A Novel Calcium-Dependent Activator of Retinal Rod Outer Segment Membrane Guanylate Cyclase†

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ABSTRACT: The membrane guanylate cyclase in retinal rod outer segments (ROS-GC) is known to be negatively regulated by calcium; when the calcium concentration is reduced below the dark-adapted level of about 500 nM, the enzyme is activated by a soluble protein. We now report that the enzyme is also positively regulated by calcium; a novel soluble protein is identified and purified from bovine retina which activates ROS-GC, with half-maximal activation occurring at 2−5 μM calcium. The activation is dose-dependent, and at its maximum, cyclase is stimulated up to 25-fold. The activator has a molecular mass of about 40 kDa and is a multimer of a 6−7 kDa peptide.

Guanylate cyclase (GC) catalyzes the formation of cyclic GMP from GTP. Both soluble and membrane-associated cyclase activities are found in several tissues (Kimura & Murad, 1974). Of the membrane-associated cyclases whose mechanism of regulation is known, two groups can be identified on the basis of the peptides regulating their activity. Members of one group, often referred to as receptors (Schultz et al., 1989; Sharma et al., 1994; Garbers & Lowe, 1994), are regulated by peptides presumably binding to their extracellular domains. These include cyclases in several tissues serving as receptors for natriuretic hormones, the intestinal cyclase activated by guanylins or heat-stable enterotoxins, sea urchin sperm cyclase activated by egg peptides such as resact and speract, and other peptide−hormone-regulated enzymes. Membrane-associated enzymes in the other group do not have any known hormone regulators but are activated by cytosolic proteins. These include cyclases from *Paramecium* and *Tetrahymena* and the vertebrate retinal rod outer segments (ROS). The *Tetrahymena* and *Paramecium* enzymes are activated by calmodulin (Kakiuchi et al., 1981; Klumpp & Schultz, 1982), and as is the case with other calmodulin-activated enzymes, the activation occurs when the calcium concentration is increased from the submicromolar to the micromolar range (Wolff & Brostrom, 1979). However, the ROS guanylate cyclase (ROS-GC) is known to be regulated in the opposite manner; its activation occurs when the free calcium concentration is decreased from the submicromolar to the nanomolar range (Koch & Stryer, 1988). Two protein mediators of this activation have recently been described (Gorczyca et al., 1994; Dizhoor et al., 1994). It has been suggested that such activators of ROS-GC are calcium-bound and inactive at higher calcium concentrations present in dark-adapted ROS and that they stimulate the cyclase only upon dissociation from calcium at the lower, nanomolar, calcium concentrations (Koch & Stryer, 1988; Stryer, 1991). This adaptation appears to fit the physiology of the ROS. A light flash on dark-adapted ROS leads to hydrolysis of cyclic GMP and reduction in its free concentration (Cote et al., 1984; Cohen & Blazynski, 1988; Pugh & Lamb, 1993). The light flash also results in a lowering of the ROS free calcium concentration (Yau & Nakatani, 1985; McNaughton et al., 1986; Gray-Keller & Detwiler, 1994) which triggers activation of the cyclase and restoration of the cyclic GMP concentration, the dark current, and the calcium concentration to the dark-adapted levels (Hodgkin & Nunn, 1988).

In contrast to this well-established negative regulation by calcium, we now report the identification and purification of a soluble protein from retina which activates ROS-GC in a calcium-dependent manner, with little or no influence at nanomolar calcium concentrations where the previously reported proteins activate the enzyme. The possible physiological role for this protein is discussed.

MATERIALS AND METHODS

Preparation of ROS and Washed ROS Membranes. Fresh bovine eyes were obtained from Wolverine Packing Co., Detroit, MI, and dark adapted for 3−4 h at room temperature. Intact ROS were isolated in the dark (Schnetkamp et al., 1979). To prepare ROS membranes free of soluble and peripheral proteins, ROS were washed three times in the dark in 10 mM Tris-HCl (pH 7.5) at 0.1 mg of protein per milliliter. ROS and washed ROS membranes were stored in aliquots at −80 °C. The preparations were thawed before use and suspended by vortexing.

ROS-GC Stably Expressed in Chinese Hamster Ovary (CHO) Cells. The coding region of bovine ROS-GC cDNA

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(Goraczniak et al., 1994) was ligated into *Hin*dIII/*Not*I sites of pcDNA3 expression vector (Invitrogen), a plasmid-carrying neomycin resistance. CHO cells were transfected with the expression construct using the calcium phosphate coprecipitation technique (Chen & Okayama, 1988), and after 72 h, a selective antibody, G418, was added to the culture medium. After 14 days of selection, resistant colonies were isolated and grown under the same conditions.

Guanylate Cyclase Assays. Cyclase activity in ROS or washed ROS membranes was measured in the dark in an assay mixture containing 40 mM Hepes (pH 7.8), 0.25 mM GTP, 6 μCi of [α-^{32}P]GTP, 4 mM cyclic GMP, 0.2 μCi of ^{3}H cyclic GMP, 15 mM $MgCl_2$, 1.25 mM isobutylmethylxanthin, and 2 mM EGTA or 1 mM $CaCl_2$. Usually, 20 μg of ROS or washed ROS protein was used per assay. The reactions were carried out at 30 °C for 10 min, and the cyclic GMP formed was measured by thin layer chromatography as described earlier (Sitaramayya et al., 1993). ROS-GC activity expressed in CHO cell membranes was measured as described (Paul et al., 1987).

Activator Assay. To detect the presence of guanylate cyclase activator in a test fraction, ROS-GC activity in washed ROS membranes was measured in the absence and presence of the test fraction in reaction mixtures containing 1 mM $CaCl_2$.

Preparation of the Activator. One hundred frozen bovine retinas were homogenized in 50 mL of 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL trypsin inhibitor, and 50 μg/mL benzamidine. The homogenate was centrifuged at 100000*g* for 30 min, and the resulting supernatant was brought to 50 mM in Tris-HCl (pH 8.0) and 5 mM in $CaCl_2$ and heated for 3 min at 75 °C in 3 mL aliquots (Dizhoor et al., 1994). The preparation was clarified by centrifugation, dialyzed against column buffer [CB; 10 mM Tris-HCl (pH 8.0)], and loaded on a 1 × 6 cm column of DEAE−sepharose CL-6B equilibrated in CB. The column was washed with the same buffer and eluted with a linear gradient of 0 to 500 mM NaCl in CB. Fractions of 2 mL were collected and assayed for their effect on guanylate cyclase activity of washed ROS membranes.

The active fractions from the DEAE−sepharose column were pooled, adjusted to 2.5 M in NaCl, and loaded on a 1 × 5 cm column of phenylsepharose CL-4B equilibrated in high salt buffer [HSB; 10 mM Tris-HCl (pH 7.5) and 2.5 M NaCl]. The column was washed with the same buffer, and the proteins were eluted with a linear gradient of 20 mL of HSB to 20 mL of Tris buffer [TB, 10 mM Tris-HCl (pH 7.5)], collecting 2.0 mL fractions. The effluent, wash, and fractions eluted with the gradient were desalted and assayed for their ability to stimulate ROS-GC activity.

The activator preparation after the phenylsepharose column was further purified by high-pressure liquid chromatography (HPLC) on a Biosep-sec 2000 (600 × 7.8 mm) gel filtration column (Phenomenex). The column was equilibrated and eluted in 50 mM Tris-HCl (pH 6.8) containing 50 mM NaCl at the flow rate of 0.4 mL/min. Fractions of 0.2 or 0.4 mL were collected and assayed for effect on cyclase activity.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Protein samples were electrophoresed in 10 to 20% polyacrylamide gradient gels (15 × 20 cm) (O'Farrell, 1975) or in 16.5% Tricine−SDS−polyacrylamide gels (Schaegger & von Jagow, 1987). Protein bands were visualized by Coomassie Blue or silver staining.

Regeneration of Activator Recovered after Preparative SDS−PAGE. Two hundred microliters of the activator preparation after HPLC was mixed three times with 100 μL of sample buffer and loaded into 15 lanes on a 8 × 10 cm SDS−PAGE gel. After electrophoresis, the location of the protein of interest was identified by cutting out and staining the first and last lanes of the gel. Following the identification, a 5 mm band of the entire width of the gel containing the protein was cut out, and the protein was extracted overnight in 10 volumes of 10 mM Tris (pH 7.5). The extract was put through three cycles of dilution with the same buffer and filtration through Centricon-10 to remove SDS. After concentration to a final volume of about 200 μL, aliquots were tested for activity and the molecular weight of the regenerated protein was determined by gel filtration chromatography as described above.

Detection of Calcium Binding. To test whether the purified activator binds calcium, the protein was electrophoresed, transferred to nitrocellulose membrane, and incubated with ^{45}Ca (Maruyama et al., 1984). Peptides used as molecular mass standards (Sigma) were treated identically to serve as controls. Washed membranes were dried and autoradiographed.

Calcium Concentrations. For routine measurements, "low" and "high" calcium concentrations were obtained by the addition of 2 mM EGTA or 0.1−1 mM $CaCl_2$, respectively, to the GC assay mixtures. To obtain the desired free calcium concentrations, $CaCl_2$ and EGTA were mixed in the proportions calculated using the software, Maxchelator, version 6.5 (Bers et al., 1994). In doing so, the pH, temperature, ionic strength, and Mg and nucleotide concentrations in the assay were taken into account. The actual $CaCl_2$ and EGTA concentrations, respectively, and the expected free calcium concentrations (parentheses) are as follows: 10 μM, 6 mM (0.56 μM); 10 μM, 2 mM (2.1 μM); 20 μM, 1 mM (7.2 μM); 40 μM, 1 mM (14.6 μM); 70 μM, 1 mM (26 μM); 90 μM, 1 mM (34 μM); 120 μM, 1 mM (45.3 μM); 150 μM, 1 mM (57.4 μM); 180 μM, 1 mM (69.8 μM); 200 μM, 1 mM (78.2 μM); and 250 μM, 1 mM (99.8 μM).

RESULTS AND DISCUSSION

Retinal extracts stimulated ROS-GC activity when incubated in the presence of calcium. When an extract was partially purified by heat treatment and fractionated on a DEAE−sepharose column, the calcium-dependent activator eluted in a single peak at about 300 mM NaCl. When the active fractions were pooled and adjusted to 2.5 M NaCl in preparation for chromatography on the phenylsepharose column, the stimulatory activity was almost completely lost, though NaCl did not precipitate the activity. Desalting fully restored the activity, suggesting that the effect of salt was reversible. Additional tests revealed that the stimulation of cyclase by the activator was sensitive to ionic strength in the assay; 125 mM NaCl reduced the stimulation by 60%, and 500 mM NaCl reduced it by 90%.

The activator from the DEAE−sepharose column was applied to the phenylsepharose column and eluted with a salt gradient. Assaying the fractions after desalting revealed that the activator eluted at about 1.5 M NaCl. The activator

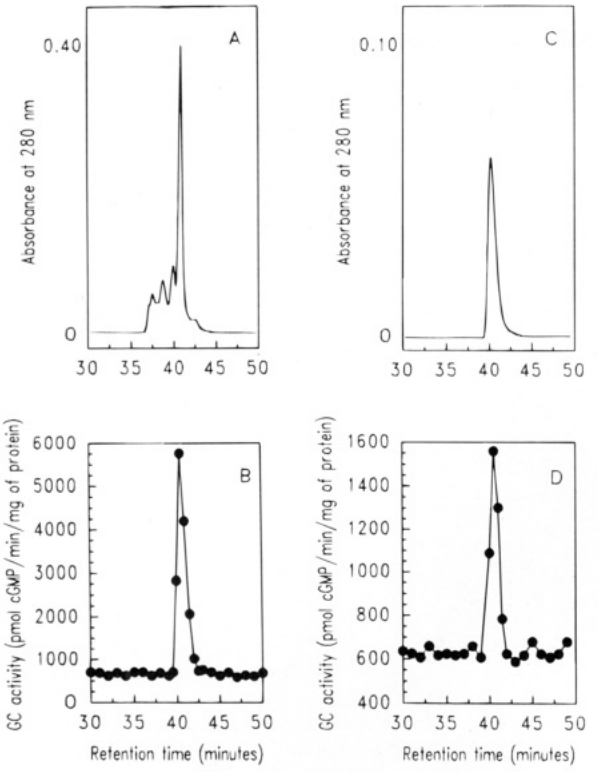

FIGURE 1: Gel filtration chromatography of native and regenerated activator. The activator preparation after phenylsepharose chromatography was subjected to gel filtration by HPLC in the final step of the purification. Part A shows the elution of proteins from the gel filtration column, and part B shows the elution of the activity. The activity coincided with a major protein peak. The gel filtration column was calibrated with the following molecular mass standards: ovalbumin (44 kDa), retention time of 39.4 min; carbonic anhydrase (29 kDa), retention time of 43.6 min; and myoglobin (19 kDa), retention time of 45.9 min. On the basis of the calibration, the apparent molecular mass of the activator eluted from the column was about 40 kDa. About one-fifth of the protein from the peak fractions (B) was electrophoresed, and the 6−7 kDa peptide (Figure 2) band was cut out of the gel, extracted, regenerated, and chromatographed again on the gel filtration column. Part C shows the elution of protein, and part D shows the elution of activity from the column.

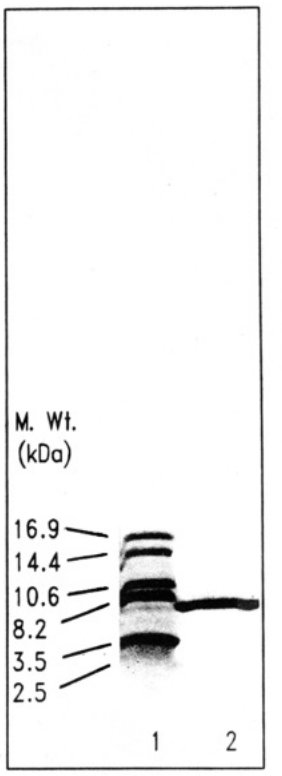

FIGURE 2: Electrophoresis of the purified activator. Four micrograms of the activator protein eluted from the HPLC gel filtration column was electrophoresed in a 10−20% gradient SDS−polyacrylamide gel and stained with Coomassie Brilliant Blue (lane 2). Lane 1 shows peptide standards with their molecular masses shown on the left.

from the phenylsepharose column was further purified by size exclusion chromatography on a Biosep-sec 2000 column. Figure 1 shows the elution of proteins (A) and GC stimulatory activity (B) from the gel filtration column. The activity eluted as a single peak with a retention time of 40.5 min (varied between 40.5 and 40.6 min in 12 experiments), corresponding to a protein with a molecular mass of about 40 000 Da (the details of column calibration are in the figure legend). The total amount of purified protein obtained from 100 frozen retinas was about 150 μg.

Figure 2 shows SDS−PAGE of the activator after the gel filtration column. The preparation consisted of a single protein band of 6−7 kDa molecular mass. However, as shown in Figure 1, the molecular mass of the purified activator in the native state, as eluted from the gel filtration column, was about 40 kDa. This suggested that the protein in the native state is a multimer of the 6−7 kDa peptide. In order to test this possibility, the 6−7 kDa protein was extracted from a SDS−PAGE gel, SDS was removed, and the regenerated preparation was chromatographed again on the HPLC gel filtration column. Figure 1C shows the elution of protein from the column in a single peak with a retention time of 40.5 min, identical to that of the native protein as seen in Figure 1A. It also coincided with the elution of the GC stimulatory activity from the column (Figure 1D). These results demonstrate that the 6−7 kDa peptide polymerizes to form the 40 kDa stimulator of GC. This is similar to S-100P, a calcium-binding protein, which has a molecular mass of 10 000 kDa as determined by SDS−PAGE and 33 000 Da as determined by gel filtration (Emoto et al., 1992).

The stimulation of ROS-GC by the activator was dose-dependent and saturable (data not shown). In three experiments in which the assays were done in the presence of 0.1 or 1 mM $CaCl_2$, the maximal stimulation obtained was 22−25-fold greater than the basal activity. The maximal activation was observed at 2.0−3.7 μM concentrations of the activator (based on molecular mass of 40 000 kDa). The activator preparation by itself had no cyclase activity.

Figure 3 shows the calcium dependence of ROS-GC stimulation by the activator. The activation was maximal at a free calcium concentration of about 40 μM, with the half-maximal activation occurring at about 2 μM. In two other experiments, half-maximal activation was observed at about 3 and 4−5 μM. Calmodulin activation of guanylate cyclases from *Tetrahymena* and *Paramecium* is also known to be regulated by calcium in the micromolar range (Kakiuchi et al., 1981; Klumpp & Schultz, 1982). The half-maximal activation of *Paramecium* cyclase occurs at 8 μM calcium (Klumpp & Schultz, 1982) and that of *Tetrahymena* cyclase at 3 μM (Kudo et al., 1981). In view of the calcium dependence of this activator in the stimulation of cyclase activity, we refer to it as calcium-dependent guanylate cyclase activator protein (CD-GCAP). This name distinguishes it

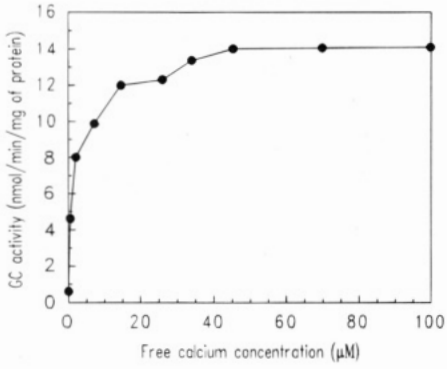

FIGURE 3: Effect of calcium concentration on the activation of guanylate cyclase by the activator. Cyclase activity in washed ROS was measured in the presence of activator at different free calcium concentrations obtained as described in Materials and Methods. Half-maximal activation was observed at about 2 μM Ca. Four micrograms of the activator protein was used in the assays.

from another protein reported to stimulate cyclase at nanomolar calcium concentrations and named guanylate cyclase activating protein (GCAP) (Gorczyca et al., 1994).

In order to test if calmodulin simulates the activity of CD-GCAP, calmodulins from bovine testes, *Saccharomyces*, and spinach (Sigma) were tested for their effect on guanylate cyclase. None of them had any influence. Protein kinase C was reported to cause about a 2-fold increase in bovine ROS and rat colonic particulate guanylate cyclase activities in the presence of ATP (Wolbring & Schnetkamp, 1995; Khare et al., 1994). This increase is prevented by protein kinase inhibitors. The observed stimulation of ROS-GC activity by CD-GCAP was in the absence of ATP, and staurosporin (Sigma), a protein kinase inhibitor, at concentrations up to 250 nM, did not reduce it (data not shown). This indicates that CD-GCAP stimulates ROS-GC independent of protein kinase C and also of ATP.

As would be expected of a protein dependent upon calcium for its biological activity, the 6−7 kDa protein bound ^{45}Ca. The binding was specific, as suggested by a lack of binding to standard proteins used as molecular mass markers (Figure 4).

As reported by other investigators (Koch & Stryer, 1988), an endogenous activator (GCAP) in ROS stimulates GC activity only at low calcium concentrations. We also observed this activation in ROS which were isolated intact and contained the endogenous soluble proteins; cyclase activity at low calcium concentrations was about 5-fold higher than at high calcium concentrations (lanes 1 and 3 in Figure 5a). Addition of CD-GCAP to ROS at low calcium concentrations did not further enhance the GC activity (lanes 3 and 4). In contrast, at higher calcium concentrations, CD-GCAP stimulated the activity (lanes 1 and 2 in Figure 5a). The extent of stimulation depended upon the amount of CD-GCAP added. In washed ROS membranes depleted of endogenous activator, the GC activity was unaffected by calcium concentration (lanes 5 and 7 in Figure 5b). Addition of CD-GCAP to washed ROS membranes at higher but not lower calcium concentrations greatly stimulated the cyclase activity (lanes 6 and 8 in Figure 5b).

To rule out the possibility that the CD-GCAP-dependent activation of ROS-GC was artifactual, the effect of CD-GCAP was tested on cloned ROS-GC. The activator stimulated the cloned cyclase only at higher calcium

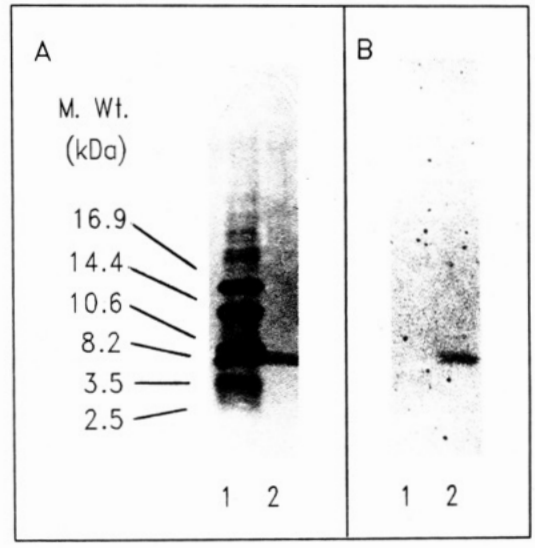

FIGURE 4: ^{45}Ca binding to the activator protein. "Two micrograms of the activator protein was electrophoresed in a Tricine−SDS−polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated with ^{45}Ca, washed, and autoradiographed. Part A shows amido black-stained proteins on nitrocellulose membrane, and part B shows the autoradiogram. Lane 1: peptide standards which served as molecular mass markers as well as controls for ^{45}Ca binding; lane 2: activator protein.

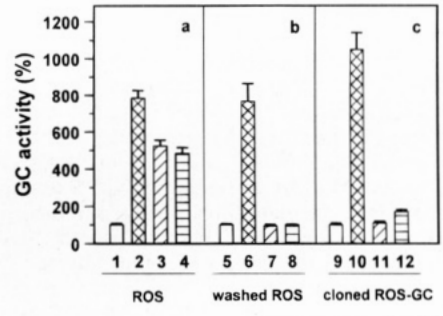

FIGURE 5: Effect of the activator on cyclase activity of ROS, washed ROS membranes, and cloned ROS-GC. Guanylate cyclase activity of ROS (a), washed ROS membranes (b), and cloned ROS-GC (c) was measured in the absence (lanes 1, 3, 5, 7, 9, and 11) and in the presence (lanes 2, 4, 6, 8, 10, and 12) of the activator. The assays were done in the presenc of high (lanes 1, 2, 5, 6, 9, and 10) or low calcium concentrations (lanes 3, 4, 7, 8, 11, and 12) as described in Materials and Methods. The data shown are mean ± standard deviation from three experiments done with partially purified CD-GCAP. Similar results were obtained with purified CD-GCAP, and in all cases, the extent of activation depended upon the amount of CD-GCAP added.

concentrations, just as it did the enzyme in ROS membranes (Figure 5c).

In the presence of saturating amounts of CD-GCAP, cloned ROS-GC was activated about 25-fold (data not shown), again similar to the enzyme in ROS membranes. Furthermore, the activity of retinal Type C natriuretic factor receptor guanylate cyclase (Duda et al., 1993) was unaffected by CD-GCAP in the presence of calcium (data not shown), indicating the specificity of CD-GCAP for ROS-GC.

Studies reported to date on the regulation of ROS-GC activity show that the enzyme is only activated when calcium concentrations are reduced to nanomolar levels (Koch & Stryer, 1988; Gorczyca et al., 1994; Dizhoor et al., 1994). Our discovery of an activator in retina which stimulates the enzyme when the calcium concentration is raised to micromolar levels is quite unexpected and suggests that ROS-GC

is a dual-regulated enzyme capable of activation by one type of stimulator at low calcium concentrations and by another at high calcium concentrations. In order to test if such a dual activation takes place in ROS, we tested the cytosolic fraction of ROS for CD-GCAP activity and found none, indicating that it either is in a very small concentration or is easily lost from intact ROS. We also considered the possibility that CD-GCAP regulates ROS-GC-like guanylate cyclases outside of the ROS. This hypothesis is supported by recent observations of immunoreactivity to ROS-GC-specific antibodies in non-ROS layers of retina, specifically the outer and inner synaptic (plexiform) layers (Liu et al., 1994; Yoshida et al., 1995). Additional support for this hypothesis comes from the earlier observation (Orr et al., 1976) that the cyclic GMP concentration is higher in the dark-adapted retinal photoreceptor synaptic layer than in the light-adapted one. Photoreceptor terminals depolarize in the dark, and depolarization of neurons is known to cause localized elevation of internal free calcium concentrations in the range of 10−100 μM (Augustine & Neher, 1992). The calcium concentration required for activation of ROS-GC by CD-GCAP is well within this range. It could therefore be suggested that CD-GCAP regulates a ROS-GC-like cyclase in retinal synaptic layers.

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