Factors that Determine Ca^{2+} Sensitivity of Photoreceptor Guanylyl Cyclase. Kinetic Analysis of the Interaction between the Ca^{2+}-Bound and the Ca^{2+}-Free Guanylyl Cyclase Activating Proteins (GCAPs) and Recombinant Photoreceptor Guanylyl Cyclase 1 (RetGC-1)†

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ABSTRACT: We explored the possibility that, in the regulation of an effector enzyme by a Ca^{2+}-sensor protein, the actual Ca^{2+} sensitivity of the effector enzyme can be determined not only by the affinity of the Ca^{2+}-sensor protein for Ca^{2+} but also by the relative affinities of its Ca^{2+}-bound versus Ca^{2+}-free form for the effector enzyme. As a model, we used Ca^{2+}-sensitive activation of photoreceptor guanylyl cyclase (RetGC-1) by guanylyl cyclase activating proteins (GCAPs). A substitution Arg^{838}Ser in RetGC-1 found in human patients with cone−rod dystrophy is known to shift the Ca^{2+} sensitivity of RetGC-1 regulation by GCAP-1 to a higher Ca^{2+} range. We find that at physiological concentrations of Mg^{2+} this mutation increases the free Ca^{2+} concentration required for half-maximal inhibition of the cyclase from 0.27 to 0.61 μM. Similar to rod outer segment cyclase, Ca^{2+} sensitivity of recombinant RetGC-1 is strongly affected by Mg^{2+}, but the shift in Ca^{2+} sensitivity for the R838S mutant relative to the wild type is Mg^{2+}-independent. We determined the apparent affinity of the wild-type and the mutant RetGC-1 for both Ca^{2+}-bound and Ca^{2+}-free GCAP-1 and found that the net shift in Ca^{2+} sensitivity of the R838S RetGC-1 observed *in vitro* can arise predominantly from the change in the affinity of the mutant cyclase for the Ca^{2+}-free versus Ca^{2+}-loaded GCAP-1. Our findings confirm that the dynamic range for RetGC regulation by Ca^{2+}/GCAP is determined by both the affinity of GCAP for Ca^{2+} and relative affinities of the effector enzyme for the Ca^{2+}-free versus Ca^{2+}-loaded GCAP.

Guanylyl cyclase activating proteins, GCAP-1[1] and GCAP-2, regulate the activity of photoreceptor guanylyl cyclase, RetGC, in a calcium-sensitive manner, a process required for recovery of photoreceptor cells from photoexcitation (*1*, *2*). At low Ca^{2+} concentrations, a characteristic of illuminated photoreceptors, GCAPs trigger activation of cGMP synthesis by RetGC and stimulate opening of cGMP-gated Na^+/Ca^{2+} channels, while at higher free Ca^{2+} concentrations, typical for dark-adapted rods and cones, GCAPs suppress the activity of the cyclase thus preventing the excessive opening of the cation channels (*3*−*6*). A number of mutations in RetGC and GCAPs have been found in association with various forms of retinal degeneration (*3*, *7*). Among this group is an autosomal dominant cone−rod dystrophy (adCORD), an inherited progressive disease that causes initial deterioration of the cone photoreceptor cells followed by loss of rods, often resulting in blindness. Genetic studies have identified several mutations linked to adCORD in the gene for retinal guanylyl cyclase 1 (RetGC-1): single mutations, R838C (*8*, *9*) and R838H (*9*); a double mutation, E837D/R838S (*10*); and a triple mutation, E837D/R838C/T839M (*11*). All of the mutations that include substitution at Arg^{838} have been localized to the putative dimerization domain of RetGC-1. Recent findings strongly implicate Arg^{838} as playing a key role in the formation and stability of the coiled-coil structure potentially involved in RetGC-1 dimerization and catalytic activity (*12*). In recombinant RetGC-1, the mutations at the Arg^{838} affect GCAP-1-mediated RetGC-1 activation by shifting its Ca^{2+} sensitivity to a higher than normal free Ca^{2+} range (*12*−*14*). This may result in constitutively accelerated cGMP synthesis in darkness and by doing so drive the intracellular calcium in photoreceptor cells to higher than normal levels, thus creating pathological conditions. The R838S substitution was observed in the families with severe cases of adCORD (*9*). It has been demonstrated that the mutation, R838S, increased apparent affinity of the cyclase for the Ca^{2+}-free GCAP-1 (*12*, *13*). However, the affinity of neither the wild-type nor the mutant cyclase to the Ca^{2+}-loaded GCAPs was ever estimated, which complicates

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[1] Abbreviations: GCAP, guanylyl cyclase activating protein; RetGC, photoreceptor membrane guanylyl cyclase; EGTA, ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca]_{1/2}$, free Ca^{2+} concentration required for half-maximal inhibition of RetGC; $[Ca]_f$, free Ca^{2+} concentrations; MOPS, 4-morpholinopropanesulfonic acid; $[Mg]_f$, free Mg^{2+} concentrations.

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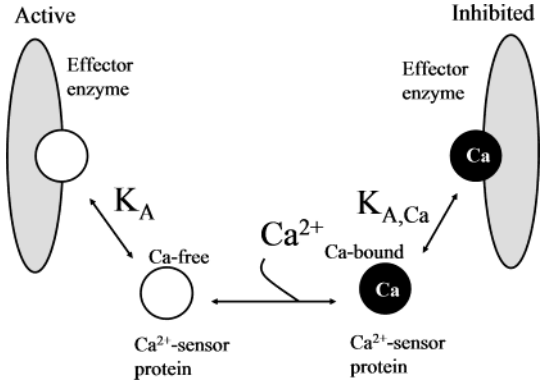

FIGURE 1: When Ca^{2+}-loaded and Ca^{2+}-free forms of the same Ca^{2+}-sensor protein produce opposite effects on the effector enzyme and/or compete with each other for the effector enzyme, then the overall Ca^{2+} sensitivity of the effector enzyme depends not only on the affinity of the Ca^{2+}-sensor protein for Ca^{2+} but also on the relative affinities of the effector enzyme for the Ca^{2+}-bound ($K_{A\ Ca}$) versus Ca^{2+}-free (K_A) form of the sensor protein. In this example, the Ca^{2+}-free form of the Ca^{2+}-sensor protein is designated the activator, and the Ca^{2+}-bound form is designated the inhibitor, as in the case of RetGC/GCAP complex. Other explanations are in the text.

attempts to analyze the interaction between the cyclase and GCAP and model the mechanism of the altered Ca^{2+} sensitivity. For example, the affinity of the Ca^{2+}-bound GCAP-1 for the cyclase may also increase as the result of the mutation, which could potentially negate the increase in affinity of Ca^{2+}-free GCAP-1. Therefore, it appears vitally important to determine the relative affinity of the Ca^{2+}-bound GCAPs for the wild-type and the R838S cyclase to correctly interpret the results of the original findings by Ramamurthy et al. (*12*).

This seems to be important not only for understanding of the RetGC regulation by Ca^{2+}/GCAPs but also for the general concept of the effector enzyme regulation by Ca^{2+}-sensor proteins. It is obvious that the affinity of a Ca^{2+}-binding sensor protein for Ca^{2+} is crucial for the regulation of the effector enzyme, and it can also be potentially affected by the interaction with the effector enzyme. However, in the cases when a Ca^{2+}-sensor protein undergoes a transition between the inhibitor and the activator forms, which can compete with each other (*15*, *16*), the actual tuning of the Ca^{2+} sensitivity of the effector enzyme should also depend on the relative affinities of the effector enzyme for the Ca^{2+}-free versus Ca^{2+}-bound form of the sensor protein (Figure 1).

We determined the apparent affinity of both Ca^{2+}-free and Ca^{2+}-bound GCAPs for the wild type and the R838S mutant of RetGC-1 and found evidence that the cyclase affinity for both forms of GCAP-1 increases as a result of this mutation. Analysis of the model demonstrates that changes in relative affinity of the Ca^{2+}-free versus Ca^{2+}-bound GCAP for RetGC-1 can effectively explain the shift in the net Ca^{2+} sensitivity of the R838S RetGC-1 mutant linked to adCORD.

MATERIALS AND METHODS

Recombinant GCAP-1 and GCAP-2. The cDNA corresponding to the GCAP-1(WT), GCAP-1(2,3,4), or GCAP-2(WT) coding region was expressed from pET11d vector (Novagen/Calbiochem) in a BLR (DE3) *Escherichia coli* strain (Novagen/Calbiochem) carrying a pBB131 plasmid that encoded *N*-myristoyl transferase (NMT) as previously described (*15*−*17*). To produce the constitutively active GCAP-1 mutant, GCAP-1(2,3,4), a substitution, Asp/Asn, that prevents high-affinity Ca^{2+} binding (*18*, *19*) was introduced in the first position of the each 12 amino acid Ca^{2+}-coordinating loops of the EF-hands 2, 3, and 4 by the polymerase chain reaction using Pfu polymerase and the protocol described previously in detail elsewhere (*16*). The cells were grown in standard LB media containing 40 mg/mL kanamycin and 100 mg/mL ampicillin. Free myristic acid was added into the suspension of bacterial cells to a final concentration of 50 μg/mL from a concentrated ethanol stock solution 30 min prior to induction with 1 mM isopropyl-β-D-thiogalactopyranoside. After 3 h of induction, the bacterial pellet was harvested and the recombinant GCAPs were then purified as described previously in detail (*20*). To produce unmyristoylated GCAP-1, it was expressed in the *E. coli* strain that did not contain NMT, as described in ref *20*. The concentration of GCAPs was measured using $A_{280}(0.1\%) = 1.12$ for GCAP-1(WT) and GCAP-1(2,3,4) and $A_{280}(0.1\%) = 1.48$ for GCAP-2 in 20 mM phosphate buffer (pH 6.5) and 6 M guanidine hydrochloride. The extinction coefficient at 280 nm for individual GCAP was computed from its amino acid composition (*21*) using the software of ExPASy proteomic World Wide Web server from Swiss Institute of Bioinformatics (*22*).

Mass Spectroscopy. Purified myristoylated GCAP-1 was desalted in 0.05% trifuoroacetic acid/50% acetonitrile at a concentration of 0.8 mg/mL and after a 2-fold dilution in 0.1% formic acid/95% acetonitrile was infused in a Therma-Finnegan LCQ DECA XP Plus electrospray ionization mass spectrometer at approx 3 μL/min for approximately 5 min. The results of the analyses were processed using Therma-Finnegan BioWorks 3.1 software.

Expression of RetGC-1 in Human Embryonic Kidney (HEK) 293 Cells. The wild-type or R838S RetGC-1 cDNA were inserted into the *Eco*RI/*Hind*III sites of the pRCCMV vector (Invitrogen) and transiently transfected into 293 F cells (Invitrogen) using a Promega ProFection kit (calcium phosphate protocol). Prior to the transformation, the 293 F cells were maintained in stationary culture for at least two passages in high-glucose Dulbecco's modified Eagle media supplemented with 10% fetal bovine serum (Invitrogen) at 5% CO_2. In 48 h after the transformation, the cells were harvested, rinsed with Dulbecco's 1× phosphate-buffered saline (PBS), detached from the dishes, washed in PBS, and pelleted by centrifugation at 500*g*. The cells were then swollen in prechilled hypotonic buffer solution A (10 mM Tris at pH 7.5, 1.0 mM $MgCl_2$, 7.5 mM 2-mercaptoethanol, and 25 μg/mL leupeptin) on ice for 10 min. The cells were lysed by homogenization in Potter−Elvehjem (Teflon−glass) grinder. A 500*g* supernatant was collected and centrifuged at 36000*g* for 30 min. The resulting pellet was resuspended in the buffer A containing 20% glycerol, and the aliquots were frozen in liquid nitrogen and stored at −70 °C.

Guanylyl Cyclase (GC) Assay. The assay mixture (25 μL) contained 30 mM MOPS/KOH at pH 7.2, 60 mM KCl, 4 mM NaCl, 1 mM DTT, different concentrations of $MgCl_2$ to vary the free Mg^{2+} between 0.1 and 7 mM, 2 mM Ca/EGTA buffer, 0.3 mM ATP, 4 mM cGMP, 1 mM GTP, 1 μCi of [α-^{32}P] GTP, 0.1 μCi of [8- ^{3}H]cGMP, and HEK 293 membranes containing equal amounts of expressed wild-type

or R838S mutant RetGC-1. The reaction mixture was incubated for 40 min at 30 °C, stopped by heating for 2.5 min at 95 °C, and the aliquots were analyzed by thin-layer chromatography using fluorescent plastic-backed polyethylenimine cellulose plates (Merck) as described previously (*23*). In all experiments, the time course of the reaction was linear within the period of the assay. Ca/EGTA buffers were prepared according to the procedure described in ref *24* and verified using fluorescent indicator dyes Fluo-3 and X-rhod FF (K_d of 325 nM and 17 μM, respectively) (Molecular Probes/Invitrogen). The free Ca^{2+} concentrations were calculated according to the method of Brooks and Stoney (*25*) using the algorithm of Marks and Maxfield (*26*). To determine the K_A and $K_{A\ Ca}$, increasing concentrations of GCAPs were added into assay mixture and the experimental data were fitted by the equation, $V = V_{max}[GCAP]^n/((K_{1/2})^n + [GCAP]^n)$, where V is the activity of RetGC-1, V_{max} is the maximal activity of RetGC-1, $K_{1/2}$ is the concentration of GCAP required for half-maximal activation of either wild-type or R838S RetGC-1 in the absence or presence of Ca^{2+}, and n is the Hill coefficient. All data shown were from several independent experiments producing similar results.

RESULTS

The specific activity of recombinant wild-type RetGC-1 produced in HEK 293 cells remains very low in the absence of GCAPs, while GCAP-1 efficiently stimulates the cyclase activity in a Ca^{2+}-sensitive manner (Figure 2A). Recently, we found that GCAPs are both Ca^{2+}- and Mg^{2+}-sensor proteins and that binding of Mg^{2+} by GCAPs is critical for the dynamic range of RetGC regulation in outer segment membranes by GCAPs at physiological levels of free Ca^{2+} ($[Ca]_f$) (*27*). Similarly, in the case of recombinant RetGC-1, its Ca^{2+} sensitivity also depends on the concentration of free Mg^{2+} ($[Mg]_f$) (Figure 2A). The $[Ca]_{1/2}$ increases proportionally with the increase of $[Mg]_f$ (Figure 2B), while the cooperativity of RetGC-1 inhibition by Ca^{2+}/GCAP-1 remains close to 2.0 at all $[Mg]_f$ tested in our experiments. The maximal activity of RetGC-1 sharply increases between 0 and 1 mM $[Mg]_f$ but only slightly changes with further increase in $[Mg]_f$ (Figure 2C).

We also examined the effect of Mg^{2+} on Ca^{2+} sensitivity of the R835S mutant RetGC-1 compared to that of the wild-type. Wild-type RetGC-1 and its R838S mutant were produced in transfected HEK 293 F cells under identical conditions and with the same level of expression (Figure 3A). Similar to the wild type, activation of the R838S RetGC-1 mutant requires Ca^{2+}-free GCAPs and is inhibited in a Ca^{2+}-sensitive manner, and the $[Ca]_{1/2}$ for the inhibition depends on $[Mg]_f$ (parts B and C of Figure 3). However, the mutation, R838S, increases the $[Ca]_{1/2}$ and decreases the cooperativity of RetGC-1 regulation by Ca^{2+} (parts B and C of Figure 3). Importantly, the $[Mg]_f$ affects the absolute values of $[Ca]_{1/2}$ of the R838S mutant to the same extent as the wild-type RetGC-1; hence, the shift in Ca^{2+} sensitivity for the R838S mutant relative to that of the wild-type remains at both low and high $[Mg]_f$. At the same time, the change in $[Mg]_f$ above 1 mM only slightly affects the absolute activity of the cyclase and does not affect the cooperativity of inhibition by Ca^{2+} (parts B and C of Figure 3). Thus, the actual value of $[Mg]_f$ in the GC assay is not crucial when it comes to the effect of R838S mutation on changing properties of RetGC-1. However, at $[Mg]_f$ close to the physiological level [0.8−0.9 mM (*28*)], the activity of the cyclase is not completely saturated (Figure 2C), and we found that the remaining difference, no matter how small it is, could interfere with the accuracy of the kinetic analysis described below. Therefore, to achieve the maximal accuracy in the analysis of the kinetic model for the RetGC-1/GCAPs interaction, we used the saturating concentration of $[Mg]_f$, as high as 7.0 mM, in the experiments and then applied the predictions made by the model to lower $[Mg]_f$ as will be shown below.

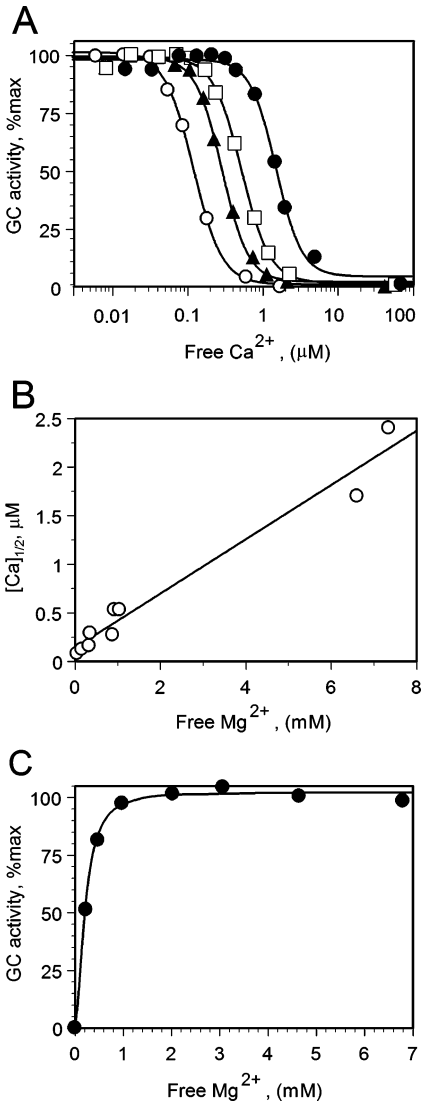

FIGURE 2: Ca^{2+} sensitivity of RetGC-1 regulation by GCAP-1 as a function of $[Mg]_f$. (A) Recombinant wild-type RetGC-1 was assayed for GC activity at various $[Ca]_f$ and $[Mg]_f$ as described under the Materials and Methods. The concentration of GCAP-1 in the reaction was 8 μM. The $[Mg]_f$ in different series was 0.17 mM (○), 0.36 mM (▲), 1.0 mM (□), and 6.6 mM (●). The data were fitted by the equation, $V = (V_{max} - V_{min})/(1 + ([Ca]_f/[Ca]_{1/2})^n) + V_{min}$, where V is the activity of RetGC-1, V_{max} and V_{min} are the maximal and minimal activities of RetGC, respectively, and n is the Hill cooperativity coefficient. (B) $[Ca]_{1/2}$ for RetGC-1 as a function of $[Mg]_f$. (C) Activity of RetGC-1 stimulated by 10 μM GCAP-1 at different $[Mg]_f$.

In photoreceptors, RetGCs are regulated by two Ca^{2+}-binding proteins, GCAP-1 and GCAP-2, that activate RetGC in a Ca^{2+}-sensitive manner. It is not immediately apparent to what extent GCAP-1 and GCAP-2 regulate specifically RetGC-1, but because it appears that both GCAP-1 and

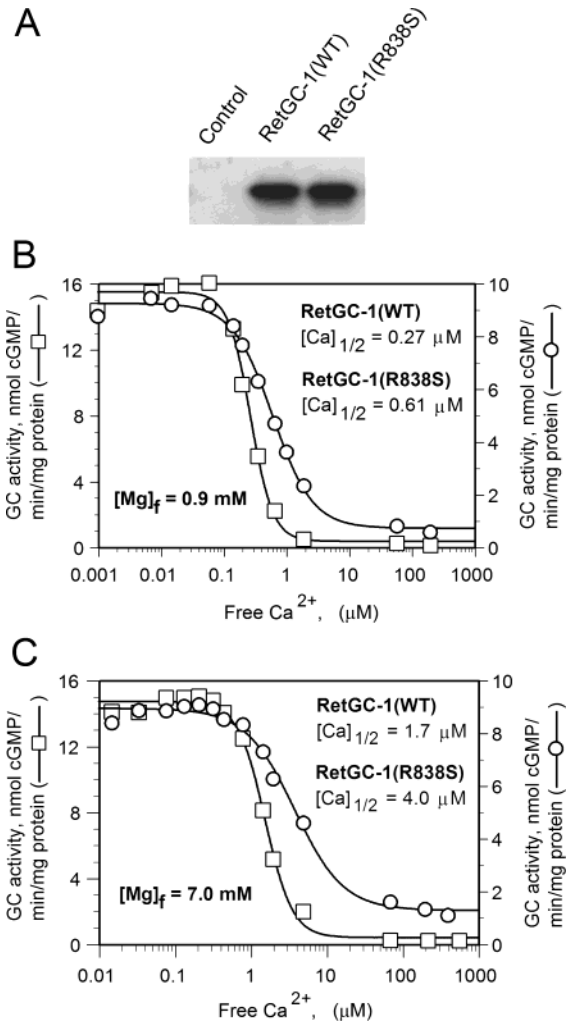

FIGURE 3: (A) Immunoblot of equal amounts of the total membrane proteins from HEK 293 F cells probed with an antibody to the RetGC-1 catalytic domain. (B and C) Ca^{2+} sensitivity of R838S (○) and wild-type RetGC-1 (□) stimulated by 8 μM GCAP-1 at 0.9 mM of $[Mg]_f$ (B) or 7.0 mM $[Mg]_f$ (C).

GCAP-2 can activate RetGC in photoreceptors of transgenic mice (*29*, *30*), we investigated the effect of R838S mutation on the Ca^{2+}-dependent regulation of RetGC-1 by both GCAP-1 and GCAP-2. The mutation, R838S, decreases the level of maximal stimulation of RetGC-1 by both GCAP-1 and GCAP-2 at low Ca^{2+} (Table 1). A decrease in the maximal GCAP-1-stimulated specific activity was also reported earlier in the case of the RetGC-1 double mutant (E837D/R838S) (*12*), whereas other authors observed an increase in specific activity for the R838S mutant (*13*). Yet, it is very difficult to conclude whether the mutation actually decreases the intrinsic activity of the enzyme or just affects, for example, the pool of correctly folded recombinant enzyme produced in HEK 293 F cells. Therefore, it is more relevant to rely on normalized rather than absolute activity of recombinant RetGC-1 when addressing the Ca^{2+} sensitivity of its regulation. Indeed, in good agreement with the earlier observations (*12*, *13*), the substitution, R838S, increases GCAP-1-stimulated activity at elevated free Ca^{2+} concentrations ($[Ca]_{1/2}$ is 1.7 ± 0.15 μM for wild-type RetGC-1 and 4.0 ± 0.25 μM for the R838S mutant) and decreases the cooperativity of RetGC-1 inhibition by Ca^{2+} (2.0 versus 1.3, respectively) (Figure 3C).

Remarkably, even at $[Ca]_f$ as high as 300 μM, GCAP-1 does not inhibit activity of recombinant RetGC-1 completely to the basal level (defined as the activity of the recombinant cyclase in the absence of GCAPs). In the case of recombinant wild-type RetGC-1, the activation is very weak (~1.0% compared to the Ca^{2+}-free GCAP-1) but measurable, because nonstimulated basal activity of the cyclase corresponded to 0.057 nmol min^{-1} mg^{-1} and increased to 0.25 (±0.03) nmol min^{-1} mg^{-1} in the presence of Ca^{2+}-saturated GCAP-1. Although this value appears rather small, it cannot be attributed to the remaining Ca^{2+}-free GCAP-1, because at this free Ca^{2+} concentration, the fractional saturation of GCAP-1 by Ca^{2+} calculated using the experimental value of $[Ca]_{1/2}$ = 1.7 μM and the cooperativity coefficient of 2.0 is ~0.99996, meaning that at $[Ca]_f$ = 300 μM only less than 0.004% of GCAP-1 can remain in the Ca^{2+}-free form. The fractional activity of the fully Ca^{2+}-loaded GCAP-1 in the case of the R838S mutant becomes much more substantial (~15%). Hence, when it comes to the recombinant cyclase, instead of being a complete inhibitor of the cyclase fully Ca^{2+}-loaded form of GCAP-1 remains a weak RetGC-1 activator.

We took the advantage of low basal activity of the recombinant RetGC-1 to determine the apparent affinity of GCAP-1 to the wild-type and mutant cyclase not only in the absence but also in the presence of saturating Ca^{2+}, as shown in Figure 4. We have found that in the case of wild-type RetGC-1 the apparent affinity of GCAP-1 to the cyclase remains the same regardless of GCAP-1 being in its Ca^{2+}-free (K_A) or Ca^{2+}-loaded ($K_{A\ Ca}$) form (Figure 4 and Table 1). Interaction of GCAP with the target membrane cyclase *in vitro* may include direct binding of GCAP to the cyclase from solution as well as first binding to the membrane and then binding to the cylase via lateral diffusion. It is impossible to study the native complex between cyclase and GCAP after its solubilization in detergent (*31*) and therefore to determine the actual dissociation constant for the cyclase/GCAP complex. Hence, we can only evaluate the integral, apparent, affinity. This creates an obvious limitation for a

Table 1: Activation of Wild-Type and Mutant RetGC-1 by GCAPs at Low and High $[Ca]_f$ in the Presence of 7.0 mM $[Mg]_f$

	RetGC-1(WT)		RetGC-1(R838S)	
GCAP/$[Ca]_f$	$K_{1/2}$[a] (μM)	V_{max}[b] (nmol cGMP min^{-1} mg^{-1})	$K_{1/2}$[a] (μM)	V_{max}[b] (nmol cGMP min^{-1} mg^{-1})
GCAP-1/EGTA	2.0 ± 0.4 ($K_{A\ WT}$)	19.6 ± 1.2	0.4 ± 0.05 ($K_{A\ R838S}$)	10.3 ± 1.1
GCAP-1/200 μM $[Ca^{2+}]_f$	2.0 ± 0.3 ($K_{A\ Ca\ WT}$)	0.19 ± 0.03	1.2 ± 0.15 ($K_{A\ Ca\ R838S}$)	1.5 ± 0.2
GCAP-2/EGTA	1.8 ± 0.2	15.6 ± 1.0	1.0 ± 0.15	4.8 ± 0.25
GCAP-1(2,3,4)/70 μM $[Ca^{2+}]_f$	5.4 ± 0.2	14.0 ± 0.7	0.56 ± 0.06	7.8 ± 0.3

[a] $K_{1/2}$ represents the concentration of GCAP required for half-maximal activation of RetGC-1. [b] V_{max} represents the maximal level of RetGC-1 activation by GCAP at specified $[Ca]_f$. Basal activity of 0.057 nmol min^{-1} mg^{-1} was subtracted.

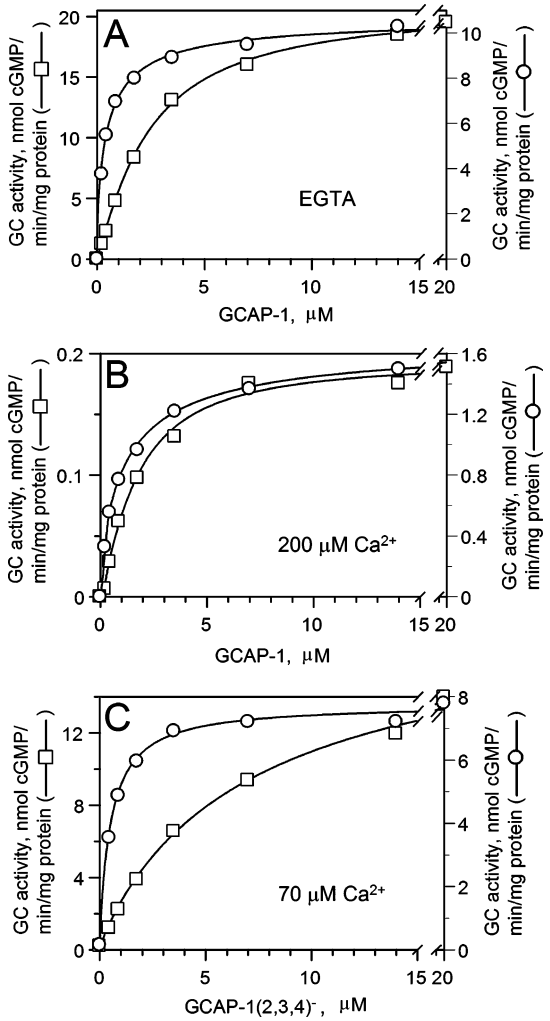

FIGURE 4: Dose dependence of the wild-type (□) and R838S RetGC-1 mutant (○) on GCAP-1(WT) and GCAP-1(2,3,4) at low (A) or high (B and C) $[Ca]_f$. The $[Mg]_f$ in the reaction was 7.0 mM. The data were fitted by the Hill equation, $V = V_{max}[GCAP]^n/((K_{1/2})^n + [GCAP]^n)$, where V is the activity of RetGC-1, V_{max} is the maximal activity of RetGC-1, $K_{1/2}$ is the concentration of GCAP required for half-maximal activation of RetGC-1, and n is the Hill coefficient. The values of V_{max} and $K_{1/2}$ are summarized in Table 1.

more detailed kinetic analysis of the GCAP/RetGC-1 interaction. However, because (a) we expressed both the wild-type and R838S cyclase using identical conditions and in the same batch of the HEK 293 cells, (b) the level of expression was the same (Figure 3), and (c) the concentration of both membranes in the assay mixture was the same, then the only difference between K_{WT} and K_{R838S} both in the presence and absence of Ca^{2+} must reflect the difference in the affinity of GCAP-1 for the corresponding cyclases. When the R838S mutant is compared to the wild-type RetGC-1, the R838S mutant interacts with higher affinity with both Ca^{2+}-free and Ca^{2+}-bound GCAP-1. Even more importantly, the mutation changes the *relative* affinity of the cyclase for the Ca^{2+}-free versus Ca^{2+}-bound form nearly 3-fold (Table 1). The difference in K_A between the wild-type and R838S remains at higher $[Ca]_f$ in the case of the constitutively active GCAP-1 mutant, GCAP-1(2,3,4); hence, this difference reflects GCAP-1/RetGC-1 interaction being altered by the mutation rather than Ca^{2+} having a nonspecific effect on the mutant cyclase (Figure 4C and Table 1).

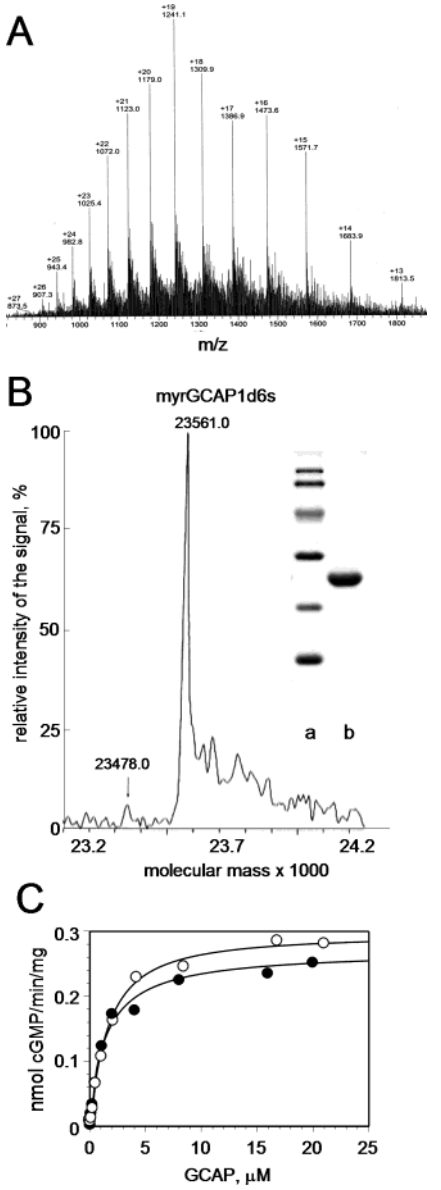

FIGURE 5: (A) Purified recombinant GCAP-1 was isolated and analyzed by electrospray ionization mass spectrometry as described under the Materials and Methods. (B) Reconstituted profile of molecular masses from *m*/*z* species shown in A. The molecular mass of the major peak corresponds to the myristoylated d6sGCAP1 (23 561 versus the calculated average isotopic mass of myristoyl-GCAP-1, 23 560). The arrow indicates the expected position of the nonmyristoylated protein (average isotopic molecular mass = 23 348). (Inset) 15% Polyacrylamide SDS gel stained with Coomassie blue. (a) Molecular mass markers (top to bottom, kDa): 94, 67, 43, 30, 20, and 14. (b) Purified myristoylated GCAP-1 (5 μg). (C) Activation of RetGC-1 by GCAP-1 from the preparation shown in B before (●) and after (○) adding 5% purified recombinant nonmyristoylated GCAP-1.

It has been reported (*32*) that nonmyristoylated GCAP-1 retains low cyclase-stimulating activity in the presence of outer segment membranes and high Ca^{2+} concentrations. Our preparation of GCAP-1 had no more than 5% of nonmyristoylated protein in it (parts A and B of Figure 5). At such a low concentration, nonmyristoylated GCAP-1 did not alter the activation of RetGC-1 by Ca^{2+}-saturated myristoylated GCAP-1 (Figure 5C). Moreover, for the small amount of nonmyristoylated GCAP-1 to be able to affect the $K_{A\ Ca}$ for the 95% myristoylated GCAP-1, the affinity of the nonmyristoylated protein had to be an order of magnitude higher

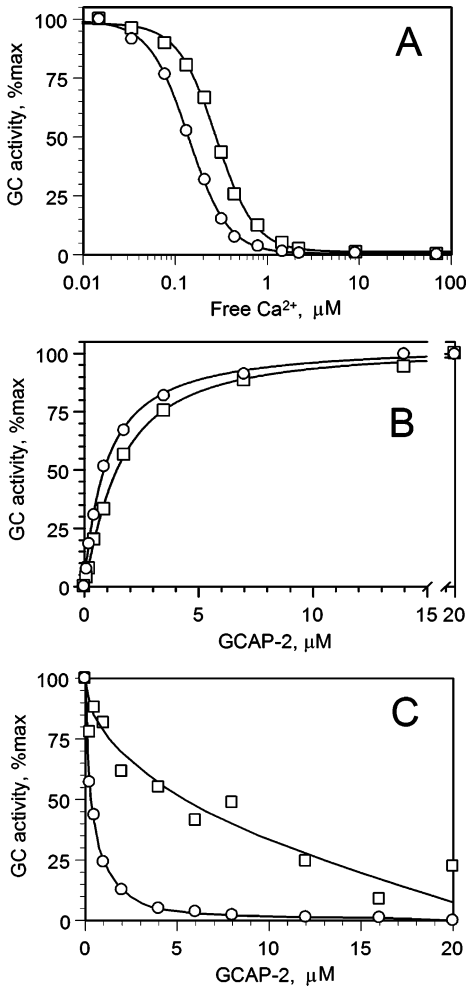

FIGURE 6: (A) Ca^{2+} sensitivity of R838S (○) and wild-type RetGC-1 (□) stimulated by 8 μM GCAP-2. (B) Dose dependence of R838S (○) and wild-type RetGC-1 (□) on GCAP-2 in the presence of 2 mM EGTA. (C) Activity of wild-type RetGC-1 (□) or R838S (○) mutant activated by 5.0 μM GCAP-1 at 200 μM Ca^{2+} in the presence of various amounts of GCAP-2 as a competitor. The $[Mg]_f$ in the reaction was 7.0 mM.

than that of myristoylated GCAP-1. However, in separate experiments (data not shown), we found that nonmyristoylated GCAP-1 had the apparent affinity similar to the myristoylated GCAP-1, ca. 1.5 μM. If the activation of RetGC-1 at saturating Ca^{2+} merely reflected the presence of the small fraction of nonmyristoylated GCAP1, then the $K_{A\ Ca\ WT}$ had to be at least 10-fold lower than we observed. Hence, contribution to the $K_{A\ Ca}$ from the nonmyristoylated GCAP-1 present in our preparation of myristoylated GCAP-1 (Figure 4 and Table 1) was negligible.

Unlike GCAP-1, no activation of RetGC-1 was observed at high $[Ca]_f$ in the case of GCAP-2. In addition, the maximal stimulation of the R838S mutant was partially suppressed (Table 1). A similar, although even more dramatic effect was reported by Duda et al. (*33*) and Tucker et al. (*14*) for the Arg to Cys substitution in this position. However, in addition to the decreased maximal stimulation of the RetGC-1(R838S) cyclase by GCAP-2, we have also found that Ca^{2+} sensitivity of the mutant cyclase by GCAP-2 was *reversed* compared that by GCAP-1 (Figure 6A). In contrast to GCAP-1, GCAP-2 completely inhibited both the mutant and wild-type cyclase at $[Ca]_f > 1$ μM. The $[Ca]_{1/2}$ decreased from near 0.26 μM for wild-type RetGC-1 to 0.13 μM for the R838S mutant (Figure 6A), but the cooperativity of the Ca^{2+}-dependent inhibition did not change. We also tested whether RetGC-1 activation by GCAP-1 at high $[Ca]_f$ could be reduced by increasing concentrations of Ca^{2+}-saturated GCAP-2 (which does not activate RetGC-1 under these conditions). We found that GCAP-2 competes effectively with the "weak activator" form of Ca^{2+}-bound GCAP-1 at high $[Ca]_f$ (Figure 6C). When RetGC-1 was activated by 5 μM GCAP-1 at 200 μM $[Ca]_f$, the concentration of GCAP-2 required for the half-maximal inhibition of the wild-type and R838S RetGC-1 was 5.3 and 0.5 μM, respectively (Figure 6C).

DISCUSSION

We have found it possible to evaluate the apparent affinity of GCAP-1 for the recombinant cyclase in both the Ca^{2+}-bound and Ca^{2+}-free/Mg^{2+}-bound (*27*) forms. These parameters can be used to further elucidate the question about the mechanism by which mutations in the dimerization domain of guanylyl cyclase, RetGC-1, associated with cone−rod dystrophy may cause the abnormal regulation of the cyclase by GCAPs.

A series of previous publications by other authors (*12*−*14*, *33*) demonstrated that a number of mutations in R838 within a human RetGC-1 putative dimerization domain found in the families with adCORD (CORD6 group) result in abnormal Ca^{2+} sensitivity of the cyclase regulation by GCAP-1. Evidently, the substitution of the crucial Arg^{838} in RetGC-1 to Cys, Ser, or Ala may affect the function of a putative cyclase dimerization domain, and this may result in a decrease in RetGC-1 sensitivity to the inhibitory effect of Ca^{2+}-bound GCAP-1 (*12*). On the basis of the models described in previous publications (*12*−*14*, *33*), the abnormal Ca^{2+} sensitivity of the R838S RetGC-1 could be attributed to a higher probability of the transition of the cyclase/GCAP complex into the active state because of the enhanced dimerization of the cyclase. We found that the $K_{A\ WT}$ and $K_{A\ R838S}$ were almost 2- and 6-fold lower than the corresponding values originally reported in ref *13*, perhaps because the method of GCAP expression and purification in ref *13* was different. Our data essentially support the original observation from that group that the K_A decreases in the case of the mutant cyclase (*12*, *13*). However, they also strongly indicate that the altered Ca^{2+} sensitivity of the GCAP-1/RetGC-1(R838S) is unlikely to be primarily a result of the spontaneous transition of the mutant cyclase into active state because of the following. Although GCAPs may interact with different sites on the cyclase (*46*), both GCAPs activate the cyclase via enhancing its dimerization (*37*). If the R838S mutation merely shifted the equilibrium between the active and inactive states in favor of the active state, one should reasonably expect a similar effect from this mutation in the case of GCAP-2 and likely an increase in basal activity for the mutant cyclase. However, in the case of GCAP-2, the Ca^{2+} sensitivity of R838S RetGC-1 in our experiments increased rather than decreased (Figure 6A) and no increase in basal activity was detected. As yet another possibility, this mutation could merely decrease the GCAP-1 affinity for Ca^{2+} in the GCAP-1/RetGC-1 complex. However, in that case, RetGC activity should have reached the basal level at certain Ca^{2+} concentrations, but this does not happen (Figure 3).

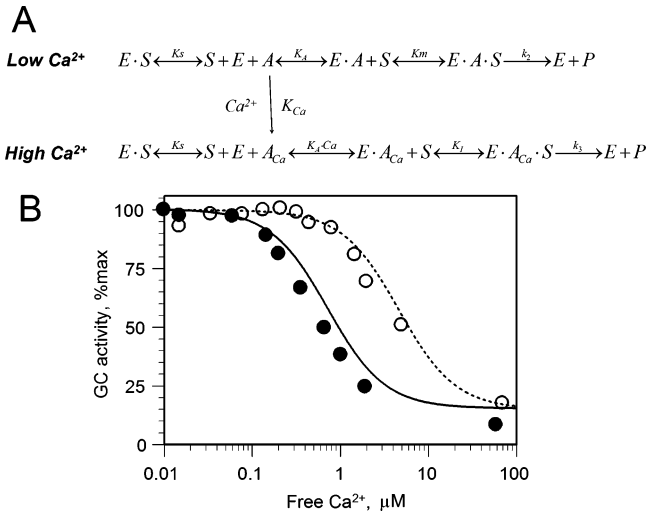

FIGURE 7: (A) Model of the regulation of RetGC by GCAP as described in the Discussion. (B) Ca^{2+} sensitivity of RetGC-1 stimulated by 8 μM GCAP-1 in the presence of 0.9 mM $[Mg]_f$ (●) or 7.0 mM $[Mg]_f$ (○). Data points are taken from parts B and C of Figure 2. Lines are theoretical curves plotted using eq 3, where $V_{max} = 100\%$ (RetGC activity at low $[Ca]_f$), $V_{min} = 15\%$ (RetGC activity at high $[Ca]_f$), $n = 1.3$ (cooperativity factor), and $[Ca]_{1/2\ R838S} = 4.6$ μM (···) or 0.74 μM (—).

The overall mechanism appears to be 2-fold. First, GCAP-1, even when fully loaded with Ca^{2+}, remains a weak activator of a recombinant RetGC-1 *in vitro*. In the case of wild-type RetGC-1, this effect is hardly detectable, especially when compared to activity of the cyclase stimulated by the Ca^{2+}-free GCAP-1, but its contribution becomes much more significant in the case of the mutant cyclase. Second, the net change in the Ca^{2+} sensitivity of RetGC-1 regulation by GCAP-1 by the mutation, R838S, is a result of a change in the relative affinities of the cyclase for the Ca^{2+}-free versus Ca^{2+}-bound form of GCAP-1. Interestingly, the Ca^{2+}-loaded forms of a homologous protein, neurocalcin, or another EF-hand protein, S100b, have been shown to also activate RetGC-1 *in vitro* (*34*, *47*). Evidently, the mutation, R838S, makes RetGC-1 more susceptible to the weak stimulating effect of the GCAP-1 in its Ca^{2+}-bound conformation.

The net shift in Ca^{2+} sensitivity of the R838S RetGC-1 mutant observed *in vitro* may arise solely from increased affinity of the mutant for the Ca^{2+}-free (Mg^{2+}-bound) GCAP-1 relative to Ca^{2+}-loaded GCAP-1. To ascertain if the change in the protein/protein affinity can indeed explain the observed shift in Ca^{2+} sensitivity of RetGC-1 activation by GCAP-1, we employed the model of the regulation of RetGC shown in Figure 7A, in which "A" represents Ca^{2+}-free GCAP-1, "A_{Ca}" represents Ca^{2+}-loaded GCAP-1, "E" represents RetGC-1, "S" represents the substrate (GTP), and "P" represents the product of the reaction (cGMP), for the cGMP synthesis rate constants $k_2 \gg k_3$. We base this model on an important assumption that the interaction between GCAPs and the cyclase, with either the wild type or mutant, does not drastically affect the GCAP affinity for Ca^{2+} (K_{Ca}). We consider this a reasonable assumption because Ca^{2+}-dependent tryptophan fluorescence and Ca^{2+} sensitivity of RetGC regulation by GCAP-1 are both observed within the similar submicromolar physiological range of free Ca^{2+} concentrations (*27*, *42*). By applying this model, we can calculate $[Ca]_{1/2}$ for inhibition of RetGC using the formula (1)

$$[Ca]_{1/2} = \frac{K_{Ca}\left(1 + \frac{[A_o]}{K_A}\right)}{\left(1 + \frac{[A_o]}{K_{A\ Ca}}\right)} \quad (1)$$

where K_A and $K_{A\ Ca}$ are apparent affinity constants of RetGC-1 for Ca^{2+}-free and Ca^{2+}-loaded GCAP-1, respectively, K_{Ca} is an apparent affinity constant of GCAP-1 for Ca^{2+}, and $[A_o]$ is the total concentration of GCAP-1. Because the K_{Ca} is not known, we cannot directly calculate $[Ca]_{1/2}$ for the inhibition of wild-type and R838S mutant RetGC-1 ($[Ca]_{1/2\ WT}$ and $[Ca]_{1/2\ R838S}$, respectively) using this formula. However, we can calculate the ratio, $[Ca]_{1/2\ R838S}/[Ca]_{1/2\ WT}$, using only the apparent RetGC-1/GCAP-1 affinity constants

$$\left(\frac{[Ca]_{1/2\ R838S}}{[Ca]_{1/2\ WT}}\right) = \frac{\left(1 + \frac{[A_o]}{K_{A\ R838S}}\right)\left(1 + \frac{[A_o]}{K_{A\ Ca\ WT}}\right)}{\left(1 + \frac{[A_o]}{K_{A\ Ca\ R838S}}\right)\left(1 + \frac{[A_o]}{K_{A\ WT}}\right)} \quad (2)$$

The parameters for $[A_o]$, $K_{A\ R838S}$, $K_{A\ WT}$, $K_{A\ Ca\ R838S}$, and $K_{A\ Ca\ WT}$ were assigned the experimental values taken from Table 1: $[A_o] = 8.0$ μM, $K_{A\ R838S} = 0.4$ μM, $K_{A\ WT} = 2.0$ μM, $K_{A\ Ca\ R838S} = 1.2$ μM, and $K_{A\ Ca\ WT} = 2.0$ μM. The ratio $[Ca]_{1/2\ R838S}/[Ca]_{1/2\ WT}$ calculated with these parameters is 2.74. We assumed that the ratio $[Ca]_{1/2\ R838S}/[Ca]_{1/2\ WT}$ does not dramatically depend on $[Mg]_f$. In this case, if the values of $[Ca]_{1/2\ WT}$ are 1.7 μM at 7.0 mM $[Mg]_f$ and 0.27 μM at 0.9 mM $[Mg]_f$, then the calculated values of $[Ca]_{1/2\ R838S}$ at low and high $[Mg]_f$ are $(1.7 \times 2.74) = 4.6$ μM and $(0.27 \times 2.74) = 0.74$ μM, respectively, which is indeed in good agreement with the experimental data taken from parts B and C of Figure 3 (4.0 and 0.61 μM). Figure 7B shows that experimental values for R838S RetGC-1 activation by GCAP-1 at different $[Ca]_f$ and $[Mg]_f$ taken from parts B and C of Figure 3 fit well with the theoretical curves calculated using the equation

$$y = \frac{V_{max} - V_{min}}{1 + \left(\frac{x}{[Ca]_{1/2\ R838S}}\right)^n} + V_{min} \quad (3)$$

which fairly accurately describes the RetGC/GCAP complex activity as a function of $[Ca]_f$. Therefore, the effect of $[Mg]_f$ on the ratio $[Ca]_{1/2\ R838S}/[Ca]_{1/2\ WT}$, if any, is indeed rather small.

It was not possible to measure directly the affinity of Ca^{2+}-loaded GCAP-2 for wild-type RetGC-1 because we could not detect any RetGC activity at high $[Ca]_f$. However, as shown in Figure 6C, equal concentrations of GCAP-2 are required to half-inhibit GCAP-1-activated wild-type RetGC-1 at high $[Ca]_f$. The simplest interpretation of this result is that both GCAPs have similar affinity for the wild-type RetGC-1 at high $[Ca]_f$, which is about 2.0 μM, close to the affinity of the Ca^{2+}-free GCAP-2 to the wild-type RetGC-1 (see Table 1). On the other hand, under the same conditions, GCAP-2 inhibits GCAP-1-activated R838S RetGC-1 much more

effectively compared to wild-type RetGC-1 (Figure 6C). The apparent affinity of Ca^{2+}-loaded GCAP-1 for the R838S RetGC-1 is 1.2 ± 0.15 μM; thus, the affinity of Ca^{2+}-loaded GCAP-2 for the mutant cyclase must be better than 1.0 μM (which is the affinity of Ca^{2+}-free GCAP-2 for the mutant RetGC-1, Table 1). In other words, the R838S mutation likely increases the cyclase affinity for the Ca^{2+}-loaded GCAP-2 relative to the Ca^{2+}-free GCAP-2 (Table 1). Hence, according to our model, this should result in increased Ca^{2+} sensitivity of the mutant RetGC-1 to regulation by GCAP-2, which is in agreement with the experimental data shown in Figure 6A.

From the good correlation between the experimental and calculated values shown in Figure 7, it does not appear that the affinity of GCAP for Ca^{2+} is drastically altered by the cyclase, unlike calmodulin and its effector enzymes (*43*). This is not surprising, because the three-dimensional structure of GCAPs and other recoverin-like proteins very much differs from that of calmodulin (*44*, *45*). Although it is impossible to verify this by direct measurement of the affinity of the GCAP-1/RetGC-1 complex for Ca^{2+}, if the K_{Ca} was dramatically affected by the interaction with wild-type versus R838S RetGC-1, we would expect the experimental data to contradict our model and deviate from the theoretical plot shown in Figure 7. While small deviation between the experimental data and the theoretical curve in Figure 7 could be attributed, for example, to our not taking into account the possible small change in the GCAP affinity for Ca^{2+} as a result of its interaction with the cyclase, its contribution is quite small compared to the change in the GCAP/RetGC-affinity component.

While regulation of the cyclase in photoreceptors is a complex process that includes at least two isoforms of the RetGC (RetGC-1 and RetGC-2, also known as ROS GC-1 and GC-2 or GC-E and GC-F), a number of other possible regulatory elements, and a transition between flexible and stable RetGC dimer (*34*−*41*), in this paper, we only focus on one such regulatory element: the Ca^{2+} sensitivity of RetGC-1 isozyme as a function of its interaction with the activator and inhibitor form of the Ca^{2+}-sensor protein, GCAP.

We have demonstrated that the shift in Ca^{2+} sensitivity of the RetGC-1(R838S) regulation by GCAP-1 and GCAP-2 is likely a result of a change in the affinity of RetGC-1 to the Ca^{2+}-free versus Ca^{2+}-loaded GCAPs rather than a change in the affinity of the GCAP/RetGC-1 complex for Ca^{2+}, an increase in the spontaneous transition of RetGC-1 into the active (dimer) state, or a simple increase in the affinity of the cyclase for the Ca^{2+}-free form. Thus, our observations indicate that the Ca^{2+} sensitivity of RetGC-1 regulation is set by three major factors: the intrinsic affinity of GCAP for Ca^{2+}, the free Mg^{2+} concentrations, and the affinity of the Ca^{2+}-free versus Ca^{2+}-loaded GCAP for RetGC. While the affinity of GCAP for Ca^{2+} and Mg^{2+} determines the dynamic range for the GCAP transition between the activator and the inhibitor state, the relative affinity of Ca^{2+}-free versus Ca^{2+}-loaded GCAP sets the actual Ca^{2+} sensitivity of RetGC regulation by Ca^{2+}/GCAPs.

To summarize, we only considered one particular model in which the Ca^{2+}-free form of a Ca^{2+}-sensor protein is the activator and the Ca^{2+}-bound form is the inhibitor. However, these conclusions must be generally applicable to any situation when Ca^{2+}-loaded and Ca^{2+}-free forms of the same Ca^{2+}-sensor protein have opposite effects on the effector enzyme activity and/or compete with each other for the effector enzyme. In all such cases, the Ca^{2+} sensitivity of the effector enzyme under physiological conditions may strongly depend not only on the affinity of the Ca^{2+}-sensor protein for Ca^{2+} but also on the relative affinities of the effector enzyme for the Ca^{2+}-bound versus Ca^{2+}-free form of the sensor protein.

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