O₂ Sensing by Airway Chemoreceptor-derived Cells

PROTEIN KINASE C ACTIVATION REVEALS FUNCTIONAL EVIDENCE FOR INVOLVEMENT OF NADPH OXIDASE*

(Received for publication, June 14, 1999, and in revised form, September 24, 1999)

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Accumulating evidence suggests that neuroepithelial bodies are airway O₂ sensors. Recently, we have established the H-146 small cell lung carcinoma line as a suitable model to study the biochemical basis of neuroepithelial cell chemotransduction. Here we explore the possibility that hypoxic modulation of K⁺ channels is intimately linked to activity of NADPH oxidase. Graded hypoxia caused graded inhibition of whole cell K⁺ currents, which correlated well with membrane depolarization. Pretreatment with the phorbol ester, 12-O-tetradecanoyl (TPA), inhibited K⁺ currents at all potentials. Although 4α-phorbol 12,13-didecanoate and TPA in the presence of bisindolylmaleimide were also able to depress K⁺ currents, only TPA could significantly ameliorate hypoxic depression of these currents. Thus, protein kinase C (PKC) activation modulates the sensitivity of these cells to changes in pO₂. Furthermore, because the addition of H₂O₂, a downstream product of NADPH oxidase, could only activate K⁺ currents during hypoxia (when endogenous H₂O₂ production is suppressed), it appears likely that PKC modulates the affinity of NADPH oxidase for O₂ potentially via phosphorylation of the p47phox subunit, which is present in these cells. These data show that PKC is an important regulator of the O₂-transduction pathway and suggests that NADPH oxidase represents a significant component of the airway O₂ sensor.

Ventilation-perfusion matching is essential for efficient oxygenation of pulmonary blood in the face of varying demands of the body’s tissues. Numerous physiological processes have long been known to ensure optimization of ventilation-perfusion matching, but the cellular mechanisms underlying such processes are only now beginning to be understood. One such important mechanism is that of O₂ chemoreception, i.e. an active cellular response to an acute general and/or regional reduction of pO₂. Most studies to date have focused on the carotid body arterial chemoreceptors. Type I cells of these sensors express O₂-sensitive K⁺ channels, and exposure to hypoxia causes rapid channel inhibition. This leads to cell depolarization, Ca²⁺ entry via voltage-gated Ca²⁺ channels, and subsequent triggering of neurosecretion, an essential step in the initiation ofafferent information, which leads to reflex increases of ventilation (1, 2). In vascular smooth muscle, O₂-sensitive K⁺ channels (3) and Ca²⁺ channels (4) have more recently been reported, and their rapid modulation by hypoxia evokes appropriate vasodilatation or vasoconstriction (depending on the location of the blood vessel (5)). Compelling evidence is emerging that neuroepithelial bodies (NEBs), located in clusters at bronchial bifurcations throughout the lung, serve as airway chemoreceptors (6, 7). Like their vascular counterparts in the carotid body, NEB cells possess numerous transmitters, which appear to be released in hypoxia (8). These transmitters (particularly serotonin) may act to initiate afferent nerve activity to medullary respiratory centers and may also act to control local vasomotor tone, hence directly influencing local ventilation-perfusion matching (9). Compared with type I cells of the carotid body, our understanding of O₂ chemoreception by NEB cells is in its infancy, due largely to the technically demanding nature of their isolation/primary culture or to the difficulty in recording currents in situ using the lung slice. However, it has been established that they are electrically excitable and, most importantly, possess O₂-sensitive K⁺ channels (7, 10), suggesting that they may respond, at least superficially, like carotid body type I cells. In recent studies, we have built on the suggestions of others to establish that small cell lung carcinoma (SCLC) cells, which are believed to be derived from NEB cells (11) and with which they share numerous similarities (12), represent immortal airway chemoreceptors. Thus, we have recently identified, in the SCLC cell line H-146, a specific component of the whole cell K⁺ current, which is sensitive to hypoxia and influences membrane potential (13, 14). This current is 4-aminoypyridine- (4-AP) and Ca²⁺-insensitive, quinidine-sensitive, and may be a novel member of the tandem P-domain family of K⁺ channels. The ease of use of cell lines as compared with isolated NEB cells or lung slices suggests that they may be exploited to understand the molecular and biochemical mechanisms underlying rapid effects of hypoxia on chemoreceptor cells.

The mechanism(s) which couple a fall of pO₂ to K⁺ channel inhibition remain elusive, but recent work has implicated the involvement of NADPH oxidase as an O₂ sensor in both NEB cells and SCLC cells (15). The NADPH oxidase model for O₂ chemoreception suggests that, under normoxic conditions, the oxidase tonically generates superoxide from O₂, which is rapidly converted to H₂O₂ by a number of enzymes including superoxide dismutase and catalase. This H₂O₂ is believed to promote channel activity. It follows, therefore, that H₂O₂ levels will decline in hypoxia, causing a concomitant reduction in

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channel activity. Several independent lines of evidence support this idea: (i) NEB and SCLC cells express the mRNA encoding K\(^+\) channels known to be modulated by H\(_2\)O\(_2\); (ii) in situ hybridization and immunohistochemistry have indicated that specific subunits of NADPH oxidase (gp91\(_\text{phox}\) and p22\(_\text{phox}\)) are present in both cell types; (iii) H\(_2\)O\(_2\) generation in NEB cell clusters is significantly greater than in surrounding tissue, can be enhanced by protein kinase C (PKC) activation using phorbol ester (which specifically phosphorylates another subunit, p47\(_\text{phox}\), of the oxidase complex), and can be suppressed by diphenyleneiodonium, a known NADPH oxidase inhibitor and; (iv) K\(^+\) currents can be enhanced (albeit transiently) by application of H\(_2\)O\(_2\) (10, 15). Collectively, these observations support the idea that hypoxic inhibition of K\(^+\) channels in airway chemoreceptor cells and SCLC cells involves reduced levels of H\(_2\)O\(_2\) derived from NADPH oxidase activity, but a direct link between the observations is still lacking. Indeed, it has, to date, never been demonstrated directly that hypoxia causes a fall of H\(_2\)O\(_2\) levels in these cells.

In the present study, we demonstrate for the first time that H\(_2\)O\(_2\) production declines during hypoxia and have exploited the fact that NADPH oxidase activity can be regulated by PKC-dependent phosphorylation (16) to test further this model for O\(_2\) chemoreception in SCLC cells. Our results provide compelling functional evidence to support a central role for NADPH oxidase in this important process, and we propose that PKC may modulate chemoreception by altering the affinity of the oxidase for O\(_2\).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

The small cell lung carcinoma cell line, H-146, was purchased from American Tissue Type Cell Collection (Rockville, MD) and was of unknown passage number. Cells were grown in suspension culture in RPMI 1640 medium (containing L-glutamine) supplemented with 10% fetal calf serum, 2% sodium pyruvate, and 2% penicillin/streptomycin (all from Life Technologies, Inc.) in a humidified atmosphere of 5% CO\(_2\)/95% air at 37 °C. Medium was changed every 2 days and cells were passaged every 6–7 days by splitting in the ratio 1:5. Cells were used between nominal passage numbers 1 and 6.

**Electrophysiology**

Solutions and Chemicals—Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma. Standard pipette solution was K\(^+\)-rich and contained: 10 mM NaCl, 117 mM KCl, 2 mM MgSO\(_4\), 10 mM HEPES, 11 mM EGTA, 1 mM CaCl\(_2\), 2 mM Na\(_2\)ATP, pH 7.4, with KOH; free [Ca\(^{2+}\)] = 112 nM. Standard bath solution was Na\(^+\)-rich and contained: 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl\(_2\), 5 mM HEPES, 2.5 mM CaCl\(_2\), 10 mM D-glucose, pH 7.4, with NaOH. 4-AP was added to the bath solution where indicated, and osmolality was maintained by isoosmotic substitution of NaCl. Where indicated, cells were preincubated with 100 nM of the phorbol esters 12-O-tetradecanoylphorbol-13-acetate (TPA) and 4\(_\alpha\)-phorbol 12, 13-didecanoate (4\(_\alpha\)-PDD) and in some cases with the PKC inhibitor, bisindolylmaleimide (BIM, 1 \(\mu\)M), for 10 min at room temperature. Where used, 1 \(\mu\)M BIM was also included in the pipette solution.

All tubing was gas-permeant (Tygon tubing BDH, Atherstone, Berkshire, UK). Normoxic solutions were equilibrated with room air. Solutions were made hypoxic, where appropriate, by bubbling with N\(_2\)\(_{95}\) for at least 30 min prior to perfusion of cells. This procedure produced no shift in pH. Solution flow rate was ~5 ml/min\(^{-1}\). Graded hypoxia was achieved by splicing variable lengths of gas-permeant tubing into the
perfusion lines. $pO_2$ was measured (at the cell) using a polarized (-800 mV), calibrated carbon fiber electrode (16); for the experiments reported herein, the $pO_2$ values were 150 (normoxia), 85, 45, 25, and 15 (hypoxia) mm Hg.

**Whole Cell Recording**—Following trituration (10 passes through a 1-ml automatic pipette tip), cells were allowed to adhere at 37 °C for at least 1 h to poly-l-lysine-coated glass coverslips before being placed in a temperature-controlled perfusion chamber (Brook Industries, Lake Villa, IL) mounted on the stage of a Nikon TMS inverted microscope. All experiments were carried out at 21 ± 1 °C. Patch pipettes were manufactured from standard walled borosilicate glass capillary tubing on a two-stage Narishige PP-83 pipette puller (Narishige Scientific Instrument Lab, Kasuya, Tokyo, Japan), were heat-polished on a Narishige microforge, and had measured tip resistances of 3–8 MΩ (when filled with K⁺-rich pipette solution).

Resistive feedback voltage-clamp was achieved using an Axopatch 200A amplifier (Axon Instruments, Forster City, CA). Voltage protocols were generated, and currents were recorded using pClamp 6.0.3 software employing a Digidata 1200 A/D converter (Axon Instruments). Data were filtered (4-pole Bessel) at 2 kHz and digitized at 5 kHz. Following successful transition to the whole-cell recording mode (12), capacitance transients were compensated for and measured. Where necessary, series resistance compensation was used at 100%.

To evoke ionic currents in H-146 cells, two voltage protocols were used: (a) ramp protocol: holding potential = −70 mV, −100 to +60 mV, ramp duration = 1 s, frequency = 0.1 Hz; (b) time series: holding potential = −70 mV, single increment to 0 mV, step duration = 50 ms, frequency = 0.1 Hz.

**Fig. 3.** The effects of phorbol esters in normoxic conditions. A–D, mean current-voltage relationships (± S.E.; dotted lines) recorded using the ramp protocol in normoxia after no pretreatment (A, $n = 11$), 10-min pretreatment with 100 nM TPA (B, $n = 8$), 100 nM 4α-PDD (C, $n = 5$), and 100 nM TPA in the presence of 1 μM BIM (D, $n = 10$). E, mean current amplitudes recorded in normoxia at 0 mV with no bath additions (Control) and following pretreatment with the agents shown beneath each bar. Number of observations in each condition is as stated in A–D.
Fast current clamp was achieved using the same amplifier, and the solutions were the same as those used in the voltage-clamp experiments. Cells were clamped at $I = 0$ pA and the recorded voltage was filtered at 1 kHz and digitized at 2 kHz.

**Single Cell Fluorescence**

H-146 cells were loaded for 30 min at 37 °C with 10 μM (in 0.01% dimethyl sulfoxide) H$_2$O$_2$ fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA, Molecular Probes, Leiden, Netherlands) during adherence to poly-L-lysine-coated coverslips (17). Coverslips were mounted on the stage of Nikon Diaphot 300 and perfused with the standard normoxic (150 mm Hg) bath solution (see above for experimental conditions) during adherence to poly-L-lysine-coated coverslips (17). Coverslips were mounted on the stage of Nikon Diaphot 300 and perfused with the standard normoxic (150 mm Hg) bath solution (see above for composition). H$_2$DCFDA was excited with light of wavelength 488 nm using a monochromator and emitted fluorescence (>510 nm) collected by a CCD camera (Hamamatsu, C4880-80). Cells were imaged using Openlab software at 2-s intervals, and pixel intensity was calculated by a CCD camera (Hamamatsu, C4880–80). Cells were imaged using the same amplifier, and the recorded voltage was filtered at 1 kHz and digitized at 2 kHz.

**Data Handling and Calculations**

**Electrophysiology**—The magnitude of the steady-state outward currents (time-series protocol) was measured as the current between 46 and 49 ms of the voltage-pulse. In the time-series plots, the currents were normalized to the maximum current at each point by the mean of the first five currents in that series; in the figures, the numbers represent the mean (± S.E.); $n$ refers to the number of cells. Current amplitudes were not corrected for cell size, because this did not vary between the four groups of cells studied, as determined by membrane capacitance measurements; control cells, 5.0 ± 0.13 picoFarad ($n = 93$); TPA-treated cells, 4.8 ± 0.23 picoFarad ($n = 57$); 4α-PDD-treated cells, 4.7 ± 0.21 picoFarad ($n = 29$); TPA- and BIM-treated cells, 5.7 ± 0.50 picoFarad ($n = 16$). Statistical comparisons were made using the paired or unpaired Student’s $t$ test, as appropriate, with $p < 0.05$ being considered significant.

**Fluorescence**—H$_2$DCFDA is effectively trapped within cells and, in common with most other fluorescence indicators, undergoes photo-bleaching. Because it is not a dual excitation or emission dye, this artifact cannot be removed by signal ratioing. Thus, during the experimental period, fluorescence intensity declines exponentially with time. Nevertheless, a change in the rate of decay is indicative of a change in cellular H$_2$O$_2$ content/production. Mean pixel intensity was calculated from each cell area at intervals of 2 s, background fluorescence was subtracted, and the subtracted intensity was plotted against time. The rate of change of fluorescence was calculated as a rolling average of slope of five consecutive frames for the entire duration of the experiment. To account for any differential dye loading, slope data from each cell were normalized such that the first average from each protocol was unity. In Fig. 2, the mean data plot shows this rolling average versus time. A downward deflection (indicative of an increase in normalized slope value) represents a decrease in H$_2$O$_2$ production/content. Exemplar, nonadjusted data are shown in the insets of Fig. 2.

**RT-PCR**—Total RNA was extracted from pelleted H-146 cells using the RNeasy Micro Kit (Qiagen, Crawley, W. Sussex, UK). The extracted RNA was then divided; 50% was treated (cleaned) with RQ-1 RNase-free DNase (1 unit/μg RNA; Promega, Southampton, Hampshire, UK) to remove genomic DNA contamination, before re-extraction using the RNeasy Micro Kit. The remaining 50% was kept in an untreated (uncleaned) state. The yield, purity, and integrity of the RNA was verified by spectrophotometry at 260/280 nm, followed by electrophoresis on 1% agarose, and was then stored in aqueous solution at ~80 °C. RT was performed on 1-μg aliquots of both cleaned and uncleaned RNA using the reverse transcription system A3500 (Promega), comprising Avian Myeloblastosis Virus reverse transcriptase and oligo(dT) (15) primers (42 °C, 15 min). The resulting cDNA was amplified by PCR and...
Fig. 5. Effect of PKC activation on hypoxic depression of 4-AP-insensitive K⁺ currents. A and B, exemplar currents under control conditions and then before (4-AP), during (4-AP + Hypoxia), and after (4-AP + Recovery) hypoxia (15 mm Hg) in the presence of 10 μM 4-AP. Traces were recorded from untreated (A) and TPA-pretreated (B) cells. Currents were recorded during 50-ms step depolarizations from a holding potential of -70 to 0 mV. C, mean time series plots of current amplitudes (with vertical S.E. bars, averaged for eight control cells (open circles) and eight TPA-treated cells (closed circles)) evoked by repeated step depolarizations from -70 to 0 mV (50-ms duration, 0.2 Hz). 4-AP and hypoxia were applied for the periods indicted by the horizontal bars. n = 8 cells in both conditions. D and E, bar graphs (with vertical S.E. bars) showing effect of TPA treatment on proportion of current inhibited by 10 μM 4-AP (D) and proportion of 4-AP-resistant current, which is inhibited by hypoxia (E, 15 mm Hg). Adapted from the same data as shown in C.

RESULTS
Response to Graded Hypoxia—In H-146 cells, we have recently shown that acute hypoxia (pO₂ ~15 mm Hg) causes rapid and reversible inhibition of whole cell K⁺ currents and membrane depolarization (13). Fig. 1 extends our earlier observations to show that reducing perfusate pO₂ in a graded manner (between 150 and 15 mm Hg) results in correspondingly graded inhibition of these K⁺ currents and also causes graded membrane depolarization (Fig. 1A). In this series of experiments, a pO₂ of 15 mm Hg significantly reduced mean outward K⁺ current amplitudes (measured at 0 mV) from 232.2 ± 28.9 pA to 149.6 ± 41.2 pA (p < 0.005, n = 7), a reduction of ~33%. The same degree of hypoxia caused a significant depolarization from -47.9 ± 1.3 mV to -38.4 ± 3.8 mV (p < 0.05, n = 7), a reduction of 9.5 mV. These values are similar to our previously published data (13). Fig. 1B shows a plot of the mean current amplitude versus membrane potential at the five grades of hypoxia examined. Hypoxic reduction in whole cell K⁺ current correlates well with membrane potential (r = 0.96) and strongly suggests, therefore, that the O₂-sensitive current contributes to the resting membrane potential in this airway chemoreceptor cell model.

Effects of Hypoxia on H₂O₂ Production in H-146 Cells—Central to the hypothesis that NADPH oxidase acts as the O₂ sensor in airway chemoreceptors is the notion that H₂O₂ levels are decreased during hypoxia. Fig. 2 demonstrates that this is indeed the case in H-146 cells. During normoxia, fluorescence
intensity gradually declined because of photobleaching (Fig. 2A and inset). However, perfusion with the hypoxic solution (15 mm Hg) caused a far greater decline in fluorescence intensity, approaching twice the base-line bleaching rate (observed under normoxic conditions (Fig. 2B and inset)). This effect was maximal around 30 s after exchange to the hypoxic solution and was reversible on reperfusion with normoxic solution. The time-course of this effect was similar to that of hypoxic inhibition of K+ currents (see Ref. 13 and also Figs. 3 and 6). These data show clearly that H2O2 levels in H-146 cells are regulated by environmental pO2 and suggest that K+ channels are under tonic control by a H2O2-generating system.

Effects of Phorbol Esters on H-146 K+ Currents—Preincubation of H-146 cells for 10 min with the membrane-permeable PKC activator, TPA (100 nM), caused a significant (p < 0.05, n = 11 control cells and 8 TPA-treated cells; unpaired Student’s t test) reduction in ramp currents at all potentials (Figs. 3, A and B). To determine the contribution which PKC activation made to this reduction in current, we employed: (a) a biologically inactive phorbol ester, 4a-PDD (100 nM); and (b) TPA together with the PKC inhibitor, BIM (1 μM bath and pipette). These maneuvers resulted in smaller, nonsignificant (p > 0.29, n = 5; p > 0.52, n = 10, respectively) reductions in currents (Fig. 3, C–E), than were seen with TPA. This suggests that the inhibition caused by TPA was attributable to both nonspecific actions of phorbol esters and activation of PKC.

Effects of PKC Activation on Hypoxic Inhibition of K+ Currents in H-146 Cells—Fig. 4A shows currents evoked by ramp depolarizations from a typical H-146 cell before, during, and after exposure to two levels of acute hypoxia. These currents exemplify the reversible, graded inhibition caused by hypoxia, which is summarized in Fig. 1A. In contrast to these findings, currents recorded in cells pretreated with 100 nM TPA, although reduced in amplitude (see above), were virtually unaffected by hypoxia (e.g., Fig. 4B). Average current amplitudes (measured at 0 mV) at all levels of hypoxia examined are plotted in Fig. 4C. Thus, TPA markedly and significantly (p < 0.01, n = 6 control cells; n = 4 TPA-treated cells) suppressed hypoxic inhibition of K+ currents in H-146 cells. The results shown in Fig. 4D (determined from currents recorded at 0 mV during normoxia and hypoxia at 15 mm Hg) indicate that this was attributable to PKC activation, because neither 4a-PDD (p = 0.18, n = 4) nor TPA in the presence of BIM (p = 0.87, n = 5) could significantly suppress the level of hypoxic inhibition of currents.

We have previously shown that the O2-sensitive K+ current is resistant to 4-AP (14). Following treatment with TPA, the proportion of the whole cell current, which was 4-AP-insensitive, remained essentially unchanged compared with control (Fig. 5, A and D). However, in the presence of a maximally effective concentration of 4-AP (10 mM), hypoxia (15 mm Hg) caused over 50% inhibition of the residual current (e.g. Fig. 5, A, C, and D). In contrast, hypoxic inhibition of the 4-AP-resistant current was significantly (p < 0.005, n = 8) reduced to around 25% following TPA treatment (e.g. Fig. 5, B, C, and E). Fig. 5C shows the mean time course of these effects. This observation shows that although TPA caused a generalized reduction in current (see also Fig. 3), the 4-AP-resistant component was less O2-sensitive in these cells when PKC was activated.

Although there are many PKC-dependent mechanisms that could account for these data, one potential site of action for PKC might be the NADPH-oxidase complex, which has been recently implicated in NEB cell O2 sensing (15). If this is the case in our NEB cell model, we would predict that products downstream of NADPH oxidase activity would regulate the O2-sensitive K+ channel. We addressed this possibility by applying one such product, H2O2, in the presence of 4-AP. Fig. 6A shows that under normoxic conditions, H2O2 was without effect. However, during hypoxic inhibition of the K+ current, H2O2 caused a dramatic and transient reactivation of the current. The time course of this effect was similar to that previously reported in NEB cells (15).

A probable site of PKC-dependent phosphorylation of the
heteromeric enzyme, NADPH oxidase, is p47phox (16). However, although there is good evidence for the presence in both native NEB cells and H-146 cells of some of the components of the enzyme (15), evidence for the presence of p47phox in H-146 cells is still lacking. Fig. 7 shows the use of RT-PCR to address this question directly. Amplification of a product compatible with the presence of p47phox (130 base pairs) in genomic DNA (uncleaned lanes) shows that the PCR protocol that we employed can detect p47phox (Fig. 7A). Using this protocol, we could detect only a small signal from cDNA reverse transcribed from DNase-treated H-146 RNA (cleaned lanes). However, a turbo PCR reaction produced a strong signal (130 base pairs) from the cleaned sample (Fig. 7A), indicating that p47phox was indeed being transcribed by these cells (confirmed by sequencing, Fig. 7B) but that its expression was low because of either rapid degradation or low copy number of p47phox mRNA; PCR cannot distinguish between these two possibilities. Regardless, p47phox is present and is, therefore, a possible site of PKC-dependent regulation of the oxidase in our model airway chemoreceptor cells.

DISCUSSION

Electrophysiological studies have demonstrated that acute reductions in pO2 suppress K+ currents in airway chemoreceptor NEBs (7) in a manner comparable to their vascular counterparts, the type I cells of the carotid body (1, 19). Such an effect underlies membrane depolarization, which presumably leads to activation of voltage-gated Ca2+ channels, thereby permitting Ca2+ influx to trigger neurotransmitter release in both cell types (8, 20). Previous studies, including our own, have demonstrated that SCLC cell lines (such as H-146 used here), which are derived from the same precursor pool as NEBs (11), provide an excellent model for studying mechanisms underlying hypoxic inhibition of NEB cell K+ channel inhibition (13, 14). This is an important advance given the present limitations of acutely isolated, primary cultured NEB cells.

An important aim of the present study was to use PKC activation as a method by which to investigate the transduction pathway responsible for hypoxic suppression of K+ currents in our recently established model of NEB O2 sensing. In NEB cells, it has been proposed, but not directly substantiated, that NADPH oxidase is an important upstream component of the chemoreception pathway (6, 15). NADPH oxidase is a heteromultimeric enzyme that contains, importantly, a PKC-activatable subunit, p47phox (16). Fig. 7 shows, for the first time, that mRNA for this subunit is present in H-146 cells. This observation has allowed us to exploit both PKC-regulation of NADPH oxidase and the fact that the candidate K+ channel is not selectively regulated by PKC per se (21) to test the hypothesis that O2 transduction relies, at least in part, on the sensing of pO2 by NADPH oxidase. Clearly, there are many targets for PKC-dependent phosphorylation. However, there is accumulating evidence in NEB and NEB-derived cells that, in terms of O2 signal transduction, NADPH oxidase activity, and its regulation by PKC, are of paramount importance. First, H2O2, a product of the sequential actions of NADPH oxidase and catalase/superoxide dismutase, appears to provide the required oxidized environment needed for tonic K+ channel activity in normoxia (15); Fig. 2 shows clearly that production of H2O2 by H-146 cells is suppressed during hypoxia with a time course similar to that of hypoxic K+ current inhibition (13). Second, phorbol 12-myristate 13-acetate treatment (another PKC-stimulating phorbol ester) causes a dramatic increase in intracellular H2O2 concentration; presumably via activation of p47phox (15). Based upon these observations, we would predict that any agent that increases the affinity of NADPH oxidase for substrate will ameliorate the inhibitory action of hypoxia.

To investigate the potential involvement of PKC in O2 sensing by H-146 cells, it was important first to characterize more completely the O2 sensitivity of hypoxic inhibition of K+ currents in these cells. Fig. 1A shows that the inhibition of K+ currents (measured at a test potential of 0 mV) by hypoxia was clearly graded, with significant inhibition only observed at a pO2 of 45 mm Hg or lower. This is in accordance with studies of the [Ca2+]i response of arterial chemoreceptor cells (20) and further validates our own model system of airway chemoreceptor cells. Most importantly, we found an excellent correlation between hypoxia-induced K+ current suppression and hypoxia-evoked membrane depolarization (Fig. 1B). This finding justifies our use of measuring current amplitude at 0 mV (which provides an acceptable compromise between signal to noise ratio and resolution of the physiological action of hypoxia) as a reliable indicator of events occurring at resting membrane potential, as we have earlier suggested (14).

Phorbol ester activation of PKC often requires several minutes to become maximal, and so to investigate the effects of PKC activation on K+ currents in H-146 cells, we pretreated cells for 10 min with such agents. Nonspecific inhibitory effects of phorbol esters on ion channels have been documented previously (22), and the data shown in Fig. 3 agree with this finding; phorbol ester treatment suppressed mean current amplitude but this can be divided into both PKC-dependent and
PKC Regulation of $K^+$ Current $O_2$ Sensitivity

 FIG. 8. Kinetic analyses of hypoxic inhibition of $K^+$ currents. The main figure shows data from Figs. 1A and 3C normalized and fitted to the Michaelis-Menton equation by iterative fitting using the method of least squares. Open circles, control cells; filled circles, TPA-pre-treated cells. The inset shows the same data transformed as a double reciprocal plot as described by the axes. Both analyses predict that preincubation of cells with TPA decreases the calculated $K_{in}$ of the system for $O_2$ approximately 6-fold.

-independent components. Because the effects of TPA were partially reversed by inhibiting PKC with BIM, it appears that the PKC-dependent component is significant. However, 4α-PDD, a phorbol ester that is inactive with respect to PKC activation, also caused some current depression. Although these data contrast with recent studies in type I carotid body cells, which indicate that TPA selectively inhibits $O_2$-sensitive Ca$^{2+}$-dependent $K^+$ channels solely via PKC activation (23), the $O_2$-sensitive $K^+$ current in H-146 cells is clearly not Ca$^{2+}$-dependent (13). Indeed, our most recent study (14) has shown that the $O_2$-sensitive $K^+$ current in H-146 cells is likely to be a member of the recently characterized tandem-P domain family of $K^+$ channels (TWIK (24), TREK (25) TASK-1 (21), TASK-2 (26), and TRAAK (27)) and shows structural similarities to TASK. Furthermore, our data in Fig. 5D show very clearly that proportionally the 4-AP-insensitive current (of which the $O_2$-sensitive current is a large component) is unaffected by phorbol ester treatment. Therefore, although TPA causes a generalized depression of $K^+$ currents, it does not affect the $O_2$-sensitive component per se, but, instead, it specifically ameliorates the hypoxia sensitivity of that component.

Indeed, the most important observation of the present study is that TPA modulates the $O_2$ sensitivity of $K^+$ currents in H-146 cells (Figs. 4 and 5), and such a finding has important implications for the frequently proposed involvement of NADPH oxidase in $O_2$ chemoreception in various tissues, including airway chemoreceptors (15), arterial chemoreceptors (28), and pulmonary vascular smooth muscle (29). This model proposes that $O_2$ sensing is dependent on the generation of $H_2O_2$ by NADPH oxidase under normoxic conditions. This enzyme has been shown to produce $H_2O_2$ tonically, and both native and recombinant subtypes of $K^+$ channels (including those found in NEB cells and SCLC cells (15)) have been shown to be enhanced by $H_2O_2$, raising the attractive concept that hypoxia inhibits $K^+$ currents by reducing $H_2O_2$ production by NADPH oxidase simply by limiting the enzyme’s substrate, $O_2$. We have now provided direct evidence that acute hypoxia reduces $H_2O_2$ production (Fig. 2). The ability of NADPH oxidase to generate $H_2O_2$ can be enhanced by the phorbol ester phorbol 12-myristate 13-acetate, which is another known activator of PKC. Our findings indicate that the PKC-activating phorbol ester, TPA, suppressed the ability of hypoxia to inhibit $K^+$ currents in H-146 cells. This specific action of TPA can be attributed to PKC activation, because it was not mimicked by another phorbol ester, 4α-PDD (which does not activate PKC) and was prevented by the PKC inhibitor, BIM. Because the nonspecific suppressing effects of TPA on $K^+$ currents measured under normoxic conditions were largely because of a PKC-independent action of TPA (see above), the PKC-dependent suppression of hypoxic inhibition is likely to occur at a site upstream of the channel itself. Furthermore, the channel which is likely to underlie the hypoxia-sensitive $K^+$ current in H-146 cells is possibly TASK-related, and TASK is insensitive to the actions of phorbol esters (21). The cytosolic protein p47phox is one of several subunits comprising the NADPH oxidase complex and is a known substrate for PKC (16). Its stimulation by phorbol 12-myristate 13-acetate (presumed to be mediated by PKC phosphorylation) has been suggested to account for the enhanced $H_2O_2$ production under normoxic conditions in NEB cells, as determined by rhodamine 123 fluorescence (15). In the context of this finding, our results provide compelling evidence that supports the idea that NADPH oxidase is the $O_2$ sensor in the hypoxic signal transduction pathway. In this model, the tonic generation of $H_2O_2$ in H-146 cells provides the appropriate environment for normoxic channel activity. That this activity is maximal in normoxia is evidenced by the ability of exogenously applied $H_2O_2$ to enhance the $O_2$-sensitive current amplitude only during hypoxia (Fig. 6B) and not during normoxia (Fig. 6A). This is in contrast to results reported for native NEB cells where $H_2O_2$ transiently activates $K^+$ currents in normoxia (15). This may be because of differences between NEB and H-146 cell NADPH oxidase turnover or specific class of $K^+$ channel targeted. Although this may suggest limitations of our model, it does not change the picture of the basic transduction pathway per se. Thus, following PKC activation, $H_2O_2$ levels are likely to be enhanced in H-146 cells as they are in NEB cells (15) due possibly to increased affinity of NADPH oxidase for $O_2$. Evidence for a PKC-dependent increase in substrate affinity is shown by the kinetic analyses of depression of $K^+$ currents during graded hypoxia. These analyses, which are summarized in Fig. 8, show that PKC increases $O_2$ affinity ~6-fold and provide an explanation for how $H_2O_2$, and therefore, channel open state probability is maintained in the face of reduced $pO_2$.

In summary, PKC activation reduces the sensitivity of H-146 cells to changes in $pO_2$. Furthermore, because the addition of $H_2O_2$, a downstream product of NADPH oxidase, only activates $K^+$ currents during hypoxia (when $H_2O_2$ production is sup-
pressed), it appears likely that PKC-dependent phosphorylation modulates the affinity of NADPH oxidase for O$_2$, and the PKC substrate p47phox (a component of the functional NADPH oxidase) is clearly present in these cells. Taken together, these data show that PKC is an important regulator of the O$_2$-transduction pathway and provide functional evidence that suggests that NADPH oxidase is a major airway O$_2$ sensor.

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J. Biol. Chem. 2000, 275:7684-7692.
doi: 10.1074/jbc.275.11.7684

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