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Stimulation of Guanylyl Cyclase-D by Bicarbonate

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Abstract

Guanylyl cyclases (GCs) catalyze the conversion of GTP to the second messenger cGMP. While some transmembrane GCs are receptors for extracellular ligands, other transmembrane GCs such as retinal-specific GC-E and GC-F are stimulated by cellular proteins. GC-D is expressed in a special group of olfactory sensory neurons. However, the direct regulatory mechanism of GC-D activity is not completely understood. Here we have demonstrated that bicarbonate directly increases the activity of purified GC-D. Bicarbonate also increases the cGMP levels in cells expressing GC-D. These results identify bicarbonate as a small molecule that regulates GC-D.

Regulation of cell functions by a wide variety of signals involves the second messenger cGMP. The physiological importance of cGMP has been highlighted by the efficacious clinical treatment of erectile dysfunction with Viagra or other cGMP phosphodiesterase inhibitors (1). cGMP controls diverse physiological functions such as relaxation of vascular smooth muscles, phototransduction, epithelial electrolyte transport, bone growth, leukocyte migration, axonal guidance, sperm motility, platelet spreading, and vascular permeability (2-10). cGMP acts on cGMP-dependent protein kinases, cyclic nucleotide-gated ion channels, and cyclic nucleotide phosphodiesterases (9).

The conversion of GTP to cGMP is catalyzed by guanylyl cyclases (GCs). There are two types of GCs in mammals: the soluble and the membrane-bound GCs (9,11,12). The soluble GCs are generally activated when nitric oxide binds to the attached prosthetic heme group. Seven membrane-bound GCs (also named transmembrane or particulated GCs) have been identified in the human genome (9). GC-A and GC-B are natriuretic peptide receptors. GC-C can be activated by bacterial heat-stable enterotoxins, guanylin, and uroguanylin. The extracellular ligands for GC-D, GC-E, GC-F, and GC-G are not known; although GC-D expressing neurons could be activated by uroguanylin and guanylin (13). GC-E and GC-F, found in the retina, can be modulated by a group of retinal-specific cellular proteins named guanylyl cyclase activating proteins in a calcium-dependent manner (14). Saturating amounts of guanylyl cyclase activating proteins could increase the activity of GC-E and GC-F by 2 to 10-fold; this increase of cGMP plays a major physiological role in the recovery phase of the retinal photoreceptor light response and in the process of light adaptation (15). Recently, we have uncovered a new cellular signaling pathway linking the small GTPase Rac to cGMP increase via p21-activated protein kinase and transmembrane GCs (16). This new pathway provides a mechanism by which all transmembrane GCs could be regulated by cellular proteins.

GC-D was first cloned from a rat olfactory cDNA library and found to be specifically expressed in a small, randomly dispersed population of neurons that is within a single topographic zone in the olfactory neuroepithelium (17). GC-D has been proposed to participate in odor

recognition (13,17-19). However, the molecular mechanism regulating the activity of GC-D is not completely known. Here we report that bicarbonate directly stimulates GC-D.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture

The constructs for pCMV5-GC-D and pAXNEO-GC-A had been described in (16). GC-D-intra (residues 546 to 1071) and GC-A-intra (residues 495 to 1061) were PCR subcloned into pGEX-2T. CHO-GC cells were generated by stably transfecting CHO-K1 cells with plasmids encoding GCs (16). CHO-K1 cells were cultured in F12K nutrient mixture (Kaighn's modification) containing 10% fetal bovine serum (Invitrogen). Transfections of CHO-K1 cells were performed with Transfast (Promega).

Protein Purification

GST-tagged intracellular domains of GC-D and GC-A (GST-GC-D-intra and GST-GC-A-intra) were purified from *E. coli*. Two liters of BL21(DE3)/pGEX-GC-intra were grown at 20°C until the turbidity reached $OD_{600} = 1.2$. Protein expression was induced with 10 μ M IPTG (Sigma) for 10 hr at 20°C. Cells were harvested by centrifugation and the pellet was resuspended in ice-cold 1X PBS containing 1 mg/ml lysozyme, 1 mM DTT, 100 μ g/ml DNase I, 0.5 μ g/ml leupeptin, 1 μ g/ml pepstatin, 2 μ g/ml aprotinin and 1 mM PMSF. After sonication, Triton X-100 (1%) was added to the lysate and the mixture was stirred for 30 min at 4°C. The lysate was then cleared of debris by centrifugation and applied to glutathione agarose beads (Sigma). After washing, GST-tagged protein was eluted with glutathione.

Guanylyl Cyclase Activity Assay

In vivo cGMP concentrations were determined from cell lysates with the Monoclonal anti-cGMP antibody based cGMP Assay Kit from NewEast Biosciences Inc. CHO-GC cells were routinely cultured in DMEM/F12 medium containing 10% fetal bovine serum. Cells were seeded in a 6-well culture plate. When the cell density reached 90%, they were changed to bicarbonate-free DMEM/F12 medium buffered with 20 mM Hepes, pH 7.5, and incubated under ambient CO₂. After 16~18 hr of bicarbonate-starvation, the cells were treated with 0.5 mM IBMX for 30 min and 40 mM sodium bicarbonate for 10 min. The cells were then harvested and lysed in 0.5% Triton X-100, and cGMP concentration was determined as described previously (16).

For the *in vitro* guanylyl cyclase activity assay, purified proteins were mixed in 50 μ l of reaction buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 1 mM GTP) and incubated for 15 min at 30°C. GTP was added last to start the reaction (there was no detergent in these reactions). The reaction was terminated by adding HCl to a final concentration of 0.1 M. The mixture was diluted 5-10 fold in 0.1 M HCl and the cGMP content was determined with the Monoclonal anti-cGMP antibody based cGMP Assay Kit from NewEast Biosciences Inc.

For membrane preparations, CHO-GC cells were cultured in one 10-cm tissue culture plate and incubated for 16~18 hr in bicarbonate-free medium. Cells were detached by incubating with 1X PBS containing 5 mM EDTA. After centrifugation, the cell pellet was resuspended in 1 mL buffer containing 150 mM NaCl, 50 mM Tris, pH 8, 1 mM EDTA and protease inhibitors. Cells were passed through a 26-G needle several times. After centrifugation at 2,500 rpm on a microcentrifuge, membrane fraction was prepared by centrifugation at 13,000 rpm for >2 hr at 4°C. 0.5 mM of IBMX was included in the GC assay buffer when membrane preparations were used.

NG108-15 cells and PC12 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. Cells in a 10-cm culture plate were transiently transfected with pCMV5/GC-D plasmid using Lipofectamine 2000 (Invitrogen). A control transfection with GFP plasmid DNA showed the transfection efficiency to be 60-70%. Twenty-four hours after transfection, the cells were split into 6-well plates. After the cells attached to the plate, they were changed to bicarbonate-free DMEM medium buffered with 20 mM Hepes, pH 7.5, and incubated at 35°C under ambient CO₂. After 16-18 hr of bicarbonate-starvation, the cells were treated with 0.5 mM IBMX for 30 min. When indicated, the cells were treated with 40 mM sodium bicarbonate for 10 min. The cells were then harvested and lysed in 0.5% Triton X-100, and cGMP concentration was determined. The measured cGMP production was mainly due to GC-D since the cGMP level in the GFP control cells was undetectable under the same condition. The cGMP levels were determined with the Monoclonal anti-cGMP antibody based cGMP Assay Kit from NewEast Biosciences Inc.

Statistical Analysis

Data are expressed as means $\pm$ S.D. from at least three experiments and analyzed by one-way analysis of variance followed by Dunnett's multiple comparison test with significance defined as $p < 0.05$.

RESULTS

Direct Stimulation of Purified GC-D-intra by Bicarbonate

During our investigation of the regulation of transmembrane GCs by intracellular signaling pathways, we had established CHO cell lines stably expressing various transmembrane GCs (16). We noticed that cells expressing GC-D showed different cGMP levels when these cells grew with or without CO₂. Since soluble adenylyl cyclase could be directly stimulated by bicarbonate and it was proposed to sense CO₂ in cells (20), we tested the possibility that GC-D could be regulated by bicarbonate and CO₂.

We purified the intracellular domain of GC-D (residues Val-546 to Gly-1071). This GC-D-intra was without the extracellular domain and the membrane-spanning domain (Fig. 1a) (16). The purified recombinant GC-D-intra had a specific activity of ~ 6 pmol/mg/min (Fig. 1b). Addition of bicarbonate increased the catalytic activity of GC-D-intra to ~ 50 pmol/mg/min (Fig. 1b). The effect of bicarbonate on the catalytic activity of GC-D was specific since bicarbonate had no stimulatory effect on GC-A, another transmembrane guanylyl cyclase (Fig. 1b). The median effective concentration (EC₅₀) of bicarbonate was ~ 25 mM (Fig. 1c). This EC₅₀ of bicarbonate was within the physiological concentrations of bicarbonate (22 $\sim$ 32 mM) (20). Either the sodium salt or potassium salt of bicarbonate had the stimulatory effect, while NaCl had no effect, indicating the effect was through bicarbonate ion (Fig. 2a). Furthermore, although high concentrations (~ 40 mM) of bicarbonate could have small changes (~ 0.1) of the pH values of the reaction solution, this small pH change was not responsible for the stimulation of GC-D-intra by bicarbonate since GC-D-intra was rather insensitive to pH changes (Fig. 2b). Therefore, bicarbonate has the chemical ability to directly increase the catalytic activity of GC-D.

Bicarbonate Increases the Activity of Full-length GC-D

To study the effect of bicarbonate on the activity of full-length GC-D, we used the membrane preparations from cells stably expressing the full-length GC-D (16). Membrane preparations from untransfected CHO cells (in the absence or presence of bicarbonate) had undetectable GC activity. Expression of full-length GC-D in CHO cells gave rise to membrane preparations with a specific activity of ~ 33 pmol cGMP/mg of total membrane proteins (Fig. 3a). Addition of bicarbonate to this membrane preparation with GC-D expression increased the specific

activity to ~77 pmol cGMP/mg of total membrane proteins (Fig. 3a). In contrast, bicarbonate did not increase the activity of GC-A from membrane preparations of CHO cells expressing full-length GC-A (Fig. 3b). GC-A was functional in CHO cells since its ligand ANP (atrial natriuretic peptide) increased its activity (Fig. 3b). Hence, bicarbonate can increase the activity of full-length GC-D.

Bicarbonate Increases GC-D Activity in Cells

To verify the bicarbonate effect on GC-D activity in cells, we measured the cellular cGMP levels in GC-D expressing CHO cells before and after bicarbonate treatment. Before the addition of bicarbonate, GC-D expressing cells had ~28 pmol cGMP/mg of total cellular proteins (Fig. 4a). Addition of bicarbonate increased the cellular cGMP level to ~75 pmol cGMP/mg of total cellular protein (Fig. 4a). On the other hand, bicarbonate did not increase the cGMP levels from CHO cells expressing other transmembrane GCs including GC-A, GC-B, GC-C, GC-E, GC-F or the soluble GC (sGC) (Fig. 4a). These results indicate that bicarbonate could specifically stimulate the activity of GC-D, leading to the increase of cellular cGMP levels.

To further confirm this increase of cellular cGMP by bicarbonate through GC-D, we examined the cGMP response in GC-D expressing and GC-D non-expressing neurons. NG108 neuronal cells had undetectable level of cellular cGMP. NG108 neurons transfected with GC-D had a cGMP level of ~17 pmol of cGMP/mg of total neuronal proteins (Fig. 4b). Addition of bicarbonate increased (by ~3-fold) the cellular cGMP level (Fig. 4b). Similarly, CO₂ also increased the cGMP levels in these cells (Fig. 4b). However, this cellular cGMP increase induced by CO₂ was blocked by the pretreatment of these cells with acetazolamide (AZ), an inhibitor of carbonic anhydrase (Fig. 4b). Therefore, these results show that the cellular increase of cGMP by bicarbonate requires GC-D, and that the stimulatory effect of CO₂ depends on its conversion to bicarbonate. Furthermore, bicarbonate increased GC-D activity when GC-D was exogenously expressed in PC12 neuronal cells (Fig. 4c). Therefore, these results confirm that bicarbonate could increase the activity of GC-D in different types of cells.

Bicarbonate Increases GC-D Activity by Increasing $V_{\max}$ without Affecting K_m

To gain mechanistic insights into how bicarbonate regulates the activity of GC-D, we have studied the effect of bicarbonate on the kinetic parameters of GC-D. Regulation of enzymatic activity can be studied in terms of changes in K_m (Michaelis constant) and $V_{\max}$ (maximum velocity). We determined that GC-D-intra has a K_m for GTP ~58 μ M and a $V_{\max}$ of ~10 pmol cGMP/mg/min (Fig. 5). We found that the major effect of bicarbonate was to increase the $V_{\max}$ to ~35 pmol cGMP/mg/min (Fig. 5). K_m for GTP (~45 μ M) was not significantly affected. These data bolster the idea that bicarbonate changes the conformation of GC-D and allosterically activates GC-D.

DISCUSSION

Regulation of Cyclases by Bicarbonate

Bicarbonate regulation of the activity of a cyclase was first reported with a mammalian soluble adenylyl cyclase (20). An increase in $V_{\max}$ of this soluble adenylyl cyclase by bicarbonate was also found to be the biochemical mechanism (21). The nature of the interaction between bicarbonate and cyclases remains to be explored. In subsequent studies with a cyanobacterial soluble adenylyl cyclase, the structures of soluble adenylyl cyclases without or with treatment of bicarbonate were solved (22). Because soaking crystals in bicarbonate destroyed the crystals, crystals were flash-soaked with bicarbonate and quickly frozen. Even though no bicarbonate was found in the structure, bicarbonate treatment induced a conformational change in soluble adenylyl cyclase that helps coalesce the active site (22). Indeed manipulation of the active site

loops has been proposed to be an ancient and conserved mechanism for allosteric control of class III cyclase activity (23). GC-D likely functions as a homodimer. There are two active sites formed at the interface of the dimer. A recent X-ray crystal structure of the dimer of a bacterial GC revealed that one active site is in the conformation to accommodate GTP, while a conformational change of the side-chain of one residue in the other active site made the GTP binding unfavorable (24). It is likely that bicarbonate allosterically changes the conformation of GC-D, allowing the formation of more efficient active site.

The fold of stimulation of GC-D activity by bicarbonate with membrane preparations or in cells is smaller than that observed with purified GC-D proteins. There could be several potential reasons. One possibility is that in cells or in membrane preparations, GC-D was modified by other cellular factors and thus existed in a different state than the GC-D-intra protein purified from *E. coli*. This might indicate the crosstalk among different mechanisms regulating GC-D activity in cells. It has been reported that two peptide ligands for GC-C, guanylin and uroguanylin, could increase cellular cGMP levels in GC-D expressing cells (13,25).

The inhibitory effect of bicarbonate on GC-A activity is reproducible with membrane preparations and in cells. However, the inhibitory effect on the activity of purified GC-A-intra protein purified from *E. coli* was less apparent. This might due to the different modifications of GC-A in CHO cells and in *E. coli*, the lower *in vitro* activity of GC-A-intra, or other reasons.

A Potential Biochemical Basis for CO₂ Sensing in Mammals

There was one report that GC-D might be involved in CO₂ sensing in mice (19). If this observation is confirmed, our data could provide a possible biochemical mechanism for the involvement of GC-D in CO₂ sensing. CO₂ is not only essential for the maintenance of biosphere homeostasis, but also a critical signaling molecule for the regulation of diverse cell and organ functions (26,27). CO₂ is an odorant to many animals. The detection of CO₂ in mice was proposed to be mediated by a special group of olfactory sensory neurons that specifically express GC-D (17,19). This small population of olfactory neurons project to necklace glomeruli in the olfactory bulb and contribute to chemosensory function (13,18). cGMP and a cGMP-gated cation channel are also essential for the sense of CO₂ (19,28). CO₂ is converted to bicarbonate in cells by carbonic anhydrase, and the gene deletion of the specific isoform (type II) of carbonic anhydrase expressed in GC-D-expressing olfactory neurons impairs the detection of CO₂ (19,29). However, it is not understood how bicarbonate is biochemically connected to cGMP increase. Our data demonstrate that bicarbonate could directly increase the activity of GC-D *in vitro* and the cellular cGMP levels in cells through GC-D. These results could provide a potential biochemical mechanism for the CO₂ sensing in some animals. Thus, CO₂ is converted to bicarbonate in cells by carbonic anhydrase. Bicarbonate activates GC-D, leading to the increase of cellular cGMP. cGMP in turn opens the cGMP-sensitive cyclic nucleotide-gated channels, leading to the excitation of GC-D expressing olfactory neurons.

While our manuscript was under review, a paper reporting a similar observation was published from a group with whom we had shared our unpublished results (30). Although the conclusions were similar that bicarbonate could stimulate GC-D, there were several major differences. We observed that the activation of purified GC-D-intra (residues Val-546 to Gly-1071) by bicarbonate is dose-dependent and saturable (Fig. 1c), which is consistent with a direct and specific interaction between these GC-D and bicarbonate. Sun et al. showed a continuous increase of activity of the GC-D cyclase domain (residues 850 to 1110) by bicarbonate (Fig. 4E in ref. 30). While the specific activity of our GC-D-intra protein was ~ 6 pmol cGMP/mg/min, the specific activity for the GC-D cyclase domain used in the study of Sun et al. was < 0.5 pmol cGMP/mg/min. In our hands, the isolated GC-D cyclase domain (residues 836 to 1071) was inactive (well below the detection limits of the assays), similar to other reports of the isolated cyclase domains of transmembrane guanylyl cyclases (31). Further investigation

is required to understand the exact physiological function of GC-D and the detailed regulatory mechanism of GC-D.

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Abbreviations

GCs, Guanylyl cyclases; AZ, acetazolamide.

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Figure 1.

Direct stimulation of purified GC-D by bicarbonate. **a**, Coomassie blue staining of purified GST-GC-D-intra and GST-GC-A-intra from *E.coli*. **b**, Effect of bicarbonate on the activity of purified GC-D-intra and GC-A-intra. **c**, Dose-response curve of the activation of purified GC-D-intra by bicarbonate. Data are shown as mean $\pm$ s.d. of three experiments.

Figure 2.

Bicarbonate is responsible for the stimulation of GC-D. **a**, Effect of KHCO₃, NaHCO₃ and NaCl on the activity of purified GC-D-intra. **b**, Effect of pH (from 7.1 to 7.9) on the activity of purified GC-D-intra in the absence or presence of NaHCO₃. The pH of the buffers was adjusted to the desired value with HCl at room temperature. The pH values shown were before the addition of bicarbonate. After addition of 40 mM sodium bicarbonate, the pH values were: 7.35, 7.41, 7.48, 7.54, 7.61, 7.69, 7.77, 7.86, and 7.94. The increase was 0.24, 0.21, 0.17, 0.14, 0.11, 0.09, 0.07, 0.05, 0.04, respectively. Data are shown as mean $\pm$ s.d. of three experiments.

Figure 3.

Bicarbonate increases the activity of full-length GC-D in membrane preparations. **a**, Membrane preparations were made from CHO cells stably expressing GC-D. The GC-D activity was measured in the absence or presence of 40 mM bicarbonate. **b**, Membrane preparations were made from CHO cells stably expressing GC-A. The GC-A activity was measured in the absence or presence of 40 mM bicarbonate, or in the presence of 10 μM ANP. Data are shown as mean $\pm$ s.d. of three to five experiments.

Figure 4.

Bicarbonate increases the cellular cGMP levels in cells expressing GC-D. **a**, Bicarbonate increased cellular cGMP levels in CHO-GC-D cells, while had no effect on cellular cGMP levels in CHO cells expressing GC-A, GC-B, GC-C, GC-E, GC-F or sGC. **b**, Bicarbonate and CO₂ increased the cellular cGMP levels of NG108 neuronal cells transfected with GC-D. The effect of CO₂ was sensitive to acetazolamide (AZ), an inhibitor of carbonic anhydrase. **c**, Bicarbonate increased the cellular cGMP levels of PC12 neuronal cells transfected with GC-D. Data are shown as mean $\pm$ s.d. of five experiments.

Figure 5. Activation mechanism of GC-D by bicarbonate. The velocity versus substrate concentration curve. Initial velocity of GC-D-intra activity was measured in the absence or presence of 40 mM bicarbonate. Data are shown as mean $\pm$ s.d. of three experiments.