Effects of low glucose on carotid body chemoreceptor cell activity studied in cultures of intact organs and in dissociated cells

Teresa Gallego-Martin,* Silvia Fernandez-Martinez,* Ricardo Riginal, Ana Obeso, and Constanco Gonzalez

Departamento de Bioquímica y Biología Molecular y Fisiología, Instituto de Biología y Genética Molecular y Centro de Investigación Biomédica en Red de Enfermedades Respiratorias, Universidad de Valladolid, Consejo Superior de Investigaciones Científicas e Instituto de Salud Carlos III, Valladolid, Spain

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The carotid body (CB) is a paired organ located in the vicinity of the carotid artery bifurcations. CBs are formed by clusters of parenchymatous chemoreceptor cells surrounded by a dense net of capillaries embedded in supporting connective tissue. Chemoreceptor cell clusters are innervated by sensory nerve endings of the carotid sinus nerve (CSN), a branch of the glossopharyngeal nerve. Central projections of the fibers of the CSN terminate in the nucleus of the tractus solitarius (26).

The CBs are arterial chemoreceptors whose cells detect blood-borne stimuli. Upon their stimulation, chemoreceptor cells release neurotransmitters that activate CSN endings. A decrease in arterial Po2 (hypoxic hypoxia) and an increase in arterial PCO2/[H+] (hypocapnia/acidosis) are the main natural stimuli to chemoreceptor cells. Hypoxia causes a reversible inhibition of specific K+ conductances in chemoreceptor cells, leading to their depolarization, activation of voltage-dependent Ca2+ channels, and release of neurotransmitters, which drive the sensory nerve endings of the CSN (25, 34). Hypocapnia/acidosis would activate chemoreceptor cells by comparable mechanisms (7, 42; but see Ref. 46). The intact CB is also sensitive to osmotic changes as small increases in plasma (or superfusing solutions in in vitro preparations) osmolarity increase action potential frequency in the CSN (19). At the systemic level, the CB is the origin of homeostatic and adaptative reflexes aimed to maintain arterial blood gases and osmolarity. Second-order neurons of the nucleus of the tractus solitarius project to other brain stem and hypothalamic nuclei, where ventilation, cardiovascular function, and osmoregulation are integrated (16, 49).

Recent evidence obtained in simplified preparations, namely cultured CB slices or isolated chemoreceptor cells, would indicate that chemoreceptor cells also are glucoreceptors (21, 40, 59). Although the cellular mechanisms proposed to be involved in glucose sensing and/or chemoreceptor cell activation by low glucose are controversial (see Ref. 33), the observation is of great physiological relevance, as it would imply that the CB in the intact animal can detect glycemia levels and contribute to glucose homeostasis. In fact, several studies carried out in intact animals have claimed that the CB is indeed a glucose sensor, having a role on glucose homeostasis (2, 32, 51, 54). Unfortunately, there is a nearly even number of studies performed both in intact animals and in intact isolated CB preparations denying the role of chemoreceptor cells as glucoreceptors (1, 4, 5, 11, 52). Without neglecting a role for the CB in glucose homeostasis, the concept on contention is the nature of the CB as a glucoreceptor.

The origin of the discrepancies regarding the glucosensing properties of the CB remains elusive, but some considerations might shed light into the problem. First, data arising from whole-animal models are extremely difficult to interpret; consequently, whole animals are not ideal preparations to define the CBs as glucoreceptors. The reason is that the putative primary action of hypoglycemia as a chemostimulant is compounded with at least the stimulant effect of counterregulatory hormones secreted in response to hypoglycemia (27; see Refs. 41, 57) and the similarly chemostimulant effect of the general...
exercise-like hypermetabolic status created by hypoglycemia (4, 5). Therefore, the dissection of direct effect of hypoglycemia as a chemostimulant is extremely difficult, if not impossible. And second, since the glucosensing properties are evidenced in cultured chemoreceptor cells and CB slices (21, 40, 59) and not in freshly isolated intact CB and/or CB-CSN preparations (1, 4, 5, 11), it would appear, as suggested by Kumar (33), that the observed capacity of chemoreceptor cells to sense glucose levels represents a phenotypic change occurring in culture conditions.

Since CB glucose-sensing capacity and its potential significance in glucose homeostasis is a question of prime physiological importance, our laboratory has made a further effort directed to solve the existing controversy. We have developed a new preparation of intact CB in culture aiming to maintain the organotypic association of CB elements and thereby likely preserve their phenotype, minimizing potential phenotypic changes (10, 18). In other words, in developing an organotypic preparation, we have tried to establish a bridge between in vitro and in vivo experiments (13), or, as stated by Randall et al. (43), our expectancies are that organotypic culture may provide experimental solutions to problems in which both cell culture and in vivo models have been unable to deliver conclusive and convincing findings. In this preparation, we have monitored the activity of chemoreceptor cells as their release of catecholamine (CA) by a radio-isotopic method, during stimulation with hypoxia, aglycemia, aglycemia + hypoxia, and high external K⁺. In another set of experiments using 24-h cultured chemoreceptor cells, we measured chemoreceptor activity, as voltammetric release of CA, and intracellular Ca²⁺ transients by microfluorometry in response to the same stimuli used with the intact organs. Our findings indicate that chemoreceptor cells from intact CBs cultured 0–6 days retained their capacity to respond to hypoxia, but did not respond to aglycemia. In 24-h-old intact CB in culture and in dissociated cells cultured during 24 h, aglycemia (but not 1 mM glucose) potentiated the responses to hypoxia. Data indicate that chemoreceptor cells do not naturally exhibit glucosensing properties.

MATERIAL AND METHODS

Animals and anestheisa. In this study, we used adult Wistar rats of both sexes of 300–380 g body weight. Animals were anesthetized with pentobarbital sodium (60 mg/kg ip) dissolved in physiological saline. Animals were euthanized by an intracardiac overdose of pentobarbital sodium. In handling the animals, we followed the European Community Council directive of 24 November 1986 (86/609/EEC) for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Committee of the University of Valladolid for Animal Care and Use.

Surgical procedures and intact CB cultures. The experiments were performed in the morning at around 9 AM. Animals were tracheostomized, and, after adequate dissections, bilateral pieces of tissue containing the carotid bifurcations were removed and placed in a lucite dissection chamber filled with ice cold O₂-saturated Tyrode solution (in mM: 140 NaCl, 5 KCl, 2 CaCl₂, 1.1 MgCl₂, 10 HEPES, 5 glucose; pH = 7.40). The CBs were identified, cleaned of surrounding tissues, and freed of the CSN with the aid of a dissecting microscope and maintained in ice-cold Tyrode. After dissecting the 6–10 CBs used in each experiment, the organs were washed twice with 1 ml of ice-cold DMEM, supplemented with 10% fetal bovine serum, 1% l-glutamine, and 1% penicillin-streptomycin-fungizone. After the second wash, an additional aliquot of 1 ml DMEM was added to the vial containing the CBs. Working in sterile conditions and with the aid of a silanized pipette, the intact organs were transferred to a 35 mm petri dish that was brought to a tissue culture chamber (37°C) with a 20% O₂/5% CO₂ atmosphere. Total DMEM in the petri dish was 2 ml. Intact CBs were maintained in culture for up to 12 days, renewing the incubation media every 3 days.

Isolated chemoreceptor cell culture. The CBs (usually 2) were incubated (12 min) in nominally Ca²⁺- and Mg²⁺-free Tyrode’s solution (pH = 7.2) containing collagenase (2.5 mg/ml, type IV, Sigma) and bovine serum albumin (6 mg/ml, Fraction V, Sigma). After the solution was removed, the CBs were incubated in a new Tyrode solution containing trypsin (1 mg/ml, type II, Sigma) and bovine serum albumin (6 mg/ml) and maintained in this solution for an additional 17-min period. The solution was removed and the tissues were subjected to mechanical disruption by aspiration through a P1000 pipette in 2 ml of culture medium (DMEM), supplemented as above. After centrifugation (2,000 rpm, 7 min), the cells were placed in 100 µl of culture medium. Dispersed cells were plated at 10–20 µl drops on small poly-l-lysine-coated coverslips kept in 12-well plates and maintained in a humidified incubator (37°C; 5% CO₂ in air). Once the cells attached, 1.5 ml of culture medium were added to maintain the cells until use (20–28 h later).

Immunohistochemistry and immunocytochemistry. To identify the chemoreceptor cells, we used tyrosine hydroxylase (TH) as a cell marker both, in CB sections and dissociated chemoreceptor cells. For immunohistochemistry studies, anesthetized rats were perfused by gravity (100 cm) through a thin nylon tube inserted into the left ventricle with 0.01 M heparinized phosphate-buffered saline (PBS), followed by freshly prepared fixative solution (4% paraformaldehyde in 0.1 M PBS). The pair of CBs was then removed under a dissecting microscope and immersed in the same fixative for additional 6–8 h at 4°C. In other occasions, freshly obtained CBs and all cultured organs were fixed by immersion in the same fixative for 24 h at 4°C. In all of the cases, the CBs were transferred to 30% sucrose in PBS at 4°C for 24 h. Each CB was serially cut at 10-µm sections on a cryostat, mounted on slides, and blocked for 10 min in blocking solution (0.1 M PBS containing 2% normal goat serum and 0.1% Triton X-100). Sections were incubated overnight (4°C) with mouse monoclonal anti-TH (TH 1:1000; Abcam). Then sections were washed with 0.01 M PBS, then reincubated in the dark with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (1:1000) in PBS containing 2% normal goat serum for 2 h at room temperature; the incubating medium also contained 4',6-diamidino-2-phenylindole (1:1000). After washed in PBS and distilled water, coverslips were mounted in a photobleaching protective medium (Vectorshield H-1000, Vector Laboratories), and the sections were examined with the appropriated filters for immunofluorescence. Dissociated cells were similarly stained for TH (8). Incubation without primary antibodies yielded only background levels of signal (data not shown). To confirm specificity, primary antibodies were preincubated for 120 min with antigenic peptides. After this step, cells were washed and incubated with secondary antibodies conjugated with fluorescent probes. Coverslips were mounted on glass slides, as described above.

Sections and cells were photographed by fluorescence microscopy with appropriated filters, using a Zeiss Axiocopt 2 (not plus) microscope equipped with a digital camera (CoolSnap cf) and analyzed with Metamorph 6.3 software.

Measurement of Glucogenic CA content, rate of [³H]CA synthesis, and release in the CB. General procedures for the synthesis experiments have been described in previous publications (15, 50). Analytic procedures have been described in detail in Conde et al. (11). To measure [³H]CA release by intact CBs, whether freshly isolated or maintained in culture, their CA deposits were previously labeled by incubation for 2 h in Tyrode solution containing high specific activity CA precursor ([3,5-³H]tyrosine, 40–50 Ci/mmol; Amersham). Following the labeling of [³H]CA stores, individual CBs were transferred to a scintillation glass vial containing 4 ml of precursor-free Tyrode.
bicarbonate solution (composition as above, except for the substitution of 24 mM NaCl by 24 mM NaHCO₃). Vials were kept in a shaker bath at 37°C for the entire experiment. Solutions were continuously bubbled with 20% O₂/5% CO₂, balance N₂, saturated with water vapor (P O₂ ± 136–140 mmHg), except during hypoxic stimulation (see below). During the first hour, the incubating solutions were renewed every 20 min and discarded. Thereafter, solutions were collected every 10 min for analysis of [³H]CA content. Hypoxic and depolarizing stimuli consisted of 10-min incubations, with low PO₂-equilibrated (7% O₂; P O₂ ± 246 mmHg) and high K⁺-containing solutions (35 mM; equiosmolar Na⁺ was removed). Low-glucose solutions were identical, except for the reduction of glucose concentration, as indicated in each case, and were applied in specific protocols (see RESULTS section). Collected solutions were acidified with glacial acetic acid to pH = 3 and maintained at 4°C to prevent degradation of the [³H]CA until analysis. The analysis included the following: adsorption to alumina (100 mg) at pH = 8.6, washing of alumina with distilled water, bulk elution of all [³H]catechols (1 ml, 1 N HCl), and scintillation counting. Raising of pH in the collected solutions from 3 to 8.6 was made by the addition to the vials under continuous shaking 3.6 ml of 2 M Tris buffer at a pH of 8.7. CBs from release experiments were analyzed as in the synthesis experiments.

Measurement of CA release by single isolated chemoreceptor cells by microampereometry. Poly-lysine coated coverslips with attached cells (15 mm of diameter) were mounted in a RC-25F recording chamber (volume, 150 μl), assembled in a PH-4 platform (Warner Instruments, Handem, CT). At the entrance of the chamber, there was a heating system (SF28, Warner Instruments) that brings inflowing prewarmed perfusing solutions to 37°C. Perfusing solutions were equilibrated with the desired gas mixture, prewarmed at 37°C, and driven to the recording chamber by gravity at a flow of 3 ml/min trough gas-impermeable Tygon lines (1/16 in. inner diameter × 1/8 in. outer diameter, Warner Instruments). Each perfusing line has intercalated an electrovalve, allowing instantaneous changes of the perfusing solutions.

PH-4 platform was placed on the stage of a microscope (Nikon TMS, Nikon, Tokyo, Japan) inside a Faraday chamber placed over an antivibration table (TMC, Peabody, MA). Measurement of the released CA was made with a carbon fiber microelectrode (5-μm diameter) coated with polypyrrole (ProCFFE, Dagan Instruments, Minneapolis, MN). With a micromanipulator (MHW-3, Narishige International, Tokyo, Japan), the electrode was located nearby an isolated cell or a cluster of cells. Recording was made with an EPC7 amplifier (List Electronic, Darmstadt, Alemania, Germany) using an oxidation potential of +500–600 mV vs. an Ag/AgCl reference electrode (EP1, WPI, Sarasota, FL) immersed in the recording chamber. Signals were recorded at a sampling rate of 500 Hz, digitized with a Digidata 1322A digitizer, and processed with AxoScope 9.2 software (Axon Instruments, Foster City, CA).

Final analysis and presentation of data were made offline with programs MiniAnalysis 6.0.3 (Synaptosoft, Decatur, GA), Origin 7 (OriginLab, Northampton, MA), and GraphPad Prism 4 (GraphPad Software, La Jolla, CA).

Intracellular Ca²⁺ measurements. Coverslips were incubated with 10 μM fura 2-AM (Molecular Probes) diluted in Tyrode solution at 20°C for 30 min. After fura 2 loading and washing the excess with Tyrode for 30 min, coverslips were mounted in a perfusion chamber placed on the stage of a Nikon Diaphot 300 inverted microscope. Cells were superfused with a solution of composition (mM; Tyrode bicarbonate): 116 NaCl, 5 KCl, 1.1 MgCl₂, 2 CaCl₂, 23 NaHCO₃, 5 glucose (pH 7.4 bubbling with 5% CO₂/20% O₂/75% N₂), and maintained at 37°C. Dual-wavelength measurements of fura 2 fluorescence were made using the two-way wavelength illumination system DX-1000 (Solamere Technology Group). Light source was a 100-W Hg lamp (OptiQuip). Light was focused and collected through a Nikon Fluor 40/1.30 objective. Dye wavelength excitation was alternated between 340 and 380 nm, and fluorescence at 540 nm was collected with a SensiCam digital Camera (PCO CCD imaging). A binning 4 × 4 was applied to get ratio images of 320 × 256 pixels (12 bits/pixel) at 0.5 Hz. Illumination system and camera were driven by Axon Imaging Workbench 4.0 (Axon Instruments) running in a Pentium computer. Hypoxia, low (zero) glucose, combination of both, and high external KCl were used as stimuli.

At the end of the experiment, cells were fixed with 2% paraformaldehyde for 15 min at 20°C, permeabilized, and blocked with permeabilizing-blocking solution (PBS containing 0.1% Triton X-100 and 2% nonimmunized goat serum) for 10 min. Anti-TH antibodies were diluted in blocking solution and incubated with the cells for 30 min. After several washes in permeabilizing solution, cells were incubated with FITC-conjugated goat anti-mouse secondary antibodies for 30 min. After washing with PBS, TH labeling was examined with the appropriate set of filters.

Statistics. All data are expressed as means ± SE. Statistical analysis was performed by two-tailed Student’s t-test for unpaired data to compare two groups and by one-way ANOVA when comparing the means of three or more groups. Values of P < 0.05 were considered to indicate statistical significance.

RESULTS

CA content and rate of synthesis in freshly isolated and cultured CB. Figure 1, A and B, shows the mean norepinephrine (NE) and dopamine (DA) contents of CBs acutely processed [time 0 (T0)] and cultured for 1–12 days (T1–T12). Note first that freshly processed CBs (T0) contained about twice the amount of DA than NE (83.2 ± 7.2 and 43.8 ± 7.0 pmol/mg tissue, respectively). Note secondly that the NE content dropped drastically from T0 to day 1 (T1), remaining low at larger ages of the cultures. This marked decrease observed (>80%) must be due to the degeneration of the intraglomic sympathetic endings, since it is known that chronic superior cervical ganglion sympathetomy in vivo in the rat reduced by an equivalent amount NE content (37). Note thirdly that DA content remained fairly constant at all ages of the CB cultures.

Figure 1, C and D, shows, respectively, the rates of [³H]NE and [³H]DA synthesis from their natural tritiated precursor. Note that, despite the marked decrease in NE content in the CBs cultured for 1 day (T1) and later days, there are not significant differences in the rate of [³H]NE synthesis between T0 (3.2 ± 0.35 pmol·mg tissue⁻¹·2 h⁻¹) and the cultures of any age. This observation would indicate that most of the synthesis was taking place in chemoreceptor cells. The rate of [³H]DA synthesis at T0 (32.9 ± 3.8 pmol·mg tissue⁻¹·2 h⁻¹) was roughly 10 times larger than that of [³H]NE. It increased with the age of the CB cultures up to T6, when it reached levels of 93.0 ± 19.9 pmol·mg tissue⁻¹·2 h⁻¹, nearly three times larger than at T0. In CBs cultured for 8 and 12 days (T8 and T12), the rate of synthesis decreased progressively to reach, at T12, values not different from those observed at T0.

Overall data on CA content and synthesis would suggest that the intact CBs in culture, although viable for up to at least 12 days, would reduce their functionality after day 6. CA release by cultured CB in response to hypoxia and high external K⁺. Figure 2 shows the release of labeled CA from freshly isolated CBs (T0) and CBs cultured for 1–12 days (T1–T12). Figure 2, A (control CBs, T0) and B (CBs cultured for 6 days, T6), illustrates the experimental protocol followed, as well as the time course of the release all along the experiments. The release in normoxic conditions (incubation in 21%
O₂ equilibrated solutions; open bars) decreased slowly in a time-dependent manner. The release in response to hypoxia (10-min incubation in 5% O₂ equilibrated solutions; dashed bars) occurs during the period of stimulation and the period immediately poststimulus, reflecting a slow washing out of the released [³H]CA. A comparable, although more intense and faster, release response is observed in response to stimulation by incubation (10 min) with a depolarizing solution (containing 35 mM K⁺/H₁₁₀₀₁). Comparison of Fig. 2, A and B (note different scales), evidences that, while basal and hypoxic release is

![Fig. 2. Release of [³H]catecholamine in freshly isolated CBs and in CBs cultured for up to 12 days. A and B: the experimental protocol and the time course of the release process in freshly isolated CBs (T₀; A) and in CBs cultured during 6 days (T₆; B). Note the different scales in the ordinates of both figures. C: mean basal release measured in control samples immediately before hypoxic and high K⁺ stimuli, i.e., at 30 and 80 min in the experiments. D and E: mean evoked release (equivalent to release above dashed line in A) by hypoxic (D) and a high-external K⁺ (E) in freshly isolated CBs (T₀) and in CBs cultured for different times (T₁–T₁₂). Note that, up to T₆, the cultured organs respond to hypoxia with a response of magnitude comparable to that seen at T₀ in freshly isolated organs. The response to high-external K⁺ was higher in cultured CBs of any age than in freshly isolated organs. C, control; Ps, post stimulus. Values are means ± SE of n (10–13) individual values. *P < 0.05; **P < 0.01; ***P < 0.001.]
comparable in T0 and T6 CBs, 35 mM K\(^+\) elicited a more intense release response in CB cultured during 6 days (T6 organs) than in freshly isolated CBs. Figure 2C shows the mean basal release measured immediately before hypoxic and high K\(^+\) stimulation for CB at T0 and up to T12. At T0, basal release amounted to 1.86 ± 0.11% of tissue content/10 min. Compared with control freshly isolated CBs (T0), basal release of \(^{3}H\)CA decreased in CBs cultured 1 and 2 days (T1 and T2 organs) and tended to be higher in CBs cultured during 8 and 12 days (T8 and T12). The release induced by hypoxia (Fig. 2D) was maintained up to T6 at levels comparable to those obtained at T0 (5.0 ± 0.6% tissue content/10 min). More aged cultured CBs exhibited marked and significantly lower release responses to hypoxia. Contrary to that, the release elicited by high external K\(^+\) (Fig. 2E) was higher in cultured CBs (T1 to T12) than in freshly isolated CBs (T0), when the release amounted to 6.0 ± 0.8% tissue content/10 min.

Basic immunohistochemistry and cytochemistry of cultured CBs. In addition to the data presented in previous figures, which correspond to the function of the entire organs, we wanted to study basic immunohistochemistry and cytochemistry of the cultured CBs to detect possible abnormalities in their basic structure and in the appearance of dissociated cells. Figure 3, A–B' and C–D', compares bright-field and TH-stained sections from perfused and T0 immersion-fixed CBs. There are not striking differences in the overall structures of the tissues, with the clusters of TH-positive chemoreceptor cells being evident in both cases. Figure 3, E and E', shows the typical appearance of a primary culture 24 h old obtained from a freshly isolated CB. In this particular example, dissociation processes yielded mostly isolated clusters (see Ref. 8). Figure 3, F–G', shows sections and chemoreceptor cells isolated from T1 CBs (i.e., CBs were cultured as intact organs for 1 day, and cells were obtained by dissociation of 1-day cultured intact CBs and maintained dissociated in culture for an additional day), and Fig. 3, H–J' correspond to sections and dissociated cells from T6 CBs. Appearance of tissue sections and dissociated cells was normal in all the cases.

Effects of glucose-free solutions on the release of CA from intact freshly isolated (T0 organs) and intact CBs cultured for 1 to 6 days (T1–T6 organs). Conde et al. (11) showed that 0, 1, and 3 mM glucose did not alter the basal normoxic release of CA from freshly isolated CBs. They also showed that 1 mM glucose did not potentiate the release of CA elicited by a hypoxic stimulus of moderate intensity (10-min incubation with 7% \(O_2\)). The present experiments were made with pairs of freshly isolated CBs and pairs of intact organs cultured for 1–6 days, following the protocols shown in Fig. 4, A and B, which correspond to CBs cultured for 1 day. One of the CBs of the pair (control) was incubated with 5 mM containing solutions all along the experiment, except for two 10-min periods, as labeled in Fig. 4A, and subjected to two hypoxic stimuli (SI and
SII). The other member of the pair (experimental) was similarly treated, except for the second hypoxic stimulus (SII) that was applied and preceded by incubation in glucose-free solution. From these figures, it is evident that, in CBs cultured for 1 day (T1 organs), aglycemia in normoxic solutions did not elicit a release response. The same was true in T0, T2, T4, and T6 CBs. As it occurs with the basal release, the hypoxia-induced release tends to decrease as the experiment proceeds, so that, in control CBs, the ratio of the evoked release in the second hypoxic stimulus (SII) to that elicited in the first presentation (SI) is usually lower than one (8). Comparison of Fig. 4, A and B, evidences that, in T1 organs, the incubation in glucose-free solutions enhances the release induced by the second hypoxic stimulus. Figure 4C shows a comparison of the SII-to-SI ratios for control and experimental CBs at T0, T1, T2, T4, and T6. SII-to-SI ratios in control CBs oscillated between 0.6 and 1.1 (except in T2, where it reached 1.9). Statistical comparisons evidenced that, in CBs cultured for 1 day (T1), but not in freshly isolated, nor in organs cultured for other periods of time, hypoxic stimulation in glucose-free solution resulted in a highly significant increase in the hypoxic release response as SII-to-SI ratios increased from 0.62 ± 0.11 to 5.3 ± 0.7.

Effects of glucose-free solutions on the release of CA from isolated chemoreceptor cells cultured for 1 day. Findings in intact CBs cultured for 24 h drove us to study, in some detail, the responses in chemoreceptor cells also isolated for 24 h. Figure 5 shows amperometric recordings made in single isolated chemoreceptor cells in different conditions. In Fig. 5A, as the upper trace, the actual recording from a cell subjected to two stimuli, an intense and a mild hypoxia (perfusing solution equilibrated with 0% O2, actual PO2 in the recording chamber ±20 mmHg and 5% O2; PO2 ±55 mmHg) is shown; lower trace shows the integrated signal. At the recording gain used in our experiments, each spike-like event would represent the signal generated by one or multiple secretory vesicles released simultaneously. Note that, during perfusion with 20% O2 equilibrated solution (normoxia), the frequency of secretory events is very low, and, therefore, the slope of the integrated signal is similarly low. Figure 5B shows a sandwich-type experiment in which two intense hypoxic stimuli had, in between a compound stimulus, zero glucose + intense hypoxic stimulus. Note that zero glucose per se does not augment the frequency of secretory events or the slope of the integrated signal, but yet it potentiates the release induced by intense hypoxia. Figure 5C shows that hypoglycemia (superfusion with solution containing 1 mM glucose) produced minimal or no change in the release induced by intense hypoxia and did not modify normoxic release. Finally, Fig. 5D shows that the potentiation of the hypoxic release by zero glucose is still present, if the perfusing solution contained 10 mM pyruvate as an alternative energetic substrate (39).

Figure 6A shows that superfusion of chemoreceptor cells (n = 13) with solutions containing 1 mM glucose vs. control perfusion with 5 mM glucose solutions does not alter basal or hypoxia-induced release of CA from cultured cells. Figure 6B shows mean values obtained in 17 chemoreceptor cells tested for the effects of superfusion with glucose-free solutions: note that glucose-free superfusion did not affect basal normoxic release of CA, but strongly potentiated hypoxia-induced release. Figure 6C shows comparable results obtained in 11 cells perfused with zero glucose-10 mM pyruvate; results are nearly identical to those obtained in the absence of pyruvate. Finally, in Fig. 6D, we present a new group of experiments in which cultures were made with full rat serum obtained in sterile
conditions. The aim of this group of experiments was to test if the potentiating effect of the hypoxic response produced by aglycemia in 24-h cultured CBs (or dissociated cells) was due to the absence of some endogenous factor present in rat serum.

Data indicate that chemoreceptor cells cultured with standard tissue culture medium and rat serum behave identically, i.e., zero glucose superfusion did not activate chemoreceptor cells in normoxia, but potentiated hypoxic responses.

Fig. 5. Sample records of the release of catecholamine measured amperometrically in isolated rat cultured chemoreceptor cells. Cells were cultured 24 h. A, B, C, and D correspond to different cells in which hypoxia and zero glucose separately, or in combination, were applied, as indicated. In each cell, bottom traces correspond to the analytic procedure followed, consisting in the representation of the cumulated current during the recording period. Pyr, pyruvate; AU, arbitrary units.

Fig. 6. Mean release responses to different stimuli obtained in isolated rat chemoreceptor cells cultured during 24 h. A: superfusion with hypoglycemic solutions (1 mM) did not elicit a release response, nor potentiated the release elicited by hypoxia. B: superfusion of the cells with zero glucose in normoxic conditions did not elicit a release response. However, zero-glucose superfusion and concomitant application of hypoxia resulted in a potentiation of the release response elicited by hypoxia in normoglycemic solutions. C: it is shown that addition of 10 mM pyruvate to the aglycemic solutions did not prevent the potentiating effects of aglycemia on the release response elicited by hypoxia. D: the behavior of chemoreceptor cells is comparable, whether cultured in standardized medium or in rat serum. Values are means ± SE of n individual values. *P < 0.05; **P < 0.01; ***P < 0.001.
Effects of glucose-free solutions on intracellular Ca\(^{2+}\) in isolated chemoreceptor cells cultured for 1 day. Figure 7, A and B, shows sample and mean fluorescence at 340- to 380-nm wavelength emission ratio (F\(_{340}/F_{380}\)) obtained in fura 2-loaded cells perfused with normoxic normoglycemic (20% O\(_2\)-equilibrated, 5 mM glucose), normoxic hypoglycemic (20% O\(_2\), 1 mM glucose), normoxic aglycemic (20% O\(_2\), zero glucose), and hypoxic normoglycemic (0% O\(_2\), 5 mM glucose). Under basal conditions (normoxia and normoglycemia), there is a large variation in the temporal pattern of F\(_{340}/F_{380}\) between individual cells, ranging from high-frequency asynchronous oscillations to quiescence; in any case, it is important to note that in no recorded cell was there observed an ascending drift of fluorescence signal that would indicate cell damage. In the sample recording, in Fig. 7A the F\(_{340}/F_{380}\) fluorescent emission has been pulled down to zero ratio to indicate that, in quantifying the emission ratios, the signal were integrated with the zero value at the start of the experiment. The augmentation of integrated signal for any desired experimental period was measured and plotted as in Fig. 7B (see Ref. 24). Data from these two figures indicate hypoxia produced fast transients in fluorescence emission ratio. In this particular cell, and in 3 additional cells of the 31 recorded, normoxic hypoglycemic (1 mM) superfusion produced a minor increase in F\(_{340}/F_{380}\) encountered in normoxic normoglycemic conditions (Fig. 7B). Normoxic aglycemic superfusion caused a slow and progressive increase in fluorescence ratio, peaking at the end of the superfusion with glucose-free solution. The overall increases elicited by aglycemia approached those produced by hypoxia; and pulse perfusion with 60 mM K\(^+\) solutions caused a fast and large increase in the F\(_{340}/F_{380}\). Figure 7, C and D, shows comparable experiments in which zero glucose and zero glucose + hypoxia were sandwiched between two hypoxic stimuli. As in previous experiments, hypoxia caused a fast transient increase in F\(_{340}/F_{380}\). Glucose-free solution in the sample cell shown increased the rate and amplitude of spontaneous oscillations. Application of hypoxia while perfusing with glucose-free solutions resulted in an augmentation of the hypoxic F\(_{340}/F_{380}\).

Figure 8A shows that, in Ca\(^{2+}\)-free solutions, the F\(_{340}/F_{380}\) decreased and flattened, and their spontaneous oscillations disappeared. Additionally, the recording shows that, in Ca\(^{2+}\)-free solutions, zero glucose does not cause any change in the emission ratios, indicating that both spontaneous Ca\(^{2+}\) oscillations in chemoreceptor cells and the increase in Ca\(^{2+}\) elicited by zero glucose are dependent on extracellular Ca\(^{2+}\). Figure 8B shows mean increases in the F\(_{340}/F_{380}\) obtained in 36 cells. Data evidence that, in Ca\(^{2+}\)-free solutions, which causes the disappearance of the spontaneous oscillations, the time-dependent increase in emission ratios drops to zero and remains zero while the cells are perfused with Ca\(^{2+}\) and glucose-free solutions. Note also that, on Ca\(^{2+}\) and glucose reintroduction, there was a rebound in the increase in the fluorescent emission ratios. Contrary to that, Figs. 8C show that nifedipine, a dihydropyridine blocker of L-type Ca\(^{2+}\) channels, did not alter either the spontaneous oscillations or the zero glucose-induced change in F\(_{340}/F_{380}\). Figure 8D shows mean data obtained in 49 cells evidences a small (15%), nonsignificant inhibitory effect of nifedipine. Since L-type Ca\(^{2+}\) channels mediate most of the depolarization and hypoxia-triggered Ca\(^{2+}\) entry in chemoreceptor cells (7), and nifedipine at the concentration used completely blocks Ca\(^{2+}\)-dependent low-PO\(_2\)-induced release of CA (9), findings indicate that the increase in the F\(_{340}/F_{380}\) produced by aglycemia is not mediated by voltage-operated Ca\(^{2+}\) channels (i.e., aglycemia does not depolarize as to significantly recruit voltage-operated Ca\(^{2+}\) channels). In a

![Fig. 7](http://ajpcell.physiology.org/)

Mean fluorescence at 340- to 380-nm wavelength emission ratio (F\(_{340}/F_{380}\)) from isolated rat chemoreceptor cells cultured for 24 h and loaded with fura 2. A: the recording of the fluorescence ratio has artificially been driven to zero to evidence more clearly the time course and magnitude of the response. Note that superfusion with solutions containing 1 mM glucose caused, in this particular cell, a small increase in the F\(_{340}/F_{380}\). B: mean emission ratios obtained in 30–49 cells. C: sample recording from a cell in which a combined hypoxic + aglycemic stimulus was applied between two hypoxic stimuli in normoglycemic solutions. D: mean results obtained in 30 cells stimulated as in C. ΔR, increment of the running integral. Values are means ± SE of n individual values. *P < 0.05.
new group of experiments, we studied the effect of 10 μM KBR7943, a selective inhibitor of the reverse mode of the Na⁺/Ca²⁺ exchanger, alone or in combination with nifedipine (2 μM). Figure 8D shows the recording of the F₃₄₀/F₃₈₀ obtained in a cell in which both drugs were added simultaneously. From this recording, it is evident that combination of KBR7943 and nifedipine abolished the increase in the F₃₄₀/F₃₈₀ elicited by aglycemia, being also noticeable that, upon removal of the two drugs and reintroduction of glucose, there is a rebound in the fluorescence that recalls the response to aglycemia itself. In Fig. 8F are shown mean F₃₄₀/F₃₈₀ obtained in a total of 22 cells: 9 treated with KBR7943 alone, and 13 treated with KBR7943 and nifedipine. KBR7943 alone caused a nonsignificant 25% decrease in the mean fluorescence, but both drugs combined reduced by ~85% the fluorescent signal (P < 0.001) produced by aglycemia in drug-free solutions.

**DISCUSSION**

The aim of present study has been to solve the controversy on whether CB chemoreceptor cells are or not glucoreceptors. Controversy emerges because, in intact preparations, the CB does not respond to hypoglycemia, but, in simplified preparations, such as cultured cells or cultured CB slices, it has been reported that chemoreceptor cells exhibit a graded sensitivity to low glucose, from 3 to 0 mM. To link the discordant findings in intact CB vs. simplified preparations, we have developed a new preparation of intact CB in culture to study the putative glucosensing properties of chemoreceptor cells, and, in addition, we have used isolated chemoreceptor cells cultured for 24 h. Chemoreceptor cells from intact CBs cultured for up to 6 days respond to hypoxia with a release of [³H]CA, not different from the cells of freshly isolated intact organs, although they exhibit an exaggerated release response to external K⁺. The absence of glucose per se does not activate neurosecretion in chemoreceptor cells in fresh CBs or in cultured organs of any age, but in CBs cultured for 24 h, application of hypoxia in glucose-free solutions generates a clear potentiation of the neurosecretory response elicited by hypoxia. In dissociated chemoreceptor cells also cultured for 24 h, superfusion with glucose-free solutions does not activate amperometrically measured neurosecretion, but, as it happens in 24-h cultured intact CBs, neurosecretion elicited by hypoxia was potentiated in glucose-free solutions. In the same preparation of isolated chemoreceptor cells, absence of glucose elicits a slow increase in intracellular Ca²⁺ (measured as fura 2 F₃₄₀/F₃₈₀) and augments the amplitude of the Ca²⁺ transient elicited by hypoxia. Low glucose (1 mM) does not mimic the effects of aglycemia (see Figs. 5C and 7A; see also Ref. 11). As a whole, the present findings indicate chemoreceptor cells are not glucoreceptors. Rather, it would appear that, in vitro preparations at certain stages, complete absence of glucose creates a metabolic compromise in chemoreceptor cells that generates a slow increase in intracellular Ca²⁺ and a potentiation of the hypoxic responses.

At the outset of our discussion, we want to justify the need for development of the preparation of the intact CB in culture. In the Introduction, we mentioned that Kumar (33) has proposed that the capacity of chemoreceptor cells to sense glucose levels seen in reduced preparations (isolated cells and CB slices) might represent a phenotypic change.
Kumar’s suggestion, Gauda (22) and Gauda et al. (23) in situ hybridization histochemistry and immunohistochemistry did not detect cholinergic traits in chemoreceptor cells, but they were seen in other elements contained in the same histological sections, namely adjacent microganglion cells and some nerve fibers innervating the rat CB. Contrary to that, Zhang et al. (58) gave positive evidence for the presence of cholinergic traits in chemoreceptor cells in their chemoreceptor cell-petrosal ganglion neuron co-cultures. Along the same lines, Reyes et al. (44, 45), in vivo and freshly isolated in vitro preparations, found that a combination of nicotinic and purinergic blockers only partially reduces the activity elicited by hypoxia in the CSN, while Zhang et al. (58) in their co-culture preparation found that the same mixture of blockers completely abrogates the hypoxic responses. Thus the need to develop an organotypic culture to link in vivo and in vitro conditions (13, 18) appeared as a requirement to solve the debate on the glucose-sensing properties of chemoreceptor cells.

Morphologically, the cultured CBs exhibit a normal organization, and, when they are dissociated, the yield of cells is also normal in appearance and number. Cultured organs seem to function properly for at least 6 days, as their chemoreceptor cells maintain their capacity to store and synthesize CA, as well as their capacity to release CA in basal conditions and under hypoxic stimulation. Longer culture times of intact CBs appear to reduce the sensitivity to hypoxia of their chemoreceptor cells. However, the response to high external K⁺ is potentiated, suggesting that, under culture conditions, some of the isoforms of Ca²⁺ channels participating in the high K⁺-induced release are overexpressed and/or the spatial relationship of these plasma membrane channels and secretory vesicles is altered (Ref. 47; see below).

Findings requiring further commentaries include the following: 1) the increase in intracellular Ca²⁺ produced by glucose-free solutions in normoxia; 2) the dissociation between this increase in intracellular Ca²⁺ and the absence of neurosecretory response; 3) the mechanism(s) of potentiation between aglycemia and hypoxia; and 4) some general aspects of the putative zero glucose response.

Zero glucose produces an increase in intracellular Ca²⁺, which, in some cells, proceeds slowly during the entire duration of glucose superfusion, and, in other cells, it is manifested by an augmentation in the frequency of spontaneous oscillations of F₃₄₀/F₃₈₀. The zero-glucose Ca²⁺ response has the additional property of being much slower (see Fig. 7A) than the response elicited by the depolarizing hypoxic and high external K⁺ stimuli, suggesting that it does not cause enough cell depolarization as to significantly activate the L-type voltage-operated Ca²⁺ channels expressed in rat chemoreceptor cells (7, 9). Consistent with this suggestion, the Ca²⁺ response elicited by zero glucose is minimally sensitive to dihydropyridine blockers of L-type Ca²⁺ channels, although it is completely dependent on the presence of Ca²⁺ in the extracellular milieu. This last fact implies that zero glucose activates some pathway for Ca²⁺ entry into the cells. We believe that our findings allow us to propose that the zero-glucose-induced Ca²⁺ entry occurs via Na⁺/Ca²⁺ exchanger working in reverse mode (46), suggesting the interpretation of our results that follow in the lines below. It would appear that, in chemoreceptor cells cultured for 24 h, whether dissociated or the intact organ, the reserves of glucose (as glycogen?) are lessened, and/or, as Kumar (33) has suggested, chemoreceptor cells become more dependent on glycolysis. The net result would be that, upon glucose removal, there would be a partial failure of the Na⁺/K⁺-dependent ATPase, a progressive increase in intracellular Na⁺, and reversal of the Na⁺/Ca²⁺ exchanger. The electrogenic operation of the exchanger triggers the entry of Ca²⁺, the increase in the F₃₄₀/F₃₈₀, and prevents or minimizes chemoreceptor cell depolarization, and, therefore, the insensitivity of the fluorescent signal to antagonists of the voltage-operated Ca²⁺ channels. When the Na⁺/Ca²⁺ exchanger is inhibited, the exchanger-mediated Ca²⁺ entry is prevented, and, at the same time, Na⁺ accumulation in the cell interior causes a progressive cell depolarization, Ca²⁺ channels are recruited, and Ca²⁺ flows into the cells. This interpretation explains the minor sensitivity of the Ca²⁺ signal to the inhibitor of the exchanger given in isolation and its complete sensitivity to the combined application of inhibitors (KBR7943 plus nifedipine). Our interpretation is strongly suggested by the additional observation (Fig. 8A) that, in the experiments with Ca²⁺-free solutions, we assist, on reintroduction of Ca²⁺, to the classical rebound response observed in this type of experiments in many structures where the involvement of the Na⁺/Ca²⁺ exchanger is well documented (6, 14, 35, 46). The slower time course of the rebound seen upon removal of KBR7943 and nifedipine (Fig. 8E) would be due to the slower wash out of the drugs.

Second point on discussion is the apparent dissociation between intracellular Ca²⁺ and neurosecretory response. As suggested by many authors (for references, see Ref. 20), the spatial organization of Ca²⁺ microdomains, not solved with fura 2 measurements, are critical to determine the efficacy of Ca²⁺ to elicit the release of neurotransmitters. Thus an initial factor that might contribute to the inability of aglycemia to elicit a release response is that the increase of Ca²⁺ occurs in microdomains different from the active zones of the synapses. In this regard, we should mention that, in rabbit chemoreceptor cells, L-type and N-type Ca²⁺ currents represent each ~20–30% of total Ca²⁺ current. Yet, while Ca²⁺ entering via L-type channels supports over 60% of the release response elicited by moderate hypoxia, Ca²⁺ entering via N-type channels does not seem to participate in the hypoxic release (47). However, we believe that the main factor in the inefficacy of the Ca²⁺ rise triggered by zero glucose to activate the release of neurotransmitters is its slow time course. In fact, in a detailed study aimed to describe the relationship between Ca²⁺ transients and secretory capacity of different secretagogues carried out in chro-maffin cells, Heldman et al. (28) concluded that the rate of Ca²⁺ influx, rather than the absolute level of intracellular Ca²⁺ concentration, determines the rate and extent of CA release. Neher and Sakaba (38), in a very elaborated review, put forward the same notion, and additionally they proposed three distinct roles for the slow [Ca²⁺] rises: first, accelerating “molecular priming” (vesicle docking and the build-up of a release machinery); second, tightening the coupling between releasable vesicles and Ca²⁺ channels; and third, the higher basal Ca²⁺ concentration. It is also expected to lead to higher peak Ca²⁺ concentration during action potentials and, consequently, to a higher probability of release during subsequent stimulation. This last effect by itself would explain the potentiation we observe between zero glucose and hypoxia, both on Ca²⁺ signal and on release (Figs. 6 and 7D).
If interpretation for the aglycemia-elicited increase in intracellular $\text{Ca}^{2+}$ given above, i.e., a partial failure of the Na$^+$ pump, is correct, should not pyruvate prevent the increase in $\text{Ca}^{2+}$ produced by zero glucose? The inability of pyruvate to reverse zero glucose effect (i.e., the inability of pyruvate to mimic glucose) fits the suggestion put forward by Kumar (33) on the high dependence of cultured cells on glycolysis. It also fits observations made in other systems, as, for example, in $\beta$-cells of the pancreas. In these cells, the metabolic status reflected by the ATP-to-ADP ratio is the signal controlling their physiological functionality (36) via regulation of the ATP-sensitive $K^+$ channel opening probability, in such a manner that an increase in cell metabolism and ATP closes the channel, leading to cell depolarization and insulin secretion. Yet pyruvate does not elicit a secretory response, or it does so at a very low rate and requiring very high pyruvate concentrations (30, 48). Apparently, the main reason for this inability is the apparent low rate of pyruvate transport inside $\beta$-cells (30). However, this does not appear to be the case in the CB (at least in the cat CB; Ref. 39), but it might also be due to the very significant role of glycolysis on the normal functioning of chemoreceptor cells as it is the case in $\beta$-cells (3). In summary, although pyruvate can support normal functioning of many cell types, by no means is this a property common to all cells, and rat chemoreceptor cells cultured for 24 h would be among cell types in which pyruvate does not support normal functioning.

In an attempt to prevent the effects of zero glucose in the cell cultures, we performed a group of experiments using rat serum, on the premise that it could be a soluble factor responsible for the change. As evidenced by the results, our hypothesis was not correct, because chemoreceptor cells cultured with rat serum responded to aglycemia identically to cells cultures in standard culture medium (Fig. 6). Taking into account the findings of Zhang et al. (59) in petrosal ganglion and chemoreceptor cell cocultures, lack of a trophic factor coming from the sensory neurons should also be excluded as responsible for the phenotypic changes. Given the importance of mechanical factors (stiffness of culture bed, application of oscillating pressures/tensile forces, etc.) in the rate of expression of many traits and activities to CO$_2$, or in other words, for a given level of PCO$_2$ the CB drive for ventilation is increased. Two additional sets of observations are needed to fully understand how the CB can contribute to the homeostasis of glycemia without direct glucose sensing. First, counterregulatory hormones (e.g., adrenalin and noradrenaline) are well-known stimulants of the CSN activity and ventilation (27; see Refs. 41, 57), and then hormones secreted by direct affects on hypoglycemia on the hypothalamus (53) are capable of stimulating the CBs mediating or contributing to the increased sensitivity of the CB to CO$_2$ (the same is true in exercise; see Ref. 26). And second, since CB stimulation increases plasma levels of CA, ACTH, cortisol, and vasopressin (17), it follows that counterregulatory hormone stimulation of the CB potentiates their own release. This explains that an immediate consequence of CB denerva-
tion in hypoglycemia is a decrease in the plasma levels of counterregulatory hormones. It also explains the observed need for higher rates of glucose infusion to maintain any glycomic level in CB-denervated animals. Indeed, the diminished levels of hormones that act to elevate the glycomica force the administration of higher amounts of exogenous glucose to reach the desired level of glycomica.

In conclusion, chemoreceptor cells in freshly isolated CBs are not activated by low- or zero-glucose levels in the superfusing solutions. Similarly, zero glucose does not activate chemoreceptor cells of intact CBs cultured for up to 6 days. In intact CBs cultured during 1 day, but not in freshly isolated CBs or organs cultured for longer periods of time, aglycemia potentiates the release of CA elicited by hypoxia. Dissociated cells cultured during 1 day behave like intact CBs cultured for 1 day regarding the release of CA. Measures of intracellular Ca2+ in isolated cells provide results compatible with the notion that, in our culture conditions, chemoreceptor cells become transiently highly dependent on glycolysis, and, therefore, aglycemia acquires the capacity to potentiate the hypoxic response. Different dissociation protocols or culture conditions used by other authors might cause the appearance of glucosensing properties with different thresholds and different intimate ionic mechanisms (40, 59). However, experimental evidence guarantees a significant role for the CB in the maintenance of glycomica. Hormones counterregulatory to insulin-induced hypoglycemia stimulate the CB in this homeostatic role.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

T.G.-M., S.F.-M., R.R., and A.O. performed experiments; T.G.-M., S.F.-M., R.R., and A.O. analyzed data; T.G.-M., S.F.-M., R.R., and A.O. prepared figures; T.G.-M., S.F.-M., R.R., A.O., and C.G. edited and revised manuscript; T.G.-M., S.F.-M., R.R., A.O., and C.G. approved final version of manuscript; C.G. conception and design of research; C.G. drafted manuscript.

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