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A ROTENONE-SENSITIVE SITE AND H_2O_2 ARE KEY COMPONENTS OF HYPOXIA-SENSING IN NEONATAL RAT ADRENOMEDULLARY CHROMAFFIN CELLS

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Abstract—In the perinatal period, adrenomedullary chromaffin cells (AMC) directly sense PO_2 and secrete catecholamines during hypoxic stress, and this response is lost in juvenile (~2 week-old) chromaffin cells following postnatal innervation. Here we tested the hypothesis that a rotenone-sensitive O_2-sensor and ROS are involved in the hypoxic response of AMC cultured from neonatal and juvenile rats. In whole-cell recordings, hypoxia (PO_2=5–15 mm Hg) inhibited outward current in neonatal AMC; this response was reversed by exogenous H_2O_2 and mimicked and occluded by intracellular catalase (1000 units/ml), as well as the antioxidants, *N*-acetyl-L-cysteine (NAC; 50 μM) and Trolox (200 μM). Acute hypoxia decreased ROS levels and stimulated ATP secretion in these cells, as measured by luminol and luciferin–luciferase chemiluminescence, respectively. Of several mitochondrial electron transport chain (ETC) inhibitors tested, only rotenone, a complex I blocker, mimicked and occluded the effects of hypoxia on outward current, cellular ROS, and ATP secretion. Succinate donors, which act as complex II substrates, reversed the effects of hypoxia and rotenone in neonatal AMC. In contrast, in hypoxia-insensitive juvenile AMC, neither NAC nor rotenone stimulated ATP secretion though they both caused a decrease in ROS levels. We propose that O_2-sensing by neonatal AMC is mediated by decreased ROS generation via a rotenone-sensitive site that is coupled to outward current inhibition and secretion. Interestingly, juvenile AMC display at least two modifications, i.e. an uncoupling of the O_2-sensor from ROS regulation, and an apparent insensitivity of outward current to decreased ROS.

Key words: oxygen-sensing, mitochondria, K^+ channel, secretion, luminescence, electron transport chain.

Hypoxia-induced catecholamine (CA) secretion by neonatal adrenomedullary chromaffin cells (AMC) functions critically in supporting the transition from fetal to extrauterine life via maintenance of cardiac conduction, initiation of surfactant secretion, and stimulation of alveolar fluid reabsorption (Slotkin and Seidler, 1988). Rodent AMC express a developmentally-regulated O_2-sensing mechanism that mediates CA release from neonatal (postnatal day (P)1) but not juvenile (P14–21) cells (Seidler and Slotkin, 1985; Thompson et al., 1997, 2002; Thompson and Nurse, 1998; Mojet et al., 1997; Rico et al., 2005). In cultured neonatal AMC, hypoxia modulates at least four K^+ conductances, resulting in enhanced membrane depolarization, broadening of spontaneous action potentials, voltage-gated Ca^{2+} influx through L-type Ca^{2+} channels and CA secretion (Thompson et al., 1997, 2002; Thompson and Nurse, 1998; Mojet et al., 1997; Keating et al., 2001, 2005). Whereas the ion channel complement appears to be retained as AMC undergo postnatal maturation, hypoxic sensitivity is lost in juvenile cells in concert with the acquisition of sympathetic innervation (Seidler and Slotkin, 1985; Thompson et al., 1997, 2002; see also Rico et al., 2005).

In recent years, studies on a variety of O_2-sensitive cells have focused on the site of O_2-sensing and the signaling pathway that couples the O_2-sensor to modulation of ion channels in the plasma membrane. Several controversial models for hypoxic signaling have been proposed and include a *decrease* in reactive oxygen species (ROS) generation, via either a plasma membrane phagocytic-like NADPH oxidase (e.g. airway neuroepithelial bodies) or the mitochondrial electron transport chain (ETC) (Fu et al., 2000; Michelakis et al., 2002). We reported, however, that the same NADPH oxidase that functioned as the key O_2-sensor in airway neuroepithelial bodies (Fu et al., 2000), was unlikely to do so in neonatal rodent adrenal medulla based on the observation that hypoxic responses in AMC from transgenic mice, deficient in the $gp91^{phox}$ subunit of NADPH oxidase, were indistinguishable from wild-type controls (Thompson et al., 2002). On the other hand, an *increase* in mitochondrial-derived ROS has been proposed as the intermediary signal during hypoxia in several other preparations (Michelakis et al., 2002; Chandel et al., 1998; Waypa et al., 2001) and though controversial, these data raise the possibility that the direction of ROS changes during acute hypoxia may be tissue specific (Michelakis et al., 2002). Additionally, other O_2-sensing models which converge on K^+ channel inhibition and secretion have been proposed for the specialized receptor cells of the mammalian carotid body (Lopez-Barneo et al., 2001). These include a membrane delimited mechanism with the putative O_2-sensor, hemeoxygenase-2 linked to large conductance Ca^{2+}-dependent K (BK) channels (Wil-

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Abbreviations: AMC, adrenomedullary chromaffin cell; ANOVA, analysis of variance; BK channel, large conductance Ca^{2+}-dependent K^+ channel; CA, catecholamine; ETC, electron transport chain; k_d, apparent binding constant; NAC, *N*-acetyl-L-cysteine; P, postnatal day; RLU, relative light unit; ROS, reactive oxygen species; SEM, standard error of the mean; TTX, tetrodotoxin.

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liams et al., 2004), mitochondrial heme protein(s) linked to alterations in ATP/ADP ratio (Wyatt and Buckler, 2004), and an extra-mitochondrial rotenone-sensitive protein acting as the O_2-sensor (Ortega-Saenz et al., 2003).

In the present study, we tested the hypothesis that a rotenone-sensitive site linked to changes in mitochondrial-derived ROS mediates hypoxic sensitivity in neonatal rat AMC. We provide evidence that hypoxia acts via a rotenone-sensitive mitochondrial site, closely linked to decreased ROS generation which in turn acts as a second messenger signal leading to outward current inhibition and secretion. Furthermore, we propose that the loss of O_2-sensing by juvenile AMC likely involves at least two modifications, one at the level of the O_2 sensor, and the other at the level of K^+ channel response to changes in ROS.

EXPERIMENTAL PROCEDURES

Cell culture and electrophysiology

Primary cultures enriched in adrenal chromaffin cells were prepared from neonatal (P0–P2 day old) and juvenile (P14–P21 day old) Wistar rats (Charles River, Saint-Constant, QC, Canada; Harlan, Madison, WI, USA) after stunning and decapitation of the animal as previously described (Thompson et al., 1997). All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC); accordingly, all efforts were taken to reduce the number of animals used and to prevent their suffering. All chemicals were obtained from Sigma (Oakville, ON, Canada) and solutions were made fresh on the day of recording. Rotenone and myxothiazol were diluted into standard recording saline from 10 mM stock solutions in DMSO. Application of vehicle (DMSO) at the concentrations used in these experiments (0.1–1%) had no effect on the cell responses under study. For example, the outward current density at +30 mV was 70.3±2.4 pA/pF without DMSO and 69.7±2.5 pA/pF (n=5) in 0.5% DMSO added to the bathing solution (see below); these values were not significantly different (P>0.05).

In the majority of experiments voltage and current clamp data were obtained using the nystatin perforated-patch configuration of the whole-cell patch clamp technique as previously described (Thompson and Nurse, 1998; Thompson et al., 2002). The pipette solution contained in mM: K gluconate, 95; KCl, 35; NaCl, 5; $CaCl_2$, 2; Hepes, 10; at pH 7.2, and nystatin (300–450 μg/ml). In a few experiments (indicated in the text) conventional whole-cell recording was used as previously described (Thompson et al., 1997). In experiments carried out at room temperature (20–22 °C), the bathing solution contained in mM: NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; and Hepes, 10, at pH 7.4, supplemented with 0.5 μM tetrodotoxin (TTX). In other experiments (as indicated in figure legends), the bathing medium consisted of a TTX-free, 24 mM HCO_3^-/5% CO_2-buffered solution at 35 °C as previously described (Zhang et al., 2000). Hypoxic solutions (PO_2=5–15 mm Hg) were generated by bubbling the bathing solution with 100% $N_{2(gas)}$ for at least 1 h, and were applied by gravity perfusion at a rate of 2–4 ml/min as previously described (Thompson et al., 1997; Zhang et al., 2000). All data are expressed as mean±standard error of the mean (SEM) and were compared using analysis of variance (ANOVA) (Microcal Origin version 6.0; Microcal Software, Northampton, MA, USA), with the level of significance set at P<0.01. Dose-response data for mitochondrial ETC blockers were collected after exposure of neonatal AMC to the appropriate concentration of drug for at least 5 min. The apparent binding constant (k_d) of each ETC blocker was determined by fitting a plot of percent inhibition of outward current at +30 mV versus blocker concentration with Hill's equation.

Luminol and luciferase chemiluminescence

Prior to determination of ROS levels by luminol, and ATP release by luciferase chemiluminescence, the growth medium was removed from the cultures and replaced with 800 μl of the Hepes-buffered bathing solution used in the electrophysiological experiments. Following the addition of 200 μl of luciferin–luciferase solution (ATP determination kit; Molecular Probes, Eugene, OR, USA, # A22066), or 120 μM luminol, the dish was placed in a Labsystem Luminoskan™ luminometer (Labsystem, Helsinki, Finland) connected to a Pentium III computer as previously described (Buttigieg and Nurse, 2004). Dishes were modified 24-well plates with glued, opaque glass rings to separate each well and prevent light spreading between wells. Luminescence readings (relative light units; RLU) were collected every 4 s for 3 min with the luminometer, stored on a personal computer, and analyzed using Labsystems, Ascent Software.

The hypoxic bathing solution was prepared as described above, and applied by manually replacing the 800 μl of normoxic (control) bathing solution with an equal volume of hypoxic solution (PO_2=15–20 mm Hg) using a pipette. The PO_2 of the solution was continually monitored in parallel 'bench-top' experiments on the same dishes using a World Precision Instruments Dissolved O_2 reader (Sarasota, FL, USA, WPI ISO2-A) and found to remain hypoxic (<25 mm Hg) for up to 10 min, which was longer than the duration of the experiment (3 min). The exchange of solutions required an ~1 min break in data acquisition, and this is indicated by a break in the plotted luminescence traces. All luminescence records were obtained at 37 °C. The ROS scavenger, *N*-acetyl-L-cysteine (NAC; 50 μM) or blockers of the mitochondrial ETC, i.e. rotenone (1 μM), myxothiazol (2 μM), or cyanide (2 mM) were added to the bathing medium. To control for differences in cell number among the dishes, the luminometer signal (RLU) for a given treatment was normalized to the initial control value obtained for the same dish. The data were analyzed by the statistical programs, Origin and Microsoft Excel (Microsoft, Redmond, WA, USA) using repeat ANOVA with significance set at P<0.05.

RESULTS

Evidence that hypoxia inhibits outward currents in neonatal AMC via decreased H_2O_2

As previously reported (Thompson et al., 1997; Thompson and Nurse, 1998), hypoxia (PO_2 ~5 mm Hg) inhibited outward current in neonatal rat AMC by 30–40% (step to +30 mV). The control current (1017±61 pA at +30 mV) was significantly (P<0.01) reduced by hypoxia to 401.7±14.4 pA (Fig. 1A, B; n=8). When hypoxia was concomitantly applied with 50 μM H_2O_2, the magnitude of the outward current returned to 882±77.6 pA, a value not significantly different from the control (P>0.01 versus control), indicating that exogenously applied H_2O_2 reversed the effects of hypoxia on outward current (Fig. 1A, B). Higher concentrations of H_2O_2 (~100 μM) increased outward current density beyond that seen in control conditions, and concentrations >300 μM caused irreversible cellular damage, indicated by rapid loss of the pipette-plasma membrane seal (data not shown).

To determine whether scavenging ROS can mimic hypoxia and cause inhibition of outward current we used two non-selective ROS scavengers and catalase, which specifically degrades H_2O_2. The non-selective scavenger