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Selective Modulation of Ligand-Gated P2X Purinoceptor Channels by Acute Hypoxia Is Mediated by Reactive Oxygen Species

H. S. Mason,[1] S. Bourke,[1] and P. J. Kemp[1]

School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom

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ABSTRACT

Purinergic excitatory synapses use ATP to mediate fast synaptic transmission via activation of P2X receptor cation channels, and this response can be altered by acute hypoxia. This study examined the effect of acute hypoxia on cloned homo- and heteromeric $P2X_2$ and $P2X_3$ receptors expressed in human embryonic kidney 293 cells. In cells expressing homomeric $P2X_2$ receptors, perfusion of 5 μM ATP (EC_{25}) induced an inward whole-cell current that showed little desensitization during repeated exposures under continuously normoxic conditions. Exposure to a hypoxic ATP solution (pO_2, 25–40 mm Hg) significantly reduced the whole-cell current to 49% of normoxic control. This hypoxic inhibition of $P2X_2$-mediated inward current was maintained across all potentials when a voltage-step protocol was applied. In contrast, currents mediated by homomeric $P2X_3$ receptors or heteromeric $P2X_{2/3}$ receptors were insensitive to an acute hypoxic challenge. One mechanism whereby hypoxia may modulate $P2X_2$ channels is via the production of reactive oxygen species (ROS). H_2O_2 (1.8 mM) reversibly reduced homomeric $P2X_2$ whole-cell currents to 38% of control. Furthermore, H_2O_2 attenuated the effect of hypoxia on homomeric $P2X_2$ whole-cell currents. Inhibitors of the mitochondrial electron transport chain that reduce (rotenone and myxothiazol) or increase (antimycin A) the production of ROS altered the magnitude of $P2X_2$-mediated currents. In summary, this is the first report indicating that acute hypoxia is able to regulate the activity of any ligand-gated ion channel. Furthermore, our data show that acute hypoxia selectively modulates the $P2X_2$ receptor and that the response of $P2X_2$ receptor subunits to hypoxia is mediated through the mitochondrial production of ROS.

The maintenance of cellular integrity is entirely dependent on the continuous supply of oxygen and the ability of cellular processes to adapt to changes in the pO_2 in the environment. The ability to detect changes in arterial pO_2 and respond appropriately is predominantly mediated by cells localized within specialized regions such as the carotid body, the pulmonary vasculature, and neuroepithelial bodies of the lung (for reviews, see Haddad and Jiang, 1997; Lopez-Barneo et al., 2001; Kemp et al., 2003). One of the primary responses to an acute reduction in pO_2 is a change in plasmalemmal ion channel activity, which leads to cellular depolarization, calcium entry and modifications in cellular excitability, or secretory activity. Interest in the role of purinoceptors in respiratory control has recently increased. Extracellular ATP activates sensory neurons within the carotid body (Zhang et al., 2000; Prasad et al., 2001) and the ventrolateral medulla (Thomas et al., 2001; Gourine et al., 2003), and this activation is prevented by the P2 receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid. However, relatively little is known about whether P2 purinoceptors are directly modulated in response to a hypoxic challenge.

Purinoceptors are classified as P1 (adenosine receptors, A_1–A_3) or P2 (ATP receptors) on the basis of their pharmacological, biochemical, and molecular properties. The P2 family of purinoceptors can be further divided into two subfamilies, the G protein-coupled P2Y receptor family and the ligand-gated P2X receptor family. Activation of P2X receptors results in the influx of cations such as Na^+, K^+, and Ca^{2+} across the plasma membrane, which increases the intracellular Ca^{2+} concentration. To date, seven members of the P2X family ($P2X_{1–7}$) have been cloned (North, 2002) whose structural properties differ from that of other members of the ligand-gated ion channel superfamily. The primary structure of the P2X receptor subunit consists of two potential transmembrane domains (M1 and M2), intracellular N- and C-terminal domains, and a large cysteine-rich

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[1] Current address: Cardiff School of Biosciences, University of Cardiff, Cardiff, UK.

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ABBREVIATIONS: HEK, human embryonic kidney; α,β-MeATP, α,β-methylene ATP; ROS, reactive oxygen species; ETC, electron transport chain.

extracellular domain that contains an ATP binding motif and sites for glycosylation (Valera et al., 1994; Brake et al., 1994; Surprenant et al., 1995). The second transmembrane domain (M2) and a hydrophobic H5 sequence immediately before the M2 domain is thought to form the ion pore and binding sites of the P2X receptor (Brake et al., 1994; Valera et al., 1994). Furthermore, there is increasing evidence that three P2X subunits are required to form a functional receptor (Nicke et al., 1998; Stoop et al., 1999).

P2X receptor subtypes are widely expressed in the brain and nervous system and are associated with autonomic sensory motor reflexes, sensory afferents, and olfactory and visual systems (North, 2002). Furthermore, P2X receptors are expressed in cells that respond to hypoxia, such as the carotid body, pulmonary smooth muscle, and PC12 cells (Kobayashi et al., 1998; Prasad et al., 2001; Chootip et al., 2002). Indeed, in the carotid body, hypoxia leads to an increased afferent discharge rate, and this is mediated via $P2X_2$ and $P2X_3$ receptors (Zhang et al., 2000; Prasad et al., 2001). However, the response of cloned P2X receptor cation channels to hypoxia is unknown. Therefore, the primary purpose of this study was to evaluate the effect of hypoxia on cloned $P2X_2$ and $P2X_3$ homo- and heteromeric receptors expressed in human embryonic kidney (HEK) 293 cells.

Materials and Methods

Expression Systems. HEK293 cells that stably express either homomeric rat $P2X_2$ receptors, homomeric human $P2X_3$ receptors, or rat $P2X_2$ and $P2X_3$ receptors in a bicistronic vector, thus giving rise to heteromeric $P2X_{2/3}$ receptor channels, were used in this study. These cell lines have been previously characterized (Brake et al., 1994; Evans et al., 1995; Valera et al., 1995; Kawashima et al., 1998). All the P2X cell lines were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1% antibiotic antimycotic, 100 μg/ml gentamicin, and 300 μg/ml Geneticin (G418) (all purchased from Invitrogen Ltd., Renfrew, Renfrewshire, UK) in a humidified incubator gassed with 5% CO_2/95% air. Cells were passaged every 7 days using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Invitrogen Ltd.). For the electrophysiological experiments described herein, HEK293 cells were plated onto glass coverslips and cultured at 37°C for 1 to 5 days.

Reagents. All compounds were of analytical grade and obtained from BDH Laboratory Supplies (Poole, Dorset, UK). Disodium ATP, α,β-methylene ATP (α,β-MeATP), H_2O_2, rotenone, antimycin A, myxothiazol, EGTA, and HEPES were obtained from Sigma Chemical (Poole, Dorset, UK). ATP and α,β-MeATP were dissolved at 100 mM in distilled H_2O, divided into aliquots, and stored at −20°C. For recording, ATP and α,β-MeATP were diluted to the desired concentration in bath solution. Stock solutions of the mitochondrial inhibitors were prepared in either ethanol (50 μg/ml antimycin A and 10 mM myxothiazol) or dimethyl sulfoxide (100 mM rotenone), and aliquots were stored at −20°C. Oxygen-free nitrogen gas and medical air (21% O_2) were obtained from BOC Ltd. (Guildford, Surrey, UK). All tubing was gas-impermeant (Tygon tubing; BDH).

Electrophysiology. Whole-cell currents were recorded at room temperature. Recording pipettes pulled from borosilicate glass had resistances of 4 to 6 MΩ when filled with pipette solution that contained 117 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 2 mM Na-ATP, and 11 mM HEPES, with the pH adjusted to 7.2 with KOH. The bath solution contained 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM HEPES, and 10 mM glucose, with the pH adjusted to 7.4 with NaOH. Hypoxic solutions were bubbled with N_2 gas for at least 30 min before perfusion of cells, which produced no shift in pH. Normoxic solutions were either equilibrated with room air or bubbled with medical air (21% O_2). pO_2 was measured (at the cell) using a polarized (−800-mV) carbon fiber electrode (Mojet et al., 1997). For the experiments reported herein, the pO_2 values were 150 (normoxia) and 25 to 40 (hypoxia) mm Hg.

Resistive feedback voltage clamp was achieved using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). Voltage protocols were generated and currents recorded using pCLAMP 8.0 software employing a Digidata 1322 A/D convertor (Axon Instruments Inc.). Data were filtered (4-pole Bessel) at 2 kHz and digitized at 5 kHz. After transition to the whole-cell configuration capacitance, transients were compensated and measured.

To monitor the response of P2X-mediated currents to repeated exposures of ATP under normoxic or hypoxic conditions, the following protocol was used. Having gained the whole-cell configuration, the cells were voltage-clamped at a potential of −70 mV. Control recordings were made by exposing cells to three exposures of ATP under normoxic conditions. The bathing solution was then switched to either a normoxic or hypoxic solution for 2 min before examining the response to three subsequent exposures of ATP under normoxic or hypoxic conditions. Finally, the bathing solution was switched back to normoxic conditions for 2 min, and the subsequent responses to the final three exposures of ATP under normoxic conditions were monitored. In some experiments, cells were held at a potential of 0 mV, and 200-ms voltage steps were applied from −100 to +100 mV in 20-mV increments were applied under normoxic and hypoxic conditions. To create concentration-response curves for P2X-mediated currents under normoxic and hypoxic conditions, cells were held in the whole-cell configuration at a potential of −70 mV and perfused with either normoxic or hypoxic bathing solution for 2 min. The cells were then exposed to ATP under either normoxic or hypoxic conditions. Each cell was only exposed to one concentration of ATP under either normoxic or hypoxic conditions. The P2X-mediated currents generated in response to the first application of ATP were then plotted, with each point representing at least five cells.

Data Analysis. Data analyses were performed using the pCLAMP 8.0 suite of software (Axon Instruments Inc.). Data are reported as mean ± S.E.M. values. Statistical comparisons were made using the paired or unpaired Student's t test as appropriate, with $P < 0.05$ regarded as significant. Concentration-response curves for ATP and H_2O_2 were fitted to the Hill equation using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Hypoxia Modulates Homomeric $P2X_2$-Mediated Currents. To evaluate the effect of hypoxia on $P2X_2$-mediated currents expressed in HEK293 cells, a concentration of ATP was required that would elicit responses of similar magnitude after repeated exposures to ATP. Extracellular ATP activated $P2X_2$-mediated currents in a concentration-dependent manner, with an EC_{50} of 9.59 μM and a Hill slope of 2.75 ($n = 28$, data not shown). At high concentrations of ATP (100 μM), the magnitude of the $P2X_2$-mediated current elicited in response to ATP decreased with repeated exposures caused by desensitization of the $P2X_2$ receptor (data not shown). However, at a concentration of 5 μM ATP (which equates to the EC_{25} value), $P2X_2$-mediated currents of similar magnitude could be evoked repeatedly, even in the presence of the divalent cations Ca^{2+} and Mg^{2+}. As a result of these observations, ATP concentrations of 5 μM were used in the following experiments that evaluated the effect of hypoxia on $P2X_2$-mediated currents.

Figure 1A shows exemplar ATP-induced whole-cell homomeric $P2X_2$-mediated currents recorded under normoxic and hypoxic conditions at a potential of −70 mV in the presence of external Ca^{2+} and Mg^{2+}. After exposing the cells to a

hypoxic solution for 2 min, the magnitude of the P2X$_2$-mediated current activated in response to 5 μM ATP was reduced compared with that under normoxic conditions. This reduction in the magnitude of the P2X$_2$-mediated current was partially relieved upon returning to normoxic conditions. Figure 1B summarizes the response of P2X$_2$-expressing cells when exposed to repeated exposures of 5 μM ATP under either continuously normoxic conditions or exposed to an acute hypoxic challenge. Under continuously normoxic conditions, there was no difference in the magnitude of the P2X$_2$-mediated current over time (the magnitude of the last exposure was 0.96 $\pm$ 0.09 that of the first exposure, $n = 5$). In contrast, exposure to a hypoxic challenge significantly reduced the magnitude of the P2X$_2$-mediated current activated in response to 5 μM ATP [0.51 $\pm$ 0.09 that of the current under normoxic conditions ($P < 0.01$, $n = 5$)]. The reduction in the magnitude of the P2X$_2$-mediated whole-cell current was partially reversible upon washout (0.68 $\pm$ 0.16 compared with control, $n = 5$).

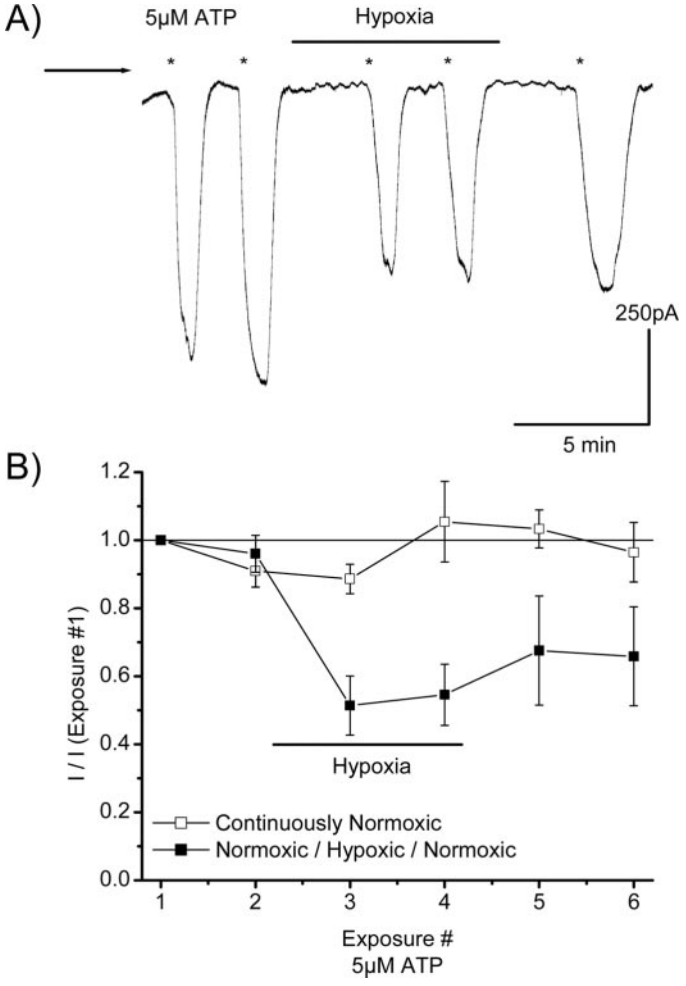

Fig. 1. Hypoxia attenuates ATP-induced currents mediated by the homomeric P2X$_2$ receptor. HEK293 cells expressing homomeric P2X$_2$ receptors were held at a potential of -70 mV in the presence of external Ca^{2+} and Mg^{2+} and repeatedly exposed to extracellular ATP. A, illustrative trace of a cell exposed to repeated exposures to 5 μM ATP under normoxic and hypoxic conditions. The stars represent the application of ATP for 10 s; the arrow indicates zero current. B, summarized data showing that exposure to an acute hypoxic challenge attenuates the magnitude of P2X$_2$-mediated currents evoked in response to 5 μM ATP (■) compared with cells held under continuously normoxic conditions (□).

The effect of hypoxia on the magnitude of the P2X$_2$-mediated current was also examined using a voltage-step protocol. Figure 2 shows illustrative traces recorded sequentially in response to 200-ms voltage steps from -100 mV to $+100$ mV in 20-mV increments from a holding potential of 0 mV under control conditions in the absence of ATP (Fig. 2A) and in the presence of 5 μM ATP under hypoxic (Fig. 2B), normoxic (Fig. 2C), and hypoxic (Fig. 2D) conditions. In the absence of ATP, the voltage-step protocol elicited small endogenous currents (Fig. 2A). Exposure to ATP under hypoxic conditions activated an inwardly rectifying P2X$_2$-mediated current (Fig. 2B). Switching the perfusate from a hypoxic to normoxic solution increased the magnitude of the P2X$_2$-mediated current (Fig. 2C), and this increase in current was fully reversible upon returning to hypoxic conditions (Fig. 2D). Figure 2E shows the mean control-subtracted data from six cells. Currents are normalized to the current evoked in response to 5 μM ATP at a potential of -100 mV under hypoxic conditions. Under hypoxic conditions, the P2X$_2$-mediated currents reversed at a potential of -8.3 mV, whereas under normoxic conditions, the currents were 38 $\pm$ 10% larger ($P < 0.05$, $n = 7$) and reversed at a potential of -4.2 mV.

A similar modulation of P2X$_2$-mediated currents by hypoxia was obtained when the reverse protocol was applied, i.e., when the cells expressing the P2X$_2$ receptor were sequentially exposed to control conditions in the absence of ATP and in the presence of 5 μM ATP under normoxic, hypoxic, and normoxic conditions. In this instance, hypoxia reduced the magnitude of P2X$_2$-mediated currents at all potentials less than -20 mV by 20% ($P < 0.05$, $n = 7$), and this was fully reversible upon returning to a normoxic solution (data not shown).

Hypoxia Does Not Modulate Homomeric P2X$_3$ Channels. The P2X-mediated current in sensory neurons is mediated principally through homomeric P2X$_3$ and heteromeric P2X$_{2/3}$ channels (Dunn et al., 2001). Therefore, the responses of homomeric P2X$_3$ and heteromeric P2X$_{2/3}$ channels to hypoxia were also examined, with the results summarized in Figs. 3 and 4. As shown in Fig. 3A, exposing cells expressing homomeric P2X$_3$ receptors to 10 μM ATP under normoxic conditions in the presence of external Ca^{2+} and Mg^{2+} activated an inward current that rapidly inactivated (peak normoxic mean P2X$_3$-mediated current, -124.1 ± 53.7 pA/pF, $n = 9$). The inactivation of the P2X$_3$-mediated current under normoxic conditions was best fit with two time constants ($\tau_1 = 1473 \pm 234$ and $\tau_2 = 214 \pm 37.5$ ms, $n = 9$; Fig. 3D). Because a second exposure to ATP significantly reduced the magnitude of the homomeric P2X$_3$ whole-cell current (data not shown), we could not perform repeated exposures to ATP on the same cell. Therefore, to examine the response to hypoxia, we used separate cell populations, and immediately after gaining the whole-cell configuration, the cells were exposed to a hypoxic bathing solution for 2 min before exposing the cells to ATP. Under hypoxic conditions, 10 μM ATP activated a P2X$_3$-mediated current whose magnitude was comparable with that activated under normoxic conditions (mean peak hypoxic P2X$_3$-mediated current, -110.4 ± 24.5 pA/pF, $n = 7$; Fig. 3C). The inactivation of the P2X$_3$-mediated current under hypoxic conditions was best fit with two time constants ($\tau_1 = 1059 \pm 119$ and $\tau_2 = 163 \pm 31.7$ ms; Fig. 3D).

Similar results were obtained when the cells were exposed to α,β-MeATP, a P2X$_1$- and P2X$_3$-selective agonist that dif-

ferentiates between $P2X_2$ and $P2X_3$ receptor subunits (data not shown). Exposure to 10 μM α,β-MeATP activated inactivating $P2X_3$-mediated currents under either normoxic or hypoxic conditions. The magnitudes of the peak $P2X_3$-mediated currents were similar under normoxic and hypoxic conditions (normoxia, -129 ± 35.6 pA/pF, $n = 8$; hypoxia, -80.6 ± 21.7 pA/pF, $n = 8$). The inactivation of the $P2X_3$-mediated currents under normoxic and hypoxic conditions was best fit with two time constants (normoxia, $\tau_1 = 1790 \pm 322$ and $\tau_2 = 390 \pm 138$ ms; hypoxia, $\tau_1 = 912 \pm 109$ and $\tau_2 = 172 \pm 18.6$ ms).

Hypoxia Does Not Modulate Heteromeric $P2X_{2/3}$ Channels. The observation that the peak activation of homomeric $P2X_2$ receptors was sensitive to hypoxia whereas that of homomeric $P2X_3$ receptors was not was intriguing. Because the sustained component of the P2X-mediated current in sensory neurons is primarily mediated through $P2X_{2/3}$ heteromeric channels (Dunn et al., 2001), we examined the response to hypoxia of $P2X_{2/3}$ heteromeric channels expressed in HEK293 cells. Figure 4A shows exemplar ATP-induced whole-cell heteromeric $P2X_{2/3}$-mediated currents recorded under normoxic and hypoxic conditions at a potential of -70 mV in the presence of external Ca^{2+} and Mg^{2+}. Figure 4B summarizes the response of $P2X_{2/3}$-expressing cells when exposed to repeated exposures of 10 μM ATP under either continuously normoxic conditions or exposed to an acute hypoxic challenge. Under continuously normoxic conditions, $P2X_{2/3}$-mediated currents reduced in magnitude over time (the magnitude of the last exposure was 0.77 ± 0.10 that of the first exposure, $n = 3$). Although exposure to a hypoxic challenge reduced the magnitude of the $P2X_{2/3}$-mediated current activated in response to 10 μM ATP (0.70 ± 0.06 that of the first exposure to ATP, $P < 0.01$, $n = 5$), this reduction was not different from that observed in cells held under continuously normoxic conditions. These data suggest that hypoxia does not modulate the activation of heteromeric $P2X_{2/3}$ receptor channels by ATP in the presence of external Ca^{2+} and Mg^{2+}.

Hypoxia Selectively Alters the Concentration-Response Curves of P2X Receptors. To further examine the effect of hypoxia on the P2X receptors used in this study, full concentration-response curves were generated under either normoxic or hypoxic conditions. Figure 5A shows the concentration-response curve of $P2X_2$-mediated currents evoked in response to the first exposure to ATP under either normoxic or hypoxic conditions. Under normoxic conditions, extracellular ATP activated $P2X_2$-mediated currents in a concentration-dependent manner with an EC_{50} of 3.73 μM. In contrast, under hypoxic conditions, the concentration-response curve

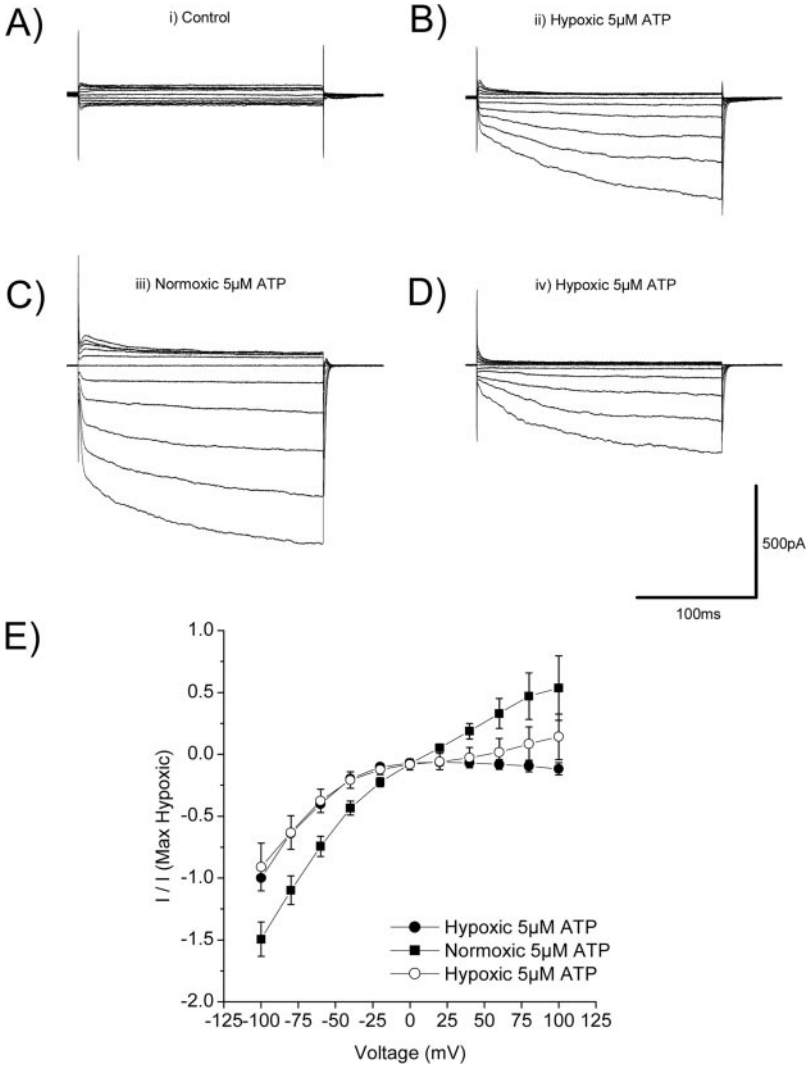

Fig. 2. Normoxia relieves the hypoxic inhibition of the ATP-induced current in cells expressing homomeric $P2X_2$ receptors. Cells expressing the $P2X_2$ receptor were held at a potential of 0 mV, and 200-ms voltage steps were applied from -100 to $+100$ mV in 20-mV increments. A–D, illustrative $P2X_2$-mediated currents evoked in response to the voltage-step protocol. Cells were initially exposed to 5 μM ATP in the presence of external Ca^{2+} and Mg^{2+} under hypoxic conditions and then normoxic conditions before returning to hypoxic conditions. E, summarized current-voltage relationship from six cells. Currents are normalized to the current recorded at a potential of -100 mV under hypoxic conditions.

was shifted to the right, with an EC_{50} of 6.34 μM. However, the magnitude of the $P2X_2$-mediated currents evoked in response to a maximal concentration of ATP (100 μM) in either normoxia or hypoxia was not significantly different.

It was possible that the concentration of ATP (10 μM, which equates to the EC_{90} value) used to examine the effect of hypoxia on the homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors could have masked any effect of hypoxia on these P2X receptors. Therefore, full concentration-response curves were also generated under normoxic and hypoxic conditions for these receptors. Figure 5, B and C, shows the concentration-response curves for heteromeric $P2X_{2/3}$- and homomeric $P2X_3$-mediated currents, respectively. Hypoxia had no effect on either heteromeric $P2X_{2/3}$- and homomeric $P2X_3$-mediated currents. Thus, in agreement with our initial findings, hypoxia selectively modulates homomeric $P2X_2$ receptors.

Hydrogen Peroxide Mimics the Effect of Hypoxia on Homomeric P2X$_2$-Mediated Currents. One of the mechanisms whereby hypoxia may modulate channel function is through the production of reactive oxygen species (ROS). We examined whether the hypoxic modulation of homomeric $P2X_2$ receptors was mediated by ROS by examining the response of $P2X_2$-mediated currents to H_2O_2 under normoxic and hypoxic conditions. Figure 6A shows the effect of perfusing 1.8 mM H_2O_2 on the magnitude of ATP-induced whole-cell $P2X_2$-mediated currents under continuously normoxic conditions at a potential of −70 mV in the presence of external Ca^{2+} and Mg^{2+}. After exposing the cells to 1.8 mM H_2O_2 solution for 2 min, the magnitude of the $P2X_2$-mediated current activated in response to 5 μM ATP was significantly reduced compared with that under control conditions (to 0.61 ± 0.15 that of the first exposure to ATP, $P < 0.05$, $n = 5$). This reduction in the magnitude of the $P2X_2$-mediated current was partially relieved upon returning to control conditions (0.76 ± 0.20 that of the first exposure to ATP, $n = 3$). The H_2O_2-induced reduction in the magnitude of the $P2X_2$-mediated current was concentration-dependent with an IC_{50} of 0.73 mM (Fig. 6B).

If hypoxia reduces the magnitude of $P2X_2$-mediated currents through the production of ROS, then pre-exposure to H_2O_2 should abolish this effect. Figure 6C shows the mean data recorded from six cells. In the presence of 1.8 mM H_2O_2 and hypoxia, the magnitude of the $P2X_2$-mediated current activated in response to 5 μM ATP in the presence of external Ca^{2+} and Mg^{2+} was 0.77 ± 0.07 that of the current under normoxic conditions ($P < 0.05$, $n = 6$). This hypoxic-induced reduction in the magnitude of the $P2X_2$-mediated current in the presence of 1.8 mM H_2O_2 was attenuated compared with that in the absence of H_2O_2 (0.51 ± 0.09, $P < 0.05$, the open symbols for comparison are taken from Fig. 1B). Thus, H_2O_2 itself mimics the effect of hypoxia on

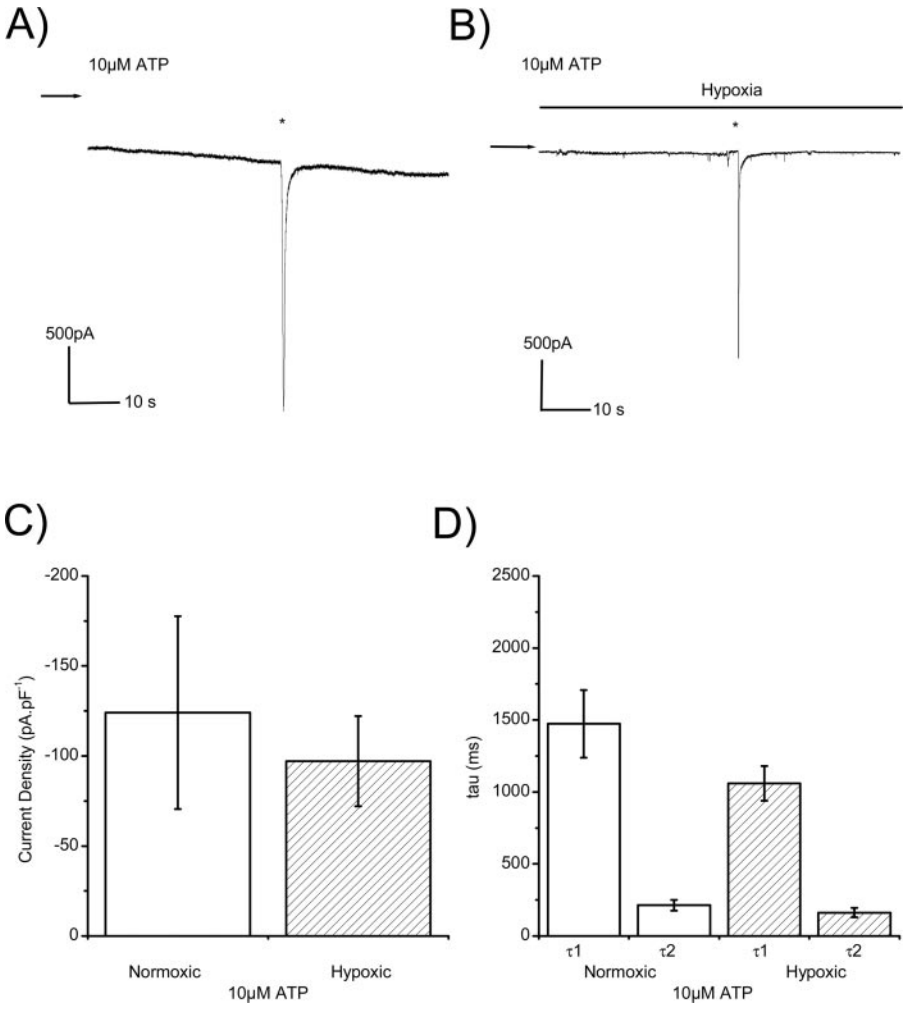

Fig. 3. Currents mediated by the homomeric $P2X_3$ receptor are insensitive to hypoxia. Illustrative $P2X_3$-mediated currents from cells held at a potential of −70 mV in the presence of external Ca^{2+} and Mg^{2+} and exposed to extracellular ATP (10 μM) under either normoxic (A) or hypoxic (B) conditions. The stars represent the application of ATP for 10 s; the arrows indicate zero current. C–D, summarized data showing that hypoxia has no effect on either (C) the magnitude of the peak ATP-induced $P2X_3$-mediated currents or (D) the inactivation time constants.

$P2X_2$-mediated currents, and the presence of H_2O_2 attenuates the effect of a subsequent hypoxic challenge.

Mitochondrial Inhibitors That Modulate the Production of ROS Mimic the Effect of Hypoxia on Homomeric $P2X_2$-Mediated Currents. To examine the source of the ROS that might underlie the hypoxic inhibition of $P2X_2$ currents, mitochondrial inhibitors of the electron transport chain (ETC) were employed; these ETC inhibitors were rotenone, antimycin A, and myxothiazol, and each block the mitochondrial ETC at a different site of the ETC. Figure 7A shows that the perfusion of cells expressing $P2X_2$ receptors with rotenone (100 nM), a complex I inhibitor that causes a reduction in ROS, significantly increased the magnitude of $P2X_2$-mediated currents in response to the first exposure to 5 μM ATP after the application of rotenone (1.22 $\pm$ 0.016 that of the first exposure to ATP, $P < 0.05$, $n = 6$). The magnitude of $P2X_2$-mediated currents in response to subsequent exposures to ATP in the presence of rotenone declined over time (sixth exposure was 0.78 $\pm$ 0.14 that of the first exposure to ATP, $n = 6$) and was irreversible upon washout (0.62 $\pm$ 0.11 that of the first exposure to ATP, $n = 5$). This was a common phenomenon with all the mitochondrial inhibitors and is presumably caused by metabolic poisoning of the cell.

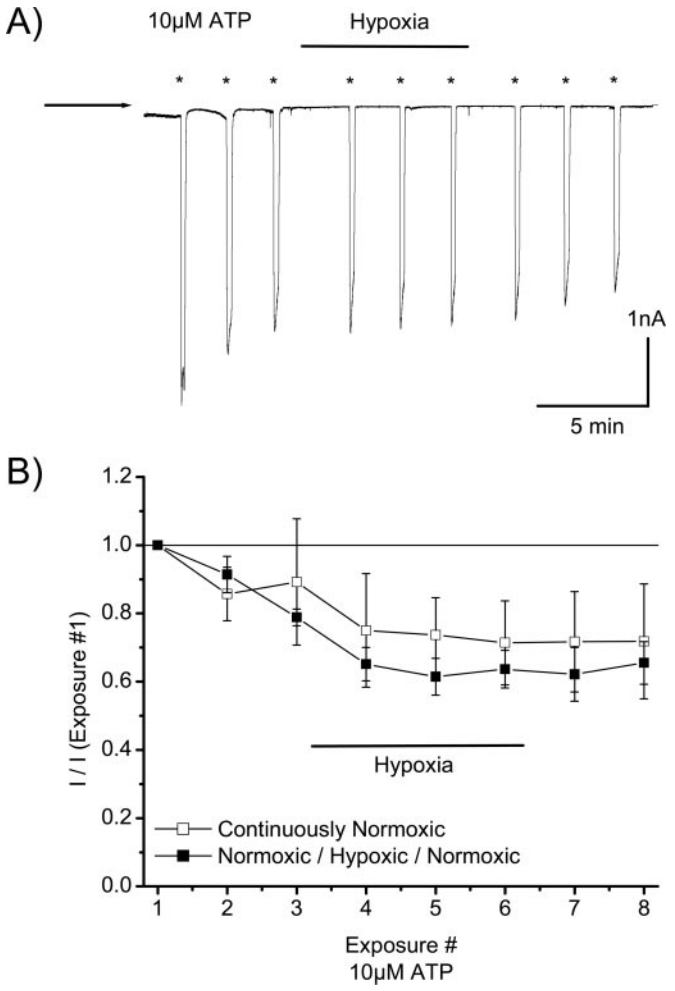

Fig. 4. Heteromeric $P2X_{2/3}$-mediated currents are insensitive to hypoxia. HEK293 cells expressing heteromeric $P2X_{2/3}$ receptors were held at a potential of -70 mV in the presence of external Ca^{2+} and Mg^{2+} and repeatedly exposed to extracellular ATP. A, illustrative trace of a cell exposed to repeated exposures to 10 μM ATP under normoxic and hypoxic conditions. The stars represent the application of ATP for 10 s; the arrow indicates zero current. B, summarized data showing that exposure to an acute hypoxic challenge has no affect on the magnitude of $P2X_{2/3}$-mediated currents (■) compared with cells held under continuously normoxic conditions (□).

If the transient increase in the $P2X_2$-mediated current is caused by the reduction in the production of ROS, then this should be overcome by providing succinate, a complex II substrate. As shown in Fig. 7A, rotenone failed to induce an increase in the magnitude of $P2X_2$-mediated currents in response to the first exposure to 5 μM ATP in the presence of 5 mM succinate. Rather, $P2X_2$-mediated currents declined over time (to 0.54 $\pm$ 0.08 that of the first exposure to ATP, $n = 3$), and this was irreversible upon washout (0.52 $\pm$ 0.03 that of the first exposure to ATP, $n = 3$). This reduction in the magnitude of the $P2X_2$-mediated currents was similar to those observed in the absence of succinate (see above).

To further elucidate the role of ROS in the modulation of $P2X_2$-mediated currents, we examined the effect of two inhibitors of complex III in the mitochondrial ETC, antimycin A and myxothiazol. These inhibitors act at different sites within complex III to increase or decrease ROS, respectively. Perfusion of antimycin A (10 μg/ml) caused a reduction in the magnitude of $P2X_2$-mediated currents over time (to 0.46 $\pm$ 0.12 that of the first exposure to ATP, $P < 0.01$, $n = 5$), and this was irreversible upon washout (0.41 $\pm$ 0.11 that of the first exposure to ATP, $n = 3$). In contrast, myxothiazol (100 nM) caused a transient increase in $P2X_2$-mediated currents similar to that observed with rotenone. The magnitude of $P2X_2$-mediated current in response to the first exposure to 5 μM ATP in the presence of myxothiazol was 1.28 $\pm$ 0.08 that of the first exposure to ATP ($P < 0.05$, $n = 4$). The magnitude of $P2X_2$-mediated currents in response to subsequent exposures to ATP in the presence of myxothiazol declined over time (sixth exposure was 0.70 $\pm$ 0.04 that of the first exposure to ATP, $P < 0.05$, $n = 4$) and was irreversible upon washout (0.58 $\pm$ 0.22 that of the first exposure to ATP, $n = 3$). Thus, together these data suggest that changes in the mitochondrial production of ROS can modulate $P2X_2$ currents.

Mitochondrial Inhibitors That Modulate the Production of ROS Do Not Affect Homomeric $P2X_3$- or Heteromeric $P2X_{2/3}$-Mediated Currents. The findings thus far suggest that hypoxia selectively modulates homomeric $P2X_2$-mediated currents and that this is mediated, in part, via the mitochondrial production of ROS. If this is true, then H_2O_2 and the mitochondria inhibitors should have no effect on either homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ receptors. Figure 8A shows the magnitude of peak and sustained currents mediated by heteromeric $P2X_{2/3}$ receptors evoked in response to the first exposure to 0.5 μM ATP (which equates to the EC_{25} value) in the presence or absence of the ROS mediators. The magnitudes of the peak and sustained $P2X_{2/3}$-mediated currents in the presence of 1.8 mM H_2O_2 (85.3 $\pm$ 10.7 pA/pF and 19.9 $\pm$ 2.5 pA/pF, $n = 10$), 100 nM rotenone (88.2 $\pm$ 15.1 pA/pF and 22.7 $\pm$ 3.8 pA/pF, $n = 11$), 100 nM myxothiazol (105.2 $\pm$ 12.1 pA/pF and 22.7 $\pm$ 5.3 pA/pF, $n = 12$), or 10 μg/ml antimycin A (70.5 $\pm$ 9.9 pA/pF and 11.6 $\pm$ 2.2 pA/pF, $n = 9$) were not found to be statistically significant from control-treated cells (97.2 $\pm$ 9.93 pA/pF and 22.0 $\pm$ 4.2 pA/pF, $n = 15$). Likewise, Fig. 8B shows the magnitude of peak and sustained currents mediated by homomeric $P2X_3$ receptors evoked in response to the first exposure to 0.5 μM ATP (which equates to the EC_{25} value) in the presence or absence

of the ROS mediators. The magnitudes of the peak and sustained $P2X_3$-mediated currents in the presence of H_2O_2 (44.4 ± 9.6 pA/pF and 1.7 ± 0.8 pA/pF, $n = 10$), rotenone (39.9 ± 9.3 pA/pF and 2.0 ± 0.4 pA/pF, $n = 11$), myxothiazol (41.9 ± 10.4 pA/pF and 2.0 ± 0.5 pA/pF, $n = 10$), or antimycin A (89.2 ± 36.4 pA/pF and 1.9 ± 0.6 pA/pF, $n = 9$) were not found to be statistically significant from control-treated cells (61.4 ± 20.0 pA/pF and 2.6 ± 0.4 pA/pF, $n = 15$). Thus, these findings support the hypothesis that hypoxia and ROS selectively modulate homomeric $P2X_2$ receptors.

Discussion

The purpose of this study was to evaluate whether hypoxia could affect the activation of homo- and heteromeric $P2X_2$ and $P2X_3$ receptor cation channels and thus be a possible mechanism contributing to the ventilatory response during a hypoxic challenge. The present findings show that hypoxia attenuates homomeric $P2X_2$-mediated currents but has no effect on homomeric $P2X_3$ or heteromeric $P2X_{2/3}$ currents. This is the first direct demonstration of acute hypoxic mod-

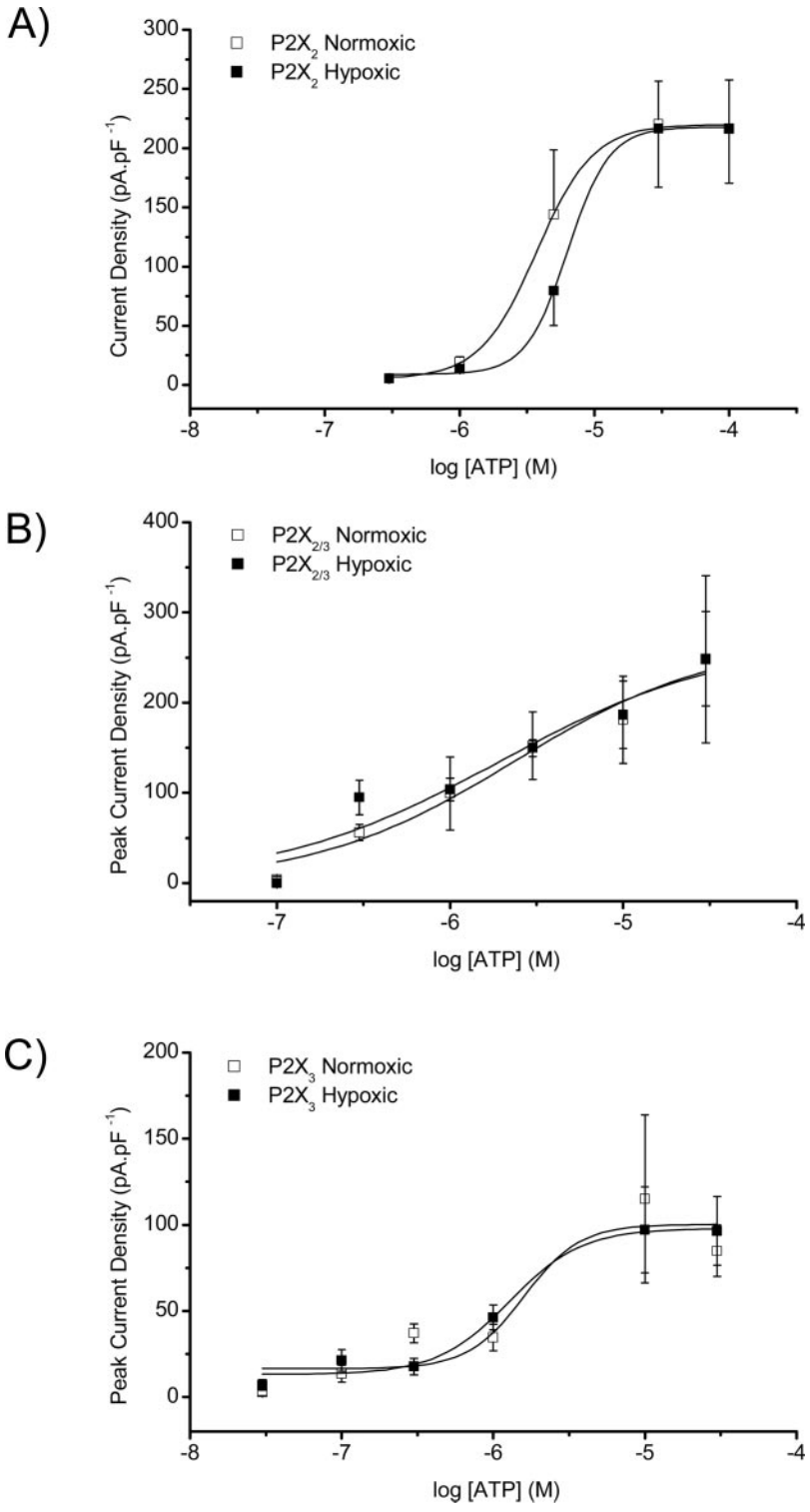

Fig. 5. The effect of hypoxia on the concentration-response curves of homomeric $P2X_2$, homomeric $P2X_3$, and heteromeric $P2X_{2/3}$ receptors. HEK293 cells expressing homomeric $P2X_2$, homomeric $P2X_3$, or heteromeric $P2X_{2/3}$ were held at a potential of −70 mV and perfused with either normoxic or hypoxic bathing solution for 2 min. The cells were then exposed to ATP in normoxic or hypoxic solution. Each cell was only exposed to one concentration of ATP under either normoxic or hypoxic conditions. A, exposure to a hypoxic solution (■) shifts the concentration-response curve of homomeric $P2X_2$ receptors to the right relative to cells exposed to normoxic solutions (□). B, concentration-response curve of heteromeric $P2X_2$ receptors under normoxic (□) and hypoxic (■) conditions. C, concentration-response curve of homomeric $P2X_3$ receptors under normoxic (□) and hypoxic (■) conditions.

ulation of a ligand-gated ion channel. Furthermore, the selective modulation of the $P2X_2$ receptor subunit is consistent with the recent report by Rong et al. (2003), who found that mice deficient in the $P2X_2$ receptor subunit showed an attenuated ventilatory response to hypoxia, whereas mice deficient in the $P2X_3$ receptor subunit were comparable with wild type. Previous studies have shown that under hypoxic conditions, ATP is coreleased with ACh from type I glomus cells and activates $P2X_2$ and $P2X_3$ receptors expressed on petrosal ganglia, which leads to an increased afferent discharge rate (Zhang et al., 2000; Prasad et al., 2001). Therefore, the combined results from this current study and that of Rong et al. (2003) suggest that the expression of homomeric $P2X_2$ receptors may play a critical role in mediating the ventilatory response to an acute hypoxic challenge within the carotid body.

A similar modulatory role of $P2X_2$ receptors may be involved in the regulation of the secretory response of PC12 to

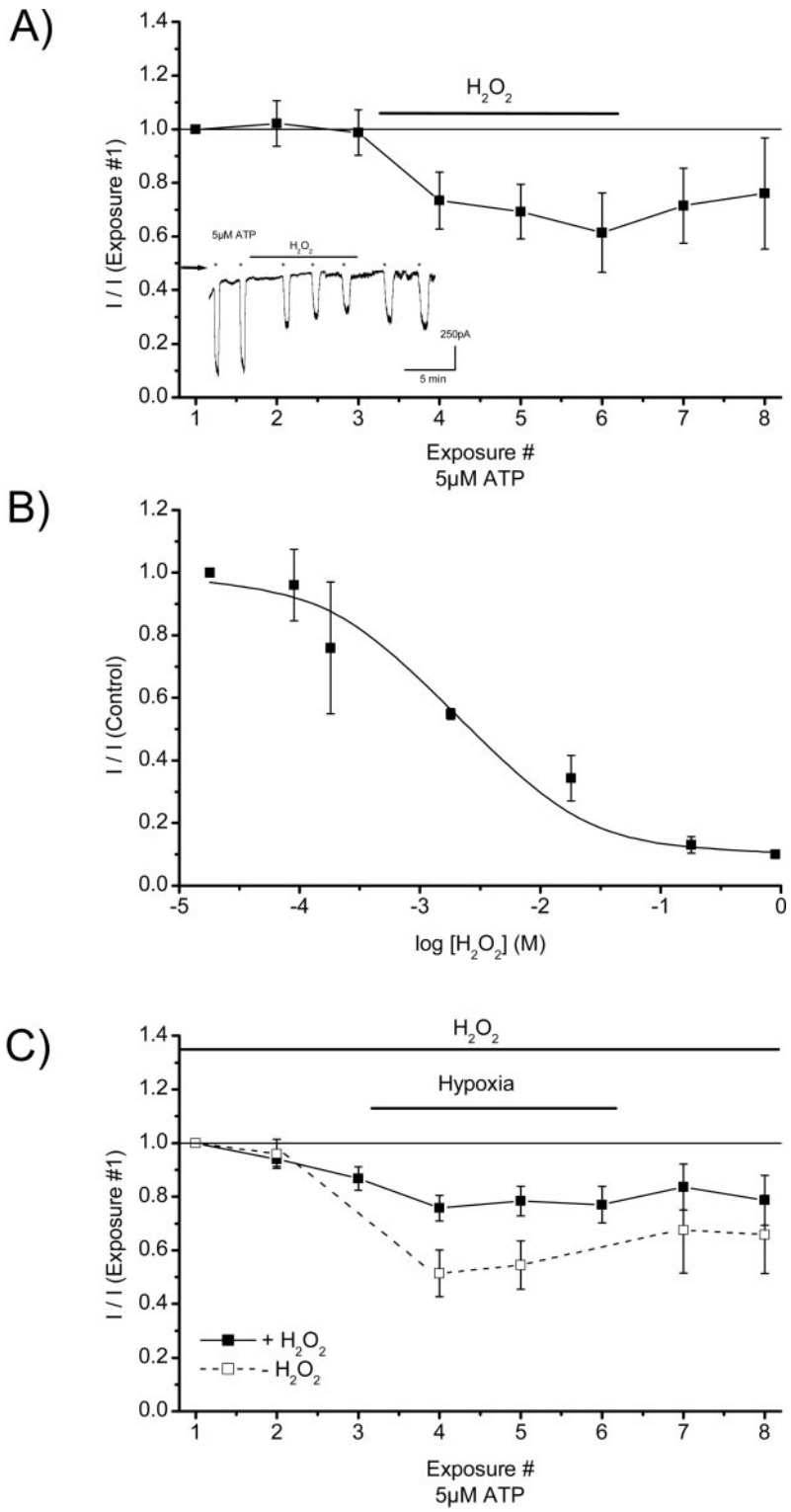

Fig. 6. H_2O_2 mimics the effect of hypoxia on homomeric $P2X_2$-mediated currents and attenuates the effect of hypoxia. A, summarized data showing that the perfusion of 1.8 mM H_2O_2 significantly reduces the magnitude of $P2X_2$-mediated whole-cell currents evoked in response to 5 μM ATP. The inset shows an illustrative trace of $P2X_2$-mediated currents in the absence and presence of 1.8 mM H_2O_2 at a holding potential of −70 mV. The stars represent the application of ATP for 10 s; the arrow indicates zero current. B, H_2O_2 concentration-response curve of $P2X_2$-mediated currents. The IC_{50} value is 0.74 mM. C, summarized data showing that pre-exposure to 1.8 mM H_2O_2 attenuates the effect of hypoxia on $P2X_2$-mediated currents evoked in response to 5 μM ATP. For comparison, the open symbols are in the absence of H_2O_2 and taken from Fig. 1B.

a hypoxic stimulus. In PC12 cells, exposure to extracellular ATP activates an inwardly rectifying current that stimulates Ca^{2+} entry through L-type Ca^{2+} channels and promotes noradrenaline secretion, and this secretion is potentiated under hypoxic conditions (Inoue et al., 1989; Nakazawa et al., 1990; Taylor and Peers, 1999; Hur et al., 2001). Further studies showed that the channel mediating the ATP-sensitive current was encoded by the $P2X_2$ receptor (Brake et al., 1994). Therefore, the attenuation of the $P2X_2$-mediated currents under hypoxia shown in this study may be a general mechanism to limit catecholamine secretion.

Although numerous types of ion channels are modulated by hypoxia (for review, see Lopez-Barneo et al., 2001), the exact mechanism whereby cells sense changes in oxygen is largely unknown. Several mechanisms have been proposed in the literature (for reviews, see Chandel and Schumacker,

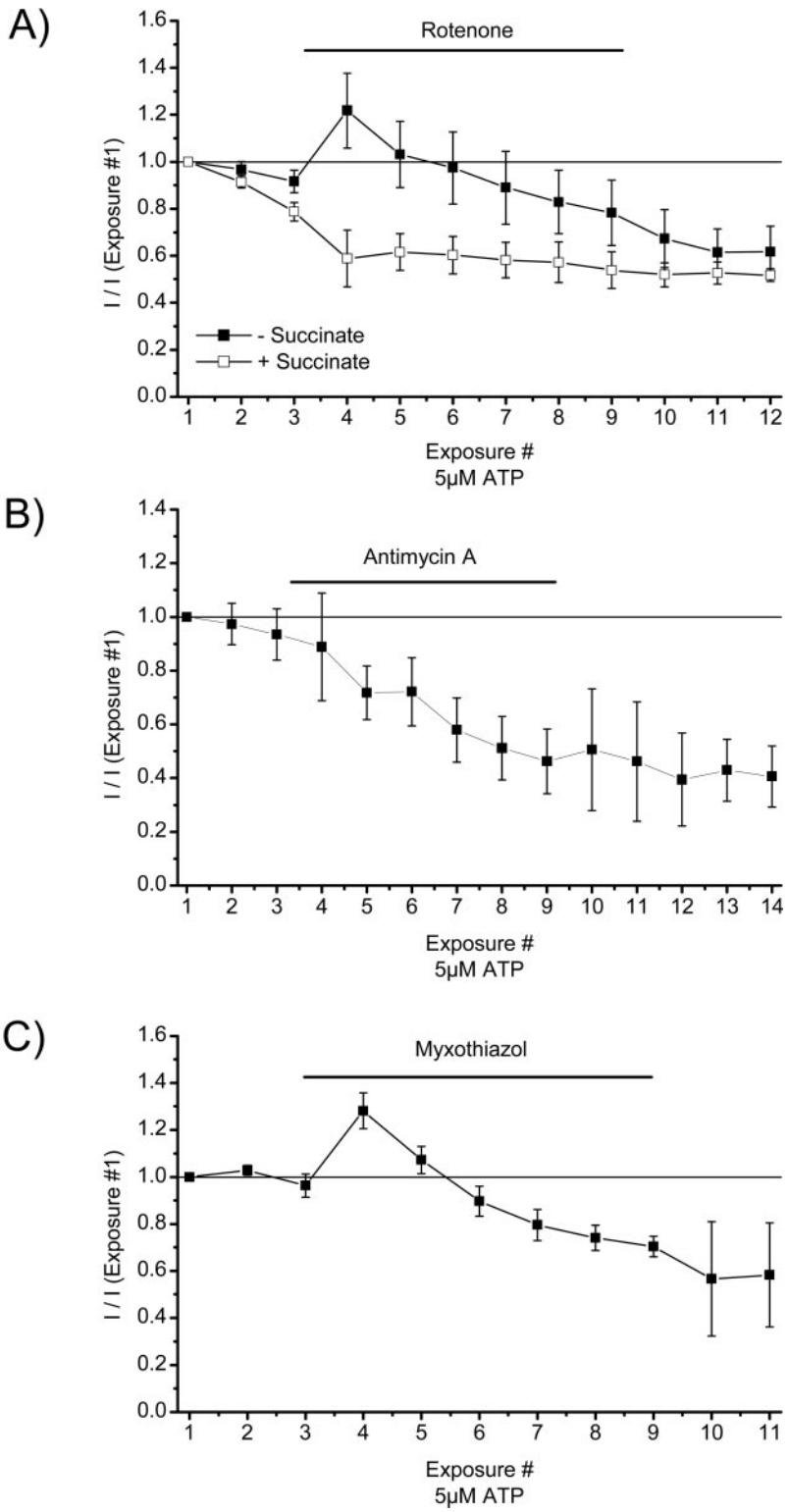

Fig. 7. Inhibitors of the mitochondrial ETC modulate $P2X_2$-mediated currents. HEK293 cells expressing homomeric $P2X_2$ receptors were held at a potential of −70 mV in the presence of external Ca^{2+} and Mg^{2+} and repeatedly exposed to extracellular ATP (5 μM). A, perfusion of 100 nM rotenone, a complex I inhibitor that decreases the production of ROS, caused a transient increase in the magnitude of $P2X_2$-mediated currents evoked in response to 5 μM ATP (■). The presence of 5 mM succinate, a complex II substrate, abolished the increase in the $P2X_2$-mediated currents (□). B, perfusion of 10 μg/ml antimycin A, a complex III inhibitor that increases the production of ROS, caused a reduction in the magnitude of $P2X_2$-mediated currents evoked in response to 5 μM ATP. C, perfusion of 100 nM myxothiazol, a complex III inhibitor that decreases the production of ROS, caused a transient increase in the magnitude of $P2X_2$-mediated currents evoked in response to 5 μM ATP.

2000; Sham, 2002). The predominant theory is that hypoxia results in either a decrease (Mohazzab and Wolin, 1994) or an increase (Leach et al., 2001; Waypa et al., 2001) in the production of ROS, which shifts the ratio of redox couples (i.e., glutathione disulfide/glutathione and NAD^+/NADH) to a more reduced state and hence alters channel function. The results from this study suggest that the hypoxic modulation of the $P2X_2$ receptor is mediated via an increase in the production of ROS, because exposure to H_2O_2 mimicked the effect of hypoxia on the $P2X_2$-mediated currents and attenuated the reduction in $P2X_2$-mediated currents when exposed to a hypoxic challenge. Although the extracellular concentration of H_2O_2 (1.8 mM) that was used in this study seems to be outside the expected physiological concentration of H_2O_2, there is increasing evidence in the literature that a H_2O_2 concentration gradient is established across biological membranes, such that the intracellular concentration of H_2O_2 may be 10-fold less than the extracellular concentration (Antunes and Cadenas, 2000; Seaver and Imlay, 2001). In this study, we have shown that currents mediated by homomeric $P2X_2$ receptors were inhibited by H_2O_2 in a concentration-dependent manner, and that the $P2X_2$-mediated currents were inhibited by extracellular H_2O_2 concentrations of $\geq$100 μM. Therefore, the perceived intracellular concentration of H_2O_2 would be in the micromolar range, which is within physiological range of H_2O_2 concentrations reported in the literature (Halliwell et al., 2000).

We further investigated the source of ROS that modulates the $P2X_2$ receptor by using mitochondrial inhibitors that either increase or decrease the production of ROS. In the presence of either rotenone or myxothiazol, which decreases the production of ROS, a transient increase in the magnitude of the $P2X_2$-mediated currents is observed. Furthermore, the transient increase in the $P2X_2$ current observed in the presence of rotenone could be abolished by the presence of succinate, which bypasses the inhibition at complex I. In contrast,

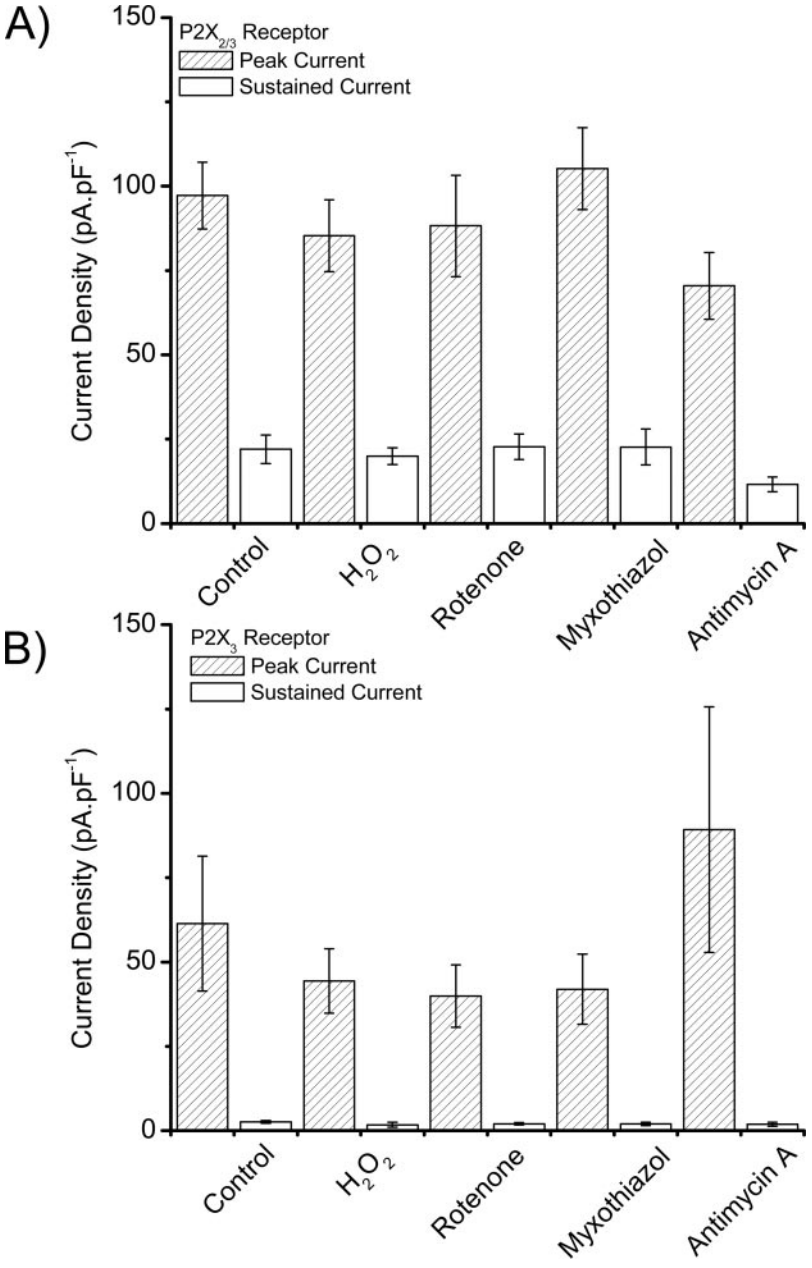

Fig. 8. Currents mediated by heteromeric $P2X_{2/3}$ or homomeric $P2X_3$ receptors are not affected by ROS. HEK293 cells expressing heteromeric $P2X_{2/3}$ or homomeric $P2X_3$ receptors were held at a potential of −70 mV in the presence of external Ca^{2+} and Mg^{2+} and perfused with 1.8 mM H_2O_2, 100 nM rotenone, 100 nM myxothiazol, or 10 μg/ml antimycin A for 2 min before being exposed to extracellular ATP (0.5 μM). A, peak and sustained $P2X_{2/3}$-mediated currents evoked by the first exposure to 0.5 μM ATP in the absence or presence of ROS generators. B, peak and sustained $P2X_3$-mediated currents evoked by the first exposure to 0.5 μM ATP in the absence or presence of ROS generators.

antimycin A, which increases ROS production, reduced the magnitude of the $P2X_2$-mediated currents. These observations support the theory that ROS can modulate the $P2X_2$ receptor. In addition, the observation that H_2O_2 and the mitochondrial inhibitors did not affect homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors supports the observation that hypoxia and ROS selectively modulate homomeric $P2X_2$-mediated currents. However, it must be noted that H_2O_2 did not entirely inhibit the hypoxia-induced reduction in $P2X_2$-mediated currents; therefore, some additional mechanism must also contribute to the modulation of the $P2X_2$ receptor in response to a hypoxic challenge. Potential additional mediators of the hypoxic modulation of $P2X_2$ receptors include nonoxidase iron proteins and/or a direct effect on the channel protein itself (Lopez-Barneo et al., 2001).

The ability of hypoxia and H_2O_2 to reduce the magnitude of $P2X_2$-mediated currents was only partially reversible upon washout, and the extent of recovery varied between individual cells (13–100%). The reason for this is unclear, but one possibility is that exposure to a hypoxic challenge may lead to a permanent modification of the $P2X_2$ receptor. Thus, exposure to a hypoxic challenge results in an increase in the mitochondrial production of ROS. The ROS subsequently passes from the mitochondria into the cytosol, where they may modify cysteine residues on the $P2X_2$ receptor subunit and hence lead to a permanent modification in the function of the $P2X_2$ receptor. Because all P2X receptors have a conserved cysteine-rich extracellular domain (Brake et al., 1994; Valera et al., 1994; Surprenant et al., 1995), the hypoxia and ROS-sensitive residue on the $P2X_2$ receptor must lie on one or more of the nonconserved residues. On the other hand, the limited reversibility of the response to hypoxia and ROS may be caused by cell-to-cell variation in the ability of individual cells to remove ROS via the action of catalases, peroxidases, and thioredoxin-linked systems. In summary, we have shown that that the magnitude of currents mediated by homomeric $P2X_2$ receptors are attenuated under hypoxic conditions and that this modulation of $P2X_2$ receptors is dependent on the production of ROS at the level of mitochondrial electron transport.

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Address correspondence to: Dr. H. S. Mason, Cardiff School of Biosciences, University of Cardiff, Biomedical Sciences Bldg., Museum Ave., Cardiff, CF10 3US, UK. E-mail: dentyh@netscape.net