Release of dopamine and norepinephrine by hypoxia from PC-12 cells

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Kumar, Ganesh K., Jeffrey L. Overholt, Gary R. Bright, Kwong Y. Hui, Hongwen Lu, Miklos Gratzl, and Nanduri R. Prabhakar. Release of dopamine and norepinephrine by hypoxia from PC-12 cells. Am. J. Physiol. Cell Physiol. 43:C1592–C1600, 1998.—We examined the effects of hypoxia on the release of dopamine (DA) and norepinephrine (NE) from rat pheochromocytoma 12 (PC-12) cells and assessed the involvement of Ca2+ and protein kinases in stimulus-secretion coupling. Catecholamine release was monitored by microvoltammetry using a carbon fiber electrode as well as by HPLC coupled with electrochemical detection (ECD). Microvoltammetric analysis showed that hypoxia-induced catecholamine secretion (P02 of medium 40 mmHg) occurred within 1 min after the onset of the stimulus and reached a plateau between 10 and 15 min. HPLC-ED analysis revealed that, at any level of PO2, the release of NE was greater than the release of DA. In contrast, in response to K+ (80 mM), DA release was ~11-fold greater than NE release. The magnitude of hypoxia-induced NE and DA releases depended on the passage, source, and culture conditions of the PC-12 cells. Omission of extracellular Ca2+ or addition of voltage-gated Ca2+ channel blockers attenuated hypoxia-induced release of both DA and NE to a similar extent. Protein kinase inhibitors, staurosporine (200 mM) and bisindolylmaleimide I (2 µM), on the other hand, attenuated hypoxia-induced NE release more than DA release. However, protein kinase inhibitors had no significant effect on K+-induced NE and DA releases. These results demonstrate that hypoxia releases catecholamines from PC-12 cells and that, for a given change in P02, NE release is greater than DA release. It is suggested that protein kinases are involved in the enhanced release of NE during hypoxia.

transmitter release; protein kinase; stimulus-secretion coupling

An adequate supply of oxygen is essential for the survival of mammalian cells. Hypoxia, i.e., decrease in oxygen availability, affects a number of cellular processes (3, 6, 7, 32), including the release of transmitters from neuronal cells (4, 8–13). Reported responses to hypoxia include both facilitation and inhibition of transmitter release. It has been reported that catecholamine release from brain cells (2, 11, 12, 22, 29) and from glomus cells of the carotid body (4, 8–10, 15, 16, 28, 32, 35) is facilitated during hypoxia, whereas release of acetylcholine from brain cells (11, 13) is inhibited during hypoxia. Furthermore, hypoxia increases catecholamine release from glomus cells of the carotid body but not from adrenal chromaffin cells (10), indicating that the effects of low oxygen are cell selective. It has also been reported that hypoxia has differential effects on the release of individual catecholamines. For a given hypoxic stimulus, release of dopamine (DA) from glomus cells of the carotid body has been shown to be greater than that of norepinephrine (NE) (15). The cellular mechanism(s) underlying the stimulus-secretion coupling during hypoxia in general and the differential release of catecholamines in particular remains largely unexplored.

Pheochromocytoma (PC-12) clonal cells are derived from rat adrenal medullary tumors. They synthesize DA and NE (17–19) and release them in response to a variety of pharmacological agents (5, 23, 26, 33). The mechanisms underlying the release of catecholamines from PC-12 cells have been extensively investigated. The mechanisms have been found to involve membrane depolarization (17, 19, 25) and elevation of cytosolic Ca2+ concentration ([Ca2+]i) (1, 3, 31, 33) that occur in response to activation of various membrane-bound receptors. In addition, recent studies have further shown that protein kinases also play an important role in transmitter release from PC-12 cells (1, 21, 31). The goals of the present study were 1) to investigate whether hypoxia releases catecholamines from PC-12 cells, 2) to determine the magnitude of DA vs. NE release for a given change in P02, and 3) to assess the importance of cytosolic Ca2+ and protein kinases in stimulus-secretion coupling during hypoxia. To accomplish these goals, we have examined the effects of different levels of ambient oxygen on the release of DA and NE from PC-12 cells. Our results demonstrate that catecholamines are released during hypoxia from PC-12 cells in a Ca2+-dependent manner and involve activation of voltage-dependent Ca2+ channels. Furthermore, at any given P02, release of NE is greater than release of DA, and a protein kinase-dependent pathway(s) seems to be associated with NE but not DA release during hypoxia.

MATERIALS AND METHODS

Cell Culture

PC-12 cells were obtained from Dr. K. Neet (Finch University of Health Sciences/Chicago Medical School; original done from Dr. L. Greene). Cells were grown in a humidified chamber circulated with 5% CO2 and 21% O2. The growth medium (RPMI-1640) was supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml of sodium penicillin G, and 100 µg/ml of streptomycin sulfate. The medium was changed every 2 days. Cells grown to ~80% confluence were used in the present study. Unless otherwise specified, the results reported in this study were obtained from cells maintained between passages 18 and 25 (see RESULTS). All experiments were performed at 37°C.
Analysis of Catecholamine Release

Microvoltammetry. Cells (4 x 10⁶) were washed twice with HEPES-buffered serum-free growth medium and placed in a glass vial containing 5 ml of the same medium equilibrated with 21% O₂. Oxygen and catecholamines in the medium were monitored simultaneously using a Clarke-type oxygen electrode and a Nafton-coated carbon fiber electrode, respectively. Catecholamines were analyzed by differential pulse voltammetry (DPV), using an electrochemical detector (Bioanalytical System). The electrodes were placed close to the cells. For DPV measurements, the electrode potential was increased from −200 to +400 mV at a rate of 20 mV/s. DPV measurements were made every 30 s. All potentials are reported with respect to an Ag-AgCl reference electrode. The height of the catecholamine peak was obtained by subtracting the linear baseline from the maximum peak at each time point. At the end of the experiment, the carbon fiber electrode was calibrated by adding known amounts of DA and NE to the medium. Both DA and NE produced a peak with a maximum at +100 mV, and they could not be further resolved into individual peaks. It is for this reason that the data are expressed as peak current (in pA) as well as in micromolar concentration of catecholamines, which included both DA and NE. The oxygen electrode was calibrated by equilibrating the medium with gas containing known amounts of oxygen and expressed as percent of oxygen in the medium.

HPLC combined with electrochemical detection. Cells were plated in tissue culture flasks in HEPES-buffered serum-free growth medium (DMEM) at a density of 5 x 10⁶ cells/flask. After exposure either to normoxia (21% O₂) or to desired levels of hypoxia (see Experimental Protocols), the cells were separated from the medium by centrifugation at 500 g for 5 min. The supernatant and the cell pellet were treated separately with 50 mg of acid-activated aluminum oxide (AAO) for 10 min. AAO, which adsorbs catecholamines, was removed from the medium by centrifugation. DA and NE were extracted by treating AAO with 100 μl of 0.1 M HClO₄ containing 0.1 M sodium metabisulfite and 0.25 mM disodium EDTA. Suitable aliquots of the perchloric acid extracts were used for the determination of DA and NE using HPLC-electrochemical detector apparatus (Shimadzu System) (20). The catecholamines were separated on an UltraspHERE ODS reverse-phase column (Beckman) by isocratic elution, using a mobile phase consisting of 4% acetonitrile, 0.1 M sodium nitrate, 0.08 M sodium dihydrogen phosphate, 0.2 mM sodium octyl sulfate, and 0.1 M EDTA, adjusted to pH 2.7 with phosphoric acid. Under these conditions, NE was eluted at ~6.3 min and DA at ~12.0 min. The chromatograms were recorded and analyzed with a Hitachi D-2500 Chromato-Integrator. The concentration of catecholamines (NE and DA) were calculated using standard curves constructed with known amounts of NE and DA. The recoveries were determined using isoproteorin as an internal standard and found to be 86 ± 4 and 82 ± 2% for DA and NE, respectively. The minimum detection limits for DA and NE were 75 and 50 fmol, respectively. DA and NE effuxes in the medium (i.e., DA and NE) were expressed as picomoles per minute per 10⁶ cells.

Assessment of Cell Viability

Lactate dehydrogenase (LDH) activity in the medium was monitored as an index of cell viability. After exposure of the cells either to normoxia (21% O₂) or to hypoxia (3% O₂ in N₂) for the desired duration, the supernatant was separated by centrifugation (500 g for 5 min). LDH activity in the medium was determined spectrophotometrically by monitoring pyruvate-mediated oxidation of NADH as described (27). The data were expressed as micromoles of NADH oxidized per minute per 10⁶ cells.

Measurement of [Ca²⁺]

[Ca²⁺], in individual PC-12 cells was monitored by microfluorometry, using the Ca²⁺-sensitive dye fura 2-AM. The procedures for the measurement of [Ca²⁺], in individual cells using this imaging technique and calibration of Ca²⁺ signals have been described previously (3). Briefly, the cells were plated on glass coverslips that were pretreated with cell adhesive (Cell-Tak, Collaborative Biomedical Products). Cells were incubated in serum-free growth medium containing fura 2-AM (5 μM) for 30 min. Subsequently, the medium was replaced with fresh medium that did not contain fura 2-AM, and the cells were allowed to recover for an additional 15 min. The coverslip containing the cells was placed in a gas-tight, temperature-regulated cell chamber and was superfused with HEPES-buffered serum-free medium equilibrated either with normoxic (21% O₂) or with hypoxic (5% O₂) gas mixtures. Images were collected every 10 s, and the results were expressed as nanomolar cytosolic Ca²⁺.

Experimental Protocols

Series 1. In the first series of experiments (n = 6), the time course of catecholamine efflux was determined during hypoxia using microvoltammetric technique. PC-12 cells were placed in glass vials containing 5 ml of HEPES-buffered DMEM at 37°C and were allowed to settle for 10 min. Catecholamine efflux and O₂ content were recorded by a carbon fiber electrode and a Clarke-type electrode, respectively, as described above (Microvoltammetry). Control efflux of catecholamine was monitored for 10 min while the medium was bubbled with 21% O₂ (normoxia). Thereafter, the medium was bubbled with 5% O₂ in N₂ (hypoxia) while the catecholamine and O₂ measurements continued. The duration of hypoxia challenge was 20 min.

Series 2. In this group of experiments (n = 8), the effects of varying intensities of hypoxia on NE and DA efflux were examined. NE and DA levels in the medium were monitored by the HPLC-electrochemical detection (ECD) method. Cells grown under identical culture conditions were plated in individual tissue culture flasks at a density of 5 x 10⁶ cells/flask in HEPES-buffered serum-free DMEM at 37°C. The medium was bubbled either with 21% O₂ or with 15, 10, 5, or 3% O₂ balanced with N₂ for 15 min. At the end of the gas challenge, an aliquot of the medium (200 μl) was collected for the determination of PO₂ by a blood gas analyzer. Cells were separated from the medium by centrifugation at 500 g for 5 min. Catecholamines (NE and DA) were extracted from the medium and the cell pellet separately, and their concentrations were determined by the HPLC-ECD method as described above (HPLC combined with electrochemical detection).

Series 3. In experiments assessing the role of extracellular Ca²⁺ (n = 6), the cells were placed in a low-Ca²⁺ medium (~0.1 mM; i.e., omission of Ca²⁺ and addition of 10 mM EGTA) and the results were compared with cells exposed to nominal Ca²⁺ (2.2 mM). Medium containing Ca²⁺ was prepared first and then bubbled either with room air (normoxia) or with hypoxic gas. Cells (5 x 10⁶) were gently dispersed and incubated in the appropriate media for a total duration of 15 min at 37°C. The influence of Ca²⁺ channel blocker on hypoxia-induced catecholamine release was assessed by incubating the cells (5 x 10⁶) with either cobalt (5 mM; n = 5) or nitrendipine (10 μM; n = 5) for 15 min before hypoxia and for an additional 15 min during hypoxia.
parallel experiments, effects of hypoxia on changes in \([Ca^{2+}]_i\) were examined using microfluorometry as described above (Measurement of \([Ca^{2+}]_i\)).

Series 4. The effects of protein kinase inhibitors were determined by incubating cells either with staurosporine (200 nM; \(n = 6\)) or with bisindolylmaleimide I (2 µM; \(n = 3\)). Cells were incubated with protein kinase inhibitors at 37°C during normoxia (control) and during hypoxia. After gas challenges, the cells were separated from the medium by centrifugation at 500 g for 5 min and NE and DA contents in the supernatant were determined by the HPLC-ECD method as described above (HPLC combined with electrochemical detection).

Data Analysis

All data are expressed as means ± SE. Statistical significance was evaluated by a paired t-test or ANOVA for repeated measures, and, if a significant interaction was indicated, the results were further compared using Tukey's test. P values <0.05 were considered significant.

RESULTS

Effects of Hypoxia on Catecholamine Release From PC-12 Cells

Time course of the response. An example illustrating the effect of hypoxia on catecholamine release, monitored by a carbon fiber electrode, is shown in Fig. 1. Under normoxia, a small but detectable release of catecholamines ranging between 0.2 and 0.4 µM was observed. In response to hypoxia (PO2 ∼40 mmHg), there was a progressive increase in catecholamine efflux. Increases in catecholamine levels could readily be detected during the first minute of hypoxia and reached a plateau between 10 and 15 min. On average, exposure to hypoxia for 15 min increased the catecholamine level sixfold (\(n = 6\)).

Fig. 1. Microvoltammetric measurements of catecholamine release from PC-12 cells by hypoxia. Top: peak current as measured by carbon fiber microelectrode (left axis, pA) and catecholamine concentration (right axis, µM). Bottom: oxygen level (%) in medium measured by a Clarke-type oxygen electrode. Arrow, time of switching from air to N2 gas bubbling. Note prompt release of catecholamines from PC-12 cells soon after onset of hypoxia and leveling of response between 10 and 15 min of hypoxic challenge.

Fig. 2. Effects of 5 levels of partial pressures of oxygen in medium on dopamine (DA; A) and norepinephrine (NE; B) release from PC-12 cells by hypoxia, as measured by HPLC-electrochemical detection. Data are means ± SE from 8 individual experiments. Note that magnitude of DA and NE release is dependent on severity of hypoxic stimulus.

DA vs. NE release during hypoxia. DA and NE are the major catecholamines in PC-12 cells (17, 33). It was not possible to distinguish between the release of individual catecholamines by microvoltammetry because the oxidation potential of DA overlaps that of NE. Therefore, in the following experiments, we used the HPLC-ECD method to distinguish the release of DA from NE. Analysis of the extracts of PC-12 cells showed that DA was the predominant catecholamine and that the DA-to-NE ratio was 8.7. These observations are in accord with other studies (33). Despite the abundance of DA, basal release of NE during normoxia was eightfold higher than release of DA. On average, during normoxia, NE release was 8.4 ± 0.2 pmol·min⁻¹·10⁶ cells⁻¹ compared with DA release of 1.0 ± 0.1 pmol·min⁻¹·10⁶ cells⁻¹ (DA vs. NE, \(P < 0.01, n = 8\)). Hypoxia enhanced the release of both DA and NE, and, as shown in Fig. 2, the effect of hypoxia depended on the severity of the stimulus. Lowering the PO2 to ∼75 mmHg had no significant effect on either NE or DA release compared
with normoxic controls. However, at a PO2 of ~40 mmHg, there was a significant increase in the release of both catecholamines. Although release of catecholamines tended to further increase at a PO2 of ~25 mmHg, the difference was not significant compared with the values at a PO2 of ~40 mmHg (P > 0.05, ANOVA). Furthermore, the magnitude of NE release at unlike hypoxia, K+ increase in the release of both NE and DA. However, PO2 of ~40 mmHg, NE release was +14.0 pmol·min⁻¹·10⁵ cells⁻¹ compared with DA release of +4.0 pmol·min⁻¹·10⁶ cells⁻¹. These observations demonstrate that hypoxia enhanced the release of catecholamines from PC-12 cells and that the basal and hypoxia-induced NE releases were greater than those of DA.

Effects of High Extracellular K+ on DA and NE Release

Previous studies have shown that high levels of extracellular K+ stimulated the release of catecholamines from PC-12 cells (17, 33). The following experiments were therefore performed to determine whether NE is released relatively more than DA in response to K+ stimulation. In response to 80 mM K+, there was an increase in the release of both NE and DA. However, unlike hypoxia, K+ preferentially induced the release of DA. Thus DA release in response to K+ averaged 11 pmol·min⁻¹·10⁵ cells⁻¹ above control, compared with NE release of 3 pmol·min⁻¹·10⁵ cells⁻¹ above control (DA vs. NE during 80 mM K+, P < 0.01, n = 6).

Effect of Hypoxia on Cell Viability

To assess whether the increased release of catecholamines during hypoxia was secondary to cell injury, we monitored LDH activity of PC-12 cells as an index of cell viability. Cells (4 × 10⁶) were exposed to either normoxia or hypoxia (PO2 ~25 mmHg, the maximum intensity of hypoxic stimulus used in the above experiments). After gas challenges, LDH activities of the medium and of the cell pellet were determined. As shown in Table 1, hypoxic exposure did not significantly alter LDH activity either in the medium or in the cells (P > 0.05, n = 5), indicating that the cell viability was unaffected by hypoxia.

Factors Influencing the Release of Catecholamines From PC-12 Cells by Hypoxia

The following series of experiments were performed to test whether the pattern of catecholamine release by hypoxia described above is influenced by factors such as passage, source, and culture conditions of PC-12 cells.

The results described above were obtained from PC-12 cells between passages 18 and 25. To assess whether the age of the cells would affect the pattern of catecholamine release, we monitored the effects of hypoxia (PO2 ~40 mmHg) on NE and DA release on cells maintained up to passage 40. The results showed that there is a progressive diminution in the release of both NE and DA from passage 25 onwards and that the release was nearly abolished in cells derived from passage 40 (Table 2). This diminution of the response occurred in spite of the fact that the cellular contents of NE and DA were comparable to those of cells at passage 18. Cells at passage 40 had no detectable basal release, and NE and DA release during hypoxia was markedly attenuated (passage 40 vs. passage 18, P < 0.05, n = 4).

In another series of experiments, we determined the effects of hypoxia on PC-12 cells obtained from the American Type Culture Collection (ATCC). NE and DA contents of ATCC cells were greater than those of our control cells (Table 2). Furthermore, as shown in Table 2, basal and hypoxia-induced NE releases were significantly less in cells from ATCC (P < 0.05, n = 4).

We also assessed whether the addition of growth factor to the culture medium influences the response of PC-12 cells to hypoxia. Nerve growth factor (βNGF; 2 ng/ml) was added to the growth medium, and the cells were maintained at 37°C in a humidified chamber for 4 days. In response to βNGF, cellular content of NE was the same as in control cells, whereas that of DA was higher than in control cells (Table 2). Basal as well as hypoxia-induced DA releases were markedly attenuated or abolished in βNGF-treated cells. On the other hand, basal release of NE was unaffected and hypoxia-induced release was attenuated by ~45% in βNGF-

Table 1. LDH activity of PC-12 cells during hypoxia

<table>
<thead>
<tr>
<th>Samples</th>
<th>LDH Activity</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell pellet</td>
<td>4.20 ± 0.18</td>
<td>4.00 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>0.26 ± 0.06</td>
<td>0.32 ± 0.10</td>
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</tbody>
</table>

Values are means ± SE of lactate dehydrogenase (LDH) activity expressed in pmol of NADH oxidized·min⁻¹·10⁵ cells⁻¹ (27); n = 5 experiments. Differences between normoxia and hypoxia were not significant.

Table 2. Factors affecting the release of catecholamines from PC-12 cells

<table>
<thead>
<tr>
<th>Catecholamines</th>
<th>Control (n = 6)</th>
<th>Passage 40 (n = 4)</th>
<th>ATCC Cells (n = 4)</th>
<th>βNGF (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Catecholamine content, nmol/10⁶ cells</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NE</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.03</td>
<td>0.20 ± 0.06*</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>DA</td>
<td>1.10 ± 0.08</td>
<td>1.08 ± 0.12</td>
<td>1.82 ± 0.21*</td>
<td>1.42 ± 0.21*</td>
</tr>
<tr>
<td>Basal release, pmol·min⁻¹·10⁶ cells⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>8.20 ± 1.10</td>
<td>ND</td>
<td>ND</td>
<td>7.8 ± 1.1</td>
</tr>
<tr>
<td>DA</td>
<td>1.10 ± 0.10</td>
<td>ND</td>
<td>0.36 ± 0.11*</td>
<td>ND</td>
</tr>
<tr>
<td>Hypoxia-induced release, pmol·min⁻¹·10⁶ cells⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE</td>
<td>22.2 ± 1.6</td>
<td>1.2 ± 0.08*</td>
<td>3.80 ± 0.12*</td>
<td>11.9 ± 0.9*</td>
</tr>
<tr>
<td>DA</td>
<td>4.7 ± 0.7</td>
<td>0.3 ± 0.06*</td>
<td>0.51 ± 0.06*</td>
<td>0.6 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE for no. of experiments (n) indicated. Control PC-12 cells were obtained from Dr. K. Neet (original clone from Dr. L. Greene). They were derived from passage 18 and cultured in absence of nerve growth factor (βNGF) in medium. For βNGF treatment, βNGF (2 ng/ml) was added to culture medium, and control cells were grown for 4 days. For hypoxia-induced release, cells were exposed to hypoxic medium of PO2 of ~38 mmHg. ND, not detected (level of catecholamines was well below detection limit of our assay); NE, norepinephrine; DA, dopamine; ATCC, American Type Culture Collection. *P < 0.05 compared with control.
treated cells compared with control cells (P < 0.05, n = 4).

Because the response to hypoxia depended critically on the age and the source of PC-12 cells, all subsequent experiments were performed on cells obtained from Dr. K. Neet between passage 18 and passage 25.

Involvement of Ca²⁺ in Hypoxia-Induced Catecholamine Release

Ca²⁺ plays an important role in the release of neurotransmitters from brain cells, synaptosomes, motor nerve endings, and PC-12 cells (12, 14, 24, 29, 34). In the following experiments, we examined the role of Ca²⁺ in hypoxia-induced catecholamine release from PC-12 cells.

Extracellular Ca²⁺. Cells were plated either in a low-Ca²⁺-medium (i.e., medium containing 10 mM EGTA with omission of 2.2 mM Ca²⁺) or in a medium containing Ca²⁺ (2.2 mM), after which they were challenged with hypoxia (P O₂ 38 ± 9 mmHg) for 15 min. Cells exposed to normoxia (P O₂ 145 ± 7 mmHg) with and without Ca²⁺ served as controls. As shown in Fig. 3, removal of extracellular Ca²⁺ markedly attenuated or abolished the hypoxia-induced release of NE and DA (P < 0.01, n = 6). In contrast, low extracellular Ca²⁺ had no effect on basal release of NE and DA during normoxia (P > 0.05, n = 6).

Effects of voltage-gated Ca²⁺ channel blockers. The results described above indicate that Ca²⁺ plays a role in hypoxia-induced catecholamine release. To test further this possibility, we monitored changes in [Ca²⁺]i during hypoxia. As shown in Fig. 4A, hypoxia resulted in a prompt elevation in [Ca²⁺]i. This increase in [Ca²⁺]i could be blocked by cobalt (5 mM), an inorganic voltage-gated Ca²⁺ channel blocker. Average data from 60 cells showed that cobalt inhibited ~75% of the hypoxia-induced increases in [Ca²⁺]i (P < 0.01; Fig. 4B).

In the following experiments, the role of voltage-gated Ca²⁺ channels in hypoxia-induced catecholamine release was assessed. PC-12 cells were treated with cobalt (5 mM) for 15 min and then exposed to either normoxia or hypoxia for an additional 15 min. During normoxia, basal release of DA was only marginally affected and NE release was unaffected by cobalt. On the other hand, hypoxia-induced NE and DA releases were attenuated or abolished by cobalt (P < 0.01, n = 5; Fig. 5).

Of the several classes of voltage-gated Ca²⁺ channels, undifferentiated PC-12 cells express predominantly L-type Ca²⁺ channels (30, 33). To test the involvement of L-type channels in catecholamine release during hypoxia, cells were exposed to nitrendipine (10 μM), a specific L-type Ca²⁺ channel blocker (n = 5). Nitrendipine had no significant effect on the basal release but attenuated NE and DA releases during hypoxia (Fig. 5). On average, NE and DA releases were inhibited by ~70% and ~80%, respectively (in pmol·min⁻¹·10⁶ cells⁻¹ above the corresponding control level of release: NE for untreated cells, 18; NE for nitrendipine-treated cells, 3; DA for untreated cells, 3.6; DA for nitrendipine-treated cells, 0.7). These observations demonstrate that activation of L-type voltage-gated Ca²⁺ channels contributes, in part, to the hypoxia-induced catecholamine release.

Influence of Protein Kinases in Hypoxia-Induced DA and NE Release

Recent studies have shown that Ca²⁺-mediated signal transduction pathways involving activation of protein kinases are associated with transmitter release from PC-12 cells (1, 21, 31). In the following experiments, we assessed the involvement of protein kinases in hypoxia-induced catecholamine release. The release of DA and NE during hypoxia was monitored in the presence and absence of staurosporine, a potent broad-spectrum protein kinase inhibitor. Staurosporine, at doses as low as 200 nM, attenuated or abolished hypoxia-induced NE release (P < 0.01, n = 6; Fig. 6B). In contrast, hypoxia-induced DA release was not significantly affected (P > 0.05, n = 6; Fig. 6A). Parallel
experiments using bisindolylmaleimide I (2 µM; n = 3), a structural analog of staurosporine, also showed a similar inhibition of hypoxia-induced NE but not DA release. On the other hand, both staurosporine (n = 6) and bisindolylmaleimide I (n = 3) had no effect on basal release of either NE or DA during normoxia (P > 0.05).

In another series of experiments, we monitored the effects of protein kinase inhibitors on catecholamine release in response to elevated levels of extracellular K+ (80 mM). As described above, high K+ resulted in modest release of NE (1.3-fold) and dramatic increases in DA (10.6-fold) release. Doses of staurosporine that blocked hypoxia-induced NE release (200 nM), however, had no significant effect on K+ -induced release of either DA or NE. On average, in the presence of staurosporine, K+-induced DA and NE release was inhibited only by 10 and 7%, respectively (P > 0.05, n = 6; Fig. 6, C and D).

DISCUSSION

The objectives of this study were to test the effects of hypoxia on the release of catecholamines from PC-12 cells and to determine the contributions of Ca2+ and protein kinases to the stimulus-secretion coupling. Our results demonstrate that hypoxia enhances catecholamine release from PC-12 cells and that, for any given oxygen level, the relative release of NE was greater than that of DA. The stimulus-secretion coupling by hypoxia involves changes in intracellular Ca2+. Mechanisms involving protein kinase(s) seem to contribute more to the release of NE than to the release of DA.

It is evident from our results that the release of catecholamines by low PO2 is rapid and can be seen within 1 min after the onset of the stimulus. Furthermore, hypoxia-induced release is not only time dependent but also depends on the severity of the stimulus. It is interesting to note that the levels of hypoxia that caused catecholamine secretion from PC-12 cells are modest (PO2 25–40 mmHg) and are often seen in many physiological and pathological conditions. It should, however, be mentioned that the effects of hypoxia critically depend on age and culture conditions of the PC-12 cells. Hypoxia has minimal effects on catecholamine release from older cells and cells exposed to bNGF (i.e., neuronally differentiated cells). We have also observed that cells obtained from ATCC have a greatly diminished response to hypoxia in comparison with those obtained from another source. These observations are in accord with those reported by Kawai et
al. (25), who also observed that the magnitude of the hypoxia-induced inhibition of $K^+$ currents is markedly less in PC-12 cells from ATCC. Taken together, these observations suggest that, in evaluating the effects of hypoxia on transmitter release from PC-12 cells, factors such as age, culture conditions, and the source of the cells need to be taken into consideration.

A major finding of the present study is that the magnitude of NE release is greater than that of DA release at any level of $P_{O_2}$ (Fig. 2). In contrast, with $K^+$ stimulation, DA release was ~11-fold higher than NE release. Taken together, these observations support the notion that NE release is preferentially affected by hypoxia. Differences in the release of individual catecholamines during hypoxia have been reported in other systems, such as the glomus cells of the carotid body (15). Gomez-Nino et al. (15) showed that hypoxia releases more DA than NE from glomus cells of the carotid body. They have attributed this difference to the relative abundance of DA over NE in the glomus tissue ($DA/NE = 10$) (15). Such an explanation in the case of PC-12 cells is unlikely because DA is the predominant catecholamine in these cells (17, 33).

Several factors, however, may have contributed to the differences in the magnitude of NE vs. DA release from PC-12 cells observed during hypoxia. These include, for instance, loss of DA and/or NE by metabolic conversions, cellular reuptake, or binding to receptors. It could be argued that a portion of DA is metabolized to dihydroxyphenyl acetic acid (DOPAC) giving rise to lower estimates of DA in the medium. However, a peak corresponding to DOPAC was not evident in the hypoxic medium during HPLC-ECD analysis, indicating that metabolic conversion of DA does not account for the relatively low level of DA release during hypoxia. Also, there is no evidence for a reuptake mechanism for DA in PC-12 cells (17). Reuptake of NE has been demonstrated in PC-12 cells, and it reached a plateau within 4–5 min (17). Despite the reuptake, NE levels in the medium were higher than DA at any level of $P_{O_2}$. The amounts of NE and DA that are bound to their respective receptors, however, cannot be ascertained...
from our study. Therefore, a more plausible mechanism for greater release of NE is that NE and DA are stored in separate vesicles and independent mechanism(s) control their release. Our observations that high K+ and low PO2 affect differentially the release of individual catecholamines support such a notion. Furthermore, protein kinase inhibitors affected the release of NE more than that of DA (see below).

The following evidence supports the notion that Ca2+ plays a major role in hypoxia-induced catecholamine release from PC-12 cells. Removal of extracellular Ca2+ blocked hypoxia-induced NE and DA release but not the basal release seen during normoxia, suggesting that low oxygen facilitates the influx of Ca2+. This assumption is further supported by the finding that [Ca2+]i is elevated during hypoxia in PC-12 cells. Blockade of voltage-gated Ca2+ channels abolished hypoxia-induced catecholamine release, indicating that activation of these Ca2+ channels is associated with stimulus-secretion coupling during hypoxia.

Multiple voltage-gated Ca2+ channels have been described in mammalian cells (30, 33, 34). Although undifferentiated PC-12 cells have been shown to express predominantly L-type Ca2+ channels (33), they seem to contribute only partly to the hypoxia-induced catecholamine release. This is based on the observation that nitrendipine, a selective L-type Ca2+ channel blocker, partially blocked the hypoxia-induced DA and NE release. This observation suggests that, in addition to L-type, other types of voltage-gated Ca2+ channels may also participate in stimulus-secretion coupling by hypoxia. Which other types of Ca2+ channels are involved, however, remains to be established. These observations on PC-12 cells are similar to those reported for carotid body glomus cells, whereby L-type Ca2+ channels are implicated in stimulus-secretion coupling during hypoxia (28, 35). As in PC-12 cells, L-type channel blockers only partially attenuate catecholamine release from glomus cells. It appears that Ca2+ influx is absolutely essential for the release of both DA and NE from PC-12 cells during hypoxia. However, changes in Ca2+ may not account for the preferential release of individual catecholamines (i.e., NE vs. DA).

Protein kinase inhibitors selectively abolished hypoxia-induced NE, but not DA, release. We believe that the effects of protein kinase inhibitors are specific because K+-induced NE or DA release was unaffected. It follows that reactions downstream from Ca2+ entry, probably involving protein kinase(s), determine release of individual catecholamines by hypoxia. What types of protein kinases are associated with hypoxia-induced NE release cannot be ascertained from our results. Future studies characterizing the types of protein kinases and the influence of hypoxia on their activation patterns are necessary to elucidate their precise roles in hypoxia-induced stimulus-secretion coupling.

In summary, the present study demonstrates that hypoxia, a physiological stimulus, induces the release of both NE and DA from PC-12 cells and that, at any given PO2, NE release is greater than DA release (Fig. 2). It is noteworthy that the levels of hypoxia inducing catecholamine release from PC-12 cells can be seen in many physiological and pathophysiological conditions and are not detrimental to the cell viability (Table 1). Furthermore, extracellular Ca2+ and voltage-gated Ca2+ channels, predominantly of the L-type, are involved in hypoxia-induced catecholamine release. The hypoxia-induced NE, but not DA, release appears to involve protein kinase(s). Because the release of neurotransmitters is essential for the maintenance of circulatory and ventilatory homeostasis during hypoxia, the results presented in this study provide evidence that PC-12 cells may serve as a potential model cell line for examining stimulus-secretion coupling during low oxygen.

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