The purpose of this study was to examine the regulation of adenosine A2A receptor (A2AR) gene expression during hypoxia in pheochromocytoma (PC12) cells. Northern blot analysis revealed that the A2AR mRNA level was substantially increased after a 3-h exposure to hypoxia (5% O2), which reached a peak at 12 h. Immunoblot analysis showed that the A2AR protein level was also increased during hypoxia. Inhibition of de novo protein synthesis blocked A2AR induction by hypoxia. In addition, removal of extracellular free Ca2+, chelation of intracellular free Ca2+, and pretreatment with protein kinase C inhibitors prevented A2AR induction by hypoxia. Moreover, depletion of protein kinase C activity by prolonged treatment with phorbol 12-myristate 13-acetate significantly inhibited the hypoxic induction of A2AR. A2AR antagonists led to a significant enhancement of A2AR mRNA levels during hypoxia, whereas A2AR agonists caused down-regulation of A2AR expression during hypoxia. This suggests that A2AR regulates its own expression during hypoxia by feedback mechanisms. We further found that activation of A2AR enhances cell viability during hypoxia and also inhibits vascular endothelial growth factor expression in PC12 cells. Thus, increased expression of A2AR during hypoxia might protect cells against hypoxia and may act to inhibit hypoxia-induced angiogenic activity mediated by vascular endothelial growth factor.

Adenosine (Ado),1 the final metabolite in the stepwise dephosphorylation of ATP, is produced and released in the central nervous system in response to ischemia and hypoxia (1, 2). Once released, Ado acts locally to decrease pre- and postsynaptic excitability, which protects neurons against the metabolic stress associated with oxygen deprivation (3, 4). Ado mediates its effects on neuronal activity via specific membrane receptors, A1, A2, and A3, that are coupled to adenylate cyclase (AC) via G proteins (5, 6). The A1 and A3 receptors are coupled to G1 protein and cause inhibition of AC, whereas the A2 receptor, which consists of the A2A and A2B receptor subtypes, is coupled to G3 protein and causes an increase in AC activity (6).

There is growing evidence that Ado receptors mediate a protective function during hypoxia (7, 8). This is based largely on the finding that the A1 receptor inhibits excitatory synaptic neurotransmission in the brain during hypoxia (7, 8). The role of the A2 receptor in modulating neuronal activity is less clear. However, the observation that A2 receptors are concentrated in brain regions that are rich in dopamine-containing cells (9, 10) suggests that the A2 receptors are involved in regulating the activity of these cells during hypoxic stress. To test this possibility, we studied the effect of A2 receptor stimulation on membrane excitability in the dopaminergic pheochromocytoma (PC12) cell line (11). PC12 cells express the Ado A2A and A2B receptors but not the A1 and A3 receptors (12, 13). We found that activation of the A2AR receptor in PC12 cells attenuated membrane excitability by activation of an outward K+ current and inhibition of an inward voltage-dependent Ca2+ current (13). Thus, the A2 receptor attenuates membrane excitability during hypoxia.

Because of the potential importance of the A2AR in mediating the cellular protection during hypoxia, we wondered if A2AR gene expression is regulated by hypoxia. The current study was undertaken to examine this possibility and to characterize further the role of the A2 receptor in regulating the cellular response to hypoxia. Briefly, we found that A2AR expression is increased during hypoxia by a mechanism that involves increased intracellular free Ca2+, protein kinase C (PKC) and de novo protein synthesis. We also found that A2AR regulates its own expression during hypoxia via a feedback-like mechanism. An important finding was that activation of A2AR increased cell viability during exposure to 1% O2 and that activation of A2AR inhibited expression of vascular endothelial growth factor (VEGF).

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**PC12 cells were purchased from the American Tissue Culture Collection and grown in Dulbecco's modified Eagle's medium/ Ham's F-12 (Life Technologies, Inc.) that contains 15 mM HEPES buffer, 2 mM L-glutamine, 10% fetal bovine serum, penicillin/streptomycin (100 units/ml, 100 μg/ml) in an incubator in which the environment (21% O2 and 5% CO2, remainder N2, 37 °C) was strictly maintained. Medium was changed twice a week. When cells reached 70% confluence, they were either exposed to continued normoxia or placed in a hypoxic incubator (Forma Scientific, Marietta, OH) that maintained a constant environment (10% O2 or 5% O2, 5% CO2, balanced with N2) for the specified exposure periods. PKA-deficient PC12 cells (A123-7) (14) were grown in Dulbecco's modified Eagle's medium with high glucose with 15 mM HEPES, 10% fetal bovine serum, 5% horse serum, and gentamicin (100 μg/ml) and an environment of 21% O2 and 10% CO2 at 37 °C.

**Northern Blot Analysis—**Total cellular RNA was extracted from PC12 cells using TRI-REAGENT (Molecular Research Center, Cincinnati, OH) according to instructions. The RNA pellets were resuspended in formamide, and the RNA concentration and purity were determined by measurement of absorbance at 260 and 280 nm with a spectrophotometer. An aliquot (20 μg) of total RNA was taken, and the volumes
were equilibrated with formamide. An equal volume of denaturation mixture (2× MOPS, 0.8 M formaldehyde) was added to each sample. The samples were then heated to 65 °C for 15 min to ensure complete denaturation and then electrophoresed in a 1% formaldehyde gel (1× MOPS buffer, 0.4 M formaldehyde, 0.1% agarose) at 100 V constant voltage until the 100-bp ladder was resolved. The DNA was visualized for 2–4 h in a buffer (0.05 M sodium phosphate, 10× SSC, 10× Denhardt’s reagent, 0.1 μg/ml denatured salmon sperm DNA, 50% formamide) and then hybridized overnight in a buffer (same as prehybridization buffer but with 10% dextran sulfate) and 1.0 × 10^6 cpm/ml radiolabeled probe. Following hybridization, the membranes were washed three times at 55 °C in 2× SSC, 0.1% SDS and then exposed on a storage phosphor screen (Molecular Dynamics, Inc., Sunnyvale, CA) for 4–5 h. The screen was scanned, and the signals were quantified (Storm, Molecular Dynamics).

A full-length cDNA encoding the Ado A2A receptor was provided by Dr. J. S. Fink (Massachusetts General Hospital, Boston, MA). After bacterial amplification of the plasmid, the EcoRI and XhoI fragment of purifying the Ado A2A receptor was isolated by electrophoresis on a low melting point agarose gel. The 3′-terminal region, which included both coding and noncoding sequence (1192 base pairs), was used as a probe for A2AR Northern blot analysis. The cDNA probe for VEGF was prepared by reverse transcriptase-polymerase chain reaction and subsequent ligation of the products into a plasmid vector, pCR® II-1 (Invitrogen, Carlsbad, CA). Primers were constructed based on the reported rat VEGF cDNA sequence (15). The sequences of primers was 5′-CCA TGA ACT TTC TGC TCT CTP-3′ and 5′-GTT GAG AGG TCT AGT TCC CGA-3′ (predicted length of the amplified DNA fragment is 630 base pairs). The polymerase chain reaction product was sequenced and confirmed to be 100% homology with the reported cDNA sequences. After bacterial amplification of the cloned VEGF cDNA in plasmid pCR® II-1, a VEGF cDNA fragment was excised using ECORI and isolated by electrophoresis on low melting point agarose gel.

The probes were labeled using a random-primed DNA labeling kit (Prime-A-Gene®, Promega, Madison, WI) and [α-32P]deoxycytidine 5′-triphosphate (NEN Life Science Products) and then purified on a Sephadex G-50 column (Roche Molecular Biochemicals).

**Immunoblotting of A2AR Protein**—The effect of hypoxia on A2AR protein level was determined by Western immunoblot analysis. Cells were washed twice with ice-cold phosphate-buffered saline and harvested by scraping the cells into 400 μl of a solution containing 0.25 M sucrose, 25 mM Tris, pH 7.2, 25 mM NaCl, and 5 mM MgCl2. Cells were centrifuged at 30,000 × g for 3 min at 4 °C, and the pellet was resuspended in 250 μl of 10 mM sodium phosphate (pH 7.0) containing 1 mM EDTA, freshly added leupeptin (0.2 μg/ml), aprotinin (2 μg/ml), and dithiothreitol (1 mM) as crude membrane fraction.

Membrane preparations were boiled for 3 min in buffer containing 50 mM Tris, pH 6.7, 2% SDS, 0.5% mercaptoethanol, and bromphenol blue as a marker. Samples containing 40 μg of protein were separated by SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell) using standard electroblotting procedures. Prestained molecular weight markers were obtained from Sigma. To reduce nonspecific binding, blots were preincubated for 1 h in a blocking mixture (3% nonfat dry milk, 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.1% Tween 20) at room temperature, and then incubated with an affinity-purified polyclonal antibody directed against the N-terminus of PC12 cell A2AR (23) at a 1:2000 dilution for 1 h at room temperature. The membranes were washed three times over 1 h in the same buffer.

Immunolabeling was detected by ECL (Amerham Pharmacia Biotech) and quantified using densitometric analysis with an ImagePro digital analysis system (Media Cybernetics, Silver Spring, MD). Ado A2AR immunoreactivity was linear over a 10-fold range of protein concentrations.

**Cell Viability Assays**—Cell viability was measured as the ability of cells to exclude trypan blue (16). PC12 cells were initially grown in 35-mm dishes and then exposed to normoxia or 1% O2 in the presence or absence of NECA or an Ado receptor antagonist 8-PT for various times. Cells were then detached by trypsinization, and resuspended in 1 ml of Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% fetal bovine serum. Cells were further dispersed by passing through a 27-gauge needle. Cells were resuspended in 1 ml of 1 × PBS, and equal volumes of cell suspension and 0.4% trypan blue (Sigma) were mixed and incubated for 10 min at room temperature. Cell viability was then counted using a hemocytometer. Cell viability was determined as the ratio of total viable cells (unstained)/total cell (unstained and stained) × 100%.

**Data Analysis**—The results were expressed as the mean ± S.E. (n represents the number of observations). The analysis of variance was used for evaluating the significance of the obtained data. Statistical significance was accepted at the conventional p < 0.05 level by two-tailed evaluation.

**Materials**—Ado, NECA, 8-PT, diltiazem, cyclohexamide, and actinomycin D were obtained from Sigma. BAPTA/AM, chelerythrine chloride, and calmidazolium chloride were purchased from Research Biochemicals International (Natick, MA).

**RESULTS**

Effect of Hypoxia on Expression of Ado A2AR Receptor mRNA and Protein in PC12 Cells—Northern blot analyses were performed to determine the effect of hypoxia on A2AR gene expression in PC12 cells. PC12 cells were exposed to a reduced O2 level (10 or 5% O2 in 5% CO2) for 3, 6, 12, or 18 h. The upper panel of Fig. 1A shows the temporal profile of Ado A2AR mRNA expression during hypoxia. It can be seen that exposure to 10 and 5% O2 led to a time-dependent increase in A2AR mRNA, which reached a peak at 12 h. The averaged results from six separate experiments for each level of hypoxia are shown in the lower panel. The increase in A2AR mRNA in cells exposed to 5% O2 was greater at all time points than that measured in cells exposed to 10% O2. These results show clearly that A2AR gene expression is stimulated in a time- and dose-dependent manner by hypoxia in PC12 cells.

We next performed immunoblot analyses to investigate the effect of hypoxia on the level of A2AR protein in PC12 cells. Fig. 1B shows the profile of A2AR during increasing duration of hypoxia (5% O2) in PC12 cells. It can be seen that the amount of A2AR protein gradually increased with hypoxia and peaked at 12 h, which is a similar time profile to that found for A2AR mRNA. The averaged results from four separate experiments are provided in the lower panel. A significant increase in A2AR receptor protein was measured at 3, 6, 12, and 18 h. Thus, hypoxia up-regulates both the A2AR mRNA and protein levels.

**Extracellular Ca2+ Is Essential for Up-regulation of A2AR Receptor mRNA during Hypoxia**—Our laboratory has previously shown that an increase in intracellular free Ca2+ is involved in the induction of tyrosine hydroxylase gene expression during hypoxia in PC12 cells (17). We wondered therefore if increased intracellular Ca2+ is involved in regulation of A2AR gene expression during hypoxia. To test this possibility, PC12 cells were exposed to hypoxia in the presence and absence of Ca2+ (5% O2 in 6% and 12 h led to a 2–4-fold increase in A2AR mRNA (Fig. 2). This increase in A2AR mRNA was markedly attenuated when the cells were tested in the absence of extracellular Ca2+ (Ca2+-free medium plus 1 mM EGTA) or chelation of intracellular free Ca2+ with BAPTA/AM (100 μM) (Fig. 2, A and B). Thus, an increase in intracellular free Ca2+ is required for induction of A2AR gene expression by hypoxia. In addition, we also found that the induction of A2AR mRNA during hypoxia was significantly reduced in the presence of diltiazem (3 μM), an L-type Ca2+ channel blocker.
channel blocker. These findings suggest that the Ca\(^{2+}\) influx from the extracellular space via L-type Ca\(^{2+}\) channels is involved in regulation of A2AR gene expression during hypoxia.

**Activation of PKC Is Required for Up-regulation of A2A Receptor mRNA during Hypoxia**—Experiments were next performed to identify the intracellular signaling pathways that mediate the Ca\(^{2+}\)-dependent induction of A2AR gene expression during hypoxia. The two major Ca\(^{2+}\)-mediated signal transduction pathways that are activated by increased cytosolic free Cu\(^{2+}\) are the calmodulin (CaM) and PKC systems (18, 19). Pharmacological studies were performed to evaluate the possible contribution of these two Ca\(^{2+}\)-activated pathways on hypoxic induction of A2AR gene expression. We found that blockade of PKC activity using chelerythrine chloride (CHL) (20 \(\mu M\)) reduced significantly the induction of A2AR gene during hypoxia (Fig. 3A). CHL is a potent and selective inhibitor of the catalytic domain of Ca\(^{2+}\)-dependent PKC isoforms (20). This finding was supported by an additional result that showed that RO-31–8220, another potent selective inhibitor of PKC (21), completely blocked the hypoxic induction of A2AR gene expression (Fig. 3A). In contrast, inhibition of CaM with calmidazolium chloride (CMZ, 20 \(\mu M\)) had no effect on the induction of A2AR gene expression during hypoxia (Fig. 3A). CMZ has been widely used to determine the role of CaM in mediating specific biological responses (22, 23). The dose of CMZ used in our experiment was sufficient to block the effect of CaM (22). We also found that a higher dose of CMZ (40 \(\mu M\)) had no effect on the hypoxia-induced up-regulation of A2AR mRNA (data not shown). These data indicate that the CaM/Ca\(^{2+}\) pathways are not involved in regulating the hypoxia-induced regulation of A2AR gene expression.

The role of PKC in hypoxia-induced enhancement of A2AR mRNA was further examined by incubating cells with 100 nm phorbol 12-myristate 13-acetate (PMA), an activator of PKC. Fig. 3C shows the effect of PMA on A2AR mRNA. The A2AR mRNA level initially increased and peaked at 3 h but then declined to a level below the original base-line level at 12 h. It is well known that prolonged incubation of cells with PMA leads to down-regulation of PKC (24). We next examined the effect of long term (6 and 12 h) incubation of PC12 cells in PMA on hypoxic activation of A2AR gene expression. Our findings show that depletion of PKC activity by prolonged PMA treatment abolished the hypoxia-induced up-regulation of A2AR message (Fig. 3D). These findings further support the involvement of PKC in hypoxia-induced regulation of A2AR gene expression. We therefore conclude that the induction of A2AR gene expression during hypoxia requires increased intracellular free Ca\(^{2+}\) and activation of PKC.

**Role of PKA Pathway in Regulation of A2A Receptor Gene Expression during Normoxia and Hypoxia**—Because the PKA is activated by depolarization and because hypoxia causes depolarization in PC12 cells (25), we tested the possibility that PKA is involved in the regulation of A2AR gene expression during hypoxia. Our strategy was to use a clonal cell line (A123.7) that was derived from PC12 cells and deficient in PKA enzyme activities (14). The induction of A2AR gene during 12-h exposure to 5% O\(_2\) was slightly greater in A123.7 cells than in the wild type PC12 cells (Fig. 4A). These results indicate that PKA is not responsible for the up-regulation of A2AR during hypoxia and that PKA might actually have an inhibitory effect on induction of A2AR gene expression during hypoxia.

We also examined the possibility that activation of PKA by 8-bromo-cAMP induces down-regulation of A2AR mRNA under normoxic conditions. This was accomplished by incubation of cells with 2 mM of 8-bromo-cAMP under normoxic conditions (21% O\(_2\)) for 3 and 6 h. Our results show that activation of PKA caused down-regulation of A2AR (Fig. 4B). We conclude therefore that PKA inhibits the expression of A2AR gene under normoxic conditions and that PKA is not involved in mediating increased A2AR gene expression during hypoxia.

**Role of the Ado Receptor in Regulation of A2AR Gene Expression during Normoxia and Hypoxia**—PC12 cells produce and
markedly attenuated in the absence of extracellular Ca\textsuperscript{2+} receptor-selective agonist NECA (10\textsuperscript{−6}M), an inhibitor of translation, for 30 min prior to exposure to hypoxia (5% O\textsubscript{2}; 6 or 12 h). We found that the hypoxic induction of A2AR gene expression was totally abolished in the presence of CHX (Fig. 6A). Thus, the regulation of A2AR gene expression during hypoxia requires de novo protein synthesis, which may include known and unknown transcription factors.

Lack of Effect of Hypoxia on Stability of A2A Receptor mRNA—Alterations in the levels of an mRNA may result from changes in gene transcription or mRNA stability or a combination of both. We examined the effect of hypoxia on the stability of A2AR mRNA. Transcription was blocked pharmacologically by pretreatment of PC12 cells with actinomycin D (3 \mu g/ml), a nonspecific blocker of RNA polymerase (28), and the time course for the decay of A2AR mRNAs was measured. In this experiment, 30 \mu g of total RNA was used for analysis. The results showed that there was no difference in the degradation time course in A2A receptor mRNA in cells exposed to either normoxia or hypoxia (Fig. 6B). Thus, hypoxic regulation of A2AR gene expression does not appear to involve an increase in A2AR mRNA stability.

Role of A2R Stimulation in Modulating VEGF Gene Expression during Hypoxia—We next examined the role of A2R stimulation in regulating the expression of VEGF, another hypoxia-inducible gene, which is induced by hypoxia in PC12 cells (29). We found that the A2R agonist, NECA (10 \mu M), caused an initial increase in VEGF gene expression during normoxia, which was followed by a progressive inhibition of VEGF mRNA levels (Fig. 7A). We also examined the hypoxia-induced regulation of VEGF gene expression in the presence of an A2 receptor antagonist (8-PT) during hypoxia. VEGF gene expression was examined in cells exposed to hypoxia (5% O\textsubscript{2}) in the presence or absence of the A2 receptor antagonist, 8-PT (10 \mu M). Our results revealed that the level of VEGF mRNA was significantly higher in the presence of 8-PT during hypoxia lasting 6 h or longer (Fig. 7B). These results indicate that activation of A2R by Ado inhibits the expression of VEGF gene expression during prolonged exposure to hypoxia in PC12 cells.

Effect of A2R Stimulation on Cellular Viability during Hypoxia—We examined the effect of A2R stimulation on cell viability during severe hypoxia exposure (1% O\textsubscript{2}), which was measured in the presence of NECA (10 \mu M) or 8-PT (10 \mu M) at various time points. We found that 12-h exposure to 1% O\textsubscript{2} caused a 30% reduction in cell viability (Fig. 8). Cell viability was significantly enhanced in the presence of NECA, while it was significantly reduced in the presence of 8-PT. These results suggest that activation of A2R by Ado inhibits the expression of VEGF gene expression during prolonged exposure to hypoxia in PC12 cells.

**DISCUSSION**

Adenosine is a potent modulator of cellular activity during hypoxia (30). We showed previously that activation of the A2AR inhibits membrane excitability during hypoxia by enhancing an outward K\textsuperscript{+} current and inhibiting an inward Ca\textsuperscript{2+} current (13). These are among the first results to show that the A2AR regulates cellular activity during hypoxia. A primary finding in the current study was that hypoxia causes a time-dependent increase in A2AR gene expression in PC12 cells. A number of enzymes, cytokines, and growth factors are inducible by hypoxia (31, 32). However, there have been few reports that support the idea that hypoxia mediates a feedback-like mechanism of gene expression via a feedback-like mechanism.

**Effect of A2R Stimulation on Cellular Viability during Hypoxia**—We next examined the role of A2R stimulation in regulating the expression of VEGF, another hypoxia-inducible gene, which is induced by hypoxia in PC12 cells (29). We found that the A2R agonist, NECA (10 \mu M), caused an initial increase in VEGF gene expression during normoxia, which was followed by a progressive inhibition of VEGF mRNA levels (Fig. 7A). We also examined the hypoxia-induced regulation of VEGF gene expression in the presence of an A2 receptor antagonist (8-PT) during hypoxia. VEGF gene expression was examined in cells exposed to hypoxia (5% O\textsubscript{2}) in the presence or absence of the A2 receptor antagonist, 8-PT (10 \mu M). Our results revealed that the level of VEGF mRNA was significantly higher in the presence of 8-PT during hypoxia lasting 6 h or longer (Fig. 7B). These results indicate that activation of A2R by Ado inhibits the expression of VEGF gene expression during prolonged exposure to hypoxia in PC12 cells.

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show that gene expression for cell surface receptors is regulated by hypoxia. One of the few examples was that α- and β-adrenergic receptor mRNAs is increased in cardiac cells during hypoxia (33, 34). Interestingly, the A1 type Ado receptor is down-regulated in rat cardiac myocytes during long term hypobaric hypoxia (35). It was also reported that A2AR mRNA levels are decreased in neonatal brain during cerebral ischemia following carotid artery ligation (36). In this study, the ischemic hemisphere de-
veloped an infarction. Therefore, it is possible that cell death in the region of the infarction was responsible for the reduction in A2AR expression. Here we present the first direct evidence that A2AR mRNA and protein levels are enhanced by hypoxia. The mild hypoxia used in the current study indicates that the regulation of A2AR may be involved not only in severe hypoxic events such as ischemic trauma but also in more physiologic processes like high altitude adaptation.

The signal transduction systems that are activated by hypoxia and eventually culminate in altered expression of O2-responsive genes are largely unknown. Previous papers from our laboratory have shown that hypoxia causes membrane depolarization and an increase in intracellular Ca2+ in PC12 cells (17, 25). Thus, activation of a voltage-dependent Ca2+ channel and a subsequent increase in intracellular free Ca2+ might be critical regulatory events in the cellular response to hypoxia in PC12 cells. An elevation of intracellular Ca2+ ion can influence a wide variety of biological processes during hypoxia. For example, we reported previously that increased cytosolic Ca2+ is required for the regulation of certain hypoxia-responsive genes (e.g. c-fos, junB, and tyrosine hydroxylase), and neurotransmitter release during hypoxia (17, 37). In the present study, we found that an increase in intracellular free Ca2+ is also involved in regulation of A2AR gene expression during hypoxia. An influx of extracellular Ca2+ through L-type Ca2+ channels is likely to be responsible, since induction of A2AR gene expression was markedly reduced by removal of extracellular free Ca2+ and by inhibition of the L-type Ca2+ channel. Moreover, chelation of intracellular free Ca2+ with BAPTA/AM also prevented activation of A2AR gene expression during hypoxia. These results strongly indicate that an increase in cytosolic Ca2+, from the extracellular space, is the trigger that mediates the induction of A2AR gene expression during hypoxia in PC12 cells.

Several laboratories have reported alterations in second messenger systems during hypoxia (16, 38). Many eukaryotic genes are regulated in a Ca2+-dependent manner through Ca2+-dependent phosphorylation/dephosphorylation of gene promo-

**FIG. 5. Effect of Ado receptor stimulation on expression of Ado A2A receptor mRNA during normoxia and hypoxia.** A, the regulation of A2AR gene expression during hypoxia was examined in the presence of 10 μM 8-PT, an Ado receptor antagonist. The upper panel shows a representative blot. The induction of A2AR mRNA during 12-h hypoxia was significantly enhanced in the presence of 8-PT (†, p < 0.05; n = 5 for each group). B, PC12 cells were incubated with 10 μM NECA, an Ado A2 receptor-selective agonist, for 6 and 12 h during normoxia. The levels of A2AR mRNA were significantly reduced from baseline (**, p < 0.01; n = 4). 30 μg of total RNA was used in this experiment. C, effect of Ado receptor activation by NECA on A2AR mRNA expression during hypoxia. Activation of Ado receptor with NECA significantly attenuated the induction of Ado receptor mRNA during hypoxia (†, p < 0.05; n = 4). Means ± S.E. are shown.

**FIG. 6. Translation and transcription dependence of regulation of A2A receptor mRNA during hypoxia in PC12 cells.** A, a representative blot showing the effect of de novo protein synthesis inhibition on regulation of A2AR expression during hypoxia. PC12 cells were pretreated with cyclohexamide (CHX; 5 μM), an inhibitor of protein translation, under normoxia for 30 min and then transferred to a hypoxic chamber (5% O2) in the presence of the same dose of CHX for 6 and 12 h. The averaged data (± S.E.) from four separate experiments are shown in the lower panel. The induction of the A2AR gene during hypoxia was totally inhibited in the presence of CHX. B, effect of hypoxia on stability of A2A receptor mRNA. PC12 cells were pretreated with actinomycin D (5 μg/ml), a RNA polymerase inhibitor, for 15 min prior to incubation in either normoxia or 5% O2 for 1, 3, 6, and 12 h. A 40-μg aliquot of total RNA was used in these experiments. There was no difference in the time course for decay of A2AR mRNA between normoxia and hypoxia. Means ± S.E. are shown (n = 3).
Role of A2 receptor stimulation in modulating VEGF gene expression during normoxia and hypoxia. A, effect of A2 receptor stimulation on VEGF mRNA levels under normoxia in PC12 cells. The level of VEGF mRNA increased temporally at 1 h and then decreased below the base-line level at 3 h and after. The averaged data (± S.E.) from four separate experiments are shown in the lower panel (**, p < 0.01 from base line). B, the regulation of VEGF gene expression during hypoxia was examined in the presence or absence of 10 μM 8-PT, an Ado receptor antagonist. The upper panel shows a representative blot for each group. Exposure to 1% O₂ induced up-regulation of VEGF mRNA, which reached a peak at 6 h and then declined at 12 and 18 h. The decline in the VEGF mRNA levels at 12 h and after was smaller in cells incubated with 8-PT. The averaged data were shown in the lower panel. The induction of VEGF mRNA during 12- and 18-h hypoxia was significantly enhanced in the presence of 8-PT (†, p < 0.05 from hypoxia without 8-PT; n = 4 for each group).

Effect of A2 receptor stimulation on cell viability during severe hypoxia in PC12 cells. Cell viability was measured as the ability of cells to exclude trypan blue dye. It was evaluated as the ratio of total viable cells (unstained) to total cells (unstained and stained) × 100. PC12 cells were exposed to 1% O₂ in the presence or absence of an A2 receptor agonist (10 μM NECA) or an Ado receptor antagonist (10 μM 8-PT) for 1, 2, 3, 6, and 12 h. Exposure to 1% O₂ significantly reduced the cell viability at 3 h and more (**, p < 0.01). The viability was significantly enhanced in the presence of A2R activation (NECA) at 12 h (†, p < 0.05), while it was reduced by the blockade of Ado receptor with 8-PT (†, p < 0.05). Data are expressed as means ± S.E. Three separate dishes of cells were used for each experiment.

Regulation of A2AR Gene Expression during Hypoxia in PC12 Cells
shown that c-fos is essential for functional activation of AP1 and subsequent activation of tyrosine hydroxylase transcription during hypoxia (53). Further studies are required to identify the molecular mechanisms by which PKC modulates the transcription of A2AR message during hypoxia.

One of the most interesting and potentially most important findings was that A2AR plays a role in the regulation of its own gene expression during hypoxia. We found that activation of A2AR with the A2 receptor agonist NECA caused down-regulation of the basal level of A2AR expression and prevented enhancement of A2AR expression during hypoxia. It was reported previously that the A2A receptor gene is regulated by A2 agonist stimulation (54). A novel finding in the present study is that the induction of A2AR gene expression during hypoxia was enhanced by the Ado receptor antagonist, 8-PT, which is known to act primarily as an Ado receptor blocker. The phophodies-erase inhibitor activity of this drug is minimal at the concentration used in this study (55). Our results suggest that expression of the A2AR gene might be inhibited by activation of A2AR in a negative feedback manner. Support for this comes from findings that showed the A2 receptor in PC12 cells is functionally activated by endogenously released Ado (26, 27). We also found that PC12 cells release Ado during hypoxia in an O2 level-dependent manner.2 We propose that Ado feedback regulation of A2AR gene expression might be an important component of the cellular responses to hypoxia, which serves to coordinate the metabolic demand with functional activities during hypoxia.

Our results also show that Ado A2 receptors play a role in protecting cells against the harmful effects of hypoxia. We showed previously that Ado attenuates the hypoxia-induced elevation of intracellular free Ca2+ in PC12 cells (13). It is generally thought that one of the most important pathophysiological factors underlying the cellular damage during ischemia is a failure to regulate intracellular Ca2+ concentration (56). We found that activation of AdoR enhances cell viability during exposure to severe hypoxia. Although the role of the A2 receptor in protection against ischemia is still controversial (57, 58), our results suggest that the protective effect of Ado receptors is mediated via modulation of intracellular Ca2+ homeostasis.

Finally, we examined the possibility that Ado modulates the expression of other hypoxia-inducible genes. We found that prolonged activation of A2AR caused an inhibition of VEGF gene expression during normoxia in PC12 cells. More importantly, we found that increased VEGF expression during hypoxia was enhanced by the Ado receptor antagonist, 8-PT. The role of Ado in mediating the induction of the VEGF gene remains controversial, but it is likely to be Ado receptor subtype-dependent (59, 60). It has been shown that Ado decreases the VEGF mRNA expression via stimulation of the A2 receptor, whereas it stimulates VEGF expression via the A1 receptor (60). Our study also reveals that Ado may modulate the hypoxic induction of VEGF mRNA. Therefore, Ado may have common roles in modulating the regulation of O2-sensitive genes including its own receptors during hypoxia. Since we have shown that hypoxia up-regulates the expression of A2AR gene and protein, it is most likely that increased A2AR during hypoxia has significant roles in mediating cellular functions such as protection of cell viability and modulation of O2-sensitive gene expression.

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