# The $A_{2B}$-$D_2$ Receptor Interaction that Controls Carotid Body Catecholamines Release Locates Between the Last Two Steps of Hypoxic Transduction Cascade

**S.V. Conde, A. Obeso, E.C. Monteiro and C. Gonzalez**

**Abstract** We have recently demonstrated that adenosine controls the release of catecholamines (CA) from carotid body (CB) acting on $A_{2B}$ receptors. Here, we have investigated the hypothesis that this control is exerted via an interaction between adenosine $A_{2B}$ and dopamine $D_2$ receptors present in chemoreceptor cells and if it is, the location of this interaction on the CB hypoxic transduction cascade. Experiments were performed *in vitro* in CB from 3 months rats. The effect of adenosine $A_{2B}$ and dopamine $D_2$ receptor agonists applied alone or conjunctly, was studied on the basal and evoked release (10% $O_2$ and ionomycin) of CA from CB. We have observed that the inhibitory action of propylnorapomorphine, a $D_2$ selective agonist, on the normoxic and 10%$O_2$-evoked release of CA was abolished by NECA, an $A_2$ agonist, meaning that an interaction between the $D_2$ and $A_{2B}$ receptors controls the release of CA from CB. Further, propylnorapomorphine inhibits the release of CA evoked by ionomycin, being this effect totally reversed by NECA. The present results provide direct pharmacological evidence that $A_{2B}$ and $D_2$ receptors interact to modulate the release of CA from rat CB between the steps of $Ca^{2+}$ entry and increase in intracellular free $Ca^{2+}$, and the activation of exocytosis and neurotransmitter release, of the stimulus-secretion coupling process.

**Keywords** Adenosine · Dopamine · A2b adenosine receptors · D2 dopamine receptors · Dopamine agonists · Adenosine agonists · Dopamine antagonists · Adenosine antagonists

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# 1 Introduction

Carotid bodies are major peripheral chemoreceptor organs sensing changes in blood $O_2$ responding by generating action potentials at the carotid sinus nerve (CSN), which are integrated in the brainstem to induce a hyperventilatory compensatory response. Hypoxia, the physiological CB stimulus increases the release of dopamine (DA) (Vicario et al. 2000) and adenosine (Conde and Monteiro 2004) from rat CB. Adenosine is an excitatory neurotransmitter at the CB, increasing CSN electrical activity and ventilation in several species (McQueen and Ribeiro 1983; Monteiro and Ribeiro 1987; Runold et al. 1990) including humans (Watt and Routledge 1985; Uematsu et al. 2000). Recently we have demonstrated that adenosine mediates up to 60% of the low $PO_2$-induced CSN activity (Conde et al. 2006). About half of this effect was postsynaptic and mediated by $A_{2A}$ receptors and the rest was presynaptic mediated by $A_{2B}$ receptors and associated to an augmentation of the release CA from chemoreceptor cells (Conde et al. 2006).

Ribeiro and McQueen (1983) in their pioneer study for the effects of adenosine in chemoreception found that adenosine potentiated the inhibitory effect of DA on cat CSN activity. Similarly, Monteiro and Ribeiro (2000) in in vivo experiments in the rat observed that the inhibitory effect of $D_2$ dopamine agonists on ventilation was attenuated or even reversed by the blockage of $A_2$ receptors. We postulated that this modulatory effect could occur due to an interaction between adenosine $A_{2B}$ and dopamine $D_2$ receptors present in glomus cells. Interactions between $A_{2A}$-$D_2$ receptors are well described in the central nervous system (Ferré et al. 1997; Fuxe et al. 2007) and these findings prompted us to investigate if interactions between $A_{2B}$ and $D_2$ receptors could be involved in the modulation by adenosine of CA release in the CB.

We have previously suggested that $A_{2B}$ receptors operate at the CB in the last steps of the stimulus-secretion coupling process, since caffeine inhibits identically the release of CA from CB in response to high $K^+$ (a non-specific depolarising stimulus) as in response to hypoxia (Conde et al. 2006). These results lead us to investigate with more detail the location of the interaction between $D_2$ and $A_{2B}$ receptors in the CB hypoxic transduction cascade.

# 2 Methods

## *2.1 Animals and Surgical Procedures*

Experiments were performed in Wistar adult rats of both sexes (250–350 g) obtained from *vivarium* of the Faculty of Medicine of the University of Valladolid. The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols. Rats were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), tracheostomized and the carotid arteries were dissected past the carotid bifurcation. The carotid bifurcation was placed in a Lucite chamber in ice-cold 95% $O_2$-equilibrated Tyrode (in mM: NaCl 140; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1.1;

HEPES 10; glucose 5.5, pH 7.40 and the CB was cleaned free of CSN and nearby connective tissue (Vicario et al. 2000). In all instances animals were killed by an intracardiac overdose of sodium pentobarbital.

### *2.2 Labelling of Catecholamines Stores: Release of $^3$H-CA*

In brief, to label CA stores of the chemoreceptor cells the CBs (8-12/experiment) were incubated during 2 hours in Tyrode solution containing the natural precursor $^3$H-tyrosine (30 μM) (specific activity of 45 Ci/mmol) and 6-methyltetrahydropterine (100 μM) and ascorbic acid (1 mM), cofactors of tyrosine hydroxylase and dopamine-β-hydroxylase, respectively. After the labelling period, individual CBs were transferred to vials containing 4 ml of precursor-free Tyrode-bicarbonate solution (composition as above except for the substitution of 24 mM of NaCl by 24 mM of $NaHCO_3$). Solutions were continuously bubbled with 20%$O_2$/5%$CO_2$/75%$N_2$ saturated with water vapour, except when applied hypoxic stimuli. The solutions of the initial incubation periods (3×20 min) were discarded to washout the precursor and the readily releasable pool of labelled CA and thereafter renewed at fixed times (every 10 min) and collected for subsequent analysis of their $^3$H-CA content. Specific protocols for stimulus and drug applications are provided in the Results. Stimulus included hypoxia 10% $O_2$-equilibrated solutions ($PO_2 \approx$ 66 mmHg) and ionomycin. The drugs used were: 5′-(N-ethylcarboxamido)adenosine (NECA, an adenosine $A_2$ agonist; 10 and 100 μM), propylnorapomorphine (a $D_2$ dopamine selective agonist, 200 nM) and ionomycin (an ionophore, 5 μM). Analysis of $^3$H-CA CB release and content was performed as previously described by Vicario et al. (2000). Release data are presented as percentage of $^3$H-CA of the tissue content; this presentation corrects for the potential variations in CB size and/or rate of $^3$H-CA synthesis.

Data were evaluated using a Graph Pad Prism Software, version 4 and were presented as mean ± SEM. The significance of the differences between the means was calculated by One-Way ANOVA with Dunnett's multiple comparison test. *P* values of 0.05 or less were considered to represent significant differences.

## 3 Results

### *3.1 Propylnorapomorphine, a $D_2$ Dopamine Agonist, Inhibits the Basal and Evoked Release of $^3$H-CA from Carotid Body and its Reversal by NECA*

After the initial washout period (see Methods) both basal-normoxic (Fig. 1A) and 10% $O_2$ (Fig. 1B) release of $^3$H-CA follows a monotonic decay. Figure 1C and D show that addition of propylnorapomorphine (200 nM), a $D_2$ selective agonist, to the incubating solutions produced a marked inhibition of the release of $^3$H-CA both

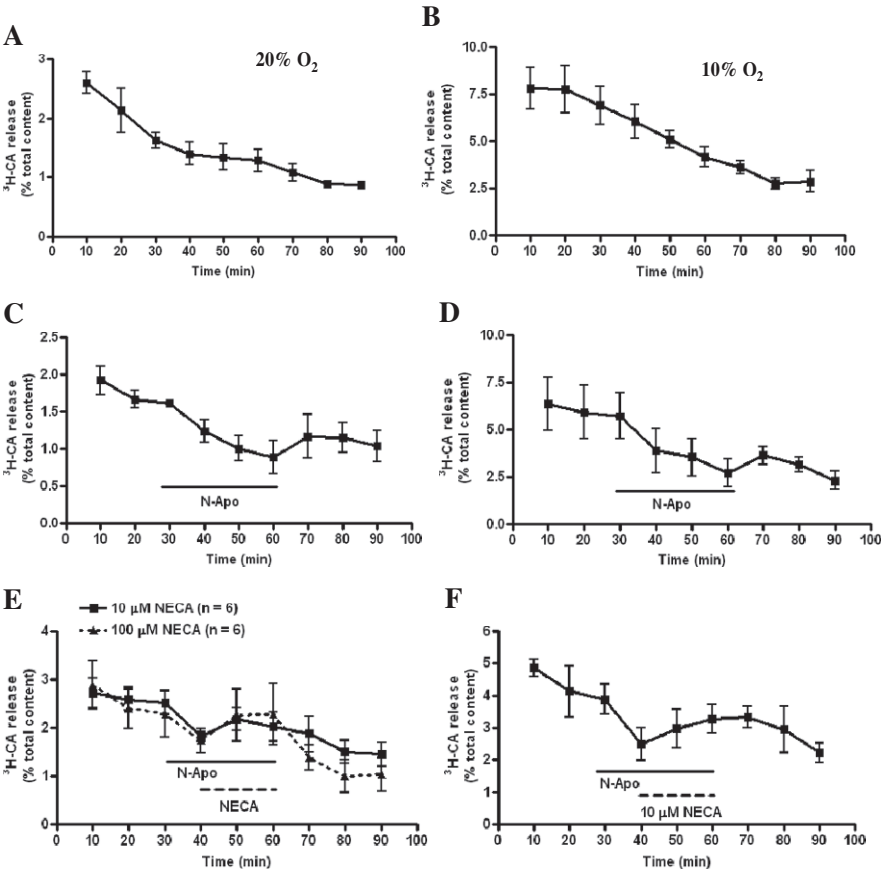


**Fig. 1** Effect of propylnorapomorphine (N-Apo, 200 nM), a $D_2$ dopamine agonist, on the release of $^3$H-CA from the carotid body in normoxic conditions (20% $O_2$) and in response to moderate hypoxia (10% $O_2$) in the absence (C and D) and in the presence of NECA (E and F). (**A** and **B**) Time courses for the release of $^3$H-CA from CB in normoxia and moderate hypoxia, respectively. (**C** and **D**) Effect of 200 nM of N-Apo on the release of $^3$H-CA from CB in normoxia and moderate hypoxia, respectively. (**E** and **F**) shows the reversal of the inhibitory effect of N-Apo on the release of $^3$H-CA from CB in normoxia and moderate hypoxia, respectively by NECA. Note that the partial reversion of inhibitory effect of N-Apo on $^3$H-CA release obtained with 10 μM of NECA is totally abolished with 100 μM of NECA Propylnorapomorphine was applied for 30 min between 30 and 60 min and NECA for 20 min between 40 and 60 min of the experimental protocol. Values represent means ± S.E.M

in normoxia and moderate hypoxia conditions, respectively, that recovered after the elimination of the drug.

In order to investigate if an interaction exists between $A_{2B}$ and $D_2$ receptors that modulate the release of $^3$H-CA from CB we tested NECA on the effect of propylnorapomorphine, on the release of this neurotransmitter from rat CB in normoxia and moderate hypoxia. We have observed that NECA, an $A_2$ agonist, when applied

in a 10 μM concentration attenuated the effect of the $D_2$ agonist on the release of $^3$H-CA from CB in normoxia (Fig. 1E) and in moderate hypoxia (Fig. 1F).

We have observed that NECA when applied in a 10 μM concentration attenuated the effect of the $D_2$ agonist on the release of $^3$H-CA from CB in normoxia (Fig. 1E) and in moderate hypoxia (Fig. 1F). It can also be observed that the inhibitory effect of the $D_2$ agonist on the basal release of $^3$H-CA is totally abolished if the concentration of NECA is increased by 10x (100 μM). These results suggest that an interaction between $A_{2B}$ and $D_2$ receptors modulate the release of $^3$H-CA from CB chemoreceptor cells both in normoxic and moderate hypoxic conditions.

## 3.2 *Effect of Ionomycin on the Release of $^3$H-CA by Carotid Body Modified by Propylnorapomorphine and NECA*

We have previously suggested that $A_{2B}$ receptors operate at the CB in the last steps of the stimulus-secretion coupling process, since caffeine produces identical effects in the response elicited by high $K^+$ and hypoxia (Conde et al. 2006). In order to investigate at which step of the stimulus-secretion process the interaction between $A_{2B}$ and $D_2$ are involved, we studied this interaction on the release of $^3$H-CA evoked by ionomycin. Ionomycin alters cell permeability producing an increase in intracellular $Ca^{2+}$ and, as a consequence, induce a $Ca^{2+}$ dependent vesicular release of CA (Dedkova et al. 2000). This effect can be observed both in Fig. 2A on the time course

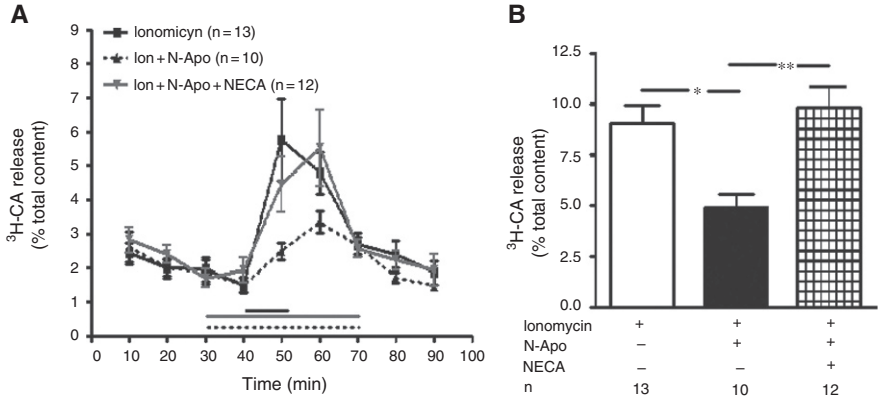


**Fig. 2** Inhibitory effect of propylnorapomorphine, a $D_2$ agonist, on the ionomycin evoked release of $^3$H-CA from CB and reversion of the inhibitory effect by NECA. Panel **A** shows the time course for the effects of 200 nM propylnorapomorphine (N-Apo) and the reversion of this effect by 10 μM of NECA on the release of $^3$H-CA evoked by 5 μM ionomycin. Ionomycin was applied for 10 min between 40 and 50 min of the experiment. N-Apo and NECA were applied 10 min prior and 20 min after stimulation with ionomycin. 0% corresponds to 1.946 ± 0.36, 1.49 ± 0.235 and 1.914 ± 0.36 % of $^3$H-CA total content respectively for ionomycin in the absence and in the presence of N-APO and N-APO plus NECA. Panel **B** shows means ± S.E.M. of the effect of N-Apo in the absence and in the presence of NECA on ionomycin evoked release of $^3$H-CA from CB. $^*P<0.05$, $^{**}P<0.01$; One-Way ANOVA with Dunnett's multi-comparison test

of the $^3$H-CA release as in the magnitude of the increase, in Fig. 2B, where the application of 5 μM of ionomycin increased the release of $^3$H-CA from rat CB by 8.78 ± 1.10%. The application of 200 nM of propylnorapomorphine decreased the $^3$H-CA release from CB evoked by ionomycin by 44% (Fig. 2B). When we tested the effect of NECA on the inhibitory effect of propylnorapomorphine on $^3$H-CA release evoked by ionomycin we observed that the application of 10 μM of NECA completely reversed the inhibition (Fig. 2A and B). These results suggest that $A_{2B}$ and $D_2$ receptors interact to modulate the release of $^3$H-CA from rat CB between the steps of $Ca^{2+}$ entry and increase in intracellular free $Ca^{2+}$, and the activation of exocytosis and neurotransmitter release, of the stimulus-secretion coupling process.

## 4 Discussion

Using a pharmacological approach we have demonstrated the existence of an interaction between $A_{2B}$ and $D_2$ receptors that modulate the release of CA from rat CB, being located between the last two steps of hypoxic transduction cascade, that is, between the steps of $Ca^{2+}$ entry and increase in intracellular free $Ca^{2+}$, and the activation of exocytosis and neurotransmitter release.

At the onset of the discussion we want to state that we have attributed all the effects of NECA to an effect on $A_{2B}$ receptors, because we have previously observed that NECA increased the release of CA both in normoxia and moderate hypoxia, an effect that is not mimicked by $A_1$ or $A_{2A}$ agonists (Conde et al. 2006).

The nature of $D_2$-$A_{2B}$ receptor effects on chemoreceptor cells should be defined as an antagonistic, because the effects of activation of $D_2$ receptors with propylnorapomorphine diminished with increasing concentrations of NECA, which activates $A_{2B}$ receptors. With the results herein described and the protocols used in this work we cannot establish if the interaction is a functional coupling between $A_{2B}$ and $D_2$ receptors, or at adenylyl cyclase level, or at both sites. Nevertheless, we can suggest that a functional coupling between $A_{2B}$ and $D_2$ receptors in chemoreceptor cells could exist, as described in the central nervous system (mostly in the striatum) between $D_2$ and $A_{2A}$ receptors (Ferré et al. 1997; Fuxe et al. 2007).

The interactions previously described between dopamine and adenosine in the CB observed when monitoring CSN activity in the cat (Ribeiro and McQueen 1983) and ventilation in the rat (Monteiro and Ribeiro 2000) have defined $D_2$ as the dopamine receptor involved, but could not identify the subtype of $A_2$ adenosine receptor participating in the interaction. Our data indicate that the subtype of adenosine receptor is the $A_{2B}$ adenosine receptor. We cannot exclude the possibility that interactions between $D_2$ and $A_{2A}$ receptors also exist in the CB. Nevertheless, the interaction herein observed is in agreement with the potentiation of the inhibitory effect of dopamine by adenosine observed in ventilation in the rat (Monteiro and Ribeiro 2000).

A final consideration relates to the fact that $D_2$ interactions, particularly in the central nervous system, are most generally described with $A_{2A}$ receptors. Nevertheless, the interaction between catecholamine autoreceptors and $A_{2B}$ receptors

is not unique to chemoreceptor cells. For example, Talaia et al. (2005) found that adenosine acting via $A_{2B}$ receptors heightens the release of noradrenalin from the sympathetic endings innervating the vas deferens, this effect being annuled by yohimbine, a blocker of presynaptic α2-adrenoreceptors.

Ionomycin is a calcium ionophore, and it is assumed that these substances directly facilitate the transport of $Ca^{2+}$ across the plasma membrane (Himmel et al. 1990; Dedkova et al. 2000). It is generally accepted that in chemoreceptor cells the stimulus-secretion coupling in response to hypoxic stimulus occurs according to following steps (Gonzalez et al. 1992, 1994): (1) $O_2$-sensing at an $O_2$-sensor → (2) activation of coupling mechanisms with $K^+$ channels → (3) change in kinetics of these $K^+$ channels resulting in a decrease in their opening probability → (4) cell depolarization → (5) activation voltage operated channels → (6) $Ca^{2+}$ entry and increase in intracellular free $Ca^{2+}$ → (7) activation of exocytosis and neurotransmitter release. The $Ca^{2+}$ signal induced by ionomycin that produces an increase in $Ca^{2+}$ intracellular concentrations, originating the release of neurotransmitters eliminates an effect of the interaction between $D_2$ and $A_{2B}$ receptors on the entire hypoxic transduction cascade until step 6 in our study. The fact that NECA abolishes the inhibitory effect of propylnorapomorphine on the ionomycin evoked release of $^3$H-CA supports the above suggestion and indicates that the interaction between the $A_{2B}$ and $D_2$ receptors present in CB chemoreceptor cells modulating CA release is between the step 6 and step 7 of the stimulus-secretion coupling process. Nevertheless, clarification of the intimate mechanisms of interaction and action on the exocytotic process would require additional experiments.

To summarise, we can assume that an antagonistic interaction between $A_{2B}$ and $D_2$ receptors exists in the CB that modulates the release of CA from chemoreceptor cells being located between the two last steps of hypoxic stimulus-secretion coupling.

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