Augmentation of L-Type Calcium Current by Hypoxia in Rabbit Carotid Body Glomus Cells: Evidence for a PKC-Sensitive Pathway

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Summers, Beth A., Jeffrey L. Overholt, and Nanduri R. Prabhakar. Augmentation of L-type calcium current by hypoxia in rabbit carotid body glomus cells: evidence for a PKC-sensitive pathway. J Neurophysiol 84: 1636–1644, 2000. Previous studies have suggested that voltage-gated Ca2+ influx in glomus cells plays a critical role in sensory transduction at the carotid body chemoreceptors. The purpose of the present study was to determine the effects of hypoxia on the Ca2+ current in glomus cells and to elucidate the underlying mechanism(s). Experiments were performed on freshly dissociated glomus cells from rabbit carotid bodies. Ca2+ current was monitored using the whole cell configuration of the patch-clamp technique, with Ba2+ as the charge carrier. Hypoxia (pO2 84: 1636 –1644, 2000. Previous studies have suggested that cytosolic Ca2+ (Ca2+) increases in glomus cells in response to hypoxia (Biscoe and Duchen 1990; Bright et al. 1996; Buckler and Vaughn-Jones 1994; Urena et al. 1994) via activation of voltage-gated Ca2+ channels (Buckler and Vaughn-Jones 1994; Gonzalez et al. 1994; Lopez-Barneo et al. 1993). Blockade of voltage-gated Ca2+ channels prevents augmentation of sensory discharge by hypoxia (Shirahata and Fitzgerald 1991b), implying that voltage-gated Ca2+ flux is required for the transduction of the hypoxic stimulus at the carotid body. Therefore it is of considerable importance to understand whether and how hypoxia regulates Ca2+ current in glomus cells.

Many investigators, however, have focused on the effects of hypoxia on K+ channels (see Gonzalez et al. 1994 for references) and have found that the K+ current is inhibited by hypoxia in glomus cells. These studies suggested an importance of K+ channel closure in producing depolarization resulting in voltage-gated Ca2+ influx as the initial steps in triggering transduction of a hypoxic stimulus at the carotid body. On the other hand, relatively little information is available on the effects of hypoxia on other ionic conductances in glomus cells. Although hypoxia has been found to increase [Ca2+]i, interestingly, several investigations have found that hypoxia has either no effect on (Hescheler et al. 1989; Lopez-Barneo et al. 1988; Lopez-Barneo et al. 1989; Peers 1990) or inhibits (Montoro et al. 1996) Ca2+ current in glomus cells. The apparent discrepancy between the effects of hypoxia on [Ca2+]i and Ca2+ channel activity prompted us to re-examine the effects of low oxygen on Ca2+ current in glomus cells.

It has been shown that bicarbonate (CO3/HCO3)-buffered solutions significantly improve the response of the in vitro carotid body preparation to hypoxia when compared with responses in a HEPES-buffered solution (Iturriaga and Lahiri 1991; Shirahata and Fitzgerald 1991a). This could in part be due to the finding that the intracellular pH in glomus cells bathed in a HEPES buffer is more alkaline than in bicarbonate buffer (Buckler et al. 1991a). All of the previous studies that examined the effects of hypoxia on the Ca2+ current in glomus cells employed a HEPES-based buffer in the medium (Hescheler et al. 1989; Lopez-Barneo et al. 1988; Lopez-Lopez et al. 1989; Montoro et al. 1996; Peers 1990). In the present study, we tested the idea that the effect of hypoxia on the Ca2+ current might be occluded in a HEPES buffer, which turns the

INTRODUCTION

The carotid bodies are the principal sensory organs that detect changes in arterial oxygen. Hypoxia increases the sensory discharge of the carotid bodies, and the ensuing reflexes are crucial for maintaining homeostasis during hypoxemia. Currently, it is believed that glomus cells, which are in synaptic apposition with sensory nerve endings, are the initial sites of sensory transduction. Several lines of evidence indicate that the transduction mechanism(s) for hypoxia involve Ca2+ influx through voltage-gated channels. It has been reported that cytosolic Ca2+ ([Ca2+]i) increases in glomus cells in response to hypoxia (Biscoe and Duchen 1990; Bright et al. 1996; Buckler and Vaughn-Jones 1994; Urena et al. 1994) via activation of voltage-gated Ca2+ channels (Buckler and Vaughn-Jones 1994; Gonzalez et al. 1994; Lopez-Barneo et al. 1993). Blockade of voltage-gated Ca2+ channels prevents augmentation of sensory discharge by hypoxia (Shirahata and Fitzgerald 1991b), implying that voltage-gated Ca2+ flux is required for the transduction of the hypoxic stimulus at the carotid body. Therefore it is of considerable importance to understand whether and how hypoxia regulates Ca2+ current in glomus cells.

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Intracellular milieu alkaline. It is possible that hypoxia augments Ca\textsuperscript{2+} current in glomus cells in a physiologically relevant CO\textsubscript{2}/HCO\textsubscript{3} -buffered extracellular medium. Therefore we examined the effects of hypoxia on the macroscopic Ca\textsuperscript{2+} current in glomus cells isolated from rabbit carotid bodies using a CO\textsubscript{2}/HCO\textsubscript{3} -buffered extracellular solution and found under these conditions that low oxygen does indeed augment Ca\textsuperscript{2+} current.

Rabbit glomus cells express multiple types of voltage-gated Ca\textsuperscript{2+} channels including L, P/Q, and N types of channels (Overholt and Prabhakar 1997). In addition, ~27% of the total macroscopic Ca\textsuperscript{2+} current in rabbit glomus cells is conducted by a channel that is resistant to specific pharmacological blockers of N, P/Q, and L-type Ca\textsuperscript{2+} channels, which we termed the “resistant” current (Overholt and Prabhakar 1997). Whether hypoxia preferentially affects one or more of these channels in glomus cells, however, has not been examined. Protein phosphorylation elicited by protein kinase C (PKC) (Hartzell 1988) and cAMP-dependent protein kinase (PKA) (Gao et al. 1997) has been shown to modulate Ca\textsuperscript{2+} channel activity in a wide variety of cell types. In the carotid body, hypoxia stimulates phospholipase C activity (Pokorski and Stroznajder 1993), which is linked to PKC activation. There is also evidence that cAMP levels are increased during hypoxia, suggesting a role for PKA in hypoxic chemotransduction (Wang et al. 1989). In the second part of the study, we identified the types of Ca\textsuperscript{2+} channels that are affected by hypoxia and examined whether PKC and/or PKA are involved in the modulation of Ca\textsuperscript{2+} current by low oxygen. Our results demonstrate that hypoxia preferentially augments L-type Ca\textsuperscript{2+} current, and the effects of low oxygen are associated with PKC, but not PKA activation.

**Methods**

**General procedures**

Experiments were performed on glomus cells freshly isolated from the carotid bodies of adult rabbits killed with CO\textsubscript{2}. Individual glomus cells were dissociated enzymatically as described previously (Overholt and Prabhakar 1997). Briefly, carotid bodies were incubated at 37°C in a medium containing trypsin (type II, 2 mg/ml, Sigma) and collagenase (type IV, 2 mg/ml, Sigma). The composition of the incubation medium was (in mM) 140 NaCl, 5 KCl, 10 HEPES, and 5 glucose, pH 7.2. The tissue was triturated with a fire-polished, glass Pasteur pipette every 10 min. After 30 min of incubation, cells were pelleted after centrifugation at 1200 rpm for 5 min. Dissociated cells were resuspended in a 50/50 mixture of Dulbecco’s modified Eagles medium (DMEM) and HAM F12 supplemented with penicillin-streptomycin (GIBCO-BRL), insulin, transferrin, selenium (ITS, Sigma), and 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a CO\textsubscript{2} incubator and were used within 36 h. All experiments were performed at room temperature. Glomus cells were identified using electrophysiological characterization as described previously (Summers et al. 1999).

**Isolation of Ca\textsuperscript{2+} current**

Ca\textsuperscript{2+} current was monitored by using the whole cell configuration of the patch-clamp technique (Hamill et al. 1981). Pipettes were made from borosilicate glass capillary tubing and had resistances of 2–3 MΩ. Currents were recorded using an Axopatch 200B voltage-clamp amplifier, filtered at 5 kHz, and sampled at a frequency of 28.6 kHz using an IBM-compatible computer with a Digidata 1200 interface and pCLAMP software (Axon Instruments). Currents were not leak subtracted. Current-voltage (I-V) relations were elicited from a holding potential of ~80 mV using 25-ms steps (5 s between steps) to test potentials over a range of ~50 to +70 mV in 10-mV increments. Current at each potential was measured as the average over a 2.5-ms span at the end of the 25-ms step.

Ca\textsuperscript{2+} current was isolated by using K\textsuperscript{+} and Na\textsuperscript{+}-free intra- and extracellular solutions. The intracellular solution had the following composition (in mM): 115 CsCl, 20 TEA-Cl, 5 MgATP, 0.2 TrisGTP, 5 EGTA, 10 phosphocreatine, and 5 N-2-hydroxyethylpiperazine-N\textsuperscript{-2}-ethanesulfonic acid (HEPES), and the pH was adjusted to 7.2 with CsOH. The HEPES-buffered extracellular solution had an osmolarity of 300 mOsm and contained (in mM) 140 NMGCi, 5.4 CsCl, 10 BaCl\textsubscript{2}, 10 HEPES, and 11 glucose, and the pH was adjusted to 7.4 with CsOH. The CO\textsubscript{2}/HCO\textsubscript{3} -buffered extracellular solution had an osmolarity of 296 mOsm and contained (in mM) 120 NGMCi, 4.8 CsCl, 10 BaCl\textsubscript{2}, 25 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, and 11 glucose, and the pH was adjusted to 7.4 by continuously bubbling with 5% CO\textsubscript{2}. The extracellular solution was changed using a fast-flow apparatus consisting of a linear array of borosilicate glass tubes (Overholt and Prabhakar 1997). In these experiments, we used Ba\textsuperscript{2+} as the charge carrier. For simplicity, Ba\textsuperscript{2+} current conducted by Ca\textsuperscript{2+} channels will be referred to as Ca\textsuperscript{2+} current. To observe Na\textsuperscript{+} current to identify a glomus cell, cells were first superfused with an extracellular solution containing Na\textsuperscript{+}, having the following composition (in mM): 140 NaCl, 5.4 KCl, 2.5 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 5.5 HEPES, and 11 glucose, and the pH was adjusted to 7.4 with NaOH.

Rundown of Ca\textsuperscript{2+} current and the effects of drugs were monitored using a wash protocol (25-ms step to 0 mV, 10 s between steps). The effects of drug agents were compensated for rundown using a linear regression of the current decrease during the wash protocol in the absence of test compounds. Rundown was negligible compared with drug effects over the same time period (e.g., 0.03 ± 0.3% per minute, mean ± SE, n = 4). Cells in which rundown was excessive or did not appear linear were excluded from the analysis. For comparison of I-V relations, Ca\textsuperscript{2+} current at each potential was normalized to the maximum value recorded during the control I-V relation in individual cells (usually 0 mV).

**Tissue collection for histological procedures**

Adult rabbits were anesthetized with a cocktail containing Ketamine, Rompun, and Acetpromazine Maleate (1 ml/kg im). Tracheal intubation was performed, and animals were ventilated with either 100% O\textsubscript{2} (normoxia) or 6% O\textsubscript{2}, 94% N\textsubscript{2} (hypoxia) for 5 min. Animals were then perfused transcardially with fixative, and tissues were removed and postfixed overnight. The fixative used was a 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Tissue samples were cryoprotected in 30% sucrose in PBS, infiltrated with a 1:1 mixture of 30% sucrose-PBS and OCT embedding medium (Sisue-Tek OCT-4583, Baxter Scientific), embedded in OCT, frozen over dry ice, and cut in a cryostat (Leica, 10-μm sections). There are three animals in the normoxic group and three animals in the hypoxic group. For each specific antibody, 2 slides with 12 tissue sections on each slide were stained for each animal in the group. On each tissue section, the glomus cells were then counted to arrive at a percentage of cells that formed ringlets under hypoxic conditions versus diffuse staining under normoxic conditions.

**Immunofluorescence labeling with PKC isofoms of carotid body tissue**

The indirect immunofluorescence technique was used to detect the presence of and to localize the cellular distribution of PKC isofoms before and after exposure to hypoxia. Fixed tissues were washed with 0.1 M PBS containing 0.2% Triton-X (PBS-Tx) for 30 min, and then blocked with 20% goat serum, 2% bovine serum albumin (BSA) in PBS-Tx for 2 h. Subsequently, tissues were incubated with monoclo-
nal, isoform-specific PKC antibodies or monoclonal TH antibody (DiaSorin) at the appropriate dilution in 2% goat serum in PBS-Tx for 24 h at 4°C. Monoclonal antibodies to seven individual PKC isoforms (α, β, γ, δ, ε, λ, and θ) were used for immunofluorescence protocols (Transduction Laboratories). After incubation with primary antibody, tissues were thoroughly washed with PBS-Tx and incubated with Texas Red goat anti-mouse immunoglobulin G (secondary antibody) diluted 1:1,000 in 2% goat serum in PBS-Tx for 2 h at room temperature. An immunocytochemical control for antibody specificity was performed by incubating the tissues with secondary antibody only. After a thorough wash in PBS-Tx, the tissues were mounted on microscope slides with Immuno-mount (Shandon). The tissue sections were viewed and imaged with a Nikon Eclipse E600 epifluorescent microscope equipped with a Diagnostic Instrument Spot camera and software.

Solutions and drugs

CO₂/HCO₃⁻-buffered extracellular solutions were made normoxic or hypoxic by continuously bubbling with either 21% O₂, 5% CO₂, 74% N₂ or 1% O₂, 5% CO₂, 94% N₂, respectively. HEPES-buffered extracellular solutions were made normoxic or hypoxic by continuously bubbling the solution with either 21% O₂, 79% N₂ or 1% O₂, 99% N₂. The pO₂ was routinely monitored with a blood gas analyzer (Laboratory Instruments) and found to be between 35 ± 5 mmHg (n = 5) for hypoxic solutions and 148 ± 3 mmHg (n = 5) for normoxic solutions. Stock solutions of staurosporine (Calbiochem), bisindolylmaleimide I (Calbiochem), and phorbol 12-myristate 13-acetate (PMA, Calbiochem) were prepared in dimethylsulfonoxide (DMSO). Nisoldipine (Miles Laboratories) was prepared as a stock solution in polyethylene glycol (PEG, MW = 400, Sigma). PKA inhibitor amide, myristoylated (Calbiochem) and ω-conotoxin MVIIIC (Alomone Labs, Jerusalem, Israel) stock solutions were prepared in sterilized, deionized water. Experiments were done in the dark when light-sensitive reagents were used (e.g., PMA, nisoldipine). The final concentrations of either DMSO or PEG were 0.1%. In control experiments (n = 4), the DMSO or PEG vehicle alone (i.e., without drug) did not effect the Ca²⁺ current.

Data analysis

All values are presented as means ± SE. Statistical significance was determined by a paired t-test or a one-way ANOVA, with Tukey’s post hoc test where appropriate. P values <0.05 were considered significant.

RESULTS

Hypoxia augments Ca²⁺ current in glomus cells

An example illustrating the effects of hypoxia (pO₂ of medium = 40 mmHg) on the Ca²⁺ current and the time course of the response in a CO₂/HCO₃⁻-buffered extracellular solution recorded from a glomus cell is shown in Fig. 1, A and B. It is obvious from these traces that hypoxia reversibly augmented the Ca²⁺ current. Figure 1B shows the time course for changes in Ca²⁺ current elicited at 0 mV from a holding potential of −80 mV. The effect of hypoxia began within tens of seconds, plateaued within 1 min, and returned to control levels within 1 min after terminating the hypoxic challenge. To assess whether hypoxia affected the I-V relationship of the Ca²⁺ current, the effects of hypoxia were tested over a broad range of membrane potentials. Figure 1C shows the average (n = 42), normalized I-V relations before and during exposure to hypoxia. Hypoxia increased the magnitude of the peak current equally over the range of potentials tested, suggesting that the effect is voltage independent. On average, the Ca²⁺ current was augmented by 24 ± 3% at 0 mV (n = 42, P < 0.05, paired t-test) when Ba²⁺ was used as the charge carrier. Similar results were obtained when Ca²⁺ was used as the charge carrier. With 2.5 mM Ca²⁺ as the charge carrier, hypoxia augmented the current by 16 ± 4% at 0 mV (n = 4, P < 0.05, paired t-test, data not shown). Since the effects of hypoxia were qualitatively and quantitatively similar (P > 0.05, ANOVA), subsequent experiments used Ba²⁺ as the charge carrier to enhance the magnitude of the current. These experiments demonstrate that hypoxia augments Ca²⁺ current in glomus cells in a CO₂/HCO₃⁻-buffered extracellular solution.

Comparison of the effects of hypoxia on the Ca²⁺ current in a CO₂/HCO₃⁻ versus HEPES-buffered extracellular solution

Previous studies have examined the effect of hypoxia on Ca²⁺ current in glomus cells using a HEPES-buffered extracellular solution (Hescheler et al. 1989; Lopez-Barneo et al. 1988; Lopez-Lopez et al. 1989; Montoro et al. 1996; Peers 1990). For comparison, we tested the effect of hypoxia on the Ca²⁺ current in a HEPES-buffered extracellular solution (pH 7.4). We found that hypoxia has no effect on the Ca²⁺ current under these conditions (n = 6). These results are shown in Fig. 2, A and B, which show the current traces and the time course,
respectively, when a glomus cell was exposed to hypoxia in a HEPES-buffered extracellular solution. These results demonstrate that hypoxia does not affect Ca$^{2+}$ current in a HEPES-buffered extracellular solution (pH 7.4).

It has been reported that switching from a HEPES- to a CO$_2$/HCO$_3^-$-buffered extracellular solution causes intracellular acidification in rat glomus cells (Buckler et al. 1991). Therefore we tested whether a change in pH could be responsible for the lack of Ca$^{2+}$ current augmentation by hypoxia when using a HEPES-buffered extracellular solution. For this purpose, we adjusted the pH of the HEPES-buffered extracellular solution to 7.0 with 1 N HCl. The current traces shown in Fig. 2C clearly show that hypoxia augments the Ca$^{2+}$ current at pH 7.0 in a HEPES-buffered external solution. The time course of the response shown in Fig. 2D resembles that seen in a CO$_2$/HCO$_3^-$-buffered extracellular solution (see Fig. 1). On average, hypoxia significantly augmented the Ca$^{2+}$ current by 20 ± 5% at 0 mV (n = 4, P < 0.05, paired t-test), which is similar to the augmentation seen in a CO$_2$/HCO$_3^-$-buffered extracellular solution (P > 0.05, ANOVA). These results demonstrate that hypoxia can augment Ca$^{2+}$ current in a HEPES-buffered extracellular solution when the pH is more acidic. Furthermore, these observations suggest that the lack of augmentation by hypoxia in a HEPES-buffered extracellular solution is not due to the presence of HEPES itself, rather secondary to changes in pH. Since hypoxia augmented Ca$^{2+}$ current in glomus cells in a CO$_2$/HCO$_3^-$-buffered extracellular solution, a more relevant physiological buffer, the remainder of the study was performed with this buffer.

We have previously reported that Ca$^{2+}$ current in rabbit glomus cells is conducted by four different types of voltage-dependent Ca$^{2+}$ channels including L, P/Q, N, and resistant channels (Overholt and Prabhakar 1997). Having characterized the conditions under which hypoxia augments the Ca$^{2+}$ current, we then asked whether hypoxia affects a particular Ca$^{2+}$ channel type. We first tested the effect of nisoldipine (2 μM NISO), an L-type Ca$^{2+}$ channel blocker, on the hypoxia-induced augmentation of the Ca$^{2+}$ current. Figure 3A shows the effect of hypoxia on the Ca$^{2+}$ current elicited by a step to 0 mV in the presence of NISO. As expected, NISO by itself blocked a portion of the basal Ca$^{2+}$ current (28 ± 7%). However, in the presence of NISO, hypoxia had little effect on the Ca$^{2+}$ current as can be seen in the current traces shown in Fig. 3A. The time course in Fig. 3B shows that hypoxia augments the Ca$^{2+}$ current prior to application of NISO, but this effect is negligible in the presence of NISO. On average, hypoxia augmentation of the Ca$^{2+}$ current was negligible (1 ± 6%, n = 6; P > 0.05, paired t-test) in the presence of nisoldipine. In another series of experiments (n = 6), we examined the effects of α-conotoxin MVIIC (MVIIC), a selective inhibitor of N- and P/Q-type Ca$^{2+}$ channels, on the hypoxia-induced augmentation of the Ca$^{2+}$ current. An example depicting the effect of hypoxia on the Ca$^{2+}$ current in the presence of 2 μM MVIIC is shown in Fig. 3C. As expected, MVIIC by itself

![Figure 2](image-url) Hypoxia augments the Ca$^{2+}$ current in a HEPES-buffered extracellular solution at pH 7.0, but not at 7.4. A: raw, whole cell Ca$^{2+}$ current recorded before (150 mmHg), during (40 mmHg), and after (Wash) exposure to a hypoxic extracellular solution (HEPES, pH 7.4). Broken line indicates 0 current level, and subscripts correspond to numbers in B indicating the position of the current traces in the time course. B: time course for changes in Ca$^{2+}$ current elicited at 0 mV under the conditions described in A. C: raw, whole cell Ca$^{2+}$ current recorded before (150 mmHg), during (40 mmHg), and after (Wash) exposure to a hypoxic extracellular solution (HEPES, pH 7.0). Broken line indicates 0 current level, and subscripts correspond to numbers in D indicating the position of the current traces in the time course. D: time course for changes in Ca$^{2+}$ current elicited at 0 mV under the conditions described in C.

**Figure 3.** Hypoxia augments L-type Ca$^{2+}$ current in rabbit glomus cells. A: raw, whole cell Ca$^{2+}$ current elicited by a step to 0 mV before (150 mmHg) and during exposure to an extracellular solution containing either 2 μM nisoldipine (NISO) or a hypoxic solution containing NISO (40 mmHg + NISO). Broken line indicates 0 current level, and subscripts correspond to numbers in B indicating the position of the current traces in the time course. B: time course for changes in Ca$^{2+}$ current as the extracellular solution is switched to and from a hypoxic solution in the absence and then in the presence of 2 μM NISO. C: raw, whole cell Ca$^{2+}$ current elicited by a step to 0 mV before and during exposure to an extracellular solution containing either 2 μM α-conotoxin-MVIIC (MVIIC) or a hypoxic solution containing MVIIC (40 mmHg + MVIIC). Broken line indicates 0 current level, and subscripts correspond to numbers in D indicating the position of the current traces in the time course. D: time course for changes in Ca$^{2+}$ current elicited at 0 mV under the conditions described in C.
blocked a portion of the basal Ca\(^{2+}\) current (17 ± 7%). However, in the presence of MVIIC, hypoxia still augmented the Ca\(^{2+}\) current, and the time course of the response resembled that of controls without MVIIC (Figs. 3D and 1B). Average data showed no significant difference in the magnitude of the response to hypoxia in the presence and absence of MVIIC (Fig. 7, hypoxia alone = 24 ± 3% vs. MVIIC + hypoxia = 26 ± 10%; \( P > 0.05 \), ANOVA). These results suggest that hypoxia selectively augments the Ca\(^{2+}\) current conducted by L-type Ca\(^{2+}\) channels, not by N- or P/Q-Ca\(^{2+}\) channel types.

PKC, but not cAMP-dependent kinase (PKA), inhibitors prevent the augmentation of the Ca\(^{2+}\) current by hypoxia

Hypoxia has been shown to increase phospholipase C (PLC) activity (Pokorski and Stroznajder 1993) and cAMP levels (Wang et al. 1989) in glomus cells. To test whether PKC and/or PKA are involved with Ca\(^{2+}\) current augmentation by hypoxia, we examined the effects of hypoxia in the presence of either PKC or PKA inhibitors. PKC inhibitors, staurosporine (STRO; 100 nM; \( n = 6 \)) and bisindolylmaleimide (BIM; 2 \( \mu M; n = 8 \)), prevented the augmentation of the Ca\(^{2+}\) current by hypoxia. As evidenced by the current traces and the time courses presented in Fig. 4, A–D, STRO and BIM augmented basal Ca\(^{2+}\) current by 18 ± 7% and 18 ± 6%, respectively. More importantly, in the presence of these inhibitors, hypoxia had no significant effect on the Ca\(^{2+}\) current (see also Fig. 7). These results suggest that PKC may be involved in the augmentation of the Ca\(^{2+}\) current by hypoxia. Next, we tested the effects of hypoxia on the Ca\(^{2+}\) current in the presence of a cell-permeable form of a PKA inhibitor (4 nM PKAi). Figure 5, A and B, illustrates the effect of PKAi, and hypoxia in the presence of PKAi on the current traces and the time course, respectively. PKAi also augmented the basal Ca\(^{2+}\) current by 14 ± 6%. However, in contrast to the PKC inhibitors, PKAi did not prevent further augmentation of Ca\(^{2+}\) current by hypoxia. In the presence of PKAi, hypoxia significantly augmented the Ca\(^{2+}\) current by 22 ± 4% (Fig. 7, \( n = 10 \), at 0 mV), implying that PKA is not associated with augmentation of the Ca\(^{2+}\) current by hypoxia.

A phorbol ester mimics the effects of hypoxia on the Ca\(^{2+}\) current in glomus cells

The results described above indicate that activation of PKC is associated with augmentation of Ca\(^{2+}\) current by hypoxia. To further establish the role of PKC in the stimulatory effect of hypoxia, we tested whether phorbol 12-myristate 13-acetate (100 nM PMA), a PKC activator, could mimic the effect of low oxygen on the Ca\(^{2+}\) current in glomus cells. Figure 6, A and B, illustrates the effect of PMA on the current traces and the time course, respectively. PMA, like hypoxia, augmented the basal Ca\(^{2+}\) current by 20 ± 3% at 0 mV (\( n = 8 \)). When cells were
Hypoxia and calcium current in glomus cells

Challenged with PMA and hypoxia together, their effects on the Ca\(^{2+}\) current were not additive \((n = 3, 29 \pm 5\%\), see Fig. 7). The possible involvement of PKC in the regulation of Ca\(^{2+}\) channels in glomus cells was further tested with long-term exposure of cells to PMA, which is known to deplete PKC (Zhong et al. 1999). For this purpose, freshly dissociated glomus cells from rabbit carotid bodies were split into two populations. One population of cells was left untreated, and the other was treated with 100 nM PMA overnight. We then compared the effect of hypoxia on the Ca\(^{2+}\) current in these two populations. In the untreated group, hypoxia increased Ca\(^{2+}\) current by 28 \(\pm\) 7\%, whereas the cells treated overnight with PMA did not respond to hypoxia (see Fig. 7, -3 \(\pm\) 4\%, \(n = 5\), at 0 mV, \(P < 0.05\), ANOVA). A summary of the average percent augmentation of the Ca\(^{2+}\) current under the various conditions are summarized in Fig. 7. Taken together, these results suggest that PKC is involved in the hypoxia-induced augmentation of the Ca\(^{2+}\) current in glomus cells.

**Hypoxia causes PKC\(\delta\) to translocate from the cytosol to the membrane in glomus cells**

Immunocytochemical analysis was performed to assess the effects of hypoxia on PKC isoforms in glomus cells. Since BIM is a specific inhibitor of \(\alpha\), \(\beta\), \(\delta\), \(\epsilon\), and \(\gamma\) isoforms of PKC, we tested whether these isoforms are present in glomus cells, and if so, which isoforms are activated by hypoxia. Sections from carotid bodies were stained for \(\alpha\), \(\beta\), \(\delta\), \(\epsilon\), and \(\gamma\) isoforms of PKC using monoclonal antibodies conjugated to Texas red as the fluorochrome. As shown in Fig. 8E, only PKC\(\delta\)-like immunoreactivity could be seen in glomus cells. To further identify glomus cells, we stained carotid body sections with monoclonal anti-tyrosine hydroxylase (TH), a well-established marker of glomus cells (Gonzalez et al. 1994). We found a similar pattern of anti-TH and anti-PKC\(\delta\) immunoreactivity in glomus cells in the normoxic tissues (Fig. 8, C and D). Figure 8E shows the control for antibody specificity in which the tissues were incubated with secondary antibody only (anti-mouse goat IgG). There was no immunoreactivity with omission of the primary antibody (negative control). On the other hand, PKC\(\gamma\)-like immunoreactivity was exclusively localized to the vascular cells of the carotid artery (arrows, Fig. 8B). These results suggest that the PKC\(\delta\) isoform of PKC is found in rabbit glomus cells, whereas the PKC\(\gamma\) isoform of PKC is found in the vasculature. In one of the three animals, PKC\(\alpha\)-like immunoreactivity was found in the carotid body vasculature (Fig. 8A).

To test whether PKC is activated by low oxygen, anesthetized animals were exposed to hypoxia (6\% O\(_2\) for 5 min, \(n = 3\)). Subsequently, animals were perfused, carotid bodies were removed, sectioned, and stained for PKC\(\delta\). As shown in Fig. 9B, PKC\(\delta\)-like immunoreactivity localized more toward the cell membrane of glomus cells forming ringlet-like structures in the carotid bodies exposed to hypoxia (arrows, Fig. 9B) compared with normoxic controls (Fig. 9A). In all three animal preparations tested for the hypoxic group, 87 \(\pm\) 3\% of the cells...
(n = 2,040 cells) under hypoxia formed ringlets of anti-PKCδ-like immunoreactivity in glomus cell clusters throughout the carotid body tissue (n = 30 sections), while the normoxic animal group (100% O₂, for 5 min, n = 3) showed no evidence of ringlet formation, but rather diffuse staining in the cytosol of the glomus cell clusters (n = 2,550 cells). These results suggest that the PKCδ isoform of PKC translocates from the cytosol to the plasma membrane during hypoxia, suggesting that PKCδ is activated during hypoxia.

**DISCUSSION**

Previous studies have shown that [Ca²⁺]ᵢ increases in response to hypoxia, and this response depends on Ca²⁺ entry through voltage-activated Ca²⁺ channels. However, several investigators have reported that hypoxia either has no effect on, or inhibits Ca²⁺ current (see references in INTRODUCTION). Therefore in this study, we re-examined the effects of low oxygen on the Ca²⁺ current. Our results demonstrate that hypoxia augments Ca²⁺ current, and this effect is primarily confined to L-type Ca²⁺ channels in glomus cells isolated from rabbit carotid bodies. The effects of hypoxia are seen in a CO₂/HCO₃⁻-buffered extracellular solution. Furthermore, the effect of hypoxia on the Ca²⁺ current appears to be associated with activation of a PKC-sensitive pathway.

**Effect of hypoxia on the Ca²⁺ current in a CO₂/HCO₃⁻-buffered versus a HEPES-buffered extracellular solution**

It can be seen from our results that hypoxia reversibly augments the Ca²⁺ current recorded in a CO₂/HCO₃⁻-buffered extracellular solution. The onset of the effects were rapid (occurring within tens of seconds after application of hypoxia) and reversible (Fig. 1). However, the present results differ from those reported by others who found that hypoxia either had no effect on (Hescheler et al. 1989; Lopez-Barneo et al. 1988) or inhibited Ca²⁺ current (Montoro et al. 1996). This discrepancy is not due to species-related differences because rabbit glomus cells were utilized in all studies. It is possible that the discrepancy between the studies is due to the use of different experimental conditions. For instance, previous studies applied hypoxia in a HEPES-buffered extracellular solution, whereas we applied hypoxia in a CO₂/HCO₃⁻-buffered extracellular solution. It has been reported that without CO₂/HCO₃⁻, tissues and cells respond differently, or even oppositely to those in the presence of CO₂/HCO₃⁻ (Thomas 1989). Also, in the carotid body, catecholamine secretion is enhanced by the presence of bicarbonate as compared with bicarbonate-free solution for the same hypoxic stimulus (Panisello and Donnelly 1998). Furthermore, several investigators have reported that the presence of CO₂/HCO₃⁻ significantly improved the response to hypoxia in the in vitro carotid body preparation as opposed to responses in HEPES (Iturriaga and Lahiri 1991; Shirahata and Fitzgerald 1991). Therefore it is possible that the presence CO₂/HCO₃⁻ in the extracellular medium also improves the responsiveness of Ca²⁺ channels to hypoxia.

Consistent with other investigators (Hescheler et al. 1989; Lopez-Barneo et al. 1988; Peers 1990), we also found no effect of hypoxia on Ca²⁺ current when using a HEPES-buffered extracellular solution at pH 7.4 (Fig. 2, A and B). However, we did not observe inhibition of the Ca²⁺ current in a HEPES-buffered medium as reported by Montoro et al. (1996). This could possibly be explained by the severity of hypoxia used in our study compared with Montoro et al. (1996). In the present study, we applied a moderate level of hypoxia (40 mmHg), whereas Montoro and colleagues (1996) applied a more severe level of hypoxia (10–20 mmHg).

How might CO₂/HCO₃⁻ improve the effects of hypoxia on
the Ca\(^{2+}\) current? It has been reported that the intracellular pH (pH\(_i\)) is significantly higher in cells exposed to a HEPES-buffered media (pH 7.8) than in bicarbonate-buffered media (pH 7.2) (Buckler et al. 1991; Thomas 1989). Therefore it is possible that the lack of an effect of hypoxia in a HEPES-buffered extracellular solution could be due to a more alkaline intracellular pH in cells under this condition. This idea is supported by our finding that hypoxia is able to augment Ca\(^{2+}\) current in a more acidic HEPES-buffered extracellular solution (pH 7.0, Fig. 2, C and D). Most importantly, these observations suggest that the lack of augmentation by hypoxia in a HEPES-buffered extracellular solution is not due to the presence of HEPES itself, rather it is secondary to changes in pH\(_i\). These secondary changes in pH\(_i\) could influence Ca\(^{2+}\) channel activity and thus mask the effect of hypoxia on the Ca\(^{2+}\) current in glomus cells under HEPES-buffered conditions at pH 7.4. In addition, secondary changes in pH\(_i\) could also influence PKC enzyme activity. For example, the association of the catalytic and regulatory domains of PKC is affected by changing pH (McFadden et al. 1989).

**Hypoxia augmentation is primarily confined to L-type Ca\(^{2+}\) channels**

Since rabbit glomus cells express multiple types of Ca\(^{2+}\) channels (Overholt and Prabhakar 1997), we examined whether the effect of hypoxia was confined preferentially to one type of Ca\(^{2+}\) channel in glomus cells. Our data indicate that the effects of hypoxia are confined to L-type Ca\(^{2+}\) current in glomus cells (Fig. 3, A and B), not N- and P/Q-type Ca\(^{2+}\) currents (Fig. 3, C and D). Sensitivity of the L-type Ca\(^{2+}\) current to hypoxia resembles that reported for L-type Ca\(^{2+}\) current in smooth muscle (Franco-Obregon et al. 1995). In a variety of cell types, including glomus cells, the L-type Ca\(^{2+}\) channel, in particular, has been found to be affected by a variety of gaseous molecules. This is supported by their sensitivity to O\(_2\) as well as another gaseous molecule, nitric oxide (Campbell et al. 1996; Franco-Obregon et al. 1995; Summers et al. 1999). Most importantly, some studies have suggested that the L-type current is involved in the hypoxia-induced neurotransmitter release from glomus cells (Gomez-Nino et al. 1994; Obeso et al. 1992). Taken together, these data suggest that augmentation of the L-type Ca\(^{2+}\) current in glomus cells by low oxygen may play a functional role by enhancing neurotransmitter release during hypoxia at the carotid body.

**Evidence for the involvement of PKC in the hypoxic-induced augmentation of the Ca\(^{2+}\) current**

Several observations from the present study provide evidence for the involvement of PKC in hypoxia-induced augmentation of the Ca\(^{2+}\) current in rabbit glomus cells. First, although kinase inhibitors (i.e., PKC and PKA inhibitors), in general, augmented basal Ca\(^{2+}\) current, only PKC inhibitors prevented hypoxia-induced augmentation of the Ca\(^{2+}\) current (Figs. 4 and 5). However, one caveat with using the whole cell configuration of the patch-clamp technique is that the intracellular milieu becomes dialyzed, allowing escape of second messengers, and therefore the possible involvement of PKA cannot be ruled out totally. Second, PMA, an activator of PKC, mimicked the effects of hypoxia (Fig. 6). Third, hypoxia had no effect on Ca\(^{2+}\) current in glomus cells following overnight treatment with PMA, which is known to deplete PKC (Zhong et al. 1999). A role for PKC in modulation of the Ca\(^{2+}\) current by hypoxia was further supported by the observation that glomus cells express PKC\(\delta\) isoform of PKC, and that hypoxia translocates PKC\(\delta\) from the cytosol to the membrane (ringlet formation), an event associated with PKC activation (Fig. 9) (Wieloch and Cardell 1993). These observations are consistent with translocation of PKC by hypoxia reported elsewhere in neural tissues (Wieloch and Cardell 1993) and activation of PKC isoforms in the pulmonary vasculature by low oxygen (Weissmann et al. 1999). In the present study, we found evidence for PKC\(\delta\) isoform of PKC in glomus cells of the rabbit carotid body. However, the present results differ from those reported by another study that found the presence of the PKC\(\alpha\) isoform in glomus cells of the normoxic cat carotid body (Faff et al. 1999). This difference could be due to the presence of different PKC isoforms in glomus cells from different species. Nonetheless, our results support the idea that activation of PKC is associated with modulation of Ca\(^{2+}\) channels by hypoxia. Our study, however, only provides a beginning step toward the characterization of what isoforms of PKC might be present in rabbit carotid body tissue, and whether hypoxia affects PKC isoforms. Further studies are needed to define the mechanisms by which hypoxia activates the different isoforms of PKC, and how hypoxia modulates the phosphorylation states of the Ca\(^{2+}\) channels in glomus cells.

In summary, we have shown that hypoxia augments Ca\(^{2+}\) current in a CO\(_2\)/HCO\(_3\)-buffered extracellular solution and this augmentation is primarily confined to L-type Ca\(^{2+}\) current and seems to be coupled to the activation of PKC.

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