

Hypoxic intensity: a determinant for the contribution of ATP and adenosine to the genesis of carotid body chemosensory activity

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Conde SV, Monteiro EC, Rigual R, Obeso A, Gonzalez C. Hypoxic intensity: a determinant for the contribution of ATP and adenosine to the genesis of carotid body chemosensory activity. *J Appl Physiol* 112: 2002–2010, 2012. First published April 12, 2012; doi:10.1152/jappphysiol.01617.2011.—Excitatory effects of adenosine and ATP on carotid body (CB) chemoreception have been previously described. Our hypothesis is that both ATP and adenosine are the key neurotransmitters responsible for the hypoxic chemotransmission in the CB sensory synapse, their relative contribution depending on the intensity of hypoxic challenge. To test this hypothesis we measured carotid sinus nerve (CSN) activity in response to moderate and intense hypoxic stimuli (7 and 0% O₂) in the absence and in the presence of adenosine and ATP receptor antagonists. Additionally, we quantified the release of adenosine and ATP in normoxia (21% O₂) and in response to hypoxias of different intensities (10, 5, and 2% O₂) to study the release pathways. We found that ZM241385, an A₂ antagonist, decreased the CSN discharges evoked by 0 and 7% O₂ by 30.8 and 72.5%, respectively. Suramin, a P₂X antagonist, decreased the CSN discharges evoked by 0 and 7% O₂ by 64.3 and 17.1%, respectively. Simultaneous application of both antagonists strongly inhibited CSN discharges elicited by both hypoxic intensities. ATP release by CB increased in parallel to hypoxia intensity while adenosine release increased preferably in response to mild hypoxia. We have also found that the lower the O₂ levels are, the higher is the percentage of adenosine produced from extracellular catabolism of ATP. Our results demonstrate that ATP and adenosine are key neurotransmitters involved in hypoxic CB chemotransduction, with a more relevant contribution of adenosine during mild hypoxia, while vesicular ATP release constitutes the preferential origin of extracellular adenosine in high-intensity hypoxia.

adenosine; hypoxia; carotid body

CAROTID BODIES (CB) are major peripheral chemoreceptor organs that release neurotransmitters in response to hypoxia, generating action potentials at the carotid sinus nerve (CSN) that are integrated in the brain stem to induce a hyperventilatory compensatory response (17). Among the neurotransmitters released in the CB are adenosine and ATP (1–3). One of the first studies on the effects of ATP on CSN activity was carried out by McQueen and Ribeiro (24) where they showed in vivo in the cat CB that ATP increased CSN activity in a dose-dependent manner. Additional experiments led them to conclude that the excitatory action of ATP was due to the adenosine formed by its extracellular degradation. More recently,

Zhang and co-workers (47) showed that the coapplication of blockers of nicotinic and P₂X receptors, hexamethonium and suramin, respectively, in cocultures of glomus cells and “juxtaped” petrosal ganglions completely abolished the hypoxia-evoked excitatory postsynaptic responses. They concluded that ATP and ACh, acting as cotransmitters, supported the synaptic transmission between chemoreceptor cells and sensory nerve endings of the CSN. The idea that ATP has an excitatory effect on chemoreception was sustained by the finding that P₂X₂ receptor knockout mice showed a markedly attenuated ventilatory response to hypoxia (36), and by the finding that ATP is released from rat CB in hypoxia (1, 2, 6).

Adenosine is both a catabolic product and a precursor of ATP and has been defined as an excitatory neurotransmitter in the CB. Exogenously applied adenosine increased CSN chemosensory discharges in the cat in vivo (24, 25) and in vitro (38), as well as in the rat (43). Adenosine and its analogs have been shown to stimulate ventilation in a dose-dependent manner in several species, including humans and rats (26, 37, 40, 45, 46), with the stimulatory effect reaching up to nearly 60% of the maximal asphyxic ventilation; this stimulation is abolished by CSN section and mediated by A₂ receptors (26; see 8). The physiological meaning of these pharmacological findings on CSN and ventilation became obvious when it was demonstrated that mild hypoxia augments adenosine release in an in vitro preparation of rat CB (2). Additional experiments (5) have shown that adenosine in the rat CB-CSN mediates ~60% of the low Po₂-induced CSN activity, half of this effect being postsynaptic and mediated by A_{2A} receptors, and the remaining half presynaptic and mediated by A_{2B} receptors.

Extracellular adenosine may have two metabolic sources, catabolism of ATP by ecto-5'-nucleotidase and adenosine transport by the equilibrative nucleoside transport (ENT) system (8, 14). Both sources have been described in the rat CB, with the latter being responsible for ~45% of the adenosine release during mild hypoxia (10% O₂) (2). In most tissues during hypoxia, or more generally when a conflict between energy supply and demand or an increased energy usage occurs, there are inverse changes in ATP and adenosine levels with subsequent increase in extracellular adenosine concentrations that tends to ameliorate or protect from potential damage (e.g., 30, 39, 42). However, tissue-specific relationships among ATP and adenosine levels might occur by several reasons such as ATP vesicular and extravesicular release whether synaptic or extrasynaptic (10, 13) and regulation of ectonucleotidases by ATP and ADP (22). The increase in energy utilization and the promotion of vesicular release of neurotransmitters induced by hypoxia in the CB (12, 17) can lead to unpredictable

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extracellular ATP adenosine relationships, and therefore to altered significance of both purines in synaptic signaling as a function of the hypoxic intensity.

Based on these facts our working hypothesis is that both ATP and adenosine are key players in the genesis of CSN activity during hypoxia, with relative contributions depending on hypoxic intensity. We have also investigated how the release of adenosine and ATP and their signaling pathways are modified by the hypoxic intensity and also the contribution of extra- and intracellular Ca^{2+} deposits on ATP release from the CB. We have found that adenosine is the main neurotransmitter involved in the communication between chemoreceptor cells and CSN sensory endings during moderate hypoxia while ATP's role is greatest in intense hypoxia. Brief accounts of some of the results in this study have been previously published (3).

EXPERIMENTAL PROCEDURES

Animals and surgical procedures. Experiments were performed in adult Wistar rats of both sexes (250–350 g) obtained from the vivarium of the Faculty of Medicine of the University of Valladolid and from the vivarium of the Faculty of Medical Sciences of the Nova University of Lisbon. The Institutional Committee of the University of Valladolid and Nova University of Lisbon for Animal Care and Use approved the protocols. The rats were kept at a constant temperature (21°C) and a regular light (0800–2000) and dark (2000–0800) cycle, with food and water ad libitum. The animals were anesthetized with pentobarbital sodium (60 mg/kg ip) and tracheostomized, and the carotid bifurcation was removed and cleared in vitro in a Lucite chamber. For adenosine and ATP release experiments, four CBs were used in each experiment due to the small dimensions of the CB (~50 μg). For the release of ATP and adenosine experiments, the carotid bifurcation was placed in a Lucite chamber in ice-cold/95% O_2 -equilibrated Tyrode (in mM: 140 NaCl, 5 KCl, 2 CaCl_2 , 1.1 MgCl_2 , 10 HEPES, 5.5 glucose, pH 7.40) and the CBs were cleared free of CSN and nearby connective tissue. Afterward the CBs were placed in 500 μl of ice-cold 95% O_2 -equilibrated Tyrode medium until the onset of the experiments that were performed in bicarbonate- CO_2 buffered solution of composition identical to above except for the absence of HEPES, substitution of 24 mM NaCl by equimolar amounts of NaHCO_3 and for the fact that all equilibrating gas mixtures contained 5% CO_2 .

For the recording of CSN activity, the preparation CB-CSN was exposed under a dissecting microscope, and a piece of tissue, including the carotid bifurcation and the glossopharyngeal nerve, was removed and placed as before in a Lucite chamber in ice-cold/100% O_2 -equilibrated Tyrode for further dissection of tissue surrounding CB and CSN. The preparation CB-CSN was digested during 3–5 min in collagenase type I (1 mg/ml) solution to loosen the perineurium to facilitate later isolation of single sensory units or paucifiber filaments of the CSN isolation and neural activity recording (35). The CB-CSN preparation was maintained in ice-cold 100% O_2 -equilibrated Tyrode until it was transferred to the recording chamber. Recording solution was Tyrode bicarbonate.

All animals were killed by an intracardiac overdose of pentobarbital sodium.

Effect of different oxygen pressures on ATP and adenosine levels released by the rat carotid body. In all ATP and adenosine release experiments, before the incubation periods, the CBs were submitted to a 30-min period of preincubation in hyperoxia (95% O_2 + 5% CO_2) at 37°C to allow recovery from the surgery. Due to their small size (CB wet weight \approx 50 μg) four CBs were used in each experiment. After preincubation, the CBs were incubated during 10 min at 37°C in 500 μl of Tyrode solution in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, an inhibitor of adenosine deaminase; 2.5

μM) equilibrated with normoxia (20% O_2 + 5% CO_2), hyperoxia (95% O_2 + 5% CO_2), and hypoxia (2%, 5%, and 10% O_2 + 5% CO_2) depending on the hypoxic intensity to be studied; in all cases balance gas was N_2 . After the incubation period the CBs were removed and adenosine and ATP were extracted from the incubation medium.

Study of the contribution of extracellular calcium and intracellular calcium deposits on the release of ATP from rat carotid body. The experiments were performed in CBs incubated during 10 min at two different hypoxic intensities, 10 and 2% O_2 + 5% CO_2 (balanced N_2) in the absence of Ca^{2+} + EDTA (10 mM), which is a Ca^{2+} -chelating agent, and in the absence of Ca^{2+} + EDTA (10 mM) + thapsigargin (1 μM), which is an inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). After the incubation period the CBs were removed and adenosine was extracted from the incubation medium.

Effect of different oxygen pressures on the metabolic pathways of adenosine production in the rat carotid body. The experiments were performed in CBs incubated during 10 min in three different hypoxia intensities, 10, 5, and 2% O_2 + 5% CO_2 (balanced N_2): only in the presence of EHNA (2.5 μM); in the presence of EHNA (2.5 μM) plus α,β -methylene ADP (AOPCP, an inhibitor of ecto-5'-nucleotidases; 100 μM); in the presence of EHNA (2.5 μM) plus *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTL, an inhibitor of ENT; 5 μM); and in the presence of EHNA (2.5 μM) plus AOPCP (100 μM) plus NBTL (5 μM). After the incubation period the CBs were removed and adenosine was extracted from the incubation medium. To investigate if ATP release in the CB was affected by NBTL and to confirm that AOPCP was indeed inhibiting ecto-5'-nucleotidase, ATP was also quantified in the same samples as adenosine.

Nucleotide extraction and adenosine and ATP quantification. Nucleotides were extracted from the incubation medium following a protocol described by Cunha et al. (9). Aliquots of the supernatant were collected and kept at -80°C until analysis of adenosine by HPLC and ATP by a bioluminescence assay. The adenosine samples were analyzed in triplicate by reverse-phase HPLC with UV detection at 254 nm as previously described by Conde and Monteiro (2). ATP samples were analyzed in triplicate during 1 min by a bioluminescence assay as previously described by Conde et al. (6).

Recording of CSN activity. The CB-CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon) and perfused with Tyrode bicarbonate equilibrated with 20% O_2 -5% CO_2 -balanced N_2 at 37°C. Extracellular recordings from single- or multiple-fiber filaments of CSN were made using a suction electrode. The pipette potential was amplified (Neurolog Digimitec, Hertfordshire, UK), filtered (1 kHz), digitized at 6 kHz (Axonscope, Axon Instruments, Molecular Devices, Wokingham, UK), and stored on a computer. Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxia (normoxia: 20% O_2 + 5% O_2 + 75% N_2 ; hypoxia: 2% or 7% O_2 + 5% CO_2 + balanced N_2). CSN unit activity was converted to logic pulses, which were summed every second and converted in a voltage proportional to the sum. The effects of suramin (50 μM) and ZM 241385 (300 nM) on the CSN activity were studied while perfusing the preparations with normoxic (20% O_2 -equilibrated) and hypoxic (2 and 7% O_2 -equilibrated) solutions. Chamber oxygen tension was quantified with a needle electrode (no. 760, Diamond Micro Sensors, Ann Arbor, MI) polarized to -0.8 V against a Ag/AgCl reference electrode placed in the recording chamber. The oxygen electrode current was also digitized in the same manner as above.

Drugs and chemicals. AOPCP, adenosine, ATP, EDTA, EHNA, NBTL, pentobarbital sodium, suramin, and thapsigargin were all from Sigma (St. Louis, MO). ZM 241385 was obtained from AstraZeneca (UK). NBTL and ZM 241385 were dissolved in DMSO in 10 mM and 5 mM stock solution, respectively. The final concentration of DMSO was always below 1/500, which by itself lacks effects on our preparations.

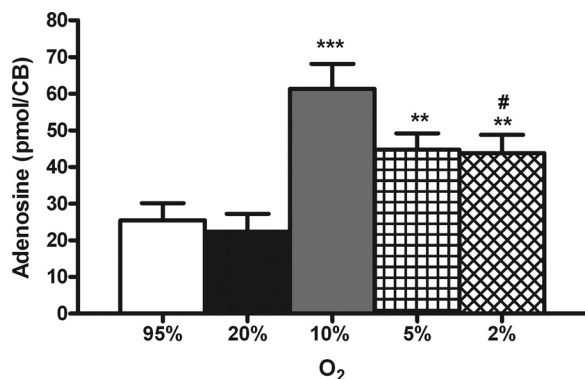


Fig. 1. Release of adenosine from the rat carotid body (CB) during a period of incubation of 10 min. The drawing shows the release response to the different oxygen percentages (2, 5, 10, 20, and 95%) present in the gas mixtures used to equilibrate the incubating solutions (all gas mixtures contained 5% CO₂ and were balanced with N₂). Experiments were performed in the presence of the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), at a concentration of 2.5 μM. Vertical bars represent means ± SE. *** $P < 0.01$, ** $P < 0.05$; one-way ANOVA with Dunnett's and Bonferroni multiple comparison test, comparing adenosine levels in response to different O₂ concentrations with adenosine levels in 20% O₂; and comparing adenosine release in 10% O₂ with other hypoxic intensities, respectively.

Data analysis. The amount of adenosine released by the CBs quantified by HPLC was expressed in picomoles per CB after division of the absolute values obtained from the chromatograms by 4 (4 CBs were used in each incubation) and correction to the volume used. Data were evaluated using a Graph Pad Prism Software, version 4, and are presented as means ± SE. The significance of the differences between the means was calculated by unpaired Student's *t*-test and by one-way ANOVA with Dunnett's and Bonferroni multiple comparison tests. *P* values of 0.05 or less were considered to represent significant differences.

RESULTS

Effect of different oxygen percentages on adenosine and ATP extracellular levels in the rat carotid body. Adenosine has a short half-life (<10 s) (28). Thereby, to avoid its degradation by adenosine deaminase, EHNA, an inhibitor of this enzyme, was added in the experiments in which adenosine was quanti-

fied. We have previously described that the CB releases adenosine when incubated with mild hypoxic solutions (10% O₂) while control tissues, superior cervical ganglion and carotid artery, did not (2). Nevertheless, the effect of high-intensity hypoxia on the release of adenosine is unknown. Figure 1 shows the effect of different oxygen percentages in the gas mixtures used to equilibrate the incubating solutions on the release of adenosine from CB. Extracellular adenosine concentrations in the incubating solutions obtained in basal conditions (20% O₂) were 22.37 ± 4.21 pmol/CB ($n = 10$) and did not significantly differ from that obtained in response to hyperoxia (95% O₂, 25.47 ± 4.68 pmol/CB; $n = 6$). As previously described, mild hypoxia (10% O₂) increased the extracellular concentrations of adenosine with the release reaching 61.31 ± 6.82 pmol/CB ($n = 10$), which represents a 174% increase above normoxic release. Hypoxia of higher intensity, like 5% and 2% O₂, increased the release of adenosine by 96% and 95%, respectively, when compared with the basal normoxic release.

Figure 2, A and B, represents the profile of ATP release from CB with the decrement of oxygen. Basal values (20% O₂) for ATP release were 3.88 ± 0.75 pmol/CB ($n = 14$), and as it happens with adenosine release, this value did not significantly differ from the one obtained in response to hyperoxia (4.53 ± 0.58 pmol/CB; $n = 15$). The histogram-like drawing in Fig. 2A evidences the release of ATP increases with the increasing intensity of hypoxia (or decreasing O₂ percentage in the gas mixtures used to equilibrate the incubating solutions). Figure 2B shows that in fact the release of ATP follows the well-known exponential-like pattern of responses of the carotid body to hypoxia, and that in all O₂ ranges from normoxia to severe hypoxia the release response is lineal (r^2 of 0.972). Mean ATP concentrations evoked by 10% O₂, 5% O₂, and 2% O₂ were, respectively, 9.59 ± 1.53 , 13.44 ± 2.41 , and 17.93 ± 1.99 pmol/CB (Fig. 2A). In another group of experiments we studied the effects of drugs that should be used in forthcoming experiments to study the origin of adenosine and its effects on the release of ATP.

Figure 2C shows that NBTI, the inhibitor of the ENT system at the concentration to be used in the experiments below, did

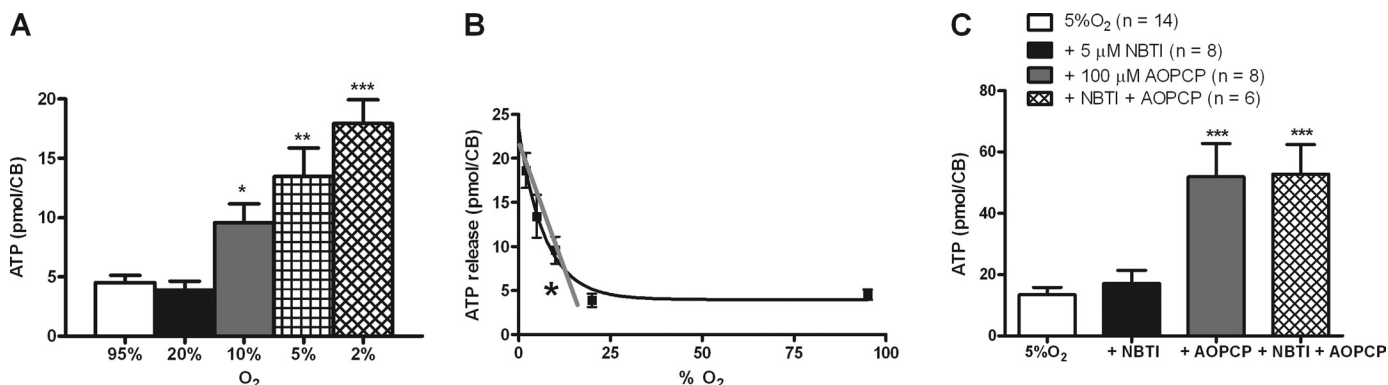


Fig. 2. Effects of low PO₂ on ATP release from the rat carotid body. A: release response to the different oxygen percentages (2, 5, 10, 20, and 95%) present in the gas mixtures used to equilibrate the incubating solutions (all gas mixtures contained 5% CO₂ and were balanced with N₂). ATP released in response to different percentages of oxygen (2, 5, 10, 20, and 95%). B: exponential relationship between PO₂ and the release of ATP. The drawing also shows that in the range of normoxia and hypoxia the release of ATP from chemoreceptor cells correlates linearly with O₂ concentrations ($r^2 = 0.972$). C: effect of NBTI (5 μM), an inhibitor of equilibrative nucleoside transport system, and AOPCP (100 μM), an inhibitor of ectonucleotidases, on the release of ATP evoked by 5% O₂ in the carotid body. Vertical bars represent means ± SE. * $P < 0.05$, ** $P < 0.01$; one-way ANOVA with Dunnett's multicomparison test between ATP levels in response to different oxygen concentrations compared with ATP levels in 20% O₂; *** $P < 0.001$.

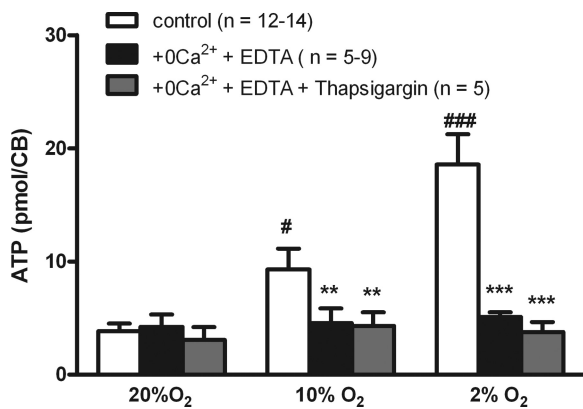


Fig. 3. Extra- and intracellular Ca^{2+} dependence of the release of ATP from rat CB in basal (20% O_2) and in mild (10% O_2) and intense (2% O_2) hypoxic conditions. Experiments were performed in Ca^{2+} -free solutions and with a Ca^{2+} -chelating agent, EDTA (10 mM), in the presence and in the absence of thapsigargin (1 μM). Experiments were performed in the presence of 5% CO_2 . Vertical bars represent means \pm SE. $**P < 0.01$ and $***P < 0.001$, and $\#P < 0.05$ and $###P < 0.001$: two-way ANOVA with Bonferroni multicomparison test between ATP levels in control conditions (Ca^{2+} -containing solutions + drugs) with ATP levels in the absence of Ca^{2+} and in the presence of drugs and ATP release in basal conditions (20% O_2) vs. ATP release in hypoxic conditions (10% and 2% O_2), respectively.

not affect the release of ATP induced by 5% O_2 . Figure 2C also shows that the AOPCP, the ecto-5'-nucleotidase inhibitor at the concentrations used in experiments that will follow, resulted in an increase in the ATP levels measured implying that in fact ecto-5'-nucleotidase is being inhibited by this compound. Combination of NBTI and AOPCP resulted in a comparable increase in the levels of ATP, confirming the lack of effect of NBTI on the release of ATP.

Effect of extracellular calcium and intracellular calcium deposits on the release of ATP from rat carotid body. ATP can be released from cells through different mechanisms that are Ca^{2+} -dependent or independent (16). The contribution of extra- and intracellular Ca^{2+} to the release of ATP from the CB was investigated in normoxia and in response to different intensities of hypoxia (10 and 2% O_2). The contribution of extracellular Ca^{2+} to ATP release in the CB was assessed measuring ATP in Ca^{2+} -free solutions in the presence of EDTA (10 mM) to chelate the free Ca^{2+} present in the extracellular incubating solution. As shown in Fig. 3, the elimination of Ca^{2+} from extracellular media and the Ca^{2+} -chelation did not modify the basal spontaneous release of ATP from the CB, meaning that extracellular Ca^{2+} did not contribute to the release of ATP during normoxia. However, the release of ATP from CB in response to mild (10% O_2) and intense (2% O_2) hypoxia was totally dependent on extracellular Ca^{2+} , since the absence of Ca^{2+} and the presence of EDTA decreased significantly the release of ATP by 50.93% and 72.53%, respectively, to reach levels similar to basal ATP release (Fig. 3). To investigate a possible contribution of intracellular Ca^{2+} deposits to the release of ATP both in normoxia and hypoxia, we have used thapsigargin (1 μM), an inhibitor of endoplasmic reticulum Ca^{2+} -ATPases (Fig. 3). As it can be observed, thapsigargin did not modify either the ATP basal release or the release evoked by hypoxia (10 and 2% O_2).

Effect of different hypoxic intensities on the metabolic pathways of adenosine production in the rat carotid body. We have previously described that during mild hypoxia (10% O_2)

$\approx 45\%$ of the adenosine present in the extracellular media comes from the release of this nucleoside per se through the ENT system (2, 8). As shown in Fig. 4A we have confirmed those results by showing that AOPCP (an inhibitor of ecto-5'-nucleotidases; 100 μM) decreased the release of adenosine evoked by 10% O_2 by 43.9%, implying that up to 55% of adenosine in the present experiments might be released to the extracellular milieu via ENT (see also Fig. 4D above 10% O_2). AOPCP (100 μM) together with NBTI (an inhibitor of ENT; 5 μM) decreased the release by 88.9%, almost completely abolishing the release of adenosine. Together, these results indicate that $\sim 11\%$ of the released adenosine to the extracellular milieu has an unidentified origin that could not be determined in the present experiments. In response to moderate hypoxia (5% O_2 ; Fig. 4B), NBTI (5 μM) caused a nonsignificant 21.7% decrease in the release of adenosine, i.e., ENT contributes to release of adenosine evoked by 5% O_2 by $\approx 20\%$. AOPCP (100 μM) at 5% O_2 decreased the release of adenosine from CB by 53.5%, indicating that extracellular catabolism of ATP contributes $\approx 54\%$ to the adenosine pool present extracellularly in hypoxic conditions (Fig. 4D above 5% O_2). Inhibition of the two pathways of adenosine production through simultaneous incubation with AOPCP plus NBTI reduced the adenosine in the extracellular media by 73.8% (Fig. 4B). These results obtained under 5% O_2 stimulation indicate that in these conditions there is around a quarter of the adenosine in the milieu whose origin remains undefined. In response to a hypoxia of higher intensity, 2% O_2 , NBTI (5 μM) did not modify the release of adenosine from the CB ($\approx 8\% \pm 5$; Fig. 4C), i.e., the contribution of ENT to release adenosine under intense hypoxia is negligible. On the other hand, AOPCP (100 μM) reduced the adenosine concentrations found in extracellular media by 73.8% (Fig. 4C), indicating that extracellular catabolism of ATP is the major contributor for the adenosine release evoked by 2% O_2 . The inhibition of the two pathways together with AOPCP plus NBTI resulted in a $\approx 80\%$ decrease in the release of adenosine, implying that $\approx 20\%$ of the adenosine released remains of undefined origin.

Effect of hypoxic intensity on the contribution of adenosine and ATP for carotid sinus nerve chemosensory activity. The next group of experiments was designed to assess the contribution of ATP and adenosine on the overall output of the CB measured as chemosensory activity (action potential frequency) in the CSN at different oxygen concentrations. Figure 5, A and B, represents typical recordings of CSN chemosensory activity in response to a high-intensity hypoxia (perfusion with a 0% O_2 -equilibrated solution) and to a mild/moderate hypoxia (perfusion with a 7% O_2 -equilibrated solution), respectively; recordings were made in control drug-free conditions and in the presence of 300 nM of ZM241385 at a concentration that blocks both $\text{A}_{2\text{A}}$ and $\text{A}_{2\text{B}}$ adenosine receptors (5) and in the presence of 50 μM of suramin, a P2X ATP receptor blocker. Note that at high hypoxic intensity suramin was more effective in inhibiting CSN activity than ZM241385; the opposite happened when using a mild/moderate hypoxic stimulus. Figure 5, C and D, represents Po_2 recorded with a microelectrode placed at the side of the preparations in the recording chamber incubated with 0% and 7% O_2 , respectively. Figure 5E shows that A_2 adenosine and P2X ATP antagonists applied alone or together did not modify basal CSN chemosensory activity. Mean basal activity was 2.27 ± 0.31 ($n = 36$) in the absence

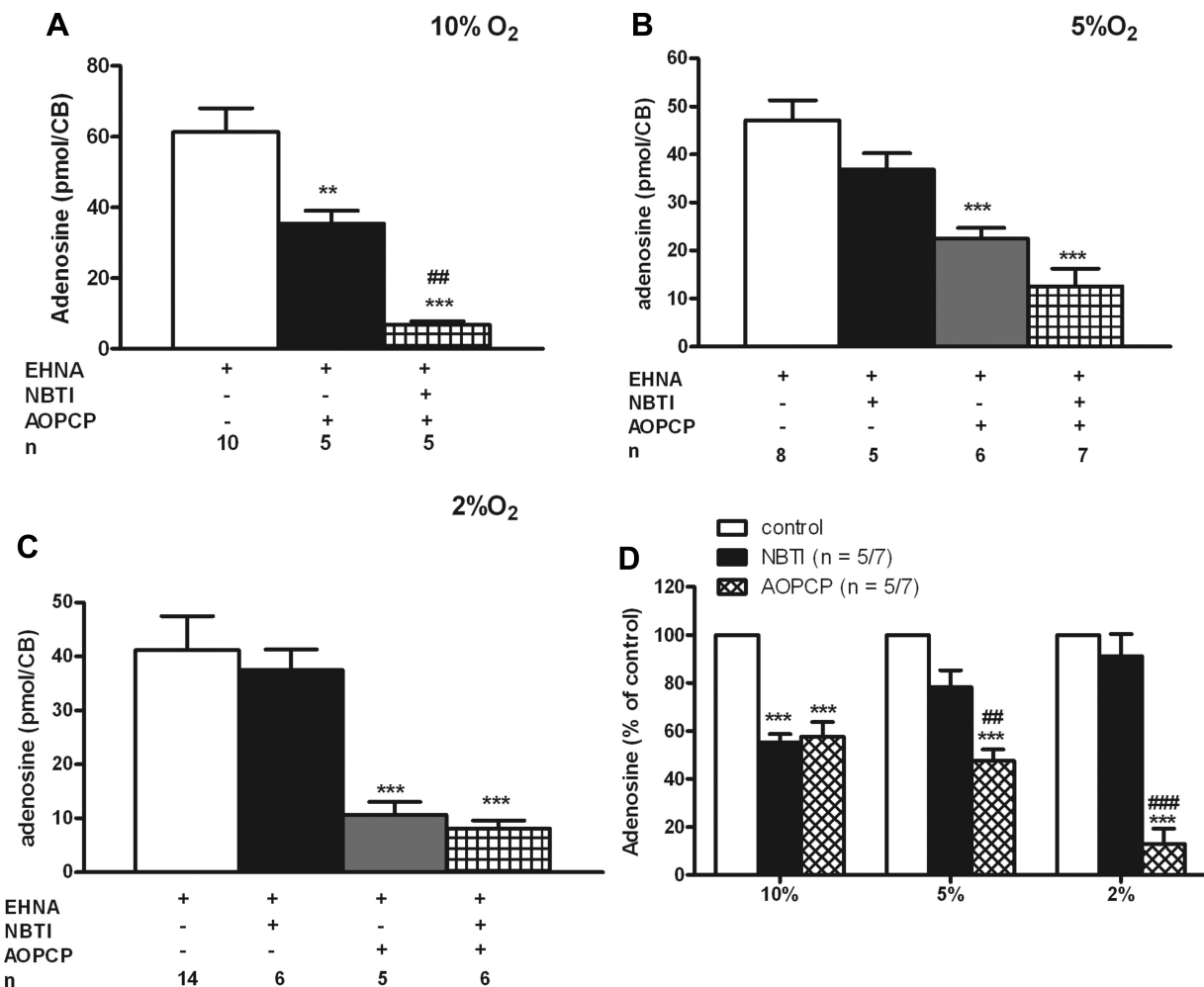


Fig. 4. Effect of an equilibrative nucleoside transport system inhibitor, *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI; 5 μ M), and an inhibitor of ecto-5'-nucleotidase, α , β -methylene ADP (AOPCP; 100 μ M), applied separately or together, on adenosine release by the rat carotid bodies in response to mild (10% O₂) (A), moderate (5% O₂) (B), and high-intensity hypoxia (2% O₂) (C). In D is represented the effect of the intensity of hypoxia on the pathways of adenosine release expressed as a % over control (adenosine release only in the presence of 2.5 μ M EHNA). Vertical bars represent means \pm SE. ** P < 0.01, *** P < 0.001, ### P < 0.01, #### P < 0.001, one-way ANOVA with Dunnett's multiple comparison test between drug-treated groups and control values and between adenosine values in the presence of NBTI and in the presence of AOPCP, respectively.

of antagonists, 2.11 ± 0.28 ($n = 13$) in the presence of ZM241385, 1.98 ± 0.39 ($n = 10$) in the presence of suramin, and 2.039 ± 0.40 ($n = 10$) when both drugs were applied together. Figure 5F represents the peak CSN frequency increase induced by both intensities of hypoxia. CSN frequency reached 50.96 ± 8.47 ($n = 17$) impulses/s in high-intensity hypoxia and 29.78 ± 3.73 ($n = 17$) impulses/s in mild/moderate hypoxia. The contribution of adenosine and ATP for CB output depended on hypoxic intensity (Fig. 5G) as the mean inhibition of the CSN chemosensory activity in mild/moderate hypoxia was 69.24, 36.75% and 88.80% in the presence of ZM241385, suramin, and both drugs applied together, respectively. During high-intensity hypoxia, inhibitions of the CSN chemosensory activity in the presence of ZM241385, suramin, and both drugs applied together were 27.51%, 83.90%, and 93.96%, respectively (Fig. 5G). The application of ZM241385 and suramin alone showed a nonsignificant tendency to increase the onset of the response (latency time) that become significant when the drugs were applied together (Fig. 5H). However, none of the drugs tested modified

significantly the time to reach the maximal activity (time to peak, Fig. 5I).

DISCUSSION

We demonstrated in the present study that adenosine and ATP are key neurotransmitters responsible for the hypoxic chemotransmission in the CB sensory synapse and that their relative contribution depends on the intensity of hypoxic challenge. Our electrophysiological data indicate that ATP contributes more than adenosine to generate CSN activity in high-intensity hypoxias with adenosine showing a more pronounced role during mild/moderate hypoxia. These findings are substantiated by the following additional observations: 1) all hypoxias tested (10%, 5%, and 2% O₂) evoked the release of ATP and adenosine from the CB; 2) adenosine is preferentially released in response to mild hypoxia than in response to higher intensity hypoxias (5% and 2% O₂); 3) ATP release from the CB correlates linearly with the intensity of hypoxia; 4) the percentage of adenosine that is produced from ATP extracellular

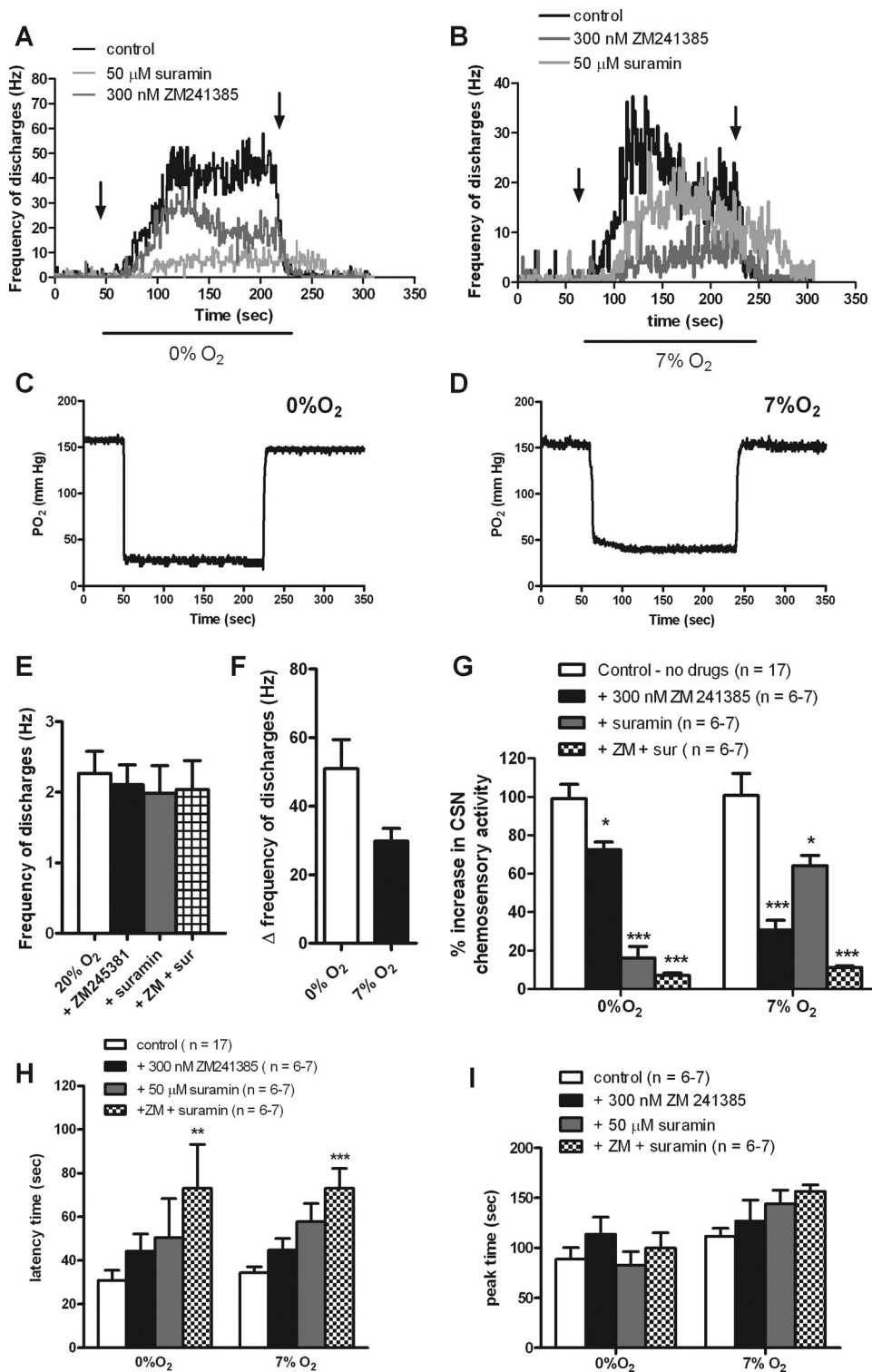


Fig. 5. Effect of ZM 241385 (300 nM), an A₂ adenosine receptor antagonist, and suramin (50 μM), a P2X ATP receptor antagonist, applied alone or together on carotid sinus nerve (CSN) chemosensory activity elicited by high (0% O₂) and mild/moderate hypoxia (7% O₂). *A* and *B*: typical recording of the effect of suramin applied alone or together with ZM241385 on the frequency of action potentials of CSN during superfusion with a solution equilibrated with 0 or 7% O₂, respectively. *C* and *D*: PO₂ curves obtained in the recording chamber during the superfusion with incubating solutions equilibrated with 0 and 7% O₂, respectively. *E–G*: mean basal frequencies, mean peak frequencies, and % of increase in CSN chemosensory activity (100% represents mean peak frequencies evoked by 0 and 7% O₂), respectively. *H* and *I*: latency (the onset of the response) and peak (time to reach maximal activity) time, respectively. Data represent means ± SE (*n* = 4–17; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, 2-way ANOVA with Bonferroni's multicomparison test).

catabolism increased as the levels of O₂ lowered. All these results combined with the fact that antagonists of A₂ adenosine receptors and P2X ATP receptors strongly blocked CSN activity in response to hypoxia suggest that adenosine and ATP are mainly responsible for the hypoxic neurotransmission in the CB, the contribution of each neurotransmitter depending on hypoxic intensity.

The observation that suramin, a P2X ATP antagonist, inhibits CB output in response to hypoxia and that this inhibition increases with the intensity of hypoxia (Fig. 5*E*) is in agreement with the previously described role for ATP as one of the key neurotransmitters involved in hypoxic chemoreception in the CB (29, 34, 36, 47) and with the more recent observations of Rong et al. (36); these authors showed that the attenuation of

the ventilatory responses in mice lacking ATP P2X₂ receptors was proportional to the intensity of hypoxia applied (i.e., the more intense the hypoxia given to the knockout mice, the greater the percentage attenuation of ventilation compared with wild-type animals). In the present study suramin did not significantly modify CSN basal activity (Fig. 5C), suggesting that ATP does not contribute to the steady basal CSN chemosensory activity in the adult rat, which is in agreement with previous observations by Reyes et al. (34) as they showed that the application of mecamylamine and suramin did not significantly modify CSN basal activity in the cat. However, recently Niane et al. (29) described that suramin decreased in 80% basal CSN chemosensory activity in newborn rats. The explanation to these differences can only rely on developmental differences. It should be noted that in the study by Niane et al. (29) the effect of ATP was age independent in the range of 4–21 days; however, previous studies of the same authors (11) have shown that basal, as well as hypoxia-induced CSN activity, increases age dependently. Thus it can be suggested that just like occurs in the inner ear [where ATP signaling initiates bursts of action potentials in auditory nerve fibers before the onset of hearing (41)], ATP in the CB can trigger activity before the synaptic connections between chemoreceptor cells and CSN terminals are fully mature, this being a developmental way of purinergic signaling substituted by strictly synaptic mechanisms in fully mature animals. This hypothesized developmental ATP signaling in the CB might have a not yet recognized significance in the maturation of the organ.

We have previously described that adenosine contributes ~55% to the CSN chemosensory activity in response to moderate hypoxia (5% O₂) through an action on A_{2A} and A_{2B} receptors (5). The results herein described are in the same line of evidence and show that the contribution of adenosine to generate hypoxic CSN chemosensory responses decreases with the intensity of hypoxia. The decrease of adenosine contribution to CSN activity according to the intensity of hypoxia correlates perfectly with the maximal adenosine release attained at 10% O₂ (Fig. 1). Since both A_{2A} and A_{2B} receptors are involved in hypoxic CSN response (5), we have used ZM241385 in a concentration that is ≥10 times higher than the described K_d for A_{2B} and 100 times higher for A_{2A} adenosine receptors and therefore we are confident that we are in the presence of a maximal inhibition of both receptor subtypes. To block ATP P2X receptors we used suramin, which blocks indistinctly all P2X receptors in a concentration previously used to inhibit ATP responses in CB-petrosal ganglion neuron cocultures (47) and intact CB (34). We cannot make any assumption on the subtype of P2X receptors involved in the CSN response to hypoxia, and in fact, a pharmacological characterization of P2X receptors would be very difficult to execute due to the absence of specific pharmacological tools to distinguish the different subtypes of P2X receptors. However, immunohistochemical studies have detected P2X₂ and P2X₃ receptors in the CB (32, 47), and it has been described that P2X₂ are involved in the CB responses to hypoxia (36). Recently, Niane et al. (29) demonstrated that P2X₃ is involved in the ventilatory and CB chemoreceptor responses in newborn rats as A317491, a new P2X₃ antagonist, inhibits those responses. Therefore, we can postulate that P2X₂, P2X₃, or P2X₂/P2X₃ heterodimers are the receptors involved in ATP actions on CB chemoreceptors. The combined application of

P2X ATP receptors and A₂ adenosine receptor antagonists strongly inhibits (93%) the hypoxic CB chemoreceptor response (Fig. 5E). We did not investigate the mechanism/neurotransmitter responsible for the remaining 7% of CSN chemosensory activity in hypoxia; nevertheless several hypothesis can be raised: 1) other neurotransmitter besides ATP and adenosine is involved in the hypoxic response in the CB; 2) the concentration of suramin used in the present study is not enough to fully inhibit P2X receptors in the CB; or 3) other ATP receptors, P2Y ATP receptors, are involved in the hypoxic CB chemosensory response. These hypotheses remain to be tested.

The release of ATP from CB was first reported by Buttigieg and Nurse (1) in the rat CB slices in response to intense hypoxic stimulations (15–20 mmHg); after that study, our laboratory also described increases in the release of ATP in response to hypoxia (2, 6). However, the effect of different hypoxic intensities on ATP CB release was lacking. In the present work we have found a hyperbolic relationship between PO₂ and ATP release in the rat CB (Fig. 2B). A comparable correlation between the release of a neurotransmitter and the intensity of the stimulus has previously been documented in the CB, in the early 1980s, for the release of catecholamines (CA), namely dopamine (DA) (12, 17, 31). Since ATP and DA are costored in chemoreceptor cell dense core-granules along with biogenic amines (20) and follow the same profile of release from the CB chemoreceptor cells, it can be suggested that these neurotransmitters are coreleased from these cells. This being the case, it should be expected that A_{2B} adenosine receptors modulate the release of ATP as they modulate the release of catecholamines from rat CB (5, 7). Vesicular costorage and corelease of ATP with classical neurotransmitters is a well-documented observation (18, 23, 44) both in the periphery and in the central nervous system. In the CB, in addition to DA-ATP corelease, acetylcholine (ACh)-ATP corelease from chemoreceptor cells (47) has been postulated; it has been also proposed that these two transmitters were responsible for the genesis of the CB response to hypoxia (see below).

It is known that mild hypoxia induces the release of adenosine from CB chemoreceptor cells in the rat (2); however, the effect of hypoxic intensity on adenosine release by the CB was not known. Herein we observed that maximal effect on adenosine extracellular concentrations was achieved with mild hypoxia (10% O₂). In fact, the profile of adenosine release from CB perfectly matches the contribution of adenosine to hypoxic CSN activity, as ZM241385, an A₂ adenosine receptor antagonist, inhibits CSN activity with higher efficacy in mild hypoxia than in intense hypoxia. The maximal release of adenosine during mild hypoxia is in agreement with the previous observation that the effects of the adenosine agonist, NECA, and adenosine nonselective receptor antagonist, caffeine, on the release of ³H-CA from the CB chemoreceptor cells, were only detectable in normoxia and in response to mild/moderate stimulus (5, 7, 8). Previously we had attributed this effect to an increased release of catecholamines from the CB during intense hypoxia that would mask the effect of adenosine on the release of DA through the interaction between A_{2B}-D₂ receptors (7, 8). However, it should now be added that the concentration of adenosine present in the extracellular media is not high enough to activate the A_{2B} receptors and to modulate the release of DA when chemoreceptor cells are

strongly stimulated. Hence, we suggest that during intense hypoxia, when adenosine concentration in the extracellular media is lower, the effect of this nucleoside will be predominantly postsynaptic on A_{2A} adenosine receptors.

Extracellular adenosine comes from the extracellular production through ATP catabolism via ecto-5'-nucleotidases and by its intracellular production and release by the bidirectional ENT system (14), as well as from cAMP that is released into the extracellular media by probenecid-sensitive transporter (see 8). Our results show that during mild hypoxia extracellular adenosine in the CB is equally derived from the extracellular ATP catabolism and from the release through the ENT (44%). Increasing hypoxic intensity reduces the percentage of adenosine that is released by the ENT. At high-intensity hypoxia, adenosine comes preferentially from ATP extracellular degradation, as AOPCP inhibits ≈74% the adenosine present extracellularly and NBTI does not modify the CB adenosine extracellular concentrations. We do not want to finish these commentaries on the origin of adenosine without referring to the small percentage whose origin is not sensitive to simultaneous inhibition of ENT and ectonucleotidases. Since it is well documented that hypoxia augments the cAMP in the CB (31), we suggest that released cAMP might represent a significant source of adenosine (see 42).

In the ongoing discussion we are focusing on adenosine and ATP because they are the subject of our study. This might give the impression that we are neglecting the fact that chemoreceptor cells express a great number of neurotransmitters (17, 20). Particularly significant is the case of dopamine, which is probably the most abundant of all neurotransmitters in chemoreceptor cells, and whose species-dependent role as “modulator,” excitatory or inhibitory neurotransmitter, is well recognized (21). Very relevant also is the case of ACh, which is the first candidate as the prime neurotransmitter between chemoreceptor cells and the CSN sensory nerve endings (for references see 17). As is the case with dopamine, there are also important species differences in the action of ACh and cholinergic agents on CSN activity (e.g., 27) as well as in the density of nicotinic vs. muscarinic receptors (19). Yet, despite these recognized differences, ACh and nicotinic receptors enjoy the favor of many researchers in the CB field as the main players in the communication between chemoreceptor cells and sensory nerve endings of the CB. Recently, however, Gauda and coworkers (15) have observed in the rat, the species used in the present study, that chemoreceptor cells do not express choline acetyltransferase, the ACh-synthesizing enzyme that in the same section was observed to be expressed in autonomic ganglion cells located in the periphery or inside the CB tissue. This finding would suggest that the observed release of ACh in intact organ preparations would in fact be released from autonomic ganglion cells, making impossible the role of ACh for a genuine neurotransmitter between chemoreceptor cell and sensory nerve endings. Additionally, as suggested by Gauda et al. (15), the inferred release of ACh from chemoreceptor cell in culture (e.g., 47) could in fact be the result of a phenotypic change of the cells in the culture conditions. It should be stated that most of the effects seen in different CB preparations in response to ACh, and in general cholinergic agents, might probably be well explained via presynaptic actions modifying the release of ATP and adenosine from chemoreceptor cells (4). As a final word of caution we want to make explicit that

extrapolation of the findings reported in our study to other species should be avoided or made very cautiously (but see Reid et al., 33).

In conclusion, in the CB in response to hypoxia there is a simultaneous release of adenosine and ATP, with the release of ATP and its extracellular catabolism to adenosine being the main origin of extracellular adenosine at high-intensity hypoxia, whereas at mild hypoxia adenosine is also released by the ENT. Also, both adenosine and ATP are the main players of the hypoxic chemotransmission in the CB sensory synapse, the contribution of each neurotransmitter depending on the intensity of hypoxia. Therefore the excitatory effects of adenosine on the CB via A_{2A} and A_{2B} receptors together with the activation of P2X ATP receptors are primordial in the genesis of CSN chemoresponses to hypoxia.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: S.V.C. and C.G. conception and design of research; S.V.C. performed experiments; S.V.C. and R.R. analyzed data; S.V.C. and E.C.M. interpreted results of experiments; S.V.C. prepared figures; S.V.C. and C.G. drafted manuscript; S.V.C., E.C.M., R.R., and A.O. edited and revised manuscript; S.V.C. and C.G. approved final version of manuscript.

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