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Hypoxia Regulates the cAMP- and Ca^{2+}/Calmodulin Signaling Systems in PC12 Cells

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Hypoxic/ischemic trauma is a primary factor in the pathology of various disease states. Yet, very little is known about the molecular mechanisms involved in cellular responses and adaptations to hypoxia. As a means of identifying intracellular signaling systems that are regulated in response to hypoxia, the effects of acute and chronic hypoxia on the activity of protein kinase A (PKA) and Ca^{2+}/CaM-dependent protein kinase II (CaMK-II) were evaluated in rat pheochromocytoma (PC12) cells. Chronic (>6 hr), but not acute exposure to hypoxia (5% O_2) significantly decreased both PKA enzyme activity and immunoreactivity compared to control levels. This effect was not due to hypoxia-induced alterations in cell number or viability. Similarly, chronic hypoxia significantly decreased CaMK-II enzyme activity and protein levels in PC12 cells. These data demonstrate that down-regulation of the cAMP and Ca^{2+}/CaM-signaling systems is a mechanism by which PC12 cells adapt to long-term hypoxia.
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Mammalian cells are critically dependent on oxygen for survival. Neurons in particular, are extremely sensitive to hypoxic/ischemic insult, and are subject to substantial injury in a very short time (1-3). The molecular mechanisms by which neurons sense and respond to a reduction in O_2 are not known. Some of the cellular effects of hypoxia in excitable cells have been shown to be mediated by O_2-sensitive voltage-dependent ion channels (for review see 4). Much of this work comes from type I cells of the carotid body, an O_2-sensing organ that controls mammalian ventilation rate during hypoxia (5). Type I cells are catecholaminergic cells that respond to hypoxia with depolarization, Ca^{2+} influx, and dopamine release (4). A small number of O_2-regulated genes have been identified in excitable cells, including tyrosine hydroxylase (6,7), vascular endothelial growth factor (VEGF) (8), and c-fos and JunB (9). One mechanism by which hypoxia regulates gene expression is via the transcription factor HIF-1 (hypoxia-inducible factor-1), which binds to a specific DNA element that has been identified in the erythropoietin gene several other O_2-sensitive genes (10-12). Very little is known about the intracellular signaling systems that control HIF-1 expression and DNA binding, though there is some general evidence that protein phosphorylation may play a role (13).

Rat pheochromocytoma (PC12) cells are a catecholaminergic, excitable cell type that has been widely used as an *in vitro* model for neural cells (14, 15). Interestingly, PC12 cells are highly sensitive to changes in O_2 levels (7, 16). Like type I cells, PC12 cells respond to hypoxia with depolarization (16, 17), Ca^{2+} influx (17, 18), and dopamine release (19). Depolarization-induced activation of tyrosine hydroxylase gene expression has been shown to require the cAMP-response element (CRE) (20). Furthermore, regulation of dopamine release during hypoxia also suggests that the cAMP signaling system may be responsive to hypoxia. Both D1 and D2 dopamine receptor transcripts are present in PC12 cells (data not shown), and these dopamine receptor subtypes are coupled to stimulation and inhibition of intracellular cAMP production, respectively (21, 22). Depolarization and the resulting increase in intracellular Ca^{2+} levels that occur during hypoxia in PC12 cells suggests that a variety of Ca^{2+}-dependent protein kinases and phosphatases may also be regulated, including Ca^{2+}/calmodulin-dependent protein kinases (20, 23). An acute reduction in oxygen tension also activates expression of a number of genes in PC12 cells, including tyrosine (7), VEGF (8), and the immediate early genes c-fos and JunB (9). However, the mechanism by which hypoxia is sensed and transduced into changes in gene expression is entirely unknown, in part because

Abbreviations used: PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; CaMK-II, Ca^{2+}-calmodulin-dependent protein kinase III; PC12, pheochromocytoma; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PBST, phosphate-buffered saline Tween-20; VEGF, vascular endothelial growth factor.

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unlike most neurotransmitters, drugs, and hormones, hypoxia does not appear to act via a classical "ligand-receptor" pathway. Still, virtually all extracellular signals mediate their biological responses at some level via regulation of protein phosphorylation systems. Given the universal role of protein phosphorylation in signal transduction, and the fact that an excitable, O_2-sensitive cell line (PC12) is depolarized by hypoxia, we hypothesized that O_2-related signaling is regulated, at least in part, by regulation of the cAMP and Ca^{2+}/CaM protein kinase pathways. These studies were undertaken to identify intracellular signaling systems involved in response and adaptation to the effects of acute and chronic hypoxia in PC12 cells.

MATERIALS AND METHODS

Cell culture. All tissue culture reagents were obtained from Life Technologies, Inc., Gaithersburg, MD. PC12 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 supplemented with 15 mM Hepes pH 7.4, 10% fetal bovine serum and with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in incubators which strictly maintained a normoxic environment of 21% O_2, 5% CO_2 balanced with N_2. When cells reached 75% confluence in 35 mm tissue culture dishes, they were exposed either to continued normoxia or placed in an oxygen-regulated incubator (Forma Scientific, Marietta, OH) in an environment of 5% O_2, 5% CO_2, balanced with N_2, for various times. In previous studies, we have shown that the partial pressure of oxygen in the media of cells exposed to 5% O_2 is in the range of 30-50 mm Hg (7).

Cell viability assays. Cell viability was measured as the ability of cells to exclude trypan blue. PC12 cells were grown in 35 mm dishes and exposed either to normoxia or hypoxia, for various times. Cells were then detatched by trypsinization, and resuspended in 1 ml DMEM/F12 medium containing 10% FBS. Cells were further dispersed by passing the suspension through a 27 gauge needle. Cells were then resuspended in 1 ml of phosphate-buffered saline, and equal volumes of cell suspension and 0.4% trypan blue (Sigma, St. Louis, MO) were mixed and incubated for 10 min at room temperature. Cells were then counted counted using a hemocytometer. Cell viability (%) was determined as the ratio of total viable cells (unstained) / total cells (unstained and stained) $\times$ 100.

PKA and CaMK enzyme assays. Cells were washed twice with ice-cold phosphate-buffered saline and harvested by scraping in 0.5 ml of a solution containing 0.25 M sucrose, 25 mM Tris pH 7.2, 25 mM NaCl and 5 mM $MgCl_2$. Cells were collected by centrifugation for 5 min at 3000 $\times$ g at 4°C. The supernatant was removed by aspiration and the cells were sonicated at 4°C for 2 seconds with a microultrasonic cell disrupter (Kontes, Vineland, NJ) in 200 μl of a solution containing 0.25 M sucrose, 10 mM sodium phosphate pH 7.0, 1 mM EDTA, and freshly added leupeptin (2 μg/ml), aprotinin (2 μg/ml) and dithiothreitol (1 mM). PKA activity was assayed for 6 min at room temperature in a final volume of 50 μl which included 25 mM Tris pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 2.5 mM NaF, 0.005% nonidet P-40, 5 mM dithiothreitol, 50 μM ATP (containing 0.1 μCi of $\gamma[^{32}P]$-ATP per assay), 75 μM kemptide, 2-10 μg of protein from crude cellular extracts, and either 10 μM 8-Br-cAMP or 10 μM PKI_{6-22}. CaMK-II enzyme activity was assayed for 6 min at room temperature in a final volume of 60 μl which included 50 mM HEPES pH 7.4, 150 mM NaCl, 100 mM $MgCl_2$, 2 mM EGTA, 0.005% nonidet P-40, 0.0002% β-mercaptoethanol (v/v), 50 μM ATP (containing 0.1 μCi of $\gamma[^{32}P]$-ATP per assay), 10 μM calmodulin, 10 μM autocamtide-2, 1-10 μg of protein from crude cellular extracts, and either 0 or 2.2 mM $CaCl_2$ ([0.2 mM] Ca^{2+}_{free}). Reactions were initiated by the addition of ATP and terminated by the addition of 20 mM EGTA (for CaMK-II assays) and spotting 40 μl aliquots on 1.5 $\times$ 1.5 cm squares of Whatman p81 phosphocellulose papers followed by immersion in 75 mM H_3PO_4. Filter papers were washed twice for 5 min in 75 mM H_3PO_4, followed by one 5 min wash in water and one 5 min wash in absolute ethanol. Filters were then air dried and subjected to liquid scintillation counting. PKA specific activity was calculated as the difference in 8-Br-cAMP-stimulated phosphorylation of kemptide and phosphorylation found in the presence of PKI_{6-22}, a specific inhibitor of PKA. 8-Br-cAMP-stimulated activity averaged 20-fold higher than that observed in the presence of PKI_{6-22}. CaMK-II activity was calculated as both basal (in the absence of Ca^{2+}) and Ca^{2+}-stimulated, which averaged 12-fold higher than that observed under basal conditions. In each experiment, kinase activity levels were normalized per μg protein. PKA enzyme activity was found to be linear over a 30-fold range of protein concentrations (between 0.5 and 15 μg protein per assay), and CaMK-II enzyme activity was linear over a 10-fold range of protein concentrations (between 1 and 10 μg protein per assay).

Immunoblotting. Crude cellular homogenates were boiled for 2 min in sample buffer containing 50 mM Tris pH 6.7, 2% SDS, 2% β-mercaptoethanol, and bromophenol blue as a marker. Samples containing 15-40 μg of protein were then run on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using standard electrophoresis and electroblotting procedures. Nitrocellulose membranes were blocked with 3% nonfat dry milk in a buffer containing 10 mM sodium phosphate (pH 7.2), 140 mM NaCl, and 0.1% Tween 20 (PBST). Blots were then imunolabeled overnight at 4°C with antibodies for either the PKA RIβ regulatory subunit (1:1000), the PKA Cα catalytic subunit (1:250), or CaMK-II (1:500), obtained from Transduction Laboratories (Lexington, KY). Immunolabeling was detected by enhanced chemiluminescence (ECL, Amersham, Chicago, IL) according to the manufacturer's recommended conditions. Immunoreactivity was quantified using densitometric analysis with an ImagePro digital analysis system (Media Cybernetics, Silver Spring, MD). At the dilutions of antibodies used, immunoreactivity for PKA-RIβ, PKA-Cα, and CaMK-II was found to be linear over a five-fold range of protein concentrations.

RESULTS

The effect of hypoxia on PKA enzyme activity in PC12 cells was examined. It was found that chronic (>6 hr) exposure to hypoxia decreased PKA activity by approximately 40% from control levels (Figure 1). Average enzyme activity levels in control PC12 cells were 3.2 $\pm$ 0.6 pmol/min/mg (basal) and 61 $\pm$ 9 pmol/min/mg (8-Br-cAMP-stimulated). Both basal (data not shown) and 8-Br-cAMP-stimulated PKA activity levels were reduced by chronic hypoxia. PKA is localized in both cytosolic and membrane-associated cellular fractions (27, 28). Enzymatic compartmentalization is presumably related to regulating phosphorylation of specific substrate proteins. Thus, it was of interest to determine whether hypoxia targeted a specific subcellular fraction of PKA activity. Cells were exposed for 72 hrs to either normoxic or hypoxic conditions and then separated into soluble and particulate fractions. The majority (~75%) of PKA enzyme activity in PC12 cells was found in the soluble fraction. In Figure 2, it can be seen that hypoxia

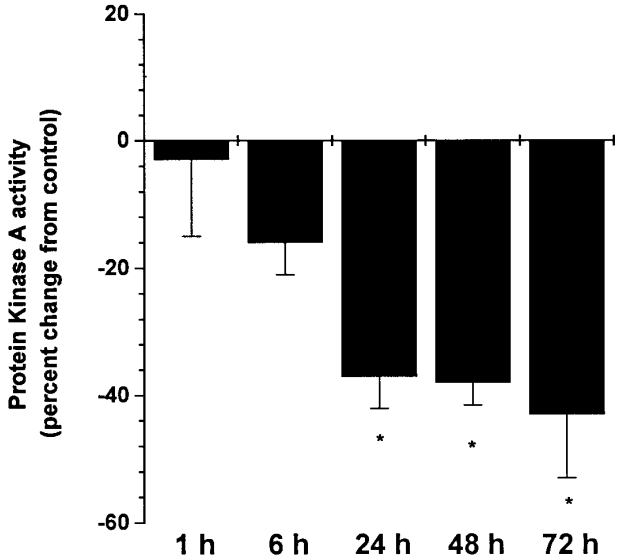

FIG. 1. Effect of hypoxia on PKA enzyme activity in PC12 cells. PC12 cells were exposed to either hypoxia (5% oxygen) or normoxia (controls, 21% oxygen) for various times, as indicated. Cells were assayed for 8-Br-cAMP-stimulated PKA activity as described in Materials and Methods. Data are expressed as percent change from control ± s.e.m., and represent N = 8 to 12 individual dishes in each group, each performed in at least 2 separate experiments. *$p < 0.005$ by χ^2 test.

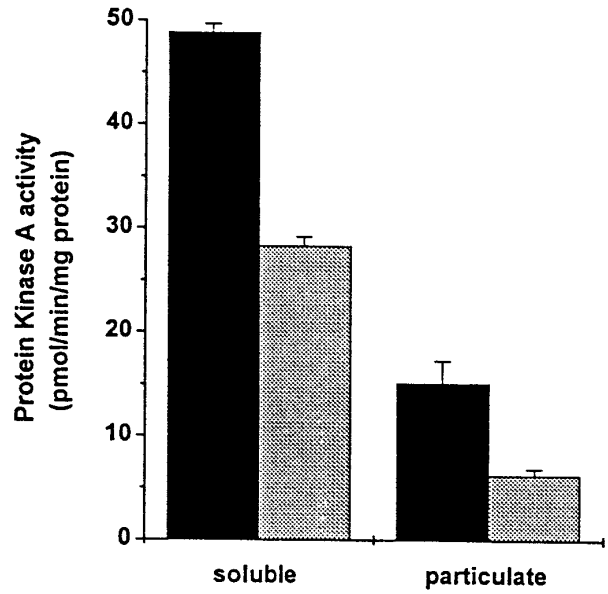

FIG. 2. Comparison of effect of hypoxia on PKA activity in soluble and particulate subcellular fractions. PC12 cells were exposed to either normoxia (*black bars*, 21% oxygen) or hypoxia (*grey bars*, 5% oxygen) for 72 hr. Cells were then separated into soluble and particulate subcellular fractions by centrifugation at 40,000*g* at 4°C for 20 min. Each fraction was assayed for PKA activity as described in Materials and Methods. Data are expressed as average activity in pmol/min/mg ± s.e.m., and represent N = 6 individual dishes in each group.

down-regulated PKA enzyme activity by approximately 50% in both the particulate and soluble fractions, similar to the effects seen in crude whole cell homogenates. These effects did not result from a hypoxia-induced decrease in cell viability. Cell viability, as measured by trypan blue exclusion, was greater than 95% in cells exposed to either normoxic or hypoxic conditions (up to 72 hours, see Table 1).

The hypoxia-induced down-regulation of PKA activity in PC12 cells could either be due to a decrease in the amount of PKA protein expressed by the cells or due to an alteration in the enzymatic activity. In order to address this question, homogenates from normoxic and hypoxic cells were analyzed by immunoblotting for the PKA regulatory (RIβ) and catalytic (Cα) subunits. It was found that hypoxia induced a down-regulation of immunoreactivity for both PKA- RIβ and PKA-Cα (Figure 3A), similar to the effects of hypoxia on PKA enzyme activity. That is, chronic (24 to 72 hr), but not acute, hypoxia decreased PKA levels by 25 to 75%, as compared to control (normoxic) levels. At all time points, the regulatory (RIβ) subunit was slightly more responsive to down-regulation by hypoxia than the catalytic (Cα) subunit (Figure 3B).

Given that one of the effects of hypoxia in PC12 and other excitable cells is to elevate intracellular Ca^{2+} levels (4, 16, 18), it is possible that Ca^{2+}-dependent signaling systems might also be responsive to hypoxia in this cell type. The effect of hypoxia on CaMK-II was therefore evaluated. It was found that chronic (>3 hr) exposure to hypoxia decreased CaMK-II enzyme activity by 30 to 40% (Figure 4), similar to the effect of hypoxia on PKA activity. The substrate used in these assays, autocamtide-2, is highly specific for CaMK-II (27). Both basal (data not shown) and Ca^{2+}-stimulated CaMK-II activity levels were significantly reduced by chronic hypoxia. We next examined the effect of hypoxia on

TABLE 1

Lack of Effect of Hypoxia on Cell Number or Cell Viability

Time exposure to hypoxia	Number cells/cm^2	% Viability
0	94,600 ± 9,200	98.3%
1 hr	94,300 ± 13,900	96.3%
24 hr	112,800 ± 5,600	98.8%
48 hr	80,000 ± 13,080	97.8%
72 hr	94,100 ± 7,000	99.4%

Cells were grown in 35 mm dishes and exposed to hypoxia (5% oxygen) for various times between 0 and 72 hours, as indicated. Cells were suspended in phosphate-buffered saline, incubated with trypan blue as described in materials and methods, and counted with a hemacytometer. Cell viability (%) was determined as the ratio of total viable cells (unstained)/total cells (stained and unstained). Data are expressed as average ± s.e.m., and represent N = 3 dishes in each group.

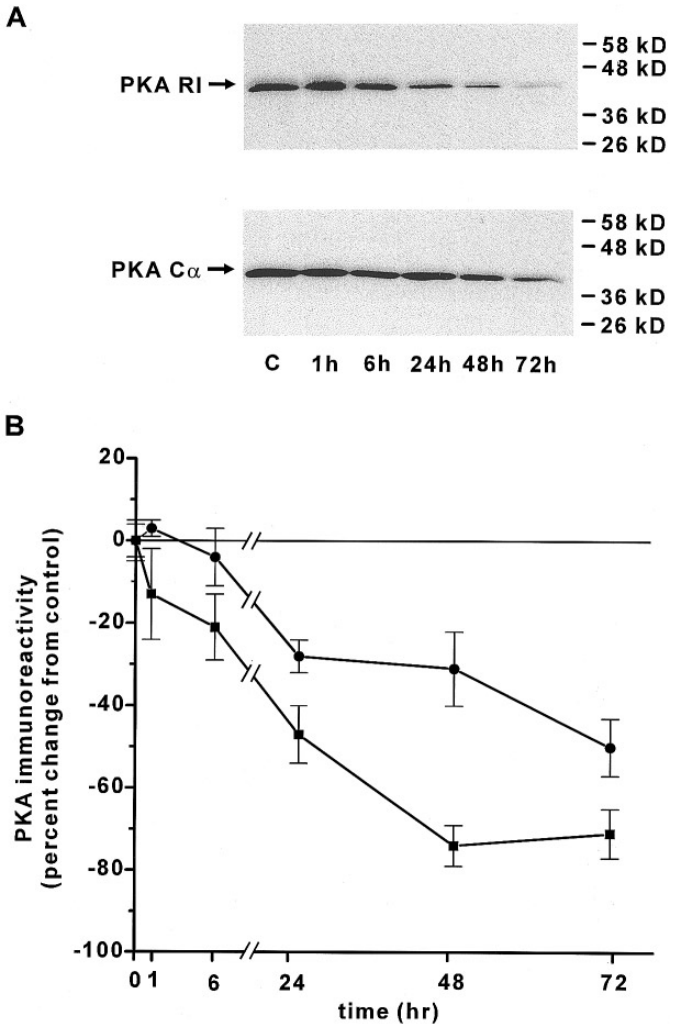

FIG. 3. Effect of hypoxia on immunoreactivity of PKA regulatory (RIβ) and catalytic (Cα) subunits. PC12 cells were exposed to either hypoxia (5% oxygen) or normoxia (controls, 21% oxygen) for various times, as indicated. Cells were immunoblotted for either PKA-RIβ or PKA-Cα as described in Materials and Methods. A. Representative immunoblots showing levels of PKA-RIβ and PKA-Cα immunoreactivity at the various time points studied. B. Levels of PKA-RIβ (■) and PKA-Cα (●) immunoreactivity as quantitated by densitometric analysis. Data are expressed as average percent change from control ± s.e.m., and represent N = 8 individual dishes in each group, each performed in 2 separate experiments.

CaMK-II protein levels. As shown in Figure 5, chronic (>6 hr), but not acute hypoxia, induced a decrease in CaMK-II immunoreactivity in PC12 cells.

DISCUSSION

The intracellular pathways involved in response to hypoxia are largely unknown, and there is even less information about the long-term effects of hypoxia. The chronic effects of hypoxia are particularly relevant to understanding the molecular processes underlying hypoxic and/or ischemic damage in the nervous system. PC12 cells are an O_2-sensitive cell line that provides a useful system to study the effects of hypoxia on catecholaminergic gene expression (7, 18, 28). PC12 cells are exquisitely sensitive to hypoxia, in that very small reductions in atmospheric O_2 (from 21% to 15% O_2) dramatically induce tyrosine hydroxylase gene expression and mRNA stability (7). Such reductions in O_2 levels also induce depolarization and inhibition of an O_2-regulated outward K^+ current (16, 17). Depolarization of PC12 cells is known to activate Ca^{2+}/calmodulin dependent protein kinases, and to stimulate cyclic AMP response element (CRE)-dependent gene transcription in this cell type (20, 23, 29). These studies were undertaken to evaluate the role of the cAMP and Ca^{2+}/CaM signaling systems in acute and chronic hypoxia in the PC12 cell line.

Oxygen is the final electron acceptor in ATP synthesis, and is thereby an absolute requirement for most biological systems. Thus, an important consideration for these studies is whether hypoxia alters protein phosphorylation systems simply by limiting cellular ATP pools. Whereas severe hypoxia (<20 mm Hg) and/or anoxia can decrease ATP production, it has been convincingly established in a number of studies that mild to moderate hypoxia, such as that used in these studies (5% O_2, PO_2 ~50 mm Hg) does not alter ATP levels (30, 31). Furthermore, hypoxia (5% O_2, up to 24 hr) does not decrease overall protein kinase activity in PC12 cells, as measured by endogenous phosphorylation assays (32).

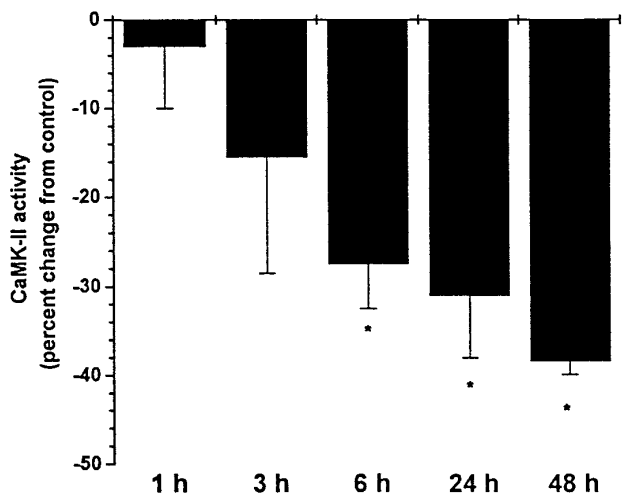

FIG. 4. Effect of hypoxia on CaMK enzyme activity in PC12 cells. PC12 cells were exposed to either hypoxia (5% oxygen) or normoxia (controls, 21% oxygen) for various times, as indicated. Cells were assayed for Ca^{2+}/CaM-stimulated kinase activity as described in Materials and Methods. Data are expressed as percent change from control ± s.e.m., and represent N = 6 to 16 individual dishes in each group, each performed in at least 2 separate experiments. *$p < 0.01$ by χ^2 test.

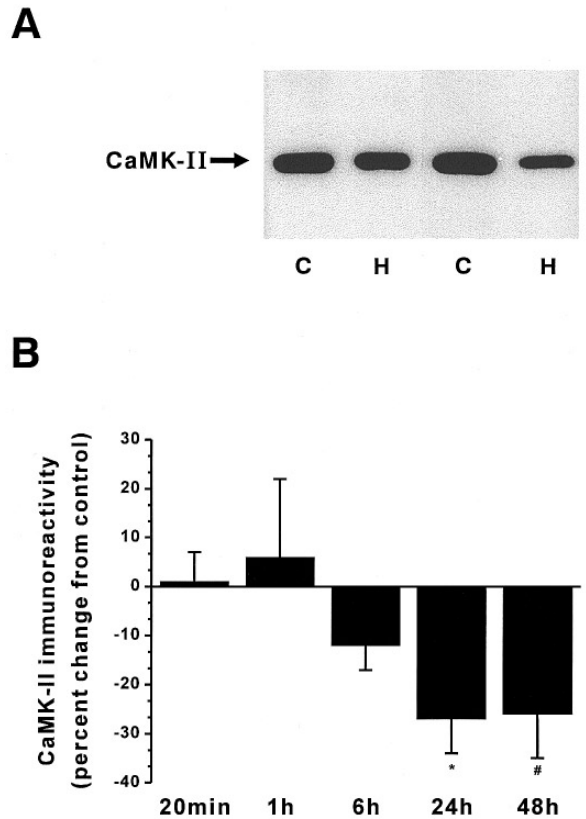

FIG. 5. Effect of hypoxia on CaMK-II immunoreactivity. PC12 cells were exposed to either hypoxia (5% oxygen) or normoxia (controls, 21% oxygen) for various times, as indicated. Total cell lysates containing 15 μg of protein were immunoblotted for CaMK-II as described in Materials and Methods. A. Representative immunoblot showing the effect of 24 h exposure to hypoxia (**H**) on CaMK-II immunoreactivity levels, as compared to control (**C**). B. Levels of CaMK-II immunoreactivity as quantitated by densitometric analysis. Data are expressed as average percent change from control ± s.e.m., and represent N = 3 to 12 individual dishes in each group. *$p < 0.05$ by χ^2 test, # not statistically significant ($0.10 < p < 0.05$) by χ^2 test.

We found that prolonged exposure to hypoxia (5% O_2, >6 hr) significantly decreased PKA enzyme activity in PC12 cells. Analysis of PKA immunoreactivity revealed a similar down-regulation of the amounts of both the regulatory (RIβ) and catalytic (Cα) subunits of the enzyme. That is, chronic (24 to 72 hr) hypoxia decreased PKA-RIβ and PKA-Cα immunoreactivity by 20 to 70% compared to control levels. The PKA regulatory subunit was somewhat more sensitive than the catalytic subunit to down-regulation by hypoxia. At all time points measured, PKA-RIβ levels were more greatly reduced by hypoxia than PKA-Cα levels. It should be noted that both the regulatory and catalytic subunits of PKA exist in multiple isoforms (33-37), and we have not determined whether all isoforms of PKA regulatory and catalytic subunits are hypoxia-sensitive. However, given the significant decrease in overall PKA activity as measured by cAMP-induced phosphorylation of kemptide, we conclude that a major portion of cellular PKA is clearly down-regulated by hypoxia. These effects were also not due to a hypoxia-induced reduction in cell viability. PC12 cells retained greater than 95% viability even after 72 hours exposure to hypoxia, as measured by trypan blue exclusion.

We also found that hypoxia exerted a similar inhibitory effect on CaMK-II activity. That is, prolonged (>3 hr) exposure to hypoxia significantly decreased CaMK-II enzyme activity levels. PC12 cells express CaMK-I and -II , but not CaMK-IV (38, 39). The decrease in CaMK-II enzyme activity was also accompanied by a decrease in CaMK-II immunoreactivity, but this effect was less pronounced than the dramatic down-regulation of PKA-RIβ and PKA-Cα by hypoxia (compare Figures 3 and 5).

Taken together, our data suggest that one mechanism by which PC12 cells adapt to reduced O_2 levels is to down-regulate the PKA and CaMK intracellular signaling systems. These signaling systems are activated during depolarization of PC12 cells (20, 23, 29), and thus down-regulation might be a compensatory response to prolonged activation of these pathways. Hypoxia-induced alterations in PKA and CaMK imply that many cellular phosphoproteins are also regulated by hypoxia. That is, a large number of PKA and CaMK substrate proteins are presumably phosphorylated to a lesser degree during conditions of hypoxia. Identification of post-receptor signaling systems and eventually specific phosphoprotein targets that are regulated by hypoxia will further our understanding of the cellular response to oxygen deprivation and, in turn, shed light on molecular mechanisms underlying the pathology of hypoxic and/or ischemic trauma.

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