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Role of the D₂ dopamine receptor in molecular adaptation to chronic hypoxia in PC12 cells

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Abstract

We have previously shown that pheochromocytoma (PC12) cells rapidly depolarize and undergo Ca²⁺ influx through voltage-dependent Ca²⁺ channels in response to moderate hypoxia and that intracellular free Ca²⁺ is modulated by activation of dopamine D₂ receptors in this cell type. The present study shows that D₂ (quinpirole-mediated) inhibition of a voltage-dependent Ca²⁺ current (*I*_{Ca}) in PC12 cells is dramatically attenuated after chronic exposure to moderate hypoxia (24 h at 10% O₂). Pretreatment of cells with pertussis toxin abolished D₂-mediated inhibition of *I*_{Ca}. The D₂-induced inhibition of *I*_{Ca} did not depend on protein kinase A (PKA), as it persisted both in the presence of a specific PKA inhibitor (PKI) and in PKA-deficient PC12 cells. Prolonged exposure to hypoxia (24 h) significantly reduced the level of G_{i/o}α immunoreactivity, but did not alter Gβ levels. Furthermore, dialysis of recombinant G_oα protein through the patch pipette restored the inhibitory effect of quinpirole in cells chronically exposed to hypoxia. We conclude that the attenuation of the D₂-mediated inhibition of *I*_{Ca} by chronic hypoxia is caused by impaired receptor–G protein coupling, due to reduced levels of G_oα protein. This attenuated feedback modulation of *I*_{Ca} by dopamine may allow for a more sustained Ca²⁺ influx and enhanced cellular excitation during prolonged hypoxia.

Keywords

Calcium current; Dopamine receptor; G protein; Hypoxia; Pertussin toxin; Quinpirole

Introduction

Our laboratory and others have extensively characterized the effects of hypoxia on the pheochromocytoma (PC12) clonal cell line. These catecholaminergic cells are derived from rat adrenal medulla, and are responsive to hypoxia in a number of ways. Many studies confirm that reduced O₂ tension depolarizes the membranes of PC12 cells and, as a consequence, releases the neurotransmitter dopamine (DA) [33,39,40]. Our laboratory has further reported that hypoxia increases intracellular free Ca²⁺ in PC12 cells through activation of voltage-dependent Ca²⁺ channels secondary to membrane depolarization [30,39]. We have also presented data that tie this hypoxia-induced increase in intracellular Ca²⁺ to hypoxia-induced gene regulation and DA release [30,40]. Thus, PC12 cells represent a valuable in vitro model system that can be used to study the cellular and molecular mechanisms which mediate oxygen sensitivity and the adaptive response to hypoxia.

Similar events have been reported to occur in type I cells of the carotid body, a small organ located at the bifurcation of the carotid artery, that serves as the mammalian oxygen chemoreceptor [3,26]. In both PC12 and type I cells, studies show that DA causes inhibition of a voltage-dependent Ca²⁺ current (*I*_{Ca}) [1,40]. In PC12 cells, DA has been shown to stimulate

D₂ receptors, inhibiting I_{Ca} and therefore attenuating the elevation of intracellular free Ca^{2+} during hypoxia [40].

The mechanism that couples the D₂ receptor to Ca^{2+} channels is not well understood, but several studies point to the involvement of G_{i/o} proteins that are sensitive to pertussis toxin (PTX) [12,16,27,36]. We know that PC12 cells adapt to prolonged exposure to hypoxia with a series of events such as increased responsiveness to acute exposure to hypoxia [5,23,34] and increased tyrosine hydroxylase gene expression [6,30]. Until now, the effects of prolonged hypoxia on the D₂-induced inhibition of I_{Ca} have not been clear.

The current study examines the effect that chronic hypoxia exerts on the D₂ receptor modulation of I_{Ca} . Our results confirm the hypothesis that the D₂ receptor modulates I_{Ca} through PTX-sensitive G_{i/o} proteins, and we show that prolonged hypoxia attenuates the observed D₂-mediated inhibition of I_{Ca} . We further demonstrate that prolonged hypoxia reduces the level of G_{i/o} α immunoreactivity, while it enhances the expression of the D₂ DA receptor in these cells. Importantly, we show that reconstitution of the G_o α protein in cells incubated in chronically hypoxic conditions recovers most of the inhibitory effect of D₂ stimulation on I_{Ca} during chronic hypoxia. We conclude, therefore, that chronic hypoxia desensitizes the D₂ modulation of I_{Ca} by a mechanism that involves down-regulation of the G_o α protein.

Materials and methods

Cell culture

PC12 cells were obtained from American Type Culture Collection (Rockville, Md., USA) and prepared as described previously [22]. Cells were maintained with Dulbecco's Modified Eagle's Medium/Ham's F12 (DMEM/F12) containing 15 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum, penicillin/streptomycin (100 U/ml and 100 μ g/ml, respectively) and grown at 37°C with 95% air/5% CO₂. PKA-deficient PC12 cells (A123.7, [10]) were grown in DMEM with high glucose (DMEM-H) with 15 mM HEPES, 10% fetal bovine serum, 5% horse serum and gentamycin (100 μ g/ml), in an environment of 21% O₂ and 10% CO₂ at 37°C. To compare the responses between PKA-deficient and parental PC12 cells, parental cells were maintained under the same conditions as A123.7 cells. Cells were passed once a week and plated at low density on sterilized 12-mm round glass coverslips for electrophysiological experiments and 35-mm tissue culture dishes for study of gene and protein expression.

PC12 cells exposed to chronic hypoxia were maintained for 24 h in an incubator equipped with an O₂ sensor that regulates the mixing of N₂ with air to give the desired level of hypoxia (10% O₂ and 5% CO₂ balanced with N₂). In a separate set of experiments, cells were incubated under hypoxic conditions for 24 h in the presence of 3 μ M butaclamol, a D₂-receptor-selective antagonist [21].

Reverse transcription-polymerase chain reaction (RT-PCR)

PC12 cells were grown on 35-mm tissue culture dishes (Corning, N.Y., USA) under normoxic (21% O₂ and 5% CO₂) and hypoxic (10% O₂ and 5% CO₂ balanced with N₂) conditions for 24 h. Total cellular RNA was isolated from PC12 cells using TRI-REAGENT (Molecular Research Center, Cincinnati, Ohio, USA). RT-PCR was performed using the GeneAmp RNA PCR kit, purchased from Perkin Elmer Cetus (Norwalk, Conn., USA). In these experiments, 3 μ g of total RNA was reverse transcribed with 2.5 μ M Oligo dT (16mer) primer, 1 mM dNTPs, 1 unit/ μ l RNase inhibitor, 2.5 unit/ μ l MuLV reverse transcriptase for 15 min at 42°C. The primers were designed to span a portion of the 3' side coding region as well as part of non-coding region of the D₂ receptor sequence (Genbank, access number M36831). The forward

primer was: 5'(1308) ACC CCA TCA TCT ACA CCA CCT T 3'; the reverse primer was: 5' TGG CGT GTT CCC TGC TTT 3'(1823). PCR was carried out for a total of 40 cycles at 1 min 95°C, 1 min 30 s at 57°C and 1 min 30 s at 72°C. The amplification product was a 533-base-pair fragment. Samples were obtained at 30, 33, 36 and 40 cycles. This primer set did not discriminate between the long and the short forms of the D₂ receptor sequence. Amplification of β -actin gene, housekeeping gene, was also performed as controls. The primers for the mouse β -actin gene were as follows: 5' GTC CCT GTA TGC CTC TGG TC 3' (forward), 5' TCG TAC TCC TGC TTG CTG AT 3' (backward). The products of RT-PCR were analyzed by electrophoresis on 1% agarose gels and verified by sequence analysis.

Electrophysiology

Detailed protocols were described previously [22]. Briefly, the coverslips with PC12 cells were transferred directly from the incubator (normal or hypoxic) into a perfusion chamber mounted on an inverted microscope (ITM-2 Olympus). The cells were immediately perfused with the normoxic recording solution. Whole-cell voltage-clamp recordings were performed in a conventional patch-clamp mode [13]. Patch pipettes had a resistance of 3–5 M Ω when filled with the internal solution and were made with a Mecnex three-stage puller (Mecnex, Switzerland) using borosilicate glass (WPI, Sarasota, Fla., USA). In the reconstitution experiments, 1- to 2-M Ω pipettes were used. Recordings were performed using an Axopatch-200A amplifier (Axon Instruments, Foster City, Calif., USA).

To study Ca²⁺ currents (I_{Ca}), Ba²⁺ was used as the charge carrier. I_{Ca} was recorded using patch-pipettes filled with (mM): 140 Cs-gluconate, 1 CaCl₂, 2 MgCl₂, 10 EGTA, 10 HEPES, 3 ATP-sodium and 0.2 GTP (pH adjusted to 7.2 with Tris base). The external solution included (mM): 122 *N*-methylglucamine-glutamate, 20 BaCl₂, 2 MgCl₂, 10 HEPES, 10 glucose (pH adjusted to 7.4 with Tris base). Currents were recorded during steps to +20 mV for 160 ms from a holding potential (HP) of –80 mV. The voltage pulses were given at 0.1 Hz. All traces were leak-subtracted using currents elicited by small hyperpolarizing pulses, filtered at 1 kHz and sampled at 10 kHz. The digitized signals were analysed on a personal computer using the pCLAMP 5.5.1 analysis programs. All experiments were carried out at room temperature (25°C).

Experiments involving dopaminergic agents were performed in a dark environment to minimize light-induced degradation of these drugs.

Preparation of cellular protein sample

PC12 cells were plated on 35-mm culture dishes and maintained in normoxic conditions. They were then exposed to hypoxia (10% O₂/5% CO₂/balance N₂) or normoxia (21% O₂) for 24 h. At the end of 24 h, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by scraping with 0.2 ml of lysis buffer which contained 10 mM Tris (pH 7.4), 1% Triton X-100, 0.2 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonylfluoride (PMSF), 2 μ g/ml leupeptin and 2 μ g/ml aprotinin. The lysate was sonicated for 2 s with a micro-ultrasonic cell disrupter (Kontes, Vineland, N.J., USA). Lysate samples were centrifuged for 5 min at 17,530 g at 4°C to remove Triton-insoluble material. The protein concentration in the sample was determined by the method of Bradford [2]. The samples were stored at –20°C in a buffer that contained final concentrations of: 50 mM Tris (pH 6.7), 2% SDS, 2% β -mercaptoethanol, and bromophenol blue marker.

Immunoblotting for G_{i/o} α , G β and DA D₂ receptor

Protein samples were boiled for 2 min and loaded on a 7.5% SDS-polyacrylamide gel, together with molecular weight marker (Sigma, St. Louis, Mo., USA). The amount of samples was 60 μ g for both G_{i/o} α and G β , and 120 μ g for D₂ receptor. Proteins were transferred to a

nitrocellulose membrane (Schleicher and Schuell, Keene, N.H., USA) using standard electroblotting procedures. The nitrocellulose membrane was treated for 1 h at 4°C with blocking solution [5% nonfat dry milk in 1× PBS which included 0.1% Tween 20 (PBST)], and then incubated with primary antibody at 4°C overnight. The membrane was later washed 4 times (15 min each wash) over 1 h with PBST at room temperature. It was then incubated with goat anti-rabbit secondary antibody linked to horseradish peroxidase (Amersham, Chicago, Ill., USA) at a 1:2000 dilution in 1% non-fat milk/PBST at room temperature for 1 h. Immunolabelling was detected using enhanced chemiluminescence (ECL) according to the manufacturer's recommended conditions. Immunoreactivity was quantitated using densitometry with ImagePro digital analysis system (Media Cybernetics, Silver Springs, Md., USA).

The primary antibodies for $G_{i/o}\alpha$ family (rabbit, polyclonal), $G\beta$ (rabbit, polyclonal) and D_2 receptor (rabbit, polyclonal) were purchased from Santa-Cruz Biotechnology (Santa Cruz, Calif., USA), Dupont NEN (Boston, Mass., USA) and Chemicon (Temecula, Calif., USA), respectively.

Reagents

(-)-Quinpirole (Quin), pertussin toxin (PTX) and butaclamol were purchased from Research Biochemicals International (RBI, Natick, Mass., USA), myristoylated, rat recombinant $G_o\alpha$ was purchased from Calbiochem, La Jolla, Calif., USA.

Data analysis

The results are expressed as the mean $\pm$ SEM (n : number of observations). 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.

Results

Effect of chronic hypoxia on D_2 -receptor-induced inhibition of voltage-dependent Ca^{2+} current in PC12-cells

Our initial experiments evaluated the effect of prolonged hypoxia on the D_2 -receptor-mediated modulation of Ca^{2+} current. Chronic exposure of PC12 cells to 10% O_2 (hypoxia) for 24 h did not change the characteristics of I_{Ca} produced by depolarizing voltage pulses. There was no significant difference in the current density of I_{Ca} among the following three groups: (1) normoxia, 13.0 ± 3.2 pA/pF; (2) hypoxia, 13.5 ± 4.0 pA/pF; or (3) hypoxia plus butaclamol (a selective D_2 agonist), 13.2 ± 2.8 pA/pF.

On the other hand, chronic hypoxia significantly decreased the I_{Ca} response to D_2 receptor activation. Figure 1 compares the effects of D_2 receptor stimulation on a control cell maintained at normoxia (21% O_2) for 24 h and in a cell maintained at hypoxia (10% O_2) for 24 h. In both cases, activation with 10 μ M Quin provided D_2 receptor stimulation. In the control group, Quin caused an average of $69 \pm 2\%$ I_{Ca} inhibition ($n=5$); in cells exposed to hypoxia, Quin caused an average I_{Ca} inhibition of only $29 \pm 2\%$ ($n=6$, $P < 0.05$). The decreased sensitivity of I_{Ca} to Quin stimulation was not caused by stimulation of DA receptors as the result of endogenous DA released during exposure to hypoxia. We measured the effect of 10 μ M Quin on I_{Ca} in cells maintained at 10% O_2 for 24 h in the presence of 3 μ M butaclamol, a selective D_2 antagonist. In this group, I_{Ca} was inhibited by $32 \pm 2\%$ ($n=6$).

PTX-sensitive G proteins mediate the D₂ receptor-induced inhibition of voltage-dependent Ca²⁺ current in PC12 cells

Several studies suggest that G_{i/o} proteins are involved in the D₂-mediated modulation of I_{Ca} in various cell types [12,27,30]. In the current studies, PC12 cells were pretreated overnight with 200 ng/ml PTX, which inactivates G_i and G_o proteins. We then measured the effect of D₂ receptor activation on I_{Ca} (Figure 2). The I_{Ca} showed little inactivation during a maintained voltage pulse of 160 ms using Ba²⁺ as the charge carrier. Treatment with PTX did not change the time-dependent properties of I_{Ca} ; however, the inhibition of I_{Ca} in response to D₂ receptor stimulation was abolished in the PTX-treated cells ($n=4$).

D₂-receptor-induced inhibition of voltage-dependent Ca²⁺ current is not mediated by PKA

To test the possibility that the effects of D₂ activation on I_{Ca} are mediated by PKA, we examined the effect of Quin on I_{Ca} in mutant PC12 cells deficient in PKA (PKA⁻) (A123.7; [10]). Figure 3A,B displays these results. To compare the effect of D₂ stimulation on I_{Ca} in parental PC12 cells and A123.7 cells, we examined the expression of D₂ receptors in A123.7 cells by RT-PCR. Figure 3A shows that A123.7 cells also express D₂ receptor mRNA. Patch-clamp results in Fig. 3B show no difference between the Quin-induced inhibition of I_{Ca} in A123.7 cells and parental PC12 cells exposed to the same conditions (21% O₂, 10% CO₂). The inhibition of I_{Ca} by Quin in parental PC12 cells ($49.3 \pm 7.8\%$) is somewhat smaller than that presented in Fig. 1. This variation might be explained by the different cell culture conditions in these experiments, that is, 10% CO₂ instead of 5% CO₂.

We also found, as shown in Fig. 3C, that Quin-induced significant changes in I_{Ca} in PC12 cells whose PKA was blocked by the PKA inhibitor fragment 6–22 amide PKI. This small heat-stable inhibitory peptide binds to the catalytic subunit of PKA and inactivates the enzyme [11]. Dialysis of cells with a pipette solution containing PKI effectively inhibits the PKA-mediated current events [22]. In the present study, PC12 cells were dialysed with 100 μ M PKI, using large-tip pipettes of 1–2 M Ω resistance for at least 6 min prior to the start of an experiment. We found that intracellular application of PKI did not abolish the effect of Quin on I_{Ca} . As a positive control, we also examined the effect of PKI on adenosine-induced modulation of I_{Ca} in PC12 cells. The adenosine-induced change in I_{Ca} was abolished by pretreatment of cells with PKI (data not shown). Taken together, these results show that the effects of D₂ activation on I_{Ca} are not mediated through the PKA pathway.

Chronic hypoxia up-regulates D₂ receptor gene expression and protein levels in PC12 cells

We hypothesized that reductions in either the associated signal transduction pathway or the D₂ receptor level could limit the effect of D₂ stimulation on I_{Ca} during prolonged exposure to hypoxia. We evaluated the effect of hypoxia on D₂ receptor gene expression using RT-PCR. Taking PCR samples at various cycles of amplification, we compared the relative signal abundance in the control group and the hypoxia-treated group (24 h, 10% O₂, $n=4$). We performed a similar comparison using PCR primers specific for mouse β -actin. As shown in Fig. 4A, the D₂-receptor mRNA level actually increased in the cells exposed to hypoxia, while the level of β -actin gene expression decreased in the chronic hypoxia group ($n=4$).

It was possible that this increase in D₂ receptor mRNA expression in the hypoxia group (Fig. 4A) resulted from chronic stimulation of the D₂ receptor by endogenously released DA. To test this possibility, we treated a new group of cells with hypoxia (10% O₂ for 24 h) with 3 μ M butaclamol, a D₂ receptor antagonist (Fig. 4B). The presence of butaclamol did not prevent the increase in hypoxia-induced D₂ receptor mRNA. We conclude that the hypoxia-induced increase in D₂ gene expression is independent of ligand activation of the D₂ receptor.

Immunoblot analyses revealed the effect of hypoxia on the D₂ receptor protein level in PC12 cells. Increasing the duration of hypoxia (10% O₂) enhanced the level of D₂ receptor protein (Fig. 4C). The averaged results from four separate experiments are shown in the lower panel of Fig. 4C. We measured a significant increase in D₂ receptor protein at 12 and 24 h. Thus, hypoxia up-regulates both the D₂-receptor mRNA and the protein levels over time during exposure to hypoxia (10% O₂).

Chronic hypoxia down-regulates G_{i/o}α proteins but not Gβ protein in PC12 cells

Many studies point to the involvement of a PTX-sensitive G protein in the modulation of *I*_{Ca} [7,12,36]. The PTX-sensitive G proteins include G_i and G_o. Our results from PTX-treated cells corroborate observations that G_{i/o}α is involved in the transduction of the effect of D₂ stimulation on *I*_{Ca}. Using a polyclonal antibody that recognizes both G_i and G_o, we evaluated the effect of hypoxia on the level of immunoreactivity in PC12 cells. Results from these experiments (Fig. 5A) showed that prolonged hypoxia decreases the level G_{i/o}α protein. The lower panel of Fig. 5A shows that the protein level was reduced by an average of 25±3% (*n*=14) in PC12 cells exposed to hypoxia for 24 h. This effect was not prevented by the presence of butaclamol. A 21±6% decrease in the G_{i/o}α protein level resulted, which is not significantly different from the hypoxia-only trial. This regulation was specific to G_{i/o}α, in that G_{αs} immunoreactivity levels in PC12 cells were not altered by exposure to chronic hypoxia [23].

Because recent studies report that β-subunits of G proteins are important for the interaction between G proteins and Ca²⁺ channels [14,17], we investigated the effect of chronic hypoxia on Gβ immunoreactivity levels in PC12 cells exposed to 10% O₂ for 6, 12 or 24 h. The anti-Gβ antibody used was broadly reactive with Gβ₁, Gβ₂, Gβ₃, and Gβ₄ isoforms. Figure 5B shows that chronic hypoxia had no effect on Gβ levels in PC12 cells. Our findings indicate that the down-regulation of the G_{i/o}α protein is a likely cause of the reduced effectiveness of D₂ activation in the inhibition of *I*_{Ca}, in cells exposed to prolonged hypoxia.

Reconstitution of G_oα protein restores the hypoxia-induced attenuation of the D₂-receptor-stimulated inhibition of *I*_{Ca} in PC12 cells

To investigate the role of reduced levels of G_oα in the hypoxia-induced attenuation of the D₂-stimulated inhibition of *I*_{Ca}, we performed reconstitution studies using dialysis via the patch pipette. Large-tip pipettes of 1–2 MΩ resistance were used for these experiments. The pipette solution for this experiment included 10 nM of recombinant rat myristoylated G_oα protein. Previous studies have shown that myristoylated G_{i/o}α dose-dependently reconstitutes the function of G_{i/o}α modulatory events, and that 10 nM is a maximal concentration, which can specifically restore function [15]. After obtaining a whole-cell configuration, *I*_{Ca} was recorded every 15 s for 15 min. After achieving a steady-state recording of *I*_{Ca}, 10 μM Quin was applied extracellularly. Figure 6A shows representative recordings of *I*_{Ca} from these experiments. The examples from left to right show the *I*_{Ca} recorded just after obtaining the whole-cell configuration (0 min), 15 min after starting perfusion, and during the application of 10 μM of Quin, respectively. No groups showed significant changes in the amplitude of *I*_{Ca} during 15 min of perfusion. The inhibition of Quin-induced *I*_{Ca} was much smaller in cells exposed to chronic hypoxia compared to the control cells. Importantly, the inhibition of Quin-induced *I*_{Ca} was almost completely restored when the cell was infused with G_oα protein. Figure 6B shows the averaged data for these experiments. We found that the intracellularly applied G_oα protein significantly recovered the D₂-receptor-stimulated inhibition of *I*_{Ca} in cells chronically exposed to hypoxia.

We conclude that the down-regulation of G_oα protein resulting from chronic hypoxia is likely to contribute to the hypoxia-induced attenuation in the D₂-receptor-stimulated inhibition of *I*_{Ca}.

Discussion

Our findings indicate that chronic hypoxia attenuates the DA D₂-receptor-mediated inhibition of I_{Ca} in PC12 cells, and they suggest that impaired receptor-G protein coupling, caused by a reduced G_o α level, is responsible for the attenuation. These findings represent the first evidence that chronic hypoxia can induce desensitization of D₂ receptor function, and the first evidence that hypoxia inhibits a D₂-receptor-mediated function by the down-regulation of a G protein.

In the current study, we found that activation of the D₂ receptor inhibits I_{Ca} in PC12 cells by means of a PTX-sensitive mechanism, and we showed that PKA is not essential for the modulation of I_{Ca} by D₂ stimulation. We used similar approaches in a previous study using A123.7 cells and PKI, and these experiments established the involvement of PKA in the adenosine-induced modulation of I_{Ca} in PC12 cells [22]. Therefore, it is possible that modulation of I_{Ca} by the activation of the D₂ receptor occurs through membrane-delimited mechanisms. Indeed, it is already established that membrane-delimited G protein regulation of Ca²⁺ channels is common in neurons and that the G protein involved in this inhibition is PTX sensitive [7,12,16,36]. Growing evidence strongly implicates G_o rather than G_i in this regulation, which could also affect the suppression of neurotransmitter release that is mediated by receptors on presynaptic neurons [4,7,8,28]. Furthermore, membrane-delimited G protein regulation of I_{Ca} by DA has been shown in chick sensory and sympathetic neurons [27]. In addition, a study of rat anterior pituitary cells has shown that the G_o α is primarily responsible for the inhibition of Ca²⁺ currents induced by D₂ receptor activation [25]. Finally, studies conducted on SH-SY5Y cells show that DA causes inhibition of Ca²⁺ currents through D₂ receptors, where G_{o2} α , a splice variant of the G_o α subunit, is involved in the coupling of receptors to Ca²⁺ channels [15].

In the current study, we found that prolonged exposure to hypoxia desensitized the D₂-mediated inhibition of I_{Ca} in PC12 cells, and this is the first report of chronic-hypoxia-induced desensitization of D₂ DA receptor function. The expression of D₂ receptors increased at both the mRNA and protein levels. We infer from these findings that the reduced effectiveness of D₂ inhibition of I_{Ca} during chronic hypoxia is not caused by a decreased level of D₂ receptors. We did find, however, that chronic hypoxia down-regulates the levels of G_{i/o} α protein. In contrast, the levels of G β protein were not altered during persistent hypoxia. It is well established that, on activation by agonist-bound receptors, the α -subunit of G proteins binds GTP in exchange for GDP and presumably dissociates from the $\beta\gamma$ complex [12,29]. The activation is terminated by a GTPase activity intrinsic to the α -subunit. It is also widely accepted that G_o α is primarily responsible for the modulation of neuronal voltage-dependent Ca²⁺ channels [4,7,8,28], although several G $\beta\gamma$ subunits are able to transduce the signal to Ca²⁺ channels [14,17]. We found that hypoxia induced an average decrease of about 25% in the levels of G_{i/o} immunoreactivity. Although this is a relatively modest effect, others have also demonstrated that small changes in levels of G proteins and other signalling molecules can result in dramatic changes in neuronal function. For example, a 15% reduction in G_{i/o} resulted in a 50% decrement in serotonin and GABA-mediated modulation of potassium conductance of dorsal horn cells, whereas a 50% reduction in G_{i/o} resulted in a complete loss of the serotonin and GABA response in these cells [18]. Furthermore, reconstitution experiments showing that the dialysis of G_o α protein significantly recovered the inhibitory function of the D₂ receptor strongly support our conclusion that the down-regulation of G_{i/o} is responsible for the hypoxia-induced desensitization of Quin-mediated inhibition of I_{Ca} .

The effect of chronic hypoxia on receptors and the activation of signal transduction pathways outlined in this study do not generalize across all neurotransmitters and receptors. Our findings in this study suggest that the hypoxia-induced desensitization of D₂ receptor function involves the down-regulation the G_o α protein, and that this G_o α protein is, in turn, involved in coupling

the receptor with the channel that mediates I_{Ca} , rather than in down-regulating the D_2 receptor itself. We found, in fact, that both the D_2 mRNA and protein levels increase during exposure to chronic hypoxia. In a previous study, however, we showed that chronic hypoxia reduces the adenosine A_{2A} receptor-mediated inhibition of the calcium current in PC12 cells [23]. In that case, the mechanism of the reduction involved the down-regulation of PKA. Also, the hypoxia-induced desensitization of β -adrenergic receptors is reported to occur in coronary endothelial cells from rat and human [31,35], and the mechanism of desensitization appears to involve a decrease in receptor density [31,35]. Finally, desensitization of the D_2 receptor in response to agonist stimulation is also believed to involve a reduction in receptor density [32]. Thus, the desensitization of D_2 by hypoxia in the current experiments occurs via a different mechanism than has been suggested for agonist-induced D_2 receptor desensitization or for hypoxia-induced desensitization of adenosine A_{2A} receptor.

It is important to determine which of the voltage-dependent Ca^{2+} channels mediates different Ca^{2+} -dependent cellular functions during hypoxia. PC12 cells express the L-, N- and P/Q-type Ca^{2+} channels [19,24]. In the present study, we did not identify which Ca^{2+} channel subtype is coupled to the D_2 receptor. However, previous studies of other cell types have shown that the D_2 receptor modulates the activity of the N-type [20,38] and P/Q-type [37] channels. The effect of D_2 activation on L-type Ca^{2+} channels is controversial [9,20]. Interestingly, a recent study has shown that acute hypoxia evokes exocytosis of catecholamines, primarily via activation of N-type Ca^{2+} channels in PC12 cells [33]. The same authors have also shown that chronic hypoxia increases the secretory response of PC12 cells to acute hypoxia and that the contribution of each Ca^{2+} channel subtype to the secretory response may change during exposure to chronic hypoxia [34].

In summary, exposure to chronic hypoxia significantly attenuates the inhibitory effect of D_2 activation on I_{Ca} . Pretreatment with pertussis toxin abolishes the D_2 -mediated inhibition of I_{Ca} in PC12 cells, but the effect of D_2 stimulation is not prevented by an inhibitor of PKA or by use of PKA-deficient PC12 cells. Importantly, chronic hypoxia leads to an increase in D_2 receptor expression and a significant reduction in the immunoreactivity level of $G_{i/o}\alpha$ protein, while it does not affect the level of G_β . Finally, reconstitution of $G_{o\alpha}$ protein by infusion recovers most of the reduced effect of D_2 stimulation on I_{Ca} in cells exposed to chronic hypoxia. We conclude that oxygen-sensitive PC12 cells respond to hypoxia via a complex mechanism. Acute hypoxia causes excitation in PC12 cells that is manifested by depolarization, elevated intracellular free Ca^{2+} , and DA release. This initial response is modulated by the D_2 receptor, the stimulation of which results in a reduction in I_{Ca} and a decrease in the level of free intracellular Ca^{2+} [40]. Here, we present evidence that prolonged hypoxia desensitizes this D_2 -mediated feedback presumably by way of a reduction of $G_{o\alpha}$ protein. Therefore, it seems that the D_2 -mediated feedback regulatory mechanism during hypoxia is biphasic. We propose that, during acute hypoxia, this D_2 -receptor-mediated feedback mediates reduced cellular excitability by inhibiting the I_{Ca} , which lowers the concentration of intracellular free Ca^{2+} . During prolonged hypoxia, on the other hand, the feedback modulation of I_{Ca} by the D_2 receptor is significantly weakened, allowing for a more sustained influx of Ca^{2+} and enhanced cellular excitation. In this scenario, the reduction in the level of $G_{o\alpha}$ protein during chronic hypoxia reduces the effectiveness of the G_o -coupled D_2 receptor in inhibiting I_{Ca} , which in turn leads to a more excitable state during prolonged hypoxia. Thus, our findings provide further evidence that Ca^{2+} plays a major role in the regulation of cell function during hypoxia, and show that calcium signalling systems are modulated when cells or organisms are chronically exposed to hypoxia, such as in high-altitude environments and in the case of hypoxia-related disease states.

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Fig. 1.

Chronic hypoxia desensitizes D₂-receptor-mediated modulation of Ca²⁺ current (I_{Ca}) in PC12 cells. PC12 cells were exposed for 24 h to hypoxia (10% O₂) with or without the D₂ antagonist butaclamol (3 μ M). I_{Ca} were elicited at room temperature every 30 s by 160-ms depolarizing pulses from a holding potential (HP) of -80 mV to +20 mV. Representative current traces in cells maintained in different experimental conditions (indicated on the *abscissa* of the *bar graph below*) are shown in the *upper panel*. Peak currents were measured during exposure to quinpirole (Quin) 10 μ M when steady-state inhibition was achieved (*open arrow*) and compared to that measured before application of Quin (*solid arrow*). The overall inhibition of I_{Ca} by Quin 10 μ M in these cells is shown in the *lower panel*. The effect of Quin on cells grown in normoxic conditions (21% O₂) is shown as the *black bar*. Cells exposed to chronic hypoxia (10% O₂, 24 h) responded significantly less to Quin (*open bar*). A similar response was obtained in cells maintained in hypoxia (10% O₂) in the presence of butaclamol (*grey bar*). Data are expressed as average $\pm$ SEM (*n*) (**P*<0.05)

Fig. 2. Pertussis-toxin- (PTX-) sensitive G protein mediates D₂ inhibition of I_{Ca} in PC12 cells. PC12 cells were treated overnight with PTX (200 ng/ml), and the effect of D₂ receptor activation on I_{Ca} was studied. Cells with no PTX treatment were used as control (*left*). Ca²⁺ currents were elicited by depolarizing voltage pulses from a HP of -80 mV to +20 mV. I_{Ca} was measured before application of Quin (*solid arrow*) and during exposure to Quin (*open arrow*). Control cells responded to Quin 10 μ M with a marked reduction of I_{Ca} . However, in PTX-treated cells (*right*), Quin had no effect on I_{Ca} ($n=4$, $P<0.05$)

Fig. 3A–C.

D₂-induced inhibition of voltage-dependent Ca²⁺ current is not mediated by protein kinase A (PKA). **A** Expression of D₂ receptor mRNA in PKA-deficient PC12 (A123.7) cells. PCR products obtained by RT-PCR of total RNA for D₂ receptors were visualized by ethidium bromide staining (RT+). The length of the PCR product was expected to be 533 bp. The size marker, 100 bp DNA ladder, is shown in the *left lane* (the size of fragments is indicated on the *left*). Negative control PCR without reverse transcriptase was performed in order to assess any genomic DNA contamination (RT-). **B** Effect of D₂ stimulation on I_{Ca} in parental and PKA deficient (A123.7) PC12 cells. Ca²⁺ currents were elicited by depolarizing voltage steps from a HP of -80 mV to +20 mV in parental PC12 cells (*left*) and in A123.7 cells (*right*) before (*solid arrows*) and during application of Quin 10 μ M (*open arrows*). Overall inhibition of I_{Ca} by Quin in parental PC12 cells (*black bar*) and A123.7 cells (*grey bar*) is shown in the *lower panel*. **C** Effect of the PKA inhibitor peptide (PKI) on Quin-induced modulation of I_{Ca} in PC12 cells. Ca²⁺ currents were elicited by depolarizing voltage steps from a HP of -80 mV to +20 mV. The *upper panel* shows representative traces. I_{Ca} was recorded in PC12 cells, which were dialysed through large-opening pipettes (1–2 M Ω resistance) for at least 6 min with regular pipette solution (*left*) or with pipette solution including 100 μ M PKI. I_{Ca} was measured before (*solid arrow*) and during application of Quin 10 μ M (*open arrow*). Overall

inhibition of I_{Ca} induced by Quin in control cells (*black bar*) and cells dialysed with PKI (*grey bar*) are shown in the *lower panel*. The intracellularly applied PKI did not abolish the effect of Quin on I_{Ca}

Fig. 4A–C.

Regulation of D₂ receptor mRNA and protein expression during chronic hypoxia in PC12 cells. **A** Increased D₂ receptor mRNA during prolonged exposure to hypoxia. Quantitative RT-PCR studies were performed to show the D₂ receptor mRNA levels from cells exposed for 24 h to normoxia (21% O₂) or hypoxia (10% O₂) (*upper panel*). Numbers below the gel image indicate the PCR cycle number. The *lower panel* shows β-actin mRNA levels from the same groups of cells (*n*=4). **B** Effect of the D₂ antagonist butaclamol on D₂ receptor mRNA levels during prolonged hypoxia. D₂ receptor mRNA levels were compared between cells maintained in normoxia (21% O₂), 24 h in hypoxia (10% O₂) and 24 h in hypoxia in the presence or absence of 3 μM butaclamol (10% O₂ + butaclamol). Samples were collected at cycle number 36. **C** Western blot analysis of D₂ receptor protein during prolonged hypoxia. The amount of D₂ receptor protein was measured in total lysates of PC12 cells maintained in normoxia and in 10% O₂ for 6, 12 and 24 h. The D₂ receptor protein levels gradually increased with hypoxia and peaked at 12 h (*upper panel*). The averaged results from 4 separate experiments are provided in *lower panel* (**P*<0.05, ***P*<0.01)

Fig. 5A,B.

Differential regulation of $G_{i/o}\alpha$ and $G\beta$ protein expression during chronic hypoxia in PC12 cells. **A** Western blot analysis for $G_{i/o}\alpha$ immunoreactivity from PC12 cells exposed for 24 h to normoxia (21% O_2), hypoxia (10% O_2) and hypoxia in the presence of 3 μ M butaclamol (10% O_2 + butaclamol). The *lower panel* shows the magnitude of the effect, as quantified by densitometry, expressed as mean $\pm$ SEM. The number of experiments for each group is indicated in *parentheses*. There was significant reduction in the amount of $G_{i/o}\alpha$ protein during 24 h of hypoxia, $*P < 0.05$. **B** Western blot analysis for $G\beta$ protein in PC12 cells maintained in normoxia (21% O_2) and after prolonged exposure to hypoxia (10% O_2). The *upper panel* shows a representative blot showing the time profile in the amount of $G\beta$ protein during hypoxia. Overall changes in the $G\beta$ protein level during hypoxia are shown in the *lower panel* (mean $\pm$ SEM).

Fig. 6A,B.

Reconstitution of G α protein restores hypoxia-induced attenuation in D $_2$ receptor-mediated inhibition of I_{Ca} in PC12 cells. **A** Reconstitution of G α protein in PC12 cells exposed to chronic hypoxia. G α protein was delivered intracellularly by dialysis through large-opening pipettes (1–2 M Ω resistance). Ca $^{2+}$ currents (I_{Ca}) were elicited by depolarizing voltage steps to +20 mV from a HP of –80 mV. Figures show representative recordings of I_{Ca} just after obtaining whole-cell configuration (0 min), 15 min into whole-cell configuration and during application of 10 μ M quinpirole (Quin) in cells incubated under normoxia (*top panel*), chronic hypoxia (10% O $_2$, 24 h, *middle panel*) and chronic hypoxia with dialysis of G α protein (*lower panel*). **B** Effect of reconstitution of G α protein on Quin-induced modulation of I_{Ca} in PC12 cells. Average inhibition of I_{Ca} induced by Quin in cells maintained in normoxia is shown as *black bar*. The effect of Quin was significantly smaller in cells exposed to chronic hypoxia (*open bar*). In cells which had been exposed to chronic hypoxia but was infused with G α protein, the Quin-induced inhibition was significantly restored (*grey bar*). Data are expressed as average $\pm$ SEM (*n*). * P <0.05, ** P <0.01 versus normoxia, † P <0.05 versus chronic hypoxia only