The Membrane Guanylyl Cyclase, Retinal Guanylyl Cyclase-1, Is Activated through Its Intracellular Domain*

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Retinal guanylyl cyclase-1 (RetGC-1) is a membrane guanylyl cyclase found in photoreceptor outer segments. It consists of an apparent extracellular domain (ECD) linked by a single transmembrane segment to an intracellular domain (ICD). Guanylyl cyclase activating protein-2 (GCAP-2) is a Ca\(^{2+}\)-binding protein that activates RetGC-1 in a Ca\(^{2+}\)-sensitive manner. To establish whether GCAP-2 stimulates RetGC-1 through the ECD or ICD, we made deletion mutants lacking either the ECD or both the ECD and transmembrane domains (TMD) of RetGC-1. Recombinant wild type RetGC-1 and both deletion mutants were expressed in HEK 293 cells, and their sensitivities to GCAP-2, Ca\(^{2+}\), and ATP were compared. Our data demonstrate that both deletion mutants are regulated similarly to wild type RetGC-1 with indistinguishable EC\(_{50}\) values for Ca\(^{2+}\) and similar K\(_{v}\) values for activation by GCAP-2. This shows that GCAP-2 functions through the ICD of RetGC-1 and that neither the ECD nor the TMD of RetGC-1 participate in its regulation by ATP.

Photoexcitation of retinal rod cells stimulates hydrolysis of intracellular cGMP. This reduces the activity of cGMP gated cation channels in the rod outer segment plasma membrane, blocks Na\(^{+}\) and Ca\(^{2+}\) influx, and allows a Na\(^{+}\)/Ca\(^{2+}\)/K\(^{+}\) exchange to decrease the concentration of free cytoplasmic Ca\(^{2+}\) (reviewed in Lagnado and Baylor (1992)). At low concentrations of free Ca\(^{2+}\), a soluble factor stimulates guanylyl cyclase (GC)\(^{1}\) activity in photoreceptor membranes (Koch and Stryer, 1988). It has now been shown that this stimulatory activity is represented by at least two Ca\(^{2+}\)-binding proteins, GCAP-1 and GCAP-2 (Dizhoor et al., 1995; Gorczyca et al., 1995). It has been proposed that the light-induced decrease in free cytoplasmic Ca\(^{2+}\) concentration stimulates GC activity in vivo. Such a feedback mechanism would stimulate resynthesis of cGMP and enhance photoreceptor recovery and/or light adaptation following photoexcitation (reviewed in Lagnado and Baylor (1992) and McNaughton (1990)).

RetGC-1 and RetGC-2 are two photoreceptor GCs that were cloned from a human retinal cDNA library (Shyjan et al., 1992; Lowe et al., 1995).\(^2\) Homologues of RetGC-1 have also been cloned from bovine and rat eye cDNA libraries, and a homologue of RetGC-2 was isolated from a rat eye cDNA library (O’Connor et al., 1994; Yang et al., 1995). RetGC-1 and RetGC-2 are expressed in photoreceptors and associate with the membrane fraction of photoreceptor outer segments (OS) (Shyjan et al., 1992, Dizhoor et al., 1994; Lowe et al., 1995). Immunofluorescence studies suggest that RetGC-1 is localized primarily in cone OS and to a lesser extent in rod OS (Dizhoor et al., 1994; Liu et al., 1994). Electron microscopy studies further indicate that RetGC-1 is associated with the membrane-rich regions of OS (Liu et al., 1994). The GC activities of OS membranes and recombinant RetGC-1 are activated by the photoreceptor Ca\(^{2+}\)-binding proteins GCAP-1 and GCAP-2, and recombinant RetGC-2 is activated by GCAP-2 (Dizhoor et al., 1995; Gorczyca et al., 1995; Lowe et al., 1995). Activation by either GCAP is inhibited by Ca\(^{2+}\) with an EC\(_{50}\) for Ca\(^{2+}\) near 200 nM (Dizhoor et al., 1994; Gorczyca et al., 1994a, 1995; Dizhoor et al., 1995). This value agrees well with the range of bulk free Ca\(^{2+}\) (50–550 nM) recently measured in intact OS in darkness and following a flash of light (Gray-Keller and Detwiler, 1994). The localization of RetGC-1 and RetGC-2 and their sensitivity to Ca\(^{2+}\) and GCAP-2 suggest that they function in the recovery of photoreceptors from photoexcitation (Dizhoor et al., 1994; Lowe et al., 1995).

A factor that influences the Ca\(^{2+}\)-sensitive stimulation of OS GCs is ATP. It has been reported that ATP or nonhydrolyzable ATP analogues potentiate the Ca\(^{2+}\)-sensitive stimulation of OS GCs in whole OS and in washed OS reconstituted with GCAP-1 (Gorczyca et al., 1994b). Both RetGC-1 and RetGC-2 are members of the membrane GC family that includes the natriuretic peptide receptor-GCs (NPR-A/GC-A and NPR-B/GC-B), the heat stable enterotoxin or guanylin receptor-GC (StaR/GC-C), and the sea urchin sperm GCs (Shyjan et al., 1992; Garbers and Lowe, 1994; Lowe et al., 1995). It has been clearly shown that the stimulation of other members of this family is influenced by adenosine nucleotides. The stimulation of NPR-A requires (Chinkers and Garbers, 1991; Marala et al., 1991), and the stimulation of StaR is prolonged by (Vaandragher, 1993a) the presence of ATP or nonhydrolyzable ATP analogues.

Each member of the membrane GC family is a type I transmembrane protein that has a ligand-binding extracellular domain (ECD) linked by a single transmembrane domain (TMD) to an intracellular domain (ICD). Within the ICD the membrane-proximal region is homologous to protein kinases (KHD).

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\(^1\)The abbreviations used are: GC, guanylyl cyclase; RetGC, retinal guanylyl cyclase; OS, outer segment(s); GCAP, guanylyl cyclase activating protein; NPR, natriuretic peptide receptor; ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; KHD, kinase homology domain; wt, wild type; PCR, polymerase chain reaction; bp, base pairs; MOPS, 4-morpholinepropanesulfonic acid; BSA, bovine serum albumin; IC, intracellular; AMP-PNP, 5'-adenylylimidodiphosphate.
Adjacent to the K+H2O is a small domain that is likely to form an amphipathic α-helix and for which a clear role in the dimerization of the NPR-A intracellular domain has been established (Wilson and Chinkers, 1995). The C-terminal portion of the ICD contains the cyclase catalytic domain. Based on the homology between RetGC-1, NPR-A, NPR-B, and StaR and on hydropathy analysis, putative assignments have been made for the extracellular and intracellular domains of RetGC-1 (see Fig. 1) (Shyjan et al., 1992; Lowe et al., 1995; Wilson and Chinkers, 1995). The orientation of RetGC-1 in membranes has yet to be determined experimentally.

Previously characterized members of the membrane GC family are activated by the binding of peptide ligands to their extracellular domains (reviewed by Garbers (1992) and Garbers and Lowe (1994)). For example, the membrane GC NPR-A is stimulated by the binding of atrial natriuretic peptide to its ECD. In contrast, the Ca2+ sensitivity of RetGC-1 and RetGC-2 activity stimulated by GCAP-2 (Lowe et al., 1995), the absence of a signal peptide in the GCAP-2 primary sequence (Dizhoor et al., 1995), and the presence of Ca2+-binding sites on GCAP-2 all suggest that regulation of RetGC-1 and RetGC-2 by GCAP-2, Ca2+, and ATP occurs in the cytoplasm. To determine experimentally if GCAP-2 acts either through the predicted ICD of RetGC-1 or through the ECD, we expressed deletion mutants of RetGC-1 lacking the ECD or both the TMD and ECD. Our results show that RetGC-1 is regulated by GCAP-2 through the ICD and that removal of the ECD and TMD does not have a significant effect on regulation by GCAP-2, Ca2+, and ATP.

MATERIALS AND METHODS

Construction of RetGC-1 Deletion Mutants—The wt RetGC-1 cDNA, cloned into the EcoRI site of pBSκC(-)–5.5, was digested with XbaI and HindIII and ligated into a mammalian expression vector, pRC CMV (Invitrogen), digested with the same restriction enzymes. The deletion mutants JEDC RetGC-1 and ICD RetGC-1 were then constructed from wt RetGC-1 cDNA in pRC CMV using signal overlap extension PCR (Horton and Pease, 1991). Two pair of primers (primer pair: overlapping primer and flanking primer), sequential PCR reactions, and two convenient restriction sites were required for the construction of each deletion mutant. Of each pair of primers, a single primer was designed to contain an overlap that could hybridize with sequence contained by the opposing overlapping primer. The first PCR reaction was carried out with separate reactions for each primer pair, and then the resulting PCR product from each of the two reactions was gel purified and ligated into an expression vector. Large deletion mutants were removed from cell homogenates by centrifugation at 2000 × g for 10 min in a Beckman tabletop centrifuge at 4°C. To isolate membranes, homogenates were brought to 0.05 or 0.4 mM NaCl and centrifuged for 45 min in a Beckman TLA 100 tabletop ultracentrifuge at 288,000 × g in a TLA 120.3 rotor at 4°C. A pellet derived from one dish of cells was resuspended in 200–400 μl homogenization buffer. Membrane preparations and homogenates were frozen on dry ice and stored at −70°C. Protein content of membrane preparations was determined in the absence of reducing agents and in the presence of 0.1% SDS using BCA protein reagent (Pierce) with BSA as a standard. All HEK 293 cell growth and transfection procedures were carried out in a 5% CO2, 37°C humidified incubator.

Recombinant GCAP-2—Expression and Preparation—A cDNA of GCAP-2 (p24) (Dizhoor et al., 1995) was cloned into the bacterial expression vector pet 11d (Novagen). GCAP-2 was coexpressed in BL21 E. coli with yeast N-myristoyl transferase with 100 μg/ml myristic acid in Luria broth (Chen et al., 1995). Purification and activation of active myristoylated recombinant GCAP-2 from E. coli will be described elsewhere. Briefly, recombinant myristoylated GCAP-2 was insoluble and therefore was dissolved in 6 M urea and then refolded by overnight dialysis against 20 mM Tris, 1 mM EDTA, and 1 mM dithiothreitol. The recombinant GCAP-2 used in these studies had a specific activity indistinguishable from that of purified preparations of recombinal RetGC-2. The purification of retinal GCAP-2 has been described (Dizhoor et al., 1995). Protein concentrations of both retinal and recombinant GCAP-2 were determined using the Bio-Rad Protein assay reagent and protocol.

GC Assay—The measurement of GC activity was carried out essentially as described in Dizhoor et al. (1995). In brief, transiently transfected 293 cell homogenates or membranes were added to equimolar concentrations of 4 μM GC buffer (400 mM KCl, 200 mM MOPS, 28 mM 2-mercaptoethanol, 40 mM MgCl2, 32 mM NaCl, 4 mM EGTA), 12.5 μl of this mixture were added to purified retinal or recombinant GCAP-2 or BSA to reach a volume of 20 μl. The reaction was started by adding 5 μl of a 5 μM substrate solution (5 mM GTP, 25 μM cGMP, ~2 μCi of [32P]GTP, ~100,000 cpm [8-H]cGMP) and then incubated at 30°C for 30 min for wt RetGC-1 and ICD RetGC-1 or 1 h for ICD RetGC-1 because of its lower specific activity. The reaction was stopped by the addition of 100 μl of 100 mM Tris pH 8.0, heating to 40°C for 2 min. After centrifugation at 10,000 × g for 10 min to pellet the heat-denatured proteins, 32P-labeled cGMP was separated from [32P]GTP by chromatography on 6 μl of the reaction mix on a polyethyleneimine cellulose thin layer chromatography plate in 0.2 μl LiCl. Spots corresponding to cGMP were visualized on a short wavelength UV illuminator, excised, and eluted by gentle shaking for 10 min in 1 ml of 2 μl LiCl in a 20-ml scintillation vial, and both 32P and 3P were counted in 10 ml of scintillation fluid (ICN) in a Beckman model LS 3801 scintillation counter. For all assays less than 10% of the substrate was depleted. For experiments involving the effects of adenine nucleotides on GC activity, an appropriate concentration of adenine nucleotides was added to the substrate mix. For the ATP titration experiment the value of the basal GC activity of wt RetGC-1 for each of the two experiments was 1.57 ± 0.047 nmol cGMP/min/mg protein. The basal GC activity of ICD RetGC-1 for each of the two experiments was 0.422 ± 0.03 nmol cGMP/min/mg protein and for stimulated GC activity was 1.3 ± 0.2 and 0.5 ± 0.3 nmol cGMP/min/mg protein. The basal GC activity of ICD RetGC-1 for each of the two experiments was 0.367 ± 0.026 and 0.187 ± 0.013 nmol cGMP/min/mg protein. The activity was 1.307 ± 0.2 and 0.53 ± 0.047 nmol cGMP/min/mg protein.

Ca-EGTA Buffers—Ca-EGTA buffers were prepared from solutions of EGTA (Sigma) and EGTA saturated with CaCl2 (Fluka) by pH titration in strict accordance with the method of Tsien and Pozzan (1989). Free Ca2+ concentrations under the assay conditions were calculated
using a multi-factor program (Marks and Maxfield, 1991) and verified by Ca\(^{2+}\) electrode and by titration with Rhod-2 fluorescent dye (Calbiochem). In experiments where the free Ca\(^{2+}\) concentration was controlled by the addition of a Ca-EGTA buffer, no EGTA was present in the 4 x GC buffer, and 2 µl of a 20 mM Ca-EGTA buffer stock was added to the assay mixture to reach a final volume of 27 µl.

Antibody Preparation—Anti-peptide antibody RetGC-1-IC (intracellular) was generated in rabbits against a synthetic peptide corresponding to Ala\(^{642}\)–Gln\(^{655}\) from the intracellular domain of RetGC-1 and an additional cysteine added at the N terminus. The peptide was coupled to keyhole limpet hemocyanin with the cross-linking reagent, N-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce). This peptide is derived from the KHD of RetGC-1 and is distinct from any sequence of RetGC-2. The antibody was purified by specific binding to the peptide coupled to CNBr-activated Sepharose, eluted in 10 mM glycine, 0.5 M NaCl, 0.05% Tween 20 at pH 2.3, and the eluate was immediately neutralized to pH 7.5 with an aliquot of 1 M NaHPO\(_4\). The affinity purified antibody recognizes only a single band in immunoblot analysis of unwashed photoreceptor OS (data not shown).

Demonstration of Expression in HEK 293 Cells by Immunoblot Analysis—Proteins mixed with Laemmli sample buffer were electrophoresed on either 7.5 or 10% SDS tris-glycine gels and transferred to nitrocellulose membrane (Schleicher & Schuell) using the Bio-Rad Mini-PRO-TEIN system. The membrane was blocked in TTBS (Tris-buffered saline, 0.05% Tween 20) containing 10% dry milk for 1 h at room temperature and then probed with 4 nM of affinity purified RetGC-1-IC antibody in blocking buffer for 3 h at room temperature. After extensive washing in TTBS, the membrane was probed for 1 h at room temperature with a donkey anti-rabbit antibody coupled to horseradish peroxidase (Amersham Corp.), and after extensive washing with TTBS, the membrane was developed using chemiluminescent reagents (Amersham Corp.). With both transfected and untransfected HEK 293 cell homogenates or membranes isolated in low salt, three or four nonspecific bands showed up on immunoblots probed with the RetGC-1-IC antibody. However, washing the HEK 293 cell membranes with 0.4 M NaCl removed all but a single band for transfected cells and all bands for control cells. A single band corresponding to the predicted size of the recombinant variants of RetGC-1 and not these additional bands could be competed away with the peptide used to generate the antibody (data not shown).

RESULTS

To determine whether the ECD or TMD of RetGC-1 play a role in stimulation of GC activity by GCAP-2, deletion mutants were constructed that lacked either the ECD (ΔECD RetGC-1) or both the ECD and TMD domains (ICD RetGC-1) of RetGC-1 (Fig. 1). We then determined if the truncated proteins could be regulated in a Ca\(^{2+}\)-sensitive manner by GCAP-2. Both recombinant and purified retinal GCAP-2 were used to carry out these experiments. We also compared the effect of ATP on the truncated and wt forms of RetGC-1 to further gauge how these truncations affected the regulation of RetGC-1 activity.

Expression of wt RetGC-1 and ΔECD RetGC-1—HEK 293 cells were transfected with wt RetGC-1 or ΔECD RetGC-1 expression constructs, and expression of the recombinant proteins was demonstrated by immunoblot analysis (Fig. 2A). Immunoreactivity of the RetGC-1-IC antibody reveals a single band for both wt RetGC-1 and ΔECD using recombinant membranes washed in 0.4 M NaCl. Membranes were washed in 0.4 M NaCl to remove proteins that were recognized nonspecifically by the primary or secondary antibody (see “Materials and Methods”). The molecular weights of 114,656 and 71,545, predicted from the coding region of each construct, are similar to the relative molecular weights of the immunoreactive polypeptides. Both recombinant proteins are localized to the particu-
Adenine nucleotides potentiate the activation of \( \Delta \text{ECD RetGC-1} \) and wt RetGC-1 by GCAP-2. GC activity was measured for wt RetGC-1 (A) and \( \Delta \text{ECD RetGC-1} \) (B) as described under "Materials and Methods." Recombinant membranes were washed in 0.4 M NaCl and assayed in the presence of BSA (lanes a); 1.5 \( \mu \text{M} \) recombinant GCAP-2 (lanes b); BSA and 0.5 mM ATP (lanes c); 1.5 \( \mu \text{M} \) recombinant GCAP-2 and 0.5 mM AMP-PNP all in 7 mM Ca\(^{2+}\) (lanes d). All assay points contained a final free Ca\(^{2+}\) concentration of 7 mM. The bars represent the mean of duplicate data points with the range indicated by the error bars. The data shown are from one experiment and are representative of six experiments with wt RetGC-1 and two experiments with \( \Delta \text{ECD RetGC-1} \).

Sensitivity of ICD RetGC-1 to Salt Concentration—ICD RetGC-1 is regulated by both ATP and the nonhydrolyzable analogue AMP-PNP (Fig. 3). The ATP/GCAP-2-stimulated activity is approximately 2.7-fold above the effect of ATP alone. Adenosine nucleotides do not activate RetGC-1. For both wt RetGC-1 and \( \Delta \text{ECD RetGC-1} \), the effect of ATP reaches a maximum near 0.5 mM ATP then decreases. The decrease in activity above 0.5 mM ATP may be due to competition for binding at the catalytic site of RetGC-1 between ATP and the substrate, GTP. The nucleotide TTP had no effect on the stimulation of catalytic activity by GCAP-2 (data not shown).

Expression and Solubility of ICD RetGC-1—We also examined the deletion mutant, ICD RetGC-1, which lacks both the ECD and TMD. Its expression in transiently transfected HEK 293 cells is demonstrated by immunoblot analysis using the RetGC-1-1C antibody (Fig. 5A). We expected that ICD RetGC-1 would be a soluble enzyme, but the immunoreactivity of the recombinant protein resided entirely within the particulate fraction. Treatment with 1 M NaCl or 2% Triton X-100 did not remove the immunoreactivity from the particulate fraction (data not shown). However, ICD RetGC-1 can be partially solubilized (−50%) in 6 M urea without detergent. This suggests that ICD RetGC-1 no longer contains a TMD. Urea without detergent does not solubilize wt RetGC-1 and \( \Delta \text{ECD RetGC-1} \) (data not shown). These properties of ICD RetGC-1 may reflect the same interaction that makes bovine RetGC-1 insoluble in nonionic detergents (Hakki and Sitaramayya, 1990; Koch, 1991). The detergent insolubility of RetGC-1 both with and without the TMD suggests that RetGC-1 is either a large oligomeric structure or associates with the cytoskeleton.

Regulation of ICD RetGC-1 by GCAP-2/Ca\(^{2+}\)—ICD RetGC-1 is regulated by GCAP-2, Ca\(^{2+}\), and adenine nucleotides. The catalytic activity of ICD RetGC-1 is regulated by GCAP-2, Ca\(^{2+}\), and adenine nucleotides (Fig. 5B). However, its specific activity appears to be lowered significantly by the removal of the TMD. Even when immunoblot analysis suggests that ICD RetGC-1 is expressed at levels equal to or greater than recombinant wt RetGC-1 and \( \Delta \text{ECD RetGC-1} \), its specific activity remains significantly lower.

Sensitivity of ICD RetGC-1 to Salt Concentration—Like wt RetGC-1 and \( \Delta \text{ECD RetGC-1} \), recombinant ICD Ret GC-1 membranes were washed in 0.4 M NaCl to remove nonspecific immunoreactive bands. These same membranes were initially used to test ICD Ret GC-1 for Ca\(^{2+}\)-sensitive stimulation by GCAP-2. We discovered that unlike wt RetGC-1 and \( \Delta \text{ECD} \)
Regulation of RetGC-1

Expression of ICD RetGC-1 and its regulation by GCAP-2, Ca\(^{2+}\), and adenine nucleotides. A, HEK 293 cells were transiently transfected with either pRC CMV or pRC CMV ICD RetGC-1. Membranes from these cells were washed in 0.4 M NaCl, mixed with Laemmli sample buffer, and electrophoresed on a 10% SDS gel. After transfer to nitrocellulose, immunoblot analysis was carried out with the RetGC-1-IC antibody. A mass of 68,178 was calculated from the expected amino acid sequence of ICD RetGC-1 and approximates the relative molecular weight of the recombinant protein recognized by the antibody. B and C, GC activity was measured as described under "Materials and Methods" on cell homogenates from HEK 293 cells transiently transfected with pRC CMV or pRC CMV ICD RetGC-1. Assays were carried out in the presence of BSA in 7 nM Ca\(^{2+}\) (lanes a), 3 \(\mu M\) recombinant GCAP-2 in 7 nM Ca\(^{2+}\) (lanes b), 0.5 \(\mu M\) ATP and 3 \(\mu M\) recombinant GCAP-2 in 7 nM Ca\(^{2+}\) (lane c), 3 \(\mu M\) recombinant GCAP-2 in 1.2 \(\mu M\) Ca\(^{2+}\) (lane d), 0.5 \(\mu M\) ATP and 3 \(\mu M\) recombinant GCAP-2 in 1.2 \(\mu M\) Ca\(^{2+}\) (lane e), 0.5 \(\mu M\) ATP and 3 \(\mu M\) recombinant GCAP-2 in 1.2 \(\mu M\) Ca\(^{2+}\) (lane f). Cell homogenates were used in these experiments, because the ability of ICD Ret GC-1 to be stimulated by GCAP-2 is lost in membranes washed with 0.4 M NaCl. The bars represent the mean of triplicate data points with the standard deviation indicated by the error bars. The data are representative of six independent experiments.

RetGC-1, ICD RetGC-1 is inactive at high NaCl concentrations (data not shown). Homogenates and membranes isolated in 0.05 M NaCl are stimulated by GCAP-2, but membranes washed in 0.4 M NaCl lose most or all GCAP-2-stimulated activity. The lowered specific activity of ICD RetGC-1 relative to wt RetGC-1 and its heightened sensitivity to NaCl may reflect instability in its structure induced by removal of the TMD.

Effect of RetGC-1 Deletions on \(K_{1/2}\) of GCAP-2 and EC\(_{50}\) for Ca\(^{2+}\).—To determine if the ECD or TMD influenced the ability of GCAP-2 to stimulate GC activity, we compared the EC\(_{50}\) values for Ca\(^{2+}\) and for GCAP-2 stimulation of wt RetGC-1, \(\Delta\)ECD RetGC-1, and ICD RetGC-1. The Ca\(^{2+}\) sensitivity for stimulation of wt RetGC-1 and the deletion mutants overlap closely, with an EC\(_{50}\) for Ca\(^{2+}\) near 280 nM (Fig. 6). This value is in the range of previously reported EC\(_{50}\) values for Ca\(^{2+}\) of 90, 240, or 144 nM for the GC activity of OS membranes (Koch and Stryer, 1988; Dizhoor et al., 1991; Wolbring and Schnetkamp, 1995) and 200 nM for recombinant wt RetGC-1 (Dizhoor et al., 1994). The half-saturation (\(K_{V}\)) values for stimulation by GCAP-2 were also measured. Mean \(K_{V}\) values and standard deviations of 5.42 ± 2.31, 8.03 ± 2.25, and 2.27 ± 0.031 \(\mu M\) were obtained for wt RetGC-1, \(\Delta\)ECD Ret GC-1, and ICD RetGC-1, accordingly (Fig. 7). The \(K_{V}\) values obtained for the deletion mutants were each statistically different from the values obtained for wt RetGC-1 in two out of three experiments with a 95% confidence interval. However, it is clear that no gross alterations of the \(K_{V}\) value have been introduced by the removal of the ECD and TMD. Interestingly, these \(K_{V}\) values are 10–40-fold higher than the value obtained when recombinant GCAP-2 is reconstituted with washed OS membranes, but the general characteristics of regulation by GCAP-2/Ca\(^{2+}\) are clearly represented. The higher \(K_{V}\) values may reflect that recombinant RetGC-1 is differentially modified than the OS membranes of photoreceptors, is glycosylated, and is predicted from the cDNA to have a signal peptide for membrane localization on the N terminus (Dizhoor et al. 1994; Koch et al., 1994; Shyjan et al., 1992; Lowe et al., 1995). These properties of RetGC-1 suggest that the ECD is either extracellular or intracellular and that a new mechanism for regulating membrane GCs, that HEK 293 cell membranes have a different lipid composition than bovine OS membranes, or that an additional protein, present in OS membranes, which increases the potency of GCAP-2 is absent in our reconstituted system.

DISCUSSION

The data we present here show that the ECD and TMD of RetGC-1 are not required for its regulation by Ca\(^{2+}\) and GCAP-2. GCAP-2 must exert its action through the intracellular domain of RetGC-1. RetGC-1 associates with the OS membranes of photoreceptors, is glycosylated, and is predicted from the cDNA to have a signal peptide for membrane localization on the N terminus (Dizhoor et al. 1994; Koch et al., 1994; Shyjan et al., 1992; Lowe et al., 1995). These properties of RetGC-1 suggest that the ECD is either extracellular or intracellular and that a new mechanism for regulating membrane GC activity was measured on HEK 293 cell homogenates as described under "Materials and Methods." Assays of wt RetGC-1 (closed triangle), \(\Delta\)ECD RetGC-1 (open circle), and ICD RetGC-1 (open square) in the presence of retinal GCAP-2 and 0.5 mM ATP were carried out at Ca\(^{2+}\) concentrations ranging from 7 nM to 2.6 \(\mu M\) free Ca\(^{2+}\). A Ca\(^{2+}\)-EGTA buffering system was used to achieve the desired free Ca\(^{2+}\) concentrations. Duplicate data points are plotted from a single experiment and are representative of two independent experiments. GC activities were normalized for comparison.

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GCs from the cytoplasm has now been established. This does not, however, preclude RetGC-1 from also having a yet to be discovered extracellular ligand analogous to those of NPR-A, NPR-B, and StaR.

We also show that ATP potentiates but is not necessary for the stimulatory effect of GCAP-2 on RetGC-1 and that neither the ECD nor TMD are necessary for this effect. The observation that AMP-PNP also potentiates stimulation of RetGC-1 by GCAP-2 indicates that the effect of ATP is not due to phosphorylation or ATPase activity. In vivo, a 2–3-fold increase in the rate of cGMP synthesis could potentially have a large effect on the inward current of a photoreceptor OS because cGMP binding to the cGMP gated channels is cooperative (Fesenko et al., 1985). However, it has yet to be experimentally determined whether intracellular ATP levels vary enough to regulate RetGC-1 activity in vivo.

Based on available published data, it appears that the non-obligatory effect of adenine nucleotides on RetGC-1 differentiates it from NPR-A, for which ATP is a necessary cofactor for stimulation by atrial natriuretic peptide (Chinkers and Garbers, 1991; Marala et al., 1991). In contrast, ligand activation of StaR activity does not require ATP but is potentiated approximately 2-fold by ATP in a manner similar to our findings with RetGC-1 (Vaan drager et al., 1993a). The activation of immunoaffinity-purified NPR-A and StaR by ATP and their extracellular ligands has been reported (Vaan drager et al., 1993b; Wong et al., 1995). This strongly suggests that ATP regulates both receptors through direct binding. Interestingly, both RetGC-1 and StaR lack a glycine-rich nucleotide-binding motif, which is conserved in protein kinases and in the KHDs of both NPR-A and NPR-B (Koller et al., 1992; Shyjan et al., 1992). Mutations in the glycine-rich sequence make NPR-A insensitive to ATP and atrial natriuretic peptide (Goraczniak et al., 1992; Duda et al., 1993). These data taken together suggest that the effect of ATP on membrane GCs may be mediated by binding of ATP to the KHDs.

It is unclear why the effect of ATP we observe for washed OS membranes is not as pronounced as when we use recombinant RetGC-1. One possibility is that the state of phosphorylation of RetGC-1 influences the effect of adenine nucleotides. Recently, it was reported that ATP has a 2-fold stimulatory effect on Ca$^{2+}$-sensitive GC activity in fresh intact OS (Wolbring and Schnetkamp, 1995). However, the effect of ATP was lost by washing the OS or by adding inhibitors of protein kinase C. The ATP effect could be restored by treating washed OS with a purified preparation of brain protein kinase C. Together with our data, these findings suggest that phosphorylation by protein kinase C may be a prerequisite for a noncatalytic role of ATP in the potentiation of RetGC-1 activation. Different states of phosphorylation of OS GC and recombinant RetGC-1 might explain the different magnitude of ATP effects observed with recombinant RetGC-1 and OS GCs.

Although we have shown that the intracellular domain of RetGC-1 is sufficient for activation, we have not shown whether or not GCAP-2 functions through direct binding to an intracellular domain of RetGC-1. The reconstitution of Ca$^{2+}$-sensitive regulation of recombinant RetGC-1 using recombinant GCAP-2 supports this model but does not rule out the involvement of an additional factor found in both OS and in HEK 293 cell membranes. For example, if the detergent insolubility of both OS and recombinant RetGC-1 is indicative of a cytoskeletal association, then we cannot rule out the involvement of a cytoskeletal protein as an intermediate for stimulation by GCAP-2. It also remains to be determined through which intracellular subdomain(s) of RetGC-1 that GCAP-2 transduces its direct or indirect stimulatory effect on catalytic activity.

RetGC-2 is another photoreceptor specific membrane GC that has been shown to be stimulated by GCAP-2 in vitro (Lowe et al., 1995). It is highly homologous to RetGC-1, and together these proteins may define a new subfamily of membrane GCs that respond to intracellular activators.

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