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EPAC signalling pathways are involved in low P_{O_2} chemoreception in carotid body chemoreceptor cells

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Chemoreceptor cells of the carotid bodies (CB) are activated by hypoxia and acidosis, responding with an increase in their rate of neurotransmitter release, which in turn increases the electrical activity in the carotid sinus nerve and evokes a homeostatic hyperventilation. Studies in isolated chemoreceptor cells have shown that moderate hypoxias ($P_{O_2} \approx 46$ mmHg) produces smaller depolarisations and comparable Ca^{2+} transients but a much higher catecholamine (CA) release response in intact CBs than intense acidic/hypercapnic stimuli (20% CO_2, pH 6.6). Similarly, intense hypoxia ($P_{O_2} \approx 20$ mmHg) produces smaller depolarizations and Ca^{2+} transients in isolated chemoreceptor cells but a higher CA release response in intact CBs than a pure depolarizing stimulus (30–35 mM external K^+). Studying the mechanisms responsible for these differences we have found the following. (1) Acidic hypercapnia inhibited I_{Ca} (∼60%; whole cell) and CA release (∼45%; intact CB) elicited by ionomycin and high K^+. (2) Adenylate cyclase inhibition (SQ-22536; 80 μM) inhibited the hypoxic release response (>50%) and did not affect acidic/hypercapnic release, evidencing that the high gain of hypoxia to elicit neurotransmitter release is cAMP dependent. (3) The last effect was independent of PKA activation, as three kinase inhibitors (H-89, KT 5720 and Rp-cAMP; ≥ 10 × IC_{50}) did not alter the hypoxic release response. (4) The Epac (exchange protein activated by cAMP) activator (8-pCPT-2′-*O*-Me-cAMP, 100 μM) reversed the effects of the cyclase inhibitor. (5) The Epac inhibitor brefeldin A (100 μM) inhibited (54%) hypoxic induced release. Our findings show for the first time that an Epac-mediated pathway mediates O_2 sensing/transduction in chemoreceptor cells.

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Abbreviations CA, catecholamines; cAMP-GEF, cAMP regulated guanine nucleotide exchange factor; CB, carotid body; CSN, carotid sinus nerve; DA, dopamine; Epac, exchange protein activated by cAMP.

Carotid bodies (CB) are small paired sensory organs located in the vicinity of the carotid artery bifurcation. These chemoreceptor organs are formed by clusters of parenchymatous cells (chemoreceptor and sustentacular) surrounded by a dense net of capillaries. Sensory nerve fibres from the carotid sinus nerve (CSN) form synaptic contacts with chemoreceptor cells. Functionally, CBs represent the main arterial chemoreceptor being responsible for the entire hyperventilation that occurs in hypoxic hypoxia (Lahiri, 1976; Honda, 1985) and contributing (∼25–40%) to the compensatory hyperventilation that occurs in situations of acidosis, both respiratory and metabolic in origin (Berkenbosch *et al.* 1979; Nattie, 1999). These reflex ventilatory responses are initiated at the level of the chemoreceptor cells, which represent the P_{O_2} and $[H^+]$ sensing elements in this chemoreceptor organ (Fidone & Gonzalez 1986; Gonzalez *et al.* 1994). Chemoreceptor cells in response to hypoxia and acidosis increase their resting rate of release of neurotransmitters, which in turn augment the action potential frequency in the CSN; central projection of the CSN into the brainstem drives ventilation. Acidic stimuli might directly stimulate the sensory nerve endings of the CSN, this direct effect also contributing to increase the CSN frequency in situations of acidosis (Rigual *et al.* 1984).

Acidic/hypercapnic stimuli, even the most intense stimuli tested (considered supramaximal) in normoxia and hyperoxia, elicit a level of activity in the CSN that oscillates between 25 and 40% of the maximal hypoxic activity (Fitzgerald & Parks, 1971; Lahiri & Delaney, 1975;

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Fitzgerald, 1976; Pepper *et al.* 1995). When the compound output of the CB chemoreceptor cells is measured as the rate of neurotransmitter release (e.g. CA release) from the intact organ, the difference is even more striking, as for example a supramaximal acidic/hypercapnic stimulus (20% CO_2-equilibrated solution/pH 6.6) causes a release response 3–4 times smaller than that elicited by a hypoxic stimulus of moderate intensity ($P_{O_2} \approx 46$ mmHg) (Rigual *et al.* 1986 *vs.* 1991; Vicario *et al.* 2000*b*; Rocher *et al.* 2005). In contrast to the integrated responses of the CB, i.e. CSN activity and release of CA, chemoreceptor cell depolarizations produced by intense hypoxia ($P_{O_2} \approx 20$–30 mmHg) are smaller (6.8 ± 0.5 mV) than those produced by acidic/hypercapnic stimulus (20% CO_2, pH 6.6; 11.8 ± 0.7 mV) in isolated rabbit chemoreceptor cells (Rocher *et al.* 2005; see Buckler & Vaughan-Jones, 1994*a*,*b* for data in the rat). Yet, the increases in intracellular Ca^{2+} produced by intense hypoxia and hypercapnic/acidic stimuli are comparable (Buckler & Vaughan-Jones, 1994*a*,*b*; Dasso *et al.* 2000). Mechanisms causing these variable relationships between cell depolarization and intracellular Ca^{2+} levels on the one hand and the intensity of exocytosis on the other are unknown. In addition, a moderate pure depolarizing stimulus (30–35 mM external K^+) elicits a CA release response comparable to that elicited by moderate hypoxia ($P_{O_2} \sim 46$ mmHg; Rocher *et al.* 2005) but an intracellular Ca^{2+} rise several times higher than that produced by hypoxia even of high intensity (Buckler & Vaughan-Jones, 1994*a*,*b*; Sanchez *et al.* 2002). Again, mechanisms for the large gain of hypoxic stimulus to elicit neurotransmitter release are unknown. In sum the level of chemoreceptor cell depolarization elicited by hypoxia is smaller than that elicited by acidic/hypercapnic stimuli and both stimuli generate comparable Ca^{2+} transients; however, more integrated responses elicited by hypoxia, i.e. release of neurotransmitters or neural activity in the CSN, are much greater than those elicited by acidic/hypercapnic stimuli. The general aim of present study has been to find the mechanisms involved in these disparate responses.

In the present study we have performed electrophysiological and neurochemical experiments aimed at providing answers to the preceding questions. Among others, specific questions addressed in our study include: why do acidic/hypercapnic stimuli which produce larger depolarizations than intense hypoxia cause comparable Ca^{2+} transients? Conversely, why do hypoxic stimuli elicit a stronger release response than intense acidic stimuli for the same Ca^{2+} signal? Why do moderate hypoxic stimuli elicit a comparable release response to 30 mM K^+ if they generate a weaker intracellular Ca^{2+} signal? Our data indicate that the weak response to the acidic stimuli is due mainly to a direct inhibition of the exocytotic machinery by the high H^+ concentrations. The higher capacity of hypoxia to elicit neurotransmitter release is due to the activation of production of cAMP, which activates exocytosis by an Epac-mediated PKA-independent mechanism.

Methods

Animals and surgery

Adult New Zealand White rabbits (1.5–2.5 kg) were anaesthetized with sodium pentobarbital (40 mg kg^{-1}; I.V., lateral vein of the ear). Animals were tracheostomized and, after adequate dissections, bilateral blocks of tissue containing the carotid bifurcations were removed and placed in a lucite chamber filled with ice cold O_2-saturated Tyrode solution (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; Hepes, 10; glucose, 5.5; pH 7.40). The CBs were identified and cleaned of surrounding tissues under a dissecting microscope. Cleaned CBs were saved in independent vials containing fresh Tyrode solution at 0–4°C until the 8–12 CBs used in a given experiment were collected. Animals were killed with an intracardiac overdose (150 mg (kg weight)$^{-1}$) of sodium pentobarbital. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the Universities of Valladolid and Miguel Hernandez.

[^{3}H]Catecholamine release experiments and analytical procedures

Groups of six CBs were incubated during 2 h in small glass vials placed in a metabolic shaker (37°C) containing 0.5 ml of Tyrode solution supplemented with 20 μM [^{3}H]tyrosine ([3–5-^{3}H]tyrosine; 30 Ci $mmol^{-1}$; Amersham Iberica, Madrid, Spain), 1 mM ascorbic acid and 100 μM 6-methyl-tetrahydropterine (Sigma, Madrid, Spain), cofactors of dopamine-β-hydroxylase and tyrosine hydroxylase, respectively (Fidone & Gonzalez, 1982).

At the end of this period of incubation to label CA stores with ^{3}H tyrosine, organs were transferred to individual vials containing 4 ml of [^{3}H]tyrosine-free Tyrode bicarbonate solution (composition as above except for equimolar replacement of 24 mM NaCl with $NaHCO_3$), which, under continuous bubbling with water vapour saturated 20% O_2–5% CO_2, balance N_2, yields a pH of 7.4. For a period of 2 h incubating solutions were renewed every 30 min and discarded, eliminating in this manner the labelled precursor and the readily releasable pool of [^{3}H]CA (Almaraz *et al.* 1986). Thereafter, incubations continued and the incubating solutions were collected every 10 min for analysis of their [^{3}H]CA content. For most of the experiments, half of the CBs used in a given experiment were used as controls, being subjected to two consecutive identical stimuli (S1 and S2, respectively),

consisting of the incubation of the organs in solutions with a low P_{O_2} (equilibrated with 7% O_2–5% CO_2–88% N_2 or 2% O_2–5% CO_2–93 N_2). In K^+-rich solutions, equimolar amounts of Na^+ were removed to maintain the osmolarity, and in solutions with high P_{CO_2} and low pH (equilibrated with 20% O_2–20% CO_2–60% N_2), the desired pH of 7.00, 6.80 or 6.60 was attained by varying the $NaHCO_3$ concentration and adjusting the NaCl accordingly to maintain osmolarity. To study the interactions between hypoxic and acidic/hypercapnic stimuli, or between high K^+ and acidic/hypercapnic stimuli, both pairs of stimuli were combined correspondingly (see Results). The other half of the CBs in any given experiment (experimental) were similarly incubated and stimulated except for the presence in the S2 incubation of a variable with respect to the corresponding controls. The use of specific drugs, as well as their protocols of application, will also be described in Results.

Immediately upon collection, incubating solutions were acidified to pH 3–3.5 with glacial acetic acid and saved at 4°C to prevent degradation of the released [^{3}H]CA. The analysis of incubating solutions for their [^{3}H]CA content consisted of specific adsorption into alumina of the labelled catechols at pH 8.6, their elution with 1 M HCl and quantification by liquid scintillation spectrometry (Almaraz *et al.* 1986; Obeso *et al.* 1992). At the end of the experiments, the CBs were homogenized in 300 μl 0.4 M perchloric acid, centrifuged for 5 min in a microfuge (Beckman) and the [^{3}H]CA present in the supernatants was analysed as incubating solutions.

The stimulus-evoked release of [^{3}H]CA in S1 and S2 was calculated as counts per minute (c.p.m.) above basal release, as shown graphically in Figs 1*B* and 2, and expressed as a percentage of the [^{3}H]CA present in the organ immediately before the application of the stimulus. Since there is a time-dependent decay in the absolute

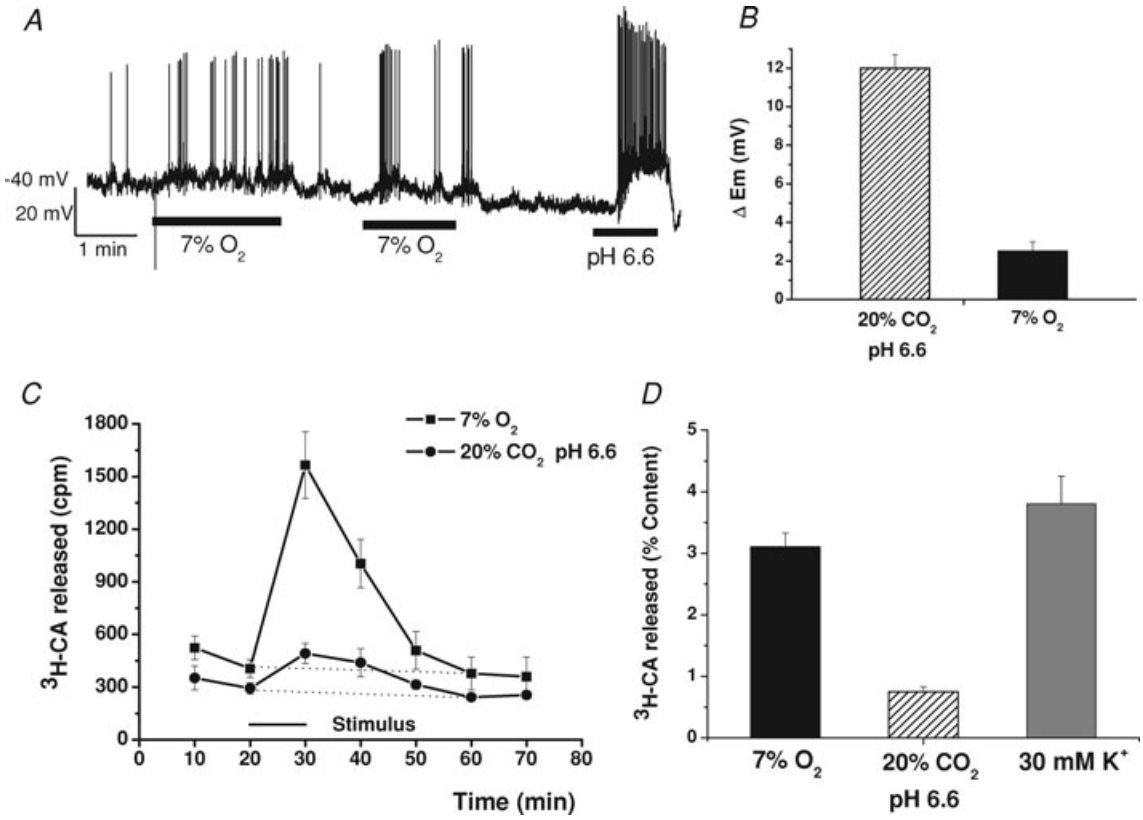

Figure 1. Effect of hypoxia and hypercapnic acidosis on chemoreceptor cell membrane potential and on the [^{3}H]CA release from *in vitro* CB

A, a sample record (perforated-patch) of membrane potential changes elicited by moderate hypoxia (7% O_2, $P_{O_2} \approx 46$ mmHg) and intense hypercapnic acidosis (20% CO_2, pH 6.6) in a dissociated chemoreceptor cell; the remaining time the cell was superfused with control solution (20% O_2-5% CO_2–75% N_2; pH 7.40). *B*, mean depolarizations produced by moderate hypoxia and intense hypercapnic acidosis ($n = 7$ and 12, respectively). *C*, the time course of stimulus-elicited [^{3}H]CA release from two groups of 6 CBs. Stimulus (7% O_2 or 20% CO_2, pH 6.6) was applied for 10 min as indicated in the drawing. *D*, total release response evoked (equivalent to c.p.m. above dotted line in *C*) by moderate hypoxia, intense acidic stimuli and 30 mM K^+. Data represent percentage of tissue CA content and are means ± S.E.M. for 6 CBs.

amount of [^{3}H]CA released, the ratios of the evoked [^{3}H]CA release in S2 to that in S1 (S2/S1) were calculated, and the S2/S1 ratios obtained in control and experimental CBs were compared. Statistical significance of the observed differences was assessed using Student's two-tailed t test for unpaired data; the significance level was established at $P < 0.05$. There were no statistically significant differences in the magnitude of S1 evoked release between control and experimental CB in any experimental condition. Results are expressed as means ± S.E.M.

Cell preparation and electrophysiological recordings

Chemoreceptor cells were obtained from rabbit CBs dissected as in the release experiments. The CBs were incubated (30 min) in nominally Ca^{2+}- and Mg^{2+}-free Tyrode solution (pH 7.2) containing collagenase (2.5 mg ml^{-1}, type IV, Sigma) and bovine serum albumin (6 mg ml^{-1}, Fraction V, Sigma). After incubation tissues were centrifuged (800 *g*, 5 min) and the pellet was resuspended in a new solution containing collagenase (1 mg ml^{-1}), trypsin (1 mg ml^{-1}, type II, Sigma) and bovine serum albumin (6 mg ml^{-1}) and incubated for an additional 15 min period. Tissues were subjected to mechanical disruption every 10 min by repeated aspiration through a fire-polished Pasteur pipette during both incubation periods. Finally, in the second incubation, dissociated cells were pelleted by centrifugation (800 *g*, 8 min), washed in an enzyme-free Tyrode solution and resuspended in 100 μl of culture medium (Dulbecco's modified Eagle's medium (DMEM)–F-12; Sigma), supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 μg ml^{-1} streptomicin and 40 μg ml^{-1} gentamicin. Drops of cell suspension of 20 μl were

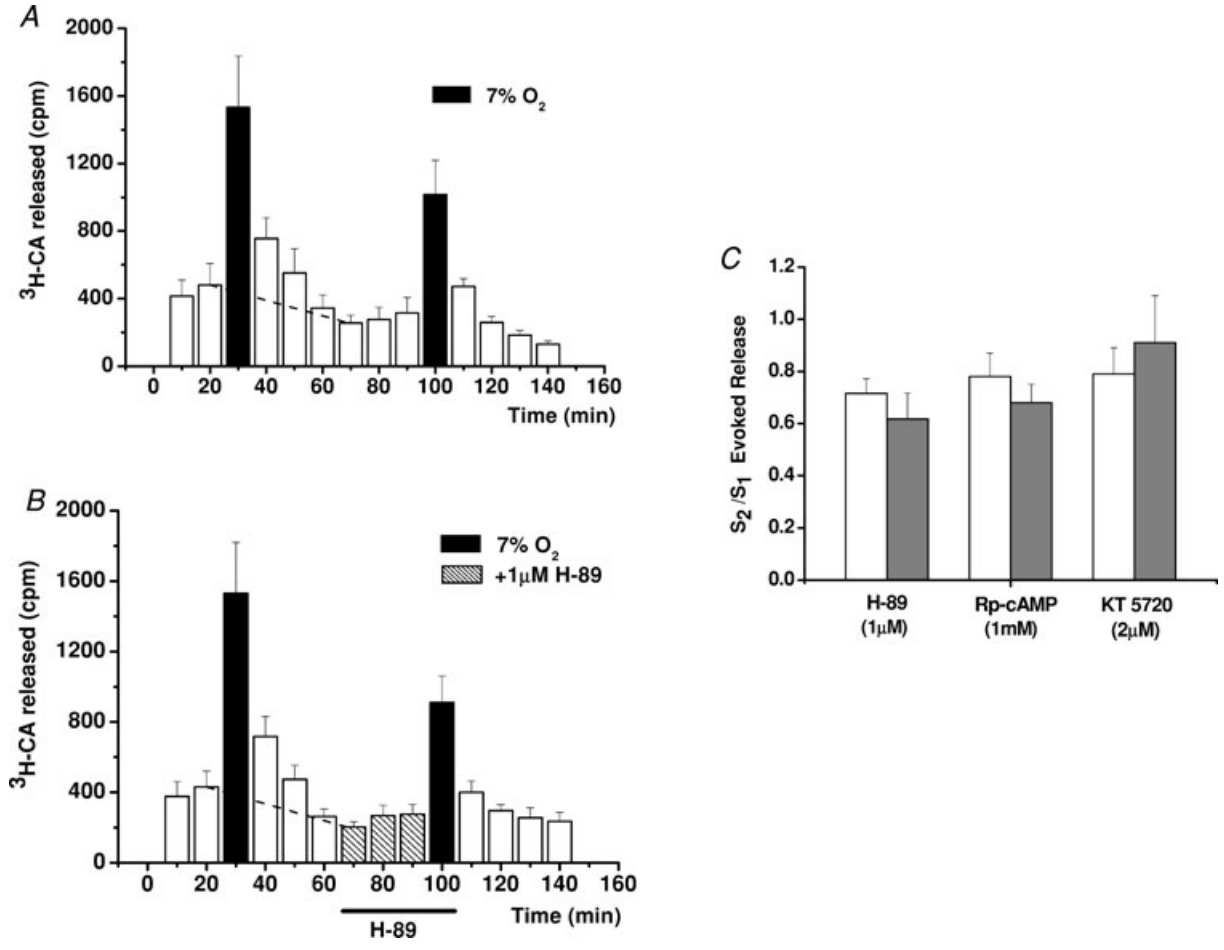

Figure 2. Effects of PKA inhibition on the release of [^{3}H]CA elicited by moderate hypoxia
A and *B* represent mean time courses of the release obtained in 6 control CBs challenged twice with a hypoxic stimulus (7% O_2-equilibrated solutions), and in 6 experimental CBs similarly stimulated except that 30 min prior to and during the second hypoxic challenge, 1 μM H-89 was present in the incubating solution. *C*, results of similar experiments using the three indicated PKA inhibitors; bars represent ratios of total evoked release obtained in the second challenge to that obtained in the first one (S2/S1) for control (open bars) and experimental CBs (grey bars). Values are means ± S.E.M. for 6–8 CBs.

plated on small poly-L-lysine-coated coverslips kept in 3.5 cm diameter Petri dishes in a humidified incubator (37°C; 5% CO_2 in air). Once the cells attached (around 60 min), 2 ml of culture medium was added to the Petri dish to maintain the cells until use (3–36 h later). Coverslips were transferred to the recording chamber (0.15 ml volume; Warner Instruments LLC, Hamden, CT, USA) on the stage of an inverted microscope (Nikon Diaphot-TMD) and superfused by gravity (1.5–3 ml min^{-1}).

The recordings of membrane potential in chemoreceptor cells were performed using the perforated-patch configuration and at 33–35°C. Bath solutions were bicarbonate-buffered and identical to those used in the [^{3}H]CA release experiments (see above). Pipette (borosilicate glass; 1.5 mm o.d.; Clark Electromedical Instruments) solution was (in mM): KCl, 35; potassium gluconate, 95; $MgCl_2$, 3; EGTA, 5; Hepes, 10; pH was adjusted to 7.2 by addition of NaOH (final sodium concentration 14 mM). Nystatin was added at a final concentration of 100–150 μg ml^{-1} (Albillos *et al.* 2000). Resistance of pipettes filled with internal solution was 2.0–3.5 MΩ. Voltage signals were recorded with an EPC-7 amplifier (List Medical, Darmstadt, Germany) or an RK 300 amplifier (BioLogic, Claix, France). Pulse generation, data acquisition and analysis were made through an A/D converter (CED 1401, Cambridge Electronic Design, Cambridge, UK) or through a Digidata 1322A (Axon Instruments, Union City, CA, USA) commanded by the software package 'Strathclyde Electrophysiology Software' (kindly provided by J. Dempster, Strathclyde University) or pCLAMP software (Axon Instruments). Voltage recordings were filtered at 2 kHz and sampled at 10–16 kHz.

Recordings of calcium currents in chemoreceptor cells were performed at room temperature (20–23°C) using the patch-clamp technique in the whole-cell configuration. When filled with intracellular solution (in mM: CsCl, 130; $MgCl_2$, 2; Hepes, 10; ethylene glycol-bis(-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10; ATP, 4; and GTP 2; pH 7.2 with CsOH), pipette resistance was 5–6 MΩ. Bath extracellular solutions containing 2 mM Ca^{2+} were identical to those used in the CA release experiments, but contain 5×10^{-7} M tetrodotoxin to block voltage-dependent sodium channels (TTX, Alomone Laboratories, Jerusalem, Israel). Calcium currents were elicited by depolarizing cells to −10 mV from a holding potential of −80 mV every 10 s (pulse duration 20 ms). The effect of hypercapnic acidosis on voltage-dependent calcium currents was tested in standard conditions as well as during superfusion of the cells in the presence of 10^{-6} M Bay K 8644 (Sigma), an activator of L-type calcium channels. Amplitudes of leak-subtracted Ca^{2+} currents were measured at the end of the voltage pulse and are expressed as means ± S.E.M.

Chemicals

[3–5-^{3}H]Tyrosine was from Amersham Iberica (Madrid, Spain); 6-methyl-tetrahydropterine, collagenase (type IV), trypsin (type II), nystatin, ionomycin, Bay K 8644, SQ-22536, H-89, KT 5720, Rp-cAMP, 8-pCPT-2′-*O*-Me-cAMP and brefeldin A were all from Sigma (St Louis, MO, USA).

Results

Effect of hypoxia and hypercapnic acidosis on chemoreceptor cell membrane potential. Comparison with the [^{3}H]CA release response

The magnitude of rabbit chemoreceptor cell depolarization induced by moderate hypoxia (superfusion with 7% O_2-equilibrated solution) and intense hypercapnic acidosis (superfusion with 20% CO_2 solution; pH 6.6) was studied in perforated-patch recorded cells. Some cells discharged spontaneous fast action potentials of 50–80 mV in amplitude in resting normoxic conditions (perfusing solution equilibrated with 20% O_2–5% CO_2; $P_{O_2} \approx$ 136–140 mmHg; Fig. 1*A*), and after switching the perfusion from normoxia to a moderate hypoxia (equilibrated with 7% O_2; $P_{O_2} \approx$ 46 mmHg) there was a small depolarization accompanied in some cells by an increase in the action potential frequency that did not follow a uniform pattern. On perfusing the cells with a hypercapnic/acidic solution the magnitude of depolarization as well as the spiking frequency was greater than with moderate hypoxia. Mean depolarizations observed were, respectively, 2.5 ± 0.5 and 12.0 ± 0.7 mV in moderate hypoxia and intense hypercapnic acidosis ($n = 7$ and 12 respectively; Fig. 1*B*), with resting membrane potential in the recorded cells oscillating between −40 and −55 mV. It has been reported previously that hypoxia of higher intensity ($P_{O_2} \approx$ 15–30 mmHg) produced more intense depolarizations of rabbit chemoreceptor cells (7–13 mV; see Perez-García *et al.* 2000; Rocher *et al.* 2005). In rabbit chemoreceptor cells as well as in rat chemoreceptor cells (e.g. Buckler & Vaughan-Jones, 1994*a*,*b*; Zhong *et al.* 1997), their spiking behaviour in response to these stimuli was variable, with some cells firing at relatively high frequencies and some others not spiking at all (Perez-García *et al.* 2000; Rocher *et al.* 2005).

Figure 1*C* shows the mean time course of the release of [^{3}H]CA elicited by the moderate hypoxic and the intense hypercapnic/acidic stimulus. Both stimuli caused a peak [^{3}H]CA release during the period of the stimulus application that was higher with the hypoxic stimulus, and for both stimuli, the [^{3}H]catechols present in the incubating solutions decreased in the post-stimulus periods to basal levels. Figure 1*D* shows the magnitude

of the evoked response, it being evident that moderate hypoxia elicited a release response considerably more intense ($3.10 \pm 0.23\%$ of total [^{3}H]CA present in the tissue) than hypercapnic acidosis ($0.75 \pm 0.08\%$ of total [^{3}H]CA present in the tissue). Thus, moderate hypoxia which produced a smaller depolarization and a smaller intracellular Ca^{2+} rise than intense hypercapnic acidosis (Buckler & Vaughan-Jones, 1994*a*,*b*) generated a more intense [^{3}H]CA release response. Figure 1*D* also shows that depolarising chemoreceptor cells with 30 mM K^+ elicited a release response comparable to that elicited by 7% O_2 and less than half of that elicited by intense hypoxia (~22 mmHg; Rocher *et al.* 2005).

Exocytosis of [^{3}H]CA elicited by hypoxia in chemoreceptor cells is enhanced by an adenylate cyclase-dependent and protein kinase A (PKA)-independent mechanism

Hypoxias of moderate intensities producing intracellular Ca^{2+} levels smaller than those generated by intense hypercapnic acidosis caused a much greater [^{3}H]CA release response. Since hypoxia increases cAMP levels in the CB and agents elevating cAMP levels in the CB increase the release of [^{3}H]CA elicited by hypoxia (Perez-García *et al.* 1990, 1991), we tested the possibility of this effect being mediated by a PKA-dependent mechanism. Figure 2*A* shows the mean time course of the release of [^{3}H]CA induced by two consecutive applications of a moderate hypoxic stimulus in control CBs, and Fig. 2*B* shows identical experiments in which prior to and during the second hypoxic stimulation CBs were incubated in the presence of 1 μM of H-89, a highly specific inhibitor of PKA, at concentration ~20 times IC_{50} ($IC_{50} = 50$ nM). There were no evident alterations either in the time course of the release caused by the PKA inhibitor or in the intensity of the release response (Fig. 2*C*) since the ratios of the evoked release in the second to the first stimulus (S2/S1 ratios) were not different in control and drug treated organs. Similarly, the widely used PKA inhibitors KT 5720 ($IC_{50} = 56$ nM) and Rp-cAMP ($IC_{50} = 15$ μM) at concentrations over 20 times the IC_{50} lacked significant effects on the [^{3}H]CA release response elicited by hypoxia (Fig. 2*C*). These and previously published observations (Perez-García *et al.* 1991) led to the next group of experiments in which we tested the effects of an adenylate cyclase inhibitor.

Figure 3*A* and *B* compares the time courses of the release of [^{3}H]CA elicited by hypoxia in the absence and in the presence of the adenylate cyclase inhibitor SQ-22536 (80 μM) evidencing that the drug caused a decrease in the intensity of the [^{3}H]CA release response, without altering the time course. The magnitude of the inhibition of the response is best appreciated by comparing the S2/S1 ratios for control and SQ-22536-treated CBs; the adenylate cyclase inhibitor reduced by nearly 50% the response elicited by the hypoxic stimulus ($P < 0.001$; Fig. 3*D*). These findings and those presented in Fig. 2 indicated that cAMP was producing its potentiating effects by a mechanism independent of the classical PKA signalling cascade, and this led us to search for the possible involvement of the recently described cascade mediated by exchange proteins directly activated by cAMP (Epacs; Seino & Shibasaki, 2005; Holz *et al.* 2006). Then, in a new group of experiments we compared the effects of the cyclase inhibitor (SQ-22536, 80 μM; $IC_{50} = 20$ μM) with the effects of the cyclase inhibitor plus the Epac activator (8-pCPT-2′-*O*-Me-cAMP, 0.1 mM; $IC_{50} = 2.2$ μM) applied simultaneously (Fig. 3*C* and *D*). This showed that the Epac activator more than reversed the effect of the adenylate cyclase inhibitor ($P < 0.05$ when compared with controls). In an additional group of experiments with moderate hypoxia as stimulus, we tested the effect of the 8-pCPT-2′-*O*-Me-cAMP alone and found that it produced a non-significant tendency to increase the response elicited by the hypoxic stimulus (Fig. 3*D*). The non-significant effect of the Epac activator was expected, because at this moderate level of hypoxic stimulation the increase in endogenous cAMP is maximal (Perez-García *et al.* 1990) and therefore the endogenous activation of the Epac pathway should be maximal or near maximal. Finally, using identical protocols we also tested the cyclase inhibitor (SQ-22536, 80 μM) on the release induced by the acidic/hypercapnic stimulus, and found that it did not cause modifications in the time course of the release (not shown) and nor did it alter the magnitude of the response (Fig. 3*D*), a finding that is consistent with the observation that acidic stimuli *per se* do not increase the cAMP levels (Perez-García *et al.* 1990). Thus, this group of experiments indicates the existence in chemoreceptor cells of Epac-mediated mechanism(s) acting as enhancer(s) of the release response to hypoxic stimuli.

A confirmation of the participation of Epacs was obtained with the use of brefeldin A, an Epac-signalling inhibitor (Huang & Hsu, 2006; Ster *et al.* 2009). As shown in Fig. 3*D*, brefeldin A inhibited the release induced by a moderate hypoxic stimulus by about the same percentage (54%) as the inhibition of adenylate cyclase.

Hypercapnic acidosis inhibits exocytosis in chemoreceptor cells

As shown in the previous figures, even after inhibiting adenylate cyclase the release response elicited by a hypoxic stimulus of moderate intensity is higher than that elicited by a acidic/hypercapnic stimulus of supra-maximal intensity. This observation lead us to suspect that hypercapnic–acidosis might have a dual effect: as a stimulus to chemoreceptor cells it would tend to promote

a Ca^{2+}-dependent release of CA (Rigual *et al.* 1991; Vicario *et al.* 2000*b*; Rocher *et al.* 2005), but at the same time, it would tend to inhibit the exocytotic machinery, with the net result of a limited release response. To directly test the hypothesis we performed the experiments shown in Fig. 4 using ionomycin (20 μM) and high external K^+ (80 mM) as releasing stimuli. Ionomycin is a Ca^{2+} ionophore which promotes Ca^{2+}-dependent transmitter release without the need of depolarization and participation of voltage operated Ca^{2+} channels (Congar *et al.* 2002), while 80 mM K^+ causes release by activating all subtypes of voltage-dependent Ca^+ channels (Rocher *et al.* 2005). In Fig. 4*A* and *B* are shown, respectively, the results obtained in a group of 10 control and 10 experimental ionomycin-treated CBs. In control organs the ionophore promoted a prompt release response that subsided very slowly due to the long wash-out time of the ionophore (Sanz-Alfayate *et al.* 2001), while in experimental organs, in which the incubation with ionomycin was carried out in acidic/hypercapnic solution, the release was greatly inhibited, appearing a frank release response only during the wash-out period after returning to normal pH solution. The percentage of inhibition if evaluated during the period of ionomycin application reached 98.3 ± 12.7% ($P < 0.001$) and if evaluated during the period of ionomycin application and wash-out

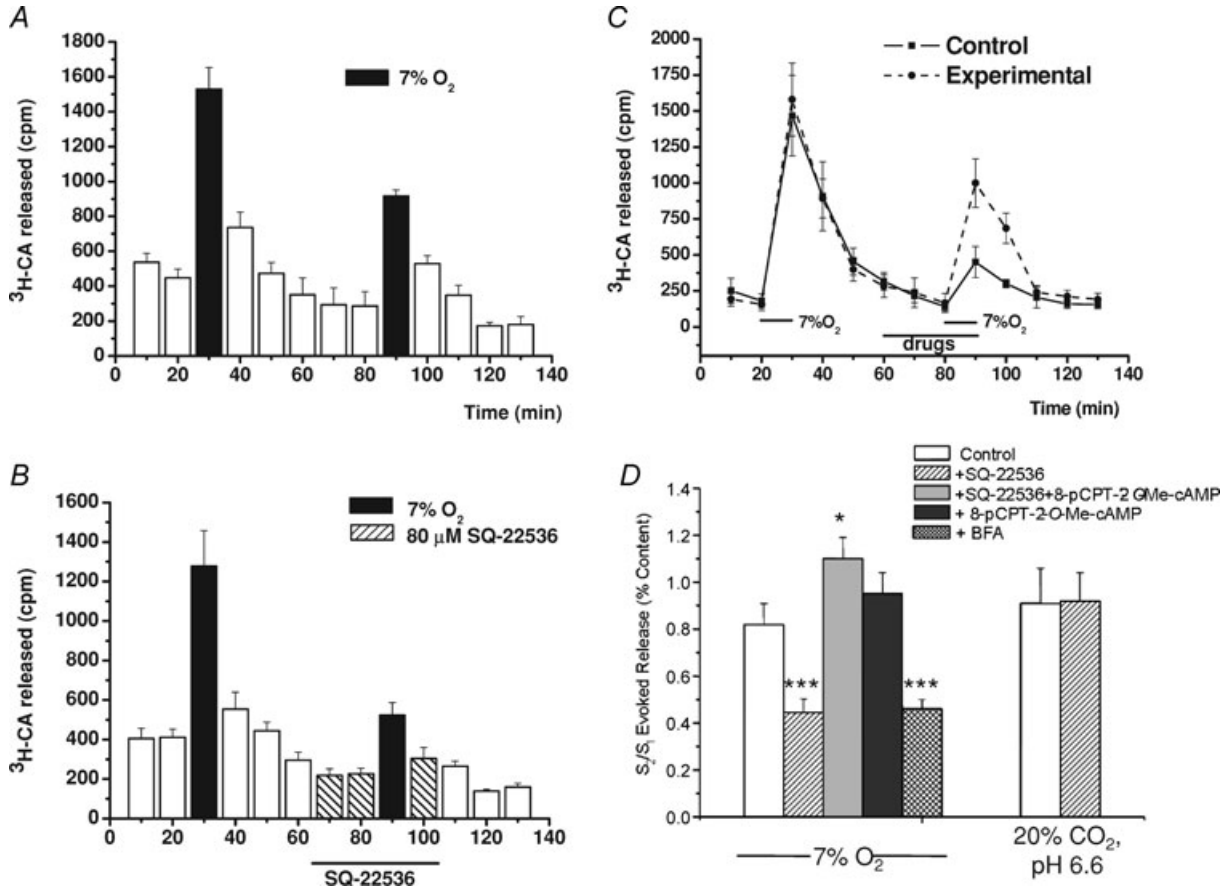

Figure 3. Effect of the adenylate cyclase inhibitor SQ-22536, the Epac activator 8-pCPT-2′-*O*-Me-cAMP and the Epac inhibitor brefeldin A on the release of [^{3}H]CA induced by low P_{O_2} and acidic/hypercapnic stimuli

A and *B*, the mean time course of release obtained in 6 control CBs challenged twice with a hypoxic stimulus (7% O_2-equilibrated solutions; black bars) as drawn, and in 6 experimental CBs similarly stimulated except that 20 min prior to and during the second hypoxic challenge and first post-stimulus, 80 μM SQ-22536 was present in the incubating solution. *C*, the mean time course of release obtained in 6 control CBs challenged twice with a hypoxic stimulus (7% O_2-equilibrated solutions) as drawn. Incubation solutions in control CBs contained 80 μM SQ-22536 during the period marked for drugs, and experimental CB incubation solution contained 80 μM SQ-22536 + 0.1 mM 8-pCPT-2′-*O*-Me-cAMP during the same period. *D*, bars represent the release evoked by hypoxia (calculated as in Fig. 2) in control (open bars) and experimental CB as labelled in the drawing (filled bars) calculated from CB in *A*–*C*. The figure also shows mean data obtained in eight individual experiments using the Epac inhibitor brefeldin A (100 μM) using protocols comparable to those in *B*. *D* also presents mean data obtained for hypercapnic acidosis with protocols identical to those described in *A* and *B*. Values are means ± S.E.M. for 6–10 CBs (*$P < 0.05$; ***$P < 0.001$).

periods it represented an inhibition of $45 \pm 7\%$ ($P < 0.001$). Figure 4*C* and *D* shows the data obtained with 80 mM external K^+. Note (Fig. 4*C*) that the acidic/hypercapnic solution did not alter the time course of the release response elicited by 80 mM K^+, but markedly reduced the intensity of the response. In fact, as shown in Fig. 4*D*, the inhibition reached 42% ($P < 0.01$).

Effects of hypercapnic acidosis on Ca^{2+} currents in isolated chemoreceptor cells

The specific effects of the Epac signalling pathways for hypoxic stimuli and the potent and direct inhibition of the exocytotic machinery exerted by acidic/hypercapnic stimuli evidenced in the experiments just described would satisfactorily explain why acidosis promotes a much weaker release response than hypoxia, for comparable levels of Ca^{2+}. However, there is an additional aspect requiring experimental exploration, namely why acidic stimuli cause Ca^{2+} transients comparable to intense hypoxia when they generate larger depolarizations. Two hints have directed our experiments to explore the possibility that acidic stimuli are inhibiting Ca^{2+} currents. First, cAMP does not activate Ca^{2+} currents in chemoreceptor cells (Lopez-Lopez *et al.* 1993), so that a specific super-activation of Ca^{2+} currents by hypoxic stimuli does not appear to be involved. Secondly, Ca^{2+} entry into chemoreceptor cells during hypoxic and acidic/hypercapnic stimulation occurs mostly via L-type Ca^{2+} channels (Buckler & Vaughan-Jones, 1994*a*,*b*; Rocher *et al.* 2005), it being well known that these channels are inhibited by low external and internal pH (Takahashi *et al.* 1993; Klöckner & Isenberg, 1994). Therefore, in the following experiments we tested the hypothesis that the acidic stimuli in spite of producing larger depolarizations than hypoxia cause

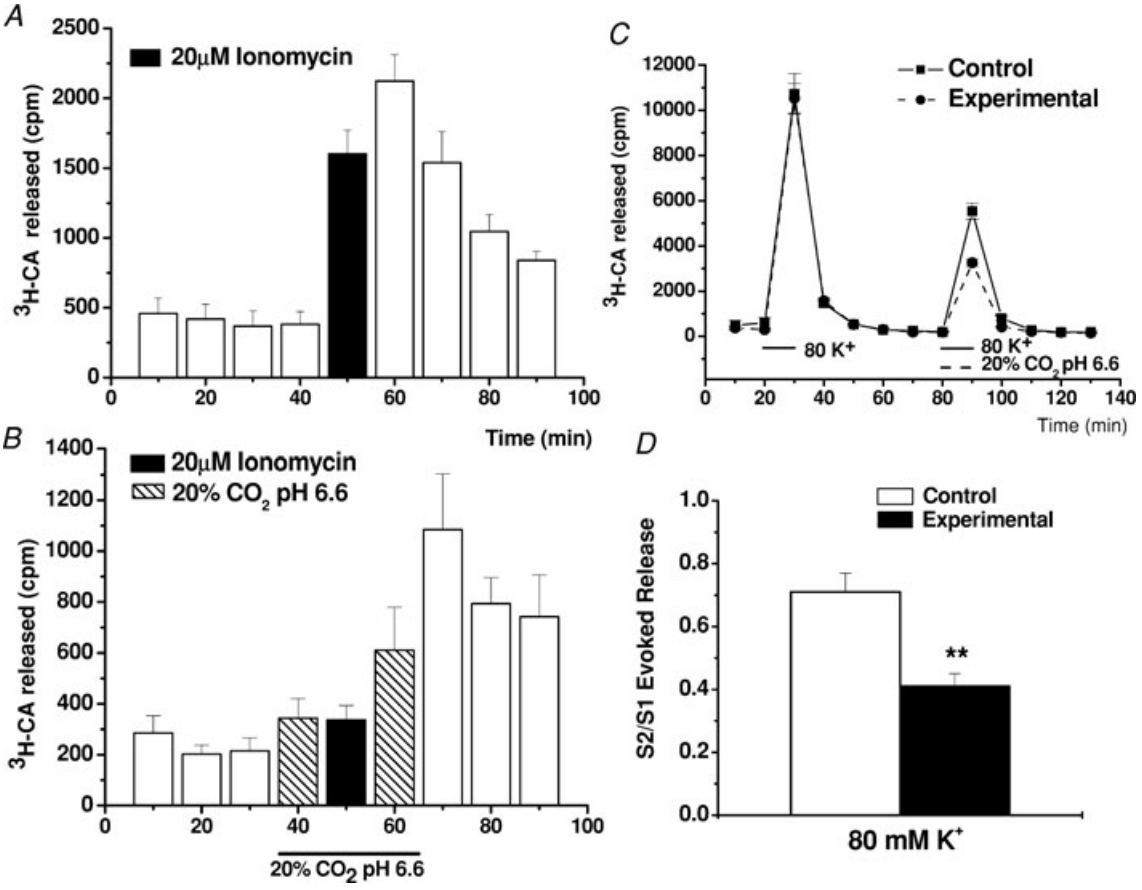

Figure 4. Effect of hypercapnic acidosis on the release of [^{3}H]CA by chemoreceptor cells evoked by a calcium ionophore and by 80 mM external K^+
A and *B*, the mean time courses ($n = 10$) of [^{3}H]CA release induced by ionomycin (20 μM, 20 min) in control and acidic incubation media. *C*, mean time courses ($n = 6$) of the release of [^{3}H]CA elicited by two consecutive applications of 80 mM K^+ in control CBs (continuous line) and in experimental CBs (dashed line); in this last group the pH of the incubation solution during the second presentation of 80 mM K^+ was 6.60 with 20% CO_2. *D*, the S2/S1 ratios for the 80 mM K^+ evoked release in control (open bars) and experimental (filled bar) CBs. Data are means ± S.E.M. ($n = 6$ CB; $^{**}P < 0.01$).

comparable Ca^{2+} transients because low pH inhibits Ca^{2+} currents.

Figure 5*A* shows a record of the Ca^{2+} current amplitude obtained at −10 mV in one cell in control, intense acidic/hypercapnic and again in control solution. It is evident that the acidic solution inhibited Ca^{2+} current in a reversible manner (see inset). In a total of 12 chemoreceptor cells recorded with this protocol we found that mean control Ca^{2+} current amplitude at −10 mV was 135.6 ± 9.5 pA and that hypercapnic acidosis produced a reversible decrease in the current to 48.0 ± 3.1 pA, representing an inhibition of $63.0 \pm 3.4\%$ ($P < 0.001$) (Fig. 5*B*). In an additional group of five cells, mean Ca^{2+} current was 119.0 ± 15.4 pA and Bay-K increased it to 168.0 ± 25.1 pA (41.4% increase; $P < 0.01$); in the same five cells hypercapnic acidosis reduced the current to 51.8 ± 9.0 pA (57% inhibition) and to 70.8 ± 8.7 (58% inhibition; $P < 0.01$), respectively, in untreated and Bay-K treated conditions. Thus, the percentage of current increase produced by Bay-K was nearly identical in normal pH and hypercapnic acid solutions (41.4 *vs.* 44.4%), and the percentage inhibition in both cases also was nearly identical, ∼57% (Fig. 5*B*), and very similar to that observed in the previous group of cells. The findings indicate that hypercapnic acidosis inhibits in a comparable extent L-type and the rest of Ca^{2+} channel subtypes because at the recording potential all Ca^{2+} channels are recruited (Rocher *et al.* 2005).

Discussion

In perforated-patch recordings we have confirmed the higher efficacy of hypercapnic acidosis (20% CO_2, pH 6.6) compared to moderate hypoxia ($P_{O_2} \approx 46$ mmHg) in depolarizing isolated rabbit chemoreceptor cells, and in neurochemical experiments we demonstrate that the acidic/hypercapnic stimulus is far less efficient than moderate hypoxia in promoting exocytotic release of [^{3}H]CA (Obeso *et al.* 1992). Finding that a cAMP-mediated mechanism potentiates the hypoxic release and not the acidic/hypercapnic release, and that hypercapnic acidosis inhibits exocytosis can satisfactorily explain these opposite relationships, i.e. high membrane depolarization/low exocytosis of [^{3}H]CA for the acidic/hypercapnic stimulus and *vice versa* for the hypoxic stimulus. The finding that hypercapnic acidosis inhibits Ca^{2+} currents provides an explanation for previous findings from other laboratories showing that the levels of intracellular Ca^{2+} produced by hypoxia and hypercapnic acidosis are comparable, even though the acidic stimulus causes a much higher depolarization. The spiking behaviour of chemoreceptor cells will be omitted in the discussion due to its variability from cell to cell. However, in spiking cells, the frequency of spikes parallels the level of depolarization (see Fig. 1*A*; see also Rocher *et al.* 2005), and therefore that omission will not detract from our considerations. As a final consideration in this summary, we want to state that our finding indicate that hypoxia as a depolarizing stimulus triggers release of CA, but as a result of its activation of adenylate cyclase and the Epac pathway, releases an amount of CA much larger than expected solely from depolarization.

A statement that we explicitly want to make at the outset of the discussion of the present findings is that the release of CA has been used as an index of the chemoreceptor cell activity with no further assumptions on the significance of CA on the genesis of the activity in the CSN. The validity of the release of CA as an index of

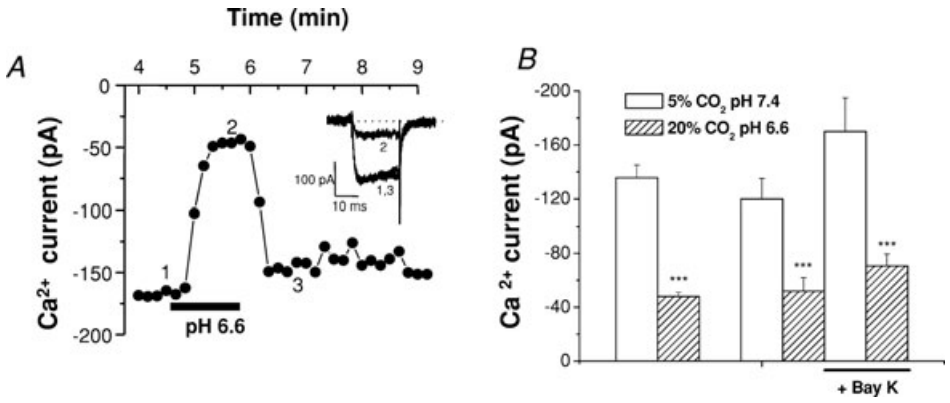

Figure 5. Effect of hypercapnic acidosis on chemoreceptor cell calcium currents
A, time course of calcium current amplitude evoked in a chemoreceptor cell by application of successive voltage pulses to −10 mV (pulse duration 20 ms; pulse frequency 0.1 Hz; $V_{hold} = -80$ mV) during superfusion with control and acidic/hypercapnic solutions (20% O_2–5% CO_2 and 20% O_2–20% CO_2 equilibrated media, respectively). The inset shows sample current traces at the times indicated (1, 2 and 3). *B*, mean calcium current amplitude found in 12 chemoreceptor cells recorded with the protocol shown in *A* (left two bars). Right four bars show mean current amplitudes obtained in 5 chemoreceptor cells recorded with an identical protocol in the absence and presence of 1 μM Bay K 8644. Data are means ± S.E.M.; ***$P < 0.001$.

chemoreceptor cell function is sanctioned for all of the stimuli tested since there is a clear parallelism between the Ca^{2+}-dependent release of CA (mostly dopamine) and the electrical activity recorded in the CSN, both being highly correlated with the intensity of the stimulus, although the ratios Δrelease/Δelectrical activity is stimulus specific, i.e., varies among different stimuli (Fidone *et al.* 1982; Obeso *et al.* 1986; Rigual *et al.* 1991; Gonzalez *et al.* 1992; Montoro *et al.* 1996). It should be acknowledged that according to the current status of our knowledge, it is most likely that several neurotransmitters (e.g. ATP, adenosine, acetylcholine and catecholamine) are involved in the genesis of electrical activity in the CSN (see Conde *et al.* 2009).

When comparing the release potency of moderate hypoxia and 30 mM K^+, it is evident that they produce comparable responses (Fig. 1*D*) in spite of the consistent observation that a high K^+ (depolarizing) stimulus causes a much larger depolarization (30 mV; Rocher *et al.* 2005) than moderate hypoxia (Fig. 1) and intracellular Ca^{2+} rises several times higher than moderate hypoxia (Buckler & Vaughan-Jones, 1994*a*,*b*). We believe that the higher gain of hypoxia to elicit neurotransmitter release is mediated by a cAMP-dependent mechanism because hypoxia, and not high K^+, produced an increase in cAMP levels in the CB (Perez-García *et al.* 1990) and because forskolin (an activator of adenylate cyclase) potentiated the release induced by hypoxia and did not affect the release elicited by high K^+ (Perez-García *et al.* 1991). These prior findings obviated testing of cyclase inhibitor and Epac agents under stimulation with high external K^+. Our finding of an endogenous cAMP-dependent mechanism enhancing the gain of the coupling of the hypoxic (and not of hypercapnic acidosis) stimulus to the exocytosis was expected, because hypoxia (and not hypercapnic acidosis) increases cAMP levels in the CB independently of the secondary actions of the released neurotransmitters (Perez-García *et al.* 1990; Cachero *et al.* 1996; see Perez-García & Gonzalez, 1997), and because forskolin augmented maximally (×5) the release of CA elicited by moderate hypoxia, very moderately the release response elicited by acidic/hypercapnic stimulus and intense hypoxia (×1.5–1.8) and did not affect the release of CA elicited by 30 mM external K^+ (Perez-García *et al.* 1991). Our present findings showing that PKA inhibitors are ineffective in modifying the hypoxic release response, while the inhibitor of adenylate cyclase reduces the response by nearly 50%, go a step further to indicate that cAMP-potentiating effects of the hypoxic Ca^{2+}-dependent release of CA is PKA independent. The regulation of cell processes, particularly Ca^{2+}-dependent exocytosis of transmitters and hormones by cAMP-dependent/PKA-independent mechanisms, has only been recognized in recent years (see Seino & Shibasaki, 2005; Holz *et al.* 2006). Experimental data indicate that the mechanisms involved in cAMP potentiation of the hypoxic release are mediated by Epacs (or cAMP regulated guanine nucleotide exchange factor; cAMP-GEF), which are known to have many cell targets including the exocytotic machinery and K^+ channels. On cAMP binding, the GEF domains of Epacs catalyse the exchange of GDP for GTP in small GTP-binding proteins leading to their activation. The facilitation of exocytosis is mediated by interactions of Epacs with Rab-3 (a member of the small GTP-binding proteins) with the intermediation of Rim-2 (a Rab-3 interacting molecule); Rab-3, which is a key modulator of exocytotic machinery (Sudhof, 2004), would be the ultimate effector of the facilitation of the release process. Overall, at a given level of intracellular calcium this mechanism increases the release probability of secretory granules already in the readily releasable pool and accelerates the refilling of this pool (Renstrom *et al.* 1997). At the same time, via a different small GTP-binding protein, Rap-1, Epac can lead to downstream activation of the extracellular-signal regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) and modulation of K^+ channels (Yuan & Chen, 2006). Then, we hypothesize that exocytotic machinery and K^+ channels are the targets for the PKA-independent regulation mediated by cAMP during hypoxic stimulation. In fact, we have previously shown that cAMP analogues (dibutyryl-cAMP) mimic hypoxia in inhibiting K^+ currents in isolated rabbit chemoreceptor cells (Lopez-Lopez *et al.* 1993). Other potential targets of Epac, such as Ca^{2+} channels and intracellular Ca^{2+} stores (Seino & Shibasaki, 2005), could be excluded in the case of chemoreceptor cells because cAMP analogues do not modify Ca^{2+} currents in chemoreceptor cells (Lopez-Lopez *et al.* 1993) and because intracellular Ca^{2+} stores appear to be of minimal significance in controlling levels of free Ca^{2+} (Vicario *et al.* 2000*a*; Conde *et al.* 2006). Anionic channels (Carpenter & Peers, 1997) and gap junctions (Abudara & Eyzaguirre, 1998) in chemoreceptor cells also are regulated by cAMP, but it is not known if this regulation is PKA dependent or independent. Finally, we should mention that preliminary data of experiments in course indicate that moderate hypoxia and the acidic/hypercapnic stimulus interact in such a manner that the release of both stimuli applied simultaneously is higher than the sum of the release of both stimuli applied independently. This behaviour of the release response is analogous to that described by many authors (Fitzgerald & Parks, 1971; Pepper *et al.* 1995) at the level of CSN discharges, but the more important aspect of our preliminary observations is that the interaction of the stimuli at the level of chemoreceptor cells and their release of DA is totally dependent on cAMP, as it is abolished by SQ-22536, the inhibitor of adenylate cyclase.

Another important finding in this study is the inhibitory effect of hypercapnic acidosis on the release of [^{3}H]CA

elicited by ionomycin (Fig. 4), indicating a direct effect of this stimulus on the exocytotic machinery. This finding can satisfactorily explain prior observations indicating that acidic/hypercapnic acidosis, which produces an increase in intracellular Ca^{2+} comparable to that of moderate hypoxia (Buckler & Vaughan-Jones, 1994*a*,*b*; Dasso *et al.* 2000), produces a much smaller Ca^{2+}-dependent exocytosis of CA (Vicario *et al.* 2000*b*; Rocher *et al.* 2005). Interestingly enough, Krüger *et al.* (1995) have described a nearly identical inhibition of acidosis for the nicotine-induced Ca^{2+}-dependent release of noradrenaline from guinea pig (inhibition reached 70% at pH 6.8 and 87.8% at pH 6.0), human atrium (76% inhibition at pH 6.0) and bovine adrenal chromaffin cells (inhibition was 75% at pH 6.0). Acidosis also inhibits insulin secretion in β cells (Bigner *et al.* 1996; Mack, 1998) and vasopressin and oxytocin release in neurohypophyseal synaptosomes (Cazalis *et al.* 1987); however, the pH sensitivity of the release machinery appears to be different in different systems. Quite recently, Ahdut-Hacohen *et al.* (2004) have described a mechanism that can explain the acid-mediated inhibition of exocytosis. Using the attached configuration of the patch clamp technique on synaptic vesicles obtained from the electric organ of *Torpedo*, these authors found that acidification of the recording medium produced a dramatic reduction of the opening frequency (>90% at pH 6.5 *vs.* the control pH 7.25) of the non-specific ion channels present in the vesicles without modifying their voltage sensitivity, implying that the overall decrease in the vesicle conductance would dramatically decrease the exocytotic vesicle emptying (Ahdut-Hacohen *et al.* 2004).

The inhibition of voltage-operated Ca^{2+} channels by the acidic/hypercapnic stimulus (Fig. 5) in chemoreceptor cells conforms with the pH sensitivity of several subtypes of Ca^{2+} channels in other cell types (Krafte & Kass, 1988; Tytgat *et al.* 1990; Chen *et al.* 1996; Kiss & Korn, 1999). It should also be noticed that in the aforementioned study of Krüger *et al.* (1995) there was also observed a decrease in the intracellular Ca^{2+} rise evoked by nicotine in bovine chromaffin cells at acidic pH, likely to be related to the pH effect on Ca^{2+} channels. This finding provides an explanation for prior observations from other laboratories showing that an acidic stimulus, in spite of producing a much higher depolarization than moderate hypoxia, raises intracellular Ca^{2+} to comparable levels (see Introduction). It must be acknowledged, however, that the correlation between the level of stable depolarization and intracellular Ca^{2+} levels might be an oversimplification for at least two reasons: first, chemoreceptor cells produce action potentials both during hypoxic and during hypercapnic acidosis stimulation but the variability of the spiking behaviour with both stimuli (Buckler & Vaughan-Jones, 1994*a*,*b*; Zhong *et al.* 1997; Perez-García *et al.* 2000; Rocher *et al.* 2005) precludes any further consideration; and second, intracellular acidification would produce a decrease of intracellular Ca^{2+} buffering-extrusion with the result that for a given amount of Ca^{2+} entering the cells the actual increase in intracellular Ca^{2+} measured is higher the lower the intracellular pH (Kohmoto *et al.* 1990; Cairns *et al.* 1993).

Findings using 80 mM extracellular K^+ as stimulus are comparable to those obtained with ionomycin, i.e. the inhibition observed would result mostly from the inhibition of the exocytosis by acidosis. This interpretation is based on prior observations that the intensity of release of neurotransmitters, including the release of CA in the CB (Almaraz *et al.* 1986), in response to 60 and 80 mM is maximal and nearly undistinguishable (Vargas & Orrego, 1976; Dismukes *et al.* 1977). Thus, even if Ca^{2+} channels are inhibited by acidosis it could be expected that the levels of intracellular Ca^{2+} needed to produce maximal release are still achieved (see Blaustein, 1975).

In summary, our study demonstrated that moderate hypoxia and strong hypercapnic acidosis depolarized chemoreceptor cells in such a manner that the acidic stimulus more than tripled the depolarizing effect of moderate hypoxia. On the contrary, the acidic/hypercapnic stimulus elicited a neurotransmitter release response that was about a third of the release response elicited by hypoxia. Our data also indicate that the low potency of the acidic/hypercapnic stimulus to elicit neurosecretion is the result of at least two factors: a direct effect of the high proton concentration of the exocytotic machinery and a direct inhibition of voltage-dependent Ca^{2+} currents. On the contrary, the high gain of the hypoxic stimulus on the exocytosis is mediated by cAMP, via a PKA-independent, Epac-mediated mechanism. This study is the first one implicating Epacs in the control of the O_2 chemoreception cascade and opens a completely new path to explore the intimate mechanisms of O_2 sensing and its regulation.

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Author contributions

A.R. and A.I.C. performed the neurochemical experiments including the analyses of the data and figure drawing. L.A. performed the electrophysiological experiments including analysis of data and drawings. C.G. was the main designer of the study and prepared the original versions of the manuscript. All authors contributed to successive amelioration of the original version to finally approve of the present version.

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