

**SELECTIVE MODULATION OF LIGAND-GATED P2X PURINOCEPTOR
CHANNELS BY ACUTE HYPOXIA IS MEDIATED BY REACTIVE OXYGEN
SPECIES**

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Running Title: ROS Mediates Hypoxic Modulation of P2X₂ Receptors

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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 P2X₂ and P2X₃ receptors expressed in HEK293 cells. In cells expressing homomeric P2X₂ receptors, perfusion of 5μM ATP (EC₂₅) induced an inward whole-cell current that showed little desensitization during repeated exposures under continuously normoxic conditions. Exposure to an hypoxic ATP solution (P_{O2} 25-40 mmHg) significantly reduced the whole-cell current to 49% of normoxic control. This hypoxic inhibition of P2X₂-mediated inward current was maintained across all potentials when a voltage step protocol was applied. In contrast, currents mediated by homomeric P2X₃ receptors or heteromeric P2X_{2/3} receptors were insensitive to an acute hypoxic challenge. One mechanism whereby hypoxia may modulate P2X₂ channels is via the production of reactive oxygen species (ROS). H₂O₂ (1.8mM) reversibly reduced homomeric P2X₂ whole-cell currents to 62% of control. Further, H₂O₂ attenuated the effect of hypoxia on homomeric P2X₂ 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 P2X₂-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 P2X₂ receptor and that the response of P2X₂ receptor subunits to hypoxia is mediated through the mitochondrial production of ROS.

INTRODUCTION

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 partial pressure of oxygen (P_{O_2}) in the environment. The ability to detect changes in arterial P_{O_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 (see reviews by (Kemp *et al.*, 2003;Lopez-Barneo *et al.*, 2001;Haddad and Jiang, 1997). One of the primary responses to an acute reduction in P_{O_2} is a change in plasmalemmal ion channel activity, which leads to cellular depolarization, calcium entry and modifications in cellular excitability or secretory activity. Recently, there has been increasing interest in the role of purinoceptors in respiratory control. Extracellular ATP activates sensory neurons within the carotid body (Prasad *et al.*, 2001;Zhang *et al.*, 2000) and the ventrolateral medulla (Gourine *et al.*, 2003;Thomas *et al.*, 2001) and this activation is prevented by the P2 receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS). However, relatively little is known about whether P2 purinoceptors are directly modulated in response to an 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 (P2X₁₋₇) 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 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 an hydrophobic H5 sequence immediately prior to M2 domain is thought to form the ion pore and ion-binding sites of the P2X receptor (Valera *et al.*, 1994; Brake *et al.*, 1994). Further, 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, olfactory and visual systems (North, 2002). Further, P2X receptors are expressed in cells that respond to hypoxia such as the carotid body, pulmonary smooth muscle and PC12 cells (Prasad *et al.*, 2001; Chootip *et al.*, 2002; Kobayashi *et al.*, 1998). Indeed, in the carotid body, hypoxia leads to an increased afferent discharge rate and this is mediated via P2X₂ and P2X₃ 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 P2X₂ and P2X₃ homo- and heteromeric receptors expressed in HEK293 cells.

MATERIALS AND METHODS

Expression Systems

Human embryonic kidney 293 (HEK293) cells that stably express either homomeric rat P2X₂ receptors, homomeric human P2X₃ receptors, or rat P2X₂ and P2X₃ 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;Valera *et al.*, 1995;Evans *et al.*, 1995;Kawashima *et al.*, 1998). All the P2X cell-lines were maintained in DMEM media supplemented with 10% fetal calf serum, 2mM L-glutamine, 1% antibiotic antimycotic, 100µg.ml⁻¹ gentamicin and 300µg.ml⁻¹ geneticin (G148) (all purchased from Gibco BRL, Paisley, Strathclyde, UK) in a humidified incubator gassed with 5% CO₂ / 95% air. Cells were passaged every 7 days using Ca²⁺- and Mg²⁺-free phosphate-buffered saline (Gibco BRL, Paisley, Strathclyde, UK). 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 were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.). Disodium ATP, α , β -methylene ATP (α , β -MeATP), hydrogen peroxide (H₂O₂), rotenone, antimycin A, myxothiazol, ethylene glycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid] (HEPES), were obtained from Sigma-Aldrich (Poole, Dorset, U.K.). ATP and α , β -MeATP were dissolved at 100mM in distilled H₂O, 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 (antimycin A, $50\text{mg}\cdot\text{ml}^{-1}$ and myxothiazol, 10mM) or DMSO (rotenone, 100mM) and aliquots were stored at -20°C . Oxygen-free nitrogen gas and medical air (21% O_2) were obtained from BOC Ltd (Guildford, Surrey, U.K.). All tubing was gas-impermeant (Tygon tubing, BDH, Atherstone, Berks, U.K.).

Electrophysiology

Whole-cell currents were recorded at room temperature. Recording pipettes pulled from borosilicate glass had resistances of $4\text{--}6\text{M}\Omega$ when filled with pipette solution that contained (in mM): 117 KCl, 10 NaCl, 2 MgCl_2 , 1 CaCl_2 , 11 EGTA, 2 Na-ATP and 11 HEPES with the pH adjusted to 7.2 with KOH. The bath solution contained (in mM): 135 NaCl, 5 KCl, 1.2 MgCl_2 , 2.5 CaCl_2 , 5 HEPES and 10 glucose, with the pH adjusted to 7.4 with NaOH. Hypoxic solutions were bubbled with N_2 gas for at least 30 min prior to 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). P_{O_2} was measured (at the cell) using a polarised (-800mV) carbon fibre electrode (Mojet *et al.*, 1997). For the experiments reported herein, the P_{O_2} values were 150 (normoxia) and 25–40 (hypoxia) mmHg.

Resistive feedback voltage clamp was achieved using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Voltage protocols were generated and currents recorded using pClamp 8.0 software, employing a Digidata 1322 A/D convertor (Axon Instruments). Data were filtered (4-pole

Bessel) at 2kHz and digitized at 5kHz. Following 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 -70mV. 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 minutes prior to 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 minutes 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 0mV and 200ms voltage steps were applied from -100 to +100mV in 20mV increments were applied under normoxic and hypoxic conditions. In order 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 -70mV and perfused with either normoxic or hypoxic bathing solution for 2 minutes. 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 represents at least 5 cells.

Data Analysis

Data analyses were performed using the pClamp 8.0 suite of software (Axon Instruments). Data are reported as mean \pm 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 H₂O₂ were fitted to the Hill equation using GraphPad Prism software (San Diego, California, U.S.A.).

RESULTS

Hypoxia modulates homomeric P2X₂-mediated currents

In order to evaluate the effect of hypoxia on P2X₂-mediated currents expressed in HEK293 cells, a concentration of ATP was required that would elicit responses of similar magnitude following repeated exposures to ATP. Extracellular ATP activated P2X₂-mediated currents in a concentration-dependent manner, with an EC₅₀ of 9.59 μM and a Hill slope of 2.75 ($n=28$, data not shown). At high concentrations of ATP (100μM), the magnitude of the P2X₂-mediated current elicited in response to ATP decreased with repeated exposures due to desensitization of the P2X₂ receptor (data not shown). However, at a concentration of 5μM ATP (which equates to the EC₂₅ value), P2X₂-mediated currents of similar magnitude could be evoked repeatedly, even in the presence of the divalent cations Ca²⁺ and Mg²⁺. As a result of these observations, in the following experiments that evaluated the effect of hypoxia on P2X₂-mediated currents, ATP concentrations of 5μM were used.

Figure 1A shows exemplar ATP-induced whole-cell homomeric P2X₂-mediated currents recorded under normoxic and hypoxic conditions at a potential of -70 mV in the presence of external Ca²⁺ and Mg²⁺. After exposing the cells to a hypoxic solution for 2 minutes, the magnitude of the P2X₂-mediated current activated in response to 5μM ATP was reduced compared to that under normoxic conditions. This reduction in the magnitude of the P2X₂-mediated current was partially relieved on returning to normoxic conditions. Figure 1B summarizes the response of P2X₂ expressing cells when exposed to repeated exposures of 5μM 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 P2X₂-mediated current over time (the magnitude of the last exposure was 0.96 ± 0.09 that of the first exposure, $n=5$). In contrast, exposure to an hypoxic challenge significantly reduced the magnitude of the P2X₂-mediated current activated in response to 5 μ M ATP (0.51 ± 0.09 that of the current under normoxic conditions ($P < 0.01$, $n=5$). The reduction in the magnitude of the P2X₂-mediated whole-cell current was partially reversible on washout (0.68 ± 0.16 compared to control, $n=5$).

The effect of hypoxia on the magnitude of the P2X₂-mediated current was also examined using a voltage step protocol. Figure 2 shows illustrative traces recorded sequentially in response to 200ms voltage steps from -100mV to $+100\text{mV}$ in 20mV increments from a holding potential of 0mV under (A) control conditions in the absence of ATP, and in the presence of 5 μ M ATP under (B) hypoxic, (C) normoxic and (D) hypoxic conditions. In the absence of ATP, the voltage step protocol elicited small endogenous currents (figure 2A). Exposure to ATP under hypoxic conditions activated an inwardly rectifying P2X₂-mediated current (figure 2B). Switching the perfusate from a hypoxic to a normoxic solution increased the magnitude of the P2X₂-mediated current (figure 2C), and this increase in current was fully reversible on returning to hypoxic conditions (figure 2D). Figure 2E shows the mean control-subtracted data from 6 cells. Currents are normalized to the current evoked in response to 5 μ M ATP at a potential of -100mV under hypoxic conditions. Under hypoxic conditions the P2X₂-mediated currents reversed at a potential of -8.3mV , whilst under normoxic conditions the currents were $38 \pm 10\%$ larger ($P < 0.05$, $n=7$) and reversed at a potential of -4.2mV .

A similar modulation of P2X₂-mediated currents by hypoxia was obtained when the reverse protocol was applied, i.e. when the cells expressing the P2X₂ receptor were sequentially exposed to control conditions in the absence of ATP, and in the presence of 5μM ATP under normoxic, hypoxic and normoxic conditions. In this instance hypoxia reduced the magnitude of P2X₂-mediated currents at all potentials more negative than -20mV by 20% ($P < 0.05$, $n=7$) and this was fully reversible on returning to a normoxic solution (data not shown).

Hypoxia does not modulate Homomeric P2X₃ Channels

The P2X-mediated current in sensory neurons is mediated principally through homomeric P2X₃ and heteromeric P2X_{2/3} channels (Dunn *et al.*, 2001). Therefore, the responses of homomeric P2X₃ and heteromeric P2X_{2/3} channels to hypoxia were also examined with the results summarized in figures 3 and 4. As shown in figure 3A, exposing cells expressing homomeric P2X₃ receptors to 10μM ATP under normoxic conditions in the presence of external Ca²⁺ and Mg²⁺ activated an inward current that rapidly inactivated (peak normoxic mean P2X₃-mediated current -124.1 ± 53.7 pA pF⁻¹, $n=9$). The inactivation of the P2X₃-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, $n=9$, figure 3D). As a second exposure to ATP significantly reduced the magnitude of the homomeric P2X₃ 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 minutes before exposing the cells to ATP. Under hypoxic conditions 10 μ M ATP activated a P2X₃-mediated current whose magnitude was comparable to that activated under normoxic conditions (mean peak hypoxic P2X₃-mediated current -110.4 ± 24.5 pA pF⁻¹, $n=7$, figure 3C). The inactivation of the P2X₃-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, figure 3D).

Similar results were obtained when the cells were exposed to α , β -MeATP, a P2X₁ and P2X₃-selective agonist that differentiates between P2X₂ and P2X₃ receptor subunits (data not shown). Exposure to 10 μ M α , β -MeATP activated inactivating P2X₃-mediated currents under either normoxic or hypoxic conditions. The magnitudes of the peak P2X₃-mediated currents were similar under normoxic and hypoxic conditions (normoxia: -129 ± 35.6 pA pF⁻¹, $n=8$; hypoxia: -80.6 ± 21.7 pA pF⁻¹, $n=8$). The inactivation of the P2X₃-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: $\tau_1 = 912 \pm 109$ ms and $\tau_2 = 172 \pm 18.6$ ms).

Hypoxia does not modulate Heteromeric P2X_{2/3} Channels

The observation that peak activation of homomeric P2X₂ receptors were sensitive to hypoxia whereas that of homomeric P2X₃ receptors were not was intriguing. As 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 Ca²⁺ and Mg²⁺. Figure 4B summarizes the response of P2X_{2/3} expressing cells when exposed to repeated exposures of 10μM 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). Whilst exposure to an hypoxic challenge reduced the magnitude of the P2X_{2/3}-mediated current activated in response to 10μM 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 Ca²⁺ and Mg²⁺.

Hypoxia Selectively Alters the Concentration-Response Curves of P2X Receptors

In order to examine further 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 P2X₂-mediated currents evoked in response to the first exposure to ATP under either normoxic or hypoxic conditions. Under normoxic conditions extracellular ATP activated P2X₂-mediated currents in a concentration-dependent manner, with an EC₅₀ of 3.73μM. In contrast, under hypoxic conditions the concentration response curve was shifted to the right

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

It was possible that the concentration of ATP ($10\mu\text{M}$, 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. Figures 5B and 5C show 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 hydrogen peroxide (H_2O_2) under normoxic and hypoxic conditions. Figure 6A shows the effect of perfusing 1.8mM 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 1.8mM H_2O_2 solution for 2 minutes, the magnitude of the

P2X₂-mediated current activated in response to 5μM ATP was significantly reduced compared to 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 P2X₂-mediated current was partially relieved on returning to control conditions (0.76 ± 0.20 that of the first exposure to ATP, $n=3$). The H₂O₂-induced reduction in the magnitude of the P2X₂-mediated current was concentration dependent, with an IC₅₀ of 0.73 mM (figure 6B).

If hypoxia reduces the magnitude of P2X₂-mediated currents through the production of ROS, then pre-exposure to H₂O₂ should abolish this effect. Figure 6C shows the mean data recorded from 6 cells. In the presence of 1.8mM H₂O₂ and hypoxia the magnitude of the P2X₂-mediated current activated in response to 5μM ATP in the presence of external Ca²⁺ and Mg²⁺ 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 P2X₂-mediated current in the presence of 1.8mM H₂O₂ was attenuated compared to that in the absence of (0.51 ± 0.09 , $P < 0.05$, the open symbols for comparison are taken from figure 1B). Thus, H₂O₂ itself mimics the effect of hypoxia on P2X₂-mediated currents and the presence of H₂O₂ attenuates the effect of a subsequent hypoxic challenge.

Mitochondrial Inhibitors that Modulate the Production of ROS Mimic the Effect of Hypoxia on Homomeric P2X₂-mediated Currents

In order to examine the source of the ROS that might underlie the hypoxic inhibition of P2X₂ currents mitochondrial inhibitors of the electron transport chain (ETC) were employed; these ETC inhibitors were rotenone,

antimycin A and myxothiazol and each block at a different site of the ETC. Figure 7A shows that perfusion of cells expressing P2X₂ receptors with rotenone (100nM), a complex I inhibitor that causes a reduction in ROS, significantly increased the magnitude of P2X₂-mediated currents in response to the first exposure to 5μM ATP following application of rotenone (1.22 ± 0.016 that of the first exposure to ATP, $P < 0.05$, $n=6$). The magnitude of P2X₂-mediated currents in response to subsequent exposures to ATP in the presence of rotenone declined over time (6th exposure 0.78 ± 0.14 that of the first exposure to ATP, $n=6$) and was irreversible upon wash (0.62 ± 0.11 that of the first exposure to ATP, $n=5$). This was a common phenomenon with all the mitochondrial inhibitors and is presumably due to metabolic poisoning of the cell.

If the transient increase in the P2X₂-mediated current is due to the reduction in the production of ROS then this should be overcome by providing succinate, a complex II substrate. As shown in figure 7A, in the presence of 5mM succinate, rotenone failed to induce an increase in the magnitude of P2X₂-mediated currents in response to the first exposure to 5μM ATP. Rather, P2X₂-mediated currents declined over time (to 0.54 ± 0.08 that of the first exposure to ATP, $n=3$) and this was irreversible on washing (0.52 ± 0.03 that of the first exposure to ATP, $n=3$). This reduction in the magnitude of the P2X₂-mediated currents was similar to those observed in the absence of succinate (see above).

To elucidate further the role of ROS in the modulation of P2X₂-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 ng.ml^{-1}) caused a reduction in the magnitude of P2X₂-mediated currents over time (to 0.46 ± 0.12 that of the first exposure to ATP, $P < 0.01$, $n=5$) and this was irreversible on washing (0.41 ± 0.11 that of the first exposure to ATP, $n=3$). In contrast, myxothiazol (100nM) caused a transient increase in P2X₂-mediated currents, similar to that observed with rotenone. The magnitude of P2X₂-mediated current in response to the first exposure to $5\mu\text{M}$ ATP in the presence of myxothiazol was 1.28 ± 0.08 that of the first exposure to ATP ($P < 0.05$, $n=4$). The magnitude of P2X₂-mediated currents in response to subsequent exposures to ATP in the presence of myxothiazol declined over time (6th exposure 0.70 ± 0.04 that of the first exposure to ATP, $P < 0.05$, $n=4$) and was irreversible upon wash (0.58 ± 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 P2X₂ currents.

Mitochondrial Inhibitors that Modulate the Production of ROS do not Affect Homomeric P2X₃- or Heteromeric P2X_{2/3}-mediated Currents

The findings thus far suggest that hypoxia selectively modulates homomeric P2X₂-mediated currents and that this is mediated, in part, via the mitochondrial production of ROS. If this is true then H₂O₂ and the mitochondria inhibitors should have no effect on either homomeric P2X₃ 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 $0.5\mu\text{M}$ ATP (which equates to the EC₂₅ 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 1.8mM H₂O₂ (85.3 ± 10.7 pA pF⁻¹ and 19.9 ± 2.5 pA pF⁻¹, n=10), 100nM rotenone (88.2 ± 15.1 pA pF⁻¹ and 22.7 ± 3.8 pA pF⁻¹, n=11), 100nM myxothiazol (105.2 ± 12.1 pA pF⁻¹ and 22.7 ± 5.3 pA pF⁻¹, n=12) or 10 ng.ml⁻¹ antimycin A (70.5 ± 9.9 pA pF⁻¹ and 11.6 ± 2.2 pA pF⁻¹, n=9) were not found to be statistically significant from control treated cells (97.2 ± 9.93 pA pF⁻¹ and 22.0 ± 4.2 pA pF⁻¹, n=15). Likewise, figure 8B shows the magnitude of peak and sustained currents mediated by homomeric P2X₃ receptors evoked in response to the first exposure to 0.5μM ATP (which equates to the EC₂₅ value) in the presence or absence of the ROS mediators. The magnitudes of the peak and sustained P2X₃-mediated currents in the presence of H₂O₂ (44.4 ± 9.6 pA pF⁻¹ and 1.7 ± 0.8 pA pF⁻¹, n=10), rotenone (39.9 ± 9.3 pA pF⁻¹ and 2.0 ± 0.4 pA pF⁻¹, n=11), myxothiazol (41.9 ± 10.4 pA pF⁻¹ and 2.0 ± 0.5 pA pF⁻¹, n=10) or antimycin A (89.2 ± 36.4 pA pF⁻¹ and 1.9 ± 0.6 pA pF⁻¹, n=9) were not found to be statistically significant from control treated cells (61.4 ± 20.0 pA pF⁻¹ and 2.6 ± 0.4 pA pF⁻¹, n=15). Thus, these findings support the hypothesis that hypoxia and ROS selectively modulate homomeric P2X₂ receptors.

DISCUSSION

The purpose of this study was to evaluate whether hypoxia could affect the activation of homo- and heteromeric P2X₂ and P2X₃ receptor cation channels and thus be a possible mechanism contributing to the ventilatory response during an hypoxic challenge. The present findings show that hypoxia attenuates homomeric P2X₂-mediated currents, but has no effect on homomeric P2X₃- or heteromeric P2X_{2/3} currents. This is the first direct demonstration of acute hypoxic modulation of a ligand-gated ion channel. Further, the selective modulation of the P2X₂ receptor subunit is consistent with the recent report by Rong *et al.* who found that mice deficient in the P2X₂ receptor subunit showed an attenuated ventilatory response to hypoxia, whereas mice deficient in the P2X₃ receptor subunit were comparable to wild type (Rong *et al.*, 2003). Previous studies have shown that under hypoxic conditions, ATP is co-released with ACh from type I glomus cells and ATP activates P2X₂ and P2X₃ receptors expressed on petrosal ganglia which leads to an increased afferent discharge rate (Prasad *et al.*, 2001; Zhang *et al.*, 2000). Therefore, the combined results from this current study and that of Rong *et al.* (2003) suggest that expression of homomeric P2X₂ 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 P2X₂ receptors may be involved in the regulation of the secretory response of PC12 to an hypoxic stimulus. In PC12 cells, exposure to extracellular ATP activates an inwardly rectifying current that stimulates Ca²⁺ entry through L-type Ca²⁺ channels and promotes noradrenaline secretion and this secretion is potentiated under hypoxic

conditions (Inoue *et al.*, 1989; Nakazawa *et al.*, 1990; Hur *et al.*, 2001; Taylor and Peers, 1999). Further studies showed that the channel mediating the ATP-sensitive current was encoded by the P2X₂ receptor (Brake *et al.*, 1994). Therefore, the attenuation of the P2X₂-mediated currents under hypoxia shown in this study may be a general mechanism to limit catecholamine secretion.

Whilst numerous types of ion channels are modulated by hypoxia (see (Lopez-Barneo *et al.*, 2001) for review) the exact mechanism whereby cells sense changes in oxygen is largely unknown. Several mechanisms have been proposed in the literature (see (Chandel and Schumacker, 2000; Sham, 2002) for reviews). 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 reactive oxygen species (ROS), which shifts the ratio of redox couples (i.e. GSSG:GSH 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 P2X₂ receptor is mediated via an increase in the production of ROS since exposure to H₂O₂ mimicked the effect of hypoxia on the P2X₂-mediated currents and attenuated the reduction in P2X₂-mediated currents when exposed to a hypoxic challenge. Although the extracellular concentration of H₂O₂ (1.8mM) that was used in this study appears to be outside the expected physiological concentration of H₂O₂, there is increasing evidence in the literature that a H₂O₂ concentration gradient is established across biological membranes such that the intracellular concentration of H₂O₂ 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 P2X₂ receptors were inhibited by H₂O₂ in a concentration-dependent manner, and that the P2X₂-mediated currents were inhibited by extracellular H₂O₂ concentrations of $\geq 100\mu\text{M}$. Therefore, the perceived intracellular concentration of H₂O₂ would be in the μM range, which is within physiological range of H₂O₂ concentrations reported in the literature (Halliwell *et al.*, 2000).

Further, we investigated the source of ROS that modulates the P2X₂ 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 P2X₂-mediated currents is observed. Further, the transient increase in the P2X₂ current observed in the presence of rotenone could be abolished by the presence of succinate, which by-passes the inhibition at complex I. In contrast, antimycin A, which increases ROS production, reduced the magnitude of the P2X₂-mediated currents. These observations support the theory that ROS can modulate the P2X₂ receptor. In addition, the observation that H₂O₂ and the mitochondrial inhibitors did not affect homomeric P2X₃ and heteromeric P2X_{2/3} receptors supports the observation that hypoxia and ROS selectively modulate homomeric P2X₂-mediated currents. However, it must be noted that H₂O₂ did not entirely inhibit the hypoxia-induced reduction in P2X₂-mediated currents and therefore some additional mechanism must also contribute to the modulation of the P2X₂ receptor in response to an hypoxic challenge. Potential additional mediators of the hypoxic modulation of P2X₂ receptors include non-oxidase 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 on washout and the extent of recovery varied between individual cells (13 to 100%). The reason for this is unclear but one possibility is that exposure to an hypoxic challenge may lead to a permanent modification of the $P2X_2$ receptor. Thus, exposure to an hypoxic challenge results in an increase in the mitochondrial production of ROS. The ROS subsequently pass 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. Since all P2X receptors have a conserved cysteine-rich extracellular domain (Valera *et al.*, 1994; Brake *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 non-conserved residues. Alternatively, the limited reversibility of the response to hypoxia and ROS may be due to 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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REFERENCES

Antunes F and Cadenas E (2000) Estimation of H₂O₂ Gradients Across Biomembranes. *FEBS Lett* **475**:121-126.

Brake AJ, Wagenbach M J and Julius D (1994) New Structural Motif for Ligand-Gated Ion Channels Defined by an Ionotropic ATP Receptor. *Nature* **371**:519-523.

Chandel NS and Schumacker P T (2000) Cellular Oxygen Sensing by Mitochondria: Old Questions, New Insight. *J Appl Physiol* **88**:1880-1889.

Chootip K, Ness K F, Wang Y, Gurney A M and Kennedy C (2002) Regional Variation in P2 Receptor Expression in the Rat Pulmonary Arterial Circulation. *Br J Pharmacol* **137**:637-646.

Dunn PM, Zhong Y and Burnstock G (2001) P2X Receptors in Peripheral Neurons. *Prog Neurobiol* **65**:107-134.

Evans RJ, Lewis C, Buell G, Valera S, North R A and Surprenant A (1995) Pharmacological Characterization of Heterologously Expressed ATP-Gated Cation Channels (P2X Purinoceptors). *Mol Pharmacol* **48**:178-183.

Gourine AV, Atkinson L, Deuchars J and Spyer K M (2003) Purinergic Signalling in the Medullary Mechanisms Of Respiratory Control in the Rat:

Respiratory Neurones Express the P2X₂ Receptor Subunit. *J Physiol* **552**:197-211.

Haddad GG and Jiang C (1997) O₂-Sensing Mechanisms in Excitable Cells: Role of Plasma Membrane K⁺ Channels. *Annu Rev Physiol* **59**:23-42.

Halliwell B, Clement M V and Long L H (2000) Hydrogen Peroxide in the Human Body. *FEBS Lett* **486**:10-13.

Hur EM, Park T J and Kim K T (2001) Coupling of L-Type Voltage-Sensitive Calcium Channels to P2X₂ Purinoceptors in PC-12 Cells. *Am J Physiol* **280**:C1121-C1129.

Inoue K, Nakazawa K, Fujimori K and Takanaka A (1989) Extracellular Adenosine 5'-Triphosphate-Evoked Norepinephrine Secretion Not Relating to Voltage-Gated Ca Channels in Pheochromocytoma PC12 Cells. *Neurosci Lett* **106**:294-299.

Kawashima E, Estoppey D, Virginio C, Fahmi D, Rees S, Surprenant A and North R A (1998) A Novel and Efficient Method for the Stable Expression of Heteromeric Ion Channels in Mammalian Cells. *Receptors Channels* **5**:53-60.

Kemp PJ, Searle G J, Hartness M E, Lewis A, Miller P, Williams S, Wootton P, Adriaensen D and Peers C (2003) Acute Oxygen Sensing in Cellular Models: Relevance to the Physiology of Pulmonary Neuroepithelial and Carotid Bodies. *Anat Rec* **270**:41-50.

Kobayashi S, Conforti L, Pun R Y and Millhorn D E (1998) Adenosine Modulates Hypoxia-Induced Responses in Rat PC12 Cells Via the A2A Receptor. *J Physiol* **508**:95-107.

Leach RM, Hill H M, Snetkov V A, Robertson T P and Ward J P (2001) Divergent Roles of Glycolysis and the Mitochondrial Electron Transport Chain in Hypoxic Pulmonary Vasoconstriction of the Rat: Identity of the Hypoxic Sensor. *J Physiol* **536**:211-224.

Lopez-Barneo J, Pardal R and Ortega-Saenz P (2001) Cellular Mechanism of Oxygen Sensing. *Annu Rev Physiol* **63**:259-287.

Mohazzab KM and Wolin M S (1994) Properties of a Superoxide Anion-Generating Microsomal NADH Oxidoreductase, a Potential Pulmonary Artery PO₂ Sensor. *Am J Physiol* **267**:L823-L831.

Mojet MH, Mills E and Duchon M R (1997) Hypoxia-Induced Catecholamine Secretion in Isolated Newborn Rat Adrenal Chromaffin Cells Is Mimicked by Inhibition of Mitochondrial Respiration. *J Physiol* **504**:175-189.

Nakazawa K, Fujimori K, Takanaka A and Inoue K (1990) An ATP-Activated Conductance in Pheochromocytoma Cells and Its Suppression by Extracellular Calcium. *J Physiol* **428**:257-272.

Nicke A, Baumert H G, Rettinger J, Eichele A, Lambrecht G, Mutschler E and Schmalzing G (1998) P2X₁ and P2X₃ Receptors Form Stable Trimers: a Novel Structural Motif of Ligand-Gated Ion Channels. *EMBO J* **17**:3016-3028.

North RA (2002) Molecular Physiology of P2X Receptors. *Physiol Rev* **82**:1013-1067.

Prasad M, Fearon I M, Zhang M, Laing M, Vollmer C and Nurse C A (2001) Expression of P2X₂ and P2X₃ Receptor Subunits in Rat Carotid Body Afferent Neurones: Role in Chemosensory Signalling. *J Physiol* **537**:667-677.

Rong W, Gourine A V, Cockayne D A, Xiang Z, Ford A P, Spyer K M and Burnstock G (2003) Pivotal Role of Nucleotide P2X₂ Receptor Subunit of the ATP-Gated Ion Channel Mediating Ventilatory Responses to Hypoxia. *J Neurosci* **23**:11315-11321.

Seaver LC and Imlay J A (2001) Hydrogen Peroxide Fluxes and Compartmentalization Inside Growing *Escherichia Coli*. *J Bacteriol* **183**:7182-7189.

Sham JS (2002) Hypoxic Pulmonary Vasoconstriction: Ups and Downs of Reactive Oxygen Species. *Circ Res* **91**:649-651.

Stoop R, Thomas S, Rassendren F, Kawashima E, Buell G, Surprenant A and North R A (1999) Contribution of Individual Subunits to the Multimeric P2X₂

Receptor: Estimates Based on Methanethiosulfonate Block at T336C. *Mol Pharmacol* **56**:973-981.

Surprenant A, Buell G and North R A (1995) P2X Receptors Bring New Structure to Ligand-Gated Ion Channels. *Trends Neurosci* **18**:224-229.

Taylor SC and Peers C (1999) Chronic Hypoxia Enhances the Secretory Response of Rat Pheochromocytoma Cells to Acute Hypoxia. *J Physiol* **514**:483-491.

Thomas T, Ralevic V, Bardini M, Burnstock G and Spyer K M (2001) Evidence for the Involvement of Purinergic Signalling in the Control of Respiration. *Neuroscience* **107**:481-490.

Valera S, Hussy N, Evans R J, Adami N, North R A, Surprenant A and Buell G (1994) A New Class of Ligand-Gated Ion Channel Defined by P2X Receptor for Extracellular ATP. *Nature* **371**:516-519.

Valera S, Talabot F, Evans R J, Gos A, Antonarakis S E, Morris M A and Buell G N (1995) Characterization and Chromosomal Localization of a Human P2X Receptor From the Urinary Bladder. *Receptors Channels* **3**:283-289.

Waypa GB, Chandel N S and Schumacker P T (2001) Model for Hypoxic Pulmonary Vasoconstriction Involving Mitochondrial Oxygen Sensing. *Circ Res* **88**:1259-1266.

Zhang M, Zhong H, Vollmer C and Nurse C A (2000) Co-Release of ATP and ACh Mediates Hypoxic Signalling at Rat Carotid Body Chemoreceptors. *J Physiol* **525**:143-158.

FOOTNOTES

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FIGURE LEGENDS

Figure 1: Hypoxia attenuates ATP-induced currents mediated by the homomeric P2X₂ receptor. HEK293 cells expressing homomeric P2X₂ receptors were held at a potential of -70mV in the presence of external Ca²⁺ and Mg²⁺ and repeatedly exposed to extracellular ATP. A) Illustrative trace of a cell exposed to repeated exposures to 5μM ATP under normoxic and hypoxic conditions. The stars represent the application of ATP for 10 sec. The arrow indicates zero current. B) Summarized data showing that exposure to an acute hypoxic challenge attenuates the magnitude of P2X₂-mediated currents evoked in response to 5μM ATP (-■-) compared to cells held under continuously normoxic conditions (-□-).

Figure 2: Normoxia relieves the hypoxic inhibition of the ATP-induced current in cells expressing homomeric P2X₂ receptors. Cells expressing the P2X₂ receptor were held at a potential of 0mV and 200ms voltage steps were applied from -100 to +100mV in 20mV increments. A) - D) Illustrative P2X₂-mediated currents evoked in response to the voltage step protocol. Cells were initially exposed to 5μM ATP in the presence of external Ca²⁺ and Mg²⁺ under hypoxic conditions and then normoxic conditions before returning to hypoxic conditions. E) Summarized current-voltage relationship from 6 cells. Currents are normalized to the current recorded at a potential of -100mV under hypoxic conditions.

Figure 3: Currents mediated by the homomeric P2X₃ receptor are insensitive to hypoxia. Illustrative P2X₃-mediated currents from cells held at a potential of -70mV in the presence of external Ca²⁺ and Mg²⁺ and exposed to extracellular ATP (10μM) under either normoxic (A) or hypoxic (B) conditions. The stars represent the application of ATP for 10 sec. The arrows indicate zero current. Summarized data showing that hypoxia has no effect on either (C) the magnitude of the peak ATP-induced P2X₃-mediated currents or (D) the inactivation time constants.

Figure 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 -70mV in the presence of external Ca²⁺ and Mg²⁺ and repeatedly exposed to extracellular ATP. A) Illustrative trace of a cell exposed to repeated exposures to 10μM ATP under normoxic and hypoxic conditions. The stars represent the application of ATP for 10 sec. 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 to cells held under continuously normoxic conditions (-□-).

Figure 5: The Effect of Hypoxia on the Concentration-Response Curves of Homomeric P2X₂, Homomeric P2X₃ and Heteromeric P2X_{2/3} Receptors. HEK293 cells expressing homomeric P2X₂, homomeric P2X₃ or heteromeric P2X_{2/3} were held at a potential of -70mV and perfused with either normoxic or hypoxic bathing solution for 2 minutes. 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 an hypoxic solution (-■-) shifts the concentration-response curve of homomeric P2X₂ receptors to the right, relative to cells exposed to normoxic solutions (-□-). B) Concentration-response curve of heteromeric P2X₂ receptors under normoxic (-□-) and hypoxic (-■-) conditions. C) Concentration-response curve of homomeric P2X₃ receptors under normoxic (-□-) and hypoxic (-■-) conditions.

Figure 6: H₂O₂ mimics the effect of hypoxia on homomeric P2X₂-mediated currents and attenuates the effect of hypoxia. A) Summarized data showing that perfusion of 1.8mM H₂O₂ significantly reduces the magnitude of P2X₂-mediated whole-cell currents evoked in response to 5μM ATP. The inset shows an illustrative trace of P2X₂-mediated currents in the absence and presence of 1.8mM H₂O₂ at a holding potential of -70mV. The stars represent the application of ATP for 10 sec. The arrow indicates zero current. B) H₂O₂ concentration-response curve of P2X₂-mediated currents. The IC₅₀ value is 0.74mM. C) Summarized data showing that pre-exposure to 1.8mM H₂O₂ attenuates the effect of hypoxia on P2X₂-mediated currents evoked in response to 5μM ATP. For comparison, the open symbols are in the absence of H₂O₂ and are taken from figure 1B.

Figure 7: Inhibitors of the Mitochondrial ETC Modulate P2X₂-mediated Currents. HEK293 cells expressing homomeric P2X₂ receptors were held at a potential of -70mV in the presence of external Ca²⁺ and Mg²⁺ and repeatedly exposed to extracellular ATP (5μM). A) Perfusion of 100nM rotenone, a

complex I inhibitor that decreases the production of ROS, caused a transient increase in the magnitude of P2X₂-mediated currents evoked in response to 5μM ATP (-■-). The presence of 5mM succinate, a complex II substrate, abolished the increase in the P2X₂-mediated currents (-□-). B) Perfusion of 10 ng.mL⁻¹ antimycin A, a complex III inhibitor that increases the production of ROS, caused a reduction in the magnitude of P2X₂-mediated currents evoked in response to 5μM ATP. C) Perfusion of 100nM myxothiazol, a complex III inhibitor that decreases the production of ROS, caused a transient increase in the magnitude of P2X₂-mediated currents evoked in response to 5μM ATP.

Figure 8: Currents Mediated by Heteromeric P2X_{2/3}- or Homomeric P2X₃ Receptors are not Affected by ROS. HEK293 cells expressing heteromeric P2X_{2/3} or homomeric P2X₃ receptors were held at a potential of -70mV in the presence of external Ca²⁺ and Mg²⁺ and perfused with 1.8mM H₂O₂, 100nM rotenone, 100nM myxothiazol or 10 ng.mL⁻¹ antimycin A for two minutes prior to being exposed to extracellular ATP (0.5μM). A) Peak and sustained P2X_{2/3}-mediated currents evoked by the first exposure to 0.5μM ATP in the absence or presence of ROS generators. B) Peak and sustained P2X₃-mediated currents evoked by the first exposure to 0.5μM ATP in the absence or presence of ROS generators.

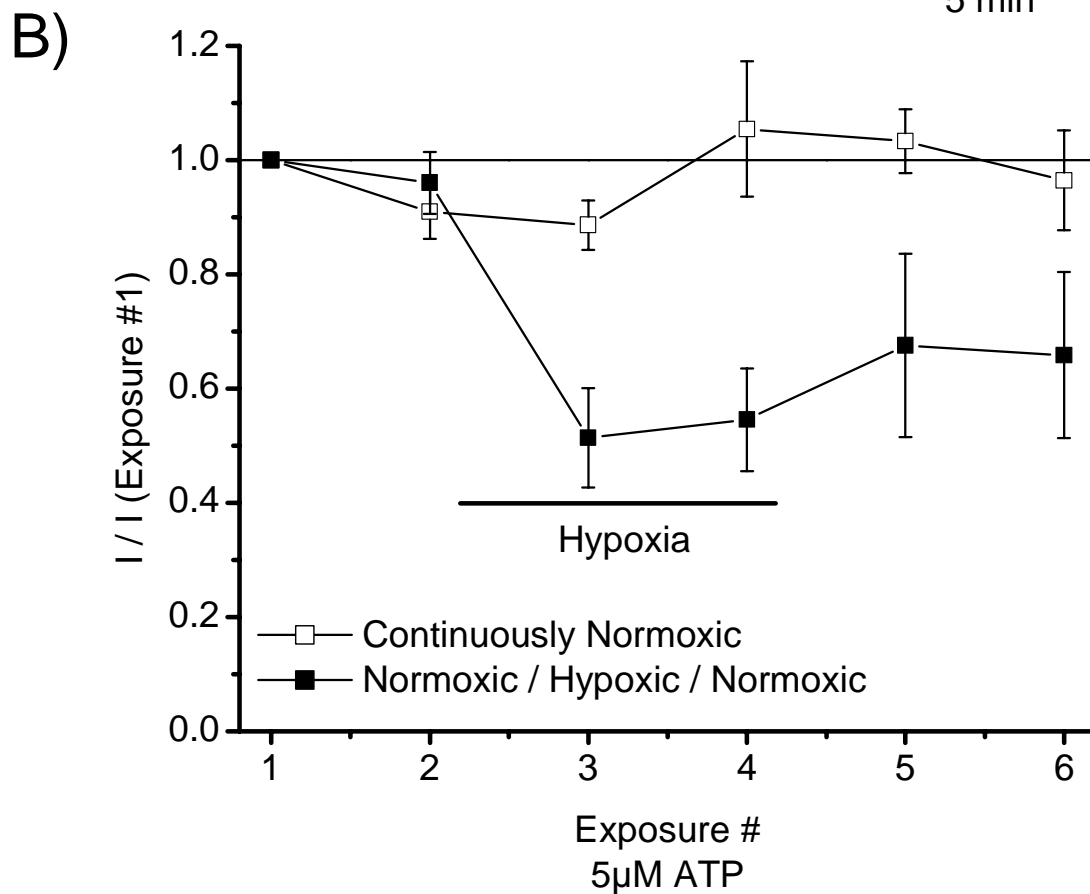
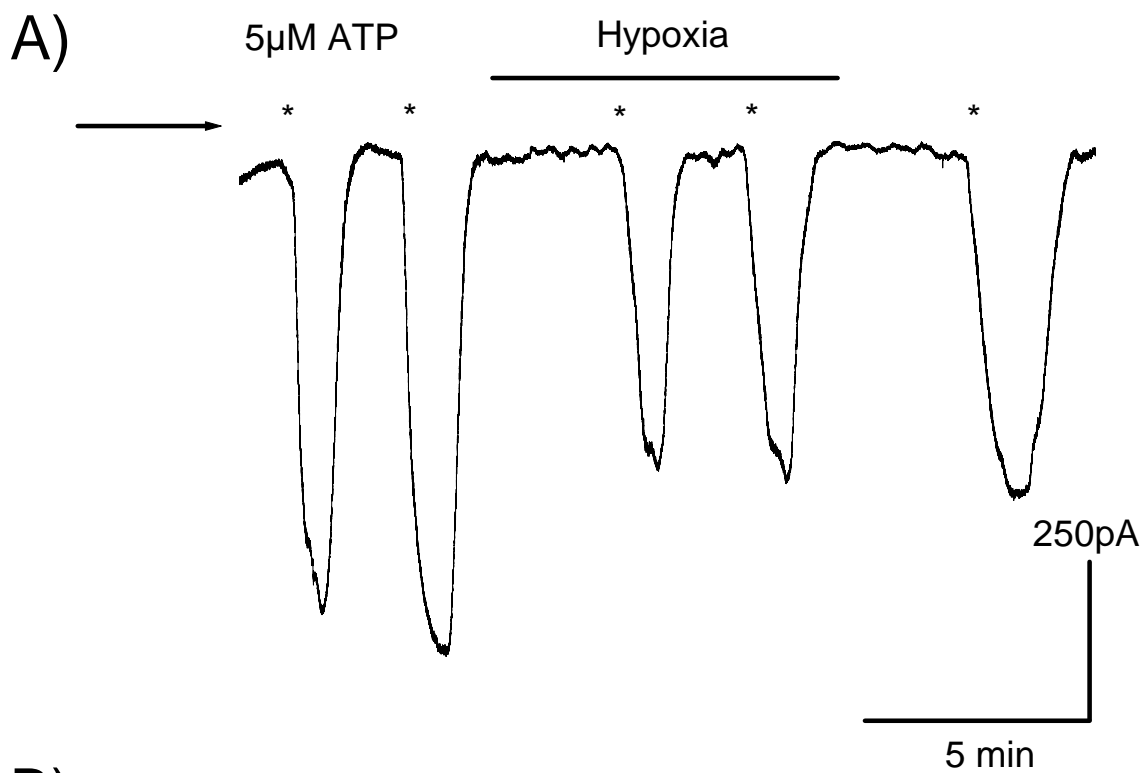


Figure 1

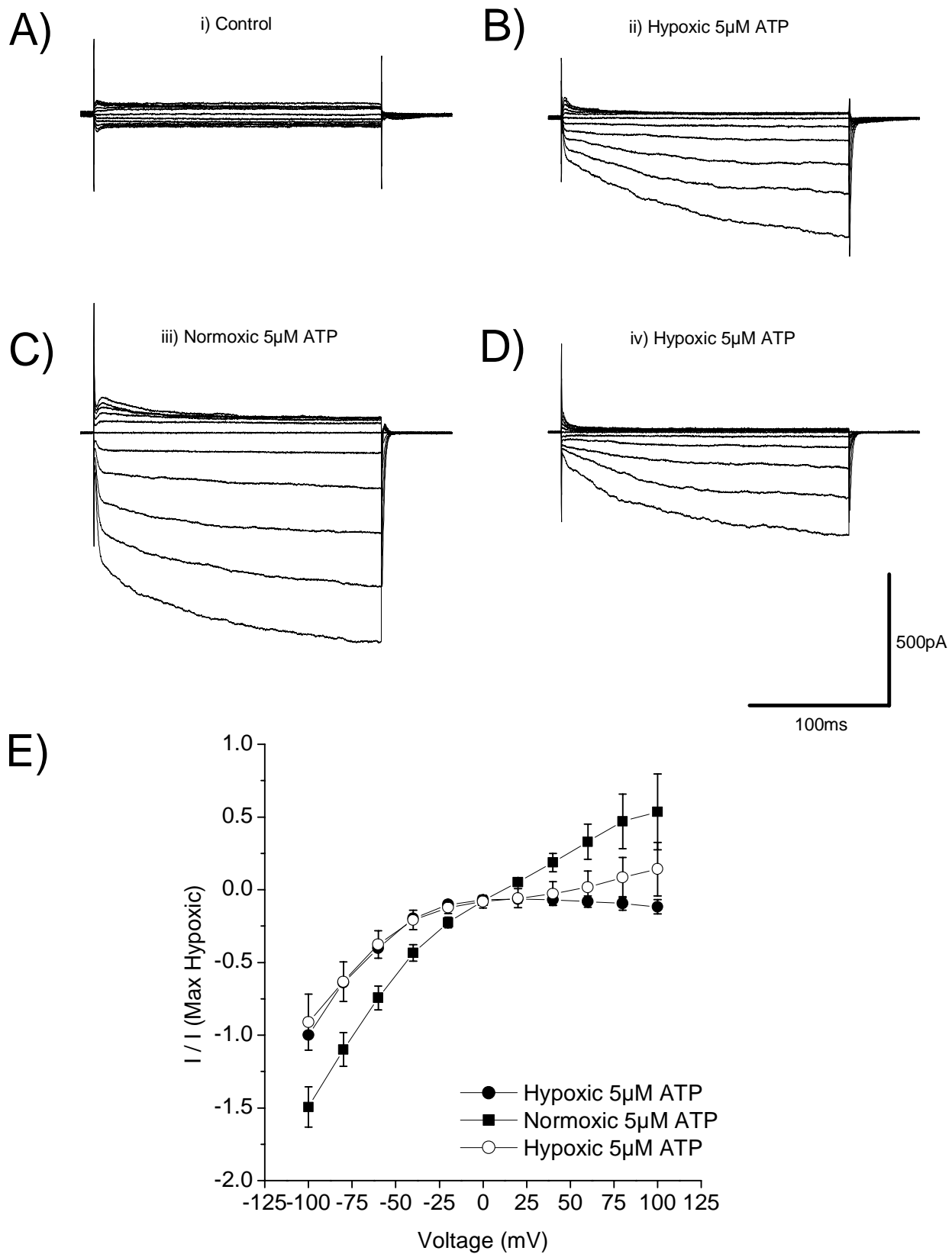


Figure 2

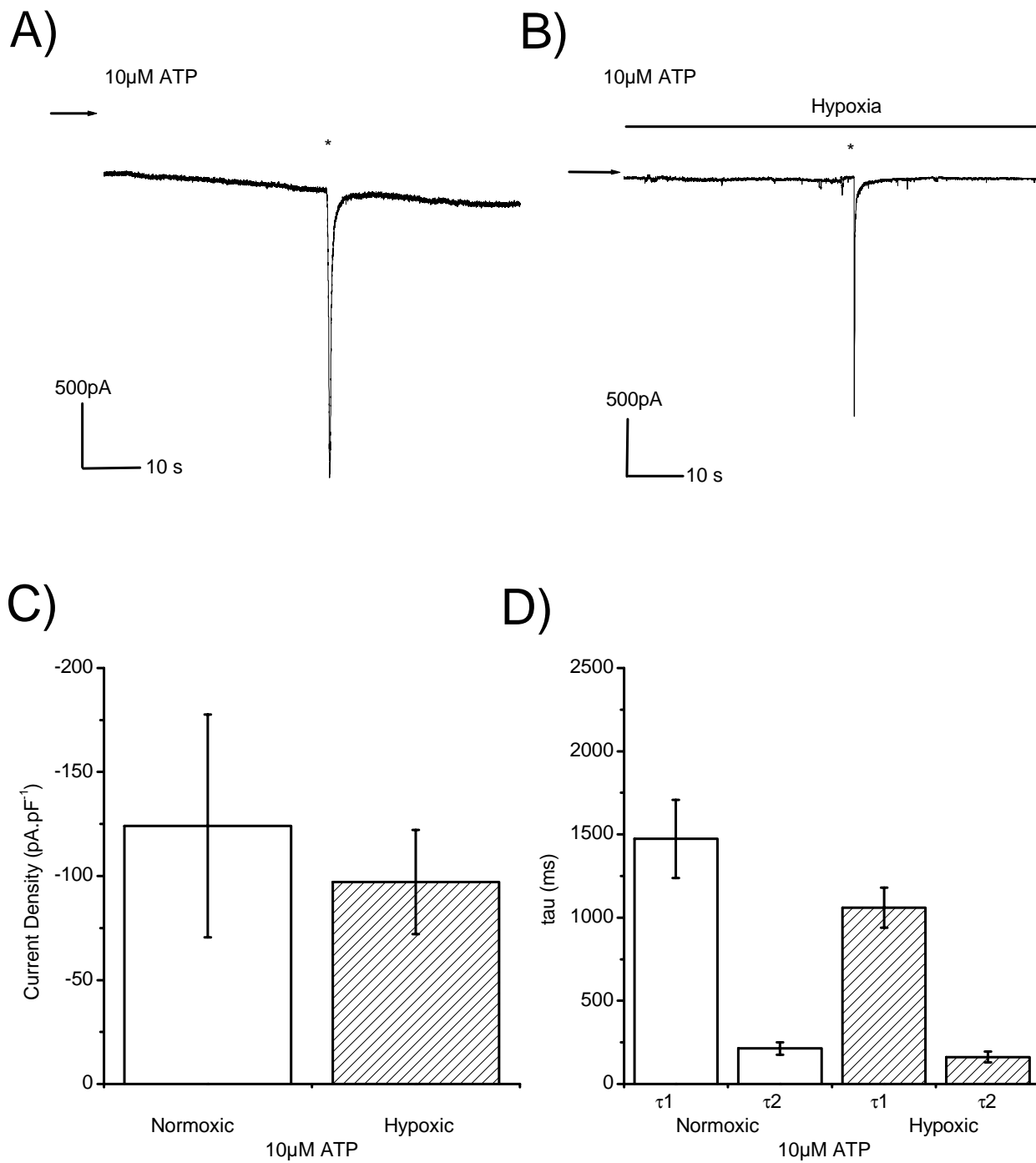


Figure 3

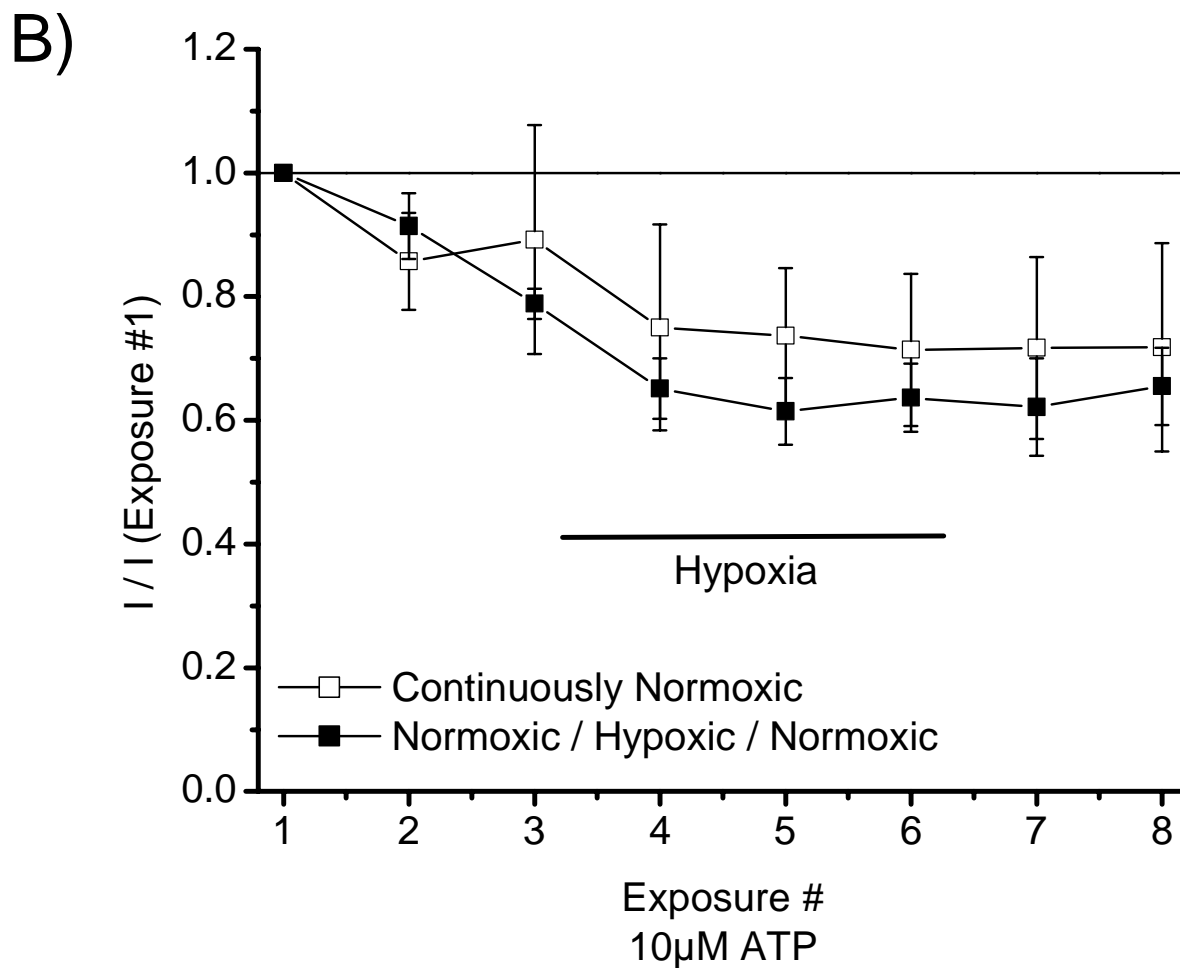
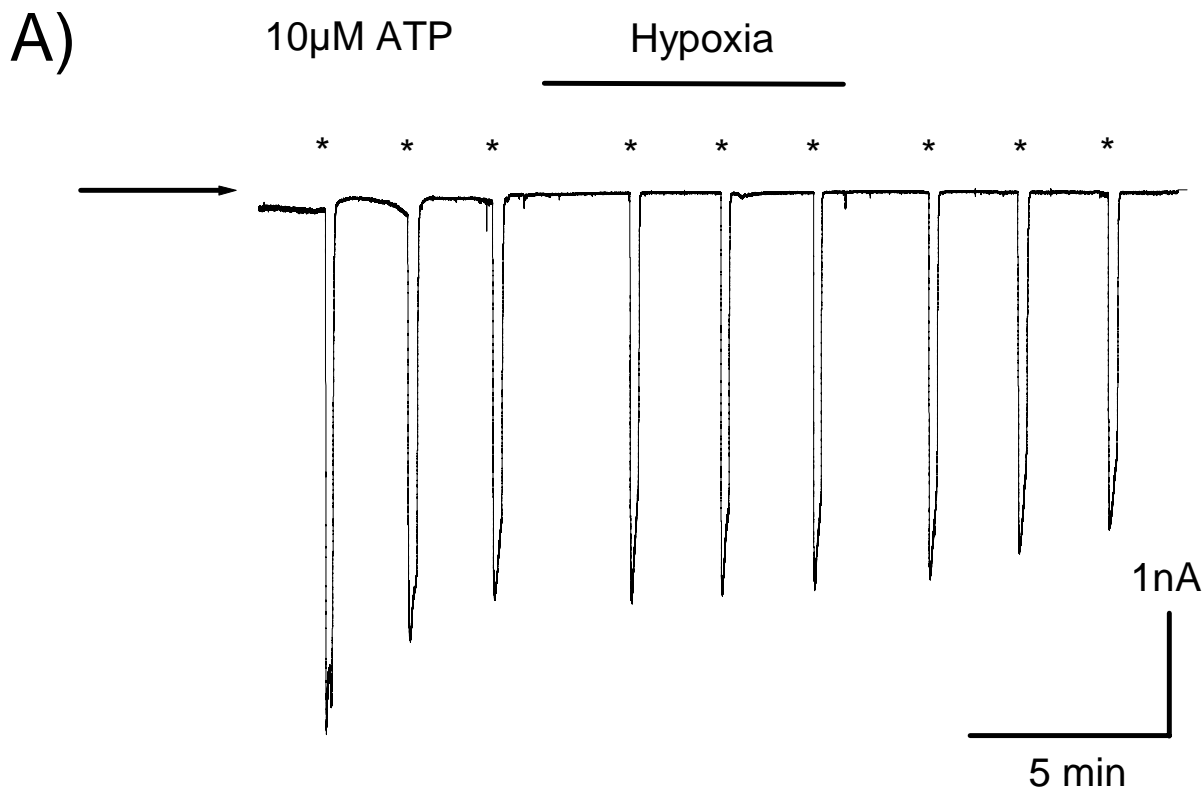


Figure 4

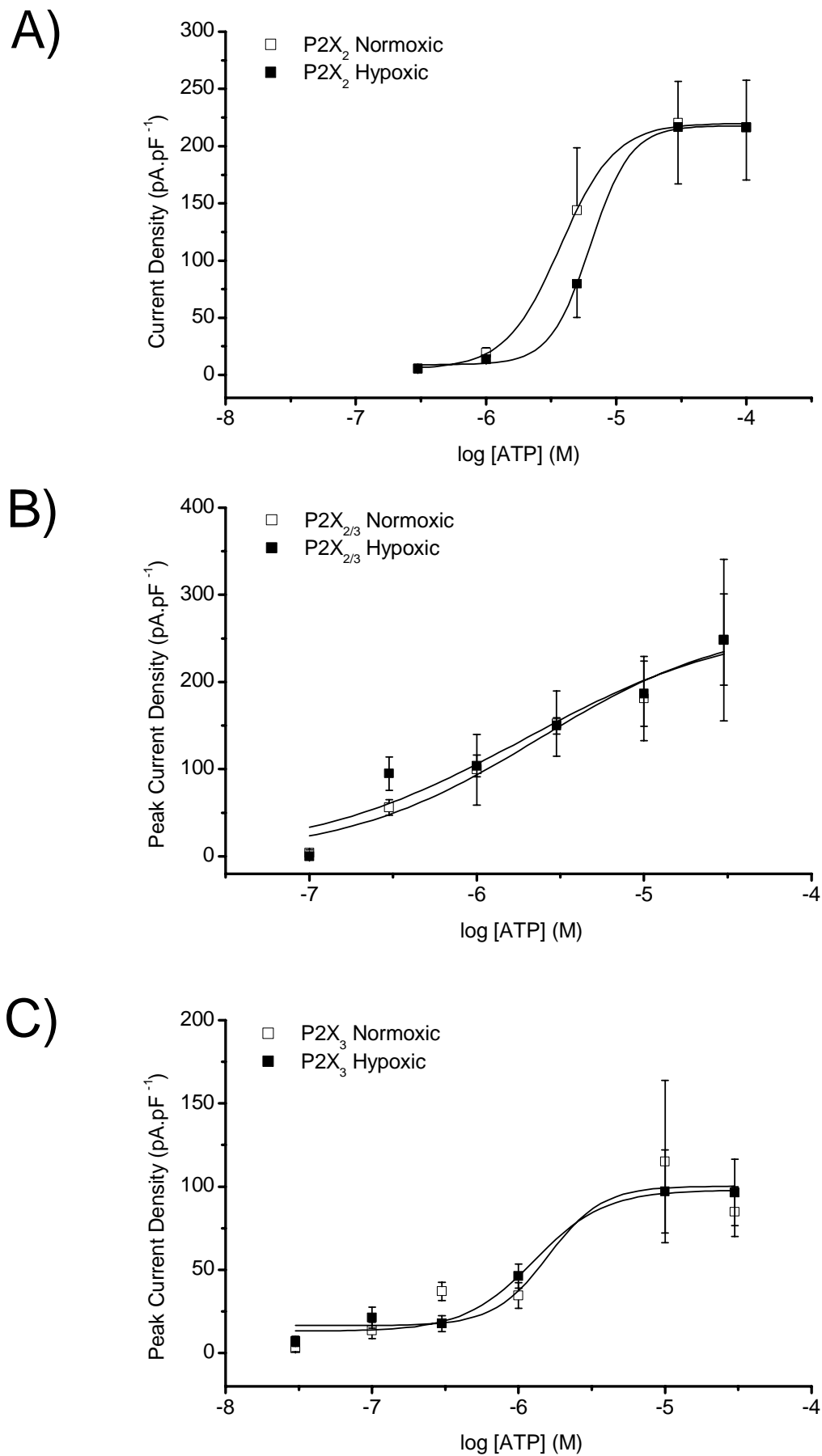
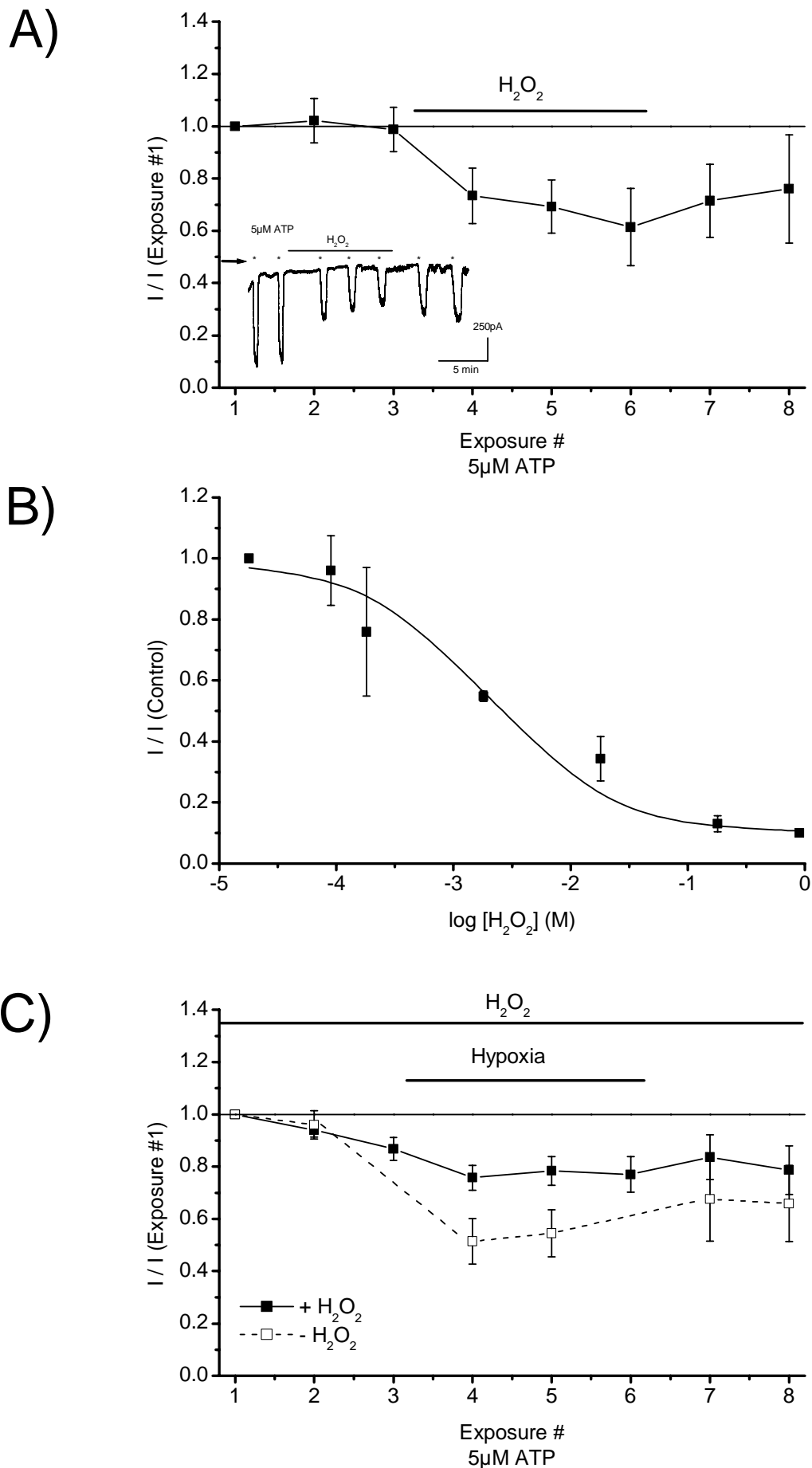


Figure 5



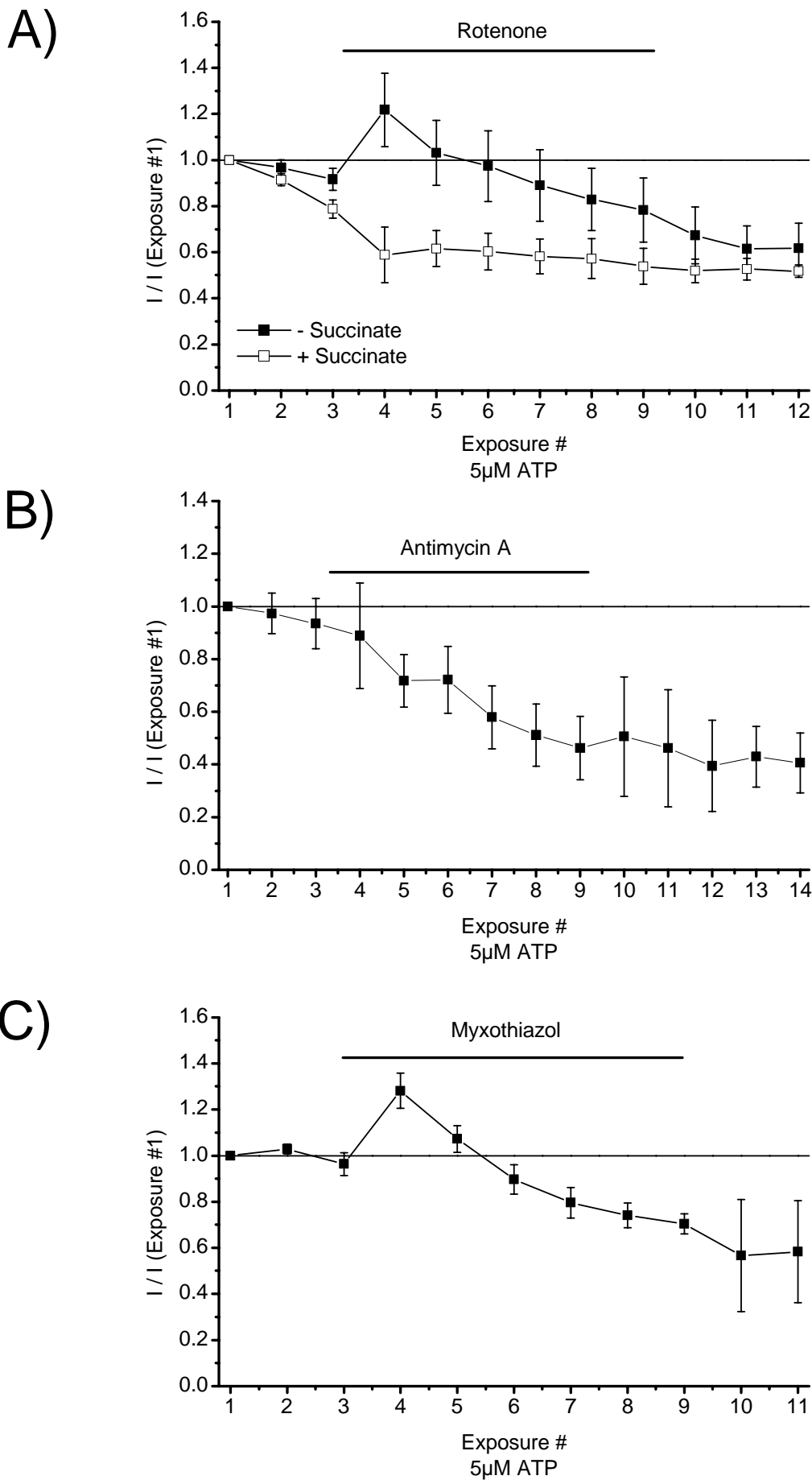


Figure 7

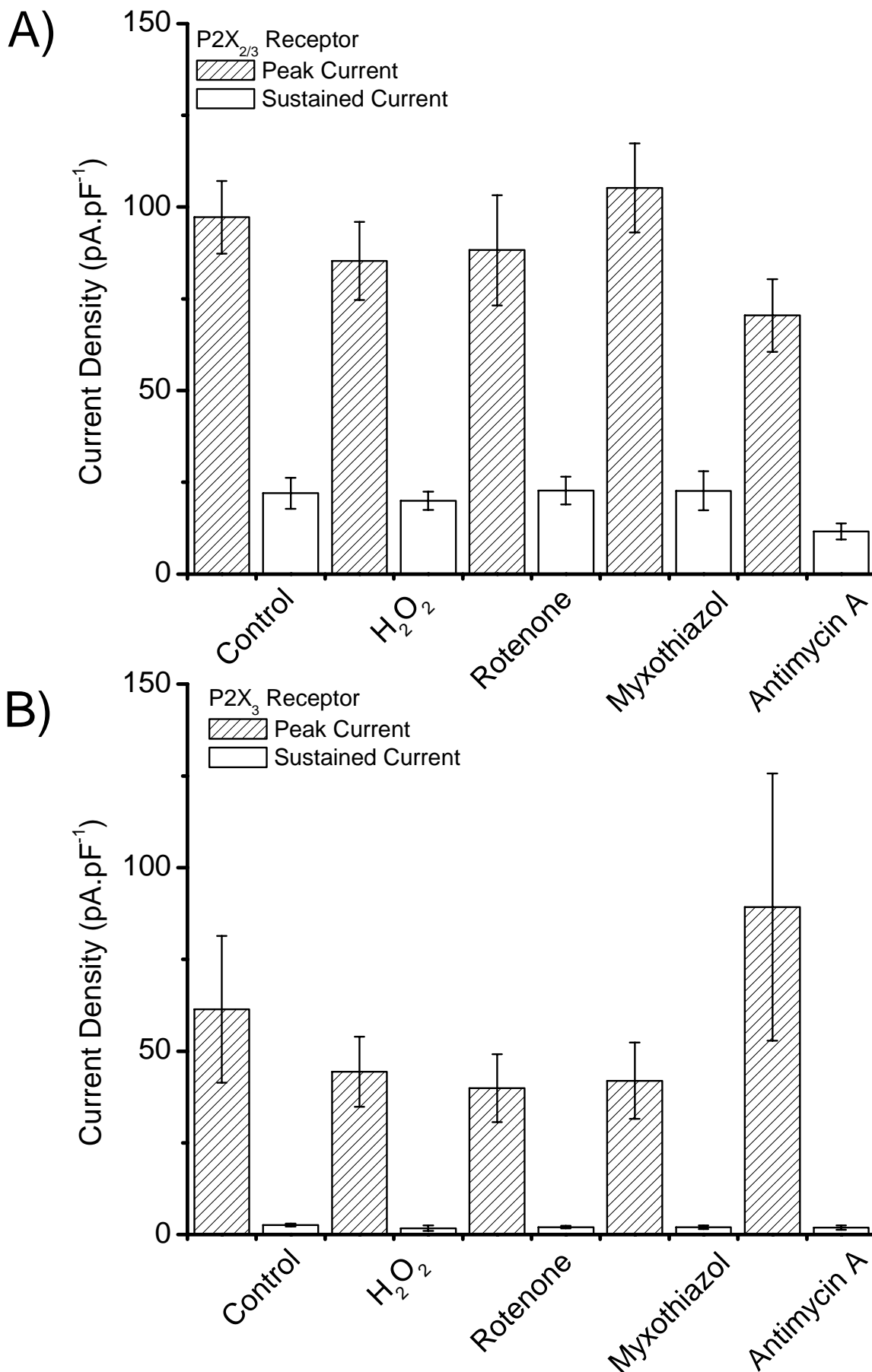


Figure 8