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Activation of Signal Transducers and Activators of Transcription by α_{1A}-Adrenergic Receptor Stimulation in PC12 Cells

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ABSTRACT

In PC12 cells stably expressing α_{1A}-adrenergic receptors (ARs), norepinephrine (NE) activates several mitogen-activated protein kinase pathways and causes differentiation (Williams et al., 1998). Using retroviral luciferase reporters, we found that NE also activated both signal transducers and activators of transcription (Stat) and γ-interferon-activated sequence-mediated transcriptional responses, with maximal effects similar to those caused by interleukin-6 (IL-6). UTP and epidermal growth factor had no effect, whereas nerve growth factor caused a small Stat activation. Responses to NE were blocked by prazosin and depended on receptor density. Responses to NE were not blocked by inhibitors of mitogen-activated protein kinase kinase (PD98059), protein kinase C (GFX203290), Src (PP2), Jak2 (AG490), or the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. The p38 mitogen-activated protein kinase inhibitors SB202190 and SB203580 blocked Stat activation by NE, the epidermal growth factor receptor inhibitor AG1478 caused a small inhibition, but the phosphoinositide 3 kinase inhibitor LY294002 potentiated both responses. Gel shifts confirmed formation of nuclear factors binding to both Stat and γ-interferon-activated sequence consensus sequences in response to NE and IL-6. Immunoprecipitation experiments showed that IL-6 increased tyrosine phosphorylation of Stat1 and Stat3 in PC12 cells, whereas NE caused a sustained increase in tyrosine phosphorylation of Stat1. These results suggest that α_{1A}-AR stimulation causes Stat-mediated transcriptional responses in PC12 cells that are not downstream of known second messenger or tyrosine kinase pathways.

α_1-Adrenergic receptors (ARs) are members of the G protein-coupled receptor (GPCR) superfamily. There are three closely related α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D}), all of which couple to the $G_{q/11}$ family of G proteins, thereby activating phospholipase Cβ and generating the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (Zhong and Minneman, 1999a). These second messengers release stored intracellular calcium and activate protein kinase C, respectively, mechanisms that are thought to underlie most cellular responses to α_1-ARs.

We have recently been studying the mitogenic actions of α_1-ARs stably expressed in PC12 cells. α_1-ARs, like other GPCRs, activate mitogenic responses in many cells, and play important roles in regulating growth and proliferation (Zhong and Minneman, 1999a). In rat PC12 cells transfected with α_{1A}-ARs, norepinephrine (NE) activates a variety of 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, 1999b). These mitogenic responses appear to be independent of inositol phosphate and calcium production (Berts et al., 1999), but are associated with activation of a variety of tyrosine kinases, particularly Pyk 2 and Src (Zhong and Minneman, 1999c).

Because α_{1A}-ARs also activate growth factor-signaling pathways, there is a possibility that these receptors might activate other signaling pathways. Cytokine receptors, including those for interleukins, interferons, and other polypeptides, are known to activate signal transducers and activators of transcription (Stats). Ligand-induced receptor dimerization results in binding of Janus tyrosine kinases and direct phosphorylation of Stats on a single tyrosine residue (Darnell, 1997). The Stats then dimerize and are translocated to the nucleus, where they bind to specific consensus DNA sequences and alter transcription. Growth factor recep-

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ABBREVIATIONS: AR, adrenergic receptor; GPCR, G protein-coupled receptor; NE, norepinephrine; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; Stats, signal transducers and activators of transcription; EGF, epidermal growth factor; IL-6, interleukin 6; IPTG, isopropyl β-D-thiogalactoside; GAS, γ-interferon activated sequence; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.

tors with intrinsic tyrosine kinase activity, such as the receptor for epidermal growth factor (EGF), also have been shown to cause Stat activation in some instances (Silvennoinen et al., 1993; Leaman et al., 1996). A few GPCRs, particularly the AT_1 receptor for angiotensin II, also have been reported to activate Stat pathways in a variety of cells (Bhat et al., 1994; Marrero et al., 1995, 1997; Peeler et al., 1996). The mechanisms underlying this activation are controversial, but may be due to a direct association of the GPCR with Jak2 (Marrero et al., 1995, 1997; Ali et al., 1997).

Due to the variety of mitogenic and transcriptional responses observed after α_{1A}-AR activation in transfected PC12 cells (Williams et al., 1998; Zhong and Minneman, 1999a,b), we wondered whether these receptors also might activate Stats. Herein, we use a variety of approaches, including luciferase reporter constructs, gel shifts, and immunoprecipitation, to demonstrate Stat activation by α_{1A}-AR stimulation in PC12 cells. Comparison with growth factors and interleukin-6 (IL-6), and use of specific inhibitors, suggests that this response is not downstream of known second messenger pathways, release of growth factors, and/or release of IL-6, but may involve p38 MAPK activation.

Experimental Procedures

Materials

Materials were obtained from the following sources: [γ-^{32}P]ATP (Amersham, Arlington Heights, IL); T4 polynucleotide kinase (New England Biolabs, Beverly, MA); poly(dI-dC) (Pharmacia, Piscataway, NJ); AG1478, AG490, SB203580, GFX203290, PP2, PD98059, and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-AM (Calbiochem, La Jolla, CA); (−)-NE bitartrate, Dulbecco's modified Eagle's medium, penicillin, streptomycin, and LY294002 (Sigma Chemical Co., St. Louis, MO); antiphosphotyrosine antibody (P-Y 99), anti-Stat antibodies, and anti-Jak2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA); enhanced chemiluminescence reagent (Amersham); and horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad, Hercules, CA).

Methods

Cell Culture. PC12 cells were propagated in 75-cm^2 flasks 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 mM HEPES, 100 mg/l streptomycin, 10^5 U/l penicillin, 10% donor horse serum, and 5% fetal bovine serum (Williams et al., 1998). Cells were detached by gentle trituration and subcultured at a ratio of 1:3 on reaching confluency. Cells were grown to confluency before use.

Preparation of Cell Lines. PC12 cells stably expressing the human α_{1A}-AR cDNA in an isopropyl β-D-thiogalactoside (IPTG)-inducible expression vector (Hirasawa et al., 1993; Esbenshade et al., 1995) were used for all experiments (Williams et al., 1998). Except where noted, cells were treated at 24 h with 1 mM IPTG to induce receptor expression. Subclone 28 characterized previously (Zhong and Minneman, 1999b) was further transfected with retrovirus coding for reporters. Sequences consisted of concatemers of Stat (two copies of GATCCAG**TTCCGGGAA**TCA; Ihle, 1996) or γ-interferon-activated sequence (GAS; four copies of GATCA**GCCTGATTTC-CCCGAAATGACGGCA**CG; Sims et al., 1993) consensus-binding sequences (in bold) upstream of a minimal human IL-2 gene promoter cloned into the promoterless retroviral luciferase plasmid pKA9 (Boss et al., 1998; Abbott et al., 2000). Phoenix-producer cells (American Type Culture Collection, Manassas, VA) 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 infected as described in Abbott et al. (2000).

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 PBS and lysed in buffer containing 25 mM Tris (pH 7.8), 4 mM EGTA, 1% Triton X-100, 10% glycerol, and 2 mM dithiothreitol (DTT). 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 mM Tris (pH 7.8), 40 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, and 1 mM DTT, and 100 μl of 0.75 mM luciferin (Life Technologies, Grand Island, NY).

Gel Shifts. Nuclear extracts were prepared by serum starving confluent PC12 cells and treating with the indicated compounds for 4 h. After aspiration of the medium, cells were suspended in 50 ml of ice-cold PBS. After centrifuging for 10 min at 2000*g*, buffer was aspirated and cells suspended in 0.5 ml of ice-cold buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.15 mM $MgCl_2$, 0.5 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Cells were allowed to swell on ice for 10 min, homogenized with a Kontes glass homogenizer (10 strokes), and centrifuged at 3750 rpm at 4°C for 15 min. The pellet was resuspended in 400 μl of buffer C [20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5

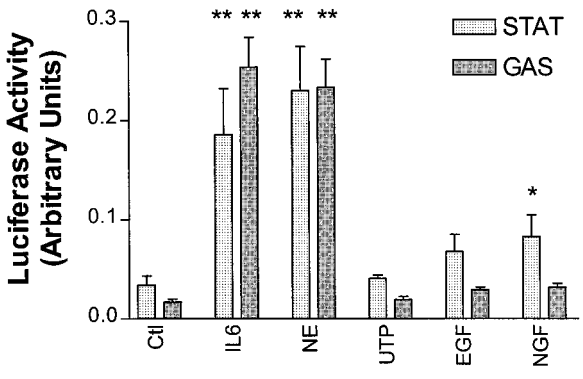

Fig. 1. Agonist-stimulated luciferase activity in α_{1A}-PC12 cells containing Stat or GAS luciferase reporters. NE (100 μM), UTP (100 μM), IL-6 (100 ng/ml), NGF (50 ng/ml), or EGF (50 ng/ml) were added to cells 4 h before harvesting for luciferase measurements. Data are the mean ± S.E. of three to four experiments performed in duplicate. $^{**}P < .001$; $^{*}P < .05$ compared with control (Ctl).

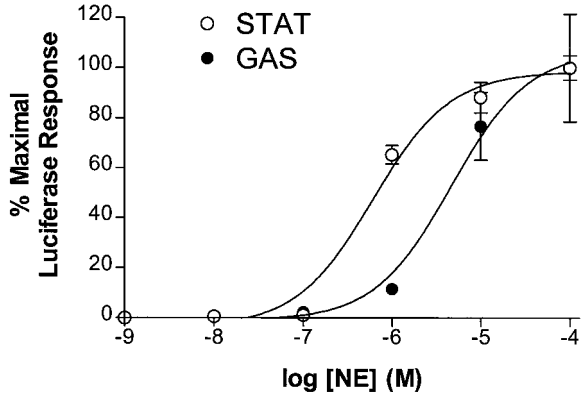

Fig. 2. Dose-response relationship for NE-stimulated luciferase activity in Stat- or GAS reporter-transfected α_{1A}-PC12 cells. Cells were treated with the indicated concentrations of NE for 4 h, harvested, and luciferase activity measured as described. Data are the mean ± S.E. of three experiments performed in duplicate and are normalized to the maximal luciferase response observed with NE.

mM DTT, and 0.2 mM PMSF), incubated with gentle rocking at 4°C for 30 min, and centrifuged at 19,000 rpm for 30 min at 4°C. The supernatant, the nuclear extract, was dialyzed against buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) for 4 to 5 h at 4°C. Samples were then microfuged at 4°C, and the supernatant was aliquoted and frozen at −70°C. For the gel shifts, ~10 μg of nuclear extract was incubated at 37° for 15 min in a 30-μl mixture containing 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 μg of poly(dI-dC), and 0.4 ng of labeled oligonucleotide. In some cases, a 100-fold excess of unlabeled oligonucleotide was added 5 min before addition of radiolabeled oligonucleotide. The unbound oligonucleotides and protein-DNA complexes were separated on a 5% polyacrylamide gel in 1× TBE at a constant 300 V. The gels were dried at 80°C for 1 h and exposed to Kodak X-Omat AR-5 at −70°C.

Immunoprecipitation. Confluent cells were serum starved for 2 h before further treatment. Cells were treated as indicated, washed twice with ice-cold PBS containing 1 mM sodium orthovanadate, and lysed on ice with RIPA lysis buffer (1% Nonidet P-40, 25 mM HEPES, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM PMSF, 1 mM NaVanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysate was centrifuged at 10,000 rpm for 15 min at 4°C in a microfuge. The supernatant containing 1 mg of protein was incubated with 10 μg of antiphosphotyrosine antibody at 4°C for 2 h followed by addition of 20 μl of protein A-agarose. After overnight incubation at 4°C, the sample was centrifuged and the immunoprecipitates washed three times with lysis buffer. After boiling in 30 μl of 2× SDS-sample buffer, 15 μl of supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Protein bands were detected by probing sequentially with the primary antibody, horseradish peroxidase-conjugated secondary antibody (1:5000), and enhanced chemiluminescence reagent.

Measurement of p38 MAPK Activity. Stat reporter transfected α_{1A}-PC12 cells were serum starved for 2 h, exposed to 100 μM NE for 15 min, and lysed. p38 MAPK activity was measured with the New England Biolabs assay kit in the presence or absence of various concentrations of SB202190. This method relies on immunoprecipitation of p38 MAPK with a phosphospecific antibody and then measurement of phosphorylation of activating transcription factor-2 in vitro, which is detected by Western blotting.

Results

Activation of Stat and GAS Reporters by Agonists in α_{1A}-AR-Transfected PC12 Cells. Retroviral luciferase reporter constructs consisted of concatemers of consensus-binding sequences for Stats (2×) or GAS (4×) upstream of a minimal IL-2 promoter, followed by the coding sequence for firefly luciferase. α_{1A}-PC12 cells were infected with retroviral reporters for either Stats or GAS and screened for responses to the cytokine IL-6. Figure 1 shows that IL-6 (100 ng/ml) caused a 5- to 15-fold increase in luciferase activity in α_{1A}-PC12 cells harboring either the Stat or GAS reporter constructs. NE (100 μM) also increased luciferase activity to a similar extent in both cell lines. However, the purinergic agonist UTP, which binds to endogenous P2Y2 receptors and also activates $G_{q/11}$-mediated responses in PC12 cells (Soltoff et al., 1998; Berts et al., 1999), had no measurable effect on luciferase activity. Likewise, activation of endogenous EGF receptors did not significantly activate either Stat- or GAS-mediated transcriptional responses, whereas NGF (50 ng/ml) caused a small 2.5-fold increase in Stat- but not GAS-mediated reporter activity (Fig. 1).

Characterization of NE-Mediated Transcriptional Activation. Concentration-response curves for NE in activating both Stat- and GAS-mediated transcriptional responses are shown in Fig. 2. The EC_{50} for NE was ~600 nM for activating the Stat reporter and ~4700 nM for activating the GAS reporter (Fig. 2). Both Stat- and GAS-mediated transcriptional responses to NE (10 μM) were blocked by the selective α_1-AR antagonist prazosin (1 μM; Fig. 3), but not by the β-AR antagonist l-propranolol (1 μM; data not shown). We also took advantage of the IPTG-inducible expression of α_{1A}-ARs in these cells (Zhong and Minneman, 1999b) to demonstrate that both transcriptional responses are dependent on receptor density. Figure 4 shows that NE had no significant effect on the activity of either reporter in the absence of IPTG when cells express a low density of α_{1A}-ARs (27 ± 5.2 fmol/mg protein). However, NE caused a 5- to 9-fold increase in luciferase expression from both reporters after a 48-h exposure to 1 mM IPTG when cells express a much higher α_{1A}-AR density (373 ± 10.4 fmol/mg protein). Intermediate responses were observed after exposure to lower concentrations of IPTG (data not shown).

Effect of Inhibitors on NE-Mediated Luciferase Expression. To begin to define the mechanism by which NE activates Stat- and GAS-mediated transcriptional responses,

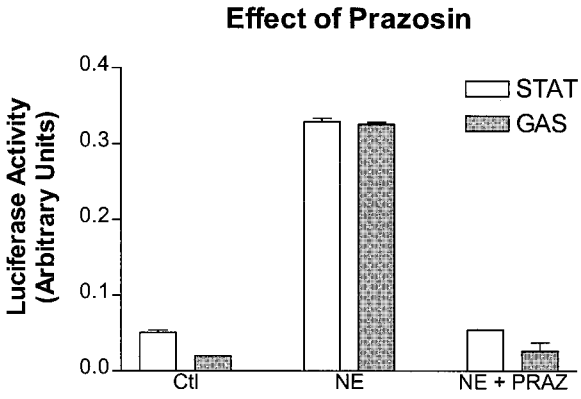

Fig. 3. Effect of prazosin on NE-stimulated luciferase activity in Stat- or GAS-transfected α_{1A}-PC12 cells. Cells were treated with or without prazosin (1 μM; PRAZ) for 30 min before addition of 10 μM NE for 4 h before harvesting. Data are the mean ± S.E. of four observations.

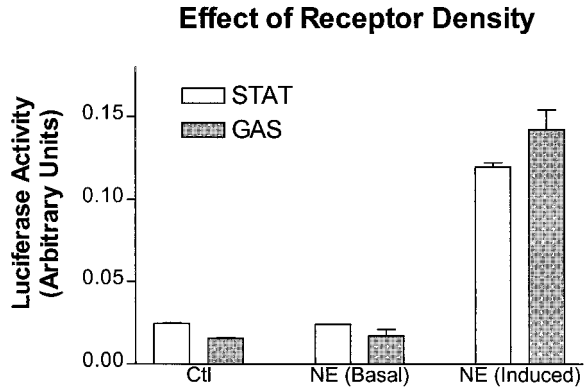

Fig. 4. Effect of increasing receptor expression on NE-stimulated luciferase activity in Stat- or GAS reporter-transfected α_{1A}-PC12 cells. Cells were treated with (induced) or without (basal) 1 mM IPTG for 48 h, and then treated with or without 100 μM NE for 4 h before harvesting and measuring luciferase activity. Receptor density was measured by radioligand binding and was 27 ± 5.2 and 373 ± 10.4 fmol/mg protein in the absence and presence of IPTG, respectively. Data are the mean ± S.E. of four observations.

a variety of specific inhibitors were examined at concentrations reported to be maximal and specific. Table 1 shows that neither the protein kinase C inhibitor GFX203290 (Toullec et al., 1991; 1 μM) nor the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (50 μM) had any effect on either Stat- or GAS-mediated transcriptional activation by NE. However, the p38 MAPK inhibitor SB203580 (Cuenda et al., 1995; 10 μM) caused a 71 ± 8% inhibition of the Stat response to NE but had no effect on the GAS response. The MAPK kinase inhibitor PD98059 (Pang et al., 1995; 10 μM) had no effect on the NE-activated Stat response, but potentiated NE-activated GAS transcription by 57 ± 35%. The selective inhibitor of EGF receptor tyrosine kinase activity AG1478 (Levitzki and Gazit, 1995; 500 nM) caused a small but significant inhibition of the NE response for both the Stat and GAS reporters. Neither the Src inhibitor PP2 (Hanke et al., 1996; 10 μM) nor the Jak2 (Meydan et al., 1996) and EGF receptor (Gazit et al., 1991) inhibitor AG490 (20 μM) significantly affected NE activation of either reporter. However, the phosphoinositide 3 kinase inhibitor LY294002 (Vlahos et al., 1994; 10 μM) significantly increased both Stat and GAS responses to NE (Table 1).

Concentration-response relationships for inhibition of NE-stimulated luciferase activity in Stat reporter-transfected cells were determined for the related p38 MAPK inhibitors SB202190 and SB 203580 (Cuenda et al., 1995). Figure 5 shows that both inhibitors blocked the NE response with potencies in the submicromolar range, and that SB202190 was slightly more potent than SB203580, as expected for inhibition of p38 MAPK. Figure 5 (bottom) demonstrates the potency of SB202190 in directly inhibiting p38 MAPK activity from these cells.

Gel Shifts. Gel shifts were performed to verify the results with the reporter constructs. Nuclear extracts were prepared from α_{1A}-PC12 cells after various treatments and incubated with ^{32}P-labeled oligonucleotides containing Stat or GAS consensus sequences. Unbound oligonucleotides and protein-DNA complexes were separated by electrophoresis and exposed to film. Figure 6 shows that treatment with either NE or IL-6 induces formation or translocation of nuclear factors that bind specifically to Stat and GAS consensus sequences. With this approach, the Stat response to IL-6 is greater than that to NE, whereas the GAS response to NE is greater than that to IL-6 (Fig. 6). In all cases, the signal is reduced by the presence of unlabeled competing oligonucleotide (Fig. 6). Other experiments showed that NE stimulation of nuclear protein binding to both Stat and GAS sequences was dependent on IPTG induction (data not shown).

Tyrosine Phosphorylation. Tyrosine phosphorylation of Stat1, 2, and 3 were examined by immunoprecipitation and Western blotting. After treatment, α_{1A}PC12 cells were lysed, and tyrosine phosphorylated proteins immunoprecipitated with an antiphosphotyrosine antibody. Precipitated proteins were separated on SDS-PAGE, transferred, and blotted with antibodies specific for Stat1, Stat2, or Stat3. In the experiment shown in Fig. 7, it is clear that NE and IL-6 increased tyrosine phosphorylation of Stat1, whereas IL-6 but not NE increased tyrosine phosphorylation of Stat3. However, longer exposure of the blots and other experiments also showed significant tyrosine phosphorylation of Stat3 in response to NE (data not shown). NGF treatment had no effect on either Stat1 or Stat3 (Fig. 7), despite the small increase in reporter activity observed in Fig. 1. UTP and EGF did not increase tyrosine phosphorylation of Stat1 or Stat3 (data not shown). No tyrosine phosphorylation of Stat2 was observed in response to any of the agonists tested (data not shown). The time course of NE stimulation of Stat1 phosphorylation in PC12 cells is shown in the lower part of Fig. 7. It is clear that this response occurs within 2 min, and is sustained for at least 20 min. Finally, Stat1 phosphorylation in response to NE was blocked by pretreatment with 10 μM SB202190, whereas Stat1 phosphorylation in response to IL-6 was unaffected by this treatment (data not shown).

TABLE 1

Effect of inhibitors on NE-stimulated luciferase activity in Stat or GAS reporter transfected α_{1A}-PC12 cells

Cells were induced with 1 mM IPTG for 48 hours, treated with inhibitors for 30 min, and then treated with 100 μM NE for 4 h in the absence of serum. Each value is the mean ± S.E. of four to eight separate observations.

Drug	%NE Response	
	STAT	GAS
1 μM GFX	111 ± 14	114 ± 19
50 μM BAPTA	119 ± 10	149 ± 12
500 nM AG1478	59 ± 1**	75 ± 8*
10 μM PP2	99 ± 4	95 ± 9
20 μM AG490	126 ± 1	148 ± 33
10 μM LY	292 ± 62**	210 ± 12**
10 μM PD98059	105 ± 18	157 ± 35**
10 μM SB202190	26 ± 6**	110 ± 15

*P < .05; **P < .01 compared with NE alone.

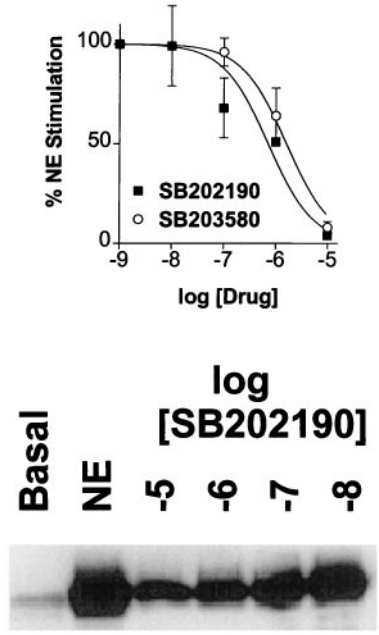

Fig. 5. Effect of p38 MAPK inhibitors SB202190 and SB 203580. Top, concentration-response curve for inhibition of NE-stimulated luciferase activity in Stat reporter-transfected α_{1A}-PC12 cells by p38 MAPK inhibitors. Cells were serum starved for 2 h and treated with the indicated concentration of inhibitor 30 min before adding 100 μM NE. Incubations were terminated after 4 h. Each point is the mean ± S.E. of four independent observations. Bottom, activation of p38 MAPK by NE in Stat-transfected α_{1A}-PC12 cells and its inhibition by SB202190. Cells were serum starved for 2 h and treated with or without 100 μM NE for 15 min. Cells were then lysed, p38 MAPK was immunoprecipitated with a phospho-specific antibody, and activity measured by phosphorylation of activating transcription factor-2 in the presence of the indicated concentrations of inhibitor as described in "Methods." The experiment shown is representative of two similar experiments.

Discussion

Stats are transcription factors activated by cytokine receptors. After receptor activation, Stats are tyrosine phosphorylated, dimerize, and translocate to the nucleus where they bind to specific DNA target sequences. Tyrosine kinase growth factor receptors also have been shown to cause Stat activation, possibly by direct tyrosine phosphorylation.

A few GPCRs also have been found to activate Jak/Stat pathways. The angiotensin AT_1 receptor has been shown to associate with Jak2 and cause Stat activation in vascular smooth muscle cells and cardiomyocytes (Bhat et al., 1994; Marrero et al., 1995, 1997; McWhinney et al., 1997). This effect appears to involve direct interaction of Jak2 with the carboxy tail of the AT_1 receptor through a YIPP motif (Ali et al., 1997). Other GPCRs, including endothelin, thrombin, and serotonin ($5HT_{2A}$), also have been reported to activate Jak/Stat pathways (Peeler et al., 1996; Bhat et al., 1997; Guillet-Deniau et al., 1997), although direct association of these receptors with Jak/Stat molecules is controversial. Interestingly, all of these receptors, like α_1-ARs, couple to $G_{q/11}$ and play an important role in smooth muscle growth.

Our results suggest that stimulation of α_{1A}-ARs leads to activation of Stat-mediated transcription in PC12 cells. We used luciferase reporters containing either the Stat or GAS consensus sequences, and found that both IL-6 and NE caused a large activation of transcription from both reporters. This effect was not observed with the endogenous P2Y2 purinergic receptors in these cells, which couple through $G_{q/11}$ to activate inositol phosphate formation and increase intracellular calcium (Soltoff et al., 1998). Activation of these receptors with UTP did not increase activity of either reporter. EGF also did not affect either reporter in our cells, whereas NGF caused a small activation of the Stat but not the GAS reporter.

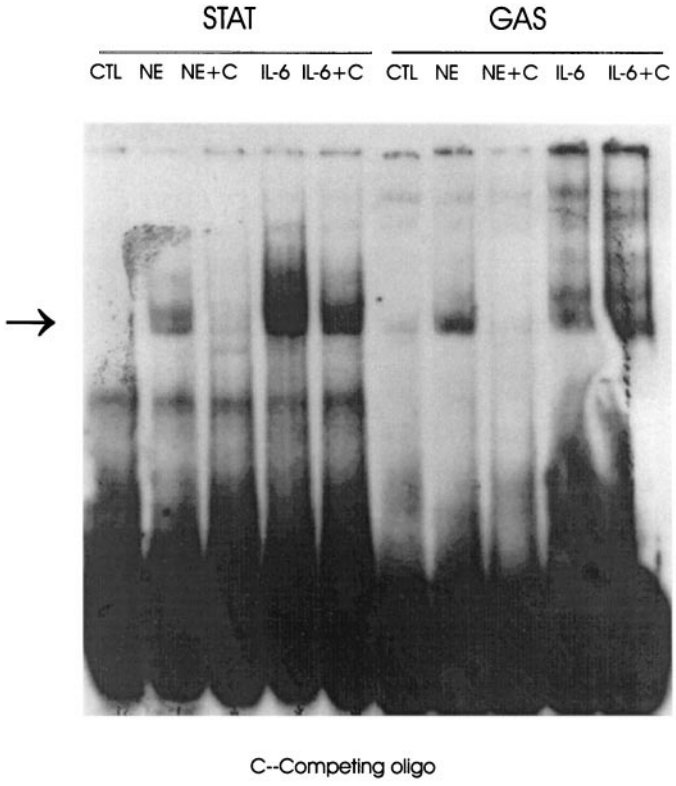

Fig. 6. Gel shifts. α_{1A}-PC12 cells were treated with or without 100 μM NE or 100 ng/ml IL-6 for 4 h before isolating nuclear extracts. Nuclear extracts containing 10 μg of protein were incubated with oligonucleotides containing consensus sequences for Stat (GATCCAGTTCCGGGAATCA) or GAS (GATCAGCCTGATTTCCCCGAAATGACGGCACG) for 15 min at 37°C, separated on a gel, and autoradiographs performed. In some experiments, unlabeled competing oligonucleotide (C) was added to demonstrate specificity of the interaction. The experiment shown is representative of three similar experiments.

Activation of both Stat and GAS reporters by NE was clearly due to α_1-AR activation. Both responses were blocked by low concentrations of prazosin and were dependent on induction of receptor expression. However, NE was more potent in activating the Stat than the GAS reporter, possibly because the GAS reporter may bind multiple transcription factors, including Stats, nuclear factor-κB, and cAMP response element-binding protein (Sims et al., 1993; Abbott et al., 2000), and may require the concerted activity of multiple transcription factors. Activation of the Stat reporter should require only Stat activation, which may explain the greater potency of NE.

The traditional second messengers mediating α_1-AR activation, inositol-1,4,5-trisphosphate and diacylglycerol, do not appear to be involved in activation of either of the reporters. UTP, which causes similar second messenger responses as NE in PC12 cells (Berts et al., 1999), causes no detectable activation of either reporter, and inhibitors of protein kinase C or increases in intracellular calcium did not block the responses. We have shown that these inhibitors are effective and specific in PC12 cells (Berts et al., 1999).

Activation of extracellular signal-regulated kinases (ERKs) has been reported to reduce Stat activation. Chung et al. (1997) showed that serine phosphorylation of Stat3 by ERK-dependent pathways negatively modulated its tyrosine phosphorylation. Sengupta et al. (1998) showed that ERK activation inhibited IL-6-induced Jak-Stat signaling in a rapid and inducible manner. We found that the MAPK kinase inhibitor PD98059 either had no effect or potentiated NE-activated responses. Because NE strongly activates

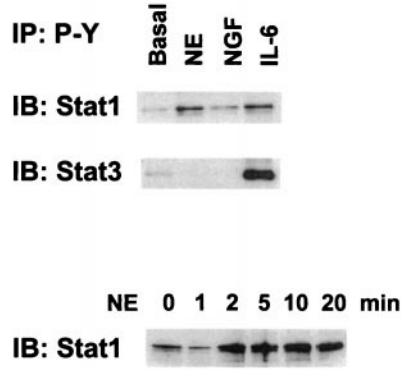

Fig. 7. Tyrosine phosphorylation of Stat proteins demonstrated by immunoprecipitation. α_{1A}-PC12 cells were induced with 1 mM IPTG for 48 h. Top, cells were serum starved for 2 h before stimulation with NE (100 μM), NGF (50 ng/ml), or IL-6 (100 ng/ml) for 5 min. Cells were lysed and 0.8 mg of total protein was mixed with 40 μl of an antiphosphotyrosine (PY-99) antibody-agarose conjugate and incubated overnight at 4°C. The immunoprecipitates (IP) were eluted and run on SDS-PAGE, and immunoblotted (IB) with anti-Stat1 or anti-Stat3 antibodies. The experiment shown is representative of three different experiments. Bottom, time course of phosphorylation of Stat1 in response to NE. Cells were serum starved for 2 h, stimulated with 100 μM NE for the times indicated, and immunoprecipitated as described above. After SDS-PAGE, blots were incubated with anti-Stat1 antibody.

ERKs in α_{1A}-PC12 cells (Williams et al., 1998) this is partly consistent with the inhibitory effects on Stat activation. It also suggests that the net effect of NE may be a balance between stimulatory and inhibitory pathways.

Activation of tyrosine kinase receptors also appears to play little role in the stimulation of Stat and GAS reporters by NE. Although these receptors often directly or indirectly activate Stats (Leaman et al., 1996), we found no activation by EGF in our cells. In addition, the EGF receptor inhibitor AG1478 caused only a small reduction in transcriptional activation by NE. The Jak2 inhibitor AG490 also inhibits EGF receptor activation (Gazit et al., 1991), and this compound had no effect on NE-mediated activation of either reporter, suggesting that EGF receptor transactivation is not involved in the NE responses. The selective Src inhibitor PP2 also did not affect NE-mediated reporter activation, suggesting that this kinase does not play a critical role.

The p38 MAPK inhibitors SB202190 and SB203580 were found to greatly diminish activation of the Stat reporter by NE over concentrations similar to those necessary to inhibit p38 MAPK. This suggests that p38 MAPK is involved in Stat activation by NE, although NE stimulation of the GAS reporter was not affected by these inhibitors. There is one report suggesting that p38 MAPK is involved in IL-6-induced transcriptional activation of Stat3 (Zauberman et al., 1999), however this area clearly needs further investigation.

Phosphoinositide 3 kinase directly interacts with Jak/Stat signaling pathways in a complex manner that depends on cell phenotype (de Groot et al., 1998). We found that the phosphoinositide 3 kinase inhibitor LY294002 strongly potentiated activation of both Stat and GAS reporters by NE. α_1-ARs, like other GPCRs, can activate phosphoinositide 3 kinase in some cells (Hu et al., 1996). Although we have not directly studied this in PC12 cells, the potentiating effect of LY294002 on NE-mediated Stat and GAS reporter activity may suggest that such an activation may be occurring, thereby inhibiting Stat and GAS reporter activity. The large size of the potentiation suggests that it may play an important modulatory role.

The mechanisms underlying α_1-AR stimulated Stat transcriptional activity in PC12 cells are likely to be complex. α_1-AR activation causes a wide range of responses, including activation of $G_{q/11}$, production of inositol phosphates and diacylglycerol, activation of protein kinase C, activation of ERKs, c-Jun NH_2-terminal kinases and p38 MAPKs, activation of a variety of tyrosine kinases, and cell differentiation (Williams et al., 1998). Interactions between pathways potentiating Stat activation (such as p38 MAPK activation) and pathways inhibiting Stat activation (such as ERK and phosphoinositide 3 kinase activation) will be critical to understanding the mechanisms involved. Although the use of specific inhibitors can give useful information, additional experiments will be required to elucidate these pathways.

Gel shifts were used to confirm that NE stimulated the binding of nuclear proteins to Stat and GAS DNA consensus sequences. Strong signals were observed in response to both NE and IL-6, consistent with the results from the luciferase reporter constructs. Interestingly, the Stat response in the gel shift assays was greater for IL-6 than NE, whereas the GAS response was greater for NE than IL-6. The reason for these differences is not clear because both reporter constructs showed similar responses to NE and IL-6.

Similarly, Western blots were used to confirm that Stat1 (and sometimes Stat3) is tyrosine phosphorylated in response to NE in α_{1A}-PC12 cells. The different patterns of phosphorylation caused by NE and IL-6 suggest that the response to NE is probably not due to local formation and release of IL-6. Stats 1 and 3 are commonly activated in response to a variety of stimuli (Darnell, 1997), particularly growth factors. Tyrosine phosphorylation of Stat1 persisted for at least 20 min after NE exposure, consistent with the sustained response observed with the gel shift protocol.

In summary, it is clear from a variety of approaches that α_{1A}-ARs activate Stat-mediated transcriptional responses in PC12 cells. These responses occur at physiological receptor densities but do not appear to be downstream of known second messengers. p38 MAPK appears to play an as-yet-undefined role in stimulation of Stat-, but not GAS-mediated transcription, and these responses may be blunted by concurrent activation of ERKs and phosphoinositide 3 kinase. This extends the already large range of signals known to be activated in response to α_1-AR stimulation to those normally involved in cytokine signaling, and provides further evidence for complex and interconnected signaling networks activated by GPCRs.

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