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A Role of p75 in NGF-Mediated Down-Regulation of the A_{2A} Adenosine Receptors in PC12 Cells

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ABSTRACT

Nerve growth factor (NGF) induces differentiation of the rat pheochromocytoma clone (PC12) by activating the high affinity receptor, $p140^{trkA}$, linked to mitogen-activated protein kinase. While the physiological role of the low affinity NGF receptor (p75) has not been clearly defined, this receptor promotes activation of nuclear factor (NF) κB in Schwann cells. PC12 cells express the A_{2A} adenosine receptor (AR), whose expression is significantly decreased by NGF treatment. In this study, we determined whether TrkA or p75 is involved in NGF-mediated regulation of A_{2A}AR expression. NGF treatment decreased A_{2A}AR in a time-dependent manner, with maximal effects observed by 1 day, and continued down-regulation of the receptor for up to 3 days in the presence of NGF. The decrease in A_{2A}AR was associated with a more delayed decrease in the steady-state levels of the A_{2A}AR mRNA. Down-regulation of the A_{2A}AR at 1 day was mimicked by activators of NFκB, such as H_2O_2, and ceramide, and was attenuated by the inhibitor pyrrolidine dithiocarbamate or following transient transfection of PC12 cells with a dominant negative IκBα mutant. Moreover, NGF stimulated nuclear accumulation of p65 subunits of NFκB (but not p50 subunits) in PC12 cells, as determined by electrophoretic mobility shift assays and by Western blotting. In contrast, inhibition of TrkA by AG879 or of TrkA-dependent mitogen-activated protein kinase mitogen-activated protein kinase kinase with PD98059 blocked PC12 cell differentiation without affecting A_{2A}AR down-regulation, suggesting dissociation between these two phenomena. Taken together, these data provide strong support for the involvement of the p75/NFκB pathway in NGF-mediated down-regulation of A_{2A}AR in PC12 cells.

Adenosine mediates a number of its physiological effects by activating adenosine receptor (AR) subtypes. Four AR subtypes, namely A_1, A_{2A}, A_{2B}, and A_3AR, have been identified by molecular cloning to date. The A_{2A}AR activates adenylyl cyclase via the guanine nucleotide stimulatory protein, G_s (Tucker and Linden, 1993). Increases in intracellular cyclic AMP levels via A_{2A}AR contribute to different actions of adenosine such as vasodilation, inhibition of platelet aggregation, and augmentation of the actions of thyrotropin on thyroid cells (Maenhaut et al., 1990; Tucker and Linden, 1993). High concentrations of A_{2A}AR are present in the striatum, where they appear to inhibit dopaminergic neurotransmission (Ferre et al., 1991). As such, blockade of A_{2A}AR functions in the striatum using selective antagonists could facilitate dopaminergic neurotransmission and prove beneficial in patients with Parkinson's disease (Ferre et al., 1992).

The rat pheochromocytoma (PC12) cell line has widely been used as a model of neuronal differentiation, survival, and apoptosis, in addition to serving as a neuronal clone for studying A_{2A}AR (Noronha-Blob et al., 1986; Nanoff et al., 1991). Activation of A_{2A}AR in PC12 stimulates adenylyl cyclase (Guroff et al., 1981; Noronha-Blob et al., 1986) and promotes the outgrowth of neurites (Guroff et al., 1981). Exposure of these cells to NGF induces differentiation into sympathetic-like neurons (Greene and Tischler, 1976). NGF interacts with a high affinity receptor, TrkA, and a low affinity receptor termed p75.

Activation of TrkA stimulates tyrosine kinase activity intrinsic to this receptor, leading to activation of downstream effectors such as Ras, mitogen-activated protein (MAP) kinase pathway, phospholipase C-γ, phosphotidylinositol-3-kinase, Akt, and *suc*-associated neurotrophic factor-induced tyrosine phosphorylated target (Kaplan and Miller, 1997). In contrast, it has been suggested that p75 enhances the affinity of TrkA for NGF through direct physical interaction between these two receptors (Bothwell, 1996). However, actions of p75 independent of TrkA include ceramide production in T9 gli-

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ABBREVIATIONS: A_{2A}AR, A_{2A} adenosine receptor; NGF, nerve growth factor; EGF, epidermal growth factor; NFκB, nuclear factor κB; PDTC, pyrrolidine dithiocarbamate; MAP, mitogen-activated protein; CGS21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol; AG 879, [α-cyano-(3,5-di-*t*-butyl-4-hydroxy)thiocinnamide]; PD98059, 2′-amino-3′-methoxyflavone.

oma cells via the sphingomyelin pathway (Dobrowsky et al., 1994) and activation of NFκB (Carter et al., 1996). Increases in ceramide production have been proposed to account for p75-mediated apoptosis in primary neuronal cultures (Wiesner and Dawson, 1996) and PC12 cells (Hartfield et al., 1998). In a separate study, NGF was shown to activate NFκB in PC12 cells, albeit with a much slower time course (Wood, 1995).

An increasing body of evidence describe "cross talk" between G protein-coupled receptors and tyrosine kinase receptors. One such example involves receptor-mediated mitogenesis involving heterotrimeric G proteins (Post and Brown, 1996). In this regard, both the α and $\beta\gamma$ subunits of G proteins have been implicated (Faure et al., 1994). $G_{\beta\gamma}$ appears sufficient to facilitate phosphorylation of src homology (SH) domains of receptor tyrosine kinases and recruit Grb2 and the guanine nucleotide exchange factor, SOS (van Biesen et al., 1995). This factor promotes Ras activation, followed by activation of downstream effectors such as MAP kinase. Regulation of G protein-coupled receptors by NGF has also been observed. NGF increased muscarinic receptor binding in PC12 cells (Jumblatt and Tischler, 1982; Lee and Malek, 1998), in part by augmenting the steady-state levels of m4 muscarinic receptor mRNA in these cells (Lee and Malek, 1998). NGF also increased the expression of the pituitary adenylyl cyclase-activating polypeptide receptor in PC12 cells (Cavallaro et al., 1995). In addition, recent evidence from our laboratory indicates that A_{2A}AR is also a target for regulation by NGF (Lee et al., 1995; Nie et al., 1995). This report has been confirmed by a recent study (Arslan et al., 1997).

The goal of this study was to determine the involvement of TrkA and p75 in NGF regulation of A_{2A}AR expression. In this report, we provide evidence for the involvement of p75 in the short-term regulation of A_{2A}AR expression in PC12 cells by NGF.

Materials and Methods

Cell Culture. Rat pheocromocytoma cells (PC12) were obtained from American Tissue Culture Collection (Rockville, MD). Culture flasks were coated with 0.1 mg/ml poly-D-lysine (Gibco-BRL, Grand Island, NY). Growth medium consisted of 85% RPMI 1640, 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 units/ml penicillin, and 25 μg/ml streptomycin (all supplies were obtained from Gibco-BRL). Cells were cultured at 37°C, in the presence of 5% CO_2 and 95% ambient air, and the medium was changed every 2 to 3 days. Murine NGF (2.5 S; Promega, Madison, WI) was administered to preconfluent PC12 cultures at a concentration of 50 ng/ml.

Membrane Preparation. Crude plasma membranes were prepared essentially as described previously (Ramkumar et al., 1990). Before performing radioligand binding studies, membranes were incubated at 37°C for 15 min with 3 units/ml adenosine deaminase (Boehringer Mannheim, Indianapolis, IN) to degrade endogenous adenosine. For quantitating A_{2A}AR by Western blotting, partially purified membranes were prepared by gently homogenizing membranes on ice in 50 mM Tris HCl buffer (pH 7.4), containing 10 mM $MgCl_2$, and 1 mM EDTA (buffer A), along with 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS; Sigma Chemical Co., St. Louis, MO) with a CHAPS:protein ratio of 2.5:1.

Membranes used for adenylyl cyclase assay were obtained after a single 40,000*g* spin for 15 min. Pellets were resuspended in 75 mM Tris buffer containing 200 mM NaCl, 12.5 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.4, at 30°C and pretreated with adenosine deaminase.

Radioligand Binding. Membranes (~75 μg/assay tube) were incubated for 1 h at 37°C with increasing concentrations of the A_{2A}AR-selective agonist [^{3}H]CGS21680 (DuPont-New England Nuclear, Boston, MA) or antagonist ^{125}I-4-(2-(7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) (Palmer et al., 1995) in a total volume of 250 μl of buffer A. Theophylline (1 mM) was included to estimate nonspecific binding. The reaction mixture was filtered over GF/B glass fiber filters (Brandel Inc., Gaithersburg, MD) and washed three times with 3 ml of ice-cold buffer A containing 0.01% of CHAPS. The radioactive content of each filter was determined using a Beckmann liquid scintillation counter (LS5801) or a Packard (5780) gamma counter. Saturation binding data were analyzed using the GraphPad Prism (GraphPad Software, San Diego, CA).

Adenylyl Cyclase Activity. Adenylyl cyclase activity was determined essentially as described previously (Ramkumar et al., 1990). Briefly, 20 μl of membranes (~100 μg of protein) were incubated with 20 μl of reaction mixture (0.14 mM ATP, 5 mM phosphocreatine, 1 μM cAMP, 30 units/ml creatine phosphokinase, 5 μM GTP, ~1.5 μCi [α-^{32}P]ATP) and 10 μl of H_2O or adenosine analogs. Incubations with all the drugs were at 37°C for 10 min, in the presence of papaverine (100 μM) to inhibit the low K_m cyclic AMP phosphodiesterase. The reaction was terminated by addition of 1 ml of ice-cold stop solution containing ~15,000 cpm of [^{3}H]cAMP, 0.3 mM cyclic AMP, and 0.4 mM ATP.

RNA Isolation and Northern Blotting. Total RNA was prepared using the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Messenger RNA was prepared using oligotex-dT (Qiagen, Chatsworth, CA) as described (Lee et al., 1995). Poly(A^+) RNA (2–5 μg) was fractionated through a 1.5% agarose, 6% formaldehyde gel and transferred to Hybond N^+ membrane (Amersham, Arlington Heights, IL). Northern blot analysis was performed essentially as described previously (Lee et al., 1995). Blots were stripped and hybridized with a rat cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH-specific RNA bands were visualized by autoradiography and quantitated by a phosphoimager with a computer interface to normalize levels of A_{2A}AR mRNA.

Preparation of Nuclear Extracts. PC12 cells were resuspended in 5 volumes of hypotonic buffer (10 mM HEPES, pH 7.9, at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) and centrifuged at 1850*g* for 5 min, followed by resuspension in 3 volumes of hypotonic buffer and allowed to swell on ice for 10 min. Cells were then disrupted using a glass homogenizer (10 up and down strokes) and centrifuged at 3300*g* for 15 min. The resulting supernatant was defined as the cytosolic extract, whereas the pellet represented the nuclear extract. The nuclear extract was resuspended in one-half volume of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and one-half volume of high salt buffer (1.2 M KCl replacing 0.02 M KCl of the low salt buffer), extracted on ice for 30 min, and centrifuged at 25,000*g* for 30 min at 4°C. The resulting supernatant was dialyzed against the dialysis buffer for 2 h and centrifuged again at 25,000*g* for 20 min. The nuclear extract was aliquoted, rapidly frozen in liquid nitrogen and stored at −70°C.

SDS-PAGE/Western Blotting. Western blotting of A_{2A}AR was performed as described previously (Palmer et al., 1992). The membrane preparations were solubilized in SDS-polyacrylamide gel electrophoresis (PAGE) buffer, and samples containing equal amounts of membrane protein were electrophoresed using SDS-PAGE. Proteins were then transferred to nitrocellulose filters. Filters were incubated in 130 mM NaCl, 2.7 mM KCl, 1.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% NaN_3, 5% low fat skim milk, and 0.1% Triton X-100 (buffer B). A polyclonal antibody against A_{2A}AR (Alpha Diagnostic International, San Antonio, TX) was then incubated with the nitrocellulose filters at a titer of 1:1000 at 4°C overnight. The filters were washed

five times with buffer B and then incubated with ^{125}I-labeled goat anti-rabbit IgG (300,000 cpm/ml) for 2 h at room temperature. This was followed by five washes with blocking solution and exposure to Kodak XAR film at −70°C for 24 to 48 h. Western blotting for different G protein subunits and for NFκB was performed similarly.

Electrophoretic Mobility Shift Assay. Nuclear extracts were incubated with a double-stranded oligonucleotide (5′-ATGTGAGGGACTTTCCCAGGC-3′) containing a consensus site for NFκB binding or a double-stranded oligonucleotide (5′-GGAATGGGGAAAGCCCAGTG-3′), which contains the putative NFκB binding sequence (located at position −240) of the rat A_{2A}AR promoter (Chu et al., 1996). Incubations were performed at room temperature for 30 min in a total volume of 15 μl of buffer containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1.0 μg of poly(dI-dC), and 10,000 cpm of oligonucleotide probes, which were labeled using T4 polynucleotide kinase. The DNA-protein complexes were resolved on a nondenaturing 5% polyacrylamide gel, performed with 0.5 × TBE running buffer (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA, pH 8.0). The binding of the labeled probes was determined using a phosphor imager after subtraction of background.

Transient Transfection of PC12 Cells. Cells were grown to about 40% confluency in culture plates and then transfected with a mixture of 5 μg of pCMX-IκBαM, 5 mg of carrier DNA, and 30 μl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (lipofectin) in a volume of 100 μl of Opti-MEM (Gibco-BRL). Cells that served as controls were incubated with the vector alone. The mixtures were allowed to stand at room temperature for 1 h before addition to the culture plates. After 6 h, regular medium (containing RPMI 1640, 10% horse serum, 5% fetal bovine serum) was added to the plates, and they were returned to the incubator for 24 h. Following this, NGF was added and the plates were incubated for an additional 24 h. Cells were harvested and membranes were prepared for radioligand binding as described above.

To determine transfection efficiency, cells were grown in 6-well culture plates and transfected with plasmid pCMV-Lac1 (1.5 μg/well; Stratagene, La Jolla, CA), containing the β-galactosidase gene. Thirty-six hours after transfection, cells were fixed and used for cytochemical staining for β-galactosidase activity using X-gal, as described previously (Ambrosini et al., 1999).

Protein Determination and Statistical Analysis. The protein concentrations of the samples were determined by the method of Bradford (Bradford, 1976). Statistical analysis was performed using Student's *t* test, one-way analysis of variance, and Dunnet's posthoc test.

Results and Discussion

Initial experiments were performed to characterize A_{2A}AR in PC12 cells. The binding of [^{3}H]CGS21680 was inhibited by the selective A_{2A}AR antagonist, ZM214385 (Palmer et al., 1995) with an inhibitory constant (K_i) of 6 nM. However, no inhibition of [^{3}H]CGS21680 binding was observed in the presence of 8-cyclopentyl-1,3-dipropylxanthine, an A_1AR-selective antagonist, at concentrations up to 1 μM. This concentration of 8-cyclopentyl-1,3-dipropylxanthine is at least 2 orders of magnitude higher than the K_i for interacting with A_1AR. This suggests that the binding sites labeled by [^{3}H]CGS21680 are likely those of A_{2A}AR.

NGF (50 ng/ml) treatment for 12 and 24 h decreased [^{3}H]CGS21680 binding, by 30 and 48%, respectively, compared with untreated controls. The decrease in binding sites was maintained essentially at the 24-h level with continued incubation with NGF for up to 6 days (Fig. 1A). To exclude the possibility that the decrease was not due to competition by NGF for [^{3}H]CGS21680 binding sites, we tested for possible interaction of NGF with these sites in competition experiments. Concentrations of NGF in excess of those that decreased [^{3}H]CGS21680 binding in cell cultures did not compete with this radioligand (data not shown). Removal of NGF from the culture media after treatment for 3 days led to substantial recovery in [^{3}H]CGS21680 binding sites by 24 h, with almost complete recovery observed by 2 days (Fig. 1A). Scatchard analyses of saturation curves performed with membranes from cells treated with NGF for 3 days indicate a 49 ± 15% decrease in the maximum binding sites (B_{max}) from a control value of 762 ± 189 ($p < .05$, paired *t* test from four determinations), with no significant change in the equilibrium dissociation constant (K_d) (Fig. 1B). K_d values were 33.2 ± 6.3 and 32.4 ± 5.2 nM for control and NGF-treated cells, respectively. Under similar conditions used for NGF treatment, epidermal growth factor (EGF), an agent that induces proliferation but not differentiation of PC12 cells, did not alter A_{2A}AR expression (Fig. 1A). This suggests that NGF mediates A_{2A}AR down-regulation by a signaling pathway not shared by EGF. Both EGF and NGF stimulate the expression of similar programs of immediate early genes. Unlike EGF, however, NGF mediates persistent activation of MAP kinase (Kaplan and Miller, 1997).

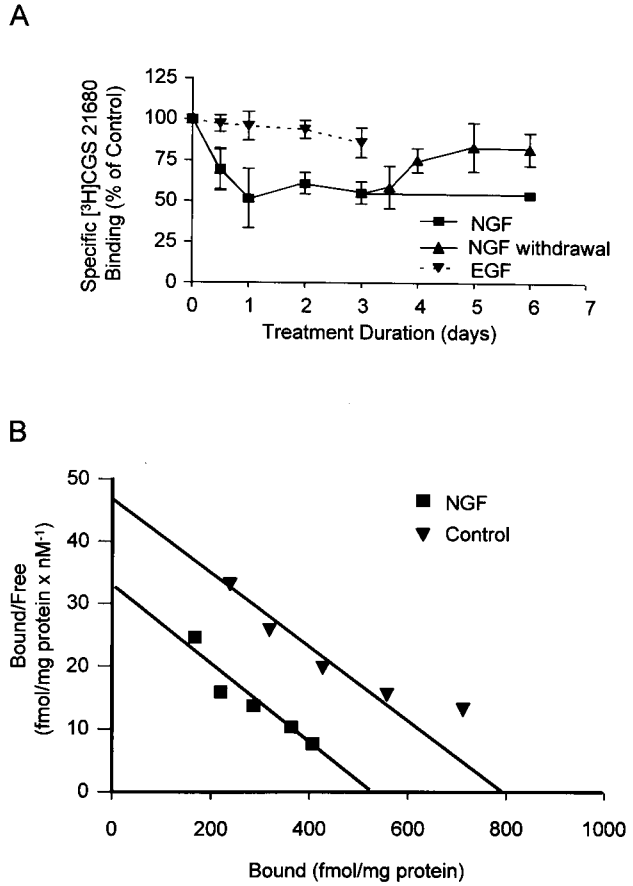

Fig. 1. NGF treatment decreased A_{2A}AR expression in PC12 cells. A, time course of NGF-mediated loss in [^{3}H]CGS21680 binding sites in PC12 cells. Cells were treated with NGF (50 ng/ml) or EGF (50 ng/ml) for the time periods indicated. For NGF withdrawal studies, the culture medium was removed by aspiration, cells were washed with phosphate-buffered saline and incubated with fresh medium without NGF. Results indicate changes in the binding of 30 nM [^{3}H]CGS21680 to membrane proteins and represent the mean ± SE of three experiments. B, representative Scatchard plots of [^{3}H]CGS21680 saturation binding. PC12 cells were treated with NGF for 3 days.

Since an agonist radioligand was used to quantitate receptor number, the observed decrease in radioligand binding could represent either a true loss in receptor number or reduced coupling of the receptor to G proteins (i.e., due to a loss in G_s proteins). To discriminate between these two possibilities, the levels of A_{2A}AR protein were assessed by Western blotting using a polyclonal antibody specific for this receptor (Marala and Mustafa, 1998). Western blotting experiments indicate labeling of a single band at approximately 45 to 48 kDa. Labeling of this band was reduced by 70 ± 6% in membranes of cells treated with NGF for 3 days, compared with untreated control cells (data not shown). Similar changes were observed using a second polyclonal antibody (data not shown) characterized by Palmer et al. (1992) as the TP/1 antibody. However, no change in the levels of G protein subunits ($G_{s\alpha}$ or β) was detected in membrane preparations from cells treated with NGF for 3 days (data not shown). These latter results are similar to those obtained previously in PC12 cells (Andreopoulos et al., 1995) but different from another study (Zubiaur and Neer, 1993). Our data suggest that the decrease in [^{3}H]CGS21680 binding by NGF likely reflects a decrease in the receptor protein and not its coupling to G_s.

Using the iodinated form of the recently available A_{2A}AR antagonist, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a]-[1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385) (Palmer et al., 1995), we obtained similar decreases in A_{2A}AR expression as with the agonist radioligand. Scatchard analysis indicated a reduction in B_{max} from 2.30 ± 0.09 pmol/mg protein to 1.40 ± 0.04 pmol/mg protein, following NGF treatment (Fig. 2B). The K_d values were relatively unchanged, being 1.6 ± 0.3 nM and 1.2 ± 0.1 nM, for control and treated cells, respectively. Furthermore, the time course for the decrease in ^{125}I-ZM241385 binding elicited by NGF closely resembled the loss in [^{3}H]CGS21680 binding under similar conditions (Fig. 2A). Taken together, these data suggest that NGF produces a true loss in A_{2A}AR expression.

Additional studies were performed to determine the effects of NGF on the steady-state levels of A_{2A}AR mRNA. NGF induced a 20 ± 4% decrease in A_{2A}AR mRNA as early as 12 h after treatment. However, the maximal decrease (80 ± 2%) was not observed until after 6 days of NGF treatment and steady-state levels for A_{2A}AR mRNA remained suppressed for up to 12 days of NGF treatment (Fig. 3). The decrease in the levels of A_{2A}AR mRNA would suggest NGF-mediated decreases in A_{2A}AR gene transcription and/or decreased mRNA stability. The slower pace of the loss of A_{2A}AR mRNA compared with the receptor protein would indicate the additional contribution of mechanisms other than transcriptional regulation and/or RNA stability in the initial phase of A_{2A}AR down-regulation.

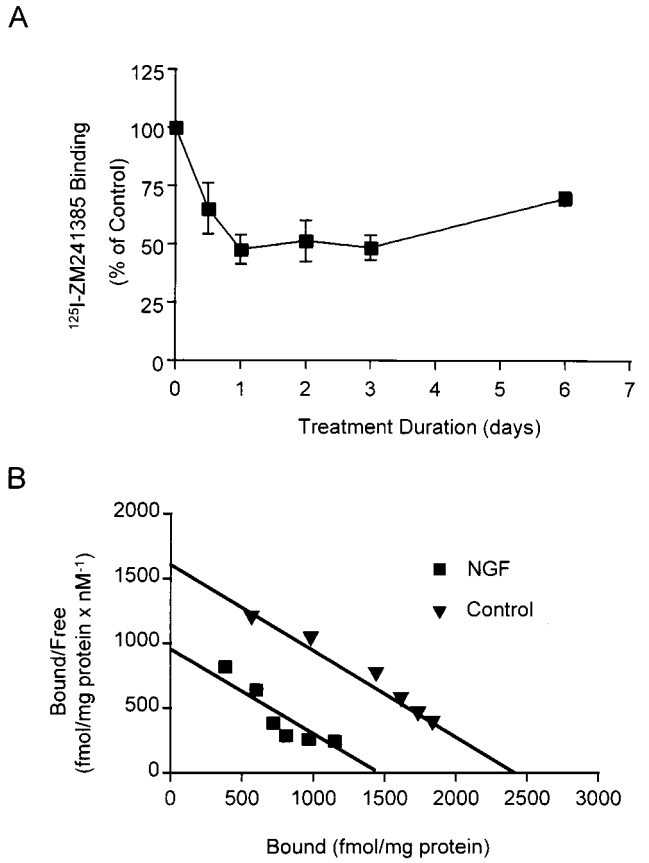

Fig. 2. NGF treatment to PC12 cells decreased the level of A_{2A}AR, as determined by the binding of the antagonist radioligand, ^{125}I-ZM241385 binding. A, time course of change in ^{125}I-ZM241385 binding following NGF treatment. Results are presented as the mean ± SE of four independent experiments. B, cells were treated with NGF for 24 h, as described above, and membranes obtained were used in saturation experiments. Results were transformed into Scatchard plots. This is a representative of three independent experiments.

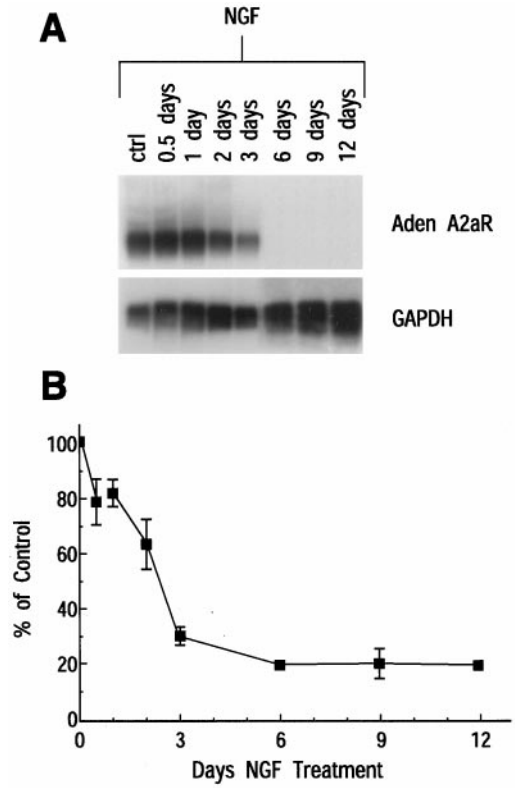

Fig. 3. NGF decrease A_{2A}AR RNA in PC12 cells. A, poly(A$^+$) RNA preparations were obtained from PC12 cells treated with NGF for the time periods indicated. Northern blotting studies were performed by electrophoresis using 2 μg of poly(A$^+$) RNA on a 1% agarose-formaldehyde gel. Filters were probed with a random-prime labeled rat A_{2A}AR-specific cDNA probe. The blots were stripped and reprobed with labeled cDNA probe for GAPDH for normalization. B, normalization of A_{2A}AR-specific RNA levels following NGF treatment as percentage of control signal (day 0). Each point represents the mean ± SE of three independent experiments.

Functionally, NGF treatment was associated with a decrease in both basal and A_{2A}AR-stimulated adenylyl cyclase activity. Basal adenylyl cyclase activity was reduced following a 24-h incubation with NGF from 2.1 ± 0.1 to 1.4 ± 0.1 pmol/min/mg protein (mean ± SE of four determinations). In untreated cells, the agonists 5′-*N*-ethylcarboxamidoadenosine (10 μM) and 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680, 10 μM) stimulated adenylyl cyclase activities to 5.5 ± 0.3 and 3.7 ± 0.7 pmol/min/mg protein, respectively. NGF treatment significantly reduced the stimulation by 5′-*N*-ethylcarboxamidoadenosine and CGS21680 to 49.1 ± 1.0% and 47.6 ± 16.5% (mean ± SE of five determinations) of their respective stimulated levels. The decrease in basal adenylyl cyclase activity following NGF treatment likely reflects a decrease in A_{2A}AR expression and/or a decrease in adenylyl cyclase expression.

NGF is known to regulate the expression of a number of different genes in PC12 cells through activation or induction of transcription factors (Kaplan and Miller, 1997). Figure 4A indicates a significant increase in DNA binding activity of NFκB consensus sequence (approximately 6-fold at the 10-μg protein concentration in the extract) in the nucleus, as determined by electrophoretic mobility shift assays, following a 2-h treatment of PC12 cells with NGF. The binding of the nuclear proteins was inhibited by 30- and 60-fold molar excesses of κB oligonucleotide probe but not by equivalent concentrations of SP-1 and AP-1 consensus sequences (Fig. 4B). Incubation of the nuclear preparations with antibody against p65 resulted in supershifts of the labeled p65-κB complex (Fig. 4C), whereas incubation with antibody against p50 protein was without effect. These data suggest that the NFκB complex that interacts with the κB sequence contains p65/p65 homodimers. This conclusion was confirmed in Western blotting studies that show a significant increase (168 ± 23% of control; $p \leq 0.05$) in the level of p65 subunit of NFκB in the nucleus and no change in p50 following NGF treatment (Fig. 4D).

To determine whether the putative NFκB sequence in the rat A_{2A}AR gene binds NFκB, an oligonucleotide probe that includes this putative sequence was tested in electrophoretic mobility shift assays. Figure 5A indicates retardation of the labeled probe on incubation with nuclear extracts from PC12 cells. Binding of the labeled probe was inhibited by an excess of either the cold κB probe or the oligonucleotide derived from the putative NFκB sequence of the A_{2A}AR promoter, but not by an excess of AP-1 or SP-1 consensus oligonucleotides. This suggests that the putative κB sequence of the A_{2A}AR gene could interact specifically with this transcription factor. As shown in Fig. 5B, increased binding of the A_{2A}AR oligonucleotide was observed in nuclear extracts of PC12 cells treated with NGF compared with the control, as would be expected if NGF increased nuclear translocation of NFκB. The difference in the degree of inhibition of labeling by the cold A_{2A}AR oligonucleotide versus the κB probe likely reflects some differences in the nucleotide sequence of these two probes. As such, the κB probe is not as effective an inhibitor at this site as the A_{2A}AR oligonucleotide at equivalent concentrations. Nevertheless, these results support a functional role of NFκB in regulating A_{2A}AR gene expression.

Other activators of NFκB, such as H_2O_2 (100 μM) and ceramide (50 μM), also down-regulate A_{2A}AR in PC12 cells (Fig. 5C). At these concentrations no loss in cell viability was observed on treating cells with H_2O_2 and ceramide, as determined by trypan blue exclusion studies. PC12 cells treated with H_2O_2 (100 μM) for 24 h showed approximately 3% nonviable cells, which is comparable to the percent nonviable cells in the controls. Similarly, cells treated with ceramide (50 μM) for 24 h showed approximately 2% nonviable cells. These results suggest that the decrease in receptor level is not due to a nonspecific toxic effect of these agents on the cells but to activation of specific signaling pathway(s). In contrast, inhibition of NFκB by pyrrolidine dithiocarbamate (PDTC, 100 μM) (Sun et al., 1995) for 1 day abrogated NGF-mediated decrease in [^{3}H]CGS21680 binding, further supporting an essential role of this transcription factor in this process. PDTC also blocked NGF-mediated decrease in the binding of ^{125}I-ZM241385 to A_{2A}AR (data not shown). At this concentration, PDTC was a potent inhibitor of NFκB, as indicated by a decrease in nuclear translocation of p65 subunits upon NGF treatment (data not shown).

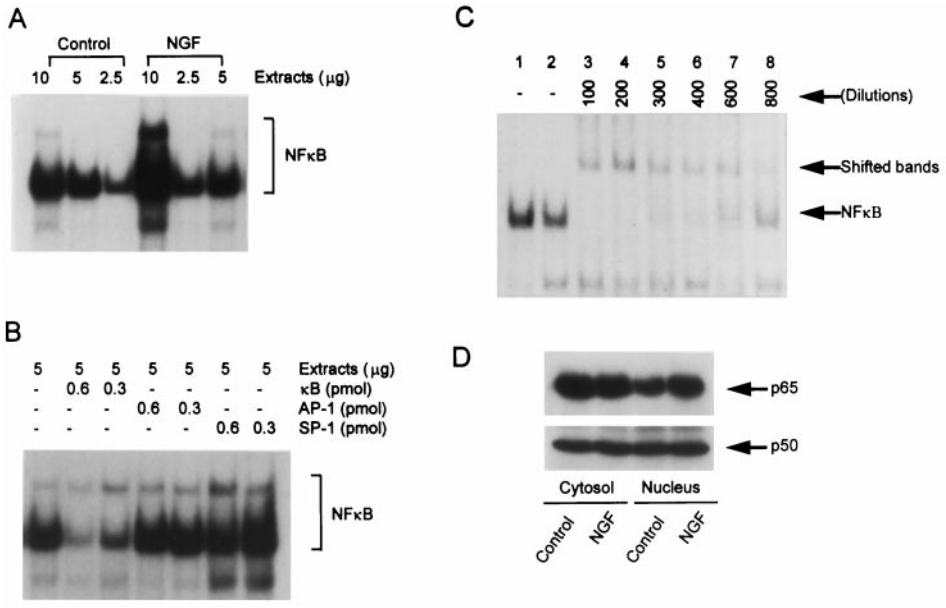

Fig. 4. NGF promotes activation of NFκB in PC12 cells. A, electrophoretic mobility shift assays were performed by incubating different amounts of nuclear extracts prepared from control and treated cells (50 ng/ml, 2 h) with ^{32}P-labeled NFκB oligonucleotide. B, specificity of the oligonucleotide-NFκB binding site. Nuclear extracts were incubated with ^{32}P-labeled NFκB oligonucleotide, in the absence or presence of increasing concentrations of competitors. Binding of the labeled κB probe was inhibited by 30- and 60-fold excess of κB probe (0.3 and 0.6 pmol, respectively) but not by equivalent concentrations of AP-1 and SP-1 probes. C, identification of the composition of NFκB complex by gel-retardation assays. Electrophoretic mobility shift assays were performed in the presence of increasing concentrations of p65 antibody. The supershifted band is indicated by the arrow. D, NGF-mediated nuclear distribution of NFκB. Western blots were performed on cytosolic (100 μg) and nuclear (50 μg) proteins and probed with polyclonal antibodies specific for p50 and p65 subunits of NFκB. Specific bands were detected using ^{125}I-labeled goat anti-rabbit IgG.

Unlike NGF, brain-derived neurotrophic factor (BDNF) exhibits high selectivity for TrkB but shares similar affinity for p75. Thus, BDNF treatment was used to determine whether another neurotrophin working via p75 could mimic the response of NGF. PC12 cells treated with BDNF showed down-regulation of A_{2A}AR, comparable to that observed with NGF. In these experiments, BDNF reduced the level of A_{2A}AR to 70.4 ± 9.1% of control, whereas similar batches of cells treated with NGF showed reduction in binding to 70.8 ± 3.9% of control.

Because TrkA is a primary target of NGF in PC12 cells (Kaplan and Miller, 1997), we tested the role of this receptor by inhibiting NGF-stimulated tyrosine autophosphorylation of TrkA using α-cyano-(3,5-di-*t*-butyl-4-hydroxy)thiocinnamide (AG 879) (Ohmichi et al., 1993). The effectiveness of AG879 treatment (50 μM) was evidenced by the observation that this drug blocked NGF-mediated neurite outgrowth. However, AG 879 treatment had no effect on the basal level of A_{2A}AR expression and did not attenuate NGF-mediated decrease in A_{2A}AR (Fig. 5C). A similar lack of effect of AG879 on A_{2A}AR RNA was observed (data not shown).

Activation of TrkA leads to, but is not limited to, stimulation of Ras, Raf, MEK, MAPK, PLC-γ1, PI-3-kinase, Akt, Rap, and Rac. To determine the role of the MAP kinase pathway in this process, we tested the effect of NGF following inhibition of MEK with PD98059 (Dudley et al., 1995). PD98059 treatment (50 μM) did not affect NGF-induced down-regulation of A_{2A}AR (Fig. 5C), further supporting a lack of involvement of the TrkA signaling pathway in this short-term regulation of A_{2A}AR. At the concentration used, PD98059 significantly inhibited NGF-mediated neurite outgrowth and MAP kinase activity in PC12 cells (data not shown).

In contrast, we have recently shown that NGF up-regulates the m4 muscarinic receptor via a MAP kinase pathway (Lee and Malek, 1998). To test that PD98059 was active in these cells, we determined the effect of this agent on NGF-mediated neurite outgrowth and the activity of MAP kinase.

To further strengthen the hypothesis that activation of NFκB was involved in NGF-mediated A_{2A}AR down-regulation, PC12 cells were transiently transfected with a dominant negative IκBα (IκBαM). The cells serving as controls were transfected with the vector alone. Cells transfected with this mutant construct showed a lack of NFκB inducibility (van Antwerp et al., 1996). Following transient transfection with IκBαM, the ability of NGF to decrease the binding of ^{125}I-ZM241385 was blunted, as compared to its effect in the control cells (Fig. 6). In these experiments, the reductions in binding obtained with NGF in the vector control and cells transfected with the IκBαM plasmid were 33.8 ± 4.2% and 12.4 ± 4.9%, respectively. Transfection efficiency in PC12 cells obtained with lipofectin, using a β-galactosidase reporter gene, was 66 ± 2%.

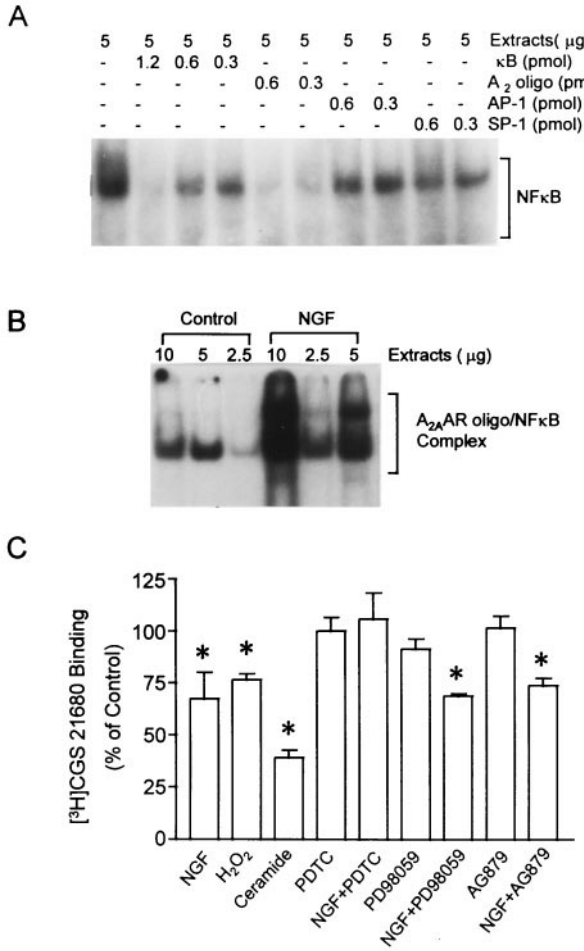

Fig. 5. The putative κB sequence of the A_{2A}AR gene binds NFκB and promotes reduction in receptor expression. A, specificity of the putative κB sequence of the A_{2A}AR gene for NFκB. Binding of the A_{2A}AR oligonucleotide was inhibited by 30- and 60-fold excess of unlabeled A_{2A}AR oligonucleotide and by increasing concentrations of κB probes (30-, 60-, and 120-fold excess) but not by the AP-1 probe. Electrophoretic mobility shift assays were performed essentially as described above, using a labeled oligonucleotide probe derived from the putative κB sequence of the A_{2A}AR gene. B, increased binding to this radiolabeled probe following NGF treatment. C, role of NFκB in regulating the expression of A_{2A}AR in PC12 cells. The concentrations of the respective agents were NGF (50 ng/ml), H_2O_2 (100 μM), ceramide (50 μM), PDTC (100 μM), PD98059 (50 μM), and AG879 (50 μM). Treatments were maintained for 1 day. Receptor binding assays were performed as described in *Materials and Methods*.

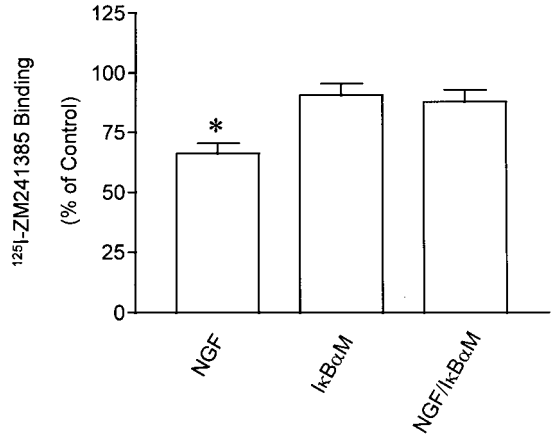

Fig. 6. Inhibition of NFκB using dominant negative mutant IκBα attenuated NGF-mediated down-regulation of A_{2A}AR. PC12 cells were transiently transfected with an expression plasmid, pCMX-IκBαM (containing the dominant negative mutant IκBα) or the plasmid pCMX (control) using lipofectin. Twenty-four hours later, NGF (50 ng/ml) was added to the different groups for an additional 24 h and membranes were harvested and used for ^{125}I-ZM241385 binding. Results are expressed as the percentage of receptors expressed in the PC12 cells transfected with pCMX alone (without NGF) and are presented as the mean ± SE of four independent experiments. The asterisk denotes statistically significant difference from control ($p < .05$).

The present data indicate that NGF down-regulates A_{2A}AR, in sharp contrast to its stimulatory action at the m4 muscarinic receptor (Lee and Malek, 1998) and the pituitary adenylate cyclase-activating polypeptide receptor (Cavallaro et al., 1995). Furthermore, the kinetics of NGF regulation of A_{2A}AR and the m4 muscarinic receptor are different. NGF-induced down-regulation of A_{2A}AR mRNA and protein appeared within 12 h and was maximal at least by day 3, whereas up-regulation of the m4 receptor peaked on day 12 of NGF treatment (Lee and Malek, 1998). Whether the decrease in A_{2A}AR mRNA reflects a decrease in transcription or a decrease in mRNA stability is presently unclear.

The major pathway for NGF-dependent regulation of A_{2A}AR delineated in this study is the p75/NFκB-dependent pathway, which appears independent of TrkA and MAP kinase. A lack of MAP kinase involvement in the response to NGF is supported by the observation that inhibition of MEK and TrkA did not affect the ability of NGF to decrease A_{2A}AR, even though it blocked neurite outgrowth (a phenomenon that appears dependent on TrkA). Furthermore, unpublished data from our laboratory indicate that NGF can induce a decrease in A_{2A}AR mRNA in nnr5 cells lacking TrkA, even though this effect required a more prolonged treatment with NGF. Since activation of MAP kinase appears critical for PC12 differentiation, it seems unlikely that A_{2A}AR down-regulation is linked to PC12 differentiation.

Clearly, p75 is critical to the down-regulation process, because regulation of the p75 effector, NFκB, led to direct regulation of A_{2A}AR expression. The mechanism by which NFκB activation regulates A_{2A}AR expression is not clear, but might involve direct interaction of this transcription factor to the putative κB binding sequence on the rat A_{2A}AR gene, leading to inhibition of transcription. In vitro experiments, described above, do support such a contention. Other genes shown to be regulated in a similar fashion by NFκB include the androgen receptor gene (Song et al., 1995), mouse κ immunoglobulin light chain (Lernbecher et al., 1993), and the major histocompatibility class II-invariant chain (Brown et al., 1994). In *Drosophila*, the homolog of NFκB, the dl gene product, inhibits developmentally important genes such as dpp and Zen (Jiang et al., 1993). Because the early decrease in A_{2A}AR protein appears to precede a significant decrease in mRNA, NGF-stimulated regulation of the translation or stability of this receptor protein may serve as an alternative mechanism for regulating the level of this receptor protein. In this respect, NFκB may play such a role in stimulating the transcription of one or more intermediate proteins whose function is to regulate the translational efficiency or stability of A_{2A}AR. While the early down-regulation may rely on these mechanisms, it is likely that maintaining this state requires the additional contribution of decreasing transcription or RNA stability. In contrast to A_{2A}AR, we have observed positive regulation of A_1AR following activation of NFκB (Nie et al., 1998). This suggests differential regulation of AR subtypes by a common stimulus.

Because A_{2A}AR is positively coupled to adenylyl cyclase, it is possible that one action of NGF is to lower the levels of cyclic AMP in the cell by reducing expression of this stimulatory receptor while increasing the expression of receptors, such as the m4 muscarinic receptor (Lee and Malek, 1998), which are negatively coupled to this enzyme. In support of this, we observed substantial reductions in the level and activity of adenylyl cyclase in membranes of cells treated with NGF, as compared with control cells (Z. Nie and V. Ramkumar, unpublished observations). However, this hypothesis is not supported by the finding that NGF treatment also sensitizes PC12 cells to pituitary adenylyl cyclase-activating factor (Cavallaro et al., 1995).

Considerable attention is currently being focused on A_{2A}AR in the striatum as a possible target for drug therapy for Parkinson's disease (Ferre et al., 1997). Drugs that block A_{2A}AR function have been proposed to be beneficial by antagonizing the inhibitory action of A_{2A}AR on D_2 dopamine receptors (Ferre et al., 1997). Our finding suggests that one class of drugs that could be useful in Parkinson's disease consists of agents that can activate NFκB in striatal neurons, leading to down-regulation of A_{2A}AR. Accordingly, down-regulation of these receptors by such agents should mimic the effect of A_{2A}AR antagonists, leading to enhanced activity of dopaminergic neurons and provide symptomatic relief in Parkinson's disease.

In summary, we have shown that NGF down-regulates A_{2A}AR in PC12 cells through a pathway involving activation of p75 and NFκB. Such an interaction of the transcription factor with the A_{2A}AR promoter could decrease the steady-state levels of A_{2A}AR mRNA. Regulation of NFκB activity provides a novel mechanism for inducing cross-regulation of A_{2A}AR, which might be important clinically.

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