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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 $P2X_2$ 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 $P2X_2$ and $P2X_3$ receptors expressed in HEK293 cells. In cells expressing homomeric $P2X_2$ receptors, perfusion of 5μM ATP (EC_{25}) 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 $P2X_2$-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 (1.8mM) reversibly reduced homomeric $P2X_2$ whole-cell currents to 62% of control. Further, 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.

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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_{O2}) in the environment. The ability to detect changes in arterial P_{O2} 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_{O2} 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 purincoeptors 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,

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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 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 $P2X_2$ 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 HEK293 cells.

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MATERIALS AND METHODS

Expression Systems

Human embryonic kidney 293 (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;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% CO_2 / 95% air. Cells were passaged every 7 days using Ca^{2+}- and Mg^{2+}-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 37ºC 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 (H_2O_2), 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 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 (antimycin A, 50mg.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 4-6MΩ 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_{O2} was measured (at the cell) using a polarised (-800mV) carbon fibre electrode (Mojet *et al.*, 1997). For the experiments reported herein, the P_{O2} 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.

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Data Analysis

Data analyses were performed using the pClamp 8.0 suite of software (Axon Instruments). 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 (San Diego, California, U.S.A.).

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RESULTS

Hypoxia modulates homomeric $P2X_2$-mediated currents

In order 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 following 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 (100μM), the magnitude of the $P2X_2$-mediated current elicited in response to ATP decreased with repeated exposures due to desensitization of the $P2X_2$ receptor (data not shown). However, at a concentration of 5μM 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, in the following experiments that evaluated the effect of hypoxia on $P2X_2$-mediated currents, ATP concentrations of 5μM were used.

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 minutes, the magnitude of the $P2X_2$-mediated current activated in response to 5μM ATP was reduced compared to that under normoxic conditions. This reduction in the magnitude of the $P2X_2$-mediated current was partially relieved on returning to normoxic conditions. Figure 1B summarizes the response of $P2X_2$ expressing cells when exposed to repeated exposures of 5μM ATP under either continuously normoxic conditions or exposed to an acute hypoxic challenge. Under continuously

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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 ± 0.09 that of the first exposure, n=5). In contrast, exposure to an hypoxic challenge significantly reduced the magnitude of the $P2X_2$-mediated current activated in response to 5μM ATP (0.51 ± 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 on washout (0.68 ± 0.16 compared to control, n=5).

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 200ms voltage steps from –100mV to +100mV in 20mV increments from a holding potential of 0mV under (A) control conditions in the absence of ATP, and in the presence of 5μM 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 $P2X_2$-mediated current (figure 2B). Switching the perfusate from a hypoxic to a normoxic solution increased the magnitude of the $P2X_2$-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 5μM ATP at a potential of -100mV under hypoxic conditions. Under hypoxic conditions the $P2X_2$-mediated currents reversed at a potential of –8.3mV, whilst under normoxic conditions the currents were 38 ± 10% larger ($P < 0.05$, n=7) and reversed at a potential of -4.2mV.

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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 5μM ATP under normoxic, hypoxic and normoxic conditions. In this instance hypoxia reduced the magnitude of $P2X_2$-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 $P2X_3$ 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 figures 3 and 4. As shown in figure 3A, exposing cells expressing homomeric $P2X_3$ receptors to 10μM 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^{-1}, n=9). The inactivation of the $P2X_3$-mediated current under normoxic conditions was best fit with two time constants (τ_1 = 1473 ± 234 and τ_2 = 214 ± 37.5 ms, n=9, figure 3D). As 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

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bathing solution for 2 minutes before exposing the cells to ATP. Under hypoxic conditions 10μM ATP activated a $P2X_3$-mediated current whose magnitude was comparable to that activated under normoxic conditions (mean peak hypoxic $P2X_3$-mediated current -110.4 ± 24.5 pA pF^{-1}, *n*=7, figure 3C). The inactivation of the $P2X_3$-mediated current under hypoxic conditions was best fit with two time constants (τ_1 = 1059 ± 119 and τ_2 = 163 ± 31.7 ms, figure 3D).

Similar results were obtained when the cells were exposed to α, β-MeATP, a $P2X_1$ and $P2X_3$-selective agonist that differentiates between $P2X_2$ and $P2X_3$ receptor subunits (data not shown). Exposure to 10μM 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^{-1}, n=8; hypoxia: -80.6 ± 21.7 pA pF^{-1}, n=8). The inactivation of the $P2X_3$-mediated currents under normoxic and hypoxic conditions was best fit with two time constants (normoxia: τ_1 = 1790 ± 322 and τ_2 = 390 ± 138 ms, hypoxia: τ_1 = 912 ± 109 ms and τ_2 = 172 ± 18.6 ms).

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

The observation that peak activation of homomeric $P2X_2$ receptors were sensitive to hypoxia whereas that of homomeric $P2X_3$ 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

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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 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 Ca^{2+} and Mg^{2+}.

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 $P2X_2$-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 3.73μM. In contrast, under hypoxic conditions the concentration response curve was shifted to the right

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

It was possible that the concentration of ATP (10μM, 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

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$P2X_2$-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 $P2X_2$-mediated current was partially relieved on 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 (figure 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 6 cells. In the presence of 1.8mM H_2O_2 and hypoxia the magnitude of the $P2X_2$-mediated current activated in response to 5μM 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 1.8mM H_2O_2 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, H_2O_2 itself mimics the effect of hypoxia on $P2X_2$-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

In order 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,

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antimycin A and myxothiazol and each block at a different site of the ETC. Figure 7A shows that perfusion of cells expressing $P2X_2$ receptors with rotenone (100nM), 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 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 $P2X_2$-mediated currents in response to subsequent exposures to ATP in the presence of rotenone declined over time (6^{th} 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 $P2X_2$-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 $P2X_2$-mediated currents in response to the first exposure to 5μM ATP. Rather, $P2X_2$-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 $P2X_2$-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 $P2X_2$-mediated currents, we examined the effect of two inhibitors of complex III in the mitochondrial ETC, antimycin A and myxothiazol. These inhibitors act at

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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 $P2X_2$-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 $P2X_2$-mediated currents, similar to that observed with rotenone. The magnitude of $P2X_2$-mediated current in response to the first exposure to 5μM 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 $P2X_2$-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 $P2X_2$ 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 0.5μM ATP (which equates to the EC_{25} value) in the presence or absence of the ROS mediators. The magnitudes of

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the peak and sustained $P2X_{2/3}$-mediated currents in the presence of 1.8mM H_2O_2 (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 $P2X_3$ receptors evoked in response to the first exposure to 0.5μM 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^{-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 $P2X_2$ receptors.

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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 an 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 modulation of a ligand-gated ion channel. Further, the selective modulation of the $P2X_2$ receptor subunit is consistent with the recent report by Rong *et al.* 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 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 $P2X_2$ and $P2X_3$ 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 $P2X_2$ 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 an 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;Hur *et al.*, 2001;Taylor and Peers, 1999). 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.

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 $P2X_2$ receptor is mediated via an increase in the production of ROS since 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 (1.8mM) that was used in this study appears 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

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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 ≥100μM. Therefore, the perceived intracellular concentration of H_2O_2 would be in the μM range, which is within physiological range of H_2O_2 concentrations reported in the literature (Halliwell *et al.*, 2000).

Further, we 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. Further, the transient increase in the $P2X_2$ 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 $P2X_2$-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 and therefore some additional mechanism must also contribute to the modulation of the $P2X_2$ receptor in response to an hypoxic challenge. Potential additional mediators of the hypoxic modulation of $P2X_2$ 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 it 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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FOOTNOTES

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FIGURE LEGENDS

Figure 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 -70mV 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 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 $P2X_2$-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 $P2X_2$ receptors. Cells expressing the $P2X_2$ receptor were held at a potential of 0mV and 200ms voltage steps were applied from -100 to +100mV in 20mV increments. A) - D) Illustrative $P2X_2$-mediated currents evoked in response to the voltage step protocol. Cells were initially exposed to 5μM 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 6 cells. Currents are normalized to the current recorded at a potential of –100mV under hypoxic conditions.

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Figure 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 –70mV in the presence of external Ca^{2+} and Mg^{2+} 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 $P2X_3$-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 Ca^{2+} and Mg^{2+} 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 $P2X_2$, 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 -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

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concentration of ATP under either normoxic or hypoxic conditions. A) Exposure to an 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.

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

Figure 7: Inhibitors of the Mitochondrial ETC Modulate $P2X_2$-mediated Currents. HEK293 cells expressing homomeric $P2X_2$ receptors were held at a potential of -70mV in the presence of external Ca^{2+} and Mg^{2+} 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 $P2X_2$-mediated currents evoked in response to 5μM ATP (-■-). The presence of 5mM succinate, a complex II substrate, abolished the increase in the $P2X_2$-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 $P2X_2$-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 $P2X_2$-mediated currents evoked in response to 5μM ATP.

Figure 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 -70mV in the presence of external Ca^{2+} and Mg^{2+} and perfused with 1.8mM H_2O_2, 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 $P2X_3$-mediated currents evoked by the first exposure to 0.5μM ATP in the absence or presence of ROS generators.

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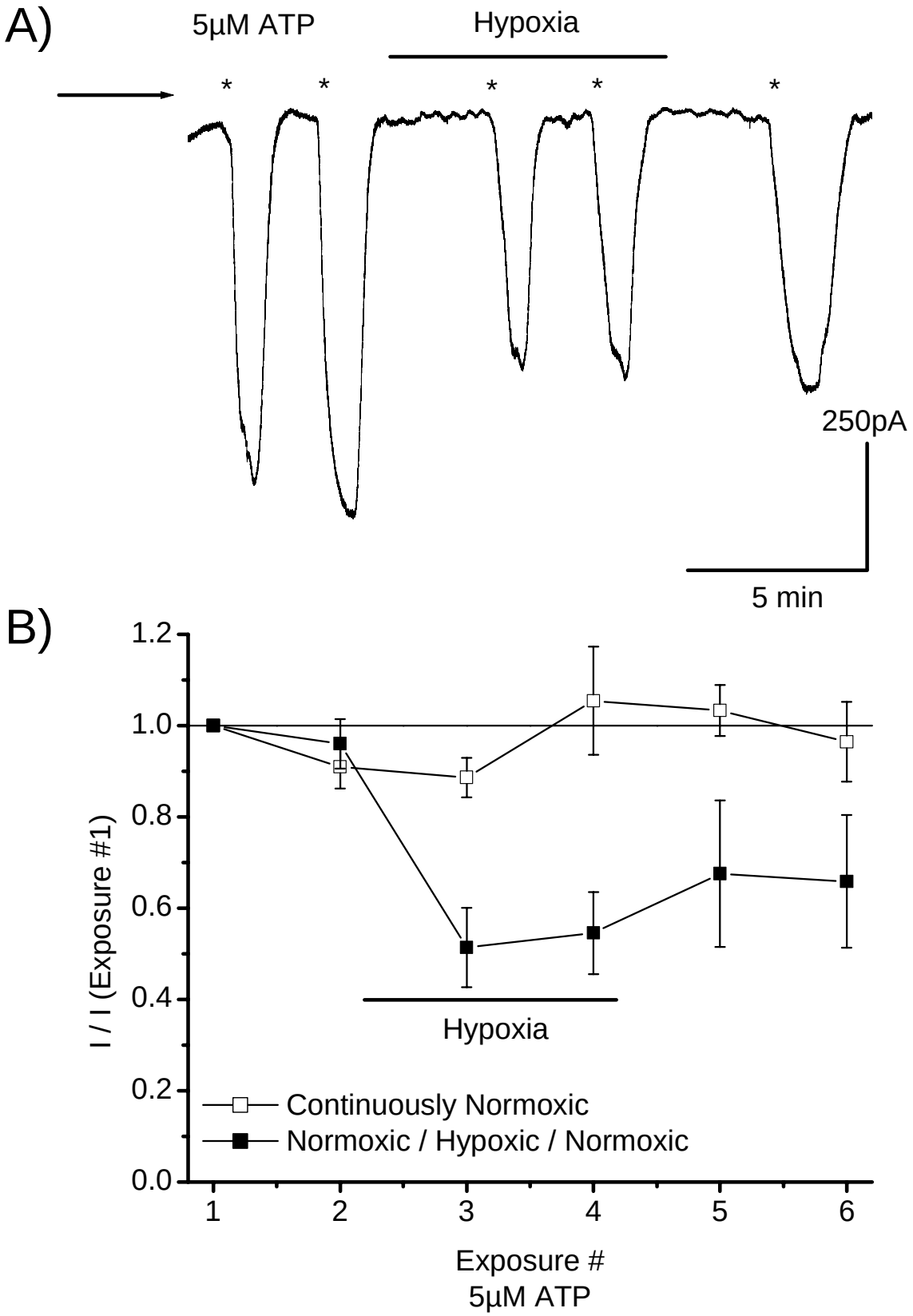

Figure 1

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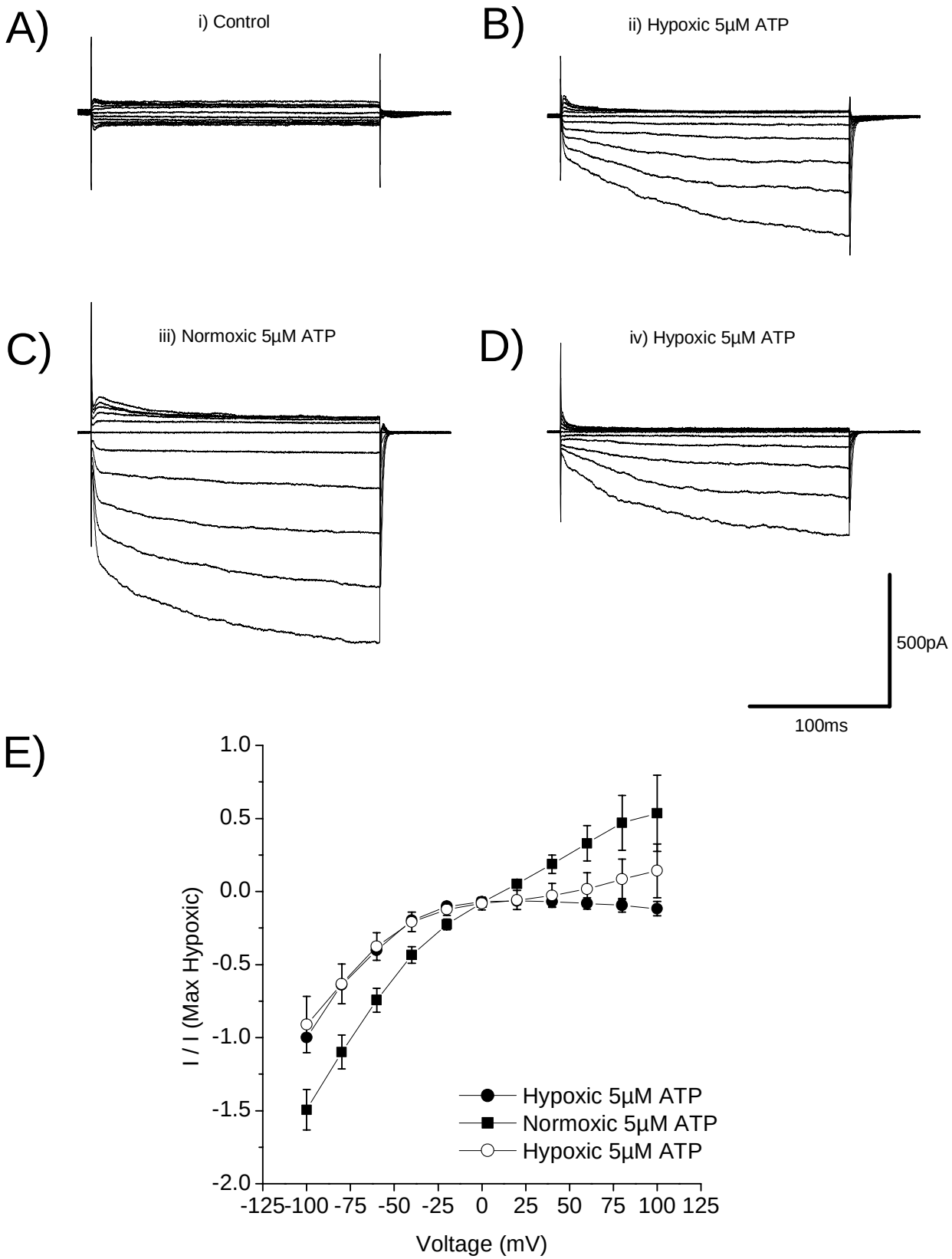

Figure 2

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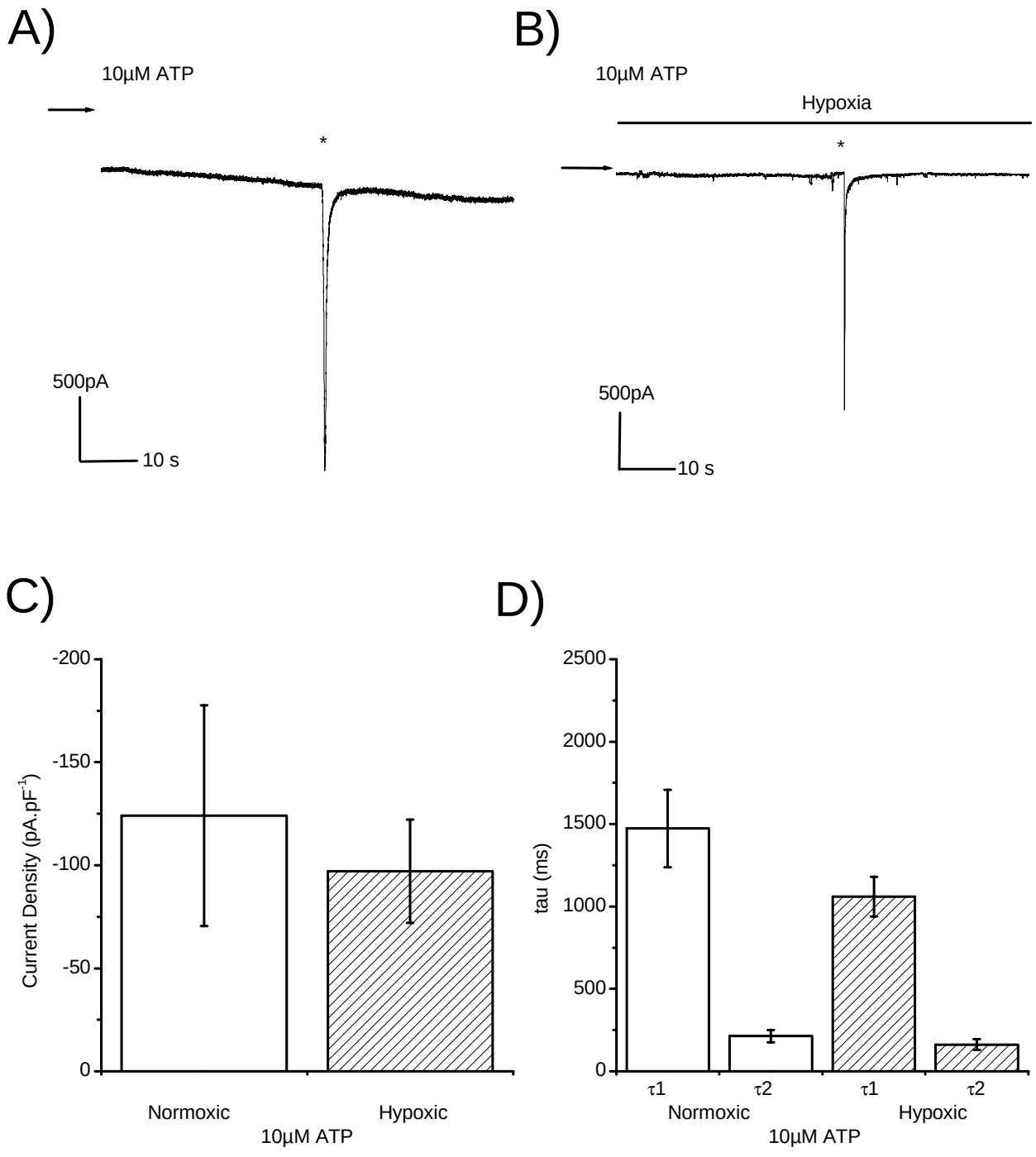

Figure 3

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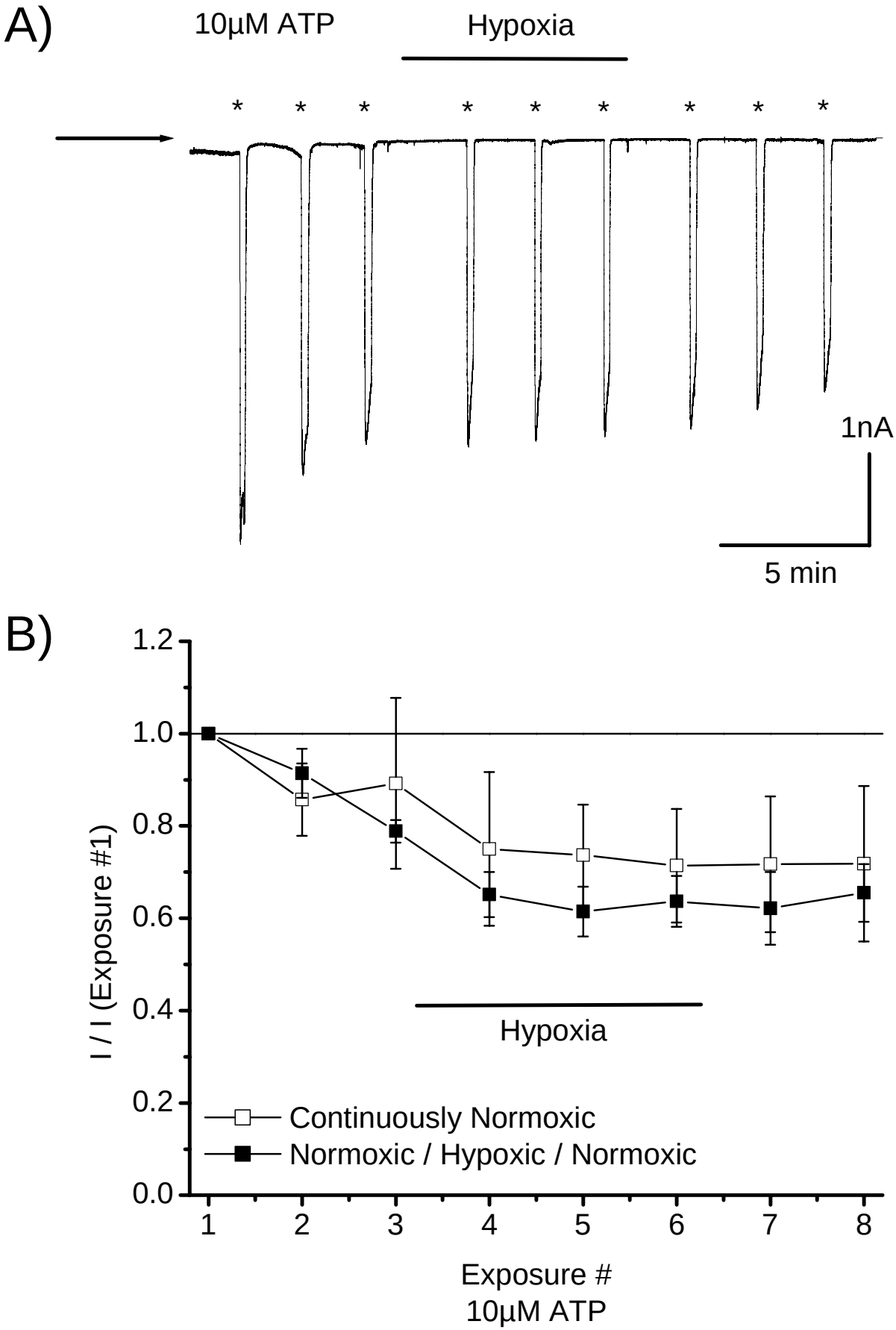

Figure 4

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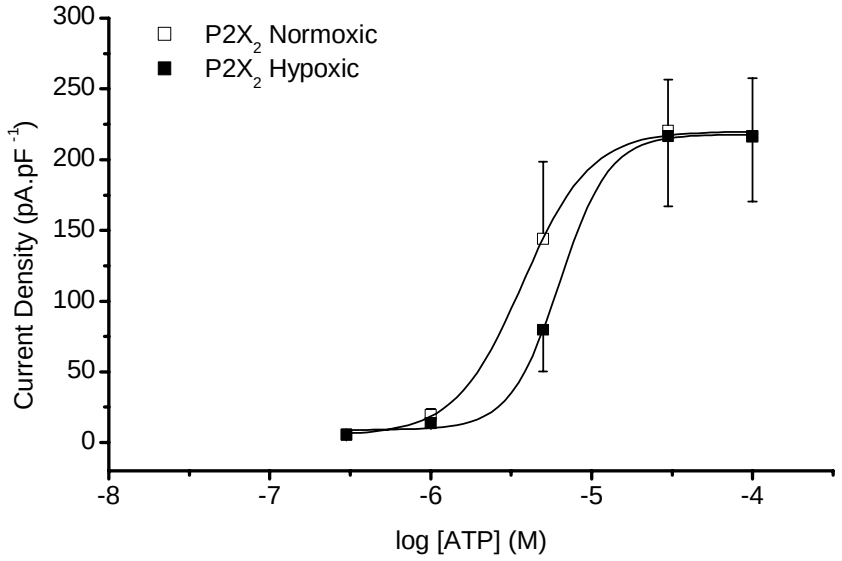

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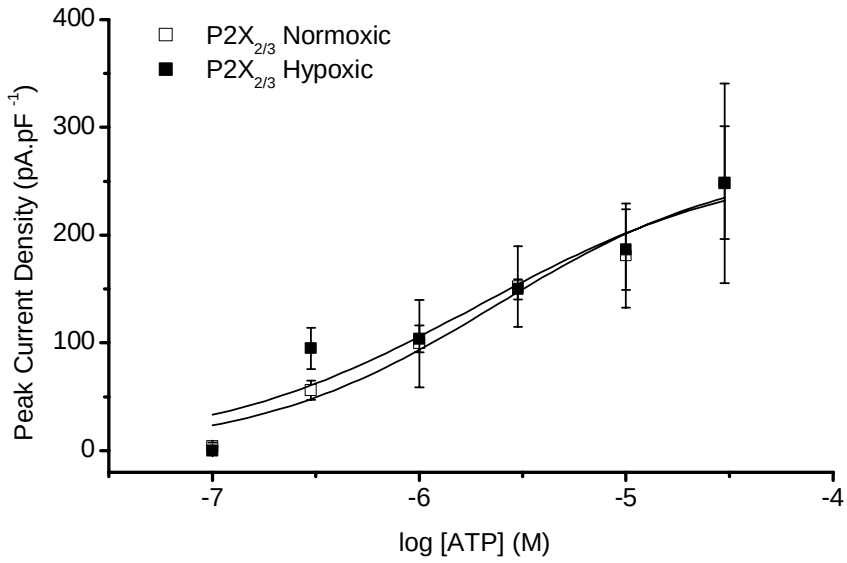

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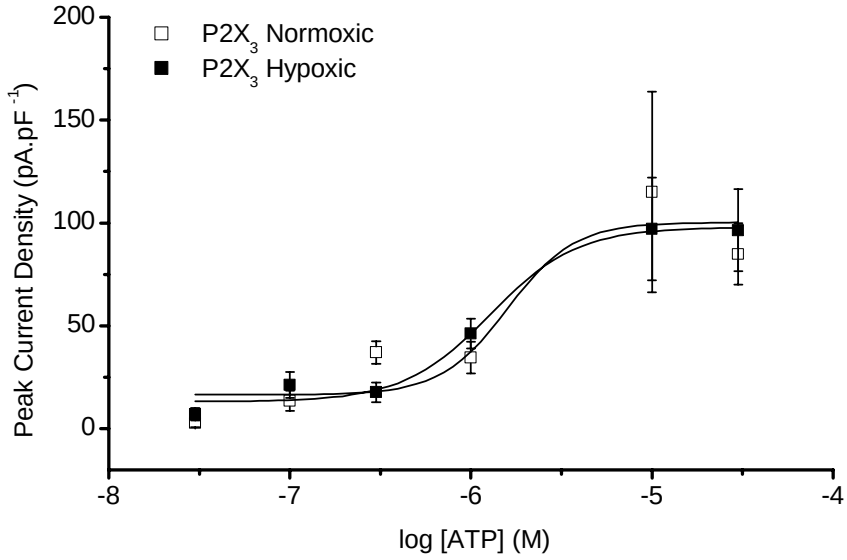

Figure 5

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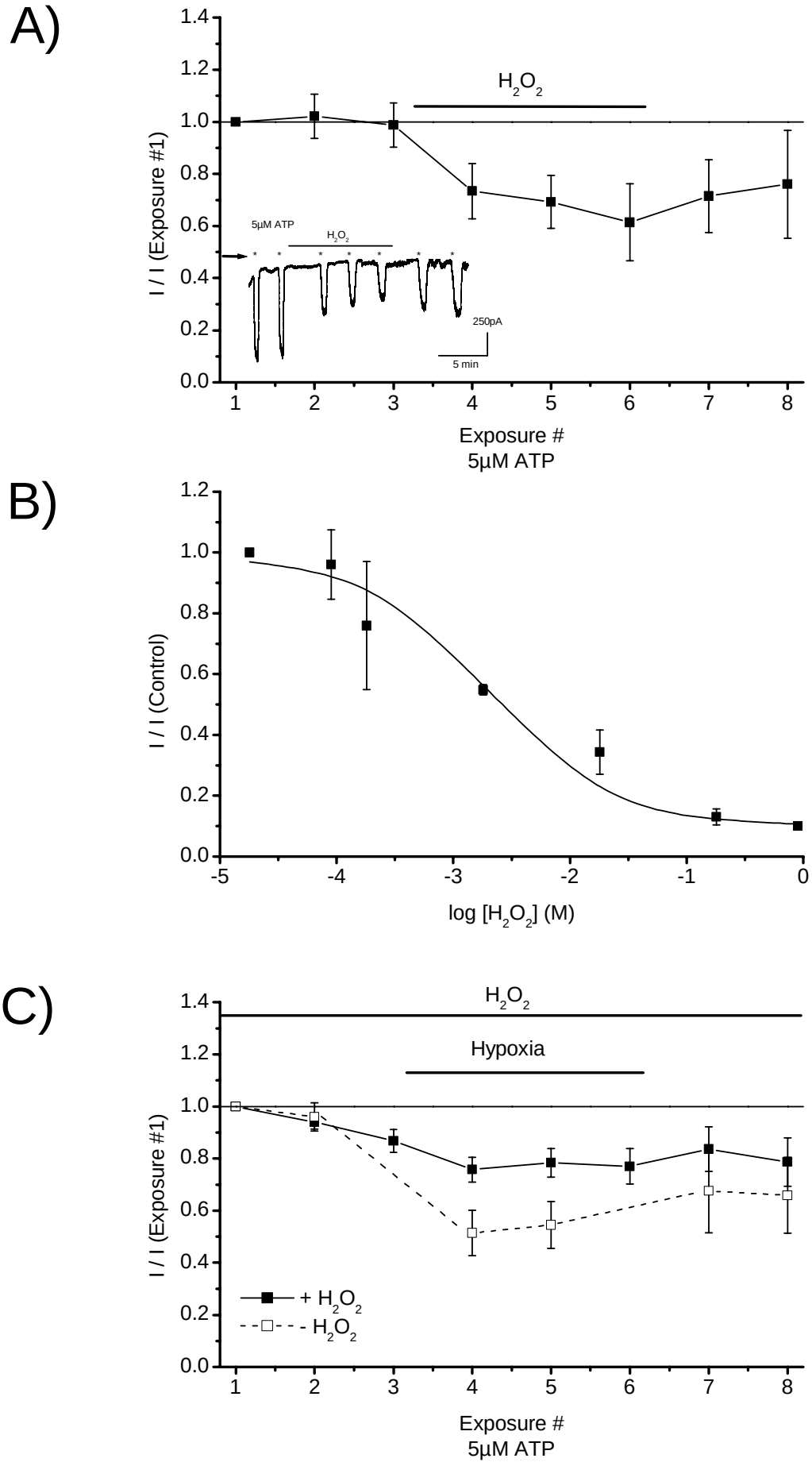

Figure 6

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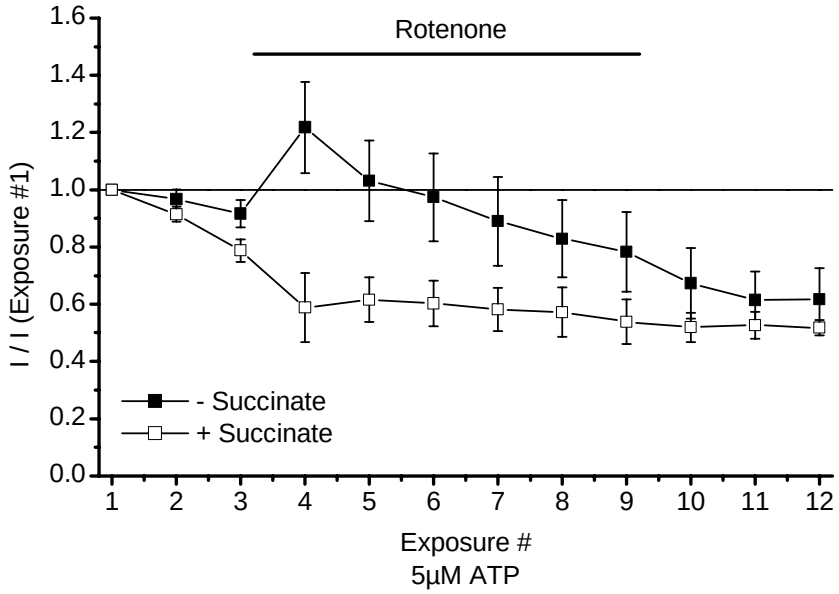

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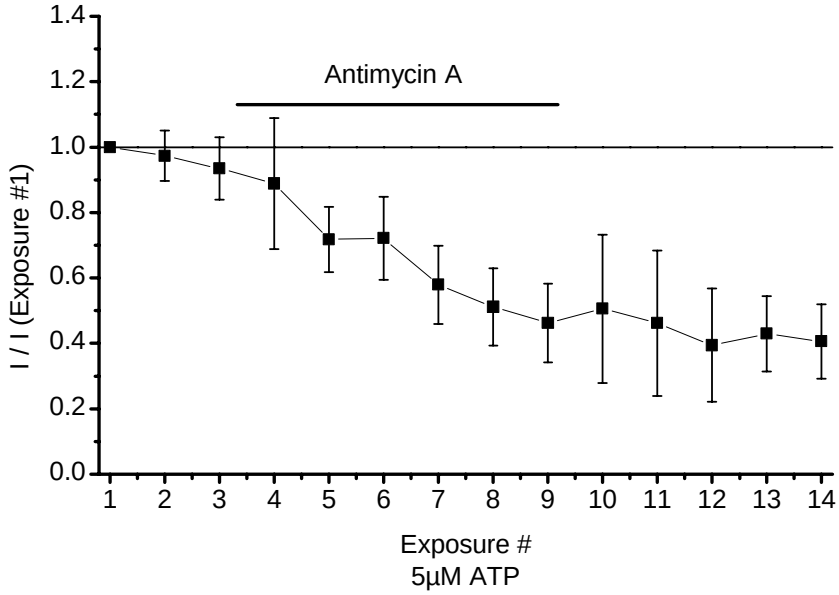

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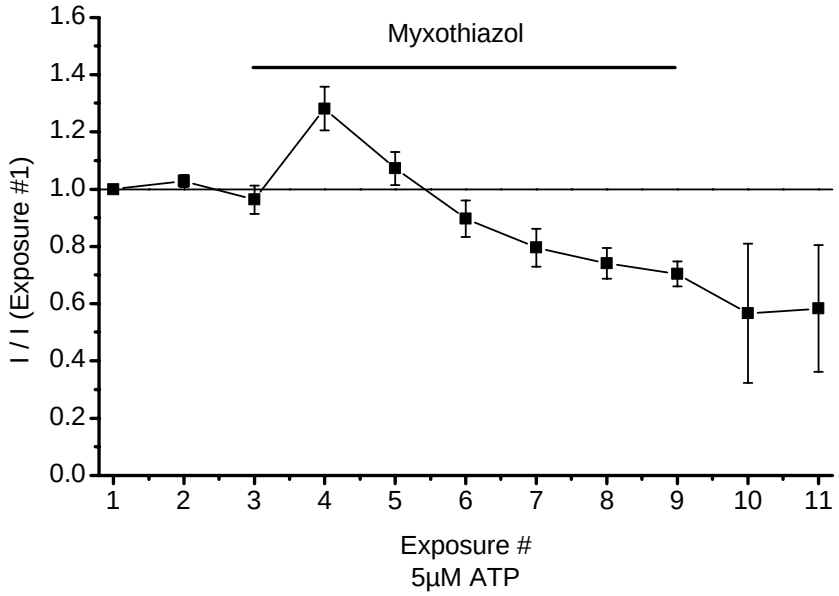

Figure 7

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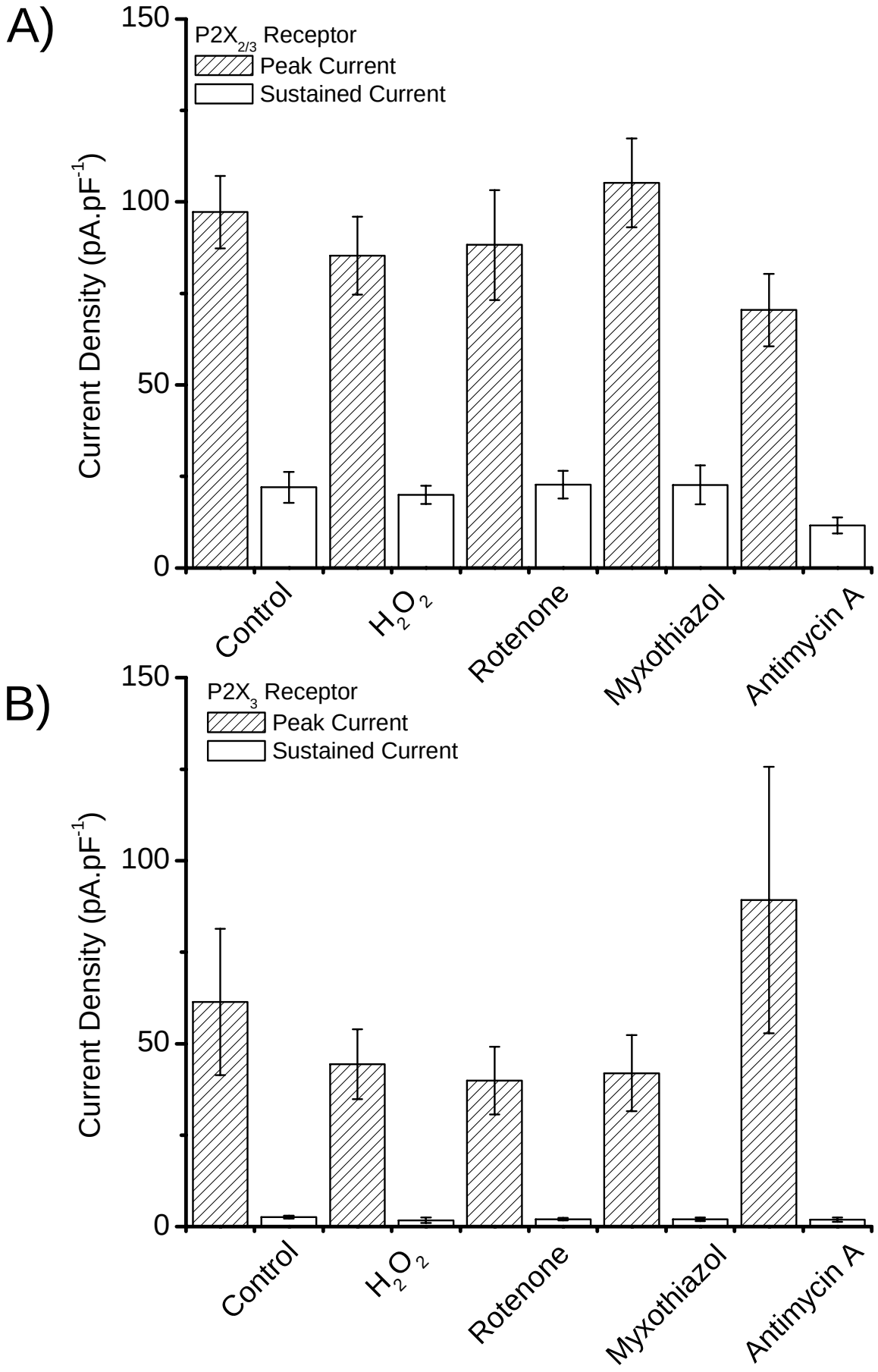

Figure 8