

# Guanylyl cyclase-D in the olfactory CO<sub>2</sub> neurons is activated by bicarbonate

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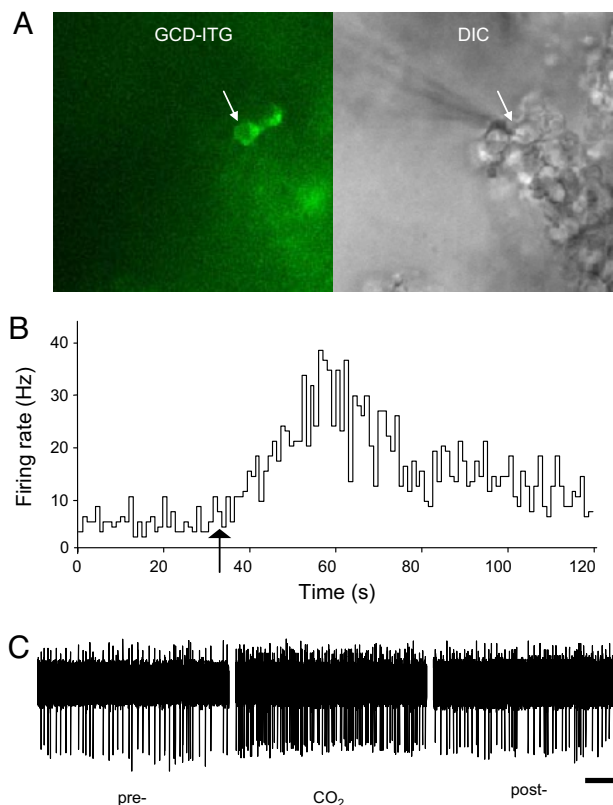
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Atmospheric CO<sub>2</sub> is an important environmental cue that regulates several types of animal behavior. In mice, CO<sub>2</sub> responses of the olfactory sensory neurons (OSNs) require the activity of carbonic anhydrase to catalyze the conversion of CO<sub>2</sub> to bicarbonate and the opening of cGMP-sensitive ion channels. However, it remains unknown how the enhancement of bicarbonate levels results in cGMP production. Here, we show that bicarbonate activates cGMP-producing ability of guanylyl cyclase-D (GC-D), a membrane GC exclusively expressed in the CO<sub>2</sub>-responsive OSNs, by directly acting on the intracellular cyclase domain of GC-D. Also, the molecular mechanism for GC-D activation is distinct from the commonly believed model of "release from repression" for other membrane GCs. Our results contribute to our understanding of the molecular mechanisms of CO<sub>2</sub> sensing and suggest diverse mechanisms of molecular activation among membrane GCs.

carbonic anhydrase | CNG channels | necklace glomeruli | transduction

Carbon dioxide (CO<sub>2</sub>) is one of the major by-products of cellular metabolism. Atmospheric CO<sub>2</sub> can be detected by the olfactory system of many animal species and mediates many fundamental behaviors including finding mates, seeking food, and avoiding predators (1–5). Recent years have witnessed substantial progresses on our understanding of the neurobiological mechanisms of CO<sub>2</sub> sensing. One emerging feature of CO<sub>2</sub> detection is that a subset of olfactory sensory neurons (OSNs) is specialized for sensing CO<sub>2</sub>. In flies, CO<sub>2</sub> is detected by OSNs that project to the glomerulus V in the antennal lobe (3, 4). Similarly, in *Caenorhabditis elegans*, the BAG neurons are believed to be critical for CO<sub>2</sub> sensing (5). Although CO<sub>2</sub> is odorless to humans, our recent study shows that the mouse olfactory system sensitively detects CO<sub>2</sub> by a subset of OSNs that project their axons to the necklace glomeruli in the olfactory bulb (1).

The exact molecular mechanisms of CO<sub>2</sub> detection remain unclear. Guanylyl cyclase-D (GC-D) is expressed exclusively in the CO<sub>2</sub>-responsive neurons, which are thus named GC-D<sup>+</sup> neurons (6–8). GC-D<sup>+</sup> neurons also express other components of signaling pathway that implicate cGMP as the second messenger, including cGMP-stimulated phosphodiesterase 2A (PDE2A) and cGMP-sensitive cyclic nucleotide-gated (CNG) channels (7, 9). Interestingly, carbonic anhydrase II (CAII) is expressed only in GC-D<sup>+</sup> neurons in the olfactory epithelium (1). As revealed by our previous study, CO<sub>2</sub> responses in the GC-D<sup>+</sup> neurons require the enzymatic activity of CAII and the opening of CNG channels (1). However, the signaling cascade that links the enzymatic activity of CAII with the opening of cGMP-sensitive CNG channels remains unknown. Presumably, CO<sub>2</sub> is a small molecule that can diffuse across cell membrane and can in turn be converted into bicarbonate ions (HCO<sub>3</sub><sup>-</sup>) and protons (H<sup>+</sup>) by CAII. Protons may be buffered by intracellular machinery to maintain physiological pH. Bicarbonates, the other product of CAII, may stimulate a GC, such as GC-D, to produce cGMP from GTP and open CNG channels. GC-D is a pseudogene in humans (10, 11), and CO<sub>2</sub> is odorless to humans (12), suggesting that GC-D may be important for CO<sub>2</sub> sensing. However, there was no evidence so far suggesting that bicarbonate could activate a GC, although it can activate soluble adenylyl cyclase



**Fig. 1.** CO<sub>2</sub> activates GC-D<sup>+</sup> neurons. (A) Targeted recording of GC-D<sup>+</sup> neurons. GC-D<sup>+</sup> cells were identified by green fluorescence in both somata and knobs in an olfactory epithelial preparation from GCD-ITG mice (Left). An electrode was guided toward a GC-D<sup>+</sup> cell (arrows) by using DIC microscopy (Right) for cell-attached extracellular recording. (B) The mean firing rate of a GC-D<sup>+</sup> cell was dramatically enhanced after CO<sub>2</sub> application in the perfusion solution (arrow). (C) Sample physiological traces showing the firing of the neurons shown in B before (pre), during (CO<sub>2</sub>), and after CO<sub>2</sub> application (post). (Scale bar, 2 s.)

(sAC) in sperm (13). In contrast, 3 membrane GCs (GC-A through GC-C) are receptors for natriuretic peptides (14–16). GC-D has also been implicated in detecting guanylin (G) and uroguanylin (UG) (17), 2 known peptide ligands of GC-C (18, 19).

In this study, we carried out biochemical assays to examine the effects of bicarbonate on GC-D. Our results demonstrate that bicarbonate directly activates GC-D by acting on its intracellular

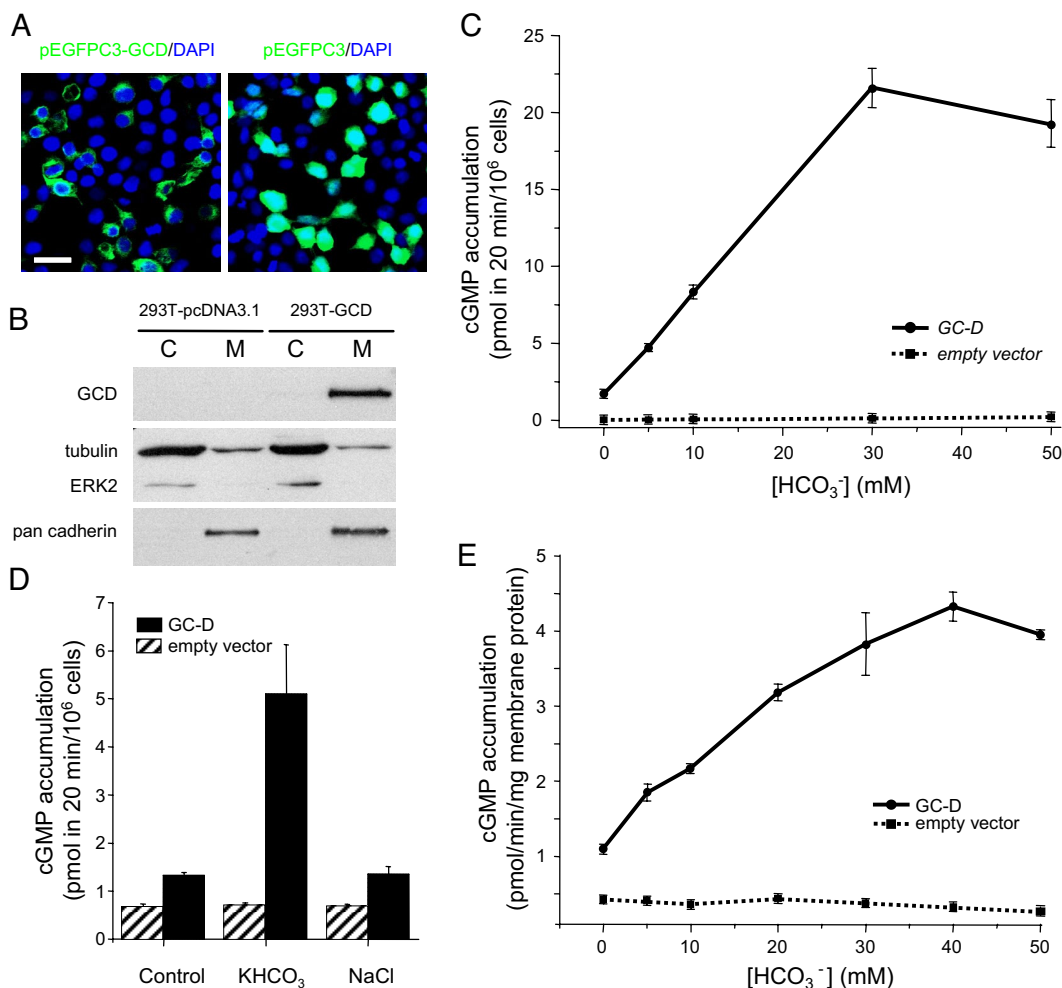
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**Fig. 2.** Bicarbonate activates the cyclase activity of GC-D. (A and B) GC-D can be heterologously expressed on cell membrane. (A) GCD-GFP fusion protein was expressed on the surface of transfected HEK-293T cells (Left), whereas GFP was expressed diffusively in cells (Right); pEGFPC3-GCD encodes the fusion protein of GFP::GC-D and pEGFPC3 encodes GFP only. DAPI indicates the DNA staining dye 4',6-diamidino-2-phenylindole and serves to mark cell nuclei. (Scale bar, 30  $\mu$ m.) (B) Western blottings showing that GC-D was present in membrane (M), but not cytosolic (C) fractions of HEK-293T cells transiently transfected with a vector containing GC-D. No GC-D was detected in HEK-293T cells transfected with empty vector pcDNA3.1. Tubulin and ERK2 were used as control for cytosolic fraction and pan cadherin was the control for membrane fraction. (C) Bicarbonate stimulated intracellular cGMP accumulation in HEK-293T cells expressing GC-D (line with circles), but had no effect on cells transfected with empty vector (dashed line with squares). (D) KHCO<sub>3</sub> (40 mM), but not NaCl (40 mM) activated GC-D. (E) Bicarbonate dose-dependently activated GC-D in vitro. Guanylyl cyclase activity at different bicarbonate concentrations was assayed by using particulate extracts from HEK-293T cells transiently expressing GC-D (line with circles) or those from cells expressing empty vector (dashed line with squares). Data are expressed as picomoles of cGMP formed per minute per milligram of total membrane protein. In this and following figures, error bars = SEM, and data were average of triplicate measurements.

cyclase domain without the need of any accessory factors. Our data further suggest that the mechanism for GC-D activation is distinct from the commonly accepted “release from repression” model for GC-A activation (20), a well characterized receptor for atrial natriuretic peptide (ANP). Thus, our data provide a framework for understanding the molecular and cellular mechanisms of CO<sub>2</sub> sensing.

## Results

Ca<sup>2+</sup> imaging in our previous study shows that CO<sub>2</sub> enhances intracellular Ca<sup>2+</sup> levels of GC-D<sup>+</sup> neurons (1). To directly test whether CO<sub>2</sub> indeed increases the frequency of action potential firing, we carried out targeted electrophysiological recording of GC-D<sup>+</sup> neurons in an intact epithelial preparation (Fig. 1A) (21). All tested GC-D<sup>+</sup> neurons responded vigorously after CO<sub>2</sub> application (Fig. 1B and C; basal firing rate, 4.8  $\pm$  1.6 Hz; peak firing rate in CO<sub>2</sub>, 13.7  $\pm$  2.9 Hz;  $n$  = 7 cells). In contrast, none of the tested GC-D<sup>-</sup> neurons were activated by CO<sub>2</sub> (basal firing rate, 5.0  $\pm$  0.2 Hz; peak firing rate in CO<sub>2</sub>, 4.9  $\pm$  0.7 Hz;  $n$  = 3 cells). Thus, our recordings directly demonstrate the selective excitatory

effect of CO<sub>2</sub> on GC-D<sup>+</sup> neurons and validate by using Ca<sup>2+</sup> imaging to measure CO<sub>2</sub> responses in GC-D<sup>+</sup> neurons.

Because CO<sub>2</sub> responses depend on both the enzymatic activity of CA and the opening of cGMP-sensitive CNG channels (1, 22), we hypothesized that bicarbonate could activate the cyclase activity of GC-D to produce cGMP. We first tested the effect of bicarbonate on cGMP production in cultured mammalian cells expressing full-length rat GC-D cDNA. GC-D expression was observed on the cell membrane of HEK-293T cells (Fig. 2A and B). Bicarbonate in the extracellular medium can be transported into HEK-293 cells within minutes, and early studies have used this method to study the bicarbonate effects on the cyclase activity of sAC (13). Extracellular application of NaHCO<sub>3</sub> stimulated cGMP accumulation in HEK-293T cells transfected with GC-D, but not in those with empty expression vector (Fig. 2C). This activation was dose-dependent for NaHCO<sub>3</sub>, with significant effects observed for NaHCO<sub>3</sub> as low as 5 mM and saturation at 30–50 mM. The effective bicarbonate concentrations are within the ranges of intracellular bicarbonate levels reported for various cells and are in accordance with earlier

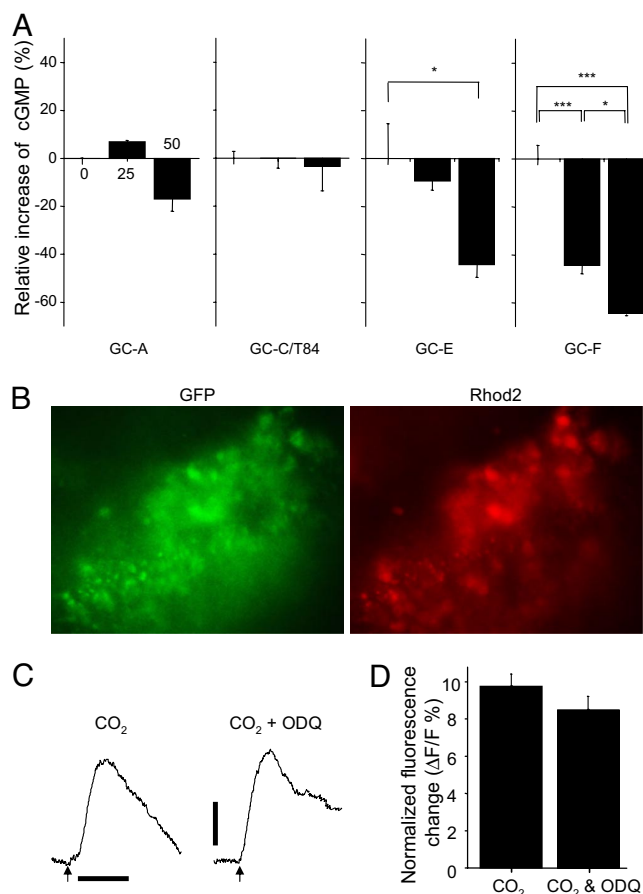
studies on the effective bicarbonate concentrations on sAC (13). Addition of  $\text{KHCO}_3$ , but not  $\text{NaCl}$ , produced stimulatory effects similar to those by  $\text{NaHCO}_3$  (Fig. 2D), suggesting that GC-D activation was specific to bicarbonate ion, but not due to  $\text{Na}^+$  or altered ionic strength. The stimulatory effects of bicarbonate on GC-D were further corroborated by using optical imaging of FRET signals sensitive to cGMP levels [supporting information (SI) Fig. S1]. Thus, these results suggest that GC-D cyclase activity can be activated by bicarbonate in a cellular context in the absence of factors specific to GC-D<sup>+</sup> neurons.

We next examined the effect of bicarbonate on GC-D protein in vitro. GC-D was transiently expressed in HEK-293T cells, and membrane protein from particulate fraction was extracted. Bicarbonate stimulated cGMP accumulation in membrane protein fraction from HEK-293T cells expressing GC-D, but not from those transfected with empty vector (Fig. 2E). This stimulation was again dose-dependent for bicarbonate, with maximum stimulatory effects at concentrations of 30–50 mM. Therefore, the cyclase activity of GC-D can be directly stimulated by bicarbonate without any accessory factors in cytosolic solutions.

Although GC-D is expressed exclusively in the  $\text{CO}_2$ -responsive OSNs, other membrane GCs and soluble (s)GCs are known to function in the olfactory epithelium (23). We tested whether several other mammalian membrane GCs could be activated by bicarbonate. Bicarbonate did not increase GC activity in HEK-293T cells expressing GC-A, GC-E, and GC-F (Fig. 3A). Bicarbonate at concentrations of 25 and 50 mM appeared to inhibit GC-E and GC-F (Fig. 3A), 2 membrane GCs expressed in retina photoreceptors (24, 25). Similarly, we found that bicarbonate did not stimulate cGMP production in T84 human colonic adenocarcinoma cells (Fig. 3A), which have been used extensively for assaying GC-C activity due to their high endogenous expression of GC-C (18, 26). As a control, GC-A and GC-C were dramatically activated after the application of their respective ligands: ANP for GC-A and G/UG for GC-C (Fig. S2). We next carried out optical imaging to test whether  $\text{CO}_2$  responses of GC-D<sup>+</sup> neurons depended on the activity of sGCs.  $\text{CO}_2$  responses were largely intact in the presence of a specific sGC inhibitor ODQ, 1H-(1, 2, 4) oxadiazolo [4, 3-a] quinoxalin-1-one (27), suggesting that bicarbonate had no stimulatory effect on sGCs (Fig. 3B–D). Thus, our results suggest that GC-D is a unique mammalian membrane GC that can be activated by bicarbonate, and bicarbonate stimulation is not a general feature of all GCs.

GC-D, like other membrane GCs, contains an extracellular domain (ex), a hydrophobic membrane-spanning region, an intracellular kinase-homology domain (kh), a coiled-coiled dimerization motif, and a C-terminal GC domain essential for its catalytic activity (cat) (Fig. 4A) (6, 15). To delineate the specific functional domain that mediates bicarbonate sensing, we engineered 3 constructs expressing mutant GC-D genes lacking the GC-D ex ( $\Delta\text{ex}$ ), kh ( $\Delta\text{kh}$ ), or both of these domains ( $\Delta\text{ex}$  and kh). Western blottings confirmed that these mutant proteins were expressed on cell membrane of HEK-293T cells (Fig. 4B). Applying bicarbonate in the extracellular medium stimulated cGMP production in HEK-293T cells expressing any of these engineered proteins (Fig. 4C), suggesting that bicarbonate sensing by GC-D is independent on its ex and kh.

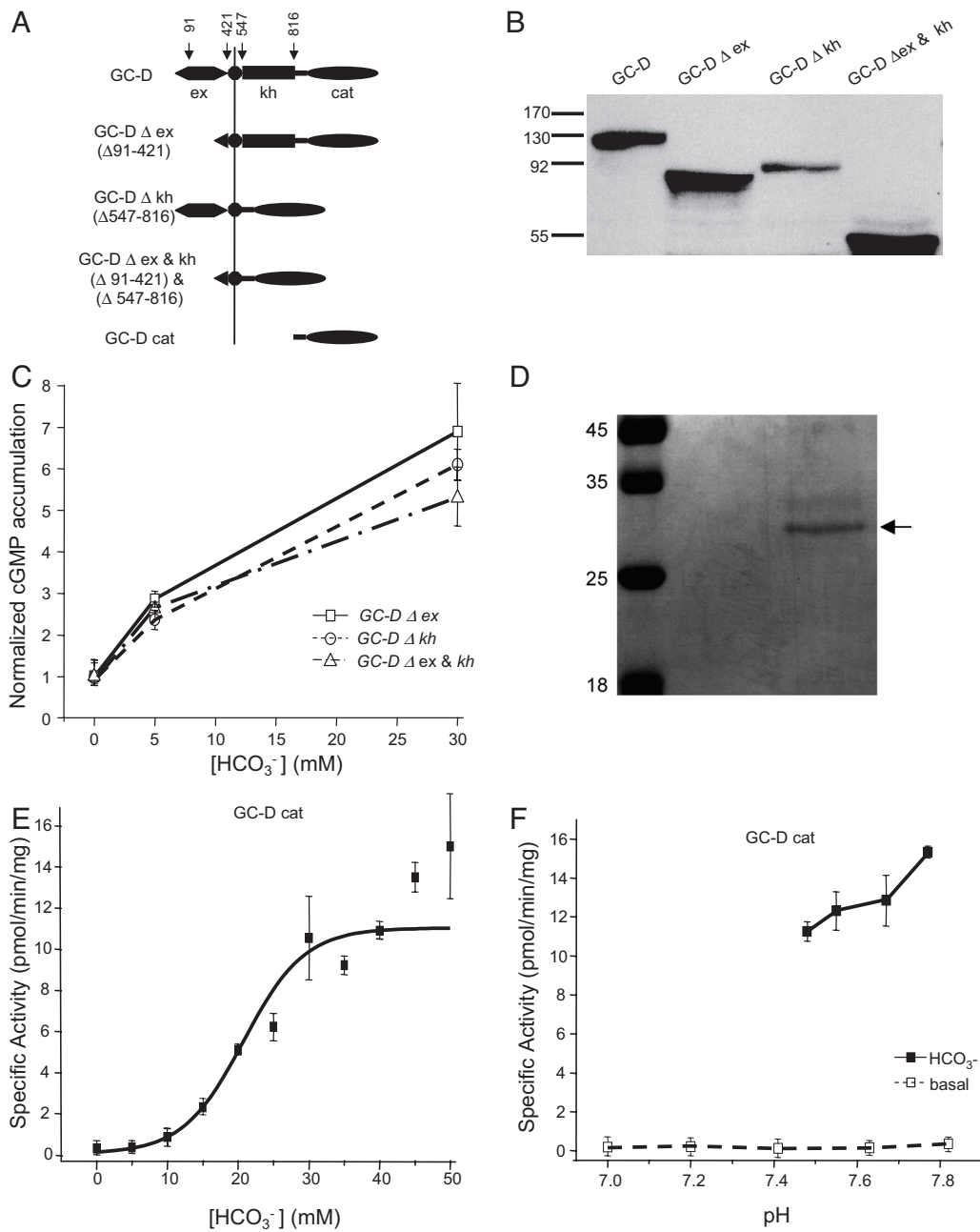
To directly test whether bicarbonate acts on GC-D cyclase domain (GC-D cat) and to further exclude the possibility of accessory factors mediating activation, we expressed the GC-D cyclase domain in insect HiFive cells and purified the recombinant protein to test the effects of bicarbonate (Fig. 4D). The cyclase activity of the purified protein was dramatically enhanced by bicarbonate in a dose-dependent manner (Fig. 4E). The median effective concentration ( $\text{EC}_{50}$ ) of this activation was  $\approx 21$  mM. Bicarbonate stimulation was not due to altered pH, because neither the recombinant protein (GC-D cat) alone nor bicarbonate-stimulated GC activities of the recombinant protein were sensitive



**Fig. 3.** Bicarbonate does not activate other membrane GCs or sGCs. (A) Bicarbonate did not stimulate the cyclase activity of cells expressing GC-A, GC-C, GC-E, or GC-F. It appeared to inhibit the basal cyclase activity of GC-E and GC-F. GC-C was assayed on T84 cells, whereas other GCs were transiently expressed in HEK-293T cells. Numbers 0, 25, and 50 in leftmost panel indicate  $\text{NaHCO}_3$  concentrations and apply to all panels. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; *t* test. (B–D)  $\text{Ca}^{2+}$  imaging reviewed that  $\text{CO}_2$  responses of GC-D<sup>+</sup> neurons were independent on the activity of sGCs. (B) GFP labeling of GC-D<sup>+</sup> neurons within an intact epithelial preparation from a GCD-ITG mouse (Left) and loading of calcium-sensitive Rhod2/AM dye into GC-D<sup>+</sup> neurons (Right). (C) Application of a selective sGC blocker, ODQ (50  $\mu\text{M}$ ), had no effect on  $\text{CO}_2$ -evoked  $\text{Ca}^{2+}$  signals. Arrows indicated the start of perfusion of  $\text{CO}_2$  solution. (Scale Bars, 5%  $\Delta\text{F}/\text{F}$  and 50 s.) (D) Group data showing that the  $\text{CO}_2$ -evoked responses before and after ODQ application were not statistically different ( $P = 0.08$ ;  $n = 27$  cells; paired *t* test).

to pH changes over the range 7.0 to 7.8 (Fig. 4F). Therefore, bicarbonate activates GC-D by directly acting on GC-D cyclase domain without the need of any accessory factors in the cell.

For GC-A, an extensively characterized membrane GC, the kh is believed to repress the cat of the cyclase domain and binding of ANP to its ex relieves this repression (15). Deleting kh from GC-A results in a constitutively active GC (20, 28). However, GC-D cyclase domain is normally inactive and can be activated by bicarbonate without the application of external peptide ligands (Fig. 4C and E). To test whether the exs and khs in GC-A and GC-D have different roles in regulating their respective cyclase domain, we engineered constructs expressing chimera proteins containing domains from these 2 proteins (Fig. 5A and Fig. S3). Replacement of GC-A cyclase domain with that of GC-D resulted in chimera proteins (A-D-1 and A-D-2) that were activated only by combined application of both bicarbonate and ANP, whereas neither bicarbonate nor ANP alone had effects (Fig. 5B and C). In contrast, replacement of GC-D cyclase domain with that of GC-A resulted in a chimera protein (D-A) that was constitutively active without the



**Fig. 4.** Bicarbonate directly activates GC-D intracellular cyclase domain and this activation is pH-independent. (A–C) Deletions of GC-D ex and its intracellular kh had no effects on the ability of GC-D to sense bicarbonate. (A) Schematic representation showing engineered GC-D mutations with deletions of ex (GC-D  $\Delta$ ex), intracellular kh (GC-D  $\Delta$ kh), and both of these domains (GC-D  $\Delta$ ex and kh). For GC-D  $\Delta$ ex, residues 91–421 were removed. Residues 1–90 were spared, because they were necessary for membrane expression. For GC-D  $\Delta$ kh, residues 547–816 were removed. GC-D cyclase domain (GC-D cat; residues 850–1,110) was purified for assay shown in *E*. (B) Western blotting by using an antibody against GC-D C-terminal peptide showing GC-D and its mutants of  $\Delta$ ex,  $\Delta$ kh, and  $\Delta$ ex and kh in membrane fractions of HEK-293T cells transfected with vectors of these proteins. (C) Deletion of ex, kh, or both had no obvious effects on the stimulation of cyclase activity by bicarbonate. (D) Coomassie Blue staining of purified recombinant GC-D cyclase domain (arrow). (E) Bicarbonate directly activated purified GC-D cyclase domain. Data are expressed as picomoles of cGMP formed per minute per milligram of protein. (F) The activation of purified GC-D cyclase domain was independent on pH when assayed alone (dashed line) or in the presence of 40 mM bicarbonate (solid black line). All of the pH values were measured in the final assay solutions.

presence of bicarbonate or ANP (Fig. 5D). Thus, GC-D cyclase domain is directly activated by bicarbonate, and GC-D activation may use a mechanism different from the release from repression model proposed for GC-A.

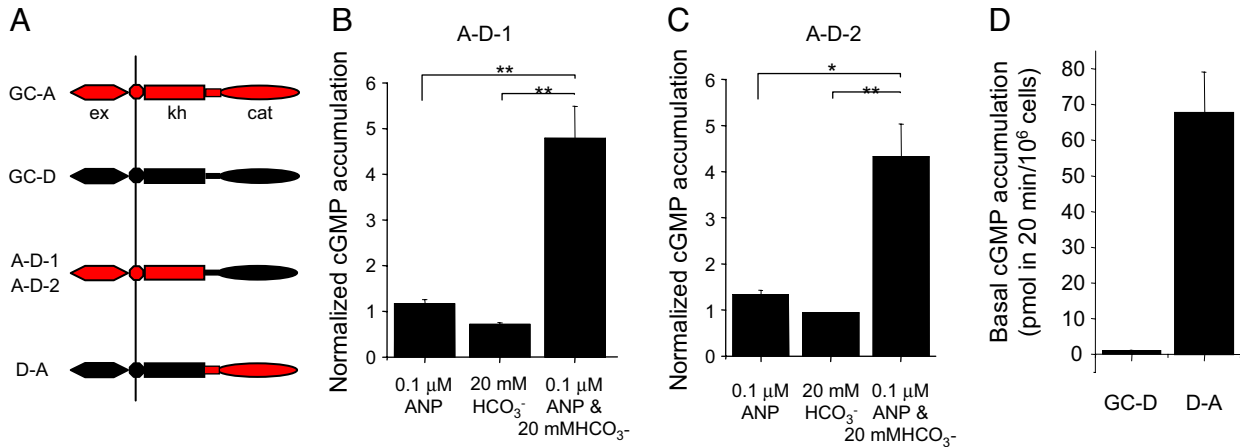
### Discussion

GC-D<sup>+</sup> neurons express rich amount of CAII and are important for sensitive detection of atmospheric CO<sub>2</sub> (1). CO<sub>2</sub> responses of these neurons also require the opening of cGMP-sensitive CNG channels (1). Our data in this study show that bicarbonate stimulates the production of cGMP by GC-D by directly acting on its intracellular cyclase domain, uncovering a key event within the signaling transduction pathway for CO<sub>2</sub> sensing.

**Intracellular Signal Transduction Cascade for CO<sub>2</sub>-Sensing.** Bicarbonate stimulates the cyclase activity of GC-D when it is heterologously expressed in cultured cells. This activation can be similarly observed

when GC-D protein is assayed *in vitro*. Most importantly, bicarbonate directly activates the purified recombinant protein containing the intracellular cyclase domain in dose-dependent and pH-insensitive manner. Therefore, GC-D can be directly activated by bicarbonate without the need of accessory factors. Bicarbonate is believed to be an important cellular “first messenger” that can be sensed by SAC (13, 29, 30). Now, GC-D should be added to the list of bicarbonate sensors.

Our results lead us to propose the following scenario of signal transduction cascade after CO<sub>2</sub> diffusion into the GC-D<sup>+</sup> OSNs (Fig. 6A). CAII catalyzes the conversion of CO<sub>2</sub> into bicarbonate ion and proton. Bicarbonate activates GC-D by directly acting on its cyclase domain, whereas proton may be quickly buffered by the cell to maintain stable intracellular pH. GC-D produces cGMPs from GTPs; cGMPs in turn open cGMP-sensitive CNG channels to allow cation influx into the GC-D<sup>+</sup> cells, resulting in neuronal depolarization to fire action potentials. This model suggests that CO<sub>2</sub> is



**Fig. 5.** Stimulation of GC activity of GC-D by bicarbonate is independent on the release of repression by ex. (A) Schematic representations showing the domain organization of native GC-A, GC-D, and chimera GC with domains from either GC-A or GC-D. A-D-1 and A-D-2 were engineered by replacing the cyclase domain of GC-A with that of GC-D starting at residue 830 or 850, respectively. D-A was engineered by replacing the cyclase domain of GC-D with that of GC-A. (B and C) The cyclase activity of both A-D-1 (B) and A-D-2 (C) was activated only by applying both bicarbonate and ANP, the peptide that binds to GC-A ex. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; *t* test. Data were normalized to the cGMP levels for A-D-1 and A-D-2 samples without the application of either ANP or bicarbonate. (D) D-A exhibited high basal GC activity. Unlike that of GC-A, the N-terminal region of GC-D does not repress the C-terminal cyclase domain, indicating that there may be no needs for any peptide as coactivator to release any potential repression in GC-D.

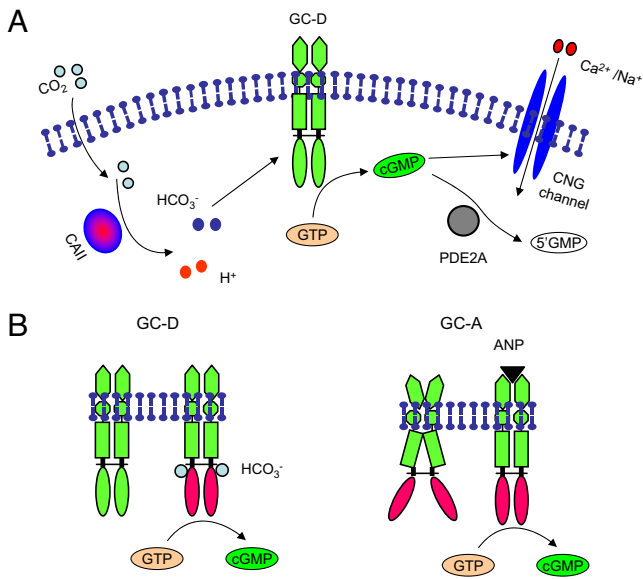
sensed by mechanisms different from those for typical odorants. In typical OSNs, odorant molecules bind to odorant receptors, which are G protein-coupled receptors, to activate  $G_{olf}$ , which in turn activates adenylyl cyclase to produce cAMPs; thus, opening cAMP-sensitive CNG channels (31–33). Our model on  $CO_2$  sensing can be further tested by future studies on the behavioral and physiological effects of both GC-D-knockout mice and the cGMP-sensitive CNG channel-knockout mice. Nevertheless, the fact that GC-D is a

pseudogene in human provides an attractive explanation on why the human olfactory system lacks ability to sense  $CO_2$  (10). Recent bioinformatics analysis indicates that GC-D is functional in most of mammals but degenerates in many primate species (11), suggesting that  $CO_2$  sensing ability might be preserved in most of mammals but largely lost in primates.

Our data also suggest that the molecular mechanisms of  $CO_2$  sensing by mammals differ from those by insects. In fly, a pair of membrane receptors, *Gr21a* and *Gr63a*, are necessary and sufficient for  $CO_2$  detection (4, 34). These 2 receptors, like other fly odorant receptors, are presumably channels themselves (35, 36). In *C. elegans*, DAF-11 (a membrane GC) and cGMP-sensitive CNG channels are essential for  $CO_2$ -mediated avoidance behavior (5). It will be interesting to test whether the mechanisms of  $CO_2$  sensing are conserved between mammals and worms, and whether DAF-11 or other membrane GCs in *C. elegans* could be activated by bicarbonate.

**Different Mechanisms of Molecular Activation Among Membrane GCs.**

Our data on domain deletions and swaps indicate that the molecular mechanism for GC-D activation differ from that for GC-A. Removal of GC-A kh results in high constitutive activity, consistent with the concept that GC-A kh represses a normally active cyclase domain (14, 28). Binding of ANP to GC-A ex is believed to induce a conformational change to release the repression; thus, activating the enzyme (Fig. 6B Right) (15). In contrast, deleting GC-D kh neither increases its basal cyclase activity nor disrupts the activating effects of bicarbonate. Similarly, GC-D without ex can be activated by bicarbonate. More importantly, purified recombinant protein of GC-D cyclase domain itself is normally inactive, but can be directly activated by bicarbonate. These results indicate that GC-D is activated by direct action of bicarbonate, rather than by binding a ligand to its ex to release the repression from kh (Fig. 6B Left). The chimera protein with the ex and kh from GC-A and the cyclase domain from GC-D can be activated only with combined application of ANP and bicarbonate. However, the chimera protein with the ex and kh from GC-D and the cyclase domain from GC-A is constitutively active. These results are consistent with the model of release from repression for GC-A (15), and a model of direct activation by bicarbonate for GC-D, suggesting diverse molecular mechanisms of activation among membrane GCs.



**Fig. 6.** Our models on the intracellular signal transduction for  $CO_2$  detection by GC-D<sup>+</sup> neurons and the molecular mechanism of bicarbonate sensing by GC-D. (A) Intracellular signal-transduction cascades in GC-D<sup>+</sup> neurons. After  $CO_2$  arrival, CAII catalyzes the conversion of  $CO_2$  into bicarbonate, which activates GC-D to produce cGMP; cGMP in turn opens the cGMP-sensitive CNG channels to activate GC-D<sup>+</sup> neurons. PDE2A restores cGMP levels by hydrolyzing it to 5'-GMP. (B) Comparison of the molecular mechanisms of activation between GC-D and GC-A. GC-D cyclase domain is normally inactive and can be activated by bicarbonate. In contrast, GC-A cyclase domain is constitutively active, but its activity is blocked by GC-A ex and kh. Binding of ANP to GC-A ex releases this blockade and activates GC-A cyclase activity.

A study using electrophysiological recordings and  $\text{Ca}^{2+}$  images suggests that GC-D can be activated by G and UG (17), 2 peptide natriuretic hormones that are known to activate GC-C. Biochemical assays from another study suggest that UG but not G activates GC-D by binding to its ex (37). Also, this study suggests that  $\text{Ca}^{2+}$  and neurocalcin can activate GC-D by binding to its intracellular domains (37). Thus, GC-D may be activated by factors other than bicarbonate. In this study, we have identified the stimulatory effects of bicarbonate on the activation of GC-D. It will be interesting to examine the effects of these additional factors on GC-D and their potential roles in behavioral and physiological functions of  $\text{CO}_2$  sensing.

## Materials and Methods

**Electrophysiological Recording,  $\text{Ca}^{2+}$  Imaging, and FRET Imaging.** The preparation of intact olfactory epithelia and calcium imaging was carried out by procedures as described previously (1, 21). Briefly, adult GCD-ITG (GCD-ires-tauGFP) mice was anesthetized and decapitated. Swatches of olfactory epithelium were peeled off and placed in oxygenated Hepes based Ringer solution (140 mM NaCl/1.5 mM KCl/1 mM  $\text{MgCl}_2$ /2.5 mM  $\text{CaCl}_2$ /11 mM glucose/10 mM Hepes, pH 7.4). In these mice, GFP is coexpressed with GC-D by bicistronic strategy that leaves the coding region of GC-D intact (8). For cell-attached patch recording, intracellular pipette solution contained (in mM) 130 K-gluconate, 5 KCl, 10 Hepes, 13 Na-phosphocreatine, and 4  $\text{MgCl}_2$  and 0.6 EGTA. Recordings were carried out at room temperature (RT;  $25 \pm 2^\circ\text{C}$ ).

For  $\text{Ca}^{2+}$  imaging, Rhod-2/AM  $\text{Ca}^{2+}$ -sensitive dye was loaded at  $34^\circ\text{C}$ . Tissues were then transferred to an imaging chamber, stabilized with a nylon net, and continuously perfused with oxygenated Ringer solution at RT. For  $\text{CO}_2$  delivery, oxygenated Ringer solution was bubbled with pure  $\text{CO}_2$  and then added into the chamber by superfusion, resulting in a final  $\text{CO}_2$  concentration of 6.4 mM.

Imaging of FRET signals for cGMP was carried out as described previously (38). Briefly, HEK-293T cells were transfected with cGMP probe cGES-DE5 either with GC-D or control empty vector. FRET signals were expressed as the intensity of YFP/CFP ratio. Transfected cells were continuously perfused with Hepes-based Ringer solution, and imaging was performed at RT.

Unless specified otherwise, all inorganic salts and peptides in this study were purchased from Sigma.

**Cell Culture and Western Blottings.** HEK-293T cells were maintained in DMEM containing 10% FBS. Transfections of HEK-293T cells were performed by using Lipofectamine2000 (Invitrogen). To examine bicarbonate sensitivity of GCs in cellular systems, transfected cells were grown in bicarbonate-free L-15 medium (GIBCO) in  $\text{CO}_2$ -deprived incubator for 24 h. Full-length GC-D cDNA was cloned from rat olfactory epithelium. For Western blottings, an anti-GC-D antibody (GeneTex) was used, because it reacted with an epitope from the GC-D C-terminal and labeled both intact and engineered GC-D proteins.

**Protein Purification and Particulate Membrane Fraction Preparation.** His-tagged intracellular cyclase domain of GC-D (His-GC-D-cyclase), consisting of GC-D residues 850–1,110, was heterologously expressed in insect HiFive cells by using the Bac-to-Bac Baculovirus Expression System (Invitrogen), and protein was purified by chromatography over  $\text{Ni}^{2+}$ -NTA Sepharose Resin (Qiagen) followed by a Superdex200 HR10/30 column (GE Healthcare). The purity of the recombinant protein was confirmed by Coomassie Blue staining (Fig. 4D). Membrane fraction of HEK-293T cells transfected with GC-D is prepared as described previously (14).

**Guanylyl Cyclase Activity Assay.** The concentrations of cGMP were determined with the cGMP ELISA Assay Kit from Assay Designs. For cell lysate assay, cells were incubated for 18–24 h after transfection before the medium was changed to serum-free medium; 24 h after transfection, cells were incubated at  $37^\circ\text{C}$  for 20 min with  $\text{NaHCO}_3$  at the indicated concentrations as well as 1 mM phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine). We empirically determined the dilution factor for each cell lysate so that the experimental readings resided in the optimal assay range of the cGMP Assay Kit.

For GC activity assay in vitro, purified recombinant proteins or particulate fractions containing heterologously expressed GCs were mixed in 50  $\mu\text{L}$  of reaction buffer (20 mM Hepes, pH 7.4/100 mM NaCl/0.4 mM  $\text{MgCl}_2$ /0.1 mM GTP) and incubated for 20 min at  $30^\circ\text{C}$ . The reaction was terminated by boiling for 5 min before its cGMP content was determined with the cGMP Assay Kit.

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