Calcium-Modulated Guanylate Cyclase Transduction Machinery in the Photoreceptor−Bipolar Synaptic Region[†]

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ABSTRACT: Rod outer segment membrane guanylate cyclase (ROS-GC) transduction system is a central component of the Ca^{2+}-sensitive phototransduction machinery. The system is composed of two parts: Ca^{2+} sensor guanylate cyclase activating protein (GCAP) and ROS-GC. GCAP senses Ca^{2+} impulses and inhibits the cyclase. This operational feature of the cyclase is considered to be unique and exclusive in the phototransduction machinery. A combination of reconstitution, peptide competition, cross-linking, and immunocytochemical studies has been used in this study to show that the GCAP1/ROS-GC1 transduction system also exists in the photoreceptor synaptic (presynaptic) termini. Thus, the presence of this system and its linkage is not unique to the phototransduction machinery. A recent study has demonstrated that the photoreceptor−bipolar synaptic region also contains a Ca^{2+}-stimulated ROS-GC1 transduction system [Duda, T., et al. (2002) *EMBO J. 21*, 2547−2556]. In this case, S100β senses Ca^{2+} and stimulates the cyclase. The inhibitory and stimulatory Ca^{2+}-modulated ROS-GC1 sites are distinct. These findings allow the formation of a new topographic model of ROS-GC1 transduction. In this model, the catalytic module of ROS-GC1 at its opposite ends is flanked by GCAP1 and S100β modules. GCAP1 senses the Ca^{2+} impulse and inhibits the catalytic module; S100β senses the impulse and stimulates the catalytic module. Thus, ROS-GC1 acts as a bimodal Ca^{2+} signal transduction switch in the photoreceptor bipolar synapse.

An important conceptual advancement is emerging in the membrane guanylate cyclase field. It predicts that the rod outer segment membrane guanylate cyclase (ROS-GC),[1] a subfamily of membrane guanylate cyclases, is the universal transduction machinery for the Ca^{2+} impulses arising in the sensory neurons. According to the cellular environment, Ca^{2+} either inhibits the machinery or stimulates it (reviewed in ref *1*). The former, and the first operational feature of the machinery, was established with studies of the photoreceptor cells in which the initial individual components of the transduction machinery were discovered (reviewed in refs *2* and *3*). ROS-GC, native to PR-OS, the site of phototransduction, was purified (*4*−*7*), sequenced in part (*7*), and then cloned, based on the sequences of its four peptides (*8*). Its functional identity to the native ROS-GC was established by its Ca^{2+}-dependent reconstitution; it mimicked the native

conditions of phototransduction (*9*), in which Ca^{2+} with a $K_{1/2}$ of ∼100 nM inhibits the guanylate cyclase (*10*, *11*). In these studies, the recombinant form of GCAP1 was used as a Ca^{2+} sensor (*9*). It was, thereby, established that the GCAP1/ROS-GC1 system is a Ca^{2+}-sensitive component of the phototransduction machinery. The studies supported an earlier prediction that a membrane-bound guanylate cyclase operates by Ca^{2+} through a Ca^{2+}-binding factor, whose identity at the time was unknown (*12*).

Phototransduction is a biochemical process, where the light signal generates an electrical signal (reviewed in refs *2* and *3*). Two interlocked critical components of the process are Ca^{2+} and cyclic GMP (*12*−*15*). The photon captured by its receptor rhodopsin creates a decline in the level of cyclic GMP, which results in closure of the CNG channel, and hyperpolarization of the photoreceptor plasma membrane. Closure of CNG-gated channels prevents Ca^{2+} from entering the cell, but a continuous operation of a $Na^+/Ca^{2+},K^+$-exchanger leads to a decrease in the intracellular Ca^{2+} concentration from ∼500 nM in the dark-adapted photoreceptor cell to <100 nM. This in turn causes the activation of ROS-GC. An increased level of synthesis of cyclic GMP, which is concomitant with inactivation of the phototransduction cascade, results in opening of the channel and, thereby, restoration of the dark current of the photoreceptors.

According to the current knowledge of the phototransduction field, ROS-GC exists in two forms: the original form

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[1] Abbreviations: CNG, cyclic nucleotide-gated; ELISA, enzyme-linked immunosorbent assay; GCAP, guanylate cyclase activating protein; GMP, guanosine monophosphate; OPL, outer plexiform layer; PR-OS, photoreceptor outer segments; ROS-GC, rod outer segment membrane guanylate cyclase.

(*8*), renamed ROS-GC1 [retGC1, GC-E (*16*, *17*)], and ROS-GC2 (*18*) [retGC2, GC-F (*17*, *19*)]. To date, the majority of the biochemical work has been focused on ROS-GC1. Functional GCAP exists in three forms: GCAP1−GCAP3 (*11*, *20*−*22*). Reconstitution studies with recombinant ROS-GC1 indicate that it can sense changes in free Ca^{2+} concentrations through either of the two GCAPs, 1 or 2 (*9*, *23*, *24*). Each GCAP binds with a specific module in ROS-GC1, residing in the intracellular region of the cyclase (*9*, *23*, *25*−*27*). In the current phototransduction model, GCAPs and ROS-GC1 form a regulatory unit in native PR-OS (reviewed in ref *2*). GCAPs sense light-dependent Ca^{2+} fluctuations and, in turn, cause proportionate changes in ROS-GC1 activity. In this way, three pivotal components of phototransduction (Ca^{2+}, GCAPs, and ROS-GC1) are physiologically connected. In the dark phase, the free Ca^{2+} concentration is 500 nM, Ca^{2+} is bound to GCAP, and the cyclase is in its basal state. Increasing levels of photoillumination intensity cause graded decreases in the concentration of Ca^{2+}. These cause concomitant increases in the cyclase activity, which, in turn, alter the cyclic GMP concentration in the photoreceptor cell.

To date, the GCAP-modulated Ca^{2+}-signaling ROS-GC1 transduction system has been established in PR-OS as a sole constituent of the phototransduction machinery. This study expands the boundaries of this system. It unequivocally establishes that the GCAP1/ROS-GC1 transduction system also exists in the photoreceptor synaptic terminus where, like in PR-OS, the system is inhibited by Ca^{2+}. Earlier studies have detected the presence of a ROS-GC, without distinguishing between ROS-GC1 and ROS-GC2, in retinal cells other than photoreceptors, including the synaptic layers (*28*, *29*). Immunolocalization studies have also suggested that GCAP1 and GCAP2 are present in synaptic regions (*30*, *31*). A more recent study has shown that the photoreceptor−bipolar synaptic region also contains the Ca^{2+}-stimulated ROS-GC1 transduction system (*34*). The system achieves the stimulatory feature via its Ca^{2+} sensor S100β, whose target sites are specifically mapped to two short regions in the C-terminal domain of ROS-GC1 (*34*). These sites are distinct from the GCAP1-modulated sites (*25*, *34*). On the basis of the findings that ROS-GC1 attains reciprocal, bimodal regulation through its defined Ca^{2+} sensor component and the component influences ROS-GC1 through its specified domain, a new topographic model of the ROS-GC1 transduction system is proposed. The model contends that the catalytic module of ROS-GC1 is flanked by GCAP1 and S100β regulatory modules. GCAP1 senses the Ca^{2+} impulse and inhibits the catalytic module; S100β senses the impulse and stimulates the catalytic module. Thus, ROS-GC1 operates as a bimodal Ca^{2+} signal transduction switch, both stimulated and inhibited by Ca^{2+} sparks in the photoreceptor−bipolar synapse region. Together with similar earlier findings in the pinealocytes (*35*) and in the olfactory system (*36*, *37*), this study suggests the universality and versatility of the Ca^{2+}-dependent ROS-GC transduction machinery in sensory neurons. Furthermore, the findings indicate that the spatial environment and the microdomain structure of the machinery determine whether Ca^{2+} waves accelerate or retard it in the formation of cyclic GMP.

EXPERIMENTAL PROCEDURES

Antibodies. Characterization of highly specific antibodies raised against ROS-GC1 and GCAP1 has been described previously (*35*−*37*). After the specificity of the reaction had been established, the ROS-GC1 and GCAP1 antibodies were enriched by precipitating the immunoglobulin fraction using ammonium sulfate. The antibodies were finally purified on antigen-affinity columns. ELISA and Western blots were used to determine the titer of the enriched antibodies. A dilution of 1:5000 was generally used for colorimetric detection of the antigen on Western blots. A monoclonal antibody against S100β (clone SH-B1) was purchased from Sigma, and the SV-2 antibody was from DBHSS.

Synthesis of Oligopeptides. The peptides corresponding to ROS-GC1 regions L503−I522, scrambled 503−522, D507−R518, and G968−G985 were synthesized as described previously (*25*, *38*).

Expression Studies. COS-7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the wild type-recombinant (wt-r) ROS-GC1 expression construct by the calcium phosphate coprecipitation technique (*39*). Sixty hours after transfection, cells were washed twice with 50 mM Tris-HCl (pH 7.5) and 10 mM buffer, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed several times with the same buffer. The resulting pellet represented crude membranes.

Preparation of the Retinal P1 Fraction. The isolation of the P1 fraction from the bovine retina was carried out according to the originally published technique (*40*), and recently used in the analysis of the outer synaptic (P1) layer as described in ref *34*. The preparation was aliquoted and stored at −150 °C until it was used.

Guanylate Cyclase Activity Assay. Membrane fractions were assayed for guanylate cyclase activity as described previously (*41*). Briefly, membranes were preincubated on an ice bath with or without GCAP1 or S100β in the assay system containing 10 mM theophylline, 15 mM phosphocreatine, 20 μg of creatine kinase, and 50 mM Tris-HCl (pH 7.5) adjusted to the appropriate free Ca^{2+} concentrations with precalibrated Ca^{2+}/EGTA solutions (Molecular Probes). The total assay volume was 25 μL. The reaction was initiated by addition of the substrate solution containing 4 mM MgCl$_2$ and 1 mM GTP and maintained by incubation at 37 °C for 10 min. The reaction was terminated by addition of 225 μL of 50 mM sodium acetate buffer (pH 6.2) followed by heating in a boiling water bath for 3 min. The amount of cyclic GMP that was formed was determined by a radio-immunoassay (*42*).

Peptide Competition Experiments. These were carried out with both native (PR-OS or P1) and recombinant (expressed in COS cells) ROS-GC1 (*4*, *11*, *34*). Respective membranes were assayed for guanylate cyclase activity in the presence of 10 nM Ca^{2+} and 4 μM GCAP1 with incremental concentrations of peptides.

Western Blot. Western blotting was carried out according to the previously published protocols (*35*, *37*). Briefly, the protein samples were transferred onto nitrocellulose membranes after electrophoresis. The membranes were incubated in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 3% bovine serum albumin (BSA) for 1 h at

room temperature followed by incubation for 1 h in the same solution containing the primary antibody. After the membranes had been washed with TBS-T, incubation was continued for the same period of time in TBS-T containing 3% BSA and the secondary antibody. Then, visualization of the immunoreactive protein bands was carried out according to the manufacturer's (Vector labs) protocol.

Cross-Linking. Cross-linking was carried out with the imidoester dimethyl suberimidate (DMS·2HCl) on membranes isolated from the P1 fraction. This noncleavable cross-linker was chosen because it is water-soluble. One hundred micrograms of protein (with or without added recombinant GCAP1) was incubated at room temperature with shaking for 60 min. The reaction was carried out in 0.2 M triethanolamine (pH 8.0) in the presence of DMS concentrations ranging from 20 to 0.2 mM. It was terminated by the addition of Tris-HCl (pH 6.8) to a final concentration of 100 mM, followed by the addition of SDS−PAGE loading dye. Samples were analyzed by SDS−PAGE (12 or 7%), followed by transfer and Western blotting. Duplicates were probed independently with highly specific antibodies against ROS-GC1 or GCAP1. Western blotting was carried out as described previously (*35, 37*).

Immunohistochemistry. Cryosections of the retina were prepared and used according to the procedure described previously (*35*). Briefly, the retina was fixed for 1 h in 4% formaldehyde at 4 °C; it was cryoprotected in 25% sucrose overnight at 4 °C and cut in sections with a thickness of 16 μm (Leica cryostat). To block nonspecific protein binding, sections were incubated in 0.1 M phosphate buffer containing 10% normal goat serum, 5% sucrose, and 0.5% Triton X-100 for 60 min at room temperature. The sections were then incubated in the same solution containing primary antibodies overnight at 4 °C, washed, and incubated with secondary antibodies conjugated to a fluorescent dye and diluted in the blocking solution. Washes were carried out at room temperature (three times for 10 min each) with 0.1 M phosphate buffer containing 5% sucrose. For detection of ROS-GC1 in the synaptic regions, the detergent was omitted from the buffer. Specimens were mounted using Vectashield mounting medium. Images were acquired using a confocal microscope (Leica). Digital images were processed using commercially available software (ImagePro Plus, Phase3 Imaging Systems and Adobe Photoshop). Controls included immunostaining reactions carried out under identical conditions except that either primary antibody was omitted or was replaced with preimmune serum. Staining was insignificant in either case.

RESULTS

The P1 Membrane Fraction Contains a Membrane Guanylate Cyclase, which Exhibits a Biphasic Type of Ca²⁺ Modulation

The P1 fraction from the bovine retina, comprising the outer plexiform layer (OPL), was isolated as described in Experimental Procedures. Incubation of the P1 membranes with incremental Ca^{2+} concentrations showed that the fraction contained a guanylate cyclase transduction system, which was either stimulated or inhibited depending on the free Ca^{2+} concentration (Figure 1). A range of Ca^{2+} concentrations from 10 nM to ∼0.4 μM caused a dose-dependent decrease in the cyclase activity, while higher Ca^{2+}
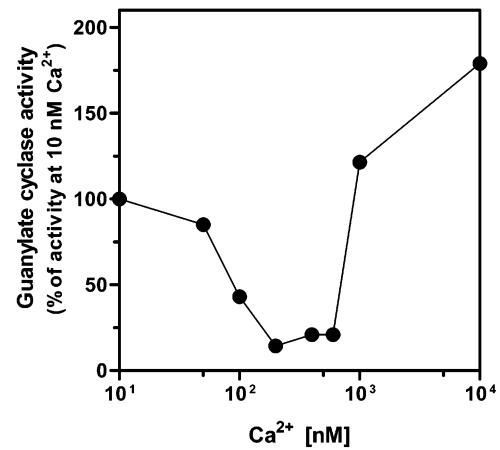

FIGURE 1: Ca^{2+} regulation of guanylate cyclase activity in membranes of the photoreceptor−bipolar cell synaptic layer (P1). The P1 fraction was prepared from the bovine retina as described in Experimental Procedures. These membranes were assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. Guanylate cyclase activity in the presence of 10 nM Ca^{2+} was 0.9 ± 0.1 nmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1}. The experiments were carried out in triplicate and repeated three times with separate membrane preparations. The results are from one representative experiment.

concentrations caused a stimulation. The inhibitory value (IC_{50}) for Ca^{2+} was 100 nM, and the stimulatory value (EC_{50}) was 0.8 μM. The identity of the Ca^{2+}-stimulated guanylate cyclase transduction system in the P1 membrane fraction has been reported recently (*34*). It consists of ROS-GC1 and its Ca^{2+} sensor component S100β (*34*). This raised a question about the identity of the Ca^{2+}-inhibited membrane guanylate cyclase transduction system. This issue has been addressed below.

The Ca²⁺-Inhibitory Transduction Component of the P1 Cyclase Is Represented by GCAP1/ROS-GC1

To determine if the Ca^{2+}-inhibited native P1 cyclase is similar to the phototransduction-linked ROS-GC, the P1 cyclase was subjected to multiple tests.

Test 1. The PR-OS and the P1 membrane fractions were isolated, and it was made certain that each does not cross contaminate the other (*34*). Side by side, these fractions were assayed for membrane guanylate cyclase activity in the presence of incremental Ca^{2+} concentrations, ranging from 10 nM to 0.7 μM. The results presented in Figure 2 show that the cyclase activity in both fractions is incrementally inhibited. The inhibition patterns of the two fractions are almost identical; the IC_{50} for Ca^{2+} in both cases is 100 nM. Thus, the native P1 and the native PR-OS guanylate cyclases are functionally indistinguishable.

Test 2. Biochemical and functional similarities between the two native, P1 and PR-OS, guanylate cyclases were further established as follows. One sequence motif in ROS-GC1 that is critical and specific for GCAP1 activation is between amino acids L503 and I522 (*25*). The core motif within this amino acid domain consists of D507−R518. To assess similarity (or dissimilarity) between the biochemical mechanisms involved in the functional Ca^{2+}-inhibited guanylate cyclase machineries of the P1 and ROS-GC1 transduction systems, the P1 and PR-OS membrane fractions, side by side, were incubated with incremental concentrations of
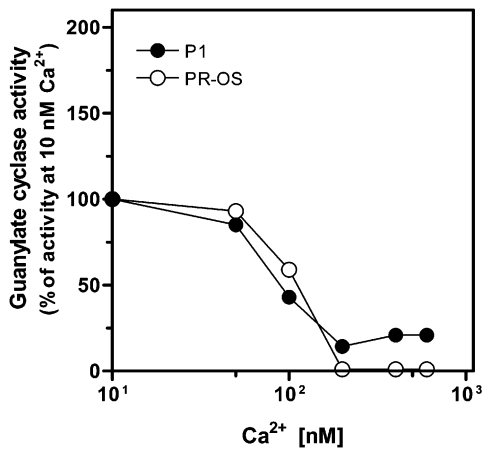
FIGURE 2: Effect of Ca^{2+} on guanylate cyclase activity in the P1 fraction and comparison with the effect of Ca^{2+} on the ROS-GC system present in the membrane fraction of photoreceptor outer segments. Membrane fractions from the P1 synaptosomal fraction (P1) and photoreceptor outer segments (PR-OS) were isolated as described in Experimental Procedures. These membranes were assayed for guanylate cyclase activity in the presence of incremental concentrations of Ca^{2+}. Guanylate cyclase activities in the presence of 10 nM Ca^{2+} were 0.9 ± 0.1 nmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1} for the P1 fraction and 9.0 ± 0.5 nmol of cyclic GMP (mg of protein)$^{-1}$ min^{-1} for the PR-OS fraction. The experiments were carried out in triplicate and repeated three times with separate membrane preparations. The results are from one representative experiment.

the L503−I522 and D507−R518 peptides at 10 nM Ca^{2+}. A peptide with the scrambled sequence of L503−I522 constituted a control. The results demonstrated a dose-dependent inhibition, reaching ∼65% for the L503−I522 peptide and ∼83% for the D507−R518 peptide in both the P1 and PR-OS fractions (Figure 3). The scrambled peptide, at concentrations of up to 0.25 mM, in both fractions only minimally (∼5−15%) inhibited the cyclase; at a concentration of 0.5 mM, the extent of inhibition was ∼20−30% (Figure 3). Furthermore, in both fractions, there was a reversal of the peptide (D507−R518)-dependent inhibition when an excess of GCAP1 was added (Figure 3C,D). These peptide competition and GCAP1 reconstitution results demonstrate that the Ca^{2+}-inhibited modulation of the cyclases in the P1 and PR-OS membrane fractions occurs by identical mechanisms, in which the modulation is mediated by GCAP1 and the modulated cyclase in both cases is the same, ROS-GC1. Hence, the operational biochemical components of the native P1 and PR-OS cyclase machineries are identical, and they are represented by GCAP1/ROS-GC1 transduction systems.

Test 3. The conclusions from test 2 were further confirmed by reconstitution experiments using recombinant ROS-GC1 and GCAP1. The recombinant ROS-GC1 was expressed in the heterologous COS cell system. At 50 nM Ca^{2+}, recombinant GCAP1 stimulated ROS-GC1 in a dose-dependent fashion with an EC$_{50}$ of 1 µM. The maximal stimulation of the enzyme was achieved at 4 µM GCAP1 (Figure 4A). The D507−R518 peptide inhibited the cyclase in a concentration-dependent fashion. The kinetics of inhibition of the recombinant ROS-GC1 were nearly identical to those of the cyclase present in the native P1 and PR-OS membrane fractions (compare Figure 4B with panels A and B of Figure 3). The control peptide, with a scrambled D507−R518 sequence,
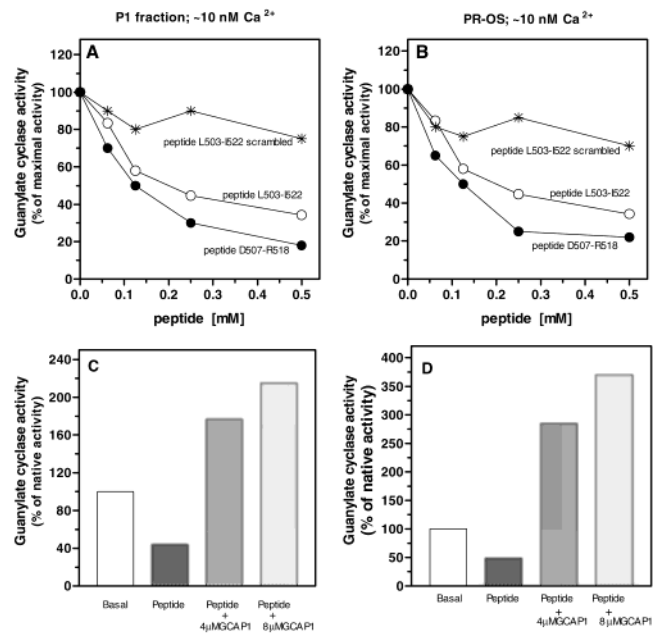

FIGURE 3: Effect of synthetic peptides derived from the ROS-GC1 sequence on GCAP1-dependent stimulation of ROS-GC1 activity. Membranes of P1 (A) or PR-OS (B) fractions were reconstituted with 2 µM GCAP1 and 10 nM Ca^{2+} and incubated with the indicated peptide concentrations. Membranes of P1 (C) or PR-OS (D) fractions were incubated with 2 µM GCAP1 and 250 µM D507−R518 peptide at 10 nM Ca^{2+} with or without the indicated concentrations of GCAP1. The experiment was carried out in triplicate and repeated three times for reproducibility. The results are from one typical experiment. Error bars are within the size of the symbols.
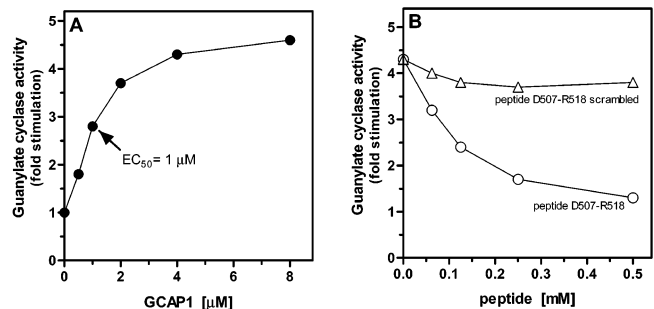

FIGURE 4: Dose-dependent response of recombinant ROS-GC1 to GCAP1 and the effect of synthetic D507−R518 and scrambled D507−R518 peptides. Membranes of COS cells expressing ROS-GC1 were used. (A) They were reconstituted with the indicated concentrations of GCAP1 and 10 nM Ca^{2+}. (B) They were incubated with 2 µM GCAP1 and increasing concentrations of either peptide at 10 nM Ca^{2+}. Experiments were carried out in triplicate and repeated at least two times for reproducibility. The data are from a representative experiment.

only very minimally inhibited ROS-GC1. These results validate the conclusions of test 2 and further show the direct participation of ROS-GC1 in GCAP1-mediated Ca^{2+} signal transduction in the P1 membrane fraction.

Test 4. The aim of this test was to directly establish the presence of GCAP1 in the P1 membrane fraction. Western blot analysis of the fraction was performed with the GCAP1-specific antibody according to a previous protocol (*35*). It is noted that this antibody has an absolute specificity for GCAP1 and does not react with the other member, GCAP2 (Figure 5A, compare rGCAP1 and rGCAP2). Purified PR-OS membrane preparations (PR-OS lane) and recombi-
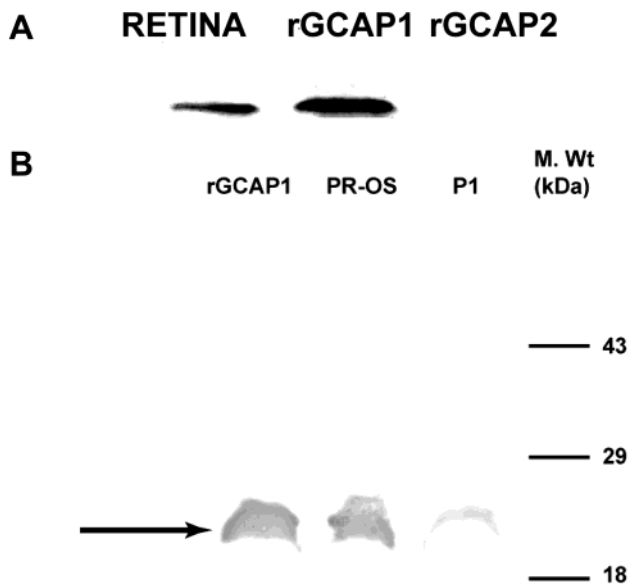
FIGURE 5: Presence of GCAP1 in the membranes of the P1 fraction and immunological analyses with the GCAP1-specific antibody. (A) Specificity of the GCAP1 antibody. Bacterially expressed and purified GCAP1 (rGCAP1), GCAP2 expressed in insect cells and purified (rGCAP2), and membranes from retina (RETINA) were electrophoresed and analyzed by Western blotting. (B) GCAP1 expression in the P1 fraction. Membranes of the P1 fraction were isolated and analyzed by Western blotting as described in Experimental Procedures. Fifty micrograms of the membrane protein was loaded in the PR-OS and P1 lanes; 0.5 μg of recombinant GCAP1 (rGCAP1) served as a control. Molecular size markers are given.

nant GCAP1 (rGCAP1 lane) served as positive controls. A single 21 kDa GCAP1 immunoreactive band, indicated with an arrow, with identical mobility was detected in each case (Figure 5B, compare rGCAP1, PR-OS, and P1). Thus, the native P1 fraction contains GCAP1.

Through four independent criteria, it is concluded that the P1 membrane fraction contains the GCAP1-modulated Ca^{2+}-inhibited ROS-GC1 transduction system and the system is functionally and biochemically identical to the one linked with the phototransduction machinery.

Cross-Linking Analysis Reveals that GCAP1 and ROS-GC1 Reside in Proximity in the Retinal P1 Fraction

To be physiologically relevant, GCAP1 and ROS-GC1 must reside in proximity. This was determined by cross-linking studies. The noncleavable imidoester DMS was used as a cross-linker. The reaction and analysis were carried out as described in Experimental Procedures, and the results are presented in Figure 6. Without the cross-linker, the ROS-GC1 antibody identified only the ROS-GC1 monomer and the GCAP1 antibody the GCAP1 monomer. In the presence of the cross-linker, both GCAP1 and ROS-GC1 antibodies identified a common immunoreactive band at ~250 kDa (indicated by a double-headed arrow). The size is that expected for a ROS-GC1 dimer−GCAP1 complex. The band was absent when the cross-linker was omitted (compare + lanes vs − lanes in GCAP1 and ROS-GC1). The mobility of the cross-linked complex suggests that the predominant interaction between ROS-GC1 and GCAP1 occurs in their dimeric forms. In reactions with the ROS-GC1 antibody, an immunoreactive band at ~120 kDa is seen and corresponds
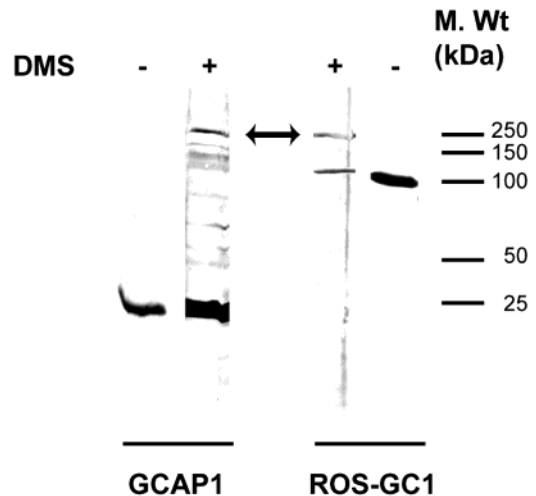

FIGURE 6: ROS-GC1 and GCAP1 are in proximity in the P1 fraction. The gel shows a cross-linking analysis. Membranes of the P1 fraction were isolated and incubated in the presence or absence of added GCAP1 (1 μM) with or without the cross-linker DMS. Cross-linking was carried out as described in Experimental Procedures. The reaction was terminated by boiling in SDS−PAGE loading buffer. Duplicate samples were electrophoresed on SDS−6% polyacrylamide gels and analyzed by Western blotting with ROS-GC1 or GCAP1 antibody. Similar results were obtained in the presence or absence of added GCAP1, except that the band intensities were enhanced in the presence of GCAP1. The results, with added GCAP1, are presented in the respective panels; a 100 μg equivalent of membrane protein was loaded in each lane. A common band, corresponding to ~250 kDa, is observed with both antibodies in the presence of the cross-linker (+ lanes) and is denoted with a double-headed arrow. Only the monomers of GCAP1 and ROS-GC1 are observed in the absence of the cross-linker (− lanes). Molecular size markers are given.

to the ROS-GC1 monomer (Figure 6, both lanes, ROS-GC1 panel). In reactions with the GCAP1 antibody, an immunoreactive band corresponding to the GCAP1 monomer is detected (Figure 6, both lanes, GCAP1 panel). Several additional bands are observed with the GCAP1 antibody probe in the presence of the cross-linker (Figure 6, + lane, GCAP1 panel), indicating that this protein may interact with several proteins other than ROS-GC1 in the retinal P1 layer. The results show that ROS-GC1 and GCAP1 reside in proximity in the retinal P1 fraction.

ROS-GC1 and GCAP1 Colocalize in Cone Synaptic Termini

To determine if ROS-GC1 and GCAP1 are localized in the outer plexiform layer, which is concentrated in the P1 fraction, immunolocalization of the two proteins was carried out on serial cryosections of the retina. Staining for ROS-GC1 was intense in both the rod and cone outer segments (Figure 7B). This is in agreement with the conclusion that the cyclase is expressed in both photoreceptor outer segments based on biochemical and genetic analyses (*4, 5, 43−45*). Staining was also observed in the inner segments, particularly those of cones. When Triton X-100 was excluded from the incubation medium, staining for the outer segment was still present; in addition, staining was also observed in the OPL and a weaker staining in the inner plexiform layer (IPL, Figure 7C). Under both conditions (with and without detergent), preadsorption of the antibody with the antigen eliminated the staining (data not shown).
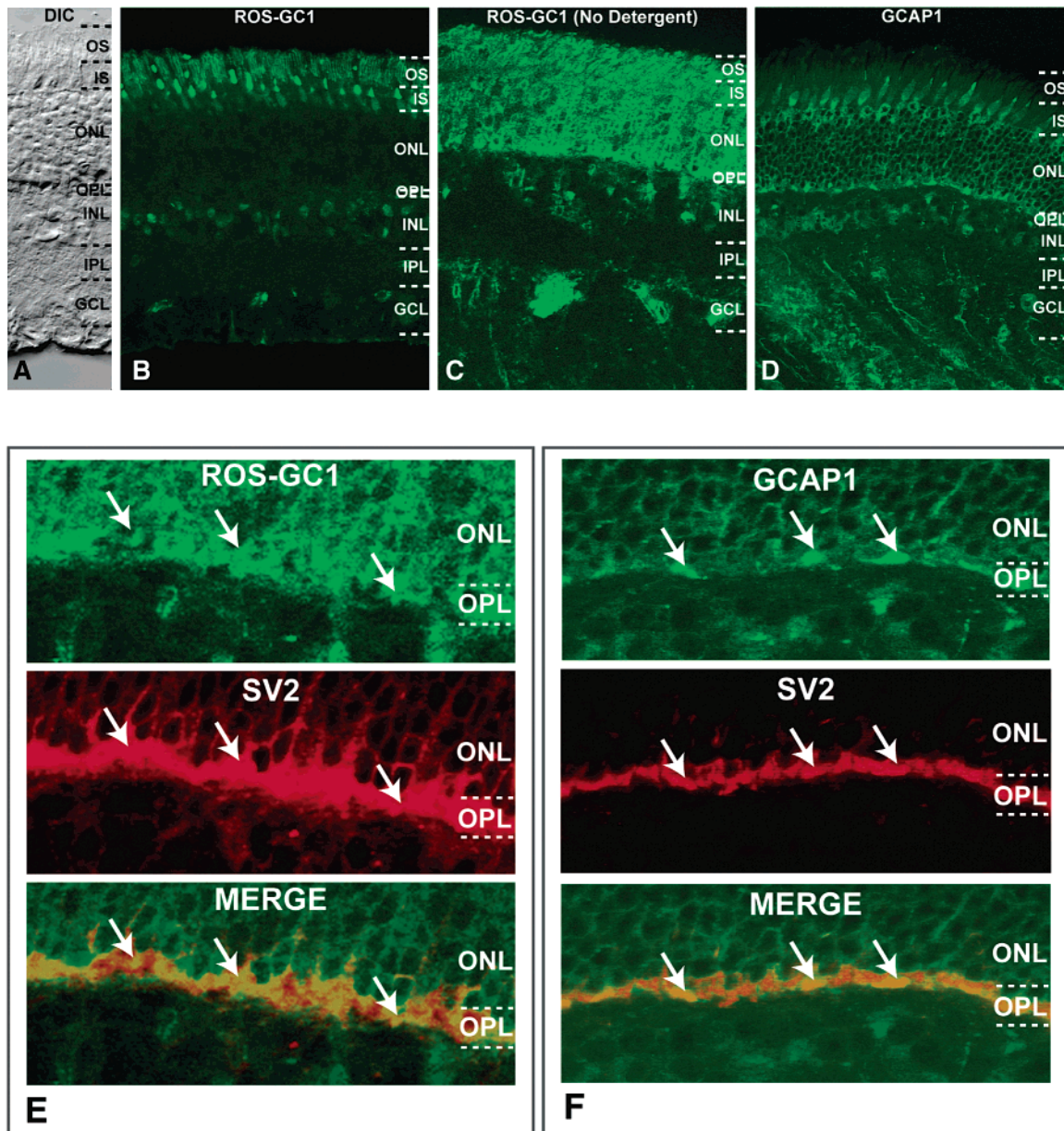
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FIGURE 7: ROS-GC1 and GCAP1 are colocalized in cone synaptic termini. Radial cryosections of bovine retina were immunostained for ROS-GC1 and GCAP1. (A) Differential interference contrast (DIC) image of a retinal section showing the retinal layers. (B) Distribution of ROS-GC1 as revealed by staining with the standard protocol. Staining is strong in rod and cone outer segments but is also present in other cells in the INL and GCL. (C) Distribution of ROS-GC1 as revealed by omitting Triton X-100. Staining is more ubiquitous, and is strong in the OPL. (D) Distribution of GCAP. Staining is strong in cone outer and inner segments and in the OPL. (E) Double staining for ROS-GC1 and SV2 (in the absence of the detergent Triton X-100). Certain OPL structures stained for SV2 (red) are also stained for ROS-GC1 (green); these appear orange-yellow in the MERGE panel. Some are denoted with arrows. The lack of total colocalization may be due to limited penetration in the ROS-GC1 staining. (F) Double staining for GCAP1 and SV2. All GCAP1-positive (green) structures in the OPL are also stained for SV2 (red) and appear orange-yellow in the MERGE panel. Some are denoted with arrows. Abbreviations: OS, outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. The boundaries of the different layers have been denoted with dashed lines.

Positive staining for GCAP1 is observed in the outer segments, inner segments, outer nuclear layer, and outer plexiform layer (Figure 7D). In the outer and inner segment layers, predominant staining was observed in the cone inner and outer segments. These observations are consistent with those from earlier studies (*10*, *11*, *30−33*). In the OPL, staining was in large structures, from which axons were ascending through the ONL to the inner segments. These are likely cone synaptic termini.

To determine which structures within the OPL are stained, the sections were doubly labeled with the antibodies against GCAP1 or ROS-GC1 and SV2, a synaptic vesicle protein that strongly stains photoreceptor termini (*46*). At higher magnification, colocalization of ROS-GC1 (in green) and SV2 (in red) is seen throughout the OPL, suggesting that ROS-GC1 is expressed in photoreceptor termini (Figure 7E). Some photoreceptor termini are indicated with arrows. The colocalization of GCAP1 with SV2 is presented in Figure 7F. Staining for GCAP1 is strong in the OPL, and it colocalizes with SV2, confirming that the GCAP1-positive structures in the OPL are cone synaptic termini. Because both ROS-GC1 and GCAP1 are localized to cone synaptic

termini, results from immunostaining, together with bio-chemical and cross-linking analyses, establish that these two proteins are coexpressed not only in the cone outer segment but also in the cone synaptic terminus, which is presynaptic to the bipolar neurons.

DISCUSSION

To date, the physiologically relevant GCAP-mediated Ca^{2+} signaling has only been found in photoreceptor outer segments. There it regulates the activity of the membrane-bound guanylate cyclase ROS-GC1 and is a critical part of a negative feedback loop that defines the recovery of a photoreceptor's light response. GCAPs sense changes in the Ca^{2+} concentration and undergo a conformational change. The change triggers activation of ROS-GC1 at low Ca^{2+} concentrations and inhibition of ROS-GC1 at high Ca^{2+} concentrations. This study provides comprehensive bio-chemical and immunological evidence to show that GCAP1-mediated Ca^{2+} signaling is also present in the presynaptic region of the photoreceptor cells, and it colocalizes with ROS-GC1.

In a recent report, Duda et al. (*34*) showed that ROS-GC1 with its Ca^{2+} sensor S100β is present in the photoreceptor–bipolar synaptic layer, and in this layer, Ca^{2+} stimulates ROS-GC1. It is, thus, concluded that the outer synaptic layer is an area of extreme sensitivity to Ca^{2+} waves and that the waves are sensed by either GCAP1 or S100β, which, in turn, stimulates or inhibits ROS-GC1. The study presented here establishes the presynaptic localization of the GCAP1/ROS-GC1 transduction system. Whether the S100β/ROS-GC1 transduction system is presynaptic, postsynaptic, or both remains to be investigated. In addition to GCAP1 and S100β, ROS-GC1 is also modulated by GCAP2 (reviewed in refs *1* and *2*). The current evidence indicates that GCAP2 is also present in the retinal synaptic layers (*30–32*), creating the possibility that the GCAP2/ROS-GC1 system may also be functional. This possibility also remains to be investigated.

At the moment, one can only speculate about how the GCAP1/ROS-GC1 system is linked to other signaling components in the presynaptic region of photoreceptors. A possible target of cyclic GMP could be a CNG channel in synaptic termini of cones (*47, 48*). Activation of these channels triggers release of glutamate from the terminus. The GCAP1/ROS-GC1 system could provide a Ca^{2+}-dependent negative feedback control of CNG channels. When the Ca^{2+} influx via voltage-dependent Ca^{2+} channels stops (for example, during hyperpolarization), the free Ca^{2+} concentration in synaptic termini would transiently decrease and in turn activate the GCAP1/ROS-GC1 system. It has been recently shown that Ca^{2+} levels fluctuate over a 10-fold range in photoreceptor synapses and reach values as low as 50 nM (reviewed in ref *49*), where the GCAP1/ROS-GC1 system is, indeed, most effective. An increase in the level of cyclic GMP would open the CNG channels, and an influx of Ca^{2+} would dampen the GCAP1/ROS-GC1 system. The CNG channels in cone synaptic termini appear to be influenced also by nitric oxide, which can activate the soluble form of a guanylate cyclase present in inner segments of photoreceptors (*50*). Thus, the complexity of cyclic GMP signaling in synaptic regions points to several spatially and temporarily restricted events and needs to be worked out in more detail.

Recent investigations have documented the presence of a Ca^{2+}-modulated membrane guanylate cyclase transduction system in three neuronal systems besides visual transduction: the pinealocytes, the cilia of the olfactory neuroepithelium, and the olfactory bulb (*35–37*). Like the photoreceptors, the pineal gland is modulated by light. The ciliated apical border of sensory neurons located in the epithelial layer is the site of odorant transduction. And, the olfactory bulb is the second neural compartment where the odorant signal is processed and is then transmitted to the olfactory cortex for smell perception. Ca^{2+}-regulated membrane guanylate cyclase resides in all three neural systems (*35–37*). One class of the pinealocytes contains the GCAP1/ROS-GC1 transduction system, and the second class contains the S100β/ROS-GC1 transduction system (*35*). The olfactory cilia contain the neurocalcin/ONE-GC [also known as GC-D (*51*)] transduction system (*36*), and the olfactory bulb contains the GCAP1/ROS-GC1 transduction system (*37*). Thus, native functional expression of ROS-GC1 is not restricted to outer segments of photoreceptor cells, and its expression may be a common feature of neurons linked with sensory perception. Its regulatory features are rather complex and depend on the specific cellular or subcellular localization.

The different modulatory features of ROS-GC1 are possible through the presence of regulatory and/or target regions in distant domains of the protein. Like other members of the membrane guanylate cyclase family, ROS-GC1 is a modular protein with the following common topography. A single helical transmembrane domain divides the protein into two portions. The N-terminus consists of an extracellular domain or intradiscal domain when present in PR-OS. The intracellular region is comprised of a juxtamembrane domain, a kinase homology domain, a dimerization domain, and a core catalytic domain. The guanylate cyclase family is subdivided into two subfamilies: the peptide hormone receptor and the Ca^{2+}-modulated ROS-GC (reviewed in ref *1*). The extracellular domain of the peptide hormone receptor subfamily contains the receptor domain. To date, in the ROS-GC subfamily, neither a ligand for nor the functional relevance of this domain has been found. As a functional unit, it is believed that all membrane guanylate cyclases exist as homodimers (reviewed in ref *1*). The ROS-GC subfamily contains an added segment at its C-terminus, which is not shared with the peptide receptor subfamily (reviewed in refs *1* and *2*). This has been termed the C-terminal extension. A recent study has shown that the C-terminal extension is the site of the S100β-modulated domain in ROS-GC1, and this domain regulates the catalytic activity of the cyclase (*34*). This domain has been mapped to two small regions: one region comprises amino acids (aa) G962–N981 and the other aa I1030–Q1041. The GCAP1-modulated domain resides at two regions, M445–L456 and L503–I522, in the jux-tamembrane domain (*25*). The domain is physiologically linked with the GCAP1-modulated activity, because a point mutation in this domain causes Leber's congenital amaurosis and loss of GCAP1-dependent ROS-GC1 regulation (*44*). Two other GCAP1 binding regions have also been reported, and they also reside at the N-terminal side of the catalytic domain; however, these regions have not been physiologically linked with GCAP1 regulation (*25*). Thus, the GCAP1-regulated module resides at the N-terminal side of the catalytic module and the S100β-regulated module at its
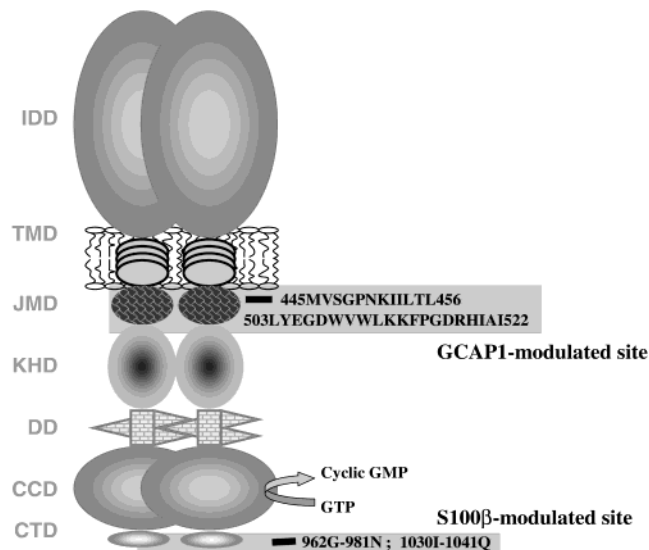
FIGURE 8: Modular domain topography of ROS-GC1. The GCAP1 and S100β modulated domains are indicated. Abbreviations: IDD, intradiscal domain; TMD, transmembrane domain; JMD, juxtamembrane domain; KHD, kinase homology domain; DD, dimerization domain; CCD, catalytic cyclase domain; CTD, C-terminal domain.

C-terminal side, and the two modules independently regulate the catalytic module. These findings allow the formation of a new topographic model of ROS-GC1 transduction (Figure 8). In this model, the catalytic module of ROS-GC1 at its opposite ends is flanked by GCAP1 and S100β modules. GCAP1 senses the Ca^{2+} impulse and inhibits the catalytic module; S100β senses the impulse and stimulates the catalytic module. In this manner, ROS-GC1 acts as a bimodal Ca^{2+} signal transduction switch in sensory neurons. The model defines reciprocal regulation of ROS-GC1 by Ca^{2+} in biochemical terms. Finally, the location of the high Ca^{2+}-regulated domain in the C-terminal extension may constitute a signature feature of the ROS-GC subfamily of membrane guanylate cyclases.

ACKNOWLEDGMENT

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