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GABA mediates autoreceptor feedback inhibition in the rat carotid body via presynaptic $GABA_B$ receptors and TASK-1

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Background K^+ channels exert control over neuronal excitability by regulating resting potential and input resistance. Here, we show that $GABA_B$ receptor-mediated activation of a background K^+ conductance modulates transmission at rat carotid body chemosensory synapses *in vitro*. Carotid body chemoreceptor (type I) cells expressed $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits as well as endogenous GABA. The $GABA_B$ receptor agonist baclofen activated an anandamide- and Ba^{2+}-sensitive TASK-1-like background K^+ conductance in chemoreceptor cell clusters, but was without effect on voltage-gated Ca^{2+} channels. Hydroxysaclofen (50 μM), 5-aminovaleric acid (100 μM) and CGP 55845 (100 nM), selective $GABA_B$ receptor blockers, potentiated the hypoxia-induced receptor potential; this effect was abolished by pre-treatment with pertussis toxin (PTX; 500 ng ml^{-1}), an inhibitor of G_i, or by H-89 (50 μM), a selective inhibitor of protein kinase A. The protein kinase C inhibitor chelerythrine chloride (100 μM) was without effect on this potentiation. $GABA_B$ receptor blockers also caused depolarisation of type I cells in clusters, and enhanced spike discharge in spontaneously firing cells. In functional co-cultures of type I clusters and petrosal sensory neurones, $GABA_B$ receptor blockers potentiated hypoxia-induced postsynaptic chemosensory responses mediated by the fast-acting transmitters ACh and ATP. Thus $GABA_B$ receptor-mediated activation of TASK-1 or a related channel provides a presynaptic autoregulatory feedback mechanism that modulates fast synaptic transmission in the rat carotid body.

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The carotid body responds to decreases in arterial P_{O_2} (hypoxia) by increasing afferent chemosensory discharge of the carotid sinus nerve, initiating changes in ventilation (Gonzalez *et al.* 1994). A popular hypothesis concerning the mechanism of hypoxic chemotransduction involves depolarisation of chemoreceptive type I cells due to hypoxic inhibition of both large-conductance Ca^{2+}-activated (Peers, 1990) and voltage-independent background (Buckler *et al.* 2000) K^+ channels, leading to activation of voltage-gated Ca^{2+} channels. The resultant influx of extracellular Ca^{2+} through these channels elevates intracellular Ca^{2+} levels, which stimulates Ca^{2+}-dependent neurotransmitter release onto sensory afferent nerve endings (Buckler & Vaughan-Jones, 1994; Gonzalez *et al.* 1994; Urena *et al.* 1994; Lopez-Barneo, 1996; Zhang *et al.* 2000). Support for this hypothesis is not universal however, particularly since in some studies inhibitors of K^+ channels fail to mimic the effects of hypoxia (Donnelly, 1997; Osanai *et al.* 1997; Lahiri *et al.* 1998). In contrast, the application of K^+ channel blockers such as TEA or iberiotoxin was sufficient to evoke extracellular Ca^{2+}-dependent release of catecholamines from thin slices of the rat carotid body (Pardal *et al.* 2000) and cultured type I clusters (Jackson & Nurse, 1997). To advance our knowledge of the mechanisms underlying carotid body function, we have developed a model of the rat carotid body in which functional synapses form *de novo* between isolated type I clusters and petrosal neurones (PNs) in co-culture (Zhong *et al.* 1997). This chemosensory complex transduces hypoxia and transmits the chemosensory response via the co-release of the excitatory neurotransmitters ACh and ATP (Zhang *et al.* 2000) onto postsynaptic nicotinic and purinergic receptors (Zhang *et al.* 2000; Prasad *et al.* 2001), giving rise to an increased discharge rate or depolarisation in the adjacent postsynaptic PN.

γ-Aminobutyric acid (GABA) is a well-characterised inhibitory neurotransmitter which acts at ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors in the central nervous system. The effects of $GABA_B$ receptor stimulation are slow and result in modulation of synaptic transmission via G-proteins and intracellular effector systems (Mott & Lewis, 1994; Kerr & Ong, 1995; Couve *et al.* 2000; Greengard, 2001) linked to Ca^{2+} and K^+ channels (Bowery & Enna, 2000). GABA is co-localised with catecholamines and 5-HT in type I cells of the mouse carotid body (Oomori *et al.* 1994). GABA-immunoreactivity has also been demonstrated in the type I (glomus) cells of other species, including chipmunk and bat (Ohtomo *et al.* 2000),

and also in neurosecretory chromaffin cells of the adrenal medulla (Oomori *et al.* 1993) where their activation is linked to the regulation of catecholamine secretion (Castro *et al.* 1989).

In carotid body type I cells, hypoxia-induced depolarisation is attributable in part to inhibition of a background K^+ channel with characteristics of a member of the tandem-pore-domain family of K^+ channels, TASK-1 (Buckler *et al.* 2000). These channels regulate resting membrane potential and help control neuronal excitability (Goldstein *et al.* 2001). Their activity is under strict regulation by several neurotransmitters, including 5-HT, noradrenaline, substance P, glutamate, thyrotropin releasing hormone (TRH) and ACh, acting at G-protein-coupled receptors (Millar *et al.* 2000; Talley *et al.* 2000; Goldstein *et al.* 2001). Since GABA is present in the carotid body, and G-protein-coupled $GABA_B$ receptors participate in autoreceptor feedback in other neurosecretory cell types (Castro *et al.* 1989), we tested the hypothesis that $GABA_B$ receptors participate in an autoregulatory feedback mechanism to regulate secretion in the rat carotid body. We found that selective inhibitors of G-protein coupled metabotropic $GABA_B$ receptors enhanced type I cell receptor potential via a G_i-and PKA-dependent pathway. Additionally, voltage-clamp experiments revealed that $GABA_B$ receptor stimulation activates a voltage-independent K^+ conductance with pharmacological and biophysical properties similar to TASK-1. Taken together, these data suggest a novel GABA-mediated autoregulatory feedback mechanism in the carotid body that modulates synaptic efficacy by converging two separate regulatory influences onto the same background K^+ conductance.

METHODS

Cell culture

Details of methods used in preparing co-cultures or separate cultures of dissociated type I cell clusters or petrosal neurones have been described previously (Zhong *et al.* 1997). Briefly, dissociated cells were obtained from carotid bodies or petrosal ganglia of 7- to 14-day-old rat pups (Wistar; Charles River, QC, Canada). Following humane killing by stunning with a blow to the head and decapitation, the carotid bifurcation and attached nodose–petrosal complex were excised. Procedures for animal handling and tissue removal were carried out in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). To produce dissociated type I cells, carotid bodies were incubated for ~45 min at 37°C in an enzymatic solution containing 0.1 % collagenase–0.1 % trypsin (GibcoBRL Life Technologies, Burlington, ON, Canada). The tissues were mechanically dissociated with forceps, and triturated to yield a suspension of dispersed single cells and type 1 cell clusters, consisting of a few to 20 or more cells. The cell suspension was plated onto a thin layer of collagen or Matrigel (Collaborative Research, Bedford, MA, USA) that was previously applied to the central wells of 35 mm tissue culture dishes. For co-cultures, petrosal neurones were obtained by enzymatically and mechanically dissociating the petrosal ganglion, as described above. Dissociated petrosal neurones were then overlaid onto cultures of carotid body type I cells, after the latter had been in culture for 3–5 days. All cultures were grown at 37 °C in a humidified atmosphere of 95 % air–5 % CO_2 in F-12 nutrient medium (GibcoBRL) supplemented with 10 % v/v fetal bovine serum (GibcoBRL), 80 U l^{-1} insulin (Sigma), 0.6 % (w/v) glucose, 2 mM L-glutamine and 1 % penicillin–streptomycin (Gibco). Electrophysiological recordings were made 2–4 days after cells were plated in the case of separate cultures, and after 3–5 days in co-cultures.

Current-clamp recordings

Methods for recording chemosensory responses from co-cultured petrosal neurones and type I cell clusters have been described elsewhere (Zhong *et al.* 1997). These recordings were made using the perforated-patch technique to preserve cytoplasmic integrity, and patch pipettes contained intracellular solution plus 300 μg ml^{-1} nystatin. Recordings were carried out at ~35°C in bicarbonate/CO_2-buffered extracellular fluid of the following composition (mM): NaCl, 115; $NaHCO_3$, 24; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; and sucrose, 12; pH 7.4 maintained by bubbling 95 % air–5 % CO_2. The intracellular solution contained (mM): potassium glutamate or gluconate, 115; KCl, 25; NaCl, 5; $CaCl_2$, 1; and Hepes, 10; pH 7.2. Hypoxia (P_{O_2} ~5 mmHg) was generated by bubbling 95 % N_2–5 % CO_2 into the extracellular fluid. P_{O_2} was measured using a commercial O_2 electrode (Diamond Electro-Tech Inc., MI, USA). Drug solutions were applied by gravity perfusion in the recording solution for at least 1 min before examining their effects, and recordings of membrane potential were only made after this period of application. Hypoxic (P_{O_2} ~5 mmHg) solutions were applied via a fast perfusion pipette placed within 300 μm of the cells under investigation. A piezoelectric switch allowed rapid changes between a barrel carrying normoxic perfusate and one containing hypoxic perfusate. With this technique, changes in P_{O_2} local to the cells of interest were reached almost instantaneously (< 3 s; Zhong *et al.* 1997) following switching. In petrosal neurones, resting membrane potential was usually more negative than −50 mV; type I cell resting potential was within the range of −38 to −58 mV.

Voltage-clamp recordings

Cultures were transferred to a perfused recording chamber mounted on the stage of a Zeiss Axiovert S100 microscope. Patch-clamp recordings were made using patch electrodes of resistance 4–7 MΩ when filled with intracellular solution. Patch electrodes were fabricated from 1.5/0.75 mm o.d./i.d. borosilicate glass (WPI) using a P-97 Brown-Flaming horizontal electrode puller (Sutter) and fire-polished. All voltage-clamp experiments were performed at room temperature (21–24 °C). Cells were voltage-clamped at −60 mV (−80 mV for Ca^{2+} currents), and currents evoked by step depolarisations to various test potentials for 100 ms at a frequency of 0.1 Hz. In some experiments, membrane potential was ramped between −60 mV and +50 mV over 1 s. Current traces were filtered at 5 kHz, digitised at 10 kHz and stored on a PC for later analysis. Capacitative transients were minimised by analog means. All analyses and voltage protocols were performed using an EPC 9 amplifier with integrated AD/DA converter and ITC-16 interface, and Pulse software (HEKA). Steady-state outward currents were measured as the average current between 90 and 99 ms of the voltage step. Inward (Ca^{2+}) currents were measured at their peak amplitude. Voltage-clamp data were analysed using Pulsefit software (HEKA). Results are expressed as means ± S.E.M., and statistical comparisons made using Student's *t* test, ANOVA or the Mann-Whitney test, as appropriate.

Solutions

K^+ currents. These currents were recorded using the perforated-patch configuration. Electrodes were filled with a solution containing (mM): NaCl, 5; KCl, 35; potassium gluconate, 95; Hepes, 10; $CaCl_2$, 2; and nystatin, 300 μg ml^{-1}. Cells were perfused with a solution composed of (mM): NaCl, 135; KCl, 5; $MgCl_2$, 1.2; Hepes, 5; $CaCl_2$, 2.5; and D-glucose, 10 (pH 7.4). In experiments using symmetrical K^+ solutions, cells were perfused with a similar solution containing 135 mM KCl and 5 mM NaCl.

Ca^{2+} currents. Ca^{2+} channel currents were recorded using the conventional whole-cell configuration, and isolated by perfusing with an extracellular solution consisting of (mM): NaCl, 125; CsCl, 5; $MgCl_2$, 0.6; $CaCl_2$ 5; Hepes, 5; D-glucose, 10; and TEA-Cl, 20 (pH 7.4 with NaOH). The pipette solution contained (mM): CsCl, 120; TEA-Cl, 20; $MgC1_2$, 2; EGTA, 10; Hepes, 10; and ATP, 2 (pH 7.2 with CsOH).

Drug solutions

Baclofen, 5-aminovaleric acid, hydroxysaclofen, H-7, H-89, chelerythrine hydrochloride and anandamide were obtained from Sigma. CGP 55845 was obtained from Tocris Cookson.

RT-PCR

Type I clusters and petrosal neurones were harvested from their culture dishes by suction into a broken glass microelectrode. Isolation of total RNA from type I clusters and subsequent DNase treatment and reverse transcription were as described for petrosal neurones (Prasad *et al.* 2001). DNA was amplified in a single PCR reaction consisting of the following (mM unless stated): Tris-HCl, 20; KCl, 50; $MgCl_2$, 1.5; each dNTP, 0.2; each primer, 0.2 μM; 5 μl template; and Platinum Taq polymerase (Gibco), 2.5 U μl^{-1}; total reaction volume 25 μl. Gene-specific primers used were (listed as sequence amplified, forward and reverse primers and expected size of product):

GABA$_{B(1)}$,	GTGACCATGATCCTTTCCAG, AACAGTCGGGACTTCTCTT,	209 bp;
GABA$_{B(2)}$,	ACTGGGGTTCTGTATGGGGA, TCTGTAGGGTCTTGGCGATG,	465 bp;
β-actin,	AAGATCCTGACCGAGCGTGG, CAGCACTGTGTTGGCATAGAGG,	327 bp.

GABA$_{B(1)}$ primers were designed against a common region and identify multiple isoforms of this subunit. The reaction was held at 94 °C for 2 min and subsequently cycled 35 times. Each cycle consisted of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a 10 min final extension at 72 °C. The specificity of the PCR primers was demonstrated by automated fluorescence sequencing (Mobix, McMaster University) of PCR products to verify their identity.

Immunofluorescence

Cryostat sections of the carotid bifurcation and cultures of type 1 cells, obtained from 7- to14-day-old rat pups, were processed for immunofluorescence. In preparation for tissue sections, animals were first anaesthetized 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–0.5 % glutaraldehyde. The carotid bifurcation was then excised and post-fixed for 1 h at room temperature in 4 % paraformaldehyde–0.5 % glutaraldehyde. The tissue was then washed in PBS (3 × 5 min each) and incubated overnight in 30 % sucrose at 4 °C. Sections (thickness, 10–12 μm) of the bifurcation containing the carotid body 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, sections were incubated overnight at 4 °C with primary antisera diluted in 1 % BSA in PBS, 0.5 % Triton X-100. Primary antibodies were guinea-pig polyclonal antisera raised against (i) GABA$_{B(1)}$ receptor N-terminal region (1:500 dilution); (ii) GABA$_{B(2)}$ receptor C-terminal region (1:500 dilution); and (iii) glutaraldehyde-conjugated GABA (1:500 dilution; all Chemicon). After rinsing in PBS (3 × 10 min each), the sections were incubated in the dark for 1 h at room temperature with the secondary antibodies diluted in blocking solution (1 % BSA/PBS, 0.5 % Triton X-100). The secondary antibody was an FITC-conjugated goat anti-guinea-pig (1:20 dilution; Jackson Immunoresearch Laboratories). Samples were washed in PBS (3 × 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 (Bio-Rad, Mississauga, ON, Canada) was used for image acquisition. In control experiments, sections were either processed as described above except that the primary antibody incubation step was omitted or, in the case of GABA immunoreactivity, the primary antibody was pre-adsorbed with excess antigen.

RESULTS

GABA$_B$ receptor blockers enhance the receptor potential in type I cells and modulate chemosensory transmission

Since GABA is localised to type I cells of the mouse carotid body (Oomori *et al.* 1994) and G-protein-coupled GABA$_B$ receptors participate in autoreceptor feedback in other neurosecretory cell types (Castro *et al.* 1989), we initially examined whether selective GABA$_B$ receptor blockade modulates receptor potential in type I cells of the rat carotid body via an autocrine–paracrine feedback mechanism. When recording type I cell membrane potential under current-clamp conditions, application of either of the GABA$_B$ receptor antagonists hydroxysaclofen (OHS; 50 μM), 5-aminovaleric acid (5-AVA; 100 μM) or CGP 55845 (100 nM) caused membrane depolarisation, consistent with the tonic activation of GABA$_B$ receptors. Membrane potential was shifted by 1.2 ± 0.6 mV (n = 6) in the presence of OHS, by 1.7 ± 0.8 mV (n = 6) in the presence of 5-AVA and by 2.7 ± 0.5 mV (n = 5) in the presence of CGP 55845. During application of these blockers, the hypoxic response was reversibly potentiated (Fig. 1*A*–*C*). Hypoxia depolarised cells by 1.6 ± 0.2 mV under control conditions, and by 3.5 ± 0.4 mV in the presence of OHS (n = 11; P < 0.001, ANOVA; Fig. 2*A*). Similarly, hypoxic depolarisation was increased by 5-AVA (from 2.4 ± 0.6 mV to 4.7 ± 1.2 mV, n = 7; P < 0.001, ANOVA; Fig. 1*B*) and by CGP 55845 (from 2.1 ± 0.5 mV to 3.3 ± 0.8 mV, n = 6; P < 0.01, ANOVA; Fig. 1*C*). In all cases, resting membrane potential was between −38 and –55 mV.

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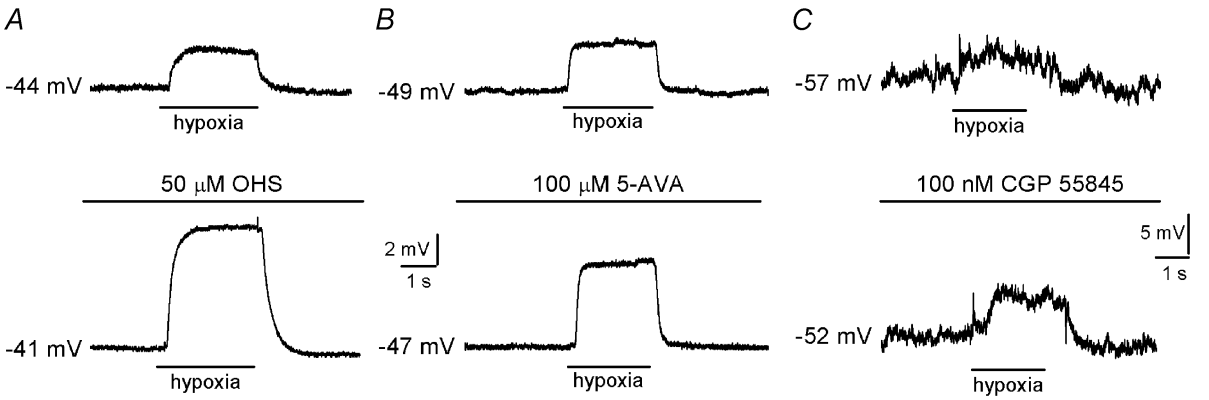

Figure 1. $GABA_B$ receptor blockade enhances receptor potential in chemoreceptor cells

A, typical current-clamp recordings made from a type I cell cluster. Application of hypoxia (P_{O_2}, 5 mmHg) is indicated by the horizontal bar below each trace. Under control conditions (upper), hypoxia induced a depolarisation which was increased in the presence of 50 μM hydroxysaclofen (OHS; lower). *B* and *C*, as in *A*, except the effects of further specific blockers of $GABA_B$ receptors, 5-aminovaleric acid (5-AVA; 100 μM) and CGP 55845 (100 nM), were examined. Note the different scale bars in *C*. In all cases, effects of $GABA_B$ receptor blockers were fully reversed on washout of the drug (not shown).

These data implicate GABA as a presynaptic modulator of chemoreceptor function via slow-acting G-protein-coupled $GABA_B$ autoreceptors. We further investigated the effect of $GABA_B$ receptor blockade on the postsynaptic response of juxtaposed petrosal neurones that functionally innervated type I clusters in co-culture. Postsynaptic depolarisation due to hypoxia was significantly and reversibly enhanced by 50 μM OHS (Fig. 2*A*). Under control conditions, hypoxia depolarised the neurones by 2.2 ± 0.3 mV; in the presence of OHS, the depolarisation due to hypoxia was increased to 6.3 ± 0.5 mV (n = 14; $P < 0.001$, ANOVA). Similarly, in the presence of 100 μM 5-AVA, hypoxia evoked a postsynaptic depolarisation of 6.5 ± 0.3 mV, a value significantly different from that recorded in the absence of the drug (2.4 ± 0.1 mV; n = 6; $P < 0.005$, ANOVA; Fig. 2*B*). Responses to both OHS and 5-AVA were fully reversed on removal of the $GABA_B$ receptor blocker. These data support the hypothesis that presynaptic $GABA_B$ receptors are involved in regulating hypoxic chemotransmission in the rat carotid body.

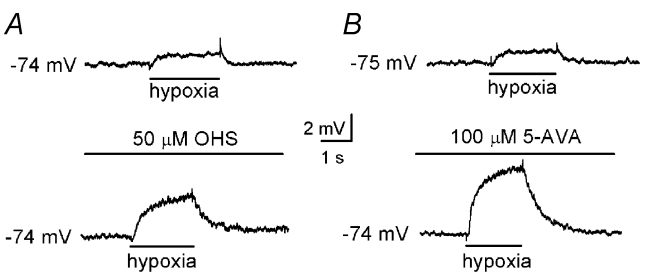

Figure 2. Postsynaptic depolarisation due to hypoxia is modulated by $GABA_B$ receptor inhibition

A, typical current-clamp recordings made from a petrosal neurone juxtaposed to a type I cluster in co-culture. Application of hypoxia (P_{O_2}, 5 mmHg) is indicated by the horizontal bar below each trace. Under control conditions (upper), hypoxia induced a postsynaptic depolarisation which was increased in the presence of the $GABA_B$ receptor antagonist, OHS (50 μM; lower). *B*, as in *A*, except the effect of a further specific $GABA_B$ receptor antagonist, 5-aminovaleric acid (5-AVA; 100 μM), was examined.

$GABA_B$ receptors regulate presynaptic excitability and release of fast excitatory neurotransmitters from type I cells

In the CNS the efficacy of synaptic transmission mediated by fast-acting neurotransmitters can be modulated by the actions of slow-acting transmitters (Greengard, 2001). Since spontaneous firing sometimes occurred in type I cells that were members of a large cluster (Zhang & Nurse, 2000) we tested whether constitutive activation of $GABA_B$ receptors may control presynaptic excitability. In the presence of 50 μM OHS, spontaneous spike activity observed under current-clamp conditions was enhanced (Fig. 3*A*). Since 'slow' transmitter receptors can alter synaptic transmission by regulating presynaptic efficacy (Greengard, 2001), it is plausible that the major presynaptic effect of GABA is to inhibit release of the fast transmitters ACh and ATP from type I cells during chemosensory stimulation (Nurse & Zhang, 1999; Zhang *et al.* 2000; Prasad *et al.* 2001). In functional chemosensory units in co-culture, perfusion of OHS (50 μM) induced spike activity in neurones juxtaposed to type I clusters (n = 6; Fig. 3*B*). This activity was reversibly reduced in the presence of either 1 μM mecamylamine or 25 μM suramin, blockers of nicotinic and purinergic receptors, respectively, and abolished in the presence of both blockers (Fig. 3*B*). This demonstrates a 'tonic' release of GABA from type I cells which reduces excitability, and that removal of this inhibition enhances release of the excitatory neurotransmitters ACh and ATP from type I cells onto postsynaptic neurones.

GABA$_B$ receptor-mediated modulation of neurotransmission occurs via G-protein coupled regulation of PKA

During current-clamp recordings of membrane potential from type I cells in clusters, enhancement of hypoxia-induced depolarisation by OHS persisted in the presence of the specific PKC blocker chelerythrine chloride (100 μM; Fig. 4*A* and *B*). In 12 experiments, hypoxia caused a depolarisation of 3.7 ± 0.9 mV under control conditions; this depolarisation increased to 7.3 ± 1.8 mV in the presence of 50 μM OHS, a value not significantly different from that seen in the presence of both OHS and chelerythrine (7.1 ± 3.2 mV; $P > 0.05$, ANOVA). Similar effects were observed using 5-AVA (not shown), and together these data suggest that modulation of PKC is not involved in the enhanced hypoxic depolarisation due to GABA$_B$ receptor blockade. In contrast, in the presence of the specific PKA blocker, H-89 (50 μM; Fig. 4*C*), or following pre-treatment for 24 h with PTX (500 ng ml^{-1}; Fig. 4*D*), the increase in the hypoxic depolarisation seen in the presence of OHS was abolished. In 11 PTX-treated cells examined, mean hypoxic depolarisation was 2.3 ± 0.4 mV under control conditions and 2.1 ± 0.5 mV in the presence of 50 μM OHS ($P > 0.05$, ANOVA). These data demonstrate that the modulation of presynaptic hypoxic depolarisation due to GABA$_B$ receptor antagonists involves inhibition of PKA coupled to the inhibitory G-protein, G$_i$.

Baclofen enhances K$^+$ current in type I cells by activating a TASK-1-like conductance

During perforated-patch recordings from type I cells in clusters using asymmetrical K$^+$ solutions, the GABA$_B$ receptor agonist baclofen (50 μM) enhanced outward current at more positive potentials (Fig. 5*A*). This enhancement was 14.0 ± 2.8 % relative to control at a test potential of +30 mV ($n = 5$). The *I–V* relationship for the baclofen-sensitive difference current was outwardly rectifying and reversed at −78.7 ± 2.5 mV ($n = 5$), close to the predicted K$^+$ equilibrium potential ($E_K = -83$ mV). Under both physiological and symmetrical K$^+$ conditions, 50 μM baclofen reversibly enhanced currents even at negative potentials, where voltage-dependent K$^+$ currents are inactive. At a test potential of −60 mV, enhancement was 8.8 ± 0.4 % ($n = 5$) under physiological conditions, and 12.1 ± 0.3 % ($n = 7$) under symmetrical K$^+$ conditions. In symmetrical K$^+$ solutions, the baclofen-sensitive (difference) current (see Fig. 5*B*) was linear and reversed at −4.8 ± 1.9 mV ($n = 7$), close to the Nernst equilibrium potential for K$^+$ ($E_K = 0$ mV under symmetrical K$^+$

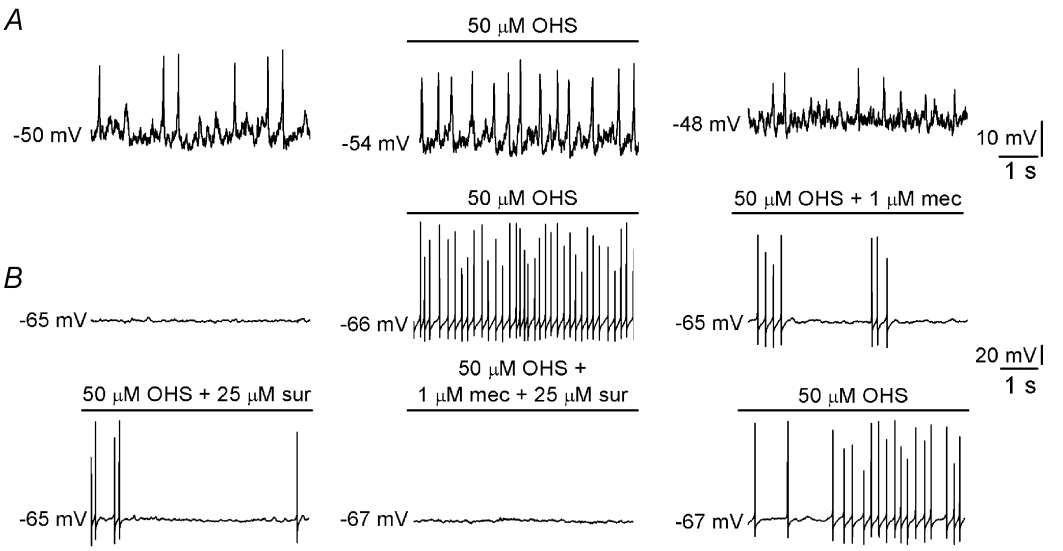

Figure 3. GABA$_B$ receptor blockers enhance excitability and release of ACh and ATP from type I cells

A, effect of 50 μM hydroxysaclofen (OHS) on membrane potential in a presynaptic type I cell. This cell exhibited spontaneous activity, which was reversibly enhanced by OHS. *B*, effect of 50 μM OHS on membrane potential in a petrosal neurone juxtaposed to a type I cluster in co-culture. Application of this GABA$_B$ receptor antagonist induced spiking in this cell, representative of 6 cells that behaved in this way. This effect is due to disinhibition of ACh and ATP release since responses were reduced in the presence of 1 μM mecamylamine (mec) or 25 μM suramin (sur), blockers of nicotinic and purinergic receptors, respectively. In the presence of both blockers OHS-induced spike activity in the neurone was abolished. Scale bars apply to all traces. Together with the data in *A*, this demonstrates a GABA$_B$ receptor-mediated 'braking' mechanism which is constitutively active and reduces presynaptic excitability and the release of fast excitatory neurotransmitters onto postsynaptic neurones.

conditions), suggesting that baclofen activates a K^+-selective, voltage-independent conductance. Enhancement of this current by baclofen was virtually abolished in the presence of 5 μM anandamide (Fig. 5*C*), a selective blocker of TASK-1 (Maingret *et al.* 2001) which appears to be expressed in rat type I cells (Buckler *et al.* 2000). Potentiation due to baclofen was reduced significantly from 15.0 ± 3.3 % in control conditions to 1.5 ± 1.3 % in the presence of anandamide (n = 9 and 7, respectively; $P < 0.01$, Mann-Whitney test). Similarly, a non-specific inhibitor of TASK-1, 10 mM Ba^{2+}, also reduced the degree of potentiation due to baclofen from, 12.4 ± 3.4 % to 3.0 ± 1.8 % (n = 4; $P < 0.05$, Mann-Whitney test).

The above data suggest that baclofen activates a K^+ current with characteristics of the TASK-1-like O_2-sensitive background K^+ channel (Buckler *et al.* 2000). To test this possibility further, whole-cell recordings were made under Ca^{2+}-free, symmetrical K^+ conditions and in the presence of 10 mM TEA, 5 mM 4-AP and 2.5 mM Ni^{2+} to block voltage- and Ca^{2+}-dependent K^+ and Ca^{2+} channels and to isolate background K^+ current (Buckler *et al.* 2000). Under these conditions, the magnitude of the hypoxia-sensitive background current (I_{KO_2}), obtained by subtracting the current obtained during hypoxia from that obtained under control conditions, was enhanced by baclofen (e.g. Fig. 5*D*). At a test potential of −60 mV, I_{KO_2} was increased from 19.5 ± 5.2 pA under control conditions to 46.0 ± 13.2 pA in the presence of 50 μM baclofen (n = 4; $P < 0.05$, Student's paired t test; see Fig. 5*D*, inset). Taken together, these voltage-clamp data demonstrate that type I cell $GABA_B$ receptor activation augments current through TASK-1-like channels, which underlie part of the O_2 sensitivity of these cells (Buckler *et al.* 2000).

In previous studies, $GABA_B$ receptor activation caused inhibition of presynaptic neuronal voltage-gated Ca^{2+} channels (Bowery & Enna, 2000). Under voltage-clamp, 50 μM baclofen was without effect on Ca^{2+} currents in 10 type I cells examined (Fig. 5*D*). Mean Ca^{2+} current induced by step depolarising to +10 mV from a holding potential of −80 mV was −38.2 ± 6.8 pA under control conditions and −41.8 ± 8.5 pA in the presence of 50 μM baclofen (n = 10; P = 0.37, Student's paired t test).

GABA and $GABA_B$ receptor subunit expression in type I cells

To verify the presence of GABA in the rat carotid body, sections of the intact carotid body and cultured type I clusters were immunostained with a GABA-specific polyclonal antibody. Positive immunoreactivity was confined to type I clusters in sections (Fig. 6*A*) and cultures (Fig. 6*B*). To test for expression of $GABA_B$ receptor subunits,

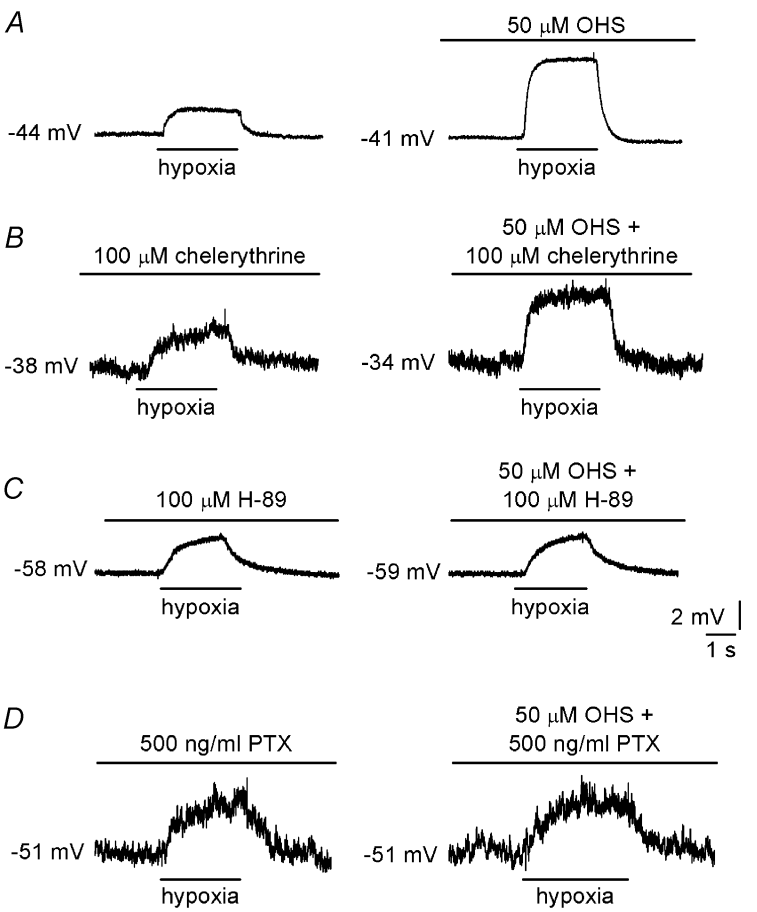

Figure 4. Mechanism of GABA-mediated regulation of synaptic transmission

A, current-clamp recordings made from a type I cell in a cluster. Application of hypoxia (P_{O_2}, 5 mmHg) is indicated by the horizontal bars below each trace. Under control conditions (left), hypoxia induced a depolarisation which was increased in the presence of 50 μM OHS (right). *B*, enhanced depolarisation still occurred in the presence of the selective PKC blocker chelerythrine chloride (100 μM, right). In contrast, enhancement was abolished in the presence of the selective PKA blocker H-89 (50 μM; *C*) or following pretreatment for 24 h with PTX (500 ng ml^{-1}; *D*). In *B* and *C*, the effects of chelerythrine and H-89 were fully reversible on washout of the kinase inhibitors (not shown).

RT-PCR and immunohistochemical techniques were used on isolated type I clusters and carotid body sections, respectively. Using gene-specific primers, PCR products corresponding to target sequences of both the $GABA_{B(1)}$ and $GABA_{B(2)}$ receptor subunits were amplified from mRNA extracted from type I cell clusters (Fig. 6*E*). Sequencing demonstrated the correct identity of PCR products compared to published sequences. When examining the localisation of $GABA_{B(1)}$ and $GABA_{B(2)}$ receptor subunits in sections of the carotid body by immunofluorescence, positive immunoreactivity for both receptor subunits was prominent in type I clusters (Fig. 6*C* and *D*, respectively). In control experiments, no staining was observed when sections were exposed to the secondary antibody without prior exposure to the primary antibody or, in the case of GABA immunoreactivity, following pre-adsorption of the primary antibody with an excess of GABA (not shown).

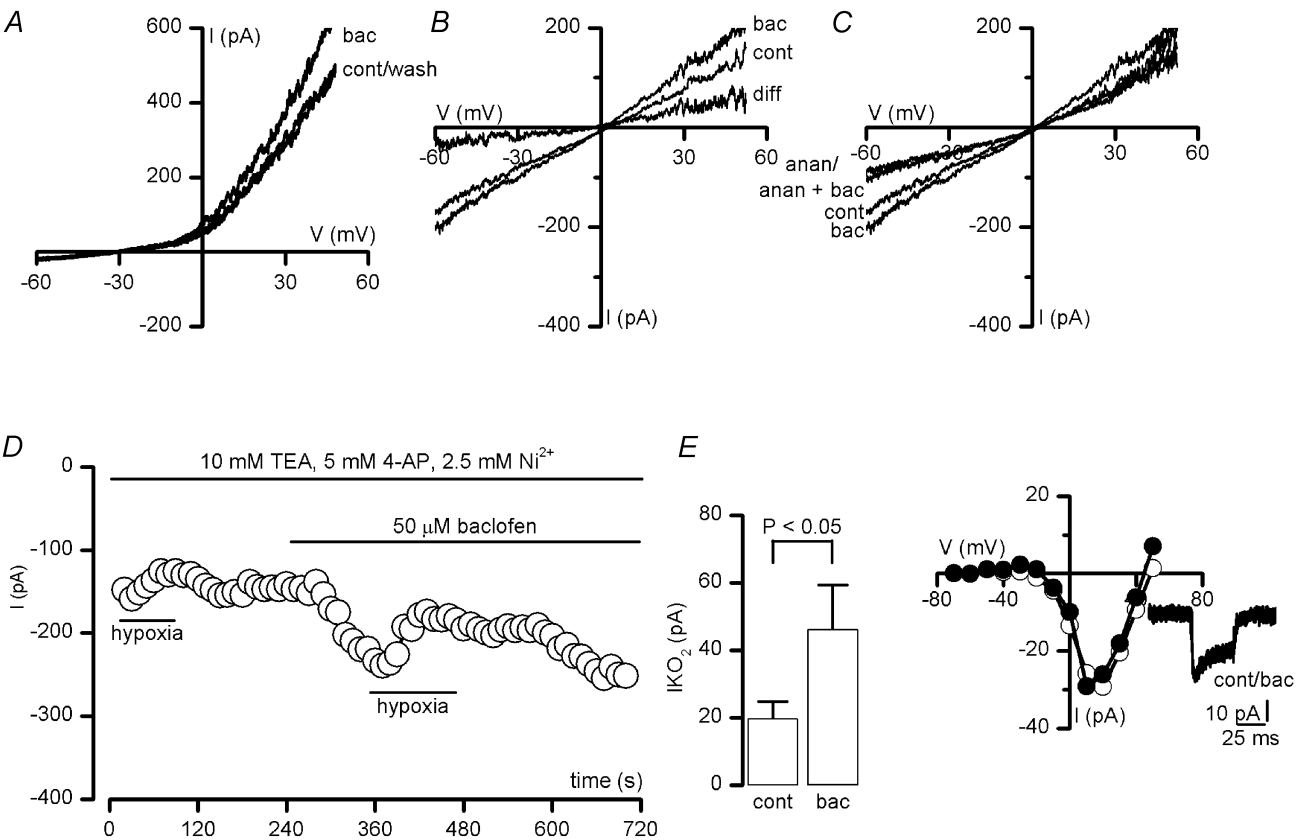

Figure 5. Baclofen activates a TASK-1-like conductance in type I cells

A, voltage-clamp recordings obtained from a type I cell in a cluster. Currents were evoked by ramp depolarisations between −60 and +50 mV under asymmetrical K^+ conditions, in the absence (cont) and presence of 50 μM baclofen (bac) and following washout (wash), as indicated. *B*, as in *A*, except currents were recorded under symmetrical K^+ conditions. Subtraction of the current obtained in the presence of baclofen from that seen under control conditions (to give the indicated difference current) shows that baclofen activates a linear K^+ conductance. *C*, the selective TASK-1 blocker anandamide (anan; 5 μM) ablated the response to baclofen. *D*, time-series recording demonstrating the enhancement of the oxygen-sensitive background K^+ current (I_{KO_2}) by 50 μM baclofen. Typical of 4 such recordings, which were made under Ca^{2+}-free, symmetrical K^+ conditions and in the presence of 2.5 mM Ni^{2+}, 10 mM TEA and 5 mM 4-AP to block voltage- and Ca^{2+}-dependent K^+ channels (Buckler *et al.* 2000). Periods of application of baclofen and hypoxia (P_{O_2}, 5 mmHg) are indicated by the horizontal bars. Currents were measured at a test potential of −60 mV. Inset shows the magnitude of the hypoxic response, obtained by subtracting the current evoked at –60 mV under normoxic conditions from that obtained in hypoxia. Plotted are mean (± S.E.M.) data obtained in 4 cells, under control conditions (cont) and in the presence of 50 μM baclofen (bac), as indicated. *E*, Ca^{2+} channel current–voltage relationships obtained from a type I cell under control conditions (○) and in the presence of 50 μM baclofen (●). Each point shows the peak current amplitude evoked by a 50 ms step depolarisation to the indicated test potential, from a holding potential of −80 mV. Ca^{2+} (5 mM) was used as charge carrier. Inset, individual current traces obtained by step-depolarising to +10 mV for 50 ms, under control conditions and in the presence of 50 μM baclofen (bac).

DISCUSSION

A role for a TASK-1-like conductance in GABAergic modulation of fast synaptic transmission

Background (leak) K^+ currents control neuronal excitability and shape action potentials by controlling the resting membrane potential (Goldstein *et al.* 2001). Enhancement of leak conductances stabilises cells at hyperpolarised potentials, while inhibition leads to membrane depolarisation and excitation. Background currents are carried by members of the K_{2P} family of tandem-pore-domain K^+ channels, of which at least 14 members have been cloned (Goldstein *et al.* 2001, 2003). K_{2P} channel activity is tightly controlled by intracellular factors such as cyclic nucleotide levels and metabolic status, and by extracellular factors such as neurotransmitters, including 5-HT, noradrenaline, substance P, glutamate, TRH and ACh (Millar *et al.* 2000; Talley *et al.* 2000; Goldstein *et al.* 2001). In hypoglossal motoneurones, where TASK-1 is abundantly expressed, native TASK-1-like currents were inhibited by agonists at several G-protein-coupled neurotransmitter receptors (Talley *et al.* 2000). This provides a mechanism whereby neurotransmitters can regulate neuronal excitability and provide slow regulation of fast neurotransmission via intracellular effectors. Here, we demonstrate that a selective agonist at metabotropic receptors for the inhibitory neurotransmitter GABA activates a K^+-selective background conductance in presynaptic chemoreceptor cells of the rat carotid body. Block of this conductance by either anandamide (Maingret *et al.* 2001) or Ba^{2+} ions, alongside data demonstrating that baclofen activates a linear K^+ conductance under conditions where voltage- and Ca^{2+}-dependent K^+ channels are inhibited, suggests that this effect is attributable to activation of a tandem-pore-domain K^+ channel with characteristics of TASK-1. This link between $GABA_B$ receptors and this background K^+ channel provides a novel mechanism for regulating neuronal excitability and synaptic signalling.

In the present study, we examined the effects of several $GABA_B$ receptor antagonists on the depolarising responses to hypoxia in both isolated type I clusters and in petrosal neurons juxtaposed to type I clusters in co-culture. The responses to hypoxia obtained in these studies were modest, particularly when compared to those obtained previously by others when recording hypoxic responses in single type I cells (e.g. Buckler & Vaughan Jones, 1994). The reasons for this are not fully understood, but one possibility is the operation of autoreceptor feedback mechanisms within clusters, such as that reported here, that serve to limit the degree of depolarisation during hypoxia. Such mechanisms are expected to be most effective when recording from clusters as opposed to single cells. In general, the receptor potential in a chemoreceptor cluster reflects a balance between inhibitory (e.g. GABA) and excitatory (e.g. 5-HT; Zhang *et al.* 2003) feedback influences, and this may vary in culture from one cluster to another.

A recent study demonstrated the activation by baclofen of a background K^+ conductance in mouse cerebellar purkinje neurones with pharmacological characteristics of the background channel THIK-1 (Rajan *et al.* 2001; Bushell *et al.* 2002). Furthermore, we recently demonstrated

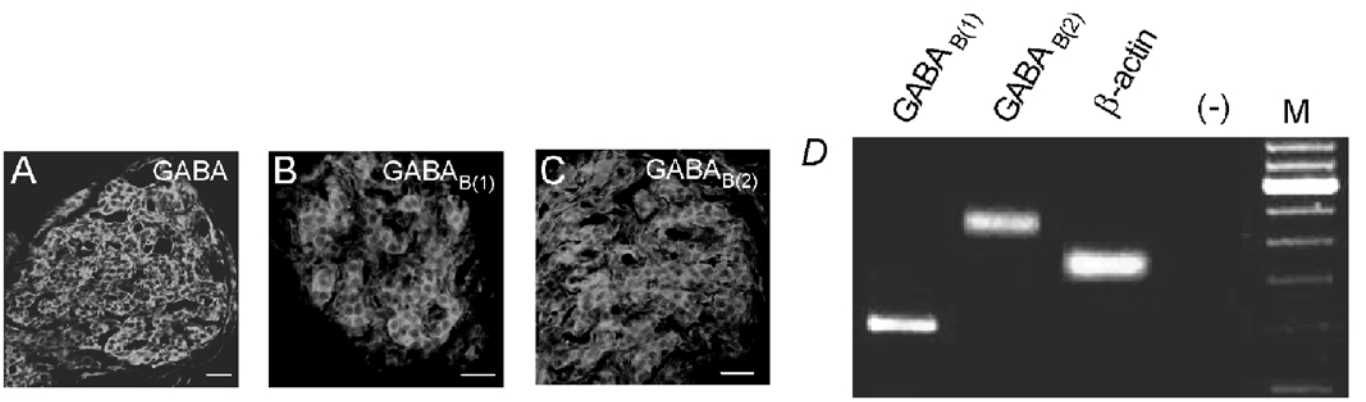

Figure 6. Presence of GABA and $GABA_B$ receptors in type I cells

Confocal images showing carotid body sections which were immunostained with a specific antibodies raised against GABA (*A*), $GABA_{B(1)}$ (*B*) and $GABA_{B(2)}$ (*C*) receptor subunits and visualised by secondary FITC fluorescence. Positive immunostaining of type I clusters is seen in each case. Scale bars represent 20 μm. In all cases, staining was abolished either when sections were exposed to the secondary antibody without prior exposure to the primary antibody, or in the case of GABA the primary antibody was pre-adsorbed with excess antigen (not shown). *D,* micrograph of a 2 % agarose gel stained with ethidium bromide and viewed under UV illumination. RT-PCR was carried out on isolated type I clusters following extraction of mRNA, and using gene-specific primers for the $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits, and β-actin. Marker lane (M) shows bands at 100 bp increments with the 600 bp fragment at increased intensity. In negative control reactions without RT (−) no PCR products were observed.

the O_2 sensitivity of a similar background K^+ current in glossopharyngeal (GPN) neurones (Campanucci *et al.* 2003). However, the THIK-1-like, O_2-sensitive K^+ current in GPN neurones is anandamide-insensitive (Campanucci *et al.* 2003), making it unlikely that these channels mediate O_2 and/or baclofen sensitivity in type I cells.

$GABA_B$ receptors linked to TASK-1 provide presynaptic autoregulatory feedback during hypoxia

GABA is a well characterised inhibitory CNS neurotransmitter and its effects at presynaptic metabotropic $GABA_B$ receptors are thought to underlie, amongst other processes, autoregulation of neurotransmitter release. In many cases regulation involves receptors coupled by G-proteins to plasmalemmal Ca^{2+} and K^+ channels (Misgeld *et al.* 1995; Bowery & Enna, 2000) or to the exocytotic machinery itself (Wu & Saggau, 1997). Here, we present immunohistochemical evidence for the presence of GABA in presynaptic type I cells of the rat carotid body. Examination of type I cell clusters in co-culture with their postsynaptic (petrosal neurone) partners revealed that selective inhibitors of $GABA_B$ receptor function markedly enhanced synaptic transmission in response to hypoxia. This is attributable, at least in part, to a presynaptic mechanism since $GABA_B$ receptor blockade also enhanced the hypoxia-induced depolarisation or receptor potential in type I cells cultured alone.

The mechanism by which $GABA_B$ receptor inhibition enhances the efficacy of hypoxic chemotransmission involves modulation of the activity of PKA, since blockers of this kinase (and not of PKC) inhibited the enhancing effect of the $GABA_B$ receptor blocker hydroxysaclofen on presynaptic depolarisation. Evidence from this study further points to a mechanism involving GABA-mediated

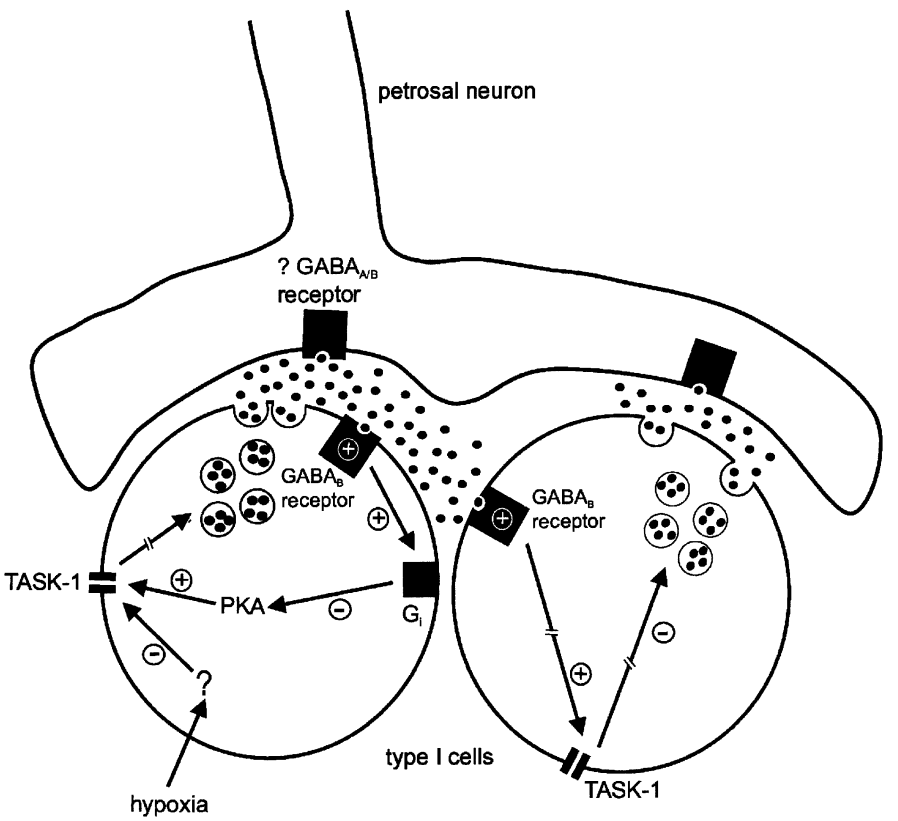

Figure 7. Schematic representation of the autoregulatory pathways involved in the GABA-mediated regulation of neurotransmitter release from type I cells during hypoxia

Via an as yet uncharacterised intracellular pathway, hypoxia inhibits TASK-1-like background channels in type I cells, leading to membrane depolarisation and ultimately (broken arrow) neurotransmitter release. In this process GABA (black circles) is released from type I cells, and acts at presynaptic $GABA_B$ receptors on either the same type I cell (autocrine) or on an adjacent type I cell (paracrine) in the cluster. This causes stimulation of the pertussis toxin-sensitive inhibitory G protein G_i, causing inhibition of protein kinase A (PKA) and subsequently activation of TASK-1. This would serve to hyperpolarise the type I cell and limit the degree of depolarisation during exposure to hypoxia, regulating the further release of transmitters. GABA may also act at postsynaptic ionotropic or metabotropic GABA receptors to modulate chemoreceptor output. For clarity, the involvement of other K^+ channels and neurotransmitters in chemotransmission, and the intracellular events leading to transmitter release, have been omitted.

activation of a background K^+ channel, with properties similar to those of TASK-1, during hypoxia. Inhibition of this TASK-1-like K^+ channel (Buckler *et al.* 2000), is thought to be at least partly responsible for the hypoxic depolarisation or receptor potential in type I cells. The rat isoform of TASK-1 possesses two C-terminal consensus sites for phosphorylation by PKA and furthermore, current through TASK-1 is inhibited by stimulation of this kinase (Leonoudakis *et al.* 1998; Lopes *et al.* 2000). Neuronal $GABA_B$ receptors couple to the inhibitory G protein, G_i (Isaacson, 1998; Leaney & Tinker, 2000), leading to K^+ channel activation (Misgeld *et al.* 1995). Moreover, G_i has also been shown to couple functionally to a $GABA_B$ receptor–adenylyl cyclase system (Nishikawa *et al.* 1997). In our system, inhibition of G_i by pretreatment with PTX abolished presynaptic sensitivity to $GABA_B$ receptor blockade. We suggest therefore that in the rat carotid body, presynaptic $GABA_B$ receptor activation by GABA released from type I cells couples to G_i, resulting in inhibition of PKA activity and activation of background K^+ channels. This pathway is enhanced during hypoxic stimulation, due to depolarisation-evoked GABA release which leads to activation of presynaptic $GABA_B$ autoreceptors and subsequently activation of the TASK-1-like conductance via G_i-mediated inhibition of PKA. This cascade gives rise to a hyperpolarisation which effectively blunts the depolarising receptor potential due to hypoxia. The end result is an autoregulatory feedback mechanism (see Fig. 7 for schematic representation of this cascade) that modulates the release of neurotransmitters from the receptor cells during hypoxia via convergence of two separate signalling pathways onto the same K^+ channels. We further suggest that the basal activation of $GABA_B$ receptors exerts control over the excitability of presynaptic type I cells, since blockade of this constitutive activity with hydroxysaclofen enhanced the excitability of presynaptic type I cells in which spontaneous activity was observed. Taken with our data, this suggests that basal $GABA_B$ receptor activation stimulates G_i, causing inhibition of adenylate cyclase and a reduction in cAMP levels, which would reduce type I cell excitability via the activation of the TASK-1-like conductance.

In the rabbit carotid body dopamine, acting at presynaptic D_2 receptors, exerts autoregulatory feedback to inhibit the further release of this transmitter (Bairam *et al.* 2000), presumably via the inhibition of voltage-dependent Ca^{2+} currents (Benot & Lopez-Barneo, 1990). The presence of dopamine and presynaptic D_2 receptors (Gauda *et al.* 1996; Donnelly, 2000) suggests that similar autoreceptor feedback loops may control neurotransmitter output in the rat carotid body, although this has not been directly tested. Since dopamine D_2 receptors are linked to inhibition of adenylate cyclase and reduce cellular cAMP levels upon activation, it is possible that dopamine acting via presynaptic D_2 receptors elicits a similar autoinhibitory feedback loop as that evoked by $GABA_B$ receptor activation. To support this possibility, D_2 receptor activation has been shown in many cases to activate neuronal K^+ conductances (Lacey *et al.* 1987; Freedman & Weight, 1988; Casteletti *et al.* 1989). On the other hand, recent studies from this laboratory indicate that paracrine release of 5-HT from clustered type I cells produces the opposite effect and augments the receptor potential via PKC-mediated inhibition of K^+ channels (Zhang *et al.* 2003).

Autoreceptor regulation of neurotransmitter release in the CNS is mediated in part by activation of presynaptic $GABA_B$ receptors which modulate voltage-gated Ca^{2+} channels (Misgeld *et al.* 1995; Bowery & Enna, 2000). However, in the present studies $GABA_B$ receptor activation was without effect on Ca^{2+} channel activity in presynaptic type I cells. In the neonatal rat carotid body, Ca^{2+} current is carried almost exclusively by L-type channels (Stea *et al.* 1995; Peers *et al.* 1996). Although the inhibitory effects of $GABA_B$ receptor activation on neuronal N- (e.g. Lambert & Wilson, 1996) and P/Q-type (e.g. Chen & van den Pol, 1998) Ca^{2+} channels are well documented, there is little evidence to suggest that L-type Ca^{2+} channels are modulated by $GABA_B$ receptors. Moreover in some neurones which express multiple Ca^{2+} channel subtypes, effects of $GABA_B$ receptor activation on L-type Ca^{2+} currents are negligible (Harayama *et al.* 1998) or non-existent (Doze *et al.* 1995) compared to effects on N- and P/Q-type channels. Also consistent with our data, there is no evidence of regulation by PKA of L-type Ca^{2+} channels in type I cells from other species (Summers *et al.* 2000). Thus, autoregulation of neurotransmitter release from type I cells is mediated by presynaptic activation of a K^+ current rather than Ca^{2+} channel inhibition. Activation of K^+ conductances by $GABA_B$ receptor agonists has mostly been described in postsynaptic CNS neurones (Misgeld *et al.* 1995), while other studies have demonstrated no effect of $GABA_B$ receptor activation on presynaptic K^+ conductances (Isaacson, 1998). However, Wagner & Dekin (1993, 1997) demonstrated the regulation by cAMP and activation by baclofen of K^+ channels in presynaptic respiratory neurones with pharmacological and biophysical characteristics similar (although not identical) to those described for background K^+ channels. Since TASK-1 is expressed in respiratory neurones (Bayliss *et al.* 2001), it is possible that $GABA_B$ receptors mediate presynaptic regulation of TASK-1 in these cells. Furthermore, background K^+ channels and $GABA_B$ receptors are co-expressed in a variety of neuronal cell types and these findings open the possibility that $GABA_B$ autoreceptors linked to background channels may be a general mechanism for controlling CNS excitability.

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