Cellular/Molecular

Rat Adrenal Chromaffin Cells Are Neonatal CO_2 Sensors

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We studied the participation of adrenal medulla (AM) chromaffin cells in hypercapnic chemotransduction. Using amperometric recordings, we measured catecholamine (CAT) secretion from cells in AM slices of neonatal and adult rats perfused with solutions bubbled with different concentrations of CO_2. The secretory activity augmented from 1.74 ± 0.19 pC/min at 5% CO_2 to 6.36 ± 0.77 pC/min at 10% CO_2. This response to CO_2 was dose dependent and appeared without changes in extracellular pH, although it was paralleled by a drop in intracellular pH. Responsiveness to hypercapnia was higher in neonatal than in adult slices. The secretory response to hypercapnia required extracellular Ca^{2+} influx. Both the CO_2-induced internal pH drop and increase in CAT secretion were markedly diminished by methazolamide (2 μM), a membrane-permeant carbonic anhydrase (CA) inhibitor. We detected the presence of two CA isoforms (CAI and CAII) in neonatal AM slices by *in situ* hybridization and real-time PCR. The expression of these enzymes decreased in adult AM together with the disappearance of responsiveness to CO_2. In patch-clamped chromaffin cells, hypercapnia elicited a depolarizing receptor potential, which led to action potential firing, extracellular Ca^{2+} influx, and CAT secretion. This receptor potential (inhibited by methazolamide) was primarily attributable to activation of a resting cationic conductance. In addition, voltage-gated K^+ current amplitude was also decreased by high CO_2. The CO_2-sensing properties of chromaffin cells may be of physiologic relevance, particularly for the adaptation of neonates to extrauterine life, before complete maturation of peripheral and central chemoreceptors.

Key words: **hypercapnia; chromaffin cells; catecholamine secretion; carbonic anhydrase; intracellular acidosis; cationic conductance**

Introduction

Catecholamine (CAT) release from the adrenal medulla (AM) is a fundamental physiologic reaction to stress situations. This response is critical in the fetus and newborn to trigger the metabolic, respiratory, and cardiovascular changes necessary for the rapid adaptation to extrauterine life (Comline and Silver, 1966; Lagercrantz and Bistoletti, 1977; Jones, 1980; Faxelius et al., 1983, 1984). CATs, especially adrenaline, are produced by chromaffin cells in the adrenal gland and secreted to the circulation after neurogenic stimulation through the splanchnic nerve. Innervation of the AM in mammals is, however, only completed during postnatal life (after approximately the first week of age in the rat) (Seidler and Slotkin, 1985, 1986). Therefore, to maintain the necessary adrenergic activity at birth, the immature adrenal gland must possess specialized, non-neurogenic, secretory mechanisms to elicit CAT release in the absence of neural input. In fact, a non-neurogenic secretory response to hypoxia has been extensively studied in adrenal glands of newborn rats (Seidler and Slotkin, 1985, 1986). Neonatal chromaffin cells have been shown to be O_2 sensors that release CATs in response to local decreases of O_2 tension (Mochizuki-Oda et al., 1997; Mojet et al., 1997; Thompson et al., 1997; García-Fernández et al., 2001). Similar to glomus cells of the carotid body (for review, see López-Barneo et al., 2001), chromaffin cells contain O_2-sensitive K^+ channels (Thompson et al., 1997; Rychkov et al., 1998; Keating et al., 2001). Inhibition of these channels by hypoxia is the signal that leads to cell depolarization, extracellular Ca^{2+} influx, and CAT secretion. The relative O_2 deficiency normally experienced by infants during delivery cannot account for the high CAT levels seen in the newborn, because a direct relation between asphyxia and CAT secretion is found only when the newborn is considerably asphyxiated (Lagercrantz and Slotkin, 1986). It has been reported that, during labor and after delivery, simultaneous to a drop in blood O_2 tension, an increase in blood CO_2 tension (P_{CO_2}) also occurs. Thus, hypercapnia could also stimulate adrenal CAT release. Hypercapnia is known to be a potent stimulus for some central neurons (for review, see Putnam et al., 2004) and for carotid body peripheral chemoreceptor cells (Iturriaga et al., 1991; Rocher et al., 1991; Buckler and Vaughan-Jones, 1994). However, whether CO_2 can directly stimulate adrenal CAT secretion has not yet been studied. This research was undertaken to determine whether adrenal chromaffin cells are CO_2 sensitive and to investigate the underlying mechanisms. We show that chromaffin cells secrete CATs in response to local, graded, increases of P_{CO_2}. CAT release elicited by hypercapnia does not require extracellular acidification, but it is paralleled by an intracellular drop of pH. Sensitivity to CO_2 is correlated with the expression of the two major isoforms of carbonic anhydrase (CA) expressed in the AM (CAI and CAII). High P_{CO_2} elicits in chromaffin cells a depolarizing receptor potential attributable to acti-

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Table 1. Composition of recording solutions

				Low Cl^-			Tyrode's–HEPES		Low Na^+	
	Control	Acidic Hpc	Isohydric Hpc	Control	Hpc (10%)	Hpc (20%)	Control	Nigericin	Control	Hpc
% CO_2	5	10	10	5	10	20			5	10
pH	7.4	7.1	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
NaCl	100	100	100	50	50	50	140	140		
Sodium gluconate	17	17		67	50				17	
KCl	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
$MgCl_2$	1	1	1	1	1	1	1	1	1	1
$CaCl_2$	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$NaHCO_3$	23	23	40	23	40	90				17
Glucose	10	10	10	10	10	10	10	10	10	10
HEPES				10	10	10	10	10		
Nigericin								4×10^{-3}		
Choline chloride									100	100
Choline bicarbonate									23	23

Compositions are given in mM. Hpc, Hypercapnia.

vation of a cationic conductance. This, in turn, induces action potential firing, transmembrane Ca^{2+} influx, and CAT secretion. These data strongly suggest that adrenal medullary cells are physiologic CO_2 sensors.

Materials and Methods

Preparation of adrenal gland slices. Wistar rats between postnatal day 0 (P0) and 8 (neonate) and older than 30 d (adult) were anesthetized by injection of 350 mg/kg chloral hydrate (FEROSA, Barcelona, Spain). The adrenal glands were quickly removed and placed on ice-cooled and O_2-saturated modified Tyrode's solution (in mM: 148 NaCl, 2 KCl, 3 $MgCl_2$, 10 HEPES, and 10 glucose), pH 7.4. Then, the adrenal glands were included in 1% (w/v) agarose (SeaKem GTG; Cambrex BioScience, Rockland, ME) in PBS, and the agarose block was glued with cyanoacrylate to the stage of a vibratome chamber and covered with the same cold, O_2-saturated modified Tyrode's solution. Slices, 200–300 μm thick, were cut parallel to the larger base of the adrenal gland, with standard razor blades on a vibratome [Lancer (St. Louis, MO) 1000 Vibratome]. The slices were incubated for 15 min at 37°C in a solution of the same composition as that used as control for the amperometric recordings (Table 1) and bubbled with 95% O_2/5% CO_2. Afterward, the slices were maintained at room temperature in the same control solution. Slices could be used for up to 6 h after cutting.

Enzymatic dispersion of chromaffin cells. Adrenal glands from neonatal rats were removed and transferred to ice-cooled extraction solution (in mM: 154 NaCl, 5.6 KCl, 11 glucose, and 10 HEPES), pH 7.4. Four to six adrenal glands were used for a cell-dispersion preparation. The cortex of the adrenal glands was dissected out, and the peeled medullas were transferred with a Pasteur pipette to a Petri dish, in which they were minced with standard razor blades. Then, the small pieces of tissue were resuspended in 3 ml of extraction solution containing 425 U/ml collagenase type IA (Sigma, St. Louis, MO) and 4–5 mg of bovine serum albumin (Sigma) and incubated at 37°C for 30 min, mixing the suspension with a blue pipette tip every 10 min. After this first digestion, cell suspension was centrifuged at room temperature for 3–4 min at 165 × *g*, the supernatant was removed, and the pellet resuspended in 3 ml of PBS containing 7650 U/ml of trypsin (Sigma) and 425 U/ml of collagenase type IA and incubated at 37°C for 10 min. To stop the digestion, 10 ml of warm DMEM culture medium (Invitrogen, Carlsbad, CA) supplemented with 1% (v/v) penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) (Bio-Whittaker, Velviers, Belgium), 2 mM L-glutamine (BioWhittaker), and 10% (v/v) fetal bovine serum (Invitrogen) were added and centrifuged at room temperature for 3–4 min at 165 × *g*. The pellet was resuspended in 100–150 μl of culture medium and plated on small pieces of glass coverslips treated with poly-L-lysine (1 mg/ml) (Sigma). Cells were then incubated at 37°C on a 5% CO_2, 20% O_2 and 75% N_2 incubator. Under these conditions, the cells could be maintained up to 72 h before the experiments.

Amperometric measurement of secretion. To measure secretory activity in chromaffin cells, an adrenal gland slice was transferred to a recording chamber (≈200 μl) and placed on the stage of an upright Zeiss (Oberkochen, Germany) microscope (Axioskop FS) equipped with long-distance water-immersion objectives. Normally, the slice was continuously perfused by gravity (flow 1–2 ml/min) with a control solution equilibrated with 5% CO_2, 20% O_2, and 75% N_2 to obtain an extracellular pH of 7.4 (normocapnia situation). The composition of this and other solutions used in the recording experiments is given in Table 1. Experiments of secretion by hypercapnia were done in two extracellular conditions, acidic and isohydric. To apply acidic hypercapnia, the standard control solution was equilibrated with 10% CO_2, 20% O_2, and 70% N_2 producing a decrease of the extracellular pH to 7.1. To obtain isohydric hypercapnia, the $NaHCO_3$ concentration in the control solution was increased, and the solution was bubbled with 10% CO_2, 20% O_2, and 70% N_2. In these conditions, extracellular pH was kept constant at pH 7.4. For the CO_2 dose–response experiments, we used low Cl^- solutions. Solutions with high K^+ were obtained by replacing equimolarly 40 mM NaCl by KCl. After switching from normocapnia to hypercapnia, complete equilibration of the new solution in the chamber was reached in <1 min. All reagents and pharmacological compounds used were obtained from Sigma. The osmolality of the solution was maintained constant at ≈275 mmol/kg, and all amperometric recordings were done at ≈36°C. In some experiments, the secretory response to hypercapnia was examined in the presence of 0.5 mM $CdCl_2$ or 2 μM methazolamide (Sigma).

For amperometric recordings, we used the procedures described previously for carotid body slices (Pardal et al., 2000). Briefly, CAT release was recorded with a 10 μm carbon-fiber electrode polarized to +750 mV, a potential more positive than the oxidation potential of CATs released from the adrenal gland. The recording electrode was placed near a cell under visual control by using a piezoelectric manipulator. Amperometric currents were recorded with an EPC 8 patch-clamp amplifier (HEKA Electronik, Lambrecht/Pfalz, Germany), filtered at 100 Hz, and digitized at 250 Hz before storage on a computer. Data acquisition and analysis of amperometric currents were done with an ITC-16 interface (InstruTech, Port Washington, NY) and PULSE/PULSEFIT software (HEKA Electronik).

Patch-clamp recording. Voltage- and current-clamp recordings were performed on dispersed single chromaffin cells using the perforated-patch whole-cell configuration of the patch-clamp technique. We also used low-resistance pipettes (1.5–3.5 MΩ), capacity compensation, and subtraction of linear leakage and capacity currents. The internal solution filling the pipette contained (in mM) 30 KCl, 100 potassium gluconate, 1 EGTA, 10 HEPES, and 1 $MgSO_4$, the pH was adjusted to 7.2 with KOH, and the osmolality was ≈275 mmol/kg. To this solution, 240 μg/ml of amphotericin B (prepared from a stock solution of 60 mg/ml in dimethyl sulfoxide) were added. The external bathing solutions for control (5% CO_2) and isohydric hypercapnia (10% CO_2) were those used in the amperometric studies (Table 1). The effect of intracellular acidification was studied by the addition of 4 μM nigericin (Table 1). The effect of extra-

Table 2. Primer sequences used for the specific amplification of CA enzymes in adrenal gland medulla cDNA

CA	Forward primer sequence	Reverse primer sequence
CAI	5′-AAACCAGCGAAGCCAAAC-3′	5′-TGTGGTGGACGGTGGTTG-3′
CAII	5′-CCGACAGTCCCCTGTGGACAT-3′	5′-GCGGAGTGGTCAGAGAGCCA-3′
CAIII	5′-GCTTGCCTCCTCCTGGTG-3′	5′-GCAGGGTGGCGTGGTGAA-3′
CAIV	5′-AGTCCCCCATCAACATCG-3′	5′-AGGCAGGGGCAAGGGTAG-3′
CAVII	5′-AACGGAGCGAGCGGACAG-3′	5′-TCGCTGAGTGGGGGTGTG-3′
CAIX	5′-CCGTTTCCCTGCCGAGAT-3′	5′-AGGACAGGACAGTTACCG-3′
CAXII	5′-TCTGATTTCCTTCTGCTA-3′	5′-AGAGGGAGGAGTAGAAGG-3′

GenBank accession numbers for the different CAs were as follows: XM226922 (CAI), NM019291 (CAII), M22413 (CAIII), L48928 (CAIV), XM226204 (CAVII), NM001216 (CAIX), and AF051882 (CAXII).

cellular Na^+ removal was tested using choline as a substitute (Table 1). Experiments were done at room temperature (≈25°C). Ionic currents were recorded with an EPC 8 patch-clamp amplifier (HEKA Electronik), filtered at 10 Hz, and digitized at 500 Hz, before storage on computer. Some voltage- and current-clamp experiments were done in the presence of 1 μM tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) and/or 2 μM methazolamide.

Microfluorimetric measurement of intracellular pH. Dispersed chromaffin cells were labeled with the acetoxymethyl ester of 2′,7′-bis (2-carboxyethyl)-5, 6-carboxyfluorescein (BCECF-AM; Molecular Probes, Eugene, OR) by incubation with 2 μM BCECF-AM in PBS at room temperature for 30 min. After that time, the coverslip containing labeled cells was transferred to fresh PBS without BCECF-AM and maintained at room temperature until measurements (<2 h). For the experiments, a coverslip with labeled cells was placed on a recording chamber (≈0.2 ml) mounted on the stage of an inverted microscope (Axiovert 35; Zeiss) equipped with epifluorescence and photometry. A monochromator (Polychrome IV; T.I.L.L. Photonics, Martinsried, Germany) was used for fluorescence excitation, and the light was deflected by a dichroic mirror (500 DCXR; Chroma Technology, Rockingham, VT) into the microscope objective (Plan-Neofluar 40×; numerical aperture, 0.75; Zeiss). Fluorescence from chromaffin cells was filtered by a Chroma Technology D 515/30 bandpass filter and detected by a CCD camera (C4880–80; Hamamatsu, Tokyo, Japan). The software used was AquaCosmos 2.0 (Hamamatsu). Alternating excitation wavelengths of 490 and 450 nm were used to obtain the 490/450 ratio. Bathing solutions were the same as those used in the hypercapnic dose–response studies.

Standard and real-time PCR. For each independent experiment, between 6 and 10 neonatal or 1–3 adult AMs were extracted and quickly frozen in liquid N_2 before homogenization of the tissue with a homogenizer (Omni 2000; Omni International, Waterbury, CT). Poly(A^+) RNA was extracted using the Dynabeads mRNA Direct micro kit (Dynal Biotech, Oslo, Norway), as indicated by the manufacturer. The reverse transcription (RT) reaction was performed immediately after the mRNA isolation using SuperScript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA). Standard PCR was done with 1–5 μl of first-strand cDNA. The primers used to amplify the different genes for CA isoforms are shown in Table 2. In every case, the band of expected size was gel purified, cloned into the pGEM-T Easy vector (Promega, Madison, WI), and its identity was determined by sequencing using the dideoxynucleotide chain-termination method with the Sequenase 2.0 kit (USB Corporation, Cleveland, OH). For CAI and CAII, partial sequences of 647 and 525 bp respectively, were amplified by PCR and cloned into pGEM-T Easy vector to be used as a template to produce the riboprobes for *in situ* hybridization experiments. Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green PCR Master Mix (Applied Biosystems) and the thermocycler conditions recommended by the manufacturer. PCRs were performed in duplicate, in a total volume of 30 μl, containing 0.1–0.5 μl of the RT reaction. Each sample was analyzed for cyclophilin to normalize for RNA input amounts and to perform relative quantifications. Primers were designed using the Primer Express software (Applied Biosystems). For CAI, the primers used (forward, 5′-GCAAGGAGAGCATCAGTCTAAGC-3′; reverse, 5′-CTCCCTCGGCACTTGACAGA-3′; GenBank accession number XM226922) amplified a band of 72 bp. Primers (forward, 5′-CAGAGAACTGGCACAAGGAGTTC-3′; reverse, 5′-GCAGTCCCGGTGTCAATGTC-3′; GenBank accession number NM019291) were generated to amplify a 75 bp fragment of rat CAII and primers (forward, 5′-GCACTGGTGGCAAGTCCAT-3′; reverse, 5′-GCCAGGACCTGTATGCTTCAG-3′; GenBank accession number NM017101) were used to amplify a band of 74 bp of the cyclophilin cDNA. Melting curve analysis showed a single sharp peak with the expected melting temperature for all samples. As control, real-time PCR for CAI and CAII was also performed after RT of RNA extracted from carotid body and kidney of neonatal and adult rats.

In situ *hybridization.* Whole adrenal glands were dissected and fixed overnight at 4°C in 4% (w/v) paraformaldehyde in PBS. After fixing, the glands were washed in PBS. *In situ* hybridization was performed in 30- to 50-μm-thick slices cut with a Lancer Vibratome as described previously (Toledo-Aral et al., 2003). Digoxigenin-UTP-labeled riboprobes against tyrosine hydroxylase (TH), CAI, and CAII were synthesized from pGEM-T Easy plasmids containing partial cDNA sequences of the three genes. After hybridization, the slices were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000; Roche Diagnostics, Mannheim, Germany). Photographs were taken with an Olympus Provis (Tokyo, Japan) microscope under Nomarski optics.

Statistical analysis. Data were presented as mean ± SEM. Data were analyzed using either Student's *t* test or one-way ANOVA followed by Tukey's test when we compared multiple paired samples. $p < 0.05$ was considered statistically significant.

Results

Secretory response of chromaffin cells to high CO_2 tension

The typical response to hypercapnia of chromaffin cells in neonatal adrenal slices is illustrated by the amperometric recording in Figure 1*A* (top trace). In the control solution (5% CO_2), the cells were silent or showed a small secretory activity characterized by spontaneous spikes representing the fusion of exocytotic vesicles (Wightman et al., 1991; Ureña et al., 1994; Pardal et al., 2000). On exposure to 10% CO_2, the frequency and amplitude of the spikes reversibly increased with a time course similar to the introduction of the new solution in the chamber. We calculated the cumulative secretion signal (Fig. 1*A*, bottom trace), which is the sum of the time integral of the amperometric spikes. From this signal, we estimated the secretion rate, which is the amount of charge transferred to the recording fiber during exposure to high CO_2 (Pardal and López-Barneo, 2002). Secretion rate (1.98 ± 0.45 pC/min; $n = 7$ cells) in normocapnia (5% CO_2) increased fourfold to fivefold during exposure to either acidic (pH 7.1) or isohydric (nonacidic; pH 7.4) hypercapnia (10% CO_2) (Fig. 1*B*). These results indicated that neonatal rat adrenal medullary cells are truly CO_2 sensors, responsive to hypercapnia independently of changes in extracellular pH. Thus, we used throughout the study isohydric hypercapnic stimulation.

To further characterize the secretory response to hypercapnia, we estimated the average quantal charge of secretory events. This value was 0.34 ± 0.03 pC ($n = 102$ events in 16 cells) in normocapnia and increased to 0.52 ± 0.03 pC ($n = 218$ events in 16 cells) during hypercapnia ($p < 0.05$). Considering that two electronic charges are transferred to the recording electrode from each molecule of CAT released, the average content of CAT molecules per vesicle was $\approx 106 \pm 9 \times 10^4$ and $\approx 163 \pm 10 \times 10^4$ in 5% CO_2 and 10% CO_2, respectively. These values are within the range of those measured in bovine chromaffin cells stimulated to secrete by different procedures (exposure to nicotine, carbamylcholine, K^+ ions, or by mechanical stimulation) (Wightman et al., 1991) and are also similar to the one calculated in neonatal rat chromaffin cells under hypoxic stimulation (García-Fernández et al., 2001). The increase of CAT content of exocytotic vesicles by hypercapnia may be explained by fusion of vesicles in the cytosol

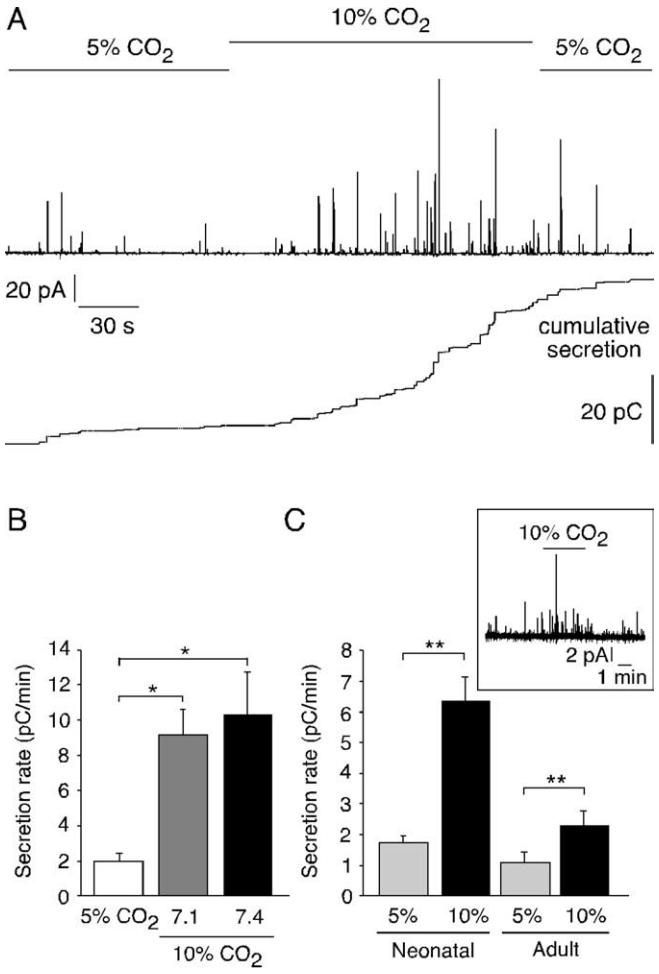

Figure 1. Secretory response to hypercapnia of rat chromaffin cells. ***A***, Top, Amperometric recording showing the secretory response to hypercapnia (10% CO_2) of a neonatal chromaffin cell in an adrenal gland slice. Bottom, Cumulative secretion signal. ***B***, Quantification of the secretory response of neonatal chromaffin cells during normocapnia (5% CO_2), acidic hypercapnia [10% CO_2, extracellular pH (pHe) 7.1], and isohydric hypercapnia (10% CO_2, pHe 7.4). Secretion rate was expressed as picocoulombs per minute (mean ± SEM; $n = 7$ cells), $^*p < 0.05$ (one-way ANOVA). ***C***, Comparison of the secretion rate (picocoulombs per minute) during 5 and 10% CO_2 exposure in responsive chromaffin cells from neonatal and adult rats (mean ± SEM; $n = 33$ for neonatal and $n = 5$ for adult rats). ***C***, Inset, Secretory response to hypercapnia of an adult chromaffin cell in an adrenal gland slice. $^{**}p < 0.005$ (Student's *t* test).

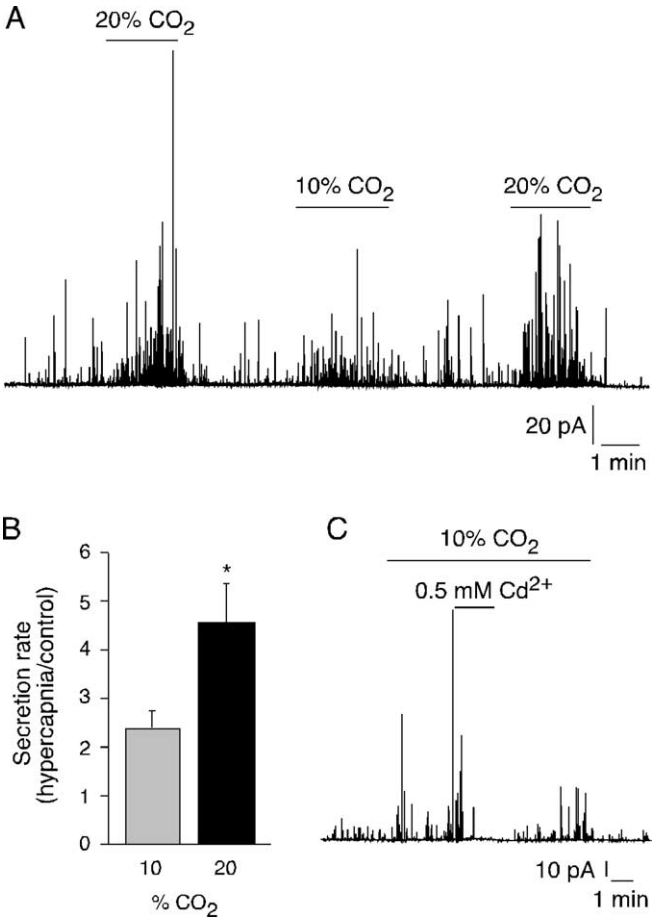

Figure 2. Dose and Ca^{2+} dependence of the secretory response of adrenal chromaffin cells to hypercapnia. ***A***, Amperometric recording of a neonatal chromaffin cell from an adrenal gland slice illustrating the secretory activity evoked by increasing CO_2 levels from control (5%) to hypercapnia (10 and 20%). ***B***, Quantification of the secretory dose–response to hypercapnia. Data are expressed as the secretion rate in hypercapnia (P_{CO_2}, 10 or 20%) divided by the secretion rate in control (P_{CO_2}, 5%) (mean ± SEM; $n = 9$), $^*p < 0.05$ (Student's *t* test). ***C***, Inhibition of the secretory response to 10% CO_2 by extracellular cadmium chloride (0.5 mM).

before they finally fuse to the cell membrane (compound exocytosis) (Ureña et al., 1994).

In neonatal rat (P0–P8) adrenal slices, 62% of the cells studied ($n = 94$) were stimulated by hypercapnia (10% CO_2), but the number of responsive cells markedly decreased in adult (P30 or older) animals (35% of cells; $n = 17$). Even in CO_2-responsive adult chromaffin cells, secretion rate (2.32 ± 0.47 pC; $n = 5$) was approximately one-third of that evoked by 10% CO_2 in neonatal glands (6.36 ± 0.77 pC; $n = 33$) (Fig. 1*C*). These differences in the secretory response to hypercapnia of neonatal versus adult cells were not explained by changes in the excitability of chromaffin cells because, in both cases, the basal secretory activity at 5% CO_2 was similar (Fig. 1*C*). Furthermore, it was also confirmed that there is no difference in the secretory responses of neonatal versus adult rats when cells were exposed to high extracellular K^+ (data not shown). Therefore, these observations suggest that the postnatal loss of responsiveness to hypercapnia is attributable to alteration of the CO_2-sensing mechanisms in adult chromaffin cells.

The secretory response of chromaffin cells to hypercapnia is dose and Ca^{2+} dependent

The secretory response to hypercapnia in neonatal chromaffin cells was dose-dependent, increasing in parallel with P_{CO_2} (Fig. 2*A*). As indicated above (Fig. 1), hypercapnia elicited a graded increase in the frequency and amplitude of the secretory events. In cells successively exposed to three P_{CO_2} levels, secretion rate increased from 3.35 ± 0.79 pC/min at 5% CO_2 to 6.55 ± 1.71 and 15.83 ± 5.56 at 10 and 20% CO_2, respectively ($n = 9$). In Figure 2*B*, we show secretion rate induced by 10 and 20% CO_2 normalized to the values obtained in the control condition. The secretory response to hypercapnia was dependent on the extracellular Ca^{2+} because it was almost completely and reversibly abolished by addition to the extracellular solution of 0.5 mM Cd^{2+}, a voltage-dependent Ca^{2+} channel blocker (Fig. 2*C*) ($n = 5$ cells). Calcium requirements for secretion in bovine chromaffin cells have been extensively studied (Augustine and Neher, 1992). In other catecholaminergic secretory cells, such as rabbit (Ureña et al., 1994) and rat (Pardal et al., 2000) carotid body glomus cells or PC12 cells (Taylor et al., 1999), the secretory response to hypoxia has also been shown to depend on extracellular Ca^{2+} influx.

Hypercapnia induces intracellular acidification

Although the hypercapnic response of chromaffin cells was independent of extracellular pH (Fig. 1*B*), we investigated whether isohydric hypercapnia produced a decrease of intracellular pH (pHi). Single isolated chromaffin cells were BCECF-AM loaded, and relative changes on the pHi were obtained from the ratio of the fluorescence (R) intensities emitted after excitation at two different wavelengths [R (F490/F450)]. On exposure of the cells to hypercapnia (10% and 20% CO_2), a clear reduction of pHi was observed (Fig. 3*A*). This effect was fast and completely reversible after removal of the stimulus. The percentage of pHi change was proportional to the level of hypercapnia, increasing when the CO_2 content of the extracellular solution was augmented. The average changes of fluorescence (ΔR) representing the difference in fluorescence ratios measured in control condition (5% CO_2) and after exposure to 10 or 20% CO_2, were 0.15 ± 0.01 and 0.29 ± 0.02 ($n = 16$; $p < 0.001$), respectively. These results indicate a close inverse correlation between extracellular P_{CO_2} and pHi and suggest that, on exposure to hypercapnia, CO_2 rapidly crosses the cell membrane and becomes hydrated, producing carbonic acid that dissociates in HCO_3^- and H^+. In fair agreement with the secretion data (Fig. 1*C*), 98% of neonatal cells (39 of 40) responded with a drop in pHi during exposure to high CO_2. In contrast, the percentage of responsive adult chromaffin cells was only 47% (8 of 17 cells tested). Although hydration of CO_2 can occur spontaneously, this process is accelerated inside the cell by the presence of CA enzyme isoforms. Then, to establish the functional participation of CAs in the hypercapnia-sensing mechanism, we tested the effect of methazolamide (2 μM), a membrane-permeable inhibitor of CA enzymatic activity (Iturriaga et al., 1991, 1993). Figure 3*B* shows that methazolamide strongly inhibited the pHi decrease induced by CO_2. ΔR in 10% CO_2 (0.26 ± 0.04) decreased to 0.07 ± 0.01 in the presence of methazolamide ($n = 8$; $p < 0.001$). For unknown reasons, methazolamide also induced a slight decrease of the fluorescence baseline.

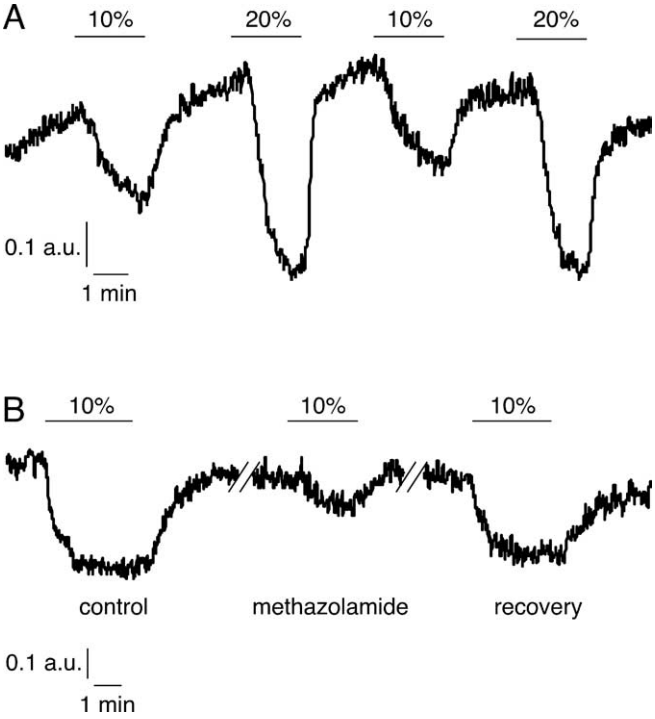

Figure 3. Intracellular acidification induced by hypercapnia and inhibition of the response by methazolamide. ***A***, Microfluorimetric measurements of changes in intracellular pH [arbitrary units (a.u.)] of isolated neonatal chromaffin cells using the acetoxymethyl ester of BCECF at a concentration of 2 μM. Relative intracellular pH expressed as the ratio of fluorescence intensities (R) measured after excitation at two wavelengths (F490 and F450). CO_2 in the external solution was changed from 5% (control) to either 10 or 20%. ***B***, Reversible inhibition of hypercapnia-induced intracellular acidification by methazolamide (2 μM). Cells were preincubated for 5 min in methazolamide before application of 10% CO_2.

The effect of methazolamide on chromaffin cells was also tested in AM slices. Figure 4 shows the secretory activity of a neonatal chromaffin cell in response to 10% CO_2 in the absence (Fig. 4*A*) or presence (Fig. 4*B*) of methazolamide (2 μM). Secretory activity in hypercapnia (expressed as ratio of secretion rate in 10% CO_2 over that in 5% CO_2) changed from 4.29 ± 0.68 to 1.85 ± 0.59 after addition of methazolamide ($n = 6$; $p < 0.005$). Therefore, methazolamide significantly reduced the secretion elicited by 10% CO_2. Furthermore, such inhibition was clearly observed despite a nonspecific increase in the basal secretory activity produced by the drug (the basal secretion rate with 5% CO_2 changed from 2.67 ± 0.64 to 4.29 ± 0.89 pC/min after addition of methazolamide; $n = 6$). Specificity of the methazolamide effect was confirmed because the secretory response elicited by high extracellular K^+ was unaltered (Fig. 4*A*,*B*, insets). Altogether, these results are consistent with the hypothesis that a decrease of pHi, dependent on CA activity, mediates the hypercapnia-induced secretory response of chromaffin cells.

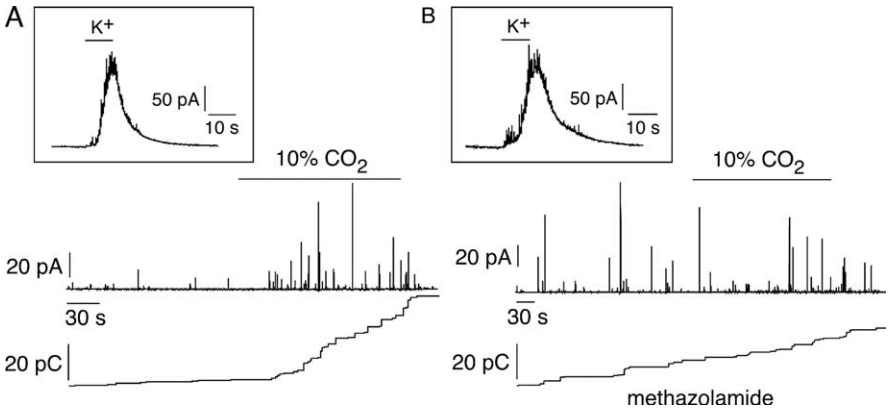

Figure 4. Participation of CA in the secretory response to hypercapnia of neonatal chromaffin cells. ***A***, Amperometric recording from a neonatal chromaffin cell during exposure to hypercapnia (10% CO_2). ***A***, Inset, Secretory response to high extracellular K^+ (40 mM) in the same conditions. ***B***, Effect of CAs inhibition by 2 μM methazolamide on the secretory response to hypercapnia of the same cell. ***B***, Inset, Secretory response to high extracellular K^+ in the presence of methazolamide.

Expression of carbonic anhydrase isoforms in neonatal and adult adrenal glands

To identify the CA isoforms present in neonatal AM, RT-PCR was done with primers for all of the isoform sequences available in the GenBank (Table 2). With this approach, only two CA isoforms, CAI and CAII, were identified. Distribution of these enzymes in the rat adrenal gland was analyzed by *in situ* hybridization, and the results demonstrated that they are located in the neonatal AM, showing, in both cases, a patchy distribution (Fig. 5*A*). Remarkably, CAI and CAII mRNAs were undetectable in the

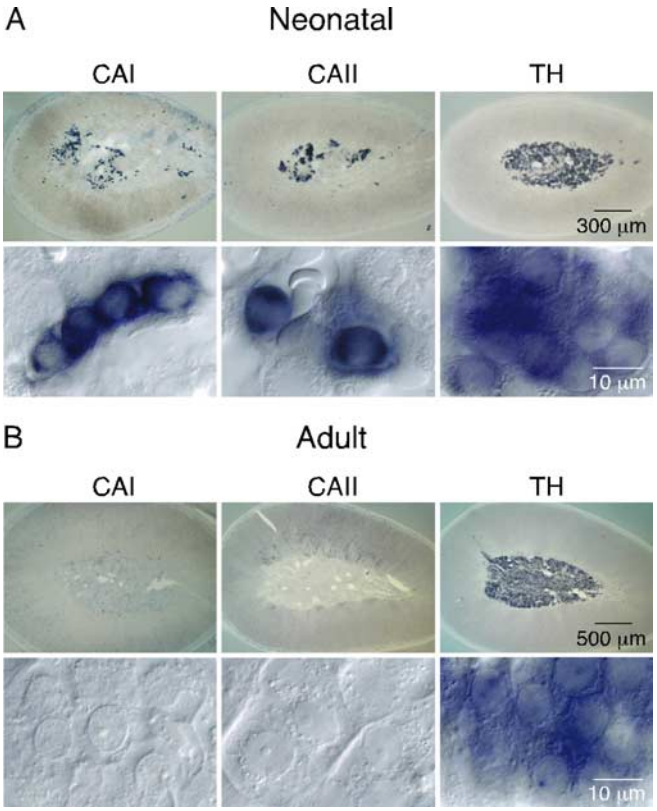

Figure 5. Expression of CA isoforms (CAI and CAII) in the AM. ***A–B***, Comparison by *in situ* hybridization of CAI, CAII, and TH mRNA levels in slices of neonatal (***A***) and adult (***B***) adrenal glands. Note the disappearance of CAI and CAII expression in adult adrenal glands. In both cases, two series of microphotographs with different magnification are shown.

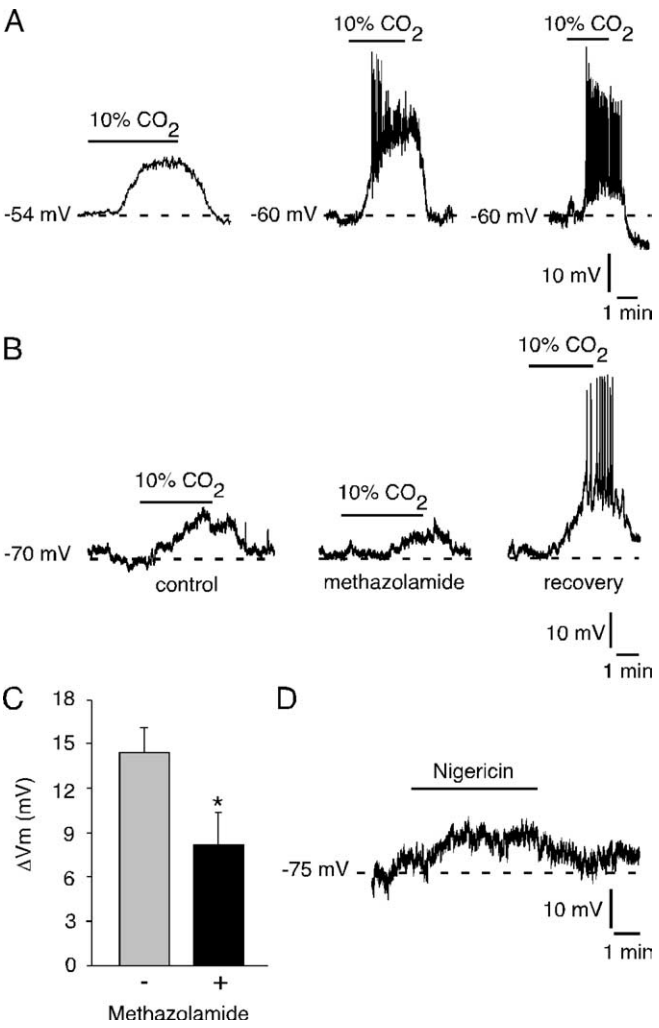

Figure 6. Hypercapnia- and acid load-elicited membrane depolarization in isolated neonatal chromaffin cells. ***A***, Examples of membrane potential changes induced by exposure to 10% CO_2 in three different isolated current-clamped neonatal chromaffin cells. The recordings were done with the perforated patch configuration of the patch-clamp technique. Broken lines indicate the spontaneous resting potential level. ***B, C***, Inhibition of hypercapnia-induced membrane depolarization by methazolamide (2 μM). Cells were preincubated for 5 min in methazolamide before application of 10% CO_2. The external solution contained TTX (1 μM). Resting membrane potential was held at −70 mV with hyperpolarizing current. Data in ***C*** represent the peak amplitude of the depolarizing response to hypercapnia (ΔV_m) before and during application of methazolamide ($n = 5$ cells; $^*p < 0.05$; Student's t test). ***D***, Membrane depolarization induced by exposure to nigericin (4 μM). Resting membrane potential was held at −75 mV with hyperpolarizing current. A representative example of three experiments is shown.

adrenal gland of adult animals (Fig. 5*B*). As control, for the mRNA stability, the expression of TH was also studied (Fig. 5*A*,*B*). These results were further confirmed by quantitative real-time PCR, comparing mRNAs extracted from AMs of neonatal versus adult rats. The quantitative analysis revealed that both enzymes were highly expressed in neonatal tissue, but their expression almost disappeared in adult animals. CAI mRNA expression was completely shut down in the adult AM (the level of neonatal expression was 1409 ± 241-fold that of the adult AM; $n = 4$). CAII levels were significantly diminished (16.6 ± 3.6-fold; $n = 6$) although not to a complete silencing of the gene. To determine whether the reduction of CAI and CAII mRNA expression seen in the adult AM is tissue specific, we analyzed the levels of expression of both CA isoforms in carotid body and kidney of neonatal and adult rats. In contrast to the findings in adrenal glands, these experiments showed that, in the carotid body, a well known peripheral chemoreceptor organ in adult life, the levels of CAII mRNA are ~1.5-fold higher in adult than in neonatal animals ($n = 2$), whereas CAI was undetectable both in neonatal and adult tissue. Similarly, the levels of CAII in kidney were approximately fivefold higher in adult than in neonatal animals ($n = 2$), and the renal expression of CAI mRNA showed no difference with the age of the animals. These results strongly suggest that the responsiveness of the neonatal AM to hypercapnia is tissue specific and depends, at least partially, on the high levels of expression of CAI and CAII in chromaffin cells. The marked decrease of CA expression in adult chromaffin cells could also help to explain why sensitivity of AM to CO_2 disappears in aged animals.

Hypercapnia-elicited depolarization and action potential firing in isolated neonatal chromaffin cells

To investigate the mechanisms underlying chromaffin cell stimulation by hypercapnia, we tested whether high P_{CO_2} can alter the membrane potential and/or the excitability of neonatal chromaffin cells. For these experiments, we used dispersed current- and voltage-clamped cells recorded with the perforated patch-clamp technique. In these experimental conditions, the resting potential of chromaffin cells averaged −50.2 ± 1.8 mV ($n = 23$). In most cells studied (76%), hypercapnia (10% CO_2) induced a reversible depolarization (14.3 ± 1.9 mV; $n = 16$ cells) that, in numerous cases, triggered the firing of action potentials (Fig. 6*A*). The effect of high P_{CO_2} on the membrane potential was fully reversible, and, when action potentials were generated repetitively, the spike burst was often followed by an afterhyperpolarization, probably

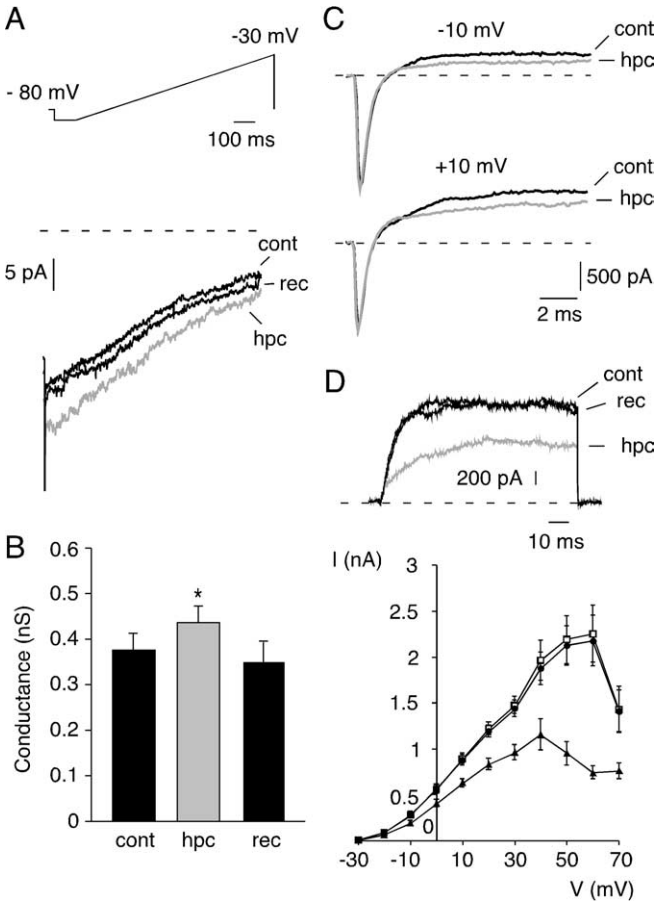

Figure 7. Changes in membrane ionic conductances induced by hypercapnia. ***A***, Representative example of whole-cell membrane currents recorded during a ramp depolarization from −90 to −30 mV in a voltage-clamped (perforated patch) neonatal chromaffin cell treated with TTX (1 μM) and exposed to hypercapnia (10% CO_2). The broken line indicates the 0 current level. ***B***, Reversible increase of slope membrane conductance (measured from −90 to −60 mV) induced by high P_{CO_2} in voltage-clamped chromaffin cells (13 measurements in 6 cells; $*p < 0.05$; Student's *t* test). ***C***, Lack of effect of hypercapnia on the voltage-dependent inward currents and inhibition of outward currents in a voltage-clamped neonatal chromaffin cell. Superimposed recordings of the currents elicited by depolarizing pulses from −80 mV to the potential indicated are illustrated. Broken lines indicate the 0 current level. ***D***, Top, Reduction of the outward K^+ current by hypercapnia elicited by a depolarizing pulse from −80 to +20 mV in a neonatal voltage-clamped chromaffin cell treated with 1 μM TTX. ***D***, Bottom, *I–V* relation for the peak outward current. Voltage steps were applied from the holding potential (−80 mV) to different test potentials (from −30 to +70 mV) in neonatal chromaffin cells treated with 1 μM TTX. Average of the peak outward current is plotted against the test potential in normocapnia (□), hypercapnia (▲), and after removal of the hypercapnic stimulus (●). Data were collected from $n = 16$ cells. cont, Control (5% CO_2); hpc, hypercapnia (10% CO_2); rec, recovery (5% CO_2).

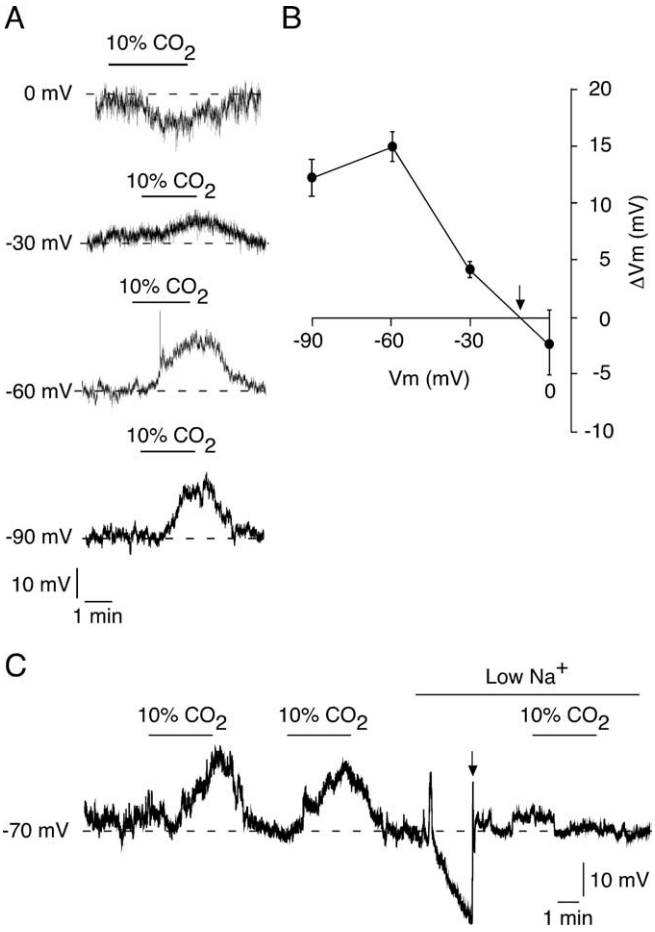

Figure 8. Cationic conductance induced by high P_{CO2}. ***A***, Hypercapnia-induced receptor potential at various resting membrane voltage levels in cells bathed with TTX (1 μM)-containing solutions to block voltage-gated Na^+ channels. The broken lines indicate the potential level before exposure to 10% CO_2. ***B***, Amplitude of the hypercapnia-induced receptor potential (ΔV_m) and reversal at approximately −10 mV (arrow) (each point represents the average of 3–9 cells tested). ***C***, Abolishment of the hypercapnia-induced depolarizing receptor potential by removal of extracellular Na^+. Resting membrane potential (broken line) was held at −70 mV with hyperpolarizing current. Hyperpolarization induced by removal of Na^+ was also compensated by application of depolarizing current (arrow). A representative example of three other experiments is shown.

because of activation of Ca^{2+}-dependent K^+ channels highly expressed in these cells (Marty and Neher, 1985; Thompson and Nurse, 1998). As shown before for the pHi and the secretory activity changes induced by hypercapnia (Figs. 3, 4, respectively), methazolamide (2 μM) also induced a marked reversible inhibition of the CO_2-dependent depolarizing potential (Fig. 6*B*,*C*). Moreover, membrane depolarization was also observed in cells exposed to either extracellular acidification (data not shown) or to intracellular acidification induced by the electroneutral H^+/K^+ exchanger nigericin (Stea et al., 1991) (Fig. 6*D*). These data suggest that intracellular acidification (either by hypercapnia or acid load) induces a depolarizing receptor potential in chromaffin cells, which is the signal leading to action potential firing, Ca^{2+} influx, and CAT secretion.

Membrane ionic conductances regulated by hypercapnia

The electrophysiological changes elicited by hypercapnia were further studied in voltage-clamped cells. Hypercapnia induced a reversible increase in the inward holding current that, at −80 mV, changed from 8.1 ± 1.7 to 9.7 ± 2.2 pA (14 measurements in seven cells; $p < 0.05$), which suggested an increase in cell conductance of ~20% at this membrane potential. Slope membrane conductance was estimated from the changes in whole-cell currents recorded during depolarizing ramps in the membrane potential range between −90 and −60 mV (Fig. 7*A*). A significant and reversible increase in membrane conductance was systematically observed during exposure to high P_{CO_2} (Fig. 7*B*). In addition to these changes in the passive membrane properties, hypercapnia also produced modifications in the macroscopic voltage-dependent ionic currents in chromaffin cells. Although inward currents, carried by Na^+ and Ca^{2+} ions (Fenwick et al., 1982), were little affected (Fig. 7*C*), the outward K^+ currents were reversibly reduced in amplitude by high P_{CO_2} in a broad range of membrane potentials (Fig. 7*C*,*D*). These observations suggested that hypercapnia induces primarily depolarization of neonatal

chromaffin cells attributable to activation of a resting membrane conductance, although the secretory response to high P_{CO_2} might be also potentiated by the slowing of action potential repolarization produced as a consequence of inhibition of voltage-gated K^+ channels.

To gain insight into the nature of the resting conductance regulated by CO_2, we performed both reversal potential and Na^+ substitution experiments. In current-clamped cells treated with TTX, to prevent the generation of Na^+-dependent action potentials, the amplitude of the hypercapnia-induced depolarization was little affected by membrane hyperpolarization but markedly reduced by depolarization. Indeed, a clear hyperpolarizing CO_2-dependent receptor potential was observed when the membrane was held at 0 mV (Fig. 8*A*). Thus, high P_{CO_2} activates a conductance with a reversal potential near −10 mV (Fig. 8*B*). Most likely, this is a cationic conductance, because the equilibrium potential for chloride ions in our solutions was −35 mV, and, in addition, substitution of 50 mM external chloride by gluconate (which shifted the chloride equilibrium potential to near 0 mV) had no major effect on the CAT secretion induced by hypercapnia. In fact, secretion rate induced by 10% CO_2 was higher using solutions with 110 mM external chloride (Fig. 1*B*) than in solutions with 60 mM chloride (Fig. 2*B*). This idea was directly confirmed by Na^+ substitution experiments, which demonstrated the disappearance of the CO_2-dependent receptor potential when most of the external Na^+ was replaced with choline (Fig. 8*C*).

Discussion

In this study, we propose the novel hypothesis that rat neonatal chromaffin cells of the AM are CO_2 sensors. We showed that hypercapnia induces in chromaffin cells a dose-dependent increase in CAT secretion attributable to cell depolarization, action potential firing, and extracellular Ca^{2+} influx. These effects are independent of extracellular acidification but are accompanied by a drop in intracellular pH. Neonatal chromaffin cells were known to be O_2 sensors (Seidler and Slotkin, 1985; Mochizuki-Oda et al., 1997; Mojet et al., 1997; Thompson et al., 1997; Rychkov et al., 1998), but, to our knowledge, the direct involvement of these cells in CO_2 homeostasis have not yet been reported.

Responsiveness to hypercapnia, both in terms of number of responsive cells as well as in the magnitude of the secretory response, was clearly reduced in adrenal gland slices from adult animals. This differential response to hypercapnia of neonatal versus adult chromaffin cells may suggest that the mechanism of CO_2 sensing is preferentially expressed in the neonatal period. In this respect, the parallel age-dependent decrease of CO_2 sensitivity and the expression of the two CA isoforms (CAI and CAII) present in chromaffin cells is highly significant . Interestingly, the decrease in the expression of these enzymes with age appears to be specific of the AM, because it does not occur in other organs such as the carotid body or the kidney. Therefore, these enzymes are likely contributing to conferring CO_2 chemosensitivity on neonatal chromaffin cells. Reduction of CAI and CAII expression could decrease the rate of CO_2 hydration and thus reduce or, at least, delay the hypercapnia-dependent lowering of intracellular pH, which, as in the carotid body glomus cells (Buckler et al., 1991a; Rigual et al., 1991; Lahiri et al., 1996) or in central chemoreceptor neurons (Putnam et al., 2004; Ritucci et al., 2005), is surely the variable that signals the cell membrane. These ideas are further supported by the decrease of hypercapnia sensitivity (in terms of pHi change, membrane depolarization, and secretion rate) of chromaffin cells treated with methazolamide, a membrane-permeant inhibitor of CA enzymes (Iturriaga et al., 1991, 1993). The direct depolarizing effect of intracellular acidification in chromaffin cells was further demonstrated by the use of nigericin, a nonelectrogenic H^+/K^+ antiporter (Stea et al., 1991). However, an additional facilitatory effect of HCO_3^- can also exist, because there are several reports indicating that bicarbonate increases CAT release (Panisello and Donelly, 1998; Iturriaga and Alcayaga, 1998) or the excitability (Buckler et al., 1991b; Iturriaga and Lahiri, 1991; Stea and Nurse, 1991) of carotid body glomus cells.

The nonuniform expression of CAI and CAII within the AM seen in the *in situ* hybridization analysis could help to explain the differences in CO_2 chemosensitivity observed among neonatal chromaffin cells. CAs, although ubiquitously expressed, are highly regulated enzymes, and, in some tissues, CAII expression changes during development, differentiation, or in the presence of hormones and growth factors (Disela et al., 1991; Buono et al., 1992; Quelo and Jurdic, 2000). The variables that determine the spatiotemporal changes in CAI and/or CAII expression and CO_2 chemosensitivity in the AM remain to be determined.

The secretory response to hypercapnia of neonatal chromaffin cells resembles, in many aspects, the responsiveness of these same cells to hypoxia. This last phenomenon also gradually disappears with postnatal maturation, because the adrenal gland is innervated by the splanchnic nerve (Seidler and Slotkin, 1985, 1986) and depends on extracellular Ca^{2+} influx (Mochizuki-Oda et al., 1997; Thompson et al., 1997; García-Fernández et al., 2001). In addition, the size of the quantal events evoked by isohydric hypercapnia (this study) and hypoxia (García-Fernández et al., 2001) are similar. However, the mechanisms underlying transduction of hypercapnia and hypoxia appear to differ in some aspects. In chromaffin cells, hypoxia produces closure of K^+ channels, thus leading to cell depolarization and secretion (Mochizuki-Oda et al., 1997; Thompson et al., 1997; Rychkov et al., 1998; Thompson and Nurse, 1998). In contrast, hypercapnia induces in chromaffin cells a depolarizing receptor potential, possibly because of activation of cation-selective channels. High P_{CO_2} also induces inhibition of the voltage-dependent K^+ channels, which may also contribute to the cell's secretory response, through broadening the action potentials and/or by increasing the duration of the depolarizing receptor potential. Interestingly, CO_2 sensing in neonatal chromaffin cells appears to differ also from the mechanisms of CO_2 chemotransduction in adult carotid body glomus cells. Reverse activation of the Na^+/Ca^{2+} exchanger (Rocher et al., 1991), activation of Ca^{2+} channels (Summers et al., 2002), or reduction of a resting K^+ conductance (Buckler and Vaughan-Jones, 1994) in response to hypercapnia (and secondary drop of intracellular pH) have been proposed to activate glomus cells. In addition, HCO_3^- export, antiporter Na^+/H^+ activation, or direct facilitation of the secretory machinery by intracellular acidification are other mechanisms proposed to be involved in hypercapnic stimulation of glomus cells (Stea et al., 1991). Although most of our experiments were done using isohydric hypercapnia, it is worth mentioning that, in carotid body cells, acidic hypercapnia produces a depolarizing receptor potential attributable to the decrease of a resting membrane K^+ conductance, which, in the absence of extracellular Na^+, can induce the firing of Ca^{2+} action potentials (Buckler and Vaughan-Jones, 1994).

The ion channel(s) type(s) involved in the hypercapnia-induced receptor potential in chromaffin cells is (are), for the moment, unknown. Intracellular pH is known to modulate the activity of numerous classes of K^+ channels. Inhibition of delayed rectifier voltage-gated K^+ channels by intracellular protons

has been observed in numerous cell preparations. For example, in carotid body cells, intracellular pH inhibits the Ca^{2+}-activated K^+ currents (Peers and Green, 1991). In addition, several cloned inward-rectifier or two-pore K^+ channel classes also respond to CO_2 and intracellular pH similarly to the K^+ currents seen in CO_2-sensitive brainstem neurons (Putnam et al., 2004). Kir 1.1 and Kir 1.2 are inhibited by lowering intracellular pH (Tsai et al., 1995; Fakler et al., 1996) and hypercapnic acidosis (Chanchevalap et al., 2000; Zhu et al., 2000). In contrast, regulation of nonselective cationic channels by intracellular pH is less studied. Nevertheless, there are some recent reports suggesting that the activity of several subclasses of nonselective cationic channels is modulated by intracellular pH (Zong et al., 2001; Andersson et al., 2004). Identification of the cationic channels expressed in chromaffin cells and their participation in CO_2 sensing should, therefore, be addressed in future experimental work.

In conclusion, we showed that rat neonatal adrenal chromaffin cells can sense CO_2 independently of changes in extracellular pH. High P_{CO_2} induces in chromaffin cells a dose-dependent drop of intracellular pH and CAT release. The effect of CO_2 does not appear to be direct but mediated by intracellular acidification. This property of neonatal chromaffin cells is gradually lost during postnatal maturation in parallel with a marked reduction in the mRNA expression of CAI and CAII isoforms. In patch-clamped chromaffin cells, hypercapnia elicits a depolarizing receptor potential, which is the signal that leads to action potential firing, extracellular Ca^{2+} influx, and CAT secretion. This receptor potential is primarily attributable to activation of a resting cationic conductance. Voltage-gated K^+ current amplitude is also decreased by high CO_2. The CO_2-sensing properties of chromaffin cells may be of physiologic relevance, particularly for the adaptation of neonates to extrauterine life, before complete maturation of peripheral and central chemoreceptors.

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