THE WELLCOME PRIZE LECTURE

On the peripheral and central chemoreception and control of breathing: an emerging role of ATP

Alexander V. Gourine

Department of Physiology, Royal Free and University College London Medical School, London NW3 2PF, UK

Peripheral and central respiratory chemoreceptors are ultimately responsible for maintenance of constant levels of arterial P_{O_2}, P_{CO_2} and $[H^+]$, protecting the brain from hypoxia and ensuring that the breathing is always appropriate for metabolism. The aim of this discussion is to shed some light on the potential mechanisms of *chemosensory transduction* – the process which links chemosensory mechanisms to the central nervous mechanisms controlling breathing. Recent experimental data suggest that the purine nucleotide ATP acts as a common mediator of peripheral and central chemosensory transduction (within the carotid body and the medulla oblongata, respectively). In response to a decrease in P_{O_2} (hypoxia) oxygen-sensitive glomus cells of the carotid body release ATP to activate chemoafferent fibres of the carotid sinus nerve which transmit this information to the brainstem respiratory centres. In response to an increase in $P_{CO_2}/[H^+]$ (hypercapnia) chemosensitive structures located on the ventral surface of the medulla oblongata rapidly release ATP, which acts locally within the medullary respiratory network. The functional role of ATP released at both sites is similar – to evoke adaptive enhancement in breathing. Understanding the mechanisms of ATP release in response to chemosensory stimulation may prove to be essential for further detailed analysis of cellular and molecular mechanisms underlying respiratory chemosensitivity.

(Submitted 4 August 2005; accepted after revision 30 August 2005; first published online 1 September 2005)
Corresponding author A. V. Gourine: Department of Physiology, Royal Free and University College Medical School, University College London, Hampstead Campus, Rowland Hill Street, London NW3 2PF UK.
Email: a.gourine@medsch.ucl.ac.uk

Seventy-five years ago, during a meeting of The Physiological Society, Corneille Heymans communicated for the first time his landmark discovery of the chemosensory function of the carotid bodies (Heymans & Bouckaert, 1930). Although the existence of peripheral chemoreceptors was first suggested by the work of Pagano in 1900 (Pagano, 1900), elegant systematic observations by Heymans triggered extensive studies designed to determine the mechanisms of peripheral chemosensitivity and the role played by the arterial chemoreceptors in the respiratory and cardiovascular responses evoked by changes in arterial P_{O_2}, P_{CO_2} and pH (reviewed in depth by Heymans & Neil (1958) and later by Michael de Burgh Daly in his Monograph for the Physiological Society (Daly M de B, 1997)).

In the 1960s studies by the groups led by Hans Loeschcke and Robert Mitchell identified the sites of CO_2 chemosensitivity within the central nervous system. First, in accord with classical reaction theory and experimental evidence, it was postulated that changes in the $[H^+]$ (pH) of the extracellular fluid that follow changes in P_{CO_2} represent the adequate and the main stimulus for the central chemosensors (for review see Loeschcke, 1982). Then, Loeschcke and Mitchell found that the primary set of CO_2 chemoreceptors was localized at, or in close proximity to, the ventral surface of the medulla oblongata (Mitchell *et al.* 1963). Recent evidence suggests an important role of intracellular pH changes in triggering responses of central chemoreceptors to CO_2 (Putnam *et al.* 2004). There are also data indicating that, in addition to the ventral medullary surface chemoreceptors, functional respiratory chemoreceptors may also be located in several other sites throughout the brainstem (Nattie, 1999, 2000).

Experiments conducted in animals in which the carotid and aortic bodies have been denervated indicated different functional roles of peripheral and central chemoreceptors. A large number of studies, impossible to list here, demonstrated clearly that under these conditions

This Wellcome Prize Lecture was given on 20 December 2004 at King's College London.

© 2005 The Author. Journal compilation © 2005 The Physiological Society DOI: 10.1113/jphysiol.2005.095968

hypoxia fails to stimulate ventilation centrally, suggesting that carotid and aortic bodies are the primary O_2-sensitive sites (Daly M de B, 1997). By contrast, although peripheral chemoreceptors are sensitive to changes in P_{CO_2} and pH, the ventilatory response to CO_2 is largely preserved in animals after denervation of peripheral chemoreceptors. Heeringa *et al.* (1979) estimated that up to 80% of the CO_2-evoked response is mediated by the action of CO_2 at the chemosensitive sites located in the brain.

In 2000 I joined the laboratory of Professor K. Michael Spyer in the Department of Physiology, University College London to investigate the role of purinoceptors in the mechanisms of CO_2/H^+ sensitivity of respiratory neurones that constitute the medullary respiratory network. This network contains neurones responsible for generation of the respiratory rhythm and pattern as well as premotor neurones responsible for transmitting this rhythm to spinal motoneurones controlling the diaphragm and intercostal muscles (Richter & Spyer, 2001; Feldman *et al.* 2003).

By this time it was already firmly established that, in addition to its known role as an intracellular energy source, ATP also functions as an extracellular signalling molecule in the central and peripheral nervous system and many peripheral tissues (Burnstock, 1997). Several subtypes of ionotropic (P2X) and metabotropic (P2Y) purinoceptors have been cloned and characterized (Ralevic & Burnstock, 1998; North, 2002); some of them were found to be $[H^+]$ sensitive and therefore could potentially confer this sensitivity onto the medullary respiratory neurones which express these receptors.

Indeed, many medullary neurones with respiratory-related activity are sensitive to changes in external pH (Kawai *et al.* 1996). The hypothesis that purinoceptors may be responsible for this chemosensitivity was proposed when Teresa Thomas demonstrated that blockade of ATP receptors within the ventral respiratory column of the medulla oblongata (microinjection of suramin) decreased resting respiratory activity and attenuated respiratory responses induced by inhaled CO_2 (Thomas *et al.* 1999). Furthermore, the ATP receptor antagonists suramin or pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) reduced baseline firing and blocked CO_2-induced increases in the activity of pre-inspiratory and inspiratory neurones (Thomas & Spyer, 2000).

Among different subtypes of P2 receptors, ionotropic $P2X_2$ receptors are quite unique in terms of their high sensitivity to changes in external pH within the physiological range 7.4–7.1 (King *et al.* 1997; Wildman *et al.* 1997). We suggested therefore that if purinoceptors are indeed responsible for central respiratory chemosensitivity, they have to be of the $P2X_2$ receptor subtype (either homomeric $P2X_2$ receptors or heteromeric receptors containing $P2X_2$ subunits).

When $P2X_2$ receptor knockout ($P2X_2^{-/-}$) mice (Cockayne *et al.* 2005) became available for our studies, they were expected to provide quick and definitive answers regarding the role of P2X receptors in central chemosensory process. However, this was not the case. In conscious freely moving mice we recorded ventilation using whole-body plethysmography and found that resting ventilation and ventilatory responses to rising levels of CO_2 in the inspired air were normal in $P2X_2^{-/-}$ mice, and were normal also in $P2X_3$ knockout ($P2X_3^{-/-}$) and in $P2X_2/P2X_3$ double receptor knockout ($P2X_{2/3}^{Dbl-/-}$) mice (Fig. 1*A*; Rong *et al.* 2003). These results suggested that either these transgenic animals effectively compensate for the loss of $P2X_2$ (and $P2X_3$) receptor subunits, or other subtypes of P2 receptors are involved in central CO_2/H^+ chemoreception.

However, an unexpected role for ATP became apparent when $P2X_2^{-/-}$ mice were exposed to hypoxic conditions. We observed that the increase in ventilation during hypoxia was markedly reduced in the $P2X_2^{-/-}$ and $P2X_{2/3}^{Dbl-/-}$ mice (Fig. 1*B*), but not in the $P2X_3^{-/-}$ mice (Rong *et al.* 2003). At 7.5% oxygen in the breathing air a profound depression of respiration was observed in mice deficient in the $P2X_2$ receptor subunit – ventilation fell well below the baseline (Fig. 1*B*). These data indicated that the $P2X_2$ receptor subunit is essential for the development of the normal ventilatory response to hypoxia and suggested that peripheral chemoreceptor function is compromised in these animals.

In adult mammals type I (glomus) cells of the carotid body are the main peripheral O_2 sensors (Gonzalez *et al.* 1994; Prabhakar, 2000; Lahiri *et al.* 2001; Williams *et al.* 2004). Upon stimulation (e.g. by hypoxia) type I cells release neurotransmitters (for a recent review see Nurse, 2005) to activate afferent nerve fibres of the carotid sinus nerve, which in turn relays this information to the CNS respiratory centres to evoke adaptive changes in breathing. The data we obtained subsequently in collaboration with Weifang Rong and Geoff Burnstock using a superfused *in vitro* carotid body–carotid sinus nerve preparation indicated that ATP may act as a key transmitter released in the carotid body by the oxygen-sensing type I cells. Our data confirmed the results of an earlier pharmacological studies conducted by Colin Nurse and colleagues using co-cultures of rat type I cells and petrosal neurones (Zhang *et al.* 2000; Prasad *et al.* 2001; Buttigieg & Nurse, 2004; Zhang & Nurse, 2004).

First, in studies using *in vitro* preparations taken from the wildtype mice we found that application of ATP evokes a dramatic increase in the carotid sinus nerve chemoafferent discharge (Rong *et al.* 2003), the effect originally described by Jarisch more than 50 years ago (Jarisch *et al.* 1952). Although, Jarisch and co-authors did not know about ‘purinergic signalling’, their conclusion about ‘nerve

© 2005 The Author. Journal compilation © 2005 The Physiological Society

endings in the carotid bifurcation' as the site of ATP action was quite right.

When the baseline carotid sinus nerve discharge in preparations taken from the wildtype and knockout animals was compared, a tonic influence of ATP on the sinus nerve chemoafferent discharge was revealed. Resting chemoafferent activity was found to be significantly lower in the $P2X_2^{-/-}$, $P2X_{2/3}^{Dbl-/-}$, as well as in $P2X_3^{-/-}$ mice compared with that in their wildtype counterparts (Fig. 2). A further reduction in resting carotid sinus nerve discharge was observed in the presence of P2 receptor antagonists PPADS or 2′ (or 3′)-*O*-(trinitrophenyl)-adenosine 5′-triphosphate (TNP-ATP) (Fig. 2).

Hypoxia-induced increases in the discharge of single chemoafferent fibres of the carotid sinus nerve were found to be dramatically reduced in the $P2X_2^{-/-}$ mice (by ∼80%) and even further in the $P2X_{2/3}^{Dbl-/-}$ mice (by ∼85%, Fig. 3) (Rong *et al.* 2003). PPADS, in a dose-dependent manner, reduced hypoxia-evoked activation of carotid chemoafferents in the preparations taken from the wildtype animals. Interestingly, afferent responses to a decrease in P_{O_2} were not affected by the $P2X_3$ receptor subunit deficiency (Fig. 3), an observation that correlates well with the results obtained using whole-body plethysmography and mentioned above. These data indicated that receptors of the $P2X_2$ subtype are essential and sufficient to mediate the effect of hypoxia on the activity of the carotid sinus nerve chemoafferents. Nevertheless, reduced resting sinus nerve discharge in $P2X_3^{-/-}$ mice (and in the presence of the potent $P2X_3$ receptor antagonist TNP-ATP), and significantly smaller hypoxia-induced chemoafferent responses in $P2X_{2/3}^{Dbl-/-}$ mice compared with that in $P2X_2^{-/-}$ mice suggested a role for the $P2X_3$ receptor subtype as well (Rong *et al.* 2003).

Finally, in accord with previous studies in rats (Prasad *et al.* 2001), we observed that in the wildtype mice both $P2X_2$ and $P2X_3$ receptor subunits are expressed in the carotid body (Rong *et al.* 2003). In collaboration with Michael Duchen we examined staining patterns of the $P2X_2$ and $P2X_3$ receptor subunit immunoreactivities using confocal microscopy and found that both subunits were confined to the afferent terminals of the sinus nerve surrounding individual glomus cells or their clusters.

Thus, ATP can now be considered as one of the key mediators of peripheral chemosensory transduction in the carotid body, playing a pivotal role in transmitting information about arterial P_{O_2} levels. We propose that during hypoxia O_2-sensitive glomus cells release ATP to activate the peripheral terminals of the carotid sinus nerve via interaction with P2X receptors that contain the $P2X_2$ subunit, with or without the $P2X_3$ subunit.

The evidence that $P2X_2^{-/-}$ mice can mount a normal respiratory response to an increase in the level of inspired CO_2 was considered insufficient to rule out the role of purinergic signalling in the central chemosensory process. As mentioned above, there was always a possibility remaining that the $P2X_2$ receptor subtype plays an important role in medullary CO_2/H^+ chemoreception, but knockout mice effectively compensate for its loss with

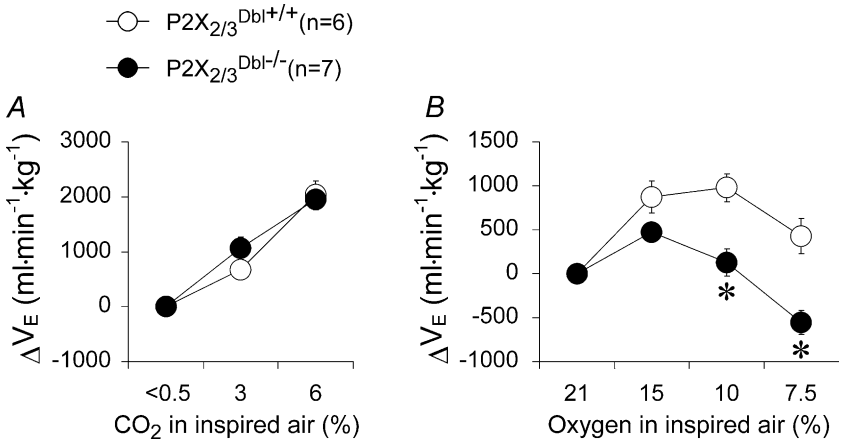

Figure 1. Respiratory responses to hypercapnia and hypoxia in mice with selective deletion of genes encoding $P2X_2$ and $P2X_3$ receptor subunits

A, changes in ventilation during hypercapnia (3 and 6% CO_2 in the inspired air) in conscious $P2X_2$ and $P2X_3$ receptor double knockout ($P2X_{2/3}^{Dbl-/-}$) and wildtype ($P2X_{2/3}^{Dbl+/+}$) mice. *B*, changes in ventilation during hypoxia (15, 10 and 7.5% O_2 in the inspired air) in conscious $P2X_{2/3}^{Dbl-/-}$ and $P2X_{2/3}^{Dbl+/+}$ mice. The resting ventilation during normocapnia/normoxia was identical in the wildtype and knockout animals. The ventilatory response to hypoxia in the $P2X_3^{-/-}$ mice was not significantly different from that in $P2X_3^{+/+}$ mice, while in the $P2X_2^{-/-}$ mice it was markedly reduced and was similar to that in the $P2X_{2/3}^{Dbl-/-}$. V_E, minute ventilation (respiratory rate × tidal volume). Data are presented as means ± S.E.M. Numbers in parentheses indicate sample sizes. *Significant difference, $P < 0.05$. Data redrawn with permission from Rong *et al.* (2003). Copyright 2003 by the Society for Neuroscience.

© 2005 The Author. Journal compilation © 2005 The Physiological Society

increased expression of other subunit(s) or up-regulation of an as yet unknown parallel mechanism. Alternatively, even if the $P2X_2$ subtype has no role at all, other P2 receptors expressed in the medulla oblongata (Yao *et al.* 2000; Thomas *et al.* 2001) could as well mediate the effect of CO_2 on breathing.

Simultaneously with the carotid body work, in collaboration with Jim Deuchars and Lucy Atkinson (University of Leeds) we investigated the extent of $P2X_2$ receptor subunit expression among the physiologically identified (using *in vivo* rat models) respiratory neurones of the medullary ventral respiratory column (Gourine *et al.* 2003). The $P2X_2$ receptor subunit was detected in ~50% of expiratory neurones and in ~20% of neurones with inspiratory-related discharge: pre-inspiratory and inspiratory (Gourine *et al.* 2003). Importantly, a substantially larger proportion of the respiratory neurones increased their discharge in response to microionophoretic application of ATP: 80% of expiratory neurones and 30% of neurones with inspiratory-related discharge were found to be rapidly excited by ATP (Gourine *et al.* 2003). These data suggested that, in addition to the $P2X_2$ receptor subtype, other P2X receptors are expressed by the medullary respiratory neurones. Furthermore, significant numbers of respiratory neurones (~30% of all tested units) were excited strongly by the P2Y receptor agonist uridine 5′-triphosphate (UTP), indicating that these neurones express functional metabotropic P2Y receptors (A. V. Gourine & K. M. Spyer, unpublished observations).

The ventral medullary respiratory network which generates breathing activity is located just above the classical CO_2/H^+ chemosensitive areas which were identified on the ventral surface of the medulla oblongata in the pioneering studies by Loeschcke and Mitchell (Mitchell *et al.* 1963). We have found that some of the medullary respiratory neurones which express the $P2X_2$ receptor subunit have long processes projecting towards the ventral medullary surface chemosensitive areas (Gourine *et al.* 2003). Thus, ATP could act on these ventrally projecting dendrites of respiratory neurones to activate the respiratory network and evoke changes in breathing. This would suggest that ATP may still have a role in the central CO_2 chemosensory process; nevertheless, definite evidence for and understanding of this role were missing.

To advance this problem further we established in our laboratory a surgical approach to the ventral surface of the medulla oblongata in anaesthetized and artificially ventilated rats. Meanwhile in the late 2002, our long-standing collaborators Nicholas Dale and Enrique Llaudet from the University of Warwick

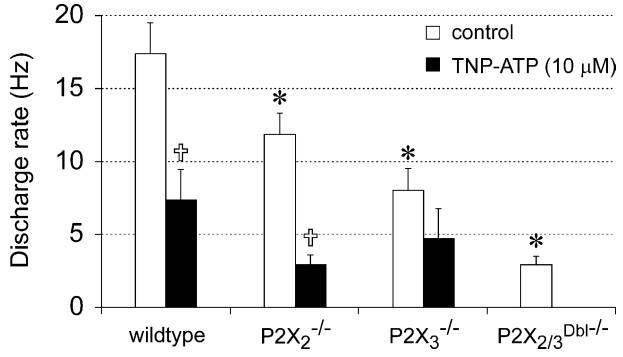

Figure 2. Effect of the P2X receptor blockade on the carotid sinus nerve baseline discharge in mice with selective deletion of genes encoding $P2X_2$ or/and $P2X_3$ receptor subunits
The plot of average baseline firing rates of the carotid sinus nerve recorded in the superfused *in vitro* carotid body–carotid sinus nerve preparations taken from the $P2X_2^{-/-}$, $P2X_3^{-/-}$, $P2X_{2/3}^{Dbl-/-}$ and wildtype mice. Note that TNP-ATP is a significantly more potent antagonist at homomeric $P2X_3$ receptors (and heteromeric receptors which contain $P2X_3$ subunit) in comparison to other P2X receptor subtypes. Accordingly, in the presence of TNP-ATP, resting carotid sinus nerve discharge in preparations taken from the wildtype mice is reduced to the level recorded in the $P2X_3^{-/-}$ mice, and in preparations taken from the $P2X_2^{-/-}$ mice, to the level seen in the $P2X_{2/3}^{Dbl-/-}$ mice. Data are presented as means ± S.E.M. *Significant difference compared with the level of baseline discharge in the preparations taken from the wildtype mice, $P < 0.05$. †Significant effect of TNP-ATP on the carotid sinus nerve discharge in preparations taken from the wildtype and $P2X_2^{-/-}$ mice, $P < 0.05$. (A. V. Gourine, W. Rong, D. A. Cockayne, A. P. Ford, G. Burnstock & K. M. Spyer, unpublished observations).

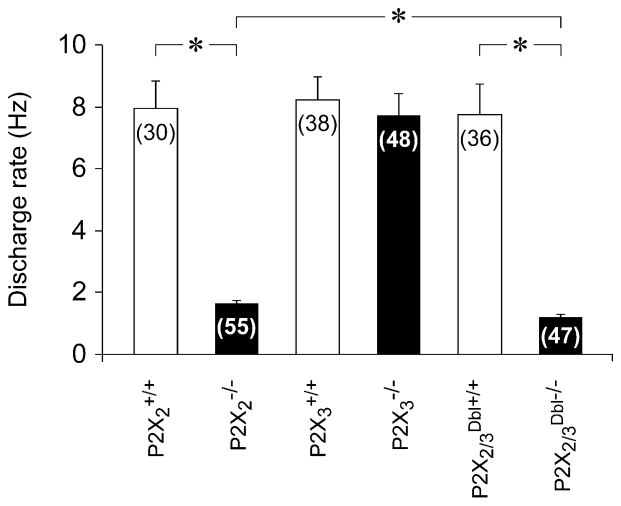

Figure 3. Hypoxia-induced changes in the carotid sinus nerve chemoafferent discharge in mice with selective deletion of genes encoding $P2X_2$ or/and $P2X_3$ receptor subunits
The plot of average hypoxia-induced peak firing rates of single chemoafferent fibres of the carotid sinus nerve recorded in the superfused *in vitro* carotid body–carotid sinus nerve preparations taken from the $P2X_2^{-/-}$, $P2X_3^{-/-}$, $P2X_{2/3}^{Dbl-/-}$ and respective wildtype mice. Note that hypoxia-induced increase in the chemoafferent discharge in the $P2X_{2/3}^{Dbl-/-}$ mice was significantly smaller in comparison to that in the $P2X_2^{-/-}$ mice. Data are presented as means ± S.E.M. Numbers in parentheses indicate sample sizes. *Significant difference, $P < 0.05$. Data from Rong *et al.* (2003).

© 2005 The Author. Journal compilation © 2005 The Physiological Society

invented enzyme-based microelectrode ATP biosensors which allowed real-time measurements of changes in extracellular concentration of ATP with unprecedented resolution and sensitivity (Llaudet *et al.* 2003, 2005; Dale *et al.* 2005).

In the very first experiment, ATP biosensors placed in direct contact with the ventral surface CO_2/H^+ chemosensitive areas of the medulla recorded an almost immediate release of ATP in response to systemic hypercapnia induced by an increase in the level of inspired CO_2 (Gourine *et al.* 2005*a*). This CO_2-induced ATP release was observed in normally breathing (isocapnic eupnoea) rats as well as in animals in which hypocapnic apnoea had been evoked (by mechanical hyperventilation to reduce blood and brain levels of P_{CO_2}). In both cases the hypercapnia-induced release of ATP from the ventral surface chemosensitive areas preceded (by ~20 s) the increase in respiratory activity (Fig. 4*A*) (Gourine *et al.* 2005*a*). Further experiments revealed that ATP release from the surface chemosensitive areas mirrors the CO_2 stimulus, is site specific (it occurs on the ventral but not on the dorsal surface of the medulla) and does not require inputs from the peripheral chemoreceptors (i.e. the amount of hypercapnia-induced ATP released in sino-aortically denervated and vagotomized rats was similar to that of the control animals) (Gourine *et al.* 2005*a*). Using miniature (125 or 250 μm in diameter) disk biosensors we were able to demonstrate that during hypercapnia ATP is released on the ventral surface of the medulla in discrete locations that correspond very closely to the classical CO_2 chemosensitive areas described in the pioneering studies by Loeschcke and Mitchell (Loeschcke, 1982) (Fig. 5*A*; Gourine *et al.* 2005*a*).

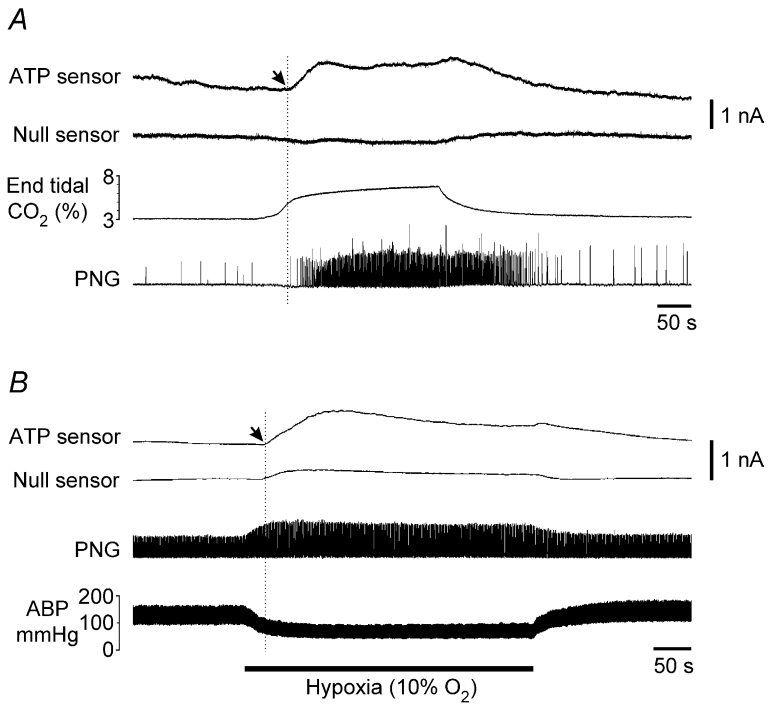

Figure 4. Release of ATP from the ventral surface CO_2 chemosensitive areas of the medulla oblongata during hypercapnia and hypoxia in rats

A, representative raw data illustrating changes in respiratory activity (phrenic nerve discharge) and concentration of ATP on the ventral surface of the medulla in response to an increase in the level of inspired CO_2 in the anaesthetized and artificially ventilated rat. To determine the temporal relationship between changes in ATP levels and CO_2-evoked enhancement in the respiratory activity, hypocapnic apnoea was induced in this animal by mechanical hyperventilation so that P_{CO_2} in the arterial blood and end-tidal levels of CO_2 were below the apnoeic threshold. *B*, representative raw data illustrating changes in the respiratory activity and concentration of ATP on the ventral surface of the medulla during systemic hypoxia (10% O_2 in the inspired air). Biosensors (> 1 mm in length; 100 μm in diameter) were placed in direct contact with a significant portion of the ventral surface CO_2 chemosensitive areas. A dual recording configuration of ATP sensor placed upon one side of the medulla along with a control (null) sensor that was placed in an equivalent position on the other side was used. The null sensor lacked the essential enzymes and thus served as a control to determine whether any 'non-specific' electroactive interferents were released and could confound ATP measurements. Arrow indicates the moment at which the concentration of ATP starts to increase above the baseline. PNG, integrated phrenic nerve activity (arbitrary units). ABP, arterial blood pressure. Data in *A* redrawn from Gourine *et al.* (2005*a*) and data in *B* redrawn with permission from Gourine *et al.* (2005*b*). Copyright 2003 by the Society for Neuroscience.

© 2005 The Author. Journal compilation © 2005 The Physiological Society

To dissect the sites of CO_2-induced ATP production further, I spent several weeks at the University of Warwick preparing together with Nick Dale horizontal slices of the rat medulla oblongata in order to isolate and study medullary chemosensitive structures *in vitro*. This approach proved to be surprisingly difficult as most of the medullary slices cut in our initial experiments exhibited no ATP release in response to an *in vitro* analog of hypercapnia (CO_2-induced acidification of the incubation media from pH 7.4 to 7.0). However, when the brainstem dissection and slicing techniques were refined to preserve the surface layers we consistently recorded marked CO_2/H^+-induced release of ATP from the most ventral slice, which contained surface chemosensitive areas (Fig. 5*B* and *C*; Gourine *et al.* 2005*a*). Interestingly, more dorsal slices in the sequence never exhibited significant ATP release in response to a decrease in pH (Fig. 5*B* and *C*). These results indicated that during hypercapnia ATP is released from the sources located at the very surface of the medulla, as preservation of the surface layers during slice preparation was essential to observe release of ATP in response to CO_2/H^+.

As yet, the functional role of ATP released during hypercapnia from the ventral surface chemosensitive areas remained unclear and back in London, using *in vivo* rat models, we investigated whether ATP applied to the ventral surface of the medulla could mimic the effect of CO_2 on breathing. Indeed, we found that application of exogenous ATP to the medullary surface chemosensitive areas (which release ATP in response to CO_2) induces an almost immediate increase in respiratory activity (Fig. 6*A*) (Gourine *et al.* 2005*a*). This effect of ATP was not affected by the adenosine receptor blockade but was markedly reduced in the presence of the P2 receptor antagonist suramin (Gourine *et al.* 2005*a*).

The functional role of CO_2-induced ATP release was finally revealed when we observed that blockade of ATP receptors within the ventral surface chemosensitive areas reduced the respiratory response to systemic hypercapnia

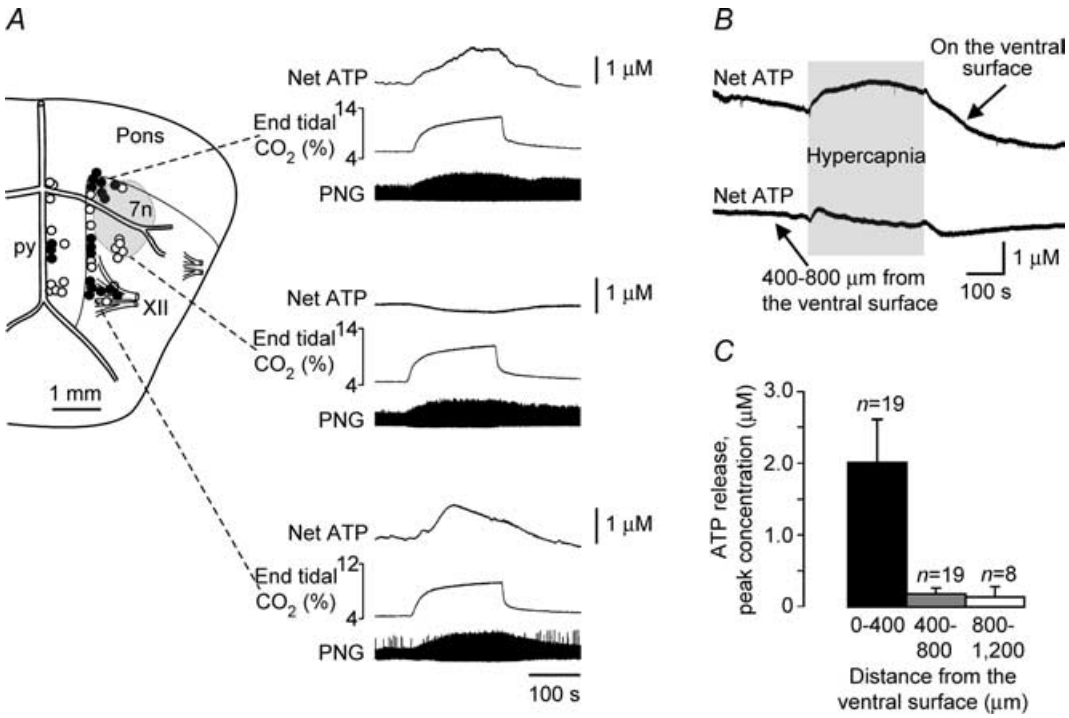

Figure 5. CO_2 induces ATP release from the classical chemosensitive areas of the ventral surface of the medulla oblongata

A, schematic drawing of the ventral aspect of the rat medulla oblongata showing sites that exhibited release of ATP in response to CO_2 (●). No ATP release was detected in locations depicted by ○. Miniature (125 or 250 μm in diameter) disk biosensors were used to map the sites of CO_2-induced ATP production. *B*, representative raw data illustrating CO_2/H^+-induced release of ATP from the ventral medullary surface *in vitro*. CO_2-induced acidification of the incubation media from pH 7.4 to 7.0 evoked marked release of ATP from the most ventral slice of the medulla oblongata, which contained surface chemosensitive areas. *C*, summary data (means ± S.E.M.) of peak CO_2/H^+-induced release of ATP from horizontal slices of the medulla. ATP release occurs predominantly within 400 μm of the ventral surface. The 'Net ATP' trace represents the difference in signal between ATP and null sensors. PNG, integrated phrenic nerve activity (arbitrary units). 7n, relative position of the facial nucleus; XII, hypoglossal nerve roots. Data reproduced from Gourine *et al.* (2005*a*).

© 2005 The Author. Journal compilation © 2005 The Physiological Society

(Fig. 6*B*). The P2 receptor antagonists PPADS or TNP-ATP when applied to the ventral surface of the medulla reduced to a similar extent both the sensitivity and the gain of the respiratory responses to rising levels of inspired CO_2 (Gourine *et al.* 2005*a*). Considering the pharmacological profiles of PPADS and TNP-ATP, speculations concerning the subtype of the P2 receptors which may mediate the effect of ATP on breathing are possible. For example, to activate the respiratory network ATP may act at $P2X_2$ receptors (PPADS and TNP-ATP are equipotent antagonists of the $P2X_2$ receptors) located on the dendrites of medullary respiratory neurones which project ventrally, towards the source of the ATP release (Gourine *et al.* 2003). However, taking into the account data obtained in the $P2X_2^{-/-}$ mice we now consider this issue to be of a secondary importance as multiple P2 receptors may be involved. This point is further strengthen by the fact that UTP applied on the ventral surface of the medulla had a delayed effect (but equivalent to ATP in magnitude, Fig. 6*A*) on respiratory activity, suggesting that metabotropic P2Y receptors may in part mediate the action of ATP on breathing (Gourine *et al.* 2005*a*).

These findings indicated that an increase in CO_2/H^+ induces immediate release of ATP from the chemosensitive structures located on the ventral surface of the medulla. After being released ATP is acting locally (presumably on the distal dendrites of the ventral respiratory column neurones that project close to the ventral surface) to evoke adaptive enhancement in breathing and therefore can be considered as one of the key mediators of central chemosensory transduction.

However, when the hypoxic stimulus was applied in the same experimental setting we obtained results which were difficult at a first glance to reconcile with the proposed model of central chemosensory transduction which involves ATP as the key mediator. It was found that during systemic hypoxia (10% O_2 in the inspired air) ATP is also released from the ventral surface chemosensitive areas of the medulla (Fig. 4*B*) (Gourine *et al.* 2005*b*). Further experiments conducted both *in vivo* and *in vitro* revealed that the amount of the hypoxia-induced ATP released from the ventral medullary surface is very similar to that released in response to CO_2 (Gourine *et al.* 2005*a*, *b*). In addition, ATP release was also present in sino-aortically denervated and vagotomized rats, in which hypoxia induces only depression of respiration (Gourine *et al.* 2005*b*).

Since hypoxia fails to stimulate ventilation centrally, ATP release from the ventral surface chemosensitive areas in these conditions seemed to present a problem for our model of central chemosensory transduction which proposes that the release of ATP constitutes a key initial event. There was, however, one major difference between hypercapnia- and hypoxia-induced release of ATP on the ventral medullary surface. As mentioned above, hypercapnia-induced release of ATP from the ventral surface chemosensitive areas always preceded (by ~20 s) the increase in respiratory activity (Fig. 4*A*) (Gourine *et al.* 2005*a*). Conversely, during hypoxia ATP release was delayed and occurred ~25 s after the initiation of enhanced respiratory activity (Fig. 4*B*) (Gourine *et al.* 2005*b*).

Interestingly, the secondary hypoxia-induced slowing of the respiratory rhythm was significantly augmented following blockade of ATP receptors in the ventral medulla (microinjection of PPADS) (Gourine *et al.* 2005*b*), revealing the functional role of ATP release during hypoxia – to maintain respiratory activity in conditions when hypoxia-induced slowing of respiration occurs. This

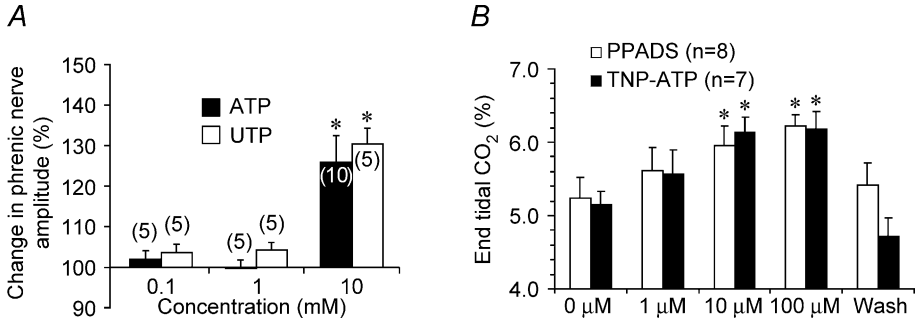

Figure 6. ATP applied to the chemosensitive areas of the ventral medullary surface mimics whereas P2 receptor antagonists attenuate the effect of CO_2 on breathing in rats

A, ATP and UTP applied (30 μl droplet) to the ventral medullary surface enhance respiratory activity. The plot of peak ATP- and UTP-induced changes in the amplitude of the phrenic nerve discharge. Note that changes in the phrenic nerve discharge induced by ATP and UTP were equivalent in magnitude. However, the effect of UTP was delayed (time to peak of response 239 ± 41 s) compared with that of ATP (time to peak 27 ± 4 s). *B*, summary data showing significant increase in mean threshold level of end-tidal CO_2 required to induce breathing from hypocapnic apnoea in the presence of P2 receptor antagonists PPADS or TNP-ATP on the ventral surface of the medulla oblongata. Data are presented as means ± S.E.M. Numbers in parentheses indicate sample sizes. *Significant difference, $P < 0.05$. Data redrawn from Gourine *et al.* (2005*a*).

© 2005 The Author. Journal compilation © 2005 The Physiological Society

conclusion is further supported by our data obtained in $P2X_2^{-/-}$ mice which in contrast to their wild-type counterparts displayed profound respiratory depression at 7.5% oxygen in the breathing air (Fig. 1*B*) (Rong *et al.* 2003). Thus, the role of ATP released in the ventral medulla during the period of hypoxia-induced depression of ventilation appears to be similar to that during hypercapnia, i.e. to stimulate breathing. However, the influence of ATP is clearly not sufficient to fully counteract other powerful mechanisms responsible for hypoxia-induced respiratory depression (Bissonnette, 2000).

The sources and the mechanisms of ATP release from the ventral surface chemosensitive areas as yet remain unknown. The evidence that both hypercapnia and hypoxia induce release of ATP which is similar in magnitude (albeit with different time courses) may be very useful for further detailed analysis of the underlying cellular and molecular mechanisms. Hypoxia has been found to induce a delayed(!) extracellular acidification in the medullary chemosensitive areas (Xu *et al.* 1992), while neurones in the ventrolateral medulla exposed to anoxia display a decrease in intracellular pH similar to that evoked by CO_2 (Chambers-Kersh *et al.* 2000; Putnam *et al.* 2004). Therefore, studies of the relationships between ATP release and intracellular pH in neurones and glial cells from chemosensitive and non-chemosensitive regions of the medulla could be a good starting point.

Further experiments are also needed to reconcile our data with the existing 'cholinergic' hypothesis of central chemosensory transduction (Loeschcke, 1982) as well as with the recent evidence indicating that both chemosensitive serotonergic neurones of the midline raphé (Richerson, 2004; Richerson *et al.* 2005) and glutamatergic neurones of the retrotrapezoid nucleus (Mulkey *et al.* 2004; Guyenet *et al.* 2005) can function as central respiratory chemoreceptors. Interestingly, we observed CO_2-induced ATP release from the ventral medullary surface sites located in close proximity to the basilar artery and lateral to the pyramidal tracts (Fig. 5*A*), where chemosensitive serotonergic medullary neurones are concentrated (Bradley *et al.* 2002). Studies designed to establish whether during hypercapnia these populations of medullary chemosensitive neurones are the sources of ATP release or the targets of ATP action could yield potentially interesting and important results.

In summary, the data obtained over the last 5 years have revealed the importance of ATP-mediated purinergic signalling in the mechanisms responsible for the control of respiration. It emerges that ATP acts as a common mediator of peripheral and central chemosensory transduction. In the carotid body ATP transmits information about oxygen levels in the arterial blood, while in the medulla oblongata it mediates the action of CO_2/H^+ on breathing. Thus, at both sites the functional role of ATP is similar – to link chemoreception to the central nervous mechanisms controlling respiratory activity (Fig. 7). The fact that the same transmitter is employed within one physiological system to mediate afferent transduction in two distinct sites, one in the periphery and one within the

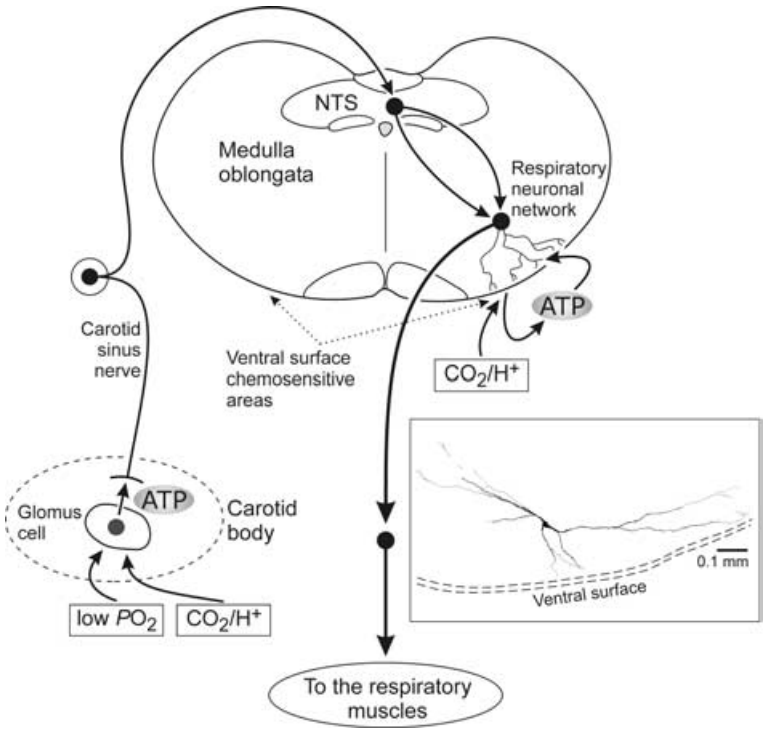

Figure 7. Hypothetical model of ATP involvement in chemosensory control of breathing
In the carotid body, a decrease in P_{O_2} or an increase in $P_{CO_2}/[H^+]$ activates glomus cells which release ATP as the main transmitter to stimulate afferent terminals of the carotid sinus nerve via interaction with P2X receptors that contain the $P2X_2$ subunit, with or without $P2X_3$ subunit. On the ventral surface of the medulla an increase in $P_{CO_2}/[H^+]$ activates primary chemosensors which release ATP to act via P2 receptors on ventrally projecting dendrites of more dorsally located secondary chemosensitive neurones and/or respiratory neurones. The activity of these neurones feeds into the respiratory network and evokes adaptive increases in breathing. The cellular sources of ATP release during hypercapnia as yet remain unknown. Other putative chemosensory transduction mechanisms (involving ACh, serotonin and others) are not shown for presentation purposes. Inset, the drawing shows a reconstruction of a representative pre-inspiratory neurone in the ventral respiratory column which was strongly activated during systemic hypercapnia. Long dendrites of this particular neurone have been found to reach the ventral surface of the medulla (A. V. Gourine & K. M. Spyer, unpublished observations). NTS, nucleus of the solitary tract.

© 2005 The Author. Journal compilation © 2005 The Physiological Society

central nervous system, seems very interesting from both physiological and evolutionary points of view.

References

Bissonnette JM (2000). Mechanisms regulating hypoxic respiratory depression during fetal and postnatal life. *Am J Physiol Regul Integr Comp Physiol* **278**, R1391–R1400.

Bradley SR, Pieribone VA, Wang W, Severson CA, Jacobs RA & Richerson GB (2002). Chemosensitive serotonergic neurons are closely associated with large medullary arteries. *Nat Neurosci* **5**, 401–402.

Burnstock G (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology* **36**, 1127–1139.

Buttigieg J & Nurse CA (2004). Detection of hypoxia-evoked ATP release from chemoreceptor cells of the rat carotid body. *Biochem Biophys Res Commun* **322**, 82–87.

Chambers-Kersh L, Ritucci NA, Dean JB & Putnam RW (2000). Response of intracellular pH to acute anoxia in individual neurons from chemosensitive and nonchemosensitive regions of the medulla. In *Oxygen Sensing: Molecule to Man*, ed. Lahiri S, Prabhakar N, Forster RE II, pp. 453–464. Kluwer Academic/Plenum, New York.

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight GE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G & Ford AP (2005). $P2X_2$ knockout mice and $P2X_2/P2X_3$ double knockout mice reveal a role for the $P2X_2$ receptor subunit in mediating multiple sensory effects of ATP. *J Physiol* **567**, 621–639.

Dale N, Hatz S, Tian F & Llaudet E (2005). Listening to the brain: microelectrode biosensors for neurochemicals. *Trends Biotechnol* **23**, 420–428.

Daly M de Burgh (1997). *Peripheral Arterial Chemoreception and Respiratory-Cardiovascular Integration*. Monograph for the Physiological Society. Oxford University Press, Oxford.

Feldman JL, Mitchell GS & Nattie EE (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* **26**, 239–266.

Gonzalez C, Almaraz L, Obeso A & Rigual R (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol Rev* **74**, 829–898.

Gourine AV, Atkinson L, Deuchars J & Spyer KM (2003). Purinergic signalling in the medullary mechanisms of respiratory control in the rat: respiratory neurones express the $P2X_2$ receptor subunit. *J Physiol* **552**, 197–211.

Gourine AV, Llaudet E, Dale N & Spyer KM (2005*a*). ATP is a mediator of chemosensory transduction in the central nervous system. *Nature* **436**, 108–111.

Gourine AV, Llaudet E, Dale N & Spyer KM (2005*b*). Release of ATP in the ventral medulla during hypoxia in rats: role in hypoxic ventilatory response. *J Neurosci* **25**, 1211–1218.

Guyenet PG, Stornetta RL, Bayliss DA & Mulkey DK (2005). Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp Physiol* **90**, 247–253.

Heeringa J, Berkenbosch A, de Goede J & Olievier CN (1979). Relative contribution of central and peripheral chemoreceptors to the ventilatory response to CO_2 during hyperoxia. *Respir Physiol* **37**, 365–379.

Heymans C & Bouckaert JJ (1930). Sinus caroticus and respiratory reflexes. Cerebral blood flow and respiration. Adrenaline apnoea. *J Physiol* **69**, 13–14P.

Heymans C & Neil E (1958). *Reflexogenic Areas of the Cardiovascular System*. Churchill, London.

Jarisch A, Landgren S, Neil E & Zotterman Y (1952). Impulse activity in the carotid sinus nerve following intra-carotid injection of potassium chloride, veratrine, sodium citrate, adenosine-triphosphate and alpha-dinitrophenol. *Acta Physiol Scand* **25**, 195–211.

Kawai A, Ballantyne D, Muckenhoff K & Scheid P (1996). Chemosensitive medullary neurones in the brainstem–spinal cord preparation of the neonatal rat. *J Physiol* **492**, 277–292.

King BF, Wildman SS, Ziganshina LE, Pintor J & Burnstock G (1997). Effects of extracellular pH on agonism and antagonism at a recombinant $P2X_2$ receptor. *Br J Pharmacol* **121**, 1445–1453.

Lahiri S, Rozanov C, Roy A, Storey B & Buerk DG (2001). Regulation of oxygen sensing in peripheral arterial chemoreceptors. *Int J Biochem Cell Biol* **33**, 755–774.

Llaudet E, Botting NP, Crayston JA & Dale N (2003). A three-enzyme microelectrode sensor for detecting purine release from central nervous system. *Biosens Bioelectron* **18**, 43–52.

Llaudet E, Hatz S, Droniou M & Dale N (2005). Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal Chem* **77**, 3267–3273.

Loeschcke HH (1982). Central chemosensitivity and the reaction theory. *J Physiol* **332**, 1–24.

Mitchell RA, Loeschcke HH, Massion WH & Severinghaus JW (1963). Respiratory responses mediated through superficial chemosenitive areas on the medulla. *J Appl Physiol* **18**, 523–533.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7**, 1360–1369.

Nattie E (1999). CO_2, brainstem chemoreceptors and breathing. *Prog Neurobiol* **59**, 299–331.

Nattie E (2000). Multiple sites for central chemoreception: their roles in response sensitivity and in sleep and wakefulness. *Respir Physiol* **122**, 223–235.

North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013–1067.

Nurse CA (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. *Auton Neurosci* **120**, 1–9.

Pagano G (1900). Sur la sensibilité du coeur et des vaisseaux sanguins. *Arch Ital Biol* **33**, 1–36.

Prabhakar NR (2000). Oxygen sensing by the carotid body chemoreceptors. *J Appl Physiol* **88**, 2287–2295.

Prasad M, Fearon IM, Zhang M, Laing M, Vollmer C & Nurse CA (2001). Expression of $P2X_2$ and $P2X_3$ receptor subunits in rat carotid body afferent neurones: role in chemosensory signalling. *J Physiol* **537**, 667–677.

Putnam RW, Filosa JA & Ritucci NA (2004). Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* **287**, C1493–C1526.

Ralevic V & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413–492.

© 2005 The Author. Journal compilation © 2005 The Physiological Society

Richerson GB (2004). Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci* **5**, 449–461.

Richerson GB, Wang W, Hodges MR, Dohle CI & Diez-Sampedro A (2005). Homing in on the specific phenotype(s) of central respiratory chemoreceptors. *Exp Physiol* **90**, 259–266.

Richter DW & Spyer KM (2001). Studying rhythmogenesis of breathing: comparison of *in vivo* and *in vitro* models. *Trends Neurosci* **24**, 464–472.

Rong W, Gourine AV, Cockayne DA, Xiang Z, Ford AP, Spyer KM & Burnstock G (2003). Pivotal role of nucleotide $P2X_2$ receptor subunit of the ATP-gated ion channel mediating ventilatory responses to hypoxia. *J Neurosci* **23**, 11315–11321.

Thomas T, Ralevic V, Bardini M, Burnstock G & Spyer KM (2001). Evidence for the involvement of purinergic signalling in the control of respiration. *Neuroscience* **107**, 481–490.

Thomas T, Ralevic V, Gadd CA & Spyer KM (1999). Central CO_2 chemoreception: a mechanism involving P2 purinoceptors localized in the ventrolateral medulla of the anaesthetized rat. *J Physiol* **517**, 899–905.

Thomas T & Spyer KM (2000). ATP as a mediator of mammalian central CO_2 chemoreception. *J Physiol* **523**, 441–447.

Wildman SS, King BF & Burnstock G (1997). Potentiation of ATP-responses at a recombinant $P2X_2$ receptor by neurotransmitters and related substances. *Br J Pharmacol* **120**, 221–224.

Williams SE, Wootton P, Mason HS, Bould J, Iles DE, Riccardi D, Peers C & Kemp PJ (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science* **306**, 2093–2097.

Xu F, Sato M, Spellman MJ Jr, Mitchell RA & Severinghaus JW (1992). Topography of cat medullary ventral surface hypoxic acidification. *J Appl Physiol* **73**, 2631–2637.

Yao ST, Barden JA, Finkelstein DI, Bennett MR & Lawrence AJ (2000). Comparative study on the distribution patterns of $P2X_1$-$P2X_6$ receptor immunoreactivity in the brainstem of the rat and the common marmoset (*Callithrix jacchus*): association with catecholamine cell groups. *J Comp Neurol* **427**, 485–507.

Zhang M & Nurse CA (2004). CO_2/pH chemosensory signaling in co-cultures of rat carotid body receptors and petrosal neurons: role of ATP and ACh. *J Neurophysiol* **92**, 3433–3445.

Zhang M, Zhong H, Vollmer C & Nurse CA (2000). Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. *J Physiol* **525**, 143–158.

Acknowledgements

I am most grateful to Professor K. Michael Spyer and Professor Nicholas Dale who have given me essential support and encouragement over the years and have made an invaluable contribution to the studies described above. I would like also to thank Enrique Llaudet, co-inventor of ATP biosensors (together with Nicholas Dale), with whom we performed all the ATP measurements; Dr Weifang Rong, with whom we conducted experiments using superfused carotid body preparations; Drs Jim Deuchars and Lucy Atkinson, who helped me with the $P2X_2$ immunohistochemistry; Professor Geoffrey Burnstock, who supported carotid body work; Drs Debra Cockayne and Anthony Ford from Roche Bioscience, who supplied P2X knockout mice; Professor Michael Duchen, who helped us with confocal microscopy; and Dr Zhenghua Xiang, who assisted with detection of the $P2X_2$ and $P2X_3$ immunoreactivities in the carotid bodies.

I am also most grateful to the members of the Physiological Society prize committee who nominated me for this award and The Wellcome Trust for the prize and the medal.

The experimental work described in this paper was supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust.

© 2005 The Author. Journal compilation © 2005 The Physiological Society