Ventilatory and carotid body chemoreceptor responses to purinergic P2X receptor antagonists in newborn rats

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Niane LM, Donnelly DF, Joseph V, Bairam A. Ventilatory and carotid body chemoreceptor responses to purinergic P2X receptor antagonists in newborn rats. J Appl Physiol 110: 83–94, 2011. First published November 4, 2010; doi:10.1152/japphysiol.00871.2010.—Adenosine triphosphate, acting through purinergic P2X receptors, has been shown to stimulate ventilation and increase carotid body chemoreceptor activity in adult rats. However, its role during postnatal development of the ventilatory response to hypoxia is yet unknown. Using whole body plethysmography, we measured ventilation in normoxia and in moderate hypoxia (12% fraction of inspired O2, 20 min) before and after intraperitoneal injection of suramin (P2X2 and P2X3 receptor antagonist, 40 mg/kg) in 4-, 7-, 12-, and 21-day-old rats. Suramin reduced baseline ventilation (~20%) and the response to hypoxia (~30%) in all rats, with a relatively constant effect across ages. We then tested the effect of the specific P2X3 antagonist, A-317491 (150 mg/kg), in rats aged 4, 7, and 21 days. As with suramin, A-317491 reduced baseline ventilation (~55%) and the hypoxic response (~40%) at all ages studied. Single-unit carotid body chemoreceptor activity was recorded in vitro in 4-, 7-, and 21-day-old rats. Suramin (100 μM) and A-317491 (10 μM) significantly depressed the sinus nerve chemosensory discharge rate (~80%) in normoxia (Po2 ~150 Torr) and hypoxia (Po2 ~60 Torr), and this decrease was constant across ages. We conclude that, in newborn rats, P2X purinergic receptors are involved in the regulation of breathing under basal and hypoxic conditions, and P2X1-containing receptors play a major role in carotid body function. However, these effects are not age dependent within the age range studied.

CAROTID BODY CHEMORECEPTORS respond to a decrease in arterial oxygen saturation by increasing action potential (AP) activity in sinus nerve afferent fibers. These fibers terminate in the brain stem, and their stimulation enhances respiratory drive and sympathetically mediated activity. Chemoreceptors undergo significant postnatal maturation. In rats, the chemoreceptor AP response to a given level of hypoxia is weak at birth and matures to adult levels over the first 3 wk of life (25). In addition, the respiratory response to hypoxia is biphasic in newborn mammals, with an initial increase in ventilation, mediated by the carotid bodies, followed by a decrease mediated by central inhibitory mechanisms and a reduction of metabolic rate (7, 17). The two phases involve different neurotransmitters, which may show an age dependence (2, 7).

Although the mechanism by which hypoxia is transduced by the carotid body to increased AP firing frequency is incompletely understood, recent data have implicated a major role of ATP acting through purinergic receptors in the adult cat, rat, and mouse (20, 33, 46). For instance, intracarotid injection of ATP to anesthetized cats increases ventilation and stimulates carotid sinus nerve discharge rate (36). In addition, similar effects are observed with ATP analogs and blocked by the ATP receptor antagonist suramin (37). Pharmacological experiments based on a culture of rat carotid body chemoreceptor cells and sensory petrosal ganglion neurons provided evidence that the effects of ATP are mainly related to activation of postsynaptic P2X2- and P2X3-containing purinergic receptors (47, 48). Furthermore, RT-PCR and immunofluorescence studies confirmed the presence of P2X receptor subunits in chemoreceptor afferent neurons (3, 21, 34, 48). Finally, the ventilatory and carotid sinus nerve responses to hypoxia were significantly decreased in mice lacking P2X2 receptors and further reduced in P2X2/3 double knockout mice (38).

By contrast, little is known regarding the contribution of the purinergic system to maturation of hypoxic chemosensitivity in the carotid body. This maturational process involves several aspects of carotid body development, including neurogenesis (44), establishment of synapses (9), expression of ion channels (26), and developmental changes of key neurotransmitters, such as dopaminergic and cholinergic systems. These developmental changes typically occur during a period overlapping late fetal development and the first 3 wk following birth in rat pups (2, 11, 31). Our laboratory recently reported that the protein expression level of P2X2 and P2X3 receptors remains stable during postnatal development in the carotid body of cats (3). However, their expression decreases in the petrosal ganglion; expression levels decrease by 50% in 2-mo-old and adult cats compared with 1- and 15-day-old cats (3). These observations led to the hypothesis that functional purinergic signaling in the carotid body changes during development and contributes to postnatal changes in the carotid body response to hypoxia. Accordingly, we designed the present study to test this hypothesis using whole body plethysmography and in vitro recordings of chemosensory activity in developing rats.

MATERIAL AND METHODS

Ethics

We used male and female Sprague-Dawley rats between postnatal (P) days 4 and 21 to conduct our studies in two different laboratories. In vivo recordings of minute ventilation (V̇E) in normoxia and hypoxia were performed at the Centre de Recherche du Centre Hospitalier Universitaire de Québec after approval by the Animal Care Committee at Laval University.
in accordance with the Canadian Council on Animal Care guidelines. Recordings of chemoreceptor activity using the in vitro preparation were performed at Yale University School of Medicine and approved by the Institutional Animal Experimentation and Use Committee.

**In Vivo Studies: Ventilatory and Metabolic Recordings**

**Animals.** All animals used were obtained from 15 litters by mating virgin female and male rats purchased from Charles River (St. Constant, Québec). Rats were supplied with food and water ad libitum and maintained under standard laboratory conditions (21°C, 12:12-h light-dark cycle: lights on at 0700 and off at 1900). Litters were culled to 12 pups on P1, with an equal number of each sex when possible.

**Measurements.** Respiratory and metabolic parameters were recorded in unrestrained male and female rat pups at P4 (n = 35), P7 (n = 31), P12 (n = 21), and P21 (n = 41) using whole body plethysmography (Emka Technologies, Paris, France), as previously described (23, 31). Using a temperature control system (Physitemp, Clifton, NJ), the temperature inside the plethysmograph was fixed at thermoneutrality for each age studied: 32°C for P4 and P7 and 30°C for P12. Relative humidity was continuously monitored from the outflowing air stream with a high-precision water vapor pressure analyzer (RH-300 Sable Systems International). The inner volume of the plethysmography chamber was 0.22 liter for P4 and P7 pups and 0.75 liter for P12 and P21 pups. Calibration of the respiratory signal was performed by injection of 0.5 or 1 ml of air (according to the size of the chamber), and tidal volume (VT) was calculated from the pressure deviation by IOX software (Emka Technologies, Paris, France). Oxygen and CO2 analyzers were calibrated following the manufacturer’s instructions (AEI Technologies, Naperville, IL). Due to the small size of young rats, body temperature was measured orally in P4 and P7 rats and rectally in P12 and P21 rats using a thermocouple (PT100 and P21) and measured continuously with a mass plethysmograph was set at 0.1 l/min (P4 and P7) or 0.2 l/min (P12 and P21) and measured continuously with a mass flowmeter (4140; TSI, Shoreview, MN). Using a dual-plethysmograph was set at 0.1 l/min (P4 and P7) or 0.2 l/min (P12 and P21) and measured continuously with a mass flowmeter (4140; TSI, Shoreview, MN). Using a dual-channel analyzer for oxygen and CO2 (AEI Technologies), respiratory gases (oxygen and CO2) were continuously measured during the entire recording time and used later to calculate oxygen consumption (VO2) and CO2 production (VCO2). Calibration was performed per the manufacturer’s specification using a one-point or two-point method with certified gases. Respiratory frequency (fR) and VT were recorded from the plethysmograph signal. VT was calculated based on barometric pressure, room and body temperature, and humidity (BTPS) using the Bartlett and Tenney (5) equation. VE was calculated as VE = fR × VT. VO2 was calculated as VO2 = flow × [(O2_in - O2_out) - O2_out × (CO2_out - CO2_in)]/[1 - O2_out] and VCO2 as VCO2 = flow × [(CO2_out - CO2_in) - CO2_out × (O2_in - O2_out)]/[1 - CO2_out], corrected to steady-state conditions, where O2_in, O2_out, CO2_in, and CO2_out are inflowing and outflowing CO2 and O2, respectively. These values were then used for calculation of the ventilatory equivalent for oxygen (VE/VO2) and carbon dioxide (VE/VCO2) (1, 17, 30). VT, VO2, and VCO2 were expressed per 100 g body wt.

**Drugs.** All drugs were purchased from Sigma Aldrich Canada (Oakville, ON, Canada). Because selective P2X3 receptor antagonists are not commercially available, we used the non-specific purinergic P2X receptor antagonist suramin (28) and the selective P2X3 receptor antagonist A-317491 (22), which acts on both homomeric P2X3- and heteromeric P2X2/3-containing receptors. Each drug was dissolved in sterile saline and injected 30 min before the recordings were started. Based on dose-response studies performed in 19- to 21-day-old rats (see below), we administered suramin and A-317491 at doses of 40 and 150 mg/kg, respectively. The choice of a 30-min delay was based on association constant data from the literature (22, 28). For in vivo studies, suramin was added to the perfusion solution at 100 μM, as previously used to assess the chemoreceptor activity in adult rats (21). A-317491 was used at a concentration of 10 μM based on the affinity constant measured for recombinant and native rat P2X1 receptors (22).

**Ventilatory dose-response curve for suramin and A-317491 in 19- to 21-day-old rats.** Because there are no data in the literature concerning the ventilatory effects of suramin or A-317491 on young rats, we first determined the ventilatory dose-response curve for each drug in normoxia [inspired O2 fraction (FIO2 = 21%) and in response to hypoxia (FIO2 = 12%)] in 19- to 21-day-old rats (data not shown). A total of 55 rats from 6 litters were used for these preliminary studies. Four doses of suramin were tested [20, 40, 80, and 100 mg/kg, injected intraperitoneally (IP), 6 rats/dose], and compared with vehicle (saline injection, n = 10 rats). Compared with vehicle, suramin had no effect on ventilation at 20 mg/kg, but decreased VT to similar levels at 40, 80, and 100 mg/kg in normoxia and hypoxia. The magnitude of this decrease was ~15 and 22% in normoxia and hypoxia, respectively. Accordingly, a dose of 40 mg/kg was chosen for the experimental series. For the selective P2X3-containing receptor antagonist A-317491, four doses were tested: 30 mg/kg (n = 3), 100 mg/kg (n = 3), 150 mg/kg (n = 6), and 300 mg/kg (n = 3) injected IP and compared with vehicle (n = 6). As with suramin, A-317491 reduced VT in normoxia and hypoxia at 150 and 300 mg/kg. Each of these doses decreased VT ~42% in normoxia and 45% in hypoxia relative to vehicle. Accordingly, the dose of 150 mg/kg was used for the experimental series. We used both males and females for the dose-response study. Because we found no sex-specific effect, we used male and female rats for recordings throughout the study, but we did not perform further sex-specific analyses.

**Experimental protocol.** To avoid pup- and litter-specific effects, rats from each litter were weighed and randomly assigned to one of three groups: vehicle (1 ml/kg, IP saline injection), suramin (40 mg/kg), or A-317491 (150 mg/kg). Treatments were given 30 min before the start of the experiment. Each rat was used for only one experiment to avoid repetitive drug/hypoxic exposures. After each rat’s body temperature was measured, it was returned to the plethysmograph. Once it laid quiet, measurements were started under normoxia (FIO2 = 21%) for 10 min. Nitrogen was mixed with air at the gas inlet to produce the desired chamber O2 pressure for hypoxia (FIO2 = 12%), which generally occurred within 1 min and was stable during the 20-min recording period. All recordings were performed under poikilocapnic conditions.
In Vitro Studies: Carotid Body Chemoreceptor Activity Recordings

Animals. Dams with their 1-day-old pups were purchased and housed in Yale University animal care facilities under standard conditions (light-dark cycle 12:12 h, food and water ad libitum). Male and female rat pups aged P3–P4 (n = 11), P7–P8 (n = 15), and P16–P21 (n = 25) from five different litters were used. For all in vitro experiments, each rat was only used for one recording (i.e., one carotid body per pup per experiment).

Single-unit chemoreceptor preparation. Each rat was deeply anesthetized with 100% CO₂, then decapitated, and the carotid body/carotid sinus nerve/petrosal ganglion complex was harvested en bloc under surgical microscopy, as previously detailed (13, 15). The connective tissue was partially digested with a mixture of collagenase type P (1 mg/ml, Roche Diagnostics, Mannheim, Germany) and protease type IX (0.2 mg/ml, Sigma Aldrich, St. Louis, MO) in Ringer buffer solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1 Na₂HPO₄, 24 NaHCO₃, 1 MgSO₄, and 5 glucose, pH = 7.4), bubbled with a mixture of 95% O₂ and 5% CO₂.

After the connective tissue was cleaned, the complex was transferred to the recording chamber (RC-22C, Warner Instruments, Hamden, CT) mounted on the stage of an inverted microscope (AxioVert 10, Carl Zeiss, Germany). The preparation was continuously superfused with 37°C Ringer solution (TC-344B in-line heater, Warner Instruments) at a rate of 3 ml/min (Peristaltic Pump Minipuls 3, Gilson, Middleton, WI). The chamber PO₂ near the preparation was continuously monitored (Oxy-Micro with PST-1 probe, World Precision Instruments, FL) during the experiment.

Single-unit chemoreceptor afferent nerve fiber activity was recorded from the soma of petrosal neurons projecting to the carotid body by placing a suction electrode in the petrosal ganglion. To facilitate unit identification and measurement of conduction time, a glass pipette filled with 1 M NaCl (0.2-MΩ impedance) was placed in the carotid body, and a cathodal electrical stimulus (100 μA, 0.1-ms duration; BSI-2, BAK Instruments, Rockville, MD) was delivered by a constant current source (BPG-1, BAK instruments) through the pipette to evoke an orthodromic AP. Electrical stimuli were delivered only until evoked single-unit activity was detected and spontaneous APs were displayed on an oscilloscope. The AP discharge activity recorded from the sensory petrosal neuron was continuously acquired, amplified (MD5, BAK instruments), filtered, digitized at a sample rate of 10 kHz (Digidata 1200, Axon Instruments, Foster City, CA), and stored on a computer (Axoscope, Axon Instruments) for further analyses.

Chemoreceptor activity recordings. Chemoreceptor activity was first recorded under control, normoxic conditions (without drug treatment) for 2–3 min (superfused with Ringer buffer equilibrated with 21% O₂ and 5% CO₂, balanced with N₂, PO₂ ~150 Torr). Then the control hypoxic response was measured for 3–4 min by superfusing the preparation with a Ringer buffer equilibrated with 5% O₂ and 5% CO₂ balanced with N₂, PO₂ ~60 Torr. After returning to normoxic conditions and stable activity, the preparation was superfused for 10 min with normoxic solution containing either suramin or A-317491, and the AP discharge rate was recorded for 2–3 min. The inflowing line was then switched to the hypoxic solution containing the same drug for 3–4 min. The drug was washed out for 10 min, and the hypoxia response retested. Only one drug was tested on a given preparation.

Data Collection and Statistical Analyses

Ventilation. Ventilatory and metabolic variables during normoxia were measured and calculated each minute during the experiment using the IOX software program (version 1.8.9 EMKA technology, Paris, France). For each rat, the last 5 min of ventilatory (fR, VT, and Ve) and metabolic (VO₂, VCO₂, VE/VO₂, and Ve/VCO₂) data were averaged. A two-way ANOVA (StatView 5.1) using the drug applied (suramin, A-317491, vehicle) and age (4-, 7-, 21-day-old rats) as treatment variables was used to test statistical significance. In these groups, the mean value of Ve in vehicle-treated rats was arbitrarily made 100. Ve of each rat in the vehicle, suramin, and A-317491 groups was calculated using the following formula: \( x = \frac{\text{individual value in normoxia}}{\text{mean value of vehicle group}} \times 100 \). This calculation was performed for each of age group studied. Values were expressed as means ± SE.

For the hypoxic response, fR and VT were obtained on a minute-by-minute basis for the 20 min of recording. We expressed the response to hypoxia as peak and steady state. The peak response is the average of the highest values observed in two successive minutes during the first 10 min of recording, and the steady-state response is the average of the last 5 min of recording. The peak and steady state fR, VT, and Ve in response to hypoxia were expressed as percent changes from normoxia.

ANOVaras were used to determine main effects of treatment and age and interactions between treatment and age. Fisher’s protected least significant difference tests were used for post hoc testing if ANOVaras yielded a P value < 0.05. Values were expressed as means ± SE.

Chemoreceptor activity. Chemoreceptor activity was analyzed using an event detection program (CLAMPFIT 9, Molecular Devices, Sunnyvale, CA). The AP occurrence times were converted to frequency (Hz) (Origin 7.5 program, Microcal Software, Northampton, MA) and plotted against oxygen concentration of the recording chamber and time to determine the activity under normoxia and hypoxic conditions. Discharge rates were averaged over the entire 3-min recording under normoxia. In contrast, discharge rates under hypoxia were quantified as the peak value of a 3-s moving average.

Significance was tested using a two-way ANOVA with treatment and age as grouping variables. If a P value < 0.05 was reported, post hoc Fisher’s protected least significant difference tests were performed. Values were expressed as means ± SE.

RESULTS

Ventilatory and Metabolic Effects of Purinergic Receptor Antagonists in Developing Rats

Normoxic ventilation. SURLAMIN. Representative respiratory recordings obtained from P4, P7, and P21 rats at 30 min after suramin injection are shown in Fig. 1, A and B, while ventilatory (fR, VT, and Ve) and metabolic variables (VO₂, VCO₂, VE/VO₂, and Ve/VCO₂) are presented in Table 1. Compared with vehicle, suramin significantly decreased fR (treatment
Table 1. Suramin effect on baseline ventilatory and metabolic parameters in developing rats

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Drug</th>
<th>n</th>
<th>Weight, g</th>
<th>Temperature, °C</th>
<th>VT, breaths/min</th>
<th>Vt, ml/100 g</th>
<th>VE, ml/min \cdot 100 g⁻¹</th>
<th>VO₂, ml/min \cdot 100 g⁻¹</th>
<th>VCO₂, ml/min \cdot 100 g⁻¹</th>
<th>V/VO₂</th>
<th>V/VE/VCO₂</th>
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<tbody>
<tr>
<td>4</td>
<td>Vehicle</td>
<td>10</td>
<td>10.2 ± 0.2</td>
<td>33.8 ± 0.2</td>
<td>166 ± 3</td>
<td>1.26 ± 0.1</td>
<td>210 ± 15</td>
<td>5.9 ± 0.2</td>
<td>4.3 ± 0.1</td>
<td>44 ± 4</td>
<td>52 ± 5</td>
</tr>
<tr>
<td></td>
<td>Suramin</td>
<td>10</td>
<td>10.8 ± 0.3</td>
<td>33.7 ± 0.4</td>
<td>157 ± 3*</td>
<td>1.11 ± 0.1</td>
<td>169 ± 6*</td>
<td>3.6 ± 0.3*</td>
<td>2.9 ± 0.3*</td>
<td>47 ± 4</td>
<td>61 ± 9</td>
</tr>
<tr>
<td>7</td>
<td>Vehicle</td>
<td>10</td>
<td>17.3 ± 0.5</td>
<td>35.2 ± 0.2</td>
<td>167 ± 2</td>
<td>0.89 ± 0.1</td>
<td>157 ± 12</td>
<td>4.6 ± 0.7</td>
<td>3.8 ± 0.6</td>
<td>42 ± 6</td>
<td>51 ± 6</td>
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<td>10</td>
<td>17.7 ± 0.5</td>
<td>34.9 ± 0.3</td>
<td>155 ± 3*</td>
<td>0.71 ± 0.1</td>
<td>110 ± 5*</td>
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<td>50 ± 6</td>
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<td>12</td>
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<td>30.2 ± 0.6</td>
<td>35.4 ± 0.2</td>
<td>157 ± 2</td>
<td>0.93 ± 0.1</td>
<td>146 ± 7</td>
<td>3.8 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>41 ± 5</td>
<td>63 ± 4</td>
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<tr>
<td></td>
<td>Suramin</td>
<td>10</td>
<td>30.5 ± 0.6</td>
<td>35.5 ± 0.2</td>
<td>137 ± 3*</td>
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<td>101 ± 5*</td>
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<td>0.90 ± 0.1</td>
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<tr>
<td></td>
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<td>2.6 ± 0.2</td>
<td>37 ± 5</td>
<td>45 ± 3</td>
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</table>

Values are means ± SE; n, no. of rats. Body temperature (°C) was recorded orally (postnatal days 4 and 7) and rectally (postnatal days 12 and 21). Shown are respiratory frequency (fR), tidal volume (VT), minute ventilation (VE), oxygen consumption (VO₂), carbon dioxide production (VCO₂), and oxygen and carbon dioxide convection ratios (V/VO₂ and VE/VCO₂) after single intraperitoneal injection of vehicle (saline) or suramin (40 mg/kg) in 4-, 7-, 12-, and 21-day-old rats. *P < 0.05 vs. vehicle.
was no significant interaction between treatment and age for $f_R$ (age \times treatment, $P = 0.3$), $V_T$ (age \times treatment, $P = 0.09$), or $Ve$ (age \times treatment, $P = 0.2$).

There was a clear treatment effect of the P2X antagonists in normoxia ($P = 0.0001$), but no interaction between age and treatment ($P = 0.9$) (Fig. 2). Furthermore, the effects of A-317491 on normoxic $Ve$ were greater than those of suramin on P4, P7, and P21 rats. This difference might be related to the difference in the dose used (3.75-fold more A-317491 than suramin) or to different binding capacities of these antagonists.

**Hypoxic response. Suramin.** Minute-by-minute ventilation data during hypoxic exposure is presented in Fig. 3A, which shows the clear effects of suramin on the hypoxic response for $Ve$ in P7 and P21 rats. Percent changes from normoxia are presented in Fig. 4, A and B, which show that suramin significantly reduced the peak (treatment effect, $P = 0.01$) and steady-state responses (treatment effect, $P = 0.001$) for $f_R$ but not $V_T$ (treatment effect, $P = 0.8$ and $P = 0.6$ for peak and steady state, respectively) in P4, P12, P7, and P21 rats. Although the peak $Ve$ was slightly reduced in P4 rats, a significant effect was only obtained in P7–P21 rats (Fig. 4A). A similar result was observed during the steady-state response to hypoxia (Fig. 4B). There was no significant interaction between age and treatment for the effects of suramin on both the peak and steady-state values for $f_R$ (age \times treatment, $P = 0.3$ and $P = 0.09$ for peak and steady state, respectively) or for $Ve$ (age \times treatment, $P = 0.9$ and $P = 0.5$, respectively). Suramin induced a significant decrease in $Ve/V_O2$ and $Ve/V_CO2$ in P7–P21 rats compared with vehicle (Fig. 4B), which indicated profound hypoventilation relative to metabolic needs in hypoxia, although suramin induced no significant interaction between age and treatment for $Ve/V_O2$ and $Ve/V_CO2$ ($P = 0.08$).

A-317491. $Ve$ during hypoxic exposure was decreased by A-317491 at all ages studied (Fig. 3B) and decreased both the peak and steady-state responses relative to vehicle (Fig. 4, A and B, respectively) (treatment effect, $P = 0.02$ and $P = 0.003$, respectively). Like suramin, there was no significant interaction between age and treatment for the effects on the peak or the steady-state responses of $Ve$ (age \times treatment, $P = 0.7$ and $P = 0.3$, respectively). As reported for normoxia, A-317491 had a stronger inhibitory effect than suramin at P4, P7, and P21 (Fig. 4). The inhibitory effects of A-317491 in hypoxia resulted in a hyperventilation, as shown by the reduced $Ve/V_O2$ and $Ve/V_CO2$ in P4, P7, and P21 rats (Fig. 4B).

**Carotid Body Response to Purinergic Receptor Antagonists in Developing Rats**

**Chemoreceptor activity in response to suramin.** For these experiments, we used 24 carotid body-carotid sinus nerve-petrosal ganglion preparations from P3–P4 ($n = 6$), P7–P8 ($n = 5$), and P16–P21 ($n = 13$) rats. Representative raw activities without (control) and with suramin (100 μM) under normoxic and hypoxic conditions are shown in Fig. 5, A–C. Compared with control, suramin decreased the chemoreceptor activity for each age group measured under conditions of normoxic isocapnic or hypoxic isocapnic superfusion. Under normoxia, the decreased chemoreceptor activity was significant only in P16–P21 (treatment effect, $P = 0.03$, Fig. 5D) rats, but in hypoxia the chemoreceptor response was significantly decreased at all ages (treatment effect, $P = 0.0001$, Fig. 5E). In line with the ventilatory data, the effects of suramin on chemoreceptor activity was not age dependent (ANOVA, age \times treatment, $P = 0.5$ and $P = 0.6$ for normoxia and hypoxia, respectively, see Fig. 8, A and B, respectively). These inhibitory effects of suramin were in line with a previous study using a similar dose that showed a decrease in the frequency of chemoreceptor activity in P19–P21 rats without a significant effect on nerve conduction velocity or AP amplitude (12).

**Chemoreceptor activity in response to A-317491.** For these experiments, we used 19 carotid body-carotid sinus nerves -

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**Table 2. A-317491 effect on baseline ventilatory and metabolic parameters in developing rats**

<table>
<thead>
<tr>
<th>Age, days</th>
<th>Drug</th>
<th>$n$</th>
<th>Weight, g</th>
<th>Temperature, °C</th>
<th>$f_R$, breaths/min</th>
<th>$V_T$, ml/100 g</th>
<th>$V_E$, ml/min⁻¹100 g⁻¹</th>
<th>$V_O2$, ml/min⁻¹100 g⁻¹</th>
<th>$V_CO2$, ml/min⁻¹100 g⁻¹</th>
<th>$Ve/V_O2$</th>
<th>$Ve/V_CO2$</th>
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</thead>
<tbody>
<tr>
<td>4</td>
<td>Vehicle</td>
<td>10</td>
<td>10.9 ± 1.1</td>
<td>33.7 ± 0.1</td>
<td>162 ± 3</td>
<td>1.23 ± 0.1</td>
<td>202 ± 13</td>
<td>5.2 ± 0.4</td>
<td>3.6 ± 0.2</td>
<td>44 ± 4</td>
<td>57 ± 5</td>
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<td>4</td>
<td>A-317491</td>
<td>5</td>
<td>9.2 ± 0.5</td>
<td>33.8 ± 0.4</td>
<td>132 ± 3*</td>
<td>0.70 ± 0.1*</td>
<td>95 ± 20*</td>
<td>2.2 ± 0.3*</td>
<td>1.5 ± 0.2*</td>
<td>41 ± 5</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>Vehicle</td>
<td>6</td>
<td>17.5 ± 1.3</td>
<td>35.7 ± 0.3</td>
<td>164 ± 3</td>
<td>0.86 ± 0.1</td>
<td>149 ± 15</td>
<td>4.5 ± 0.3</td>
<td>3.2 ± 0.2</td>
<td>39 ± 6</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>A-317491</td>
<td>6</td>
<td>16.8 ± 1.2</td>
<td>34.7 ± 0.2</td>
<td>134 ± 7*</td>
<td>0.46 ± 0.1*</td>
<td>60 ± 8*</td>
<td>1.7 ± 0.2*</td>
<td>1.4 ± 0.1*</td>
<td>37 ± 5</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>21</td>
<td>Vehicle</td>
<td>10</td>
<td>50.5 ± 1.7</td>
<td>36.7 ± 0.7</td>
<td>143 ± 2</td>
<td>0.82 ± 0.1</td>
<td>129 ± 5</td>
<td>3.7 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>34 ± 1</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>21</td>
<td>A-317491</td>
<td>6</td>
<td>47.4 ± 2.8</td>
<td>36.7 ± 0.1</td>
<td>105 ± 3*</td>
<td>0.66 ± 0.1*</td>
<td>69 ± 5*</td>
<td>2.1 ± 0.2*</td>
<td>1.4 ± 0.2*</td>
<td>35 ± 5</td>
<td>51 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, no. of rats. Body temperature (°C) was recorded orally (postnatal days 4 and 7) and rectally (postnatal day 21). Shown are $f_R$, $V_T$, $Ve$, $V_O2$, $V_CO2$, $Ve/V_O2$, and $Ve/V_CO2$ after single intraperitoneal injection of vehicle (saline) or A-317491 (150 mg/kg) in 4-, 7-, and 21-day-old rats. *$P < 0.05$ vs. vehicle.
petrosal ganglion preparations from P3–P4 (n = 5), P7–P8 (n = 5), and P16–P21 (n = 9) rats. Because no data are currently available in the literature describing the use of A-317491 on peripheral chemoreceptors, we initially tested its specificity on chemoreceptor activity. Figure 6 shows an example of the effect of A-317491 on tissue prepared from P3–P4 rats. The orthodromically evoked APs were recorded after an electrical stimulus at S1, S2, and S3 (Fig. 6A) to evaluate the nerve conduction time and AP amplitude without and with A-317491 (Fig. 6C). Neither the nerve conduction time (time lapsed from the stimulus artifact to the arrival of an afferent AP at the soma of the petrosal neuron) nor the AP amplitude (the amplitude difference between baseline and the AP peak) was affected by A-317491 (Fig. 6C), suggesting that the drug had no anesthetic effect.

Raw AP activities without (control) and with A-317491 (10 μM) under normoxic and hypoxic conditions are shown in Fig. 7, A–C. As reported for suramin, the discharge rate was inhibited by A-317491 under conditions of normoxic isocapnic or hypoxic isocapnic superfusion (Fig. 7, A–C). However, the decrease in discharge rate under normoxic superfusion was not significant due to the high variability in chemoreceptor activity relative to the low discharge frequency. During hypoxia, the discharge rate was significantly lower than control at each age studied (treatment effect, P = 0.001, Fig. 7E). In addition, there was no interaction between age and treatment (ANOVA age × treatment, P = 0.5 and 0.6, for normoxia and hypoxia, respectively; Fig. 8, A and B, respectively).

Finally, unlike the ventilatory responses (Figs. 2 and 4), there was no significant difference between suramin and A-317491 with respect to their effects on chemosensory activity in normoxia (treatment effect, P = 0.06, Fig. 8A) and hypoxia (treatment effect, P = 0.1, Fig. 8B), although the dose of suramin used was 10-fold higher than that of A-317491.

DISCUSSION

The primary result from the present study is that antagonism of P2X- (suramin) and P2X3-containing receptors (A-317491) exerts a significant effect on normoxia and hypoxia-stimulated ventilation across the range of ages studied. These actions are mediated, in part, by peripheral chemoreceptors, which are inhibited by both suramin and A-317491. This result is broadly consistent with previous studies that demonstrated a critical role of P2X receptors in sensory transduction at the carotid body in mature animals (38). Furthermore, the effects of antagonists were relatively constant across the range of ages. Thus, despite significant changes in respiratory control in early life, the contribution of P2X receptors to the overall ventilatory level remains relatively constant.

Technical Limitations

There are some technical considerations inherent in the present experiments. First, accurate measurement of VT using whole body plethysmography on very small animals is problematic (30). However, as previously discussed (23, 31), the
Use of a vehicle group for each age category studied was appropriate because all experiments were performed under similar conditions. Furthermore, rat pups were asleep for 70–80% of the time (4, 8), so we cannot deny the possibility that our recordings were made while the rats were asleep. Interestingly, the results of our in vitro experiments were in line with the plethysmography ventilation recordings. Second, the drugs tested here may have crossed the blood-brain barrier, so the ventilatory effects of the drugs may involve both peripheral and central blockade. Findings on whether suramin may cross (35) or not cross (39) the blood-brain barrier have been inconsistent. Currently available data indicate that A-317491 does not cross the blood-brain barrier (45). In addition, the efficiency of the blood-brain barrier changes developmentally (27), so central effects of the drugs may have been present at some ages and not others. This issue could be addressed using chemodenervated rat pups, but this surgery is exceptionally challenging in rats of young ages and is associated with a high mortality rate (40). Interestingly, P4 and P7 rats that received suramin or A-317491 showed respiratory patterns under normoxic condition that were similar to chemodenervated pups (40). The results of treatment with A-317491 or suramin being consistent between the in vivo and in vitro experiments suggest that the ventilatory results were mostly related to peripheral rather than central activation of purinergic receptors.

Finally, with respect to single-unit chemoreceptor recording, the number of sampled chemoreceptor neurons was relatively small and may not reflect the level of carotid chemoreceptor activity for the entire population. However, this limitation is likely to be minor because there was good agreement in the present experiments between the chemoreceptor recordings and the observed ventilatory changes produced by hypoxia or drug treatment.

Fig. 4. Percent changes from normoxia of respiratory frequency, tidal volume, and $V_{\dot{E}}$ responses to hypoxia measured at peak (A) and at steady state (B) after a single injection of vehicle (open bars) or suramin (40 mg/kg; dark shaded bars) in P4, P7, P12, and P21 rats, or after administration of A-317491 (150 mg/kg; light shaded bars) in P4, P7, and P21 rats. Percent changes from normoxia of ventilatory equivalent for oxygen and carbon dioxide responses to hypoxia were only determined at steady state. $V_{\dot{O}_2}$, oxygen consumption; $V_{\dot{CO}_2}$, CO$_2$ production. Values are means ± SE. n values for vehicle, suramin, and A-317491, respectively, are as follows: 10, 10, and 6 (P4); 6, 12, and 6 (P7); 11 (vehicle) and 10 (suramin) (P12); 10, 11, and 6 (P21). *P < 0.05 vs. vehicle (saline).
Implication of ATP and Purinergic Receptors on Respiratory Control

A role for ATP acting through purinergic receptors is well established within the mature carotid body. The chemoreceptor afferent fibers within the carotid body show immunoreactivity to P2X2 and P2X3 receptors (34). In addition, the messenger RNA for P2X receptors is present in high concentrations in the carotid body and petrosal ganglia, which contain the soma for chemoreceptor afferent neurons (3). ATP is rapidly released from the carotid body during stimulation with hypoxia (10, 16), and antagonism of P2X receptors with suramin or pyridoxal-5-phosphate-6-azophenyl-2',4'-disulfonate reduces chemoafferent activity (21, 34, 43) and postsynaptic activity in petrosal neurons cocultured with carotid body glomus cells (48). Similarly, knockout mice lacking the P2X2 receptors showed a clear decrease in the carotid sinus nerve chemoreceptor activity and in the ventilatory response to acute hypoxia. These responses are further reduced when both P2X2 and P2X3 subunits are deleted in the P2X2/3 double-knockout mice (18, 38). In petrosal neurons, αβ-methylene-ATP (a specific agonist of P2X3 receptors) produces fast and long-lasting inward depolarizing currents, demonstrating the functional presence of P2X3 receptors in these neurons (48).

All of these observations suggest an important role of activation of P2X3-containing receptors in specific neurons to affect the ventilatory and chemoreceptor activity of adult animals. In addition to mediating an excitatory role at the carotid body, ATP is rapidly released in the brain stem and plays an important role in mediating central processing of respiratory input (18, 19, 41). Purinergic receptors for ATP, present as homomeric and heteromeric forms on cellular membranes (32), have been identified in the nucleus tractus solitarius (19, 24), which receives the central projections of the carotid sinus nerve.

Purinergic Receptors Mediate Potent Tonic Drive Under Normoxia in Rat Pups

In rat pups aged P4–P21, suramin and A-317491 inhibited ventilation during normoxia, as previously reported in adult rats (19, 42). The VE/VO2 and VE/VCO2 were not altered by P2X blockade, indicating that ventilation is reduced in
proportion to the reduced \( \dot{V}_O_2 \) and \( \dot{V}_C_0_2 \). Thus, blood gases were likely not affected by the drugs. It seems likely that the reduced carotid body activity (as observed in vitro) contributed to this reduction of ventilation, but this assertion requires confirmation. The mechanism by which suramin reduces metabolic rate is not known, but suramin has been shown to decrease the activity of glycolytic enzymes in Trypanosoma Brucei (the parasite that causes African sleeping sickness) (14) and reduces respiration in isolated liver mitochondria (6). Whether these effects are also mediated by A-317491 is currently unknown.

Across all of the ages studied, the reduction in normoxic chemoreceptor activity was \( \sim 80\% \) (Fig. 8A), a level consistent with that observed in mice lacking the heteromeric forms of P2X\(_{2/3}\) receptors. In mice lacking homomeric P2X\(_2\) receptors, the decrease in resting activity was reported to be \( \sim 40\% \) (18). Hence, data obtained here, together with those in the literature, are in line with the tonic role of ATP and the implication that P2X\(_{2}\)-containing receptors regulate ventilation at rest in both newborn and adult rats. The magnitude of the inhibition in \( \dot{V}_E \) and the reduction of chemoreceptor activity by purinergic antagonists were relatively constant over the age range studied (Figs. 2 and 8A), suggesting that the contribution of ATP receptors to normoxic chemoreceptor activity is constant over development.

**Purinergic Receptors Are Involved in Hypoxic Ventilatory Responses in Rat Pups**

Both suramin and A-317491 produced a significant reduction in the ventilatory response to acute hypoxia, and this effect was most pronounced between P7 and P21. A-317491 produced a significant reduction in ventilation at P4, while suramin produced no statistically significant effect on ventilation in P4 rats. The larger effects in older rats were likely due to the enhanced peripheral chemoreceptor activity following maturation, which largely takes place over the first week of life and continues through the second and third weeks (25). The ventilatory changes were consistent with the large decrease in chemoreceptor activity during hypoxia following treatment with suramin and A-317491 (Figs. 5E and 7E). As in normoxia, the relative decrease in chemoreceptor activity following treatment with P2X blockers was relatively constant across ages, suggesting that the role of P2X stimulation is constant across early development. However, although the relative role is constant, the absolute role played by P2X receptors might actually increase with development, as shown by the change in absolute AP discharge rates following suramin or A-317491, which were greater at P16–P21 than P3–P4 (Figs. 5E and 7E). This finding suggests that ATP receptors contribute to the higher spiking activity of the mature organ.

**Perspectives**

This study clearly demonstrates that P2X receptors are involved in the hypoxic ventilatory response in developing rats and highlights the important role of P2X\(_{3}\)-containing receptors. The lack of difference between the effect of suramin and A-317491 on chemosensory neuronal activities, together with the lack of age-dependent effects of either drug, are in line with previous data showing no changes in levels of P2X\(_2\) or P2X\(_3\),
receptors in the carotid body and petrosal ganglion during the first 2 wk of life in cats (3). Because ventilation and the chemosensory response to hypoxia were not completely abolished by suramin or A-317491, other excitatory pathways, such as metabotropic P2Y receptors (29) or other transmitters (2), are necessarily involved. In particular, we have recently demonstrated that cholinergic receptors might be critically involved in postnatal maturation of the hypoxic ventilatory response (31). Furthermore, interactions between different receptors might also play a role in the process of carotid body matura-

Fig. 7. Effects of A-317491 on chemoreceptors activity in developing rats. A, B, and C: typical recordings of single-unit chemosensory activities of petrosal ganglion neurons in response to normoxic-isocapnic and hypoxic-isocapnic superfusion without (control) and with A-317491 (10 μM) in developing rats at P3–P4 (A), P7–P8 (B), and P16–P21 (C). A-317491 depressed the chemosensory discharge rate in normoxic (D) and hypoxic (E) superfusion solutions. Please note the difference in the scale between D and E. Values are means ± SE; n = 5 (P3–P4), 5 (P7–P8), and 9 (P16–P21). *P < 0.05 vs. control.

Fig. 8. Normalized chemosensory discharge activity in developing rats. For each age group studied, discharge rates during superfusion with suramin (dark shaded bars) or A-317491 (light shaded bars) were normalized to control (superfusion without drugs) for normoxic (A) and hypoxic (B) conditions.
tion, because receptor-receptor interaction has been recently suggested to play a role at rest and in response to hypoxia (46). Finally, how ATP and its receptors interact with the other modulatory factors, such as a persistent Na\(^+\) current in chemosensitive fibers (15), needs to be better understood.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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