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CO₂/bicarbonate modulates cone photoreceptor ROS-GC1 and restores its CORD6-linked catalytic activity

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Abstract

This study with recombinant reconstituted system mimicking the cellular conditions of the native cones, documents that photoreceptor ROS-GC1 is modulated by gaseous CO₂. Mechanistically, CO₂ is sensed by carbonic anhydrase (CAII), generates bicarbonate that, in turn, directly targets the core catalytic domain of ROS-GC1 and activates it to increased synthesis of cyclic GMP. This, then, functions as a second messenger for the cone phototransduction. The study demonstrates that, in contrast to the Ca²⁺-modulated phototransduction, the CO₂ pathway is Ca²⁺-independent, yet is linked with it and synergizes it. It, through R⁷⁸⁷C mutation in the third heptad of the signal helix domain of ROS-GC1, affects cone rod dystrophy, CORD6. CORD6 is caused firstly by lowered basal and GCAP1-dependent ROS-GC1 activity and secondly, by a shift in Ca²⁺ sensitivity of the ROS-GC1/GCAP1 complex that remains active in darkness. Remarkably, the first but not the second defect disappears with bicarbonate thus explaining the basis for CORD6 pathological severity. Because cones, but not rods, express CAII, the excessive synthesis of cyclic GMP would be most acute in cones.

Keywords

membrane guanylate cyclase; CO₂; Ca²⁺-sensor GCAP1; phototransduction; cone rod dystrophy

INTRODUCTION

ROS-GC1 is one of seven members of the mammalian membrane guanylate cyclase family encoded by a gene localized to human chromosome 17p13.1 [1–3]. Physiologically, it is integral to visual transduction [4, 5] [reviewed in ref. 6], the biochemical process by which rods and cones in the retina convert incoming light into an electrical signal. ROS-GC1 generates cyclic GMP, the second messenger of the signal. It controls all of the cyclic GMP production in cones and about 95% of the cyclic GMP production in the rod [7, 8], the other 5% being generated by the second ROS-GC member, ROS-GC2 [9–11]. Turnover of phototransduction and recovery of cyclic GMP levels are critical for positioning the amplitude, bestowing reproducibility, and for adjusting the duration of the photon response

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Conflict of interest

The authors declare that they do not have any conflict of interest.

[12–15]. The catalytic activity of ROS-GC1 is inversely tied to the intracellular free Ca^{2+} concentration. In darkness, cyclic GMP levels are at their basal levels, the CNG channels are open and the inward current is carried by Na^+ and Ca^{2+} . Efflux of Ca^{2+} through a Na^+/K^+ , Ca^{2+} exchanger maintains an intracellular $[\text{Ca}^{2+}]$ of ~250 nM in mouse [16] and ~800 nM in salamander outer segments [17]. Light sparks hydrolysis of cyclic GMP and closure of the CNG channels. The influx of Ca^{2+} stops, yet extrusion through the exchanger persists, decreasing the intracellular $[\text{Ca}^{2+}]$ by as much as an order of magnitude. The Ca^{2+} -sensing GCAP subunits of the ROS-GC1 complex kick-start the catalytic activation of ROS-GC1. In rods, GCAP1 is the “first responder”; the continued decline in $[\text{Ca}^{2+}]_i$ recruits GCAP2, which further stimulates ROS-GC1. As the cyclic GMP concentration recovers, CNG channels reopen to allow a rise in intracellular Ca^{2+} , which then returns ROS-GC1 catalytic activity back to its basal level.

The GCAP1-modulated Ca^{2+} signal originates at the juxtamembrane domain (JMD) whereupon it undergoes successive processing by the WTAPPELL motif in the kinase homology domain [18] and by the signaling helix domain (SHD) before converging on the core catalytic domain (CCD) (Fig. 1). An additional mode of modulation is Ca^{2+} -independent and occurs through bicarbonate, presumably formed from gaseous CO_2 . Bicarbonate raises basal enzymatic activity as well as maximal activity at low $[\text{Ca}^{2+}]_i$ by binding directly to the CCD.

Several mutations in the ROS-GC1 gene (GUCY2D) are linked with retinal diseases, Leber’s congenital amaurosis (LCA) and cone–rod dystrophies (CORDs), marked by the progressive and irreversible loss of vision [19–23]. Molecular analyses of LCA1 and CORDs reveal a common biochemical theme [24–27]. The mutations lower basal catalytic activity of the ROS-GC1 and alter the GCAPs signaling pathways [24–27]. Consequently, the light-dependent fluctuations of cyclic GMP and Ca^{2+} concentrations are negatively affected, resulting first in the death of the cone and then of the rod photoreceptors [24–27].

The R^{787}C (corresponding to human R^{838}C) mutation in ROS-GC1 gene leads to familial CORD type 6 (CORD6). R^{787} occupies the central position within the third heptad of the signaling helix domain, SHD (Fig. 1: marked in red). The mutation shifts Ca^{2+} sensitivity to higher concentrations leading to the proposal that photoreceptor degeneration results from defective negative Ca^{2+} feedback that raises intracellular cyclic GMP to abnormally high levels in darkness. However, the mutation also reduces basal and maximal enzymatic activity at low Ca^{2+} , making it difficult to reconcile the autosomal dominant nature of the disease.

The present study was designed to test (1) the CO_2 /bicarbonate signaling pathway of ROS-GC1, (2) its molecular linkage with the Ca^{2+} -modulated GCAP1 phototransduction pathway operating in cone photoreceptors, and (3) how CO_2 affects the functioning of cone photoreceptors afflicted by the CORD6-causing R^{787}C mutation in ROS-GC1.

MATERIALS AND METHODS

Reconstituted systems.

Two bovine ROS-GC1 mutants were constructed: R⁷⁸⁷C and H3. The conversion of R⁷⁸⁷→C and the deletion of the third heptad (H3; aa 784–790) of the signaling helix domain of ROS-GC1 were performed by polymerase chain reaction (PCR) using the following mutagenic primers: FW, 5'-CCTGATCCGGGAGTGCACAGAGGAGCTG-3', Rev, 5'-CAGCTCCTCTGTGCACTCCCGGATCAGG-3' for the R⁷⁸⁷C mutant and FW, 5'-AGTAACCTGGAGGACCTGCTGGAGCTGGAAAAGCAG-3', Rev, 5'-CTGCTTTTCCAGCTCCAG CAGGTCCTCCAGGTTACT-3' for the H3 mutant.

Expression in a heterologous system.—COS-7 cells (purchased from ATCC CRL-1651 and maintained in our laboratory) were induced to express ROS-GC1 or its mutants using a calcium-phosphate coprecipitation [30] or Lipofectamine (Thermo Fisher Scientific). Seventy hours after transfection, the cells were harvested and their membranes prepared [28].

Co-expression of ROS-GC1 and carbonic anhydrase type II (CAII).—Bovine ROS-GC1 and mouse CAII cDNAs were cloned into pBudCE4.1 vector (Thermo Fisher Scientific) respectively into *NotI/XhoI* sites of the EF-1a multiple cloning site and *SaII/XbaI* sites of the CMV multiple cloning site. The construct was verified by sequencing and used for ROS-GC1-CAII co-expression in COS cells.

Immunohistochemistry.

The construction of GFP-GCAP1 was described previously [18, 31]. GFP-GCAP1 was expressed together with ROS-GC1 or its R⁷⁸⁷C mutant in COS cells grown on coverslips in chambers in DMEM medium supplemented with 10% fetal bovine serum. Seventy hr after transfection the cells were fixed in 4% formaldehyde in Tris-buffered saline (TBS) for 15 min at room temperature. The fixed cells were washed with TBS, blocked in 10% preimmune donkey serum in TBS/0.5% Triton X-100 (TTBS) for 1 hr at room temperature, washed with TTBS, incubated with ROS-GC1 antibody in blocking solution overnight at 4 °C, washed with TTBS and then incubated with AlexaFluor 647-conjugated secondary antibody for 1 hr and washed with TTBS. For GFP the excitation and emission wavelengths were 475 nm and 505 nm, respectively; for AlexaFluor647 they were 643 nm and 670 nm. Images were acquired using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system, and analyzed using Olympus FluoView FV10-ASW software. Digital images were processed using Adobe Photoshop software.

Assay of guanylate cyclase activity.

Membrane samples of transfected COS cells were incubated individually without or with varying concentrations of recombinant bovine GCAP1 and/or NaHCO₃. GCAP1 was purified as described in [18]. The assay mixture (25 µl) consisted of (mM): 10 theophylline, 15 phosphocreatine, and 50 Tris-HCl; pH 7.5, and 20 µg creatine kinase (Sigma). In experiments with GCAP1, 1 mM EGTA was added to the reaction mixture; when the bicarbonate effect was tested, neither EGTA nor Ca²⁺ was added. The reaction was initiated

by addition of the substrate solution 4 mM MgCl₂ and 1 mM GTP (final concentrations) and maintained by incubation at 37 °C for 10 min. In the experiment for [GTP] dependency of guanylate cyclase activity (Fig. 3C), the GTP concentrations varied from 0 – 1.5 mM and the MgCl₂ concentration was constant, 4 mM. The reaction was terminated by the addition of 225 µl of 50 mM sodium acetate buffer, pH 6.2, followed by heating on a boiling water bath for 3 min. The amount of cyclic GMP formed was determined by radioimmunoassay [32]. All assays were done in triplicate and except where stated otherwise, were performed three times. Guanylate cyclase activity is presented as average ± SD of three experiments done in triplicate.

To correlate the catalytic changes brought about by the mutations, the activities of the mutants were compared with wild type recombinant ROS-GC1 through Michaelis plots for the ligand used by fitting the data with the Hill equation, $v = V_{\max} (S)^n / K_M + S^n$. $V_{\max}$ is the activity, S is the concentration of the ligand, K_M is the substrate concentration at which half-maximal velocity is achieved, and n is the Hill coefficient.

RESULTS

CO₂ signaling of ROS-GC1 activity

It has been shown previously that bicarbonate supplied in a solution modulates ROS-GC1 catalytic activity both of native and recombinant forms [33, 34]. To determine whether CO₂ gas in the presence of carbonic anhydrase can mimic the bicarbonate effect, these two proteins (ROS-GC1 and carbonic anhydrase) were simultaneously expressed in COS cells which were then exposed to 15% CO₂. In order to ensure that ROS-GC1 and carbonic anhydrase are indeed expressed in the same cell, their cDNAs were cloned into a vector (pBud CE 4.1) that is designed for independent expression in mammalian cells of two genes from a single plasmid. Indeed, every cell expressing ROS-GC1 expressed also carbonic anhydrase type II (CAII) (Fig. 2A). The transfected cells were incubated without, or with, CO₂ and the amount of cyclic GMP formed was assessed as a measure of ROS-GC1's catalytic activity. In the absence of carbon dioxide ROS-GC1 catalyzed the synthesis of $\sim 200 \pm 30$ pmoles of cyclic GMP (mg prot)⁻¹ regardless whether CAII was expressed or not (Fig. 2B: panel “-CO₂”). In the presence of CO₂ there was 210 ± 35 pmoles of cyclic GMP (mg prot)⁻¹ formed without CAII but 580 ± 90 , with CAII (Fig. 2B: panel “+CO₂”). These results demonstrate that (1) carbonic anhydrase without CO₂ or CO₂ without carbonic anhydrase is without any effect on ROS-GC1 activity; (2) it is only when CO₂ is transformed into bicarbonate by the action of CAII that it stimulates ROS-GC1.

The carbonic anhydrase is widely present in living organisms starting from bacteria and algae finishing on the animal kingdom including humans but it is only, in at least some cone photoreceptors (mouse, human), that the expression of ROS-GC1 is accompanied by expression of CAII [35] and only one GCAP, GCAP1 [36]. Thus, it was tempting to simulate cone-native cellular conditions in heterologous system. Accordingly, wt-ROS-GC1, CAII and GCAP1 were co-expressed in COS cells and the cells were incubated in the presence of gaseous CO₂. Cyclic GMP formed was assayed as the measure of guanylate cyclase activity. The results are presented in figure 2C. The amount of cyclic GMP formed by wt-ROS-GC1 alone was approximately one-fourth of the cyclic GMP formed by ROS-GC1 in the presence

of GCAP1 [compare: 250 pmol cGMP (mg prot)⁻¹ vs. 950]. When co-expressed with GCAP1 and CAII, the wt-ROS-GC1 synthesized 7 times higher amount of cyclic GMP than in its basal state and 2-fold higher than when co-expressed with GCAP1 alone under the same conditions. Thus, CO₂ in the presence of CAII mirrors the previously observed effect of bicarbonate [33] which potentiates the GCAP1 modulation of ROS-GC1 activity. The same will happen in these cone photoreceptors which possess CAII activity, and ROS-GC1 and GCAP1 as the only constituents of the ROS-GC/GCAP signaling system.

R⁷⁸⁷C is primarily cone photoreceptors degenerative mutation of ROS-GC1

More than 100 naturally occurring mutations in ROS-GC1 gene have been linked with retinal dystrophies, mainly Leber congenital amaurosis (LCA1) and cone-rod degeneration (CORD6). Arginine at position 787, located within the third heptad of the signaling helix domain (SHD), is likely to be the most mutation-prone residue in ROS-GC1 as four single mutations and two complex mutations are known to affect it [37]. It therefore appears that the SHD of ROS-GC1 is the hot-spot for degenerative mutations. Here we delve into the issue of how R⁷⁸⁷C mutation relates to the function of the third heptad of the signaling helix and through it of the ROS-GC1 enzyme and whether carbon dioxide ameliorates or worsens the functioning of cones affected by the mutations.

R⁷⁸⁷C and H3 ROS-GC1 mutants localize to membranes and possess

enzymatic activity—The biochemical effect of the R⁷⁸⁷ mutation was explored using recombinant bovine ROS-GC1 and its two mutant forms, R⁷⁸⁷C and H3. In H3, amino acid residues 784–790 constituting the entire third heptad of the SHD, the location of the R⁷⁸⁷ residue, were deleted. The first objective was to verify that the introduced mutations did not impair proper membrane targeting of the proteins. The wt-ROSGC1 and its mutants were expressed in COS cells and analyzed by immunostaining with anti-ROS-GC1 antibody (Fig. 3A). In all three cases, immunoreactive bodies were found predominantly in the membranes of the endoplasmic reticulum and to a lower extent in the plasma membrane and importantly, the levels of expression of these three proteins were the same. This pattern of expression in a heterologous system agrees with previous observations [18, 38]. Thus, the mutants, similar to their parental ROS-GC1, remain membrane proteins.

To assess enzymatic functionality, the wt-ROS-GC1, the R⁷⁸⁷C and H3 mutants were expressed in COS cells and their guanylate cyclase activities were determined in the cells particulate fractions (Fig. 3B). The activity of the wt-ROS-GC1 was 100 ± 21 pmol cGMP min (mg protein)⁻¹ whereas the activities of the mutants were 39 ± 11 and 36 ± 6 for the R⁷⁸⁷C and H3, respectively. When the mutants were analyzed for the dependences of their activities on GTP concentration, they showed identical relations between the activity and the substrate concentration as well as between the substrate concentration and the reaction rate (Fig. 3C). The GTP concentration at which the activities of the wt-ROS-GC1 and of both mutants were equal one-half of the maximal activity (EC₅₀) was ~0.6 mM. The Hill coefficient values calculated from the Hill equation and determined by GraphPad Prism program were similar, averaging at ~2 for all of them. The exact values were as follows (from Hill's): wt-ROSGC1, 2.15 ± 0.2; R⁷⁸⁷C, 2.2 ± 0.1; H3, 1.95 ± 0.2; from Graph Pad Prism: wt-ROS-GC1, 1.972; R⁷⁸⁷C, 2.203; and H3, 2.087. These results demonstrate that

despite exhibiting lower catalytic activities the mutants did not differ from the wild type cyclase in their basic guanylate cyclase enzymatic characteristics. At a molecular level these results imply that the R⁷⁸⁷C mutation residing in the H3 region of SHD breaks the linkage with one (or more) of the substrate binding residues of the catalytic domain -- D⁸³⁴ and D⁸⁷⁸ (Mg²⁺ binding), N⁹⁵³ (ribose positioning), E⁸⁷⁴ and C⁹⁴⁶ (guanine recognition), and/or R⁹²⁵ and R⁹⁵⁷ (triphosphate positioning), thus affecting the basic catalysis operation [39].

Reduced GCAP1- modulated Ca²⁺ signaling of ROS-GC1 mutants—GCAP1 binds ROS-GC1 molecule at aa L⁵⁰³–I⁵²² region within the JMD [40]. To determine if the mutations within SHD interfere with GCAP1 binding, the interaction was monitored in heterologous system by immunofluorescence. GCAP1 tagged with green fluorescent protein (GFP) was expressed alone or together with wt-ROS-GC1 or its R⁷⁸⁷C mutant. When expressed alone, GCAP1-GFP was evenly scattered in all cellular compartments including the nucleus (Fig. 4A). This pattern of heterologous expression of GCAP1 was compatible with the earlier observations [18, 41]. When co-expressed with ROS-GC1 or its mutant the GCAP1-linked GFP fluorescence migrated to the membranes and followed the pattern of ROS-GC1 immunofluorescence (Fig. 4 B and D for ROS-GC1 and figure 4E and G for the R⁷⁸⁷C mutant). Similar results were obtained for the H3 mutant (not shown). These results demonstrate that qualitatively the interaction between GCAP1 and any of the mutants does not differ from that between GCAP1 and wt-ROS-GC1.

Membranes of COS cell expressing the mutants or the wt-ROS-GC1 were further analyzed for GCAP1-dependent activation. They were exposed to increasing concentrations of GCAP1. To ensure that the GCAP1 is Ca²⁺-free, the standard reaction mixture was supplemented with EGTA to the final concentration of 1 mM resulting in ~10 nM free Ca²⁺. Ca²⁺-free GCAP1 stimulated the activity of the wild type and the mutant cyclases in a dose dependent fashion (Fig. 5A). The half-maximal stimulation occurred at ~1 μM GCAP1 (denoted as EC₅₀) for the mutants and the wild type cyclase and the Hill's coefficient values were also very close to each other, being 1.93 ± 0.3, 2.10 ± 0.1, and 2.16 ± 0.1 for wt-ROS-GC1, R⁷⁸⁷C and H3 mutants, respectively.

The V_{max} achieved by wt-ROS-GC1 was 490 pmol cyclic GMP/min⁻¹ (mg protein)⁻¹, by the R⁷⁸⁷C mutant, 340 and by the H3 mutant it was 320. Thus the mutants, although being activated by GCAP1, did not reach the same maximal enzymatic velocity as the wt-ROS-GC1 did. Another approach to analyze the response of a protein to any ligand is to calculate its x-fold stimulation above the basal activity, triggered by the ligand. When the mutants were analyzed this way a different picture emerged. They were twice as responsive to GCAP1 as the wt-ROS-GC1 was (Fig.5B: compare 9–10 fold stimulation for the mutants and 4-fold stimulation for the wt-ROS-GC1). Thus the mutants while exhibiting overall lower activity, were hyper-responsive to GCAP1.

The dependency of ROS-GCs catalytic activity on [Ca²⁺]_i via GCAPs is the hallmark feature of the ROS-GC/GCAP signaling system in phototransduction. To test whether the mutants adhere to this trend, the GCAP1-dependent activities of each mutant and of the wt-ROS-GC1 were determined at different free Ca²⁺ concentrations ranging from 10 nM to 10 μM. Typical profiles are shown in figure 6. The Ca²⁺ concentration at which the activity of each

cyclase is half-maximal was approximated graphically from these plots and is denoted as IC_{50} . For easier comparison of the IC_{50} values the activities were normalized; the cyclases activities at 10 nM Ca^{2+} were taken as maxima and at 10 μM as minima. Increasing concentrations of free Ca^{2+} resulted in dose-dependent lowering of GCAP1-stimulated activity of all the cyclases, the wt-ROS-GC1 and the mutants, but with evident differences. While the IC_{50} value for the wt-ROS-GC1 was ~370 nM, free Ca^{2+} , it was shifted to almost two-fold higher free Ca^{2+} concentrations, ~650 nM for the R⁷⁸⁷C and H3 mutants (Fig. 6). These results demonstrate that the R⁷⁸⁷C and H3 mutations in ROS-GC1 result in cyclases that are more resistant to Ca^{2+} -dependent inhibition.

Restorative effect of bicarbonate on mutant ROS-GC1 activity—Contrary to GCAPs, bicarbonate stimulates ROS-GC1 activity in a Ca^{2+} -independent mechanism. As a first step in examining the effect of the R⁷⁸⁷C and H3 mutations on bicarbonate signaling of ROSGC1 activity, the mutants were tested whether they respond to bicarbonate. Membranes of COS cells expressing wt-ROS-GC1 or the mutants were exposed to 50 mM bicarbonate and their activities were determined. They were 340, 134 and 148 pmol cyclic GMP min⁻¹ (mg prot)⁻¹ for the wt-ROS-GC1, R⁷⁸⁷C and H3 mutants, respectively. Thus, the mutants, although not attaining the same as wt-ROSGC1 V_{max} , were stimulated in their activities by bicarbonate ~4-fold above the basal values, identically as the wt-ROS-GC1 was. Hence the mutations did not affect the cyclases' pattern of response to bicarbonate. When the membranes were challenged with rising concentrations of bicarbonate, both mutants responded with a dose-dependent increase in activity (Fig. 7). At ~80 mM bicarbonate both mutants achieved their V_{max} 's that were 5-fold above the basal velocities; however, as the consequence of the lowered basal activity, the absolute values of the maximal activities were below the level reached by wt-ROS-GC1. The Hill coefficients were 2.46 for the R⁷⁸⁷C mutant and 2.30 for the H3, the values close to the 2.6 value observed for the wt-ROS-GC1 in these experiments as well as in previously reported [33]. These results reveal that the R⁷⁸⁷C and H3 mutations are neutral with respect to bicarbonate signaling of ROS-GC1 activity; they neither enhance nor hinder the signaling.

GCAP1 and bicarbonate together enhance each other's effect on ROS-GC1 activity and operate synergistically [28, 33]. It was, therefore, imperative to test whether the same remained true for the R⁷⁸⁷C and H3 mutants. To test this, COS cells membranes expressing individually both mutants or wt-ROS-GC1 as control, were reconstituted with increasing concentrations of GCAP1 at 10 nM Ca^{2+} in the presence and absence of 50 mM bicarbonate and the amount of cyclic GMP was assayed as a measure of the guanylate cyclase activity (Fig. 8). As expected, 50 mM bicarbonate enhanced GCAP1 mediated stimulation of wt-ROS-GC1, lowered the EC_{50} value for GCAP1 to ~0.6 μM and raised V_{max} to ~1000 pmol cyclic GMP min⁻¹ (mg prot)⁻¹. It also increased the guanylate cyclase activity over the GCAP1 concentrations tested. Surprisingly, the mutants achieved almost the same maximal velocity as wt-ROSGC1 under the same experimental conditions, ~1000 pmol cyclic GMP min⁻¹ (mg prot)⁻¹. These results show that bicarbonate further boosts the hyper-responsiveness of the mutants R⁷⁸⁷C and H3 mutants to GCAP1.

CO₂ signaling of the R⁷⁸⁷C mutant

The results presented above show that the characteristic for CORD6 ROS-GC1 mutation, R⁷⁸⁷C and deletion of the signaling helix domain third heptad where this mutation is located, affect similarly ROS-GC1 activity and its Ca²⁺-dependent and -independent modulations. Having these pieces of information gathered, an attempt was made to repeat the experiment with CO₂ and wt-ROS-GC1 but using the R⁷⁸⁷C mutant instead. The mutant was expressed in COS cells with CAII and/or GCAP1 and the cells were exposed to 15% CO₂. Cyclic GMP formed was determined as a measure of the mutant's guanylate cyclase activity. The results are shown in figure 9. In its basal, unstimulated state the R⁷⁸⁷C mutant synthesized 117 pmol cyclic GMP (mg prot)⁻¹, an amount slightly lower than one-half of the amount of cyclic GMP synthesized by wt-ROS-GC1 [compare figure 2: 270 pmol cGMP (mg prot)⁻¹]. When co-expressed with GCAP1, the mutant formed approximately 6-times more cyclic GMP, 650 ± 100 pmol cGMP (mg prot)⁻¹, but it was still less than wt-ROS-GC1 did [compare figure 2: 960 ± 40 pmol cGMP (mg prot)⁻¹]. Finally, when co-expressed with GCAP1 and CAII the amount of cyclic GMP synthesized by the mutant rose up to 1475 ± 80 pmol cGMP (mg prot)⁻¹, an amount almost 13-times over its basal value and 2-times over its GCAP1 stimulated level. Thus CO₂ in the presence of CAII mirrors the effect of bicarbonate by potentiating the GCAP1 influence on ROS-GC1 activity. Although the amount of cyclic GMP formed by the mutant did not reach the amount formed by the native cyclase [1660 ± 160 pmol cGMP (mg prot)⁻¹] as it was in the reconstitution experiment with COS cells membranes, exogenous GCAP1 and solution of sodium bicarbonate (Fig. 7) still, the numbers are close enough to allow a conclusion that CO₂ through bicarbonate formed in the presence of CAII will increase the synthesis of cyclic GMP in the CORD6 affected cone photoreceptors to the level nearing this in normal ones.

DISCUSSION

There are several highly innovative aspects of the present investigation.

(1) Gaseous CO₂ signal transduction pathway

Besides the mouse olfactory neurons [42], the gaseous CO₂-modulated membrane guanylate cyclase transduction system has not been reported in any vertebrate sensory neurons. Even more, the mouse gene, ONE-GC (alternatively named GC-D) [43, 44] is a pseudogene in humans [45, 46]. It has thus, been concluded that CO₂ is odorless for humans [47] and the CO₂-modulated membrane guanylate cyclase transduction system is in the exclusive domain of the mouse (rodent) olfactory system.

The present report demonstrates that photoreceptor ROS-GC1 is modulated by CO₂. This conclusion is arrived at through experimentation using a recombinant wt-ROS-GC1 reconstituted in a heterologous system mimicking the cellular conditions of the native cones. Co-expressed with CAII in COS cells it is stimulated more than 2.5-fold by gaseous CO₂ (Fig. 2B). In the absence of CO₂, CAII is ineffective in stimulating ROS-GC1; like-wise, CO₂ in the absence of CAII is without any effect. Because functionally CAII catalyzes generation of bicarbonate from CO₂ it is inferred that CO₂ signals via bicarbonate stimulation of ROS-GC1 catalytic activity.

(2) Bicarbonate mediates CO₂-modulated pathway

Bicarbonate is directly sensed by the photoreceptor ROS-GC1 [28, 33]. It stimulates its catalytic activity regardless of $[Ca^{2+}]_i$, the stimulation does not require the GCAPs domains and, importantly, the two pathways, bicarbonate and GCAPs, originate at the opposite ends of ROS-GC1 [33] and at the physiological level CO₂ is functional in the GCAP1- and GCAP2-double knockout mice [28]. Thus, these pathways at the biochemical and physiological levels are divergent. Together with the evidence that CO₂ stimulates the catalytic activity of ROS-GC1, and physiologically through bicarbonate enhances the circulating current, decreases sensitivity to the flashes and accelerates flash response kinetics in the salamander red-sensitive cones [33, 34], it is concluded that CO₂ modulates cone phototransduction via its mediator bicarbonate. This conclusion is in accordance with the evidence that human red- and green-sensitive cones express the CAII in their outer segments [35].

Proposed mechanism (Fig. 10).—Incorporating these facts, we propose a CO₂-modulated signal transduction mechanism for the cone phototransduction pathway. Generated in the cone outer segment or diffused in the plasma membrane, carbon dioxide is sensed by CAII which catalyzes its reaction with water molecule leading to formation of H⁺ and HCO₃[−]. The produced bicarbonate directly targets the catalytic domain (CCD) of ROS-GC1 and activates it to increased synthesis of cyclic GMP which is a two-step process. In the first step the circlet-like structure formed by two antiparallel oriented monomers of CCD [39] partially closes to attach the substrate Mg²⁺–GTP, a requirement for the basic catalysis to occur. In the subsequent step, upon receiving the ligand (bicarbonate)-generated signal the circlet tightens, and creates cyclic GMP at the cyclase's full capacity (exemplified by Fig. 3 in ref. 39)].

(3) GCAP1 signaling pathway

Presently, this pathway is misdirected by some groups. It requires critical evaluation to understand its true biochemistry and physiology.

More than two-decades of structural analyses of the recombinant ROS-GC1 [4] (reviewed in ref. 6, figure 4) have revealed the boundaries and functions of its modular blocks. Two Ca²⁺-modulated GCAPs, 1 and 2, from their independent targeted domains located, respectively, in juxtamembrane domain (JMD) and C-terminal extension (CTE) of ROS-GC1, generate two non-overlapping signaling pathways [5, 28, 39, 40, 48–50]. Both signals are translated by the catalytic domain (CCD) into the enhanced production of cyclic GMP, which, in turn, serves as the second messenger of phototransduction. Before entering CCD, the GCAP1 signal passes sequentially through the ⁶⁵⁷WTAPELL⁶⁶³ and of L⁷⁷⁰-P⁸⁰⁷ SHD micro-domains (Fig. 1). There is a critical functional interplay between these two modular domains. Remarkably, the WTAPELL and SHD modules control almost 90% of the GCAP1's regulatory activity [18, 31]. The ⁶⁵⁷W residue in the WTAPELL is the primary factor, controlling about 73% of the module's total Ca²⁺-modulated GCAP1 signaling activity. This conclusion has a critical bearing on understanding the pathology of the LCA1 and CORD6 diseases. These diseases are consequences of the ROS-GC1 specific mutations,

all residing in the Ca^{2+} -modulated GCAP1-signaling pathway, LCA1: F⁵¹⁴S [19, 51], recently described A⁶⁵⁹V [52] and CORD6: R⁷⁸⁷C [22].

(4) Signaling helix domain (SHD).

The function-based SHD term was coined for proteins containing a five-heptad conserved structural motif between two signaling domains [53]. ROS-GC1's five-heptad-SHD module, amino acid residues L⁷⁷⁰-P⁸⁰⁷ [31] meets this criterion and has 50% structural conservation, including all the most preserved residues, with the signaling helix sequence logo [53]. This domain is one of the central points in the present investigation.

In ROS-GC1, SHD module is wedged between the kinase homology domains (KHD) and the CCD (Fig. 1). Prior to the use of SHD terminology [53], a corresponding, ~ 50-amino acid linker region of ANFRGC (atrial natriuretic factor receptor guanylate cyclase) was named dimerization domain (DD) [54, 55]. It was argued that DD functioned to assemble two monomeric subunits of ANF-RGC into a homo-dimer that represents the functional cyclase. The prediction was that the DD structure represents an amphipathic helix in the ANF-RGC monomer and a coiled-coil structure in the homodimer. This ANFRGC-based concept was extended to ROS-GC1 [56, 57] for which it was proposed that the coiled-coil-DD structural unit was the critical component for ROS-GC's catalytic activity. This idea is the driving force for some groups to define and link the GCAPs-modulated Ca^{2+} signal transduction mechanisms with phototransduction, and, in turn, to explain the CORD's retinal dystrophies [56–59].

Multiple studies, including this one, do not support this idea. The so called “DD” linker region is neither a dimer forming element nor does it assume a typical coiled-coil structure [38, 39, 60–63]. The CCD module, ranging in existence from the bacterium Cya2 [64] and eukaryotic green algae *Cyg12* [65] guanylate cyclases to the mammalian ROS-GC1 [39, 63], is intrinsically dimeric and catalytically active; it does not require “DD” linker region for its basal catalytic activity.

(5) The R⁷⁸⁷ residue of heptad 3 is an amplifier of the SHD's transmission activity

Basal ROS-GC1 catalytic activity.—The prediction that SHD module, indeed, constitutes a transduction switch has been supported for its role in suppressing the catalytic activities of the guanylate cyclases - STa-RGC [62] and ROS-GC1 [31, 38]. It has thus been concluded that function of the effector ligand is to overcome the suppression and enhance the catalytic activity of CCD. Yet, puzzlingly, the R⁷⁸⁷C mutation linked with CORD6 alone causes a significant decrease (~60%) in the catalytic activity (Fig. 3B), a phenomenon also observed earlier [25, 26, 56], yet not commented upon.

The present investigation carried out with the H3 ROS-GC1 mutant further supports the above conclusion by demonstrating that the mutant also lacks most of its catalytic activity (Fig. 3C).

Ca^{2+} -dynamic changes.—Under physiological conditions in the photoreceptor outer segments ROS-GC1 activity is determined by dynamic changes in cytoplasmic Ca^{2+} concentrations through GCAP1. The activity is high at low $[\text{Ca}^{2+}]_i$ and decreases as the Ca^{2+}

concentration increases resulting in light and dark specific cyclic GMP/Ca²⁺ homeostasis. Do the R⁷⁸⁷C mutation and/or deletion of the third heptad affect this paradigm? The answer is “yes”. At low nanomolar Ca²⁺ the activities of both mutants are controlled by GCAP1 in a dose-dependent fashion (Fig. 5) however, their catalytic efficiencies are lower than that of the wt-ROS-GC1 resulting in reduced cyclic GMP synthesis. With increasing Ca²⁺ concentrations the GCAP1-dependent activity of the R⁷⁸⁷C and H3 mutants decrease but the half-maximal inhibition occurs at Ca²⁺ concentration almost two times higher than that for wt-ROS-GC1 (Fig. 6: compare ~650 nM free Ca²⁺ for the mutants vs. 370 nM, for wt-ROS-GC1). These values indicate that ROS-GC1 stays activated even in the photoreceptor dark state. Thus, it is concluded that the R⁷⁸⁷ residue residing in the third heptad of SHD controls proper amplification of the ligand-dependent activity of ROS-GC1 and therefore upholds the cyclic GMP/Ca²⁺ homeostasis in photoreceptor outer segments.

Ca²⁺-independent vs. Ca²⁺-dependent signaling.—Carbon dioxide signaling via bicarbonate is Ca²⁺ independent but most importantly the signal does not pass through any intermediate module but targets directly the catalytic domain of ROS-GC1. Do therefore, R787 and H3 have any role in signaling by CO₂/bicarbonate? In reconstitution experiments bicarbonate stimulates the catalytic activities of both mutants and of wt-ROS-GC1 ~4-fold above the basal level (Fig. 7). Hence the mutations do not affect the catalytic activities of the guanylate cyclase in response to bicarbonate. Keeping in mind that the R⁷⁸⁷C and H3 mutations lower basal ROS-GC1 activity (Fig. 3), and that GCAP1 stimulation is not sufficient to overcome this lowering (Fig. 5A), the intriguing finding is that when bicarbonate is present together with GCAP1, it brings the activities of both mutants to the level of wt-ROS-GC1 (Fig. 8). Similar “rescue” effect occurs when R⁷⁸⁷C mutant expressed in COS cells together with CAII and GCAP1 is exposed to CO₂ (Fig. 9); the specific activity achieved by the mutant is comparable to that achieved by the wt-ROS-GC1 (compare figures 2 and 9). Thus, bicarbonate being a mediator of CO₂ enhances the amplifying ability of R⁷⁸⁷ and H3 in ligand (GCAP1)-signaling of ROS-GC1 activity.

(6) Heptad 3 and the pathogenesis of CORD6

Although the primary consequence of R⁷⁸⁷C and H3 mutations is low basal catalytic activity in ROS-GC1 and its failure to be fully activated by GCAP1 at low [Ca²⁺], the CORD6 caused by the R⁷⁸⁷C mutation seems unlikely to be instigated exclusively by low cyclic GMP synthesis. Instead, the additional reason of the disease arises from the secondary cause. It is the altered Ca²⁺ sensitivity of the ROS-GC1/GCAP1 complex that maintains ROS-GC1 in an activated state even in darkness causing non-physiological elevation of intracellular cyclic GMP. Raised levels of cyclic GMP are toxic in part because they allow lethal levels of Ca²⁺ to enter photoreceptor cells [66]. Another consequence of elevated intracellular levels of cyclic GMP is an exuberant activity of cyclic GMP-dependent protein kinase (PKG), what is also detrimental [67].

(7) Global function of heptad 3 and R⁷⁸⁷ analogous conserved residue

With the presented facts that H3 of SHD and its node residue R⁷⁸⁷ are critical in tuning total modular activity in the ROS-GC, and that this residue is the central part of the “ERT” conserved signature motif in the membrane guanylate cyclase family [53], the question was

raised does R⁷⁸⁷ have a global function? Would its mutation in other guanylate cyclases cause a loss of function and lead to a serious abnormality?

The answer is yes, based on the analysis of two different subtypes of the guanylate cyclase family and both belonging to the hormone receptor subfamily: atrial natriuretic factor (ANF) receptor guanylate cyclase (ANF-RGC or NPR1) and C-type natriuretic peptide (CNP) receptor guanylate cyclase (CNPRGC or NPR2).

ANF-RGC.—By sensing ANF hormone pulsed through heart beats [68] and [Ca²⁺] levels modulated by neurocalcin δ , ANF-RGC [69, 70] is the pivotal molecule that controls blood pressure and combats hypertension (reviewed in: ref. 6). Thus, we reasoned that ANF-RGC's H3 in SHD module and specifically its R⁸⁰² residue (corresponding to R⁷⁸⁷ in ROS-GC) should also be critical in controlling catalytic activity of the guanylate cyclase. And, indeed, it is. Deletion of H3, or mutation R⁸⁰²C (corresponding to R⁷⁸⁷C in ROS-GC1), disables most of ANF-RGC's basic catalytic activity with the concomitant blunted response to ANF/ATP in the ligand-dependent activation. (Supplemental Information: Fig. S1).

CNP-RGC.—To broaden this theme, we surveyed the literature on CNP-RGC. Here, the corresponding to ROS-GC1 R⁷⁸⁷ residue is R⁸¹⁹. The R⁸¹⁹C mutation in human causes acromesomelic dysplasia, characterized by severe dwarfism [71]. Because dwarfism is also observed in the CNP-RGC- or CNP-gene knockout mice with disability to produce cyclic GMP [72, 73] we conclude that analogous to ROSGC1 H3 and its R⁷⁸⁷ conserved residue, the R⁸¹⁹ in CNP-RGC is also critical for controlling physiology and biochemistry of other members of the guanylate cyclase family. Hence the residue has a critical guanylate cyclase-specific global functional role in which it disables the catalytic activity of the guanylate cyclase.

(8) Additional possible consequences of R⁷⁸⁷C mutation

Expression of ROS-GC1 is not restricted to the retina. Outside the retina ROS-GC1 and GCAP1 signal transduction system is expressed in mitral cells of the olfactory bulb [74] and in a subpopulation of pinealocytes [75]. Any genetic mutation in ROS-GC1 will not be limited to retinal expression of ROS-GC1 but will occur in every ROS-GC1 molecule throughout the body. It is therefore an imperative, that from a clinical point of view, all individuals afflicted with CORD6 in addition to monitoring their vision are also tested for abnormalities in their sense of smell as well as in sleep and wakefulness cycles. When traveling, these individuals should be advised on the possibility of enhanced effects of jet lag, especially in eastward travel, because of disturbance in melatonin secretion by pineal gland.

The presented results, however, offer the potential that the severity of these abnormalities may be, at least to some extent, ameliorated by accessibility of CO₂/carbonic anhydrase/bicarbonate. As a powerful modulator of ROS-GC1, bicarbonate is likely to influence the penetrance and severity of diseases caused by dysregulation of cyclic GMP synthesis. If CO₂/bicarbonate were to reach sufficient levels it might support adequate cyclic GMP synthesis by ROS-GC1 thus repairing abnormalities resulting from reduced cellular cyclic GMP levels. On the other hand, inhibition of bicarbonate production by carbonic anhydrase

inhibitors may be therapeutic in attenuating disease processes caused by elevated cyclic GMP levels.

Conclusions

In summary, this study is the beginning of biochemical characterization of a novel gaseous CO₂-modulated ROS-GC signal transduction pathway in the photoreceptor sensory neurons. Via its outstanding mode of regulation, the pathway applies outside impact on the conventional cone-linked systems. To date this pathway has been denied to exist in humans and other vertebrates; only exception is in the rodents, yet the rodent membrane guanylate cyclase is represented by a pseudogene in humans. The human and the conserved ROS-GC1 gene in vertebrates exists in other sensory neurons linked with olfaction, pinealocytes and gustatory epithelium; and in all the neurons, the encoded guanylate cyclase is Ca²⁺-modulated. Do these sensory neurons, like photoreceptors, embody gaseous CO₂-modulated signaling pathway and, thus, able to sense CO₂? This study offers this possibility and opens these fields of investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Modular composition of the bovine ROS-GC1 dimer.

A 56 amino acid leader sequence (LS) precedes the extracellular domain (ExtD) in the nascent, immature protein. All signaling events occur in the intracellular domain (ICD), which is separated from ExtD by a transmembrane domain (TMD). ICD is composed of the following domains: juxtamembrane (JMD), kinase homology (KHD), signaling helix (SHD), catalytic core (CCD) and C-terminal extension (CTE). GCAP1 binds to the JMD. The SHD is expanded below the dimer to show the sequences of five heptad segments. Heptad 3 is shadowed and the R⁷⁸⁷ residue in the center of this heptad is highlighted in red. Highlighted Q and K residues in heptad 4 are also prone to mutations leading to CORD [29].

Figure 2. Carbon dioxide in the presence of carbonic anhydrase type II (CAII) mimics the effect of bicarbonate on ROS-GC1 activity.

COS cells were induced to express wt-ROS-GC1, co-express wt-ROS-GC1 and CAII, or co-express wt-ROS-GC1 (or ROS-GC1-CAII) with GCAP1. The ROSGC1-CAII construct in pBud vector is described in Materials and Methods section. (A) COS cells transfected with ROS-GC1-CAII construct. Every cell expressing ROS-GC1 expresses also CAII as verified by immunocytochemistry using anti-ROS-GC1 and anti-CAII antibodies. The cells were viewed using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system. (B) COS cells expressing ROS-GC1 or ROS-GC1 and CAII were incubated in the absence or presence of 12% CO₂ at 37 °C. (C) COS cells expressing ROS-GC1, ROS-GC1 and CAII or co-expressing GCAP1 with ROS-GC1 or ROS-GC1 and CAII were incubated in the presence of 12% CO₂ at 37 °C. After the incubation the cells were scraped from the culture dishes, homogenized by passing through 30G needle, centrifuged and cyclic GMP was determined in the supernatant by RIA. The experiment was done in triplicate.

Figure 3. ROS-GC1 R⁷⁸⁷C and ΔH3 mutants localize to membranes and are enzymatically functional.

COS cells were transfected with cDNA of wt-ROS-GC1 or its R⁷⁸⁷C and ΔH3 mutants. **(A)** Seventy-two hr after transfection, the cells were fixed and incubated with anti-ROS-GC1 antibody followed by incubation with secondary antibodies conjugated with DyLight 649 (excitation at 643 nm). The cells were viewed using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system. In all three cases the immunoreactive bodies were found predominantly in the membranes of the endoplasmic reticulum and to a lower extent in the plasma membrane. The immunostaining also shows that the levels of expression of these three proteins were the same. **(B)** Lowered basal guanylate cyclase activity in membranes of COS cells expressing ROS-GC1 or its R⁷⁸⁷C and ΔH3 mutants. **(C)** R⁷⁸⁷C and ΔH3 mutants retain their dependence on [GTP]. In both (B) and (C) membranes of COS cells transfected with wt-ROS-GC1 were analyzed in parallel. The experiments were done as described in Materials and Methods section, performed in triplicate and repeated three times with different COS cell membrane preparations. The results shown are mean ± SD. In figure (C) error bars are within the size of the symbols.

Figure 4. GCAP1 binds to ROS-GC1 and its R⁷⁸⁷C mutant.

COS cells were transfected with GCAP1-GFP cDNA or were co-transfected with cDNA of GCAP1-GFP and ROS-GC1 or its R⁷⁸⁷C mutant. 72 hr after transfection the cells were fixed and incubated with anti-ROS-GC1 antibody followed by secondary antibody as described in Materials and Methods. Expression of GFP-GCAP1 was traced by green fluorescence. The cells were viewed using an inverted Olympus IX81 microscope/FV1000 Spectral laser confocal system. (A) GCAP1-GFP expressed alone; (B) GCAP1-GFP co-expressed with ROS-GC1; (C) ROS-GC1 co-expressed with GCAP1-GFP; (D) merge of (B) and (C); (E) GCAP1-GFP co-expressed with R⁷⁸⁷C mutant; (F) R⁷⁸⁷C mutant co-expressed with GCAPGFP; (G) merge of (E) and (F).

Figure 5. GCAP1 dependent activation of ROS-GC1 and R⁷⁸⁷C or H3 mutant. COS cells were induced to express individually wt-ROS-GC1 and its mutants R⁷⁸⁷C or H3. (A) Guanylate cyclase activity was measured in the particulate fractions of these cells reconstituted with increasing concentrations of Ca²⁺-free GCAP1 (1mM EGTA in the reaction mixture lowered [Ca²⁺] to ~10 nM). (B) Plotting the results in (A) on a relative scale showed that the mutants were 2-fold more responsive to GCAP1 than the wt-ROS-GC1. The experiments were done in triplicate and repeated three times with different COS cell membranes preparations. The results shown are mean $\pm$ SD of these experiments. The error bars are within the size of the symbols.

Figure 6. Shift in Ca^{2+} -dependent modulation of ROS-GC1 mutants by GCAP1 to higher $[\text{Ca}^{2+}]$. Membranes of COS cells expressing individually wt-ROS-GC1 or its mutants R⁷⁸⁷C and

H3 were assayed for guanylate cyclase activity as a function of free Ca^{2+} concentration at a constant, 8 μM , concentration of GCAP1. For easier comparison of the IC_{50} values (free Ca^{2+} concentration at which the activity is half-maximal) the activities were normalized with activities at 10 nM Ca^{2+} were taken as maxima and at 10 μM , as minima. The maximal activities were as follows: 450, 126, and 130 $\text{pmol cGMP min}^{-1}(\text{mg prot})^{-1}$ for ROS-GC1, R⁷⁸⁷C and H3, respectively; the minimal activities were (in the same order) 100, 25, and 22 $\text{pmol cGMP min}^{-1}(\text{mg prot})^{-1}$. The values presented are representative of two experiments done in triplicate.

Figure 7. ROS-GC1 R⁷⁸⁷C and Δ H3 mutants retain responsiveness to bicarbonate. Membranes of COS cells expressing individually wt-ROS-GC1 or its mutants R⁷⁸⁷C and Δ H3 were assayed for guanylate cyclase activity as a function of bicarbonate concentration. The experiment was done in triplicate and repeated three times. Representative plots are shown.

Figure 8. Synergistic stimulation of ROS-GC1 and its mutants' activities by bicarbonate and GCAP1 at low free Ca^{2+} concentration.

Membranes of COS cells expressing individually wt-ROS GC1 or its mutants R⁷⁸⁷C and H3 were assayed for guanylate cyclase activity as a function of GCAP1 concentration at 1 mM EGTA (10 nM Ca^{2+}) without or with the presence of 50 mM bicarbonate. The GCAP1 EC₅₀ values were $\sim 1\mu\text{M}$ in the absence of bicarbonate for all three cyclases and $\sim 0.6\mu\text{M}$ in the presence of bicarbonate. The experiment was done in triplicate three times with wt-ROS-GC1 and the R⁷⁸⁷C mutant and two times with the H3 mutant. The graphs presented are from representative experiment.

Figure 9. Carbon dioxide in the presence of carbonic anhydrase type II restores the activity of R⁷⁸⁷C mutant.

COS cells were induced to (1) express R⁷⁸⁷C mutant; (2) co-express R⁷⁸⁷C mutant with GCAP1; (3) co-express R⁷⁸⁷C mutant with GCAP1 and CAII. The cells were incubated at 37 °C in the presence of 12% CO₂. After the incubation they were scraped from the culture dishes, homogenized by passing through 30G needle, centrifuged and cyclic GMP was determined by RIA in the supernatant. The experiment was done in triplicate.

Figure 10. CO₂ modulation of illumination: Model.

CO₂ passing through the plasma membrane (or metabolic CO₂ generated inside the outer segment) is sensed by carbonic anhydrase type II (CAII) which catalyzes its reaction with water producing HCO₃⁻ and H⁺. Bicarbonate targets the ROS-GC1 catalytic domain stimulating it to increased synthesis of cyclic GMP. Cyclic GMP then opens cyclic GMP gated channels (CNG channel) allowing influx of Ca²⁺. To prevent toxic increase of [Ca²⁺]_i, some cyclic GMP is hydrolyzed to 5'-GMP.