CO$_2$/pH chemosensory signalling in co-cultures of rat carotid body receptors and petrosal neurons: role of ATP and ACh

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Running Title: CO$_2$/pH-induced co-release of ACh and ATP from Type 1 cells

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The neurotransmitter mechanisms that process acid hypercapnia in the mammalian carotid body (CB) are poorly understood. Using a co-culture model, containing rat CB chemoreceptor (type 1 cell) clusters and petrosal neurons (PN), we tested the hypothesis that co-released ACh and ATP was an important mechanism. Sensory transmission from type I clusters to PN in co-culture occurred at chemical synapses via co-release of ATP and ACh since: (i) isohydric hypercapnia depolarized and/or increased firing in co-cultured PN, but not in PN cultured alone; (ii) PN chemoexcitatory responses were inhibited by decreasing the extracellular Ca$^{2+}$: Mg$^{2+}$ ratio; (iii) partial inhibition of these responses occurred during separate perfusion of cholinergic (hexamethonium or mecamylamine) and P2X (suramin) receptor blockers, though inhibition was often complete with both blockers present; (iv) rapid chemoexcitatory responses to hypercapnia were inhibited by acetazolamide (10 µM), an inhibitor of carbonic anhydrase, localized in type I cells. Acidosis (pH = 7.0, 7.2) enhanced the ATP-induced whole-cell current in functional PN, relative to that at physiologic pH (7.4), suggesting that increased sensitivity of postsynaptic P2X receptors may contribute to acid chemotransmission. Type I cells in CB tissue sections expressed vesicular acetylcholine transporter (VACHT), a cholinergic marker, as revealed by confocal immunofluorescence. Thus, co-release of ACh and ATP is an important neurotransmitter mechanism for processing isohydric and acidic hypercapnia in the rat carotid body.
INTRODUCTION

Mammalian carotid bodies (CB) are bilaterally paired organs that help maintain blood PO$_2$ and PCO$_2$/pH homeostasis via the control of ventilation (Gonzalez et al. 1994). Thus, in response to a fall in arterial PO$_2$, or an increase in PCO$_2$ or acidity, there is a compensatory increase in sensory discharge in the afferent carotid sinus nerve (CSN), which projects to the central pattern generator in the brainstem. Activation of this reflex leads to an increase in ventilation and restoration of blood PO$_2$ and PCO$_2$/pH. There is a consensus that CB glomus or type I cells are the sensory receptors for these three chemostimuli (Gonzalez et al. 1994; Peers and Buckler 1995; Lopez-Barneo 1996). In the case of low PO$_2$ or hypoxia, the depolarising receptor potential in type I cells arises primarily from the inhibition of several classes of voltage-independent (background) and voltage-dependent K$^+$ conductances which vary among different species (Peers and Buckler 1995; Buckler et al. 2000; Lopez-Barneo et al. 2001; Prabhakar 2002). This depolarisation leads to calcium entry through voltage-dependent Ca$^{2+}$ channels, and secretion of neurotransmitters that excite CSN afferent nerve terminals.

Though the transduction mechanisms for hypercapnic and acidic stimuli in type I cells have received less attention, there is strong evidence that these stimuli also produce inhibition of voltage dependent K$^+$ channels (Lopez-Lopez et al. 1989; Peers 1990; Peers and Green 1991; Stea et al. 1991) and cause membrane depolarisation and voltage-gated Ca$^{2+}$ entry (Buckler and Vaughan-Jones 1993, 1994; Roy et al. 1997). The effects of hypercapnia have long been considered to be due secondarily to acidification of intracellular pH or pH$_i$ (Travis 1971; Buckler et al. 1991; Iturriaga et al. 1991, 1993; Buckler and Vaughan-Jones 1994), however, more recent studies on rabbit type I cells suggest that CO$_2$ may also have direct effects on enhancing L-type Ca$^{2+}$ currents and presumably neurotransmitter release, independent of changes in pH$_i$ (Summers...
et al. 2002). The neurotransmitters that mediate the excitatory effects of hypercapnic and acidic stimuli in the CB are poorly understood, though it is known that both stimuli evoke catecholamine secretion from type I cells (Gonzalez et al. 1994; Buerk et al. 1998). In the rat CB, the effects of the main catecholamine, dopamine, generally appear to be inhibitory, and certainly not essential for hypoxic chemoexcitation (Donnelly 1996). Recent evidence from our laboratory based on a co-culture model of rat CB type I cell clusters and dissociated petrosal neurones, favor co-release of ATP and ACh as the principal mechanism for mediating hypoxic chemoexcitation (Zhang et al. 2000; Prasad et al. 2001).

In the present study we exploit the advantages of the co-culture model, particularly the ability to record and analyse subthreshold events in postsynaptic neurons situated close to a type I cluster, to elucidate the neurotransmitter mechanisms underlying hypercapnic and acidic chemotransmission. We present evidence that as for hypoxia, co-release of ATP and ACh appears to be an important mechanism. In addition, given that the rat CB petrosal terminals express P2X2-P2X3 purinergic receptors (Prasad et al. 2001), which are known to be pH sensitive (Stoop et al. 1997), we tested the hypothesis that acidic chemoexcitation within the physiological range of extracellular pH, may consist of a postsynaptic component involving increased sensitivity of these purinergic receptors. Finally, since recent studies in rat CB suggest that cholinergic markers may be absent from type I cells in situ (Gauda et al. 2004), we used confocal immunofluorescence to investigate whether or not vesicular acetylcholine transporter (VACHT), a cholinergic protein marker, is expressed in rat type I cells in situ.
METHODS

Co-cultures

Separate cultures of dispersed carotid body (CB) cells, or of dissociated petrosal ganglia were prepared by combined enzymatic and mechanical dissociation of the tissue as previously described (Stea and Nurse 1991, 1992; Zhong et al. 1997). The tissues were obtained from 9-14 day-old Wistar rat pups (Charles River, Quebec; Harlan, Madison, WI), after they were killed by decapitation following a blow to the head. All procedures for animal handling and care were carried out according to regulations of the Canadian Council on Animal care (CCAC) and institutional guidelines. Co-cultures were usually produced by first preparing monolayers containing CB type 1 cell clusters, and then adding an overlay of dissociated petrosal neurons 3-5 days later. These procedures were identical to those described in detail elsewhere (Zhong et al. 1997; Zhang et al. 2000). Cultures were grown in F-12 nutrient medium supplemented with various additives (Zhong et al. 1997), at 37°C in a humidified atmosphere of 95% air-5% CO₂. Electrophysiological recordings from separate petrosal (alone) or CB type I cell cultures were carried out within 3-5 days after plating; co-cultures were usually examined 3-6 days after the neurons were plated.

Electrophysiology

Nystatin perforated-patch, whole-cell recording was used to measure membrane potential (current clamp) or ionic currents (voltage clamp) with the aid of an Axopatch 1D patch clamp amplifier and a Digidata 1200 A-D converter (Axon Instruments Inc., Foster City, CA). The procedures are described in detail elsewhere (Zhong et al. 1997; Zhang et al. 2000). Current and voltage clamp protocols, data acquisition and analysis were performed using pCLAMP software (version 5.5., and
8.0.1, Axon Instruments Inc.) and Axotape (version 2.02., Axon Instruments Inc.). All recordings were carried out at ~ 34°C and the control (normocapnic) extracellular solution consisted of a bicarbonate/CO₂-buffered saline of the following composition (mM): NaCl, 115; NaHCO₃, 24; KCl, 5; CaCl₂, 2; MgCl₂, 1; glucose, 10; and sucrose, 12; at pH 7.4, maintained by bubbling 95% air-5% CO₂. The chemosensory stimuli, i.e. isohydric or acidic hypercapnia (and in a few cases, hypoxia) were applied by a ‘fast perfusion’ system utilizing a double-barrelled pipette assembly as previously described (Zhong et al. 1997). In the case of isohydric hypercapnia, the pH was kept constant at 7.4 as the CO₂ tension was increased, by elevating Na bicarbonate (substituted for same amount of NaCl) as follows: 10% CO₂ (48 mM NaHCO₃); 15% CO₂ (72 mM NaHCO₃); and 20% CO₂ (96 NaHCO₃). In the case of acidic hypercapnia, the bicarbonate concentration was held constant at 24 mM as the CO₂ tension was increased, or in a few cases, the bicarbonate concentration was reduced as the CO₂ tension was held constant at 10%. Hypoxia (PO₂ = ~5 mmHg) was obtained by bubbling N₂ gas into the control extracellular perfusate as previously described (Zhong et al. 1997; Zhang et al. 2000). Results are expressed in the text as mean ± standard error of the mean (S.E.M.). For paired and multiple comparisons of current density (pA/pF) and membrane potentials, Student’s paired t test or ANOVA was used as appropriate; the non-parametric Mann-Whitney test or ANOVA was used for comparison of ratios and percentages. The level of significance was set at p<0.05.

Drugs

Mecamylamine, hexamethonium, suramin, and acetazolamide were obtained from Sigma-Aldrich (Oakville, ON).

Confocal Immunofluorescence

Cryostat sections of the carotid bifurcation from 2- to 3-week-old rat pups were processed for
immunoreactivity against tyrosine hydroxylase (TH) and vesicular acetylcholine transporter (VACHT). Animals were first anesthetized by intraperitoneal administration of Somnotol (65 mg kg$^{-1}$), before perfusion via the aorta with phosphate-buffered saline (PBS) followed by PBS containing 4% paraformaldehyde. In a few experiments the perfusate consisted of Streck Tissue Fixative (STF; Streck Laboratories, LaVista, Nebraska). The excised carotid bifurcation was then washed in phosphate buffered saline (PBS; 3 x 5 min each) or Tris Buffered Saline (TBS) and incubated overnight in 30% sucrose at 4°C. Sections (thickness, 15-18 µm) of the bifurcation containing the CB were cut in a cryostat and collected on glass slides coated with 2% silane (Sigma). After air drying, sections were stored at –20°C until ready for immunostaining. Following rehydration in PBS (formaldehyde-fixed tissue) or TBS (STF tissue), sections were blocked for 30 min in 2% horse serum at room temperature and then incubated overnight at 4°C with primary antisera diluted in 1% BSA/PBS, 0.5% Triton-X100. The primary antisera were: (i) monoclonal mouse anti-TH antibody (1:50 dilution; Boehringer Mannheim, Montreal, Quebec) or polyclonal rabbit anti-TH antibody (1:1000 dilution; Chemicon, Temecula, CA) and (ii) polyclonal goat anti-VACHT antibody (1:200; Catalog # AB1578; Chemicon). After rinsing in PBS (3 x 10 min each), the sections were incubated in the dark for 1 hr at room temperature with the secondary antibodies diluted in blocking solution (1% BSA/PBS; 0.5 % Triton-X100). The following combinations of secondary antibodies were used: (i) N-hydroxysuccinimidy fluorophore (Cy3)-conjugated donkey anti-goat IgG (1:500 dilution) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50; Cappel, Aurora, OH); or (ii) FITC-conjugated donkey anti-goat IgG (1:50 dilution) and Texas Red-conjugated goat anti-rabbit IgG (1:100; Jackson Research Laboratories, Westgrove, PA). Samples were washed in PBS (3 x 5 min each) and covered with Vectashield Mounting Medium (Vector Laboratories,
Burlington, Ontario) before viewing under a Bio-Rad Microradiance 2000 confocal microscope, equipped with argon (two lines, 488 and 514 nm) and helium/neon (543 nm). Lasersharp software was used for image acquisition. Two types of control experiments were carried out. First, sections were processed as described above except that the primary antisera were omitted. No positive staining was observed in these cases. Second, the specificity of VACHT-immunostaining was confirmed in experiments where the primary antibody was pre-incubated (24 hr at 4°C) with 10x molar excess antigen or blocking peptide, corresponding to amino acids 511-530 of the rat VACHT sequence, before application to the sections. In these pre-absorption experiments (n =3), positive staining for VACHT immunofluorescence was abolished or markedly reduced.

**Carbonic anhydrase (CAH) cytochemistry**

The localization of carbonic anhydrase activity in carotid body cultures was carried out using a modification of Hansson’s cobalt-precipitation technique. The procedures were identical to those described in detail elsewhere (Nurse 1990).
RESULTS

Effects of hypercapnia on cultured rat type I cells

Using perforated patch whole-cell recording we first verified that after culture for several days O₂-sensitive type I cells in clusters displayed CO₂ sensitivity, as do freshly-isolated single cells (Buckler and Vaughan-Jones 1994). For these studies we examined type I cells that were members of a large cluster, in which spontaneous spike activity and voltage fluctuations are commonly recorded (Zhang and Nurse 2000). As exemplified in Fig. 1A, both hypoxia and isohydric hypercapnia significantly increased spike frequency in such cells (n = 4), and when present together the combined response was higher than that due to either stimulus acting alone. The stimulus-induced spike frequency ratio relative to control is shown for different cells exposed to one or more of these chemostimuli (Fig. 1B).

Hypcapnic signalling in co-culture depends on chemical transmission and carbonic anhydrase activity

We previously showed that functional chemosensory connections develop de novo between type 1 clusters and petrosal neurons (PN) in co-culture (Zhong et al. 1997; Nurse and Zhang 1999; Zhang et al. 2000; Prasad et al. 2001). To understand further the mechanisms underlying hypcapnic chemotransmission, the membrane potential of PN that were juxtaposed to type I clusters was monitored under current clamp, during rapid perfusion of the hypcapnic stimulus. In 86% of such recordings (37/43), isohydric or acidic hypcapnia caused PN membrane depolarisation that was sometimes accompanied by a burst of action potentials (Fig. 2 A,B). An increase in synaptic-like activity in the neuron was sometimes seen during stimulus application. This activity progressively increased as the CO₂ tension was increased from 5% to 20% (pH =
7.4) and consisted of a complex waveform of spikes and variable-amplitude, subthreshold potentials that resembled postsynaptic potentials (Fig. 2D). These responses to hypercapnia were absent in petrosal neurons cultured alone (n = 13; Fig. 2C), suggesting they depended on chemical synaptic interactions with type I cells, as previously observed for hypoxic chemotransmission (Zhong et al. 1997). Confirmation that the hypercapnia-induced responses in co-cultured PN depended on chemical transmission was obtained in experiments where the extracellular solution was switched to one containing low Ca\(^{2+}\) (0.1 mM) and high Mg\(^{2+}\) (6 mM). In all such cases (n = 7), the hypercapnic response in the neuron was markedly and reversibly inhibited (Fig. 2E; see also Prasad et al. 2001).

Carbonic anhydrase (CAH) activity is thought to play a key role in the speed and magnitude of the initial response of the carotid body to hypercapnia (Travis 1971; Hanson et al. 1981; Buckler et al. 1991; Iturriaga et al. 1991,1993; Gonzalez et al. 1994). This enzyme activity is localized intracellularly in type I cells both in situ (Ridderstrale and Hanson 1984; Rigual et al. 1985; Nurse 1990) and in culture (Fig. 3 D; see also Nurse 1990), and catalyses the hydration of CO\(_2\), producing very rapid changes in intracellular pH (pH\(_i\)) in response to extracellular changes in PCO\(_2\) (Buckler et al. 1991). As exemplified in Fig. 3A, the receptor potential recorded in type I cells during brief (~ 4 sec) application of the hypercapnic stimulus was reversibly abolished in the presence of the permeable CAH inhibitor, 10 µM acetazolamide (ACZ; n = 9). Since the magnitude of this receptor potential is expected to determine the amount of neurotransmitter release from type I cells, we predicted that ACZ should also inhibit the postsynaptic afferent response in co-cultured PN. Indeed, as shown in Fig. 3 B,C, the increase in spike discharge induced by isohydric hypercapnia (10% CO\(_2\); pH = 7.4) in co-cultured PN was reversibly abolished in the presence of 10 µM ACZ (n = 5).
Since CAH increases the rate of fall of pH, in type I cells following an increase in PCO$_2$, with little effect on the steady state pH (Buckler et al. 1991), it was of interest to contrast the effects of ACZ during a prolonged PCO$_2$ stimulus. As illustrated in Fig. 4A,B, the initial or transient response recorded in co-cultured neurons during hypercapnia was larger than that seen at the end of a prolonged stimulus (~ 1 min). The maximum response, measured as an increase in spike frequency (Fig. 4A; inset) or a subthreshold depolarisation (Fig. 4B,C), usually occurred within 12 sec of stimulus application and declined over the next 40-50 sec. In the presence of 10 µM ACZ, the transient response was considerably reduced, whereas the response near the end of the prolonged stimulus was hardly affected (Fig. 4A,B,C). These data are consistent with the notion that CAH activity contributes to the rapid onset and overshoot of the CO$_2$ response in the carotid body (Travis 1971; Buckler et al. 1991; Iturriaga et al. 1991, 1993; Gonzalez et al. 1994).

*Co-release of ATP and ACh mediates hypercapnic chemotransmission in co-culture*

Hypoxic chemotransmission in similar co-cultures is mediated principally by co-release of ACh and ATP from type I cells (Zhang et al. 2000). To investigate whether the same is true for hypercapnia, we tested the effects of nicotinic and purinergic receptor blockers separately, and in combination, on the neuronal response in co-culture. Two different nicotinic blockers, 200 µM hexamethonium or 1 µM mecamylamine, partially inhibited the response to isohydric hypercapnia (10% CO$_2$) as exemplified by the co-cultured neurons in Fig. 5A and 5B respectively. Additionally, the purinergic blocker suramin (50 µM) also partially inhibited the response (Fig. 5B; middle traces) in the same neuron that was inhibited by 1 µM mecamylamine.
Co-release of ATP and ACh appeared to be the major neurotransmitter mechanism since combined application of 50 \(\mu\)M suramin and 1 \(\mu\)M mecamylamine almost completely inhibited the hypercapnic response in this neuron (Fig. 5B; lower traces). In 12 similar cases, the mean neuronal depolarisation evoked by 10% CO\(_2\) was 12.3 ± 2.5 mV (control), compared to 4.2 ± 1.7 mV in the presence of 1 \(\mu\)M mecamylamine, 5.1 ± 1.9 mV in the presence of 50 \(\mu\)M suramin, and 0.8 ± 0.2 mV in the presence of both drugs. The same conclusion was reached in other co-cultured neurons which showed an increase in spike discharge during isohydric hypercapnia \((n = 4)\). For example in Fig. 6 (upper and middle traces), the CO\(_2\)-induced spike discharge was reduced in a dose-dependent manner when the two blockers (suramin and mecamylamine) were applied separately. However, combined application of these blockers was more effective, producing complete inhibition of the discharge at concentrations that caused only partial inhibition when applied separately (Fig. 6; lower traces). For this neuron, mean spike frequency was 11.2 ± 1.7 hz \((n = 3\) trials) in control (10% CO\(_2\)), versus 2.1 ± 0.4 hz in 1 \(\mu\)M mecamylamine, 1.6 ± 0.5 hz in 50 \(\mu\)M suramin, 0 hz in 1 \(\mu\)M mecamylamine plus 50 \(\mu\)M suramin, and 7.2 ± 1.0 hz after wash out of both drugs.

**Effects of acidic hypercapnia in co-culture**

Since the carotid body acts as a pH sensor, independent of the effects of CO\(_2\) (Biscoe et al. 1970; Lopez-Lopez et al. 1989; Peers 1990; Stea et al. 1991; Buckler and Vaughan-Jones 1993; Wilding et al. 1992; Summers et al. 2002), we tested whether or not this property is retained in the co-culture model. Indeed, at a constant CO\(_2\) tension, the chemoexcitatory neuronal response in co-culture progressively increased as the pH of the extracellular perfusate decreased following
reduction of the bicarbonate concentration (Fig. 7A). In general, the frequency of postsynaptic activity (spikes plus subthreshold e.p.s.p.’s) recorded in co-cultured neurons was enhanced during acid hypercapnia (10% CO$_2$/ pH= 7.2) relative to isohydric hypercapnia (10% CO$_2$/ pH= 7.4) as exemplified in Fig. 7B,C. The ratio of the frequency of synaptic events during isohydric and acid hypercapnia relative to control (normocapnia; 5% CO$_2$/ pH= 7.4) for a group of 6 cells is shown in Fig. 7D. It is conceivable that the increase in frequency of detectable e.p.s.p.’s under these acidic conditions may be due, at least in part, to the acid sensitivity of the postsynaptic P2X receptors on petrosal neurons (see below).

Additional evidence that acidity produced excitatory effects on co-cultured neurons, separate from those induced by CO$_2$, was obtained in experiments carried out in the presence of acetazolamide. For example, an increase in chemosensory discharge due to extracellular acidity was still detectable in the presence of acetazolamide (Fig. 8), which abolishes the rapid increase in firing due to increased CO$_2$ (Fig. 4). In 4 cells exposed to 10 µM acetazolamide, decreasing extracellular pH from 7.4 to 7.1 at constant CO$_2$ (10%) caused a significant increase in neuronal firing from $0.3 \pm 0.2$ hz to $4.2 \pm 0.7$ hz  ($p < 0.001$).

As discussed above for isohydric hypercapnia, co-release of ACh and ATP appeared to be the principal synaptic mechanism mediating the effects of acid hypercapnia. For example in Fig. 9, combined application of low doses of mecamylamine (0.5 µM) and suramin (25 µM) was sufficient to block completely postsynaptic activity, whereas only partial inhibition was obtained when either blocker was present alone ($n = 5$).

Effects of acidity on P2X receptors expressed by functional chemosensory neurons

Acidity is known to affect differentially the sensitivity of P2X receptors to ATP and purinergic
agonists depending on receptor subtype (Li et al. 1996; Stoop et al. 1997). We proposed that heteromeric P2X2-P2X3 receptors were likely to be the functional ones expressed by carotid body chemosensory neurons (Zhang et al. 2000; Prasad et al. 2001). Since the effect of ATP on these heteromeric receptors is potentiated by acidity in native nodose neurons (Li et al. 1996) and in heterologous expression systems (Stoop et al. 1997), we considered the possibility that the pH sensitivity of postsynaptic P2X receptors may contribute to the carotid body response during extracellular acidity. We first identified neurons that responded to acid hypercapnia in co-culture as in Fig. 10A, and then tested the pH sensitivity of ATP-induced whole cell currents in the same neurons. The dose of ATP (10 µM) used in these experiments was subsaturating, based on previous studies on the ATP dose-response relation for the purinergic receptors on functional petrosal neurons (Zhang et al. 2000). As shown in Fig. 10B, C, the ATP-induced inward current at a holding potential of –60 mV was potentiated by acidic pH (7, 7.2) and inhibited by alkaline pH (7.6) in identified chemosensory neurons. Thus, the pH sensitivity of postsynaptic P2X receptors on petrosal afferents may contribute to acidic chemoreception in the carotid body.

Expression of cholinergic marker VACHT in rat carotid body type I cells in situ

The evidence presented above, together with that from previous studies on similar co-cultures and the isolated carotid body-sinus nerve preparation (Zhang et al. 2000), provide strong support for the involvement of ACh as a co-transmitter during both hypercapnic and hypoxic chemotransmission in the rat carotid body. This view was recently challenged on the basis of the absence of cholinergic markers in type I cells of sectioned rat CB using in situ hybridisation and immunocytochemical techniques (Gauda et al. 2004). To validate that cholinergic protein markers are indeed expressed in rat type I cells in situ, we used confocal immunofluorescence to
localize the vesicular acetylcholine transporter (VChT), an established cholinergic marker (Eiden 1998). Using double-label immunofluorescence on tissue sections of ~2 week-old rat CB, we found that type I cell clusters identified by positive tyrosine hydroxylase (TH)-immunofluorescence (Alexa) were also immunopositive positive for VChT (Cy3) as illustrated in Fig. 11A,B respectively. This result was confirmed in tissue sections from 5 different animals, and in each case visualization at higher power verified there was co-localization of TH and VChT immunostaining. In control experiments (n = 3), a 24 hr preincubation of the primary VChT antiserum with 10x molar excess of competing or blocking peptide, corresponding to amino acids 511-530 of the VChT sequence, resulted in abolition of staining (Fig. 11C); TH immunostaining of type I cells is evident in the same section (Fig. 11D). In other control experiments, omission of either primary antibody alone resulted in abolition of all immunofluorescence even though the secondary antibodies were present. Taken together, these data demonstrate that cholinergic markers are expressed in (Wistar) rat type I cells in situ.
DISCUSSION

In this study we investigated the mechanisms underlying CO₂/pH chemotransmission in the rat carotid body (CB) using a co-culture model of chemoreceptor type I cells and petrosal sensory neurons. This model was successfully used in previous studies to elucidate the synaptic mechanisms involved in the processing of hypoxic stimuli by these polymodal receptors (Zhong et al. 1997; Nurse and Zhang 1999; Zhang et al. 2000, 2003). The main conclusion from the present studies is that CO₂/pH signalling is dependent on co-release of ATP and ACh from type I cells and, consistent with this view, these cells were shown to express the cholinergic marker, the vesicular acetylcholine transporter (VACHT), in rat CB tissue sections in situ. While expression of cholinergic markers was not detected in rat type I cells by in situ hybridisation and immunocytochemical techniques (Gauda et al. 2004), for reasons that may be related to strain and/or methodological differences, there is recent evidence that both hypercapnia and acidity stimulate ACh release from whole rabbit CB as detected by HPLC, and that ACh itself is detectable in type I cells by immunocytochemistry (Kim et al. 2003). Additionally, our data raise the possibility that the acid sensitivity of P2X2-P2X3 purinoceptors, present on petrosal neurons and their afferent terminals (Prasad et al. 2001), may contribute to the CB chemoexcitatory response evoked by extracellular acidity. Co-release of ATP and ACh was previously reported to be the main mechanism for signalling hypoxia in similar co-cultures, as well as in the intact CB-sinus nerve preparation in vitro (Zhang et al. 2000). More recently, a similar mechanism was proposed for the mediation of chemosensory responses induced by stop flow and acidity in the cat carotid body (Varas et al. 2003). A pivotal role for ATP and the purinergic P2X2 subunit in the ventilatory response to hypoxia was recently confirmed using a transgenic mouse model (Rong et al. 2003). In the latter study, however, the response to hypercapnia was unaffected.
probably because central chemoreceptors were still functional.

_Mechanisms of CO₂ signalling by afferent neurons_

Prior to investigating the neurotransmitter basis for CO₂/pH chemotransmission we first confirmed that rat type I cell clusters retained the ability to sense hypercapnia after several days in culture (see also, Sato 1994). In larger clusters, where type I cells show voltage fluctuations and occasional spike activity (Zhang and Nurse 2000), isohydric hypercapnia (10% CO₂/ pH =7.4) increased spike discharge. In co-culture, isohydric hypercapnia induced depolarising responses (spikes and/or subthreshold potentials) in many petrosal neurons. Such responses were absent in petrosal neurons cultured alone, consistent with type I cells acting as the primary receptors for this stimulus. Frequently, increased CO₂ caused a burst of action potentials in the co-cultured neuron within a few seconds of stimulus application. These responses were inhibited following reduction of the extracellular Ca²⁺: Mg²⁺ ratio, as expected if chemical transmission was involved. Moreover, the transient or rapid neuronal response to an increase in CO₂ was inhibited by the permeable carbonic anhydrase inhibitor, acetazolamide (ACZ; 10 µM), whereas the reduced response remaining after prolonged stimulus application was hardly affected. Carbonic anhydrase is expressed intracellularly in type I cells (Ridderstrale and Hanson 1984; Rigual et al 1985; Nurse 1990; see also Fig. 3D), and in the present study ACZ also abolished the rapid type I cell depolarisation due to isohydric hypercapnia, presumably via slowing the rate of fall of intracellular pH or pHᵢ (Buckler et al. 1991). These data are consistent with the previously described roles of carbonic anhydrase in regulating the speed and magnitude of the initial responses of the carotid body to CO₂ rather than the steady state responses (Buckler et al. 1991; Iturriaga et al. 1991, 1993; Gonzalez et al. 1994).
Co-release of ATP and ACh from type I cells appeared to be the main mechanism mediating CO₂ chemotransmission in co-culture, as previously reported for hypoxia (Zhang et al. 2000). In particular, separate application of nicotinic (mecamylamine or hexamethonium) and purinergic (suramin) blockers only partially inhibited the neuronal CO₂ response in co-culture (see also, Prasad et al. 2001), whereas combined application of both blockers abolished most or all of the response. The same conclusion was reached whether the neuronal response was subthreshold or suprathreshold, suggesting that conduction block in the nerve terminals was not a complicating factor. We previously reported that P2X2 and P2X3 purinoceptor subunits are expressed on petrosal chemoafferent nerve terminals in the rat CB in situ, where they appear to mediate the ATP component of the postsynaptic response during hypoxia via heteromultimeric P2X2-P2X3 receptors (Zhang et al. 2000; Prasad et al. 2001; see however, Rong et al. 2003). Though the functional subunits of the nicotinic ACh receptors (nAChR) on petrosal afferents remain to be determined, there is evidence that these neurons express a variety of nAChR subtypes (Fitzgerald 2000) and are sensitive to ACh (Zhong and Nurse 1997; Nurse and Zhang 1999; Zhang et al. 2000; Varas et al. 2003). Therefore, the simplest explanation of our results is that ATP and ACh, released from type I cells during hypercapnia, act on postsynaptic P2X2-P2X3 and nicotinic receptors to produce their excitatory effects. However, the possibility that during increased CO₂ these same neurotransmitters are also involved in autocrine-paracrine modulation of type I cell responses cannot be excluded (Nurse and Zhang, 1999; Mokashi et al. 2003; Xu et al. 2003).

Mechanisms of acidic chemotransmission

In this study we also examined the effects of varying extracellular acidity under constant CO₂ tension in co-culture. Acidity within the physiological range (pH = 7.0 – 7.2) increased the
chemosensory response recorded in co-cultured neurons when the CO$_2$ tension was maintained at 10%. In these cases the enhanced neuronal response appeared as an increased frequency of action potentials and/or subthreshold potentials that resembled excitatory post synaptic potentials (e.p.s.p.’s) seen at conventional chemical synapses. Moreover, the presence of acetazolamide did not noticeably affect the rapid responses induced by acid hypercapnia (10% CO$_2$; pH = 7.1), suggesting the effects of acidity were independent of CO$_2$.

The neurotransmitter mechanisms mediating the effects of acid hypercapnia appeared similar to those discussed above for isohydric hypercapnia. In particular, the response was partially inhibited when either mecamylamine or suramin was present alone, but was abolished when both drugs were present together. These data suggest that co-release of ATP and ACh from type I cells is the main mechanism mediating the effects of both acidic and isohydric hypercapnia in the rat CB. A similar mechanism was recently proposed as the basis for acidic chemoexcitation in the isolated cat sinus nerve-CB preparation (Varas et al. 2003), though in this case the acidic stimulus was severe (pH = 6.8 –7) and subthreshold postsynaptic events were not recorded. Additionally, our studies uncovered a potential postsynaptic mechanism for enhancing the CB response to extracellular acidity within the physiological range (pH 7-7.3). In identified chemosensory neurons that responded to acidity in co-culture, we found that the ATP-evoked inward current at –60 mV was enhanced by acidic (pH 7.0 – 7.2) and suppressed by alkaline (pH = 7.8) conditions. This reflects the expected pH sensitivity of homomeric P2X2 and heteromeric P2X2-P2X3 purinoreceptors (Stoop et al. 1997), of which the latter are the presumed functional ones in chemosensory petrosal afferent terminals (Zhang et al. 2000; Prasad et al. 2001). Therefore, the pH sensitivity of these receptors represents a likely postsynaptic mechanism by which ATP released from type I cells contributes to acidic chemoreception in the CB. Recent
studies suggest that ATP may also have presynaptic effects via autocrine-paracrine actions on
neighboring type I or type II cells (Mokashi et al. 2003; Xu et al. 2003). It remains to be
determined whether other CB neurotransmitters (e.g. 5-HT, GABA, dopamine) mediate
autocrine-paracrine modulation of the chemosensory response during acid hypercapnia, as
recently reported for hypoxia (Fearon et al. 2003; Zhang et al. 2003).

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LEGENDS

**Figure 1.** Effects of hypoxia and hypercapnia on spontaneously active type I cells. Spontaneous spikes and/or membrane potential fluctuations were sometimes seen in type I cells that were members of a large cluster (>10 cells; Zhang and Nurse 2000) as exemplified in A (upper trace). In the same cell, both hypoxia (Hox) and isohydric hypercapnia (10% CO₂) reversibly increased action potential frequency when applied separately, and the effect was additive when the two stimuli were applied together (Hox + 10% CO₂). Lower histogram shows a plot of spike frequency ratio (relative to initial control) for type I cells from different cultures exposed to one or more of these stimuli. The ratio value for hypoxia (Hox) plus 10% CO₂ was significantly greater than that for Hox alone (p<0.01) and 10% CO₂ alone (p<0.05); though there was no significant difference between Hox and 10% CO₂ (p>0.05), there was a tendency for the effects of 10% CO₂ to appear larger.

**Figure 2.** Effects of isohydric hypercapnia on petrosal neurons cultured with and without type I cells. In A,B, suprathreshold and subthreshold chemosensory responses to 10% CO₂ (pH = 7.4) were recorded in two different petrosal neurons that were juxtaposed to type I clusters in coculture; note the depolarisation preceding the robust excitatory response in A, and the increase in spontaneous activity after the stimulus was terminated. Petrosal neurons cultured in the absence of type I cells were generally unresponsive to an increase in CO₂ (C). In D, increasing CO₂ to 10, 15 and 20% under isohydric conditions caused a progressive increase in the response of a cocultured neuron, as indicated by the increase in frequency of spikes and subthreshold post-synaptic potentials. Chemosensory transmission from type I clusters to petrosal neurons depended on chemical transmission as exemplified in E, where the neuronal response was
reversibly inhibited on lowering the extracellular Ca\(^{2+}\) and elevating extracellular Mg\(^{2+}\) to 0.1 mM and 6 mM respectively. Top calibration bars on left corresponds to Fig. A, and on right to Figs. B,C; lower right calibration bars correspond to Figs. D,E.

**Figure 3.** Role of the carbonic anhydrase inhibitor, acetazolamide (ACZ), in chemotransmission during hypercapnia. In A, the rapid depolarising receptor potential induced by isohydric hypercapnia (10% CO\(_2\)) in a type I cell was reversibly inhibited by ACZ (10 \(\mu\)M). This inhibitor also blocked sensory transmission in co-culture, since the CO\(_2\)-induced increase in chemosensory discharge in co-cultured petrosal neurons was reversibly abolished by ACZ as exemplified in B,C. In D, the cultured type I cluster was selectively positive for carbonic anhydrase activity (dark staining) as revealed by Hansson’s cobalt-precipitation technique.

**Figure 4.** Role of carbonic anhydrase in transient vs steady state afferent responses during hypercapnia. In A, a prolonged hypercapnic stimulus gave rise to an initial burst of spike activity, which gradually subsided in a co-cultured neuron; upper inset shows spike frequency vs time relation for this neuron. In this same cell application of 10 \(\mu\)M acetazolamide (ACZ) abolished most of the transient response with little effect on the final steady state response (A; lower trace). In other neurons the peak transient response to hypercapnia was subthreshold as in B at time t1. However, this transient response was still sensitive to inhibition by ACZ, unlike the steady state response at later times, t2 in B,C. In C, ACZ caused a significant (***; p< 0.001) inhibition of the transient response at t1, and the response remaining after this inhibition at t1 was similar to the steady state response at t2; bars in C are means ± s.e.m. for a group of 5
neurons.

**Figure 5.** Sensitivity of the neuronal hypercapnic response to nicotinic and purinergic blockers in co-culture. In two different neurons, the response to isohydric hypercapnia was partially and reversibly inhibited by the nicotinic blockers hexamethonium (200 µM) in A, and mecamylamine (1 µM) in B (upper traces). For the cell in B, the hypercapnic response was subthreshold and partial inhibition also occurred in the presence of the purinergic blocker, suramin (50 µM; middle traces). When this cell was further exposed to nicotinic and purinergic blockers together the response was almost completely and reversibly abolished (B; lower traces). These data suggest that co-release of ACh and ATP is the principal mechanism mediating CO$_2$ chemotransmission.

**Figure 6.** Dose-dependent effects of suramin and mecamylamine on the CO$_2$-induced spike discharge in chemosensory neurons in co-culture. In this neuron, suramin caused a dose-dependent inhibition of the neuronal discharge induced by 10% CO$_2$ at concentrations of 50 and 100 µM (upper traces). Mecamylamine caused a similar dose-dependent inhibition of the discharge in the same neuron at concentrations of 1 and 2 µM (middle traces). A combination of even lower doses of suramin (25 µM) and mecamylamine (0.5 µM) produced a strong inhibition when applied together, and the discharge was completely abolished in the presence of 50 µM suramin plus 1 µM mecamylamine (lower traces). Thus, co-release of ACh and ATP also appears to be the main synaptic mechanism in neurons that show a robust response to hypercapnia in co-culture.
Figure 7. Effects of acid hypercapnia on chemosensory transmission in co-culture. In A, at a constant CO\textsubscript{2} tension (10%), an increase in acidity from pH 7.4 (HCO\textsubscript{3}\textsuperscript{-} = 48 mM) to pH 7.2 (HCO\textsubscript{3}\textsuperscript{-} = 24 mM) and 7.0 (HCO\textsubscript{3}\textsuperscript{-} = 12 mM), caused a progressive increase in the strength of the chemosensory response in the same neuron. In a different neuron (B), switching from normocapnia (5% CO\textsubscript{2}/ pH = 7.4) to isohydric hypercapnia (10% CO\textsubscript{2}/ pH = 7.4) caused the expected increase in neuronal activity (compare middle and left traces in B), whereas a subsequent switch to acidic hypercapnia (10% CO\textsubscript{2}/ pH = 7.2) caused an even further increase in activity (compare right and middle traces in B). The activity in this neuron consisted of spikes and subthreshold synaptic potentials that resembled excitatory post synaptic potentials or e.p.s.p.’s seen at chemical synapses; the lower traces in B (inset) correspond to expanded segments of the upper traces (indicated by brackets) and show more clearly the time course and size of the e.p.s.p.’s. A quantitative comparison of the activity measured as spike frequency or spike plus e.p.s.p. frequency is shown in C for the same cell as in B. Data from 6 cells are shown in D, where the activity frequency (spikes plus e.p.s.p.’s) relative to control (5% CO\textsubscript{2}/pH = 7.4) is plotted and compared for isohydric (solid bin) vs acid (open bin) hypercapnia in cells exposed to the 3 treatments. Data are mean ± s.e.m. and indicate significant (P < 0.01) effects of hypercapnia and acidity on neuronal activity. The resting potential was −73 mV in A and −67 mV in B.

Figure 8. Independent effects of extracellular pH and PCO\textsubscript{2} on chemosensory transmission in co-culture. All recordings are from same neuron of a chemosensory unit in a co-culture exposed to acetazolamide (ACZ) under conditions of isohydric hypercapnia (upper traces) and acid hypercapnia (lower traces); the CO\textsubscript{2} tension was held constant at 10% during stimulus
application. In the upper traces, ACZ strongly inhibited the rapid excitatory effects of 10% CO₂, applied during period indicated by lower horizontal bar. In the lower traces, ACZ was less effective in blocking the spike discharge and this was likely due to the independent excitatory effects of extracellular acidity (pH = 7.1) on chemotransmission.

**Figure 9.** Transmission of acid hypercapnia in co-culture is dependent on co-release of ACh and ATP. All traces are from the same chemosensory neuron that was excited by hypoxia (not shown) and acid hypercapnia (10% CO₂/ pH =7.2; left and right traces). Perfusion of the culture with 0.5 µM mecamylamine (mec; middle upper trace) or 25 µM suramin (sur; centre trace) caused partial inhibition of the chemosensory discharge. When both drugs were present together the response was completely and reversibly abolished (middle lower trace). Thus, as for isohydric hypercapnia (Fig. 6), co-release of ACh and ATP appears to be an important synaptic mechanism for signalling acid hypercapnia.

**Figure 10.** pH sensitivity of purinergic P2X receptors on chemosensory petrosal neurons. Functional neurons that responded to acid hypercapnia in co-culture were first identified as in A (resting potential = -69 mV). Inward currents, evoked at −60 mV by rapid perfusion of 10 µM ATP over the soma of such neurons, were compared at different pH as illustrated in B. The dose-response curve for a group of 6 neurons treated in this way is shown in C; vertical axis is the current amplitude ratio, relative to that at pH = 7.4. Note that acidity (pH < 7.4) within the physiological range potentiated the ATP-induced inward current, whereas alkaline pH (7.6) caused a small inhibition of the current. These data suggest a postsynaptic mechanism whereby acidity of the P2X receptors on the terminals of chemosensory petrosal neurons (Zhong et al.
2000; Prasad et al. 2001), may contribute to acidic chemotransmission.

**Figure 11.** Co-localization of tyrosine hydroxylase (TH) and vesicular acetylcholine transporter (VACHT) in carotid body type I cells in situ. The same tissue section from a 12 day-old-rat carotid body was immunostained for TH (A; FITC fluorescence) and VACHT (B; Cy3 fluorescence). Note co-localization of TH and VACHT in type I cell clusters. In control experiments for antibody specificity (n =3), the same sections were immunostained for TH (C) and VACHT (D), after overnight preincubation with a 10x molar excess of blocking peptide, corresponding to amino acids 511-530 of the rat VACHT sequence. Note the preincubation step abolished VACHT immunostaining in D, whereas TH-immunostaining was unaffected (C ).
fig. 1
fig.2
Figure 3

A. Recordings showing the effects of 10% CO₂ and 10 μM ACZ on membrane potential.

B. Comparison of control and wash conditions under 10% CO₂.

C. Frequency response over time in response to 10% CO₂ and 10 μM ACZ.

D. Microscope image of cells with a scale bar indicating 30 μm.

30 μm
**Figure 4**

**A**

- 10% CO₂

- Control: Depolarization sequence with time points t1 and t2.
- 10 µM ACZ: Reduced depolarization compared to control.

**B**

- 10% CO₂

- Control: Depolarization with time points t1 and t2.
- ACZ: Depolarization with time points t1 and t2.

**C**

- Depolarization (mV) vs. Time (s)
- t1 and t2: Comparison of depolarization with and without ACZ.

Legend:

- **ACZ**: Presence of 10 µM ACZ.
- Statistical significance indicated by asterisks: ***p < 0.001**
fig. 6
fig. 7
10% CO₂ pH 7.2  

0.5 µM mec  

wash  

25 µM sur  

0.5 µM mec + 25 µM sur  

fig. 9
fig. 10