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Ecto-5'-nucleotidase, adenosine and transmembrane adenylyl cyclase signalling regulate basal carotid body chemoafferent outflow and establish the sensitivity to hypercapnia.

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Abstract

Carotid body (CB) stimulation by hypercapnia causes a reflex increase in ventilation and, along with the central chemoreceptors, this prevents a potentially lethal systemic acidosis. Control over the CB chemoafferent output during normocapnia and hypercapnia most likely involves multiple neurotransmitters and neuromodulators including ATP, acetylcholine, dopamine, serotonin and adenosine, but the precise role of each is yet to be fully established. In the present study, recordings of chemoafferent discharge frequency were made from the isolated *in vitro* CB in order to determine the contribution of adenosine, derived specifically from extracellular catabolism of ATP, in mediating basal chemoafferent activity and responses to hypercapnia. Pharmacological inhibition of ecto-5'-nucleotidase (CD73), a key enzyme required for extracellular generation of adenosine from ATP, using α,β -methylene ADP, virtually abolished the basal normocapnic single fibre discharge frequency (superfusate $PO_2 \sim 300$ mmHg, $PCO_2 \sim 40$ mmHg) and diminished the chemoafferent response to hypercapnia ($PCO_2 \sim 80$ mmHg). These effects were mimicked by the blockade of adenosine receptors with 8-(p-sulfophenyl) theophylline. The excitatory impact of adenosinergic signalling on CB hypercapnic sensitivity is most likely to be conferred through changes in cAMP. Here, inhibition of transmembrane, but not soluble, adenylate cyclases, by SQ22536 and KH7 respectively, produced a rapid reduction in normocapnic single fibre activity and inhibited the elevation evoked by hypercapnia by approximately 50%. These data therefore identify a functional role for CD73 derived adenosine and transmembrane adenylate cyclases, in modulating the basal chemoafferent discharge frequency and in priming the CB to hypercapnic stimulation.

1 Introduction

The mammalian carotid bodies (CBs) are the primary peripheral chemoreceptors that respond to acute hypoxia, and stimulation drives the reflex hyperventilation that acts to preserve O₂ delivery and metabolism in the brain and vital organs (Kumar, 2009). However, the importance of the CB in the regulation of breathing is not limited to hypoxia. Continuous chemoafferent input from CB into the central nervous system (CNS) is thought to account for up to 60% of eupneic ventilation (Blain *et al.*, 2009). Furthermore, the CBs are activated by other blood stimuli including hypercapnia and acidosis, both of which are potentially lethal if not adequately countered.

It was originally proposed that approximately 30-50% of the reflex ventilatory response to arterial hypercapnia was mediated through direct stimulation of the CB (Heeringa *et al.*, 1979; Rodman *et al.*, 2001), with the remaining contribution arising from chemoreceptors located in the CNS (Nattie, 1999). However, a more recent investigation has shown that a complete silencing of the CB chemoafferent output significantly depresses the central CO₂ chemoreceptor sensitivity (Blain *et al.*, 2010). Therefore, in addition to direct hypercapnic CB excitation, maintenance of a tonic CB chemoafferent signal into the CNS seems to have an important role in establishing the hypercapnic sensitivity of the central chemoreceptors and further emphasises the importance of the CB in blood CO₂ and pH homeostasis.

Control over the CB chemoafferent output in basal conditions and during hypercapnia most likely involves multiple neurotransmitters and neuromodulators including ATP, acetylcholine, dopamine, serotonin and adenosine (Nurse, 2010), but the contribution of each is yet to be fully characterised. Despite adenosine being an established neuromodulatory substance in the central nervous system (Cunha, 2001), its source and physiological role in the CB remains somewhat unresolved. Up to now the majority of investigations have focused on the action of adenosine in mediating the CB response to acute changes in O₂ tension (McQueen & Ribeiro, 1986; Conde *et al.*, 2006; Conde *et al.*, 2012) or the adaptations following chronic hypoxia (Livermore & Nurse, 2013). However, the physiological importance and source of endogenous adenosine in establishing basal CB chemoafferent activity and responses to hypercapnia is unclear.

Physiologically active concentrations of adenosine, present in the synapse between the CB type I cell and the adjacent chemoafferent neurone, may originate from multiple sources. First, adenosine may be generated via extracellular catabolism of ATP; an excitatory neurotransmitter that is secreted from type I and type II cells (Zhang *et al.*, 2012; Piskuric & Nurse, 2013). This conversion requires two key enzymes; ectonucleoside triphosphate diphosphohydrolyase 1 (CD39) and ecto-5'-nucleotidase (CD73) (Bianchi & Spychala, 2003). Second, adenosine may be formed in the type I cell and then released into the synapse through the bidirectional equilibrative nucleotide transporter (ENT) (Cass *et al.*, 1998). Measurements of whole organ adenosine suggest a possible contribution of both sources to the total 'pool' of available adenosine and this may be subject to variation depending on the ambient level of O₂ (Conde & Monteiro, 2004; Conde *et al.*, 2012). This current study aims to more clearly define the

functional importance of endogenous adenosine specifically generated from extracellular catabolism of ATP in modulating the sensory neuronal output of the CB, both under basal conditions and during hypercapnia.

The four G-protein coupled adenosine receptors cloned to date (A_1 , A_{2A} , A_{2B} and A_3) all exert their actions through inhibition or excitation of transmembrane adenylyl cyclases (tmACs) and production of cAMP (reviewed in (Ribeiro & Sebastiao, 2010)). Thus any functional role of adenosine is likely to be conferred through modifications in cAMP accumulation. We have previously reported that the CB expresses numerous different tmAC mRNA transcripts (Nunes *et al.*, 2013) as well as the soluble AC (sAC) isoform (Nunes *et al.*, 2009). CB cAMP is elevated during hypercapnia (Perez-Garcia *et al.*, 1990) and, in isolated type I cells, cAMP analogues potentiate inward Ca^{2+} current in a manner that is quantitatively similar to hypercapnia (Summers *et al.*, 2002). Here we investigate whether direct targeting of tmAC or sAC impairs the CB chemoafferent outflow in normocapnia and/or hypercapnia.

2 Methods

2.1 Ethical approval

All surgical procedures were performed in accordance with project and personal licences issued under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Biomedical Services Unit at the University of Birmingham.

2.2 Extracellular recordings of single and few-fibre chemoafferent neurones

The whole carotid bifurcation along with the attached carotid sinus nerve (CSN) and CB were isolated from adult male Wistar rats (50–200 g) under inhalation anaesthesia (2–4% isoflurane in O_2). Following tissue removal, animals were immediately killed by exsanguination. The bifurcations were rapidly transferred into an ice cold bicarbonate buffered extracellular Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4 , 5 Na_2SO_4 , 1.3 $MgSO_4$, 24 $NaHCO_3$, 2.4 $CaCl_2$, 11 D-glucose, equilibrated with 95% O_2 and 5% CO_2 .

Extracellular recordings of single or few-fibre chemoafferent activity were made from the cut end of the CSN using glass suction electrodes pulled from GC150-10 capillary glass (Harvard Apparatus, Edenbridge, UK). The chemoafferent derived voltage was recorded using a CED micro1401 and visualised on a PC with Spike2 software (Cambridge Electronic Design, Cambridge, UK). The chemoafferent voltage signal was sampled at 15000 Hz. Using the in-built wavemark analysis in the Spike2 software, electrical activity originating from a single chemoafferent fibre was determined by its unique 'wavemark' signature based on frequency, shape and amplitude.

2.3 Experimental solutions and drugs.

During experimentation, whole CBs were continuously superfused with a standard bicarbonate buffered Krebs solution containing, in mM: 115 NaCl, 4.5 KCl, 1.25 NaH_2PO_4 , 5 Na_2SO_4 , 1.3 $MgSO_4$, 24 $NaHCO_3$,

2.4 CaCl₂ and 11 D-glucose. All solutions were heated to 37°C using a water bath (Grant W14; Grant Instruments, Cambridge, UK) and had a pH of 7.4 except during hypercapnic acidosis.

Chemoafferent responses to hypercapnia acidosis were induced by raising the superfusate PCO₂ from approximately 40 mmHg (pH 7.4) to 80 mmHg (pH 7.15) at a constant PO₂ (300 mmHg), as has been previously reported (Bin-Jalilah *et al.*, 2005). Since the chemoafferent response to hypercapnia is thought to peak initially and then adapt to a lower sustained frequency (Black *et al.*, 1971), measurements of chemoafferent activity were taken from the fifth minute of the hypercapnic stimulus, after a relatively steady state frequency had been achieved. CO₂ sensitivity was subsequently calculated as the increase in single fibre discharge frequency per mmHg increase in superfusate PCO₂ (Δ Hz / mmHg PCO₂), given that the rise in discharge frequency is linear over this PCO₂ range (Biscoe *et al.*, 1970; Fitzgerald & Parks, 1971; Pepper *et al.*, 1995; Vidruk *et al.*, 2001).

2.4 Analysis of data

Values are expressed as mean \pm standard error of mean unless otherwise stated. Statistical analysis was performed using i) a paired 2-tailed student's t-test or ii) repeated measures one way Analysis of Variance (ANOVA) with Bonferroni or Dunnett's post hoc analysis where appropriate (Prism v5; GraphPad Software, La Jolle, CA, USA). Significance was taken as $p < 0.05$.

3 Results

3.1 Basal chemoafferent outflow is dependent on ecto-5'-nucleotidase (CD73) activity and adenosinergic signalling.

Basal chemoafferent output from the CB provides the peripheral component of the drive to breathe. Initial experiments sought to determine whether or not adenosine generated from extracellular ATP catabolism has any part in establishing this sensory neuronal activity of the CB. Pharmacological targeting of ecto-5'-nucleotidase (CD73; a key membrane bound enzyme involved in the formation of adenosine from extracellular ATP) using the inhibitor α,β -methylene ADP (AOPCP; 100 μ M; Conde & Monteiro, 2004) had a striking effect on basal single fibre activity, diminishing the discharge frequency by $91.2 \pm 3.8\%$ (Fig. 1a *upper* & 1b). A similar ($93.9 \pm 1.9\%$) reduction in basal frequency was observed in the presence of the adenosine receptor antagonist 8-(p-sulfophenyl) theophylline (8-SPT, 300 μ M; Wyatt *et al.*, 2007) (Fig. 1a *middle* & 1b). The four G-protein coupled adenosine receptors cloned to date (A₁, A_{2A}, A_{2B} and A₃) all exert their actions through inhibition or excitation of transmembrane adenylyl cyclases (tmACs) and modification of cAMP production. In the current investigation, the presence of 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536; a tmAC inhibitor; 200 μ M, IC₅₀ = 20 μ M; Rocher *et al.*, 2009) caused a $70.2 \pm 16.0\%$ decrease in basal single fibre frequency (Fig. 1a *lower* & 1b). This is consistent with the idea that tonic generation of adenosine from ATP modulates basal CB neuronal outflow through production of cAMP.

3.2 Chemoafferent responses to hypercapnia are dependent on ecto-5'-nucleotidase (CD73) activity, adenosinergic receptor stimulation and transmembrane adenylyl cyclase production of cAMP.

Experiments were designed to establish or rule out a potential role for endogenous adenosine in mediating the heightened sensory neuronal activity of the CB in hypercapnia. Selective inhibition of CD73 with AOPCP (100 μ M) diminished the single fibre discharge frequency recorded in both normocapnic (40 mmHg PCO₂) and hypercapnic (80 mmHg PCO₂) conditions (Fig. 2a & b). Furthermore, AOPCP caused a dramatic ($98.0 \pm 1.8\%$) reduction in CB CO₂ sensitivity (Fig. 2c). This inhibitory effect was rapidly reversed once the drug was removed from the superfusate as demonstrated in the raw trace example in Fig. 2a. Similar observations were made in the presence of 8-SPT (300 μ M). 8-SPT significantly attenuated chemoafferent activity in both normocapnia and hypercapnia (Fig. 2d) and markedly decreased the CB CO₂ sensitivity by $81.5 \pm 5.8\%$ (Fig. 2e).

As with the basal discharge, the excitatory impact of adenosinergic signalling on CB hypercapnic sensitivity is most likely to be conferred through activation of tmACs coupled to the adenosine receptors. Here, addition of the tmAC inhibitor SQ22536 (200 μ M), produced a rapid reduction in normocapnic single fibre activity and inhibited the elevation evoked by hypercapnia (Fig. 3a & b). In every fibre tested the absolute increase in hypercapnic discharge was reduced in the presence of SQ22536. Accordingly, further analysis showed that SQ22536 (200 μ M) produced a $47.1 \pm 9.4\%$ decline in CB CO₂ sensitivity (Fig. 3c). Normal hypercapnic responses were readily restored following removal of the agent from the superfusate, as exemplified in Fig. 3a. In contrast, the soluble adenylyl cyclase (sAC) antagonist, 2-(1H-Benzo[d]imidazol-2-ylthio)-N'-(5-bromo-2-hydroxybenzylidene)propanehydrazide (KH7; 10 μ M; Nunes *et al.*, 2013), failed to impact on basal chemoafferent activity or the response to hypercapnia (Fig. 3d & e). KH7 (10 μ M) did not alter CB CO₂ sensitivity even after prolonged incubation (Fig. 3d & f). These data therefore suggest that cAMP production mediates a component of the functional chemoafferent response to hypercapnia and that this is selectively dependent on tmAC rather than sAC activation.

4 Discussion

4.1 CD73, adenosine and tmAC signalling mediate basal CB activity and the sensitivity to hypercapnia.

The findings presented in this study indicate that extracellular adenosine formed selectively through catabolism of ATP, in the presence of ecto-5'-nucleotidase (CD73), has an important neuromodulatory role in mediating the CB sensory neuronal discharge, both under basal conditions and during stimulation by hypercapnia. In addition we show that tmAC but not sAC activity is necessary for full expression of CB hypercapnic sensitivity.

It has been previously reported that inhibition of ATP metabolism in normoxia by AOPCP decreases CB adenosine content (Conde & Monteiro, 2004). Our observations suggest that this source of adenosine is functionally required in order to generate the basal chemoafferent outflow. The impact of adenosine is

most likely to be conferred through modifications in cAMP, given that the A_{2A} and A_{2B} adenosine receptor subtypes expressed in the CB (Gauda *et al.*, 2000; Kobayashi *et al.*, 2000; Conde *et al.*, 2006) are coupled to tmACs (Ribeiro & Sebastiao, 2010). Accordingly, in this investigation pharmacological inhibition of tmACs by SQ22536 caused a significant reduction in basal chemoafferent activity.

The basal sensory output from the CB accounts for up to 60% of the drive to breath at rest (Blain *et al.*, 2009) and may contribute to the resting sympathetic outflow to the vasculature (Kumar & Prabhakar, 2012). Furthermore, it is the chronic rise in basal CB activity following CIH that is thought to drive the hypertension in patients with OSA (Narkiewicz *et al.*, 1998; Peng *et al.*, 2003). It is probable that overall chemoafferent output in normoxia is dependent on both spontaneous pre-synaptic (type I cell) depolarisation and neurotransmitter release, along with subsequent post-synaptic receptor activation. Chemoafferent neurones may also generate a degree of spontaneous activity. In large clusters of type I cells, spontaneous cellular depolarisation is reported to be a consequence of an attenuation of the background K⁺ current, which is itself regulated by local 5-HT and PKC activation (Zhang & Nurse, 2000; Zhang *et al.*, 2003). The observations presented here suggest an additional signalling mechanism that is dependent on adenosine formation and tmAC mediated cAMP production.

It is becoming more apparent that the CB has a principal role in countering rises in arterial CO₂. As well as direct stimulation of the CB accounting for approximately 30-50% of the reflex hypercapnic ventilatory response (Heeringa *et al.*, 1979; Rodman *et al.*, 2001), it has been recently shown that the CB chemoafferent outflow is necessary in establishing the CO₂ sensitivity of the central medullary chemoreceptors (Blain *et al.*, 2010). Despite these important findings, the full transduction mechanisms that lead to an increase in CB discharge frequency during hypercapnia are still elusive. The results described here provide evidence that the heightened chemoafferent activity in hypercapnia is dependent on CD73 mediated catabolism of ATP to form adenosine. The principal origin of the synaptic ATP is most likely to be the type I cell given that ATP is secreted as a neurotransmitter in both normocapnic (Conde *et al.*, 2012) and hypercapnic conditions (Zhang & Nurse, 2004). Interestingly, in chemosensitive regions of the brainstem, hypercapnia-evoked ATP release occurs through the gap junctional protein, connexin26 (Cx26) (Huckstepp *et al.*, 2010b). Direct binding of CO₂ prevents pore occlusion and effectively 'traps' Cx26 in an open conformation (Huckstepp *et al.*, 2010a; Meigh *et al.*, 2013). In the CB, recent evidence has identified a mechanism of ATP induced ATP release through pannexin channels in type II cells (Zhang *et al.*, 2012). Whether ATP is released from type II cells in normoxia and/or hypercapnia, thus acting as a substrate for additional adenosine formation, could be an interesting area for future investigating.

We also demonstrate that inhibitory targeting of tmACs also reduced the CO₂ sensitivity of the CB. This is in agreement with the idea that adenosine partially establishes the chemoafferent sensitivity of the CB to hypercapnia through tmAC dependent cAMP production. In the later instance however, a considerable proportion of the response to hypercapnia is preserved, which was in contrast to the results described using 8-SPT and AOPCP. This may have been due to incomplete run-down in tmAC function

using SQ22536 or, more likely, that adenosine has additional actions that are independent of cAMP. These alternative downstream signalling pathways possibly involve activation of PKC or phospholipase C, as has been proposed for a component of the excitatory adenosine neuromodulation in the CNS (Cunha, 2001).

The finding that SQ22536 but not KH7 reduces chemoafferent excitation in hypercapnia, suggests that cAMP regulation of CB CO₂ sensitivity is itself determined by tmAC and not sAC enzymatic activity. Although sAC is present in the CB (Nunes *et al.*, 2009) and is directly stimulated by binding of CO₂ or HCO₃⁻ (Townsend *et al.*, 2009), it appears to have no functional contribution in moderating the chemoafferent frequency in hypercapnia. This is in accordance with our previous findings where KH7 failed to modify CB cAMP content when the organ was exposed to isohydric hypercapnia (Nunes *et al.*, 2013). Thus we propose that rises in CO₂ or intracellular HCO₃⁻ alone are insufficient to stimulate sAC enough to have functional effects on chemoafferent discharge in hypercapnia.

4.3 Conclusions

Endogenous adenosine produced from extracellular catabolism of ATP in the presence of ecto-5'-nucleotidase (CD73) is necessary for the generation of a basal chemoafferent discharge frequency. This basal discharge may be of clinical significance in a number of cardiorespiratory disorders where enhanced CB activity is associated with increased sympathetic outflow and thus with potential increase in patient morbidity and mortality. In addition this source of adenosine and tmAC generation of cAMP, acting downstream of adenosine receptors, are required for the full expression of the CB sensitivity to hypercapnia.

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Figure legends

Fig. 1 The basal chemoafferent discharge frequency of the carotid body (CB) is critically dependent on ecto-5'-nucleotidase (CD73) mediated formation of adenosine. **(a)** Example raw neuronal traces demonstrating the inhibitory impact of three different compounds on basal chemoafferent outflow: AOPCP (100 μ M; inhibitor of CD73), 8-SPT (300 μ M; non-selective adenosine receptor antagonist) and SQ22536 (200 μ M; inhibitor of transmembrane adenylate cyclases). For each trace, overdrawn action potentials are shown inset to exhibit single fibre discrimination. **(b)** Mean single fibre basal frequencies in the presence and absence of each pharmacological agent. Data presented is from 6 fibres (n=6) from 4 CB preparations (AOPCP), 6 fibres (n=6) from 4 CB preparations (8-SPT) and 5 fibres (n=5) from 4 CB preparations (SQ22536). Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control basal discharge frequency; paired Student's t-test.

Fig. 2 Carotid body (CB) responses to hypercapnia are mediated by adenosine generated from ecto-5'-nucleotidase (CD73). **(a)** An example electrophysiological recording of the CB response to 5 minutes of hypercapnia ($PCO_2 = 80$ mmHg) in the presence and absence of the CD73 inhibitor AOPCP. Raw discharge is shown (upper) along with frequency histograms (lower). Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination used to measure frequency. The inhibitory action of AOPCP was readily reversible. **(b)** Mean discharge frequencies recorded in normocapnia ($PCO_2 = 40$ mmHg) and hypercapnia ($PCO_2 = 80$ mmHg), in control conditions and following addition of AOPCP. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. **(c)** Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions and following AOPCP drug application. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; paired Student's t-test. For B) and C) data is from 6 fibres (n=6) from 5 CB preparations. **(d)** Mean discharge frequencies recorded in normocapnia and hypercapnia, in control conditions and in the presence of 8-SPT (300 μ M), the non-selective adenosine receptor antagonist. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. **(e)** Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) for control and 8-SPT groups. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; paired Student's t-test. For D) and E) data is from 7 fibres (n=7) from 3 CB preparations.

Fig. 3 Rises in chemoafferent activity in hypercapnia are dependent on transmembrane (tmAC) but not soluble adenylate cyclase (sAC) activity. **(a)** Characteristic example recording of the response to hypercapnia ($PCO_2 = 80$ mmHg) in the presence and absence of the tmAC inhibitor SQ22536 (200 μ M). Raw discharge is shown (upper) along with frequency histograms (lower). Overdrawn action potentials are shown inset to demonstrate the single fibre discrimination. The inhibitory impact of SQ22536 was fully reversible. **(b)** Mean single fibre discharge frequencies recorded in normocapnia ($PCO_2 = 40$

mmHg) and hypercapnia ($\text{PCO}_2 = 80$ mmHg), in control conditions and following addition of SQ22536 to the superfusate. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; one way repeated measures ANOVA with Bonferroni post hoc analysis. **(c)** Calculated mean CO_2 sensitivity (Δ Hz / mmHg PCO_2) in control conditions during SQ22536 drug application. Error bars indicate \pm S.E.M. * denotes $P < 0.05$ compared with control group; paired Student's t-test. For (b) and (c) the data presented is from 7 fibres ($n=7$) from 4 CB preparations. **(d)** As for (a) but in the presence of the sAC inhibitor KH7 ($10 \mu\text{M}$). KH7 was applied for ~ 30 minutes to maximise delivery and uptake into the CB before exposure to hypercapnia. **(e)** Mean single fibre discharge frequencies recorded in normocapnia ($\text{PCO}_2 = 40$ mmHg) and hypercapnia ($\text{PCO}_2 = 80$ mmHg), for control and during application of KH7. Error bars indicate \pm S.E.M. **(f)** Calculated CO_2 sensitivities (Δ Hz / mmHg PCO_2) for control and KH7 groups. Error bars indicate \pm S.E.M. For E) and F) data is from 5 fibres ($n=5$) from 4 CB preparations.





