was examined with specific inhibitors. Inhibition of protein kinase C by GFX203290 (1 μM; Toullec et al., 1991) completely blocked NE-mediated activation of the NFκB reporter, but caused a large potentiation of the CRE reporter response (Table 1). GFX203290 also caused a partial inhibition of the SRE responses to both NE and NGF. Addition of BAPTA to chelate intracellular Ca^{2+} did not substantially alter the pattern of inhibition caused by GFX203290 (Table 1), and pretreatment with BAPTA alone had no measurable effect on any of the responses (data not shown).

TABLE 1. *Effect of inhibitors of protein kinase C and Ca^{2+} on transcriptional responses to NE and NGF*

	% of control	
	GFX203290	GFX203290 + BAPTA
NE		
AP1	121 ± 13	141 ± 17
SRE	70 ± 8[a]	66 ± 1[a]
CRE	281 ± 10[a]	451 ± 92[a]
NFκB	5 ± 1[a]	34 ± 13[a]
NFAT	140 ± 30	244 ± 101[b]
NGF		
AP1	120 ± 19	164 ± 29[b]
SRE	50 ± 9[a]	50 ± 1[a]

Cells containing the indicated reporters were pretreated for 30 min with 1 μM GFX203290 alone or in the presence of 30 μM BAPTA before addition of NE (100 μM) or NGF (50 ng/ml) as described in the text. Cells were harvested 4 h later, and luciferase activity was determined. The effects of the inhibitors are expressed as a percentage of the response to agonist in the absence of inhibitor. Each value is the mean ± SEM of three to five observations.

[a] $p < 0.01$, [b] $p < 0.05$, compared with no inhibitor.

TABLE 2. *Effect of MAPK inhibitors on transcriptional responses to NE and NGF*

	% of control	
	PD98059	SB202190
NE		
AP1	23 ± 13[a]	39 ± 3[a]
SRE	70 ± 2[a]	31 ± 2[a]
CRE	192 ± 1[a]	64 ± 4[a]
NFκB	139 ± 12[b]	108 ± 8
NFAT	56 ± 8[a]	40 ± 3[a]
NGF		
AP1	43 ± 7[a]	98 ± 15
SRE	64 ± 6[a]	99 ± 8

Cells containing the indicated reporters were pretreated for 30 min with 20 μM PD98059 or 3 μM SB202190 before addition of NE (100 μM) or NGF (50 ng/ml). The effects of the inhibitors are expressed as a percentage of the response to agonist in the absence of inhibitor. Each value is the mean ± SEM of three to five observations.

[a] $p < 0.01$, [b] $p < 0.05$, compared with no inhibitor.

Effect of MAPK inhibitors

The role of MAPK pathways in transcriptional responses was examined with the selective MAPK kinase (MEK) inhibitor PD98059 (Pang et al., 1995) and the selective p38 MAPK inhibitor SB202190 (Cuenda et al., 1995) (Table 2). PD98059 (20 μM) caused a marked reduction in AP1 responses to both NE and NGF, a partial reduction in SRE responses to both NE and NGF, and a partial reduction in the NFAT response to NE. PD98059 also caused a significant potentiation of CRE and NFκB responses to NE (Table 2). The p38 MAPK inhibitor SB202190 showed a more interesting pattern. SB202190 (3 μM) inhibited AP1 and SRE activation by NE, but not by NGF. SB202190 also partially inhibited CRE and NFAT responses to NE, but had no effect on the NFκB response.

Concentration–response relationships for GFX203290 and PD98059

The potency and specificities of some of the drugs showing biphasic effects were examined with concentration–response relationships. Figure 4 shows that GFX203290 inhibits the NFκB response and potentiates the CRE response to NE over the same concentration range, with half-maximal effects occurring at ~0.3 μM in both cases. This is similar to the concentration of GFX203290 required to inhibit responses to PMA (Berts et al., 1999). Similarly, PD98059 inhibits the AP1 response and potentiates the CRE response to NE over similar concentration ranges, with half-maximal effects

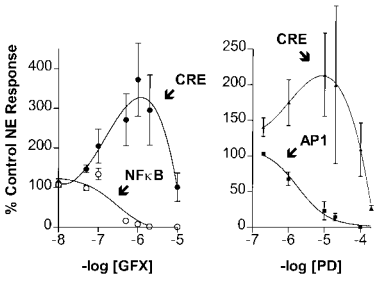

FIG. 4. Concentration–response curves for dual effects of GFX203290 (GFX) and PD98059 (PD) on different NE-stimulated transcriptional responses. α_{1A}-PC12 cells containing the indicated luciferase reporter constructs were incubated with the indicated concentrations of inhibitors for 30 min before 100 μM NE was added. After 4 h, cells were harvested and luciferase activity was measured as described. Values are expressed as a percentage of the control NE response in the absence of inhibitor and are the means ± SEM of three or four experiments.

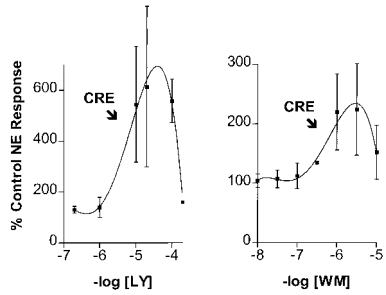

FIG. 5. Concentration–response curves for potentiation of NE-induced CRE activation by inhibitors of PI-3-kinase. α_{1A}-PC12 cells containing the CRE luciferase reporter construct were incubated with the indicated concentrations of LY294002 (LY) or wortmannin (WM) for 30 min before 100 μM NE was added. After 4 h, cells were harvested and luciferase activity was measured as described. Values are expressed as a percentage of the control NE response in the absence of inhibitor and are the means ± SEM of three or four experiments.

occurring at ~2 μM in both cases (Fig. 4). Potentiation of the CRE response to NE by both GFX203290 and PD98059 was biphasic, with substantial inhibition observed at higher (presumably nonspecific) drug concentrations.

Effect of other inhibitors

Several other compounds known to interfere specifically with particular signaling pathways were also examined. PP2 is known to potently inhibit the Src family of tyrosine kinases (Hanke et al., 1996) and was found to reduce or eliminate all transcriptional responses except CRE (Table 3). PP2 was particularly effective against the SRE responses to both NE and NGF. Cyclosporin A is a potent inhibitor of calcineurin (Liu et al., 1991) and, as expected, was very effective in reducing NE-stimulated NFAT responses, but also caused substantial inhibition of a number of other reporters (Table 3). Inhibition of phosphatidylinositol 3-kinase (PI-3-kinase) by LY294002 (Vlahos et al., 1994) and wortmannin (Powis et al., 1994) caused little or no reduction in any of the responses examined, but greatly potentiated the CRE response to NE. LY294002, but not wortmannin, also caused a smaller potentiation of the AP1 responses to both NE and NGF (Table 3).

TABLE 3. *Effect of various inhibitors on transcriptional responses to NE and NGF*

	% of control			
	PP2	Cyclosporin A	LY294002	Wortmannin
NE				
AP1	61 ± 6[a]	44 ± 3[a]	152 ± 27[b]	117 ± 9
SRE	0 ± 0[a]	76 ± 33	166 ± 17[a]	79 ± 47
CRE	157 ± 29[a]	32 ± 11[a]	664 ± 130[a]	195 ± 42[b]
NFκB	20 ± 2[a]	80 ± 36	72 ± 0.3[a]	79 ± 34
NFAT	57 ± 17[a]	2 ± 2[a]	106 ± 26	88 ± 22
NGF				
AP1	20 ± 4[a]	96 ± 30	226 ± 37[a]	144 ± 30
SRE	9 ± 5[a]	76 ± 1[a]	98 ± 3	95 ± 1

Cells containing the indicated reporters were pretreated for 30 min with 10 μM PP2, 1 μM cyclosporin A, 20 μM LY294002, or 1 μM wortmannin before addition of NE (100 μM) or NGF (50 ng/ml). The effects of the inhibitors are expressed as a percentage of the response to agonist in the absence of inhibitor. Each value is the mean ± SEM of three to five observations.

[a] $p < 0.01$, [b] $p < 0.05$, compared with no inhibitor.

Concentration–response relationships for PI-3-kinase inhibitors

Figure 5 shows the concentration dependence of potentiation of the CRE response to NE by LY294002 and wortmannin. Both drugs potentiated the CRE response over concentrations reported to be specific for inhibition of PI-3-kinase (EC_{50} values: LY294002, 3 μM; wortmannin, 0.3 μM). Again, biphasic effects on potentiation were observed, and the potentiation was lost at higher drug concentrations (Fig. 5).

Effects of inhibitors on NE- and NGF-induced PC12 cell differentiation

To determine which transcriptional events are required for PC12 cell differentiation, the effect of some inhibitors on differentiation induced by NE and NGF was examined in α_{1A}-PC12 cells. Cells were pretreated with specific inhibitors for 30 min and then exposed to NE or NGF for 2 days, and the extent of differentiation was determined morphologically. Table 4 shows that all of the inhibitors examined reduced the extent of NE-induced differentiation. These include the protein kinase C inhibitor GFX203290, the MEK inhibitor PD98059, the p38 MAPK inhibitor SB202190, and the Src inhibitor PP2. In contrast, most of these inhibitors did not significantly reduce the extent of NGF-induced differentiation. The only exception was the MEK inhibitor PD98059, which reduced NGF-induced differentiation as reported previously (Pang et al., 1995), although only by ~65% (Table 4).

TABLE 4. *Effect of inhibitors on NE- and NGF-induced PC12 cell differentiation*

	Degree of differentiation (Scale: 0–4)	
Treatment	NE	NGF
No drug	2.9 ± 0.18	3.1 ± 0.21
GFX203290 (1 μM)	1.5 ± 0.40[a]	2.5 ± 0.31
PD98059 (20 μM)	0 ± 0[a]	1.1 ± 0.21[a]
SB202190 (3 μM)	0 ± 0[a]	3.7 ± 0.23
PP2 (10 μM)	0 ± 0[a]	3.0 ± 0.24

α_{1A}-PC12 cells were grown on collagen-coated plates and induced with 1 m*M* IPTG for 2 days. Drugs were added 30 min before agonist treatment, and pictures were taken 48 h later. The length of neurites relative to the width of the cell body was quantified as follows: 0, no neurite longer than 50% of cell body width; 1, longest neurite 50–100% of cell body width; 2, longest neurite 100–150% of cell body width; 3, longest neurite 150–200% of cell body width; 4, longest neurite >200% of cell body width. Cells cultured without agonists showed no detectable differentiation. Each value is the mean ± SEM of measurements of the 15 cells in the field with the longest neurites.

[a] $p < 0.01$, compared with no drug.

Comparison of α_1-AR subtypes

To determine whether the results obtained with the α_{1A}-AR subtype were generalizable to the other α_1-AR subtypes, we transfected cell lines stably expressing each subtype at similar densities (Zhong and Minneman, 1999*b*) with each reporter construct and examined responses to NE. Figure 6 shows that the human α_{1B}- and α_{1D}-AR subtypes were less effective in activating the various reporters compared with the α_{1A}-AR subtype. In α_{1B}-PC12 cells (subclone 8), NE activated AP1 and NFAT responses almost as well as in α_{1A}-PC12 cells. However, NE caused a much smaller CRE response in α_{1B}-PC12 cells and caused no significant activation of SRE or NFκB. It is interesting that in the α_{1D}-PC12 cells (subclone 3), NE caused no significant activation of any of the reporter constructs (Fig. 6), similar to the weak second messenger responses activated by this subtype in PC12 cells (Zhong and Minneman, 1999*b*).

DISCUSSION

The recently discovered links between GPCRs and MAPK pathways has led to a growing understanding of the complex signaling networks activated by these receptors. α_1-ARs activate $G_{q/11}$ proteins, thereby activating phospholipase Cβ and generating the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. It is increasingly likely that additional signals are generated by GPCR activation, by G protein α and/or $\beta\gamma$ subunits, by activation of MAPK pathways, and/or by direct interactions of GPCRs with other proteins (Hall et al., 1998; Malbon and Karoor, 1998; Shih et al., 1999). The consequences of these interactions are still ill-defined.

We used PC12 cells stably transfected with α_1-ARs to define pathways linking these receptors to mitogenic responses. NGF acts through tyrosine kinase receptors in these cells to activate MAPK pathways and cause the cells to differentiate into a neuronal phenotype. The mechanisms involved include receptor autophosphorylation, binding of Shc and Grb2, and activation of Sos, Ras, and Raf (Davis, 1993). Stimulation of α_{1A}-ARs in PC12 cells also activates MAPK pathways and causes differentiation (Williams et al., 1998). This response involves the tyrosine kinases Pyk2 and Src (Zhong and Minneman, 1999*c*), but not increases in Ca^{2+} or activation of protein kinase C (Berts et al., 1999). Also, different α_1-AR subtypes activate different mitogenic responses (Zhong and Minneman, 1999*b*), suggesting that they may not be linearly related to $G_{q/11}$ activation.

We used a set of retroviral transcriptional reporters recently developed by Abbott et al. (2000) to compare transcriptional responses to the $G_{q/11}$-coupled α_{1A}-ARs and P2Y2 purinergic receptors, and the tyrosine kinase receptors for NGF and EGF. Each of these receptors activates MAPK pathways in PC12 cells, although only NGF and α_{1A}-ARs cause differentiation. The reporters used are based on well characterized *cis*-acting enhancer elements, designed to provide a broad survey of transcriptional coactivators that might be controlled by receptor signaling. The CRE and AP1 elements usually show preference for the CREB/activating transcription factor (ATF) and Fos/Jun families of basic leucine zipper proteins, respectively (Foletta et al., 1998), but complex patterns of heteromeric utilization are known (Hai and Curran, 1991; De Cesare et al., 1999). The SRE enhancer

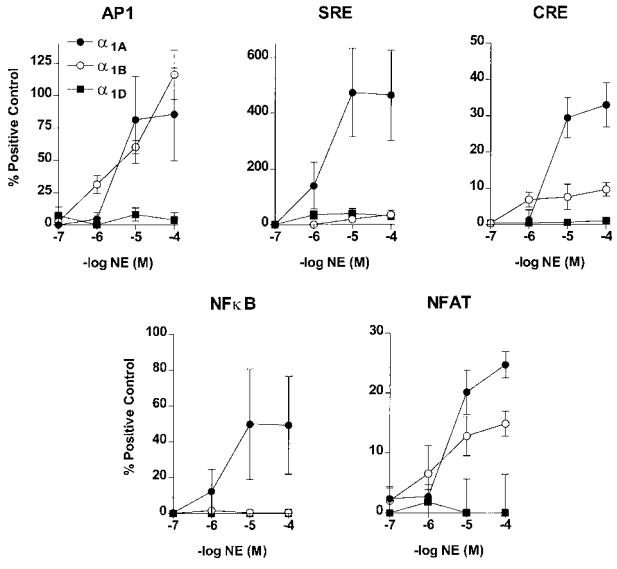

FIG. 6. Comparison of transcriptional responses to closely related α_1-AR subtypes. PC12 cells stably transfected with the human α_{1A} (subclone 3), α_{1B} (subclone 8), or α_{1D} (subclone 3)-ARs at similar densities were also transfected with the indicated transcriptional reporters. Following screening for the positive control responses, cells were treated with the indicated concentrations of NE for 4 h and harvested, and luciferase activity was determined. Values are expressed as a percentage of the positive control response and are the means ± SEM of three to six experiments.

from the c-Fos promoter assembles a coordinate complex between the serum response factor and Ets-domain proteins in several cell types (Price et al., 1995). The NFAT enhancer from the IL-2 promoter cooperatively binds the Rel-related NFAT isoforms with Fos/Jun dimers (Rao et al., 1997), whereas the NFκB enhancer from the κ-light chain gene typically shows preference for RelA (p65) and p50 NFκB subunits (Sen and Baltimore, 1986).

We found that NE activated all five reporters studied, whereas NGF activated only two. The effect of NE did not occur in the basal reporter construct with no transcriptional enhancers, suggesting that it was not a nonspecific stimulation of basal promoter activity, luciferase mRNA stability, or translation. The fact that stimulation of a GPCR causes a wider array of transcriptional responses than did stimulation of a tyrosine kinase receptor is surprising, given the traditional view of growth factor-mediated mitogenic signaling.

It was also surprising that UTP activation of P2Y2 purinergic receptors caused no reporter activation. P2Y2 receptors also couple through $G_{q/11}$ and cause increases in intracellular Ca^{2+} similar to those caused by α_{1A}-ARs. UTP also activates MAPK in PC12 cells, although this response is more transient than that caused by NE (Berts et al., 1999). This suggests that activation of $G_{q/11}$ and release of stored intracellular Ca^{2+} is not sufficient for activation of any of these reporters, although such conclusions are complicated by the possible rapid degradation of UTP, or differential desensitization of the two receptor types. As reporter assays must be conducted for hours to allow luciferase synthesis, the UTP signal may simply be too short. However, the similar Ca^{2+} responses to UTP and NE over the first few minutes suggest that this signal alone is not sufficient for reporter activation.

EGF, like NGF, activated only the AP1 and SRE reporters, consistent with the convergence of these two tyrosine kinase receptors on common mitogenic pathways. The EGF response was smaller than that to NGF or NE, consistent with the less sustained activation of MAPK pathways by EGF (Marshall, 1995), and the fact that EGF does not cause differentiation.

Several inhibitors were used to selectively block certain signaling pathways and determine whether they were involved in transcriptional responses. The inhibitors did not depress all responses nonspecifically, but caused a mixed pattern of inhibition, no effect, or potentiation. In cases where opposing effects of a drug were observed on different responses, concentration–response curves suggest that the two effects occurred over the same concentration range. In addition, some inhibitors differentially affected activation of the same reporter by NE and NGF, suggesting that the effects are specific.

Inhibition of protein kinase C and Ca^{2+} suggests that these second messenger pathways are required for NFκB and SRE activation by NE, but play an inhibitory role in CRE, NFAT, and possibly AP1 activation. It is surprising that these second messengers do not play a more prominent role in transcriptional responses to α_{1A}-AR activation, although it is consistent with our previous data showing that they play little role in MAPK activation. It is also consistent with the lack of effect of UTP on transcriptional activation discussed above. It is particularly surprising that the NFAT and CRE responses were not blocked, but were actually potentiated by these compounds, because these responses are known to require Ca^{2+} signaling. GFX203290 also reduced, but did not eliminate, the effect of NE on differentiation of α_{1A}-PC12 cells, suggesting a role for protein kinase C.

The MEK inhibitor PD98059 inhibited activation of AP1 and SRE by both NE and NGF, and reduced NFAT activation by NE. However, PD98059 potentiated CRE and NFκB activation by NE. The dual inhibition of AP1 and potentiation of CRE occurred over the range of concentrations known to inhibit MEK (Pang et al., 1995), suggesting that MEK is required for NE-stimulated AP1 activation, but that MEK activation blunts NE-stimulated CRE activation. Both NE and NGF activate extracellular signal-regulated kinases (ERKs) in α_{1A}-PC12 cells (Williams et al., 1998), consistent with a role for this MAPK pathway in these transcriptional responses. As expected, PD98059 inhibited differentiation of the cells induced by both NE and NGF, although it was more effective against NE than NGF.

NE also activates p38 MAPK in α_{1A}-PC12 cells, although NGF does not (Williams et al., 1998). It is interesting that the p38 MAPK inhibitor SB202190 markedly reduced four of the five transcriptional responses to NE, but neither of the responses to NGF. This suggests that activation of p38 MAPK plays a pivotal role in transcriptional responses to NE. This is supported by the fact that SB202190 completely blocks NE-induced differentiation of these cells, but has no effect on NGF-induced differentiation.

We showed previously that although both NE and NGF activate Src in α_{1A}-PC12 cells, the Src inhibitor PP2 only inhibits the differentiation caused by NE (Zhong and Minneman, 1999*c*). It is interesting that PP2 blocked both AP1 and SRE activation by both NE and NGF, suggesting that these responses play little role in the differentiation caused by NGF. PP2 also reduced NFκB and NFAT responses to NE, but potentiated CRE activation. The calcineurin inhibitor cyclosporin A blocked NFAT activation by NE as expected, but also reduced CRE and AP1 activation, with little or no effect on the other responses.

Finally, inhibitors of PI-3-kinase generally had few significant effects on reporter activation by NE and NGF, except for a major potentiation of the CRE response to NE at concentrations known to inhibit PI-3-kinase.

All three known α_1-AR subtypes increase inositol phosphates and intracellular Ca^{2+}, but they do so with different coupling efficiencies ($\alpha_{1A} \geq \alpha_{1B} > \alpha_{1D}$) (Theroux et al., 1996). We showed previously that α_{1A}-ARs activate all three MAPK pathways in PC12 cells, α_{1B}-ARs activate ERKs and, p38 but not c-Jun NH_2-terminal kinases, and α_{1D}-ARs only activate ERKs (Zhong and

Minneman, 1999*b*). Here we transfected these cell lines with reporter constructs to examine transcriptional responses. We found a pattern of coupling efficiency similar to that observed previously with second messenger and MAPK responses ($\alpha_{1A} \geq \alpha_{1B} > \alpha_{1D}$). α_{1A}-ARs activated all five reporter constructs, α_{1B}-ARs only three reporters, and α_{1D}-ARs did not activate any reporters. It was surprising that α_{1B}-ARs did not increase SRE activity, because α_{1A}-ARs cause such a large activation. We showed previously that α_{1B}-ARs cause almost as large an increase in inositol phosphate and Ca^{2+} as α_{1A}-ARs in PC12 cells (Zhong and Minneman, 1999*b*). This supports results obtained with the protein kinase C inhibitor and the UTP discussed above, suggesting that protein kinase C and Ca^{2+} are not sufficient for these transcriptional responses.

Complex signaling pathways shared by GPCRs and growth factor receptors may converge on immediate-early genes. Typically, the promoter regions of these genes recruit and assemble a variety of transcription factors, and the final transcriptional response reflects an integration of multiple signals (Hill and Treisman, 1995). We used the various luciferase reporters to break down promoter activation to a more manageable level, using individual enhancer elements to measure the activity of different classes of transcription factors. However, these responses still may not have a linear relationship between the receptor and a single transcription factor. For example, CREB is one of several closely related factors that can bind to a consensus CRE element, and although these proteins can function autonomously as dimers, their responsiveness is complicated further by the many different known CREB kinases (De Cesare et al., 1999). At the other extreme, SRE (Gille et al., 1996) and NFAT (Rao et al., 1997) enhancers each represent sites for combinatorial assembly of a variety of coactivator proteins, each of which is controlled by distinct patterns of signaling information. For example, NFAT is dephosphorylated by Ca^{2+} signaling and assembles with AP1 complexes that are phosphorylated by kinases such as protein kinase C and Jun kinase. Our data show clearly that substantial heterogeneity exists in how activators evoke these enhancers. It will be important to determine whether different receptors control the function of these enhancers by different transcription factors, or by regulating the same factors through different signaling pathways.

These results provide insights into the complex signaling networks activated by GPCR activation. Of particular interest is the fact that α_{1A}-AR activation in PC12 cells caused a much wider array of transcriptional responses than growth factor receptor activation. The transcriptional responses to α_{1A}-AR activation were not linearly related to second messenger generation, and could not be predicted from the known G protein coupling specificity of the receptors. As inhibitors of a variety of second messenger, MAPK, and tyrosine kinase pathways all block differentiation in response to NE, but not NGF, it appears that α_{1A}-AR-induced differentiation requires a complex interplay of signals. This supports the hypothesis that GPCR signaling pathways are not linear cascades resulting in second messenger formation and activation of specific targets, but must involve networks of interacting signals.

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