Adenosine A2A Receptor mRNA Regulation by Nerve Growth Factor Is TrkA-, Src-, and Ras-dependent via Extracellular Regulated Kinase and Stress-activated Protein Kinase/c-Jun NH2-terminal Kinase*

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We have shown previously that nerve growth factor (NGF) down-regulates adenosine A2A receptor (A2AAR) mRNA in PC12 cells. To define cellular mechanisms that modulate A2AAR expression, A2AAR mRNA and protein levels were examined in three PC12 sublines: i) PC12nnr5 cells, which lack the high affinity NGF receptor TrkA, ii) srcDN2 cells, which overexpress kinase-defective Src, and iii) 17.26 cells, which overexpress a dominant-inhibitory Ras. In the absence of functional TrkA, Src, or Ras, NGF-induced down-regulation of A2AAR mRNA and protein was significantly impaired. However, regulation of A2AAR expression was reconstituted in PC12nnr5 cells stably transfected with TrkA. Whereas NGF stimulated the mitogen-activated protein kinases p38, extracellular regulated kinase 1 and 2 (ERK1/ERK2), and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in PC12 cells, these kinases were activated only partially or not at all in srcDN2 and 17.26 cells. Inhibiting ERK1/ERK2 with PD98059 or inhibiting SAPK/JNK by transfecting cells with a dominant-negative SAPKβ/JNK3 mutant partially blocked NGF-induced down-regulation of A2AAR expression in PC12 cells. In contrast, inhibiting p38 with SB203580 had no effect on the regulation of A2AAR mRNA and protein levels. Treating SAPKβ/JNK3 mutant-transfected PC12 cells with PD98059 completely abolished the NGF-induced decrease in A2AAR mRNA and protein levels. These results reveal a role for ERK1/ERK2 and SAPK/JNK in regulating A2AAR expression.

Adenosine receptors are G-protein coupled receptors that mediate important physiological processes in both the central and peripheral nervous system, including vasodilation, respiratory depression, wakefulness, and spontaneous locomotor activity. There are four major adenosine receptor subtypes, A1, A2A, A2B, and A3; each is encoded by a distinct gene, and each has unique affinities for adenosine analogs and methylxanthine derivatives (1–3). In the developing rat brain, adenosine A2A receptor (A2AAR)1 mRNA is expressed transiently in various regions (4). Moreover, a severalfold increase in A2AAR protein levels occurs during early postnatal development in a number of brain regions, whereas a decrease in A2AAR mRNA is observed in other regions (5).

PC12 cells, derived from a rat pheochromocytoma, have been used extensively as a model for neuronal differentiation and development (6). In response to NGF, these cells differentiate into sympathetic-like neurons and extend neurites (6). The signal transduction pathways activated by NGF originate at both high (TrkA) and low (p75) affinity receptors, and downstream targets of each receptor have been implicated in regulating expression of genes involved in differentiation, neurotransmission, and neuronal function (6–13). Stimulation of the receptor tyrosine kinase TrkA results in the activation of Ras, Src, phospholipase C-γ, SNT, and phosphoinositide 3-OH kinase (14–17). In PC12 cells, active Ras triggers a cascade of phosphorylation events leading to activation of ERK1/ERK2 via Raf-1 (18–20) or p38 MAP kinase and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) via MAP kinase kinase kinase (21–23). p75 activation increases ceramide production (24) and activates NFκB (25).

Gene products regulated by NGF in PC12 cells include several G-protein coupled receptors, such as the M1 muscarinic, secretin, and adenosine A2A receptors (7, 26, 12). Using gene expression profiling (expressed sequence tags) coupled with Northern analysis, a decrease in A2AAR mRNA could be demonstrated as early as 3 days (3 d) posttreatment with NGF and levels remained depressed for up to 12 d (7). In situ hybridization with an A2AAR oligonucleotide probe detected a 50% decrease in the number of grains per cell in NGF-differentiated PC12 cells, confirming that NGF decreases A2AAR mRNA levels (26). Corresponding to the changes in mRNA levels, immunoreactive A2AAR protein declines by more than half after 7 d of NGF treatment, and the number of binding sites for the A2AAR selective antagonist, [3H]HSCH 58261, decreases by 3-fold (26). When PC12 cells are treated with A2AAR agonists, a transient down-regulation of A2AAR mRNA and protein occurs (27). Despite these observations, the specific cellular mechanisms regulating A2AAR mRNA levels have not been thoroughly delineated. In this study, we provide the first insights into the downstream pathways employed by NGF to control A2AAR expression in PC12 cells. Such pathways may likewise play an important role in the regulation of A2AAR expression during brain development.

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EXPERIMENTAL PROCEDURES

Cell Culture—Rat pheochromocytoma cells (PC12) were obtained from the American Type Culture Collection (Manassas, VA). PC12nnr5, clone 106, and srcDN2 cells were a generous gift from Gordon Guroff at NICHD, National Institutes of Health (Bethesda, MD). The dominant-negative Ras cell line, 17.26, was obtained from Robert Maue at Dartmouth Medical Center (Hanover, NH). PC12 cell lines were maintained on rat tail collagen, Type IV (Upstate Biotechnology, Saranac Lake, NY) as described previously (7). SrcDN2, 17.26, and clone 106 were cultured in the presence of 300 μg/ml Geneticin (Life Technologies, Inc.). Cells were treated with PD98059 or SB203580 (Calbiochem, San Diego, CA) and mouse 2.5S NGF (Promega, Madison, WI) as described below.

Northern Blot Analysis—Poly(A)⁺ RNA was isolated, fractionated through a denaturing agarose gel, and transferred to Hybond N⁺ membranes (NEN Life Science Products) essentially as described previously (12, 31). A 5′-labeled 2.3-kilobase pair Sal/I/HindIII fragment from a rat A₂A cDNA clone and a 1.2-kilobase pair EcoRI/HindIII fragment from a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used as probes (7). Expression levels of A₂A AR mRNA were normalized to GAPDH mRNA levels. Blots were analyzed on a Molecular Dynamics PhosphorImager.

Data are expressed as the mean ± S.E. of n independent experiments.

Ribonuclease Protection Assay—At the times indicated, total RNA was isolated from PC12nn5 cells. An RNase protection assay was performed essentially as described by Lee et al. (28). Construction of plasmid 118GAPDHspP73 for generating an antisense riboprobe of the GAPDH mRNA was described previously (12). A 5′-labeled 2.3-kilobase pair Sal/I/HindIII fragment from a rat GAPDH cDNA clone was used as the template for in vitro transcription. The antisense riboprobe was hybridized to total RNA isolated from PC12 cells under conditions that allow hybridization to GAPDH mRNA but not to A₂A AR mRNA (28). The level of hybridization was determined using a PhosphorImager. The specific activity of the antisense riboprobe was determined by measurement of radioactivity on a beta counter.

RESULTS AND DISCUSSION

To assess the contribution of individual components of the NGF signal transduction pathway leading to regulation of A₂A AR mRNA and protein, PC12 sublines lacking functional TrkA or overexpressing dominant-inhibitory forms of Ras or Src were used. The role of the three MAP kinase family members p38, ERK1/ERK2, and SAPK/JNK in NGF-mediated regulation of A₂A AR mRNA was also determined.

TrkA Is Required for Down-regulation of A₂A AR mRNA and Protein—Earlier experiments have concentrated on the effects of long term (7–12 days) NGF treatment on A₂A AR expression in PC12 cells (7, 26). In the present study, shorter periods of treatment were examined to define initial pathways responsible for NGF regulation. Steady state A₂A AR mRNA declined to 62 and 43% of untreated control cells following 1 and 3 d of NGF treatment, respectively (Fig. 1A). Correspondingly, binding of the A₂A AR antagonist 125I-ZM241385 to PC12 cells decreased by 50 and 45% following 1 and 3 d of NGF treatment, respectively (Fig. 1B). Thus, NGF-induced down-regulation of A₂A AR mRNA and protein is apparent as early as 24 h following NGF treatment, with a further decrease in A₂A AR mRNA occurring at 3 d.

The contribution of TrkA and p75 to the regulation of A₂A AR mRNA and protein were examined in PC12nnr5 cells, which are a PC12 subline that expresses p75, lacks functional TrkA receptors, and does not differentiate in response to NGF (31). As the basal steady-state level of A₂A AR mRNA is reduced in PC12nnr5 cells, a ribonuclease protection assay was performed to quantitate A₂A AR mRNA. As shown in Fig. 1, NGF failed to down-regulate both A₂A AR mRNA and protein levels. For comparison, the effects of NGF on A₂A AR expression were studied in clonal cell line 106. Clone 106, derived from PC12nnr5 cells stably transfected with TrkA, has levels of 125I-NGF binding similar to those in native PC12 cells and differentiates in response to NGF (32). When cultures of clone 106 were treated with NGF for 1 or 3 d, both A₂A AR mRNA and protein levels were down-regulated to the same extent as native PC12 cells (Fig. 1). Because TrkA is implicated in A₂A AR mRNA and protein regulation, potential roles for TrkA-associated signaling components, Src and Ras, were examined.

NGF-mediated Down-regulation of A₂A AR mRNA and A₂A AR Protein Is Impaired by Dominant-negative Src—As oncogenic Src mimics NGF-induced neurite outgrowth and phosphorylation of a similar set of cellular substrates, a role for Src in the signal transduction pathway initiated by NGF has been implicated (33). Therefore, the effects of NGF on A₂A AR mRNA and protein were examined in srcDN2 cells that overexpress a dominant-negative kinase-defective Src mutant (34). Upon treatment of srcDN2 cells with NGF for 1 or 3 d, down-regulation of steady-state mRNA was not observed (92 and 100% of untreated cells, respectively) (Fig. 2). Likewise, A₂A AR protein levels remained near control levels following 1 and 3 d of NGF treatment (110 and 81%, respectively). Thus, Src appears to be critical for NGF-induced down-regulation of both A₂A AR mRNA and protein.

Ras Mediates NGF-stimulated Down-regulation of A₂A AR

buffer and twice with kinase buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 25 mM sodium β-glycerophosphate, 2 mM sodium orthovanadate, 0.5 mM dithiothreitol) and resuspended in kinase buffer containing 100 μM ATP, 5 μM of [γ-32P]ATP and 2 μg of glutathione S-transferase-ATF-2 substrate. Reactions were incubated 30 min at 30 °C and terminated by the addition of 2 × SDS-PAGE loading buffer. Proteins were separated on a 7.5% SDS-PAGE gel and analyzed by autoradiography.

Radioligand Binding—Crude membrane preparations were obtained as described recently (30). 125I-ZM241385, an A₂A AR-specific antagonist was used to measure specific binding to A₂A ARs in crude membrane preparations. Data are expressed as the mean ± S.E. of n independent determinations.
mRNA and Protein—PC12 cells undergo a Ras-dependent transient induction of several immediate-early genes within minutes of NGF treatment that precedes neurite outgrowth (35). The delayed response genes are transcriptionally active hours to days following NGF treatment (6, 7), and the induction of several genes, such as agrin, tau, transin, and SCG10, has been shown to be Ras-dependent (8, 10, 11). Furthermore, transcriptional down-regulation of the epidermal growth factor receptor requires Ras activity (36).

The data shown in Fig. 3 demonstrate that NGF can also mediate down-regulation of A2AAR mRNA in a Ras-dependent manner. In 17.26 cells expressing a dominant-negative Ras mutant (35), steady state mRNA levels following 1 and 3 d of NGF treatment were 88 and 81% of untreated control cells, respectively, which is a less dramatic decrease than that seen in native PC12 (compare Figs. 1A and 3A). A similar impairment of NGF-induced down-regulation of protein was observed in 17.26 cells treated as in A. Data are expressed as the mean ± S.E. of four or five independent determinations. 125I-ZM241358 binding in untreated srcDN2 cells was 66 ± 21 fmol/mg.

**Mechanisms of Adenosine Receptor Regulation**

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**Fig. 1.** TrkA is required for NGF-induced down-regulation of A2AAR mRNA and protein. A, bottom, quantitation of steady state A2AAR mRNA in PC12, PC12nnr5 (nnr5), and clone 106 (106) cells treated for 0, 1, and 3 d with 50 ng/ml NGF. Data are expressed as the mean ± S.E. of four to eight independent determinations. Top, left and right panels, representative Northern blot; center panels, representative ribonuclease protection assay. B, quantitation of the percentage of change in antagonist binding to PC12, PC12nnr5, and clone 106 cells treated as in A. Data are expressed as the mean ± S.E. of three to six independent determinations. 125I-ZM241358 binding in untreated PC12, PC12nnr5, and clone 106 was 339 ± 18, 70 ± 4, and 207 ± 19 fmol/mg, respectively.

**Fig. 2.** Dominant-inhibitory Src prevents down-regulation of A2AAR mRNA and protein by NGF. A, bottom, quantitation of steady state A2AAR mRNA in srcDN2 cells treated for 0, 1, and 3 d with 50 ng/ml NGF. Data are expressed as the mean ± S.E. of five independent determinations. Top, representative Northern blot. B, quantitation of percentage of change of antagonist binding to srcDN2 cells treated as in A. Data are expressed as the mean ± S.E. of three to five independent determinations. 125I-ZM241358 binding in untreated srcDN2 cells was 66 ± 21 fmol/mg.

**Fig. 3.** Down-regulation of A2AAR mRNA and protein is blocked by dominant-negative Ras. A, bottom, quantitation of steady state A2AAR mRNA in 17.26 PC12 cells treated for 0, 1, and 3 d with 50 ng/ml NGF. Data are expressed as the mean ± S.E. of four to seven independent determinations. Top, representative Northern blot. B, quantitation of the percentage of change in antagonist binding to 17.26 PC12 cells treated as in A. Data are expressed as the mean ± S.E. of four or five independent determinations. 125I-ZM241358 binding in untreated 17.26 cells was 85 ± 20 fmol/mg.
was comparable to that found in native PC12 cells (data not shown). In agreement with our findings, srcDN2, 17.26, and native PC12 cells exhibited similar levels of $^{125}$I-NGF binding to TrkA (32). Taken together, these results indicate that both Src and Ras are necessary TrkA-signaling components that regulate $A_{2A}$AR mRNA and protein levels. Ras has multiple downstream effectors that activate divergent signaling pathways (reviewed in Ref. 37), such as Raf-1 and MAP kinase kinase kinase 1 activates SEK, which in turn activates SAPK/JNK and p38 (40–43). As such, the activity of p38, ERK1/ERK2, and SAPK/JNK was examined in PC12, srcDN2, and 17.26 cells.

**MAP Kinase Family Members Are Activated in PC12 Cells but Not in Sublines Expressing Dominant-inhibitory Src or Ras Mutants—**NGF activation of p38, ERK1/ERK2, and SAPK/JNK was assayed with phosphorylation state-specific antibodies (Fig. 4). In native PC12 cells, NGF activated p38 and ERK1/ERK2 at early time points (15 and 30 min), whereas SAPK/JNK was not activated until 3 d. In agreement with previous reports, expression of dominant-negative Ras in 17.26 cells inhibited NGF activation of ERK1/ERK2 (38, 39). More recently, Raf-independent Ras-activated MAP kinase pathways have been identified. For example, the Ras effector MAP kinase kinase kinase 1 activates SEK, which in turn activates SAPK/JNK and p38 (40–43). As such, the activity of p38, ERK1/ERK2, and SAPK/JNK was examined in PC12, srcDN2, and 17.26 cells.

**A2AAR mRNA is Regulated by MAP Kinases**—To examine potential roles of the MAP kinase family members, JNK and ERK1/ERK2, had opposing effects on tau promoter activity and affected promoter activity over different time frames (10).

**Role of ERK1/ERK2, p38, and SAPK/JNK in Regulating $A_{2A}$AR mRNA and Protein—**To examine potential roles of the individual MAP kinases in affecting down-regulation of $A_{2A}$AR mRNA by NGF, the synthetic compound SB203580 was used to inhibit p38 (44). Although p38 activation was impaired in srcDN2 and 17.26 cells, this kinase does not appear to be involved in $A_{2A}$AR mRNA regulation as SB203580 failed to inhibit NGF-induced down-regulation of $A_{2A}$AR mRNA in native PC12 cells (Fig. 5A). Similarly, another p38 inhibitor, SB202190, also failed to inhibit $A_{2A}$AR mRNA down-regulation (data not shown). Inhibition of p38 activity in PC12 cells was verified by an in vitro immunocomplex kinase assay. Whereas p38 from lysates of NGF-stimulated cells phosphorylated glutathione $S$-transferase-ATF-2 (4-fold above basal levels), p38 from NGF-stimulated cells pretreated with SB203580 did not appreciably phosphorylate its substrate (data not shown). ERK1/ERK2 activity was inhibited by employing the MAP kinase kinase inhibitor PD98059 (45). Cells co-treated with NGF and PD98059 demonstrated significantly less down-regulation of $A_{2A}$AR mRNA than cells treated with NGF alone, suggesting that ERK1/ERK2 plays at least a partial role in controlling $A_{2A}$AR mRNA steady state levels (compare Figs. 5B and 1A). As reported previously (45), PC12 cells pretreated with PD98059 did not extend neurites following 3 d NGF as did...
cells treated with NGF alone (data not shown). Western analysis also confirmed that PD98059 inhibited ERK1/ERK2, but not p38 and SAPK/JNK, activity in PC12 cells treated with NGF (data not shown).

To inhibit SAPK/JNK, PC12 cells were transiently transfected with SAPK/K55A, a kinase-defective version of SAPK/JNK3. Following 1 and 3 d of NGF treatment, PC12 cells expressing SAPK/K55A did not extend neurites, whereas empty vector-transfected PC12 cells extended neurites to the same extent as nontransfected cells (data not shown). These findings are in agreement with studies demonstrating that differentiation of PC12 cells requires the SAPK/JNK signal transduction pathway (46). As shown in Fig. 5C, PC12 cells expressing SAPK/K55A did not undergo NGF-induced down-regulation of A2AAR mRNA following 1 d of treatment (102% of untreated cells). After 3 d of NGF incubation, SAPK/K55A transfectants had a slight decrease in A2AAR mRNA (79% of untreated cells) that was not as great as that of empty vector-transfected cells. When kinase-defective SAPK/β transfectants were co-incubated with PD98059 and NGF for 3 d, down-regulation of A2AAR mRNA was completely blocked (Fig. 5D). In contrast, when cells transfected with an empty vector were treated with 3 d with NGF alone, A2AAR mRNA was down-regulated to the same extent (42%) as wild-type PC12 cells (Fig. 1A). As inhibition of either ERK1/ERK2 or SAPK/JNK individually results in partial inhibition of A2AAR mRNA regulation, these results indicate that ERK1/ERK2 and SAPK/JNK are both required for complete down-regulation of A2AAR mRNA.

The capacity of NGF to down-regulate A2AAR mRNA following inhibition of the different MAP kinases was mimicked at the protein level (Fig. 6). It will be of interest in the future to determine whether NGF utilizes other mechanisms (besides mRNA regulation) to down-regulate A2AAR protein (e.g., ubiquitin-mediated protein degradation).

Concluding Remarks—To summarize, the data presented here demonstrate that NGF-induced down-regulation of A2AAR mRNA and protein levels is TrkA-, Src-, and Ras-dependent. Furthermore, the MAP kinase family members ERK1/ERK2 and SAPK/JNK are distal signal transduction components activated by NGF and are implicated here as having important roles in mediating regulation of A2AAR mRNA. Recent reports have demonstrated a role for mitogen-activated protein kinase family members in regulating mRNA stability. NGF-induced stabilization of the M₃ muscarinic receptor mRNA requires ERK1/ERK2 (12) and p38 plays a role in stabilizing cyclooxygenase-2 mRNA (47, 48). As NGF destabilizes A2AAR mRNA transcripts,² we are currently examining the role of ERK1/ERK2 and SAPK/JNK in NGF-mediated A2AAR mRNA destabilization.

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