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Inactivation of EF-hands Makes GCAP-2 (p24) a Constitutive Activator of Photoreceptor Guanylyl Cyclase by Preventing a Ca^{2+}-induced "Activator-to-Inhibitor" Transition*

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Guanylyl cyclase activator proteins GCAP-1 and GCAP-2 (Dizhoor *et al.*, 1995, Gorczyca *et al.*, 1995) are members of a recently identified subclass of EF-hand type Ca^{2+}-binding proteins that respond to Ca^{2+} differently than any other known members of the EF-hand superfamily. GCAPs acquire an activating conformation only in their Ca^{2+}-free form. Free Ca^{2+} concentrations corresponding to levels in dark-adapted vertebrate photoreceptors inhibit the ability of GCAPs to activate photoreceptor guanylyl cyclases (RetGCs). We studied the effects of mutations that block binding of Ca^{2+} to the EF-hands of GCAP-2. Unlike other EF-hand proteins, which fail to activate their target when their EF-hands are inactivated by mutations, GCAP-2 with any single EF-hand inactivated remains active and is 3–6 times less sensitive to the inhibitory effect of Ca^{2+}. Inactivation of any two or all three EF-hands produces active forms of GCAP-2 that are insensitive to inhibition by physiological intracellular concentrations of Ca^{2+}. Unexpectedly we also found that activation of RetGCs by a Ca^{2+}-insensitive mutant is inhibited by Ca^{2+}-loaded wild type GCAP-2. We propose the following. 1) GCAP-2 can exist in two extreme functional forms: an apo form that activates RetGCs and a Ca^{2+}-loaded form that blocks activation of RetGCs. 2) All three EF-hands of GCAP-2 contribute to the inhibitory effect of Ca^{2+}. 3) Inactivation of two or three EF-hands is sufficient to shift the "activator-inhibitor" transition outside the physiological range of intracellular free Ca^{2+}.

A variety of proteins with EF-hand type Ca^{2+}-binding domains interact with their targets only when their EF-hands are occupied by Ca^{2+} (reviewed by Strynadka *et al.* (1989) and Crivici and Ikura (1995)). However, two recently discovered guanylyl cyclase-activating proteins, GCAP-1 and GCAP-2 (Dizhoor *et al.*, 1994, 1995; Gorczyca *et al.*, 1994, 1995), demonstrate remarkably different behavior. They activate their target enzyme, a photoreceptor membrane guanylyl cyclase (RetGC),[1] only in their Ca^{2+}-free or "apo" forms. The apo form of neuromodulin is the only other known example of an EF-hand protein that is capable of interacting with its target protein, calmodulin. However, even in that case the function of neuromodulin appears to "dock" calmodulin rather than imparting Ca^{2+} sensitivity to an effector enzyme (reviewed by Liu and Storm (1990) and Crivici and Ikura (1995)). The unique type of Ca^{2+} sensitivity of GCAPs accounts for their biological function as Ca^{2+} sensors in a feedback mechanism that regulates resynthesis of cGMP in vertebrate photoreceptors (Koch and Stryer, 1988).

Ca^{2+} enters outer segments (OS) of vertebrate photoreceptors through cGMP-gated Na^+/Ca^{2+} channels in the plasma membranes. These channels are open in the dark, but light closes them by stimulating cGMP hydrolysis. Channel closure lowers the intracellular free Ca^{2+} concentration because Ca^{2+} is removed from the OS by a light-independent Na^+/K^+, Ca^{2+} exchanger (reviewed by Lagnado and Baylor (1992) and Yarfitz and Hurley (1994)). The decrease in free Ca^{2+} concentration stimulates cGMP synthesis (Koch and Stryer, 1988). It has been shown *in vitro* that when free Ca^{2+} decreases from 500 nM to below 50 nM, GCAP-2 (Dizhoor *et al.*, 1994, 1995; Lowe *et al.*, 1995) and its homolog, GCAP-1 (Gorczyca *et al.*, 1994, 1995; Palczewski *et al.*, 1994), activate RetGCs. GCAPs activate RetGC via the cyclase intracellular domain (Laura *et al.*, 1996). GCAPs and GCAP-regulated membrane guanylyl cyclases have been found only in photoreceptors (reviewed by Garbers and Lowe (1995)). GCAP-1 and GCAP-2 belong to the family of recoverin-like proteins (Dizhoor *et al.*, 1995, Gorczyca *et al.*, 1995). Each member of this family has four EF-hand like domains (Fig. 1).

The present study was undertaken to understand the role of Ca^{2+}-binding domains in GCAP-2 function. We analyzed the effects of EF-hand mutations on the ability of recombinant GCAP-2 to stimulate RetGC activity in OS membranes. Inactivation of the EF-hands produced a constitutively active form of GCAP-2 that is insensitive to Ca^{2+}. We also demonstrated that Ca^{2+} induces a transition of wild type GCAP-2 into an inhibitor form that competes with constitutively active GCAP-2. This provides an additional important feedback element for regulation of RetGCs.

MATERIALS AND METHODS

Photoreceptor Outer Segments

Photoreceptor outer segments were isolated from frozen bovine retinas using sucrose gradient centrifugation (McDowell, 1993) and washed in low salt buffer in order to remove endogenous GCAPs as described (Dizhoor *et al.*, 1994, 1995).

Guanylyl Cyclase

Guanylyl cyclase was assayed using washed OS membranes containing both RetGCs as described in detail (Dizhoor *et al.*, 1995), except polyethyleneimine cellulose TLC plates were purchased from Merck.

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[1] The abbreviations used are: RetGC, photoreceptor guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; OS, photoreceptor outer segment(s).

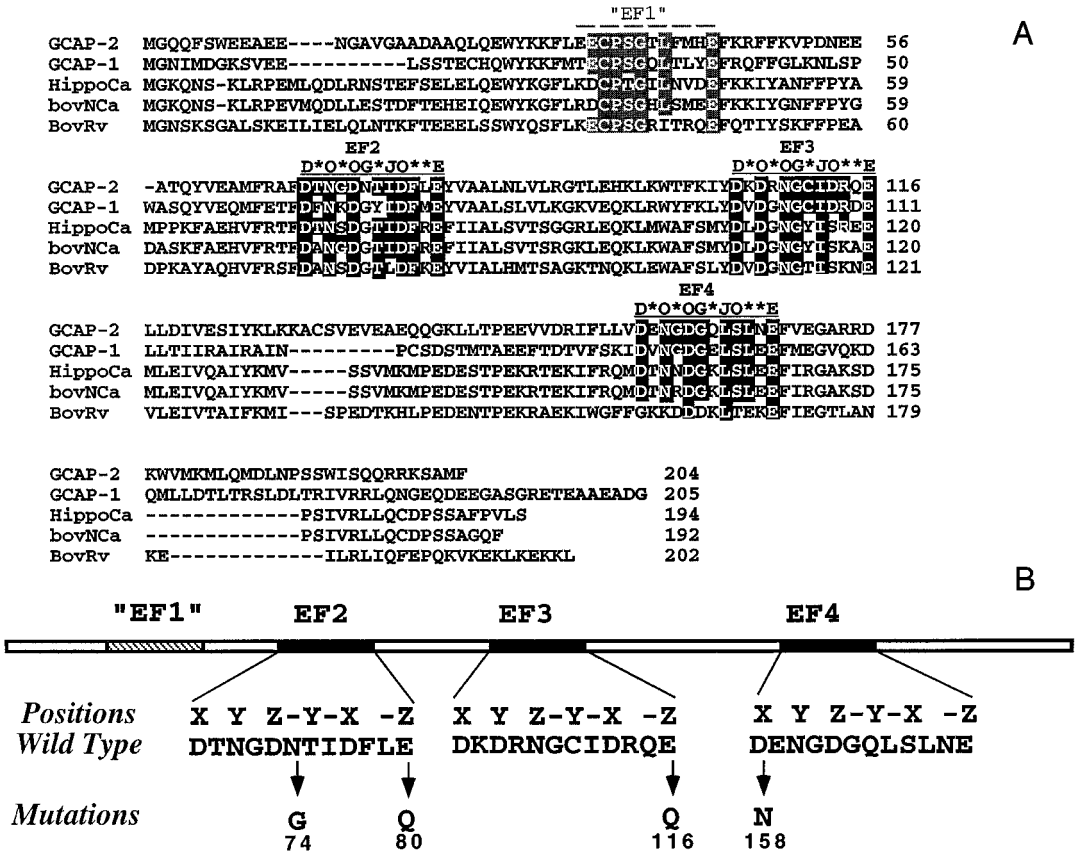

FIG. 1. **EF-hands of GCAP-2 and mutations used in this study.** *A*, a homology comparison between EF-hand related regions of recoverin-like proteins: bovine GCAP-2 (Dizhoor *et al.*, 1995), bovine GCAP-1 (Palczewski *et al.*, 1994), rat hippocalcin (Kobayashi *et al.*, 1992), bovine neurocalcin (Okazaki *et al.*, 1993), and bovine recoverin (Dizhoor *et al.*, 1991). A consensus motif for the EF-hands: *o*, amino acid with an oxygen-containing side chain; *J*, Ile, Leu, or Val; *, any amino acid. *EF1–EF4*, EF-hand-related sequences. *EF1* is lacking two oxygen-containing side chain amino acids. *EF2* in wild-type GCAP-2 has Asn-74 instead of a consensus G. *B*, mutations introduced into EF-hands of GCAP-2: N74G, to make EF2 a complete match to the consensus sequence; E80Q, to inactivate EF2 ($EF2^-$ mutant); E116Q- to inactivate EF3 ($EF3^-$ mutant); or D158N, to inactivate EF4 ($EF4^-$ mutant). The following combinations of inactivating mutations were used to generate double or triple mutations: *E80Q*/*E116Q*, $EF(2;3)^-$; *E80Q*/*D158N*, $EF(2;4)^-$; *E116Q*/*D158N*, $EF(3;4)^-$; *E80Q*/*E116Q*/*D158N*, $EF(2;3;4)^-$.

Expression of Recombinant GCAP-2

Eukaryotic Expression—GCAP-2 cDNA and its mutant forms were expressed in HEK293 cells under control of the β-actin promoter as described (Dizhoor *et al.*, 1995). As a negative control, cells were mock-transfected with vector DNA only. The amount of expressed GCAP-2 in the soluble cell fraction was evaluated using immunoblotting as described (Dizhoor *et al.*, 1995).

Prokaryotic Expression—The GCAP-2 cDNA coding region (Dizhoor *et al.*, 1995) was inserted into the *Nco*I/*Bam*HI sites of the pET11d vector (Novagen) and expressed under control of the isopropyl-1-thio-β-D-galactopyranoside-dependent promoter in the BL21(DE3)pLysS *Escherichia coli* strain carrying a p88131 plasmid encoding yeast *N*-myristoyl transferase (a gift from Dr. J. Gordon). As an *N*-myristoyl donor, free myristic acid was added into the suspension of bacterial cells to 50 μg/ml 30 min before the induction with isopropyl-1-thio-β-D-galactopyranoside as described (Dizhoor *et al.*, 1993). The expressed GCAP-2 and its mutants were always found in the inclusion bodies. The cells were disrupted by three cycles of ultrasonication, and the insoluble material was washed three times with a 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 7 mM 2-mercaptoethanol, 100 μM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin (Buffer A). GCAP-2 was extracted from the pellet by homogenization in buffer A containing 6 M freshly deionized urea for 30 min and dialyzed twice against 300-1000 volumes of Buffer A overnight at 4 °C. Precipitate was removed by centrifugation at 30,000 × *g* for 10 min. Final purification of recombinant protein was achieved by gel filtration on a Superdex 75 column. The molecular mass of recombinant myristoylated GCAP-2 was determined using electrospray ionization mass spectrometry (Dizhoor *et al.*, 1992). It corresponded to the calculated molecular mass of GCAP-2 (Dizhoor *et al.*, 1994, 1995) having its N-terminal Met removed and the remaining Gly-2 myristoylated. Within the accuracy of the method, it was identical to the mass of GCAP-2 isolated from bovine retinas.

Mutagenesis of GCAP-2

Polymerase chain reaction was used to generate site-directed mutants using a "splicing by overlap extension" approach (Horton and Pease, 1991). Nucleotide substitutions were incorporated into overlapping polymerase chain reaction primers 24–30 base pairs long during their chemical synthesis. High concentrations (~100–200 ng) of GCAP-2 cDNA and *Pfu* polymerase (Promega) were used to decrease the possibility of random mutations. The positions of mutations were verified by cDNA sequencing, and the masses of recombinant mutant proteins expressed in *E. coli* were also verified by mass spectrometry.

Ca-EGTA Buffers

Ca-EGTA buffers were calculated using a multifactor program "Bound and Determined," based on the algorithm of Marks and Maxfield (1994), and were prepared according to the method of Tsien and Pozzan (1989), and free Ca^{2+} concentrations were verified using Rhod-2 Ca^{2+}-sensitive dye (Calbiochem) and a Ca^{2+}-selective electrode (Orion).

RESULTS AND DISCUSSION

There are four EF-hand like regions in GCAP-2 (Fig. 1, *EF1–EF4*) (Dizhoor *et al.*, 1995), as in other members of the recoverin family. Strictly speaking, "EF1" in GCAP-2 is not a true EF-hand. It cannot bind Ca^{2+} because it lacks two oxygen-containing side chain amino acids at Ca^{2+} coordinating positions "*Y*" and "−*X*" (positions in EF-hand are given according to Strynadka *et al.* (1989) and Babu *et al.* (1992)), important for Ca^{2+} binding. It also has Glu instead of Asp in the *X* position. No metal ion was bound within the similarly disrupted EF-hand related region of recoverin (Flaherty *et al.*, 1993). The other three EF-hands of GCAP-2 have all the proper amino acid

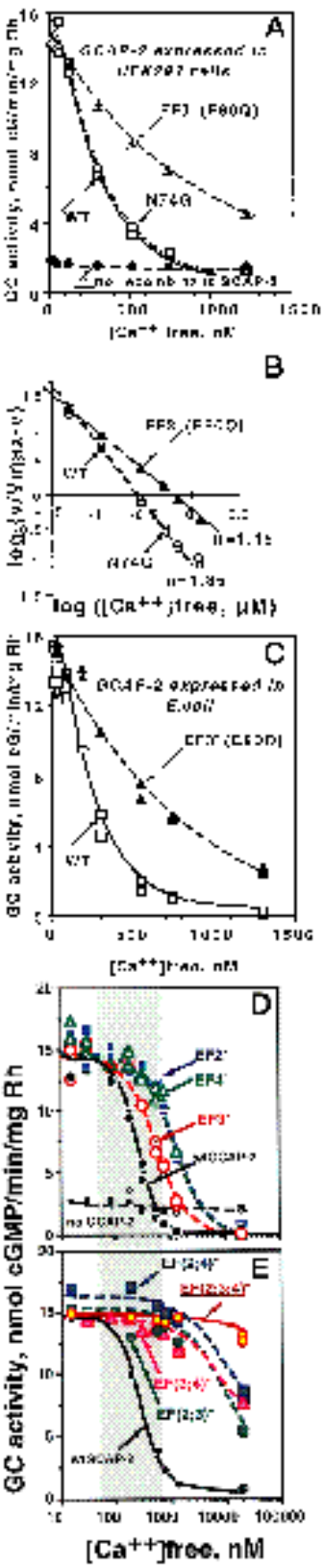

FIG. 2. **The Ca^{2+} sensitivities of wild type and mutated GCAP-2.** *A*, wild type and mutant GCAP-2 cDNA (N74G or E80Q) were expressed in HEK293 cells and soluble extracts (~50 μg of total protein) from the cells containing equal amounts of the expressed proteins were reconstituted with washed outer segment membranes to assay overall (both RetGC-1 and RetGC-2) guanylyl cyclase activity. As a control, an extract from cells transfected with the vector without GCAP-2 cDNA was used. *B*, a Hill plot using data presented in *panel A* demonstrates the effect of N74G or W80Q substitutions on the cooperativity of the inhibitory effect of Ca^{2+}. *C*, wild type GCAP-2 or E80Q GCAP-2 were expressed in *E. coli*. 2 μg of either protein were reconstituted with washed OS membranes. *D*, the effect of single EF-hand inactivations. Mutant recombinant proteins (5 μg each) expressed in *E. coli* were reconstituted with washed OS membranes and assayed for their GC stimulating activities as a function of free Ca^{2+} concentration. Rabbit serum albumin instead of GCAP-2 was used as a control to demonstrate basal activity of RetGC ("no GCAP-2" curve). At free Ca^{2+} concentration > 10 μM, wild-type GCAP-2 and single EF-hand knock-out mutants decrease basal activity of the cyclase. In *panel A* the maximal concentration of free Ca^{2+} was only 1.2 μM. *E*, mutants with any two or all three EF hands inactivated. The *shadowed area* in *D* and *E* corresponds to the range of free Ca^{2+} concentrations found in vertebrate photoreceptors upon their transition between light- and dark-adapted states (Gray-Keller and Detwiler, 1994).

side chains for coordinating Ca^{2+}. EF2 has a single deviation from the consensus sequence, Asn-74 instead of the usual Gly between the coordinating residues "*Z*" and "−*Y*" of the EF-hand motif (Strynadka *et al.*, 1989). We used site-directed mutagenesis of the three EF-hands of GCAP-2 to study their functional significance in imparting Ca^{2+} sensitivity to RetGC.

We found that GCAP-2 expressed either in HEK293 cells or in *E. coli* activates OS GC in a Ca^{2+}-sensitive manner indistinguishable from activation by GCAP-2 isolated from bovine retina. In both cases the EC_{50} for the inhibitory effect of Ca^{2+} is ~250 nM with a Hill coefficient of 1.7–1.9 (Fig. 2, *A* and *C*). Half-saturation of RetGCs activity in our assay system containing OS membranes occurs at ~500 nM recombinant GCAP-2, consistent with previous results using GCAP-2 isolated from bovine retina (Dizhoor *et al.*, 1994, 1995; Gorczyca *et al.*, 1995). These results validate our use of recombinant GCAP-2 from both eukaryotic and prokaryotic cells for the *in vitro* mutagenesis studies reported here.

One might expect that the substitution N74G (Fig. 1*B*) would improve EF2 and increase the Ca^{2+} sensitivity of GCAP-2. However, we find that the Ca^{2+} sensitivity of N74G GCAP-2 expressed in HEK293 cells is indistinguishable from that of the wild type GCAP-2 (Fig. 2, *A* and *B*). This suggests that either EF2 does not participate in Ca^{2+} sensitivity of GCAP-2 or that EF2 is important but the naturally occurring Asn-74 effectively substitutes for Gly. We resolved that issue by using a different mutation that inactivates EF2. As shown below, we find that EF2 is, indeed, important for the Ca^{2+} sensitivity of GCAP-2.

It has been established that carboxyl side chains of the first Asp (position *X*) or the last Glu (position −*Z*) of an EF-hand are essential for coordinating Ca^{2+}. Their substitution with Asn or Gln, respectively, hampers Ca^{2+} binding and inactivates an EF-hand as a regulatory domain (Strynadka *et al.*, 1989, Babu *et al.*, 1992). In order to inactivate EF2, EF3 or EF4 domains of GCAP-2 we introduced point mutations, E80Q, E116Q, or D158N, respectively, as indicated in Fig. 1*B*. Inactivation of EF3 (E116Q) decreases Ca^{2+} sensitivity and cooperativity (Fig. 2, *A* and *B*). E116Q GCAP-2 expressed either in HEK293 cells or in *E. coli* gave equivalent results (Fig. 2, *A* and *C*). This confirms that expression in *E. coli*, solubilization/dialysis and purification do not alter the functional properties of GCAP-2. Fig. 2*D* demonstrates that inactivating any one of the three EF-hands does not interfere with activation of OS GC at low Ca^{2+} concentrations, but substantially (3–6-fold) reduces the Ca^{2+} sensitivity of GCAP-2. Mutations in EF2 or EF4 reduce Ca^{2+} sensitivity more effectively than a mutation in EF3.

A precise value of the intracellular free Ca^{2+} level in mammalian photoreceptors has not yet been reported. In lower vertebrate photoreceptors, however, it was most recently evaluated using Ca^{2+}-sensitive fluorescent dyes (Gray-Keller and Detwiler, 1994). The bulk free Ca^{2+} concentration in dark-adapted photoreceptors is near 500 nM, and after light adaptation it decreases to as low as 50 nM. On this basis we refer to

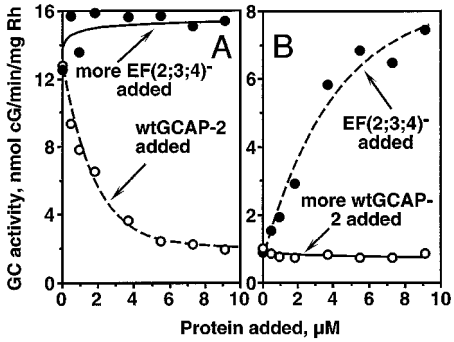

FIG. 3. **Effect of the Ca^{2+}-loaded form of GCAP-2 on RetGC stimulation by a Ca^{2+}-insensitive mutant of GCAP-2.** *A*, washed OS membranes were reconstituted with 2.75 μM GCAP-2 EF(2;3;4)$^-$ at 1.2 μM free Ca^{2+}. Increasing concentrations of either GCAP-2 EF(2;3;4)$^-$ or wild-type GCAP-2 were then added into the assay mixture as indicated on the *x* axis. *B*, washed OS membranes were reconstituted with 2.75 μM wild-type GCAP-2 at 1.2 μM free Ca^{2+}. Increasing concentrations of either wild type GCAP-2 or the EF(2;3;4)$^-$ mutant were then added into the assay mixture as indicated on the *x* axis.

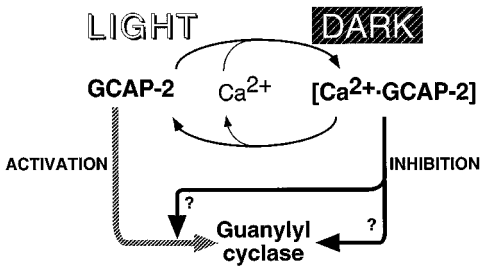

FIG. 4. **A model for the transition of GCAP-2 between the two functional states that regulate photoreceptor GC activity.** In the light, when the intracellular Ca^{2+} concentration is low, GCAP-2 activates GC. An increase in free Ca^{2+} concentration as a result of reopening cGMP-gated channels causes the transition of GCAP-2 into its inhibitory Ca^{2+}-loaded form, which also competes with the remaining active form of GCAP-2 to facilitate RetGC inactivation.

submicromolar free Ca^{2+}concentrations as the "physiological range" (*shadowed area* in Fig. 2, *D* and *E*). Regulation of RetGCs by Ca^{2+}*in vitro* takes place within that range (Koch and Stryer, 1988; Dizhoor *et al.*, 1994, 1995; Gorczyca *et al.*, 1995; Lowe *et al.*, 1995). However, the mutant forms of GCAP-2 shown in Fig. 2*D* are only partially inhibited by free Ca^{2+} within its physiological range.

In order to evaluate the contribution of each individual EF-hand to the overall Ca^{2+} sensitivity of GCAP-2, we generated double mutations that inactivate pairs of EF-hands: EF2 and EF3 (E80Q/E116Q), EF2 and EF4 (E80Q/D158N), EF3 and EF4 (E116Q/D158N). We also inactivated all three EF-hands (E80Q/E116Q/D158N). Mutating any pair of EF-hands makes GCAP-2 insensitive to the inhibitory effect of submicromolar Ca^{2+} (Fig. 2*E*). However, the double mutants of GCAP-2 are ~50% inhibited by very high (>10 μM) free Ca^{2+}. The most dramatic effect on Ca^{2+} sensitivity of GCAP-2 occurred in the triple mutant E80Q/E116Q/D158N, which is fully active within the whole range of free Ca^{2+} concentrations between 15 nM and 20 μM (Fig. 2*E*). Importantly, this mutant, like all the other GCAP-2 mutants used in this study, is not impaired in its ability to fully activate RetGCs.

According to the original model by Koch and Stryer (1988), photoreceptor membrane guanylyl cyclase is regulated by a Ca^{2+}-binding protein that is active at low Ca^{2+} and inactive at high Ca^{2+}concentrations. By using the EF-hand mutations described above, we have found that there is an important additional feedback element. The Ca^{2+}-loaded form of GCAP-2 effectively inhibits stimulation of RetGCs by the active form of GCAP-2 (Fig. 3). OS membranes reconstituted with the Ca^{2+}-insensitive GCAP-2 triple mutant E80Q/E116Q/D158N demonstrate high RetGC activity, even at 1.2 μM free Ca^{2+}. Further addition of the mutant protein produces little or no effect on cyclase activity. However, addition of wild type GCAP-2 at 1.2 μM Ca^{2+} inhibits RetGC activity that was stimulated by the triple mutant (Fig. 3*A*). Conversely, when OS membranes were initially reconstituted with wild type GCAP-2 at 1.2 μM Ca^{2+}, only basal GC activity was detected. Additional wild type GCAP-2 had no effect, but addition of the constitutively active triple mutant GCAP-2 stimulated cyclase activity (Fig. 3*B*). In the presence of 1.2 μM free Ca^{2+}, wild type GCAP-2 increased the EC_{50} for the triple mutant from ~0.5 to ~ 6 μM (not shown). Apparently, the Ca^{2+}-loaded form of GCAP-2 is not only incapable of activating the cyclase itself, it also strongly competes with the active form of GCAP-2. The Ca^{2+}-loaded form of GCAP-2 may also affect basal cyclase activity. At Ca^{2+} concentrations above 1 μM, basal RetGC activity in washed OS membranes is higher than in the presence of GCAP-2 (Fig. 2*D*). Based on these results, we propose a model for regulation of RetGC by GCAP-2 (Fig. 4).

High free Ca^{2+} (~500 nM) in dark-adapted photoreceptors maintains GCAP-2 in the Ca^{2+}-loaded form incapable of activating GC. After illumination, when the intracellular free Ca^{2+} concentration is low, GCAP-2 transforms into its active "apo" form and stimulates cGMP resynthesis. When cGMP in the cell is restored and cGMP gated channels open, the free Ca^{2+} concentration increases and transforms GCAP-2 back into its Ca^{2+}-loaded form. This form no longer activates RetGC, but instead inhibits it. This facilitates the turn-off of cGMP resynthesis. The ability of Ca^{2+}-loaded GCAP-2 to inhibit GC could also have an additional function such as preventing any spontaneous activation of RetGC in dark-adapted photoreceptors. It is remarkable that GCAP-2 can be either an activator or inhibitor of RetGC. The Ca^{2+}-loaded form of GCAP-2 strongly competes with the active protein (Fig. 3). There are at least two possible mechanisms for such competition. The inhibitory form of GCAP-2 might interact with the target RetGC and thus outcompete the active apo form. Alternatively, the inhibitory effect may reflect oligomerization. For example, the Ca^{2+}-loaded form of GCAP-2 may interact directly with the apo form to form an inactive complex.

In other EF-hand proteins, such as calmodulin, not all EF-hands are equally involved in regulating their targets (reviewed by Strynadka *et al.* (1989)). GCAP-2 is remarkably different in this regard. Our study has focused on the role of EF hands in regulating GCAP-2 activity. As a criteria for functional significance, we compared the ability of GCAP-2 mutants with inactivated EF-hands to stimulate RetGC. Surprisingly, each mutant demonstrates a substantial loss of Ca^{2+} sensitivity. Our data suggest that each EF hand contributes to the overall regulation of a cyclase-activating domain in GCAP-2. In one model for this regulation, each EF-hand would directly interact with such a domain. Alternatively, only one EF-hand might interact with the cyclase-activating region, but the activity of this EF-hand would depend on cooperative Ca^{2+} binding involving other EF-hand(s) (Waltersson *et al.*, 1993). The loss of cooperativity in Fig. 2*B* indicates that EF-hands in GCAP-2 may interact. At least two functional EF-hands are required for GCAP-2 to operate within the physiological range of free Ca^{2+}. Introduction of the Ca^{2+} insensitive GCAP-2 double or triple EF-hand mutants into intact photoreceptors would provide a useful model for studying the physiological role of Ca^{2+} feedback in photoreceptor recovery and light adaptation.

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