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Mg²⁺/Ca²⁺ cation binding cycle of guanylyl cyclase activating proteins (GCAPs): role in regulation of photoreceptor guanylyl cyclase

Alexander M. Dizhoor, Elena V. Olshevskaya, and Igor V. Peshenko

Department of Basic Sciences, Pennsylvania College of Optometry, Salus University, 8360 Old York Road, Elkins Park, PA 19027, USA

Abstract

Photon absorption by photoreceptors activates hydrolysis of cGMP, which shuts down cGMP-gated channels and decreases free Ca²⁺ concentrations in outer segment. Suppression of Ca²⁺ influx through the cGMP channel by light activates retinal guanylyl cyclase through guanylyl cyclase activating proteins (GCAPs) and thus expedites photoreceptors recovery from excitation and restores their light sensitivity. GCAP1 and GCAP2, two ubiquitous among vertebrate species isoforms of GCAPs that activate retGC during rod response to light, are myristoylated Ca²⁺/Mg²⁺-binding proteins of the EF-hand superfamily. They consist of one non-metal binding EF-hand-like domain and three other EF-hands, each capable of binding Ca²⁺ and Mg²⁺. In the metal binding EF-hands of GCAP1, different point mutations can selectively block binding of Ca²⁺ or both Ca²⁺ and Mg²⁺ altogether. Activation of retGC at low Ca²⁺ (light adaptation) or its inhibition at high Ca²⁺ (dark adaptation) follows a cycle of Ca²⁺/Mg²⁺ exchange in GCAPs, rather than release of Ca²⁺ and its binding by apo-GCAPs. The Mg²⁺ binding in two of the EF-hands controls docking of GCAP1 with retGC1 in the conditions of light adaptation and is essential for activation of retGC. Mg²⁺ binding in a C-terminal EF-hand contributes to neither retGC1 docking with the cyclase nor its subsequent activation in the light, but is specifically required for switching the cyclase off in the conditions of dark adaptation by binding Ca²⁺. The Mg²⁺/Ca²⁺ exchange in GCAP1 and 2 operates within different range of intracellular Ca²⁺ concentrations and provides a two-step activation of the cyclase during rod recovery.

Keywords

Photoreceptor; Guanylyl cyclase; Retina; Ca²⁺; Mg²⁺; GCAP; Calcium-binding proteins

GCAPs and guanylyl cyclase activation in photoreceptor recovery

In visual phototransduction in vertebrate rods and cones, photon absorption by rhodopsin or cone visual pigments triggers hydrolysis of cGMP by activating a transducinphosphodiesterase 6 (PDE6) cascade, which results in closure of the cGMP-gated cation channels (CNG) in the plasma membrane and membrane hyperpolarization (Fig. 1, reviewed in [1-3]). In order to reset the sensitivity of photoreceptor, cGMP levels are quickly restored in photo-bleached rods [4-7] by retina-specific guanylyl cyclase (retGC), a membrane enzyme present in rod and cone outer segments [8-10]. Whereas other members of membrane guanylyl cyclase family are regulated by their extracellular ligands, such as peptide hormones (reviewed in [11,12]), in the

photoreceptor cyclase its extracellular segment (most of which is likely to be exposed into the intradiskal space of the photoreceptor disks) does not reveal the ability to bind extracellular peptide hormones. Instead, retGC is a Ca^{2+} -regulated enzyme whose activity is controlled via the intracellular domains [13-15], conferred by soluble EF-hand sensor proteins, called guanylyl cyclase activating proteins (GCAPs) [7,8,16-19].

The feedback triggering activation of cGMP resynthesis results from a steep decline in free Ca^{2+} levels in photoreceptors between dark and light (Fig. 1). Photoexcitation, via closure of CNG channels and Ca^{2+} removal by $\text{Ca}^{2+}/\text{Na}^{+}$ exchanger, decreases the free intracellular Ca^{2+} in rods and cones nearly 10-fold [20,21], in mouse rods—from ~250 nM in the dark to ~25 nM in the light [22,23]. Catalytic activity of retGC in the dark is negatively controlled by Ca^{2+} -bound GCAPs [4,24-26] and requires the release of Ca^{2+} from GCAPs in the light to convert GCAPs in the form that stimulates retGC instead of inhibiting it [4,8,16-18,24,27]. Activity of cGMP production in rods stimulated by GCAPs in the light increases 10–12-fold [4,5] and is the most important reaction controlled by the Ca^{2+} feedback during a single-photon response [2,4]. Activation of retGC by GCAPs restrains the amplitude of a single-photon response and therefore begins during the rising phase of the photoresponse, virtually as soon as Ca^{2+} starts to fall in response to illumination [4,27].

Activation and inhibition of the photoreceptor guanylyl cyclase between light and dark is a result of $\text{Mg}^{2+}/\text{Ca}^{2+}$ exchange in GCAPs

Several GCAP variants have been identified in vertebrate retinas [8,17,28,29], and two of these homologs, GCAP1 and GCAP2, are found in all tested vertebrate species. GCAPs, related to a group of recoverin-like proteins, are *N*-fatty acylated and contain one non-metal binding EF-hand-like domain (EF1) and three metal binding EF-hands (EF2-4) (Fig. 2) capable of binding Ca^{2+} or Mg^{2+} [26]. Since Ca^{2+} and Mg^{2+} coordination involves slightly different polar groups from the amino acid residues in the EF-hand loop, we used mutations that either block Ca^{2+} binding without blocking Mg^{2+} binding or block Mg^{2+} binding as well (Fig. 3a) to determine the role of these cations in retGC regulation by GCAP1 [26,30]. Inactivation of Ca^{2+} binding in EF-hand loops by point mutations makes GCAP1 and -2 into constitutive activators of RetGC, capable of accelerating RetGC and insensitive to the inhibition by Ca^{2+} [24,26,30, 31] (Fig. 3). In contrast to that, mutations that disable not only Ca^{2+} but also Mg^{2+} binding [30] eliminate the GCAP ability to stimulate RetGC (Fig. 3a, b). It is therefore binding of Mg^{2+} to the apo-form of GCAP1 what makes it into a RetGC activator state, and not only dissociation of Ca^{2+} but also its replacement by Mg^{2+} is required to convert GCAP into a RetGC activator in the light. Mg^{2+} binding makes GCAP1 conformation drastically different from its apo form, as revealed by Trp fluorescence, microcalorimetry, and NMR analyses [26,30,32]. Trp fluorescence spectrum of GCAP1 also changes differently in response to Mg^{2+} versus Ca^{2+} binding, by lacking a component that correlates with the transition of GCAP1 into a Ca^{2+} bound, inhibitory state [30,33]. It is important to emphasize that blocking of Mg^{2+} binding in any individual EF-hand by point mutations does not cause a non-specific misfolding of GCAP1 as a result of those mutations, but rather specifically prevents the mutated EF-hand from assuming its proper cation-bound conformation, because: (i) different combinations of point mutations that block Mg^{2+} binding in the same EF-hand affect GCAP properties in a similar fashion, (ii) mutating any particular EF-hand does not eliminate conformational changes in GCAP1 as a result of cation binding in other parts of the molecule detected by Trp fluorescence, and (iii) mutating an EF-hand in one semi-globule of GCAP1 does not eliminate either Ca^{2+} or Mg^{2+} binding in EF-hands located in the opposite, non-mutated half of the molecule [26,30,32].

While GCAPs bind Ca^{2+} within submicromolar range ($K_{d\text{Ca}} \sim 0.06\text{--}0.2 \mu\text{M}$), they also efficiently bind Mg^{2+} within submillimolar range ($K_{d\text{Mg}} \sim 0.2 \text{ mM}$ for GCAP1) [26,30,32,

33] and, therefore, can be turned into Mg^{2+} -bound form at the physiological intracellular Mg^{2+} concentrations in vertebrate rods and cones (~ 0.9 mM; [34]). Despite its much lower intracellular concentrations, Ca^{2+} binds with much higher affinity to GCAPs, and in the dark its concentration is high enough to efficiently compete with Mg^{2+} and replace it in EF-hands. However, because the free Ca^{2+} concentrations sharply fall after illumination and those of Mg^{2+} remain near 1 mM [34], Mg^{2+} becomes able to replace Ca^{2+} in EF-hands in the light and thus create the state of GCAP required for stimulation of RetGC activity [26,30,33]. At the physiological concentrations of Mg^{2+} and Ca^{2+} , GCAP1 in photoreceptors simply cannot stably exist as an apo-protein [33]. Therefore, as photoreceptors repeatedly respond to the decrease and increase of illumination by opening and closing their cGMP-gated channels, which is translated into the rise and fall in their free Ca^{2+} concentrations, GCAPs must correspondingly undergo cyclic changes between their Ca^{2+} -bound (retGC inhibitor) and Mg^{2+} -bound (retGC activator) states (Fig. 3c) resulted from the competition between Mg^{2+} and Ca^{2+} for the same binding centers in GCAP molecule.

Recent observations directly indicate that cation binding in EF-hands is critical for proper docking of GCAP1 with RetGC, such that if GCAP1 did not have the ability to bind Mg^{2+} , a dissociation of Ca^{2+} would merely render GCAP1 inactive in the light, rather than allow it to maintain a complex with the cyclase as its activator. There are two lines of evidence in support of this model: (i) the apparent affinity of RetGC for GCAP1 drops when the Mg^{2+} binding is blocked in EF-hand 3 and especially EF-hand 2 [26] and (ii) when Mg^{2+} binding is blocked in EF-hand 2, GCAP1 is unable to efficiently co-localize with RetGC1 when both proteins are co-expressed in transfected HEK293 cells (Fig. 4) [35].

Binding of Mg^{2+} versus Ca^{2+} causes a characteristic change in the intrinsic Trp fluorescence of GCAP1 [33,36,37] corresponding to its activator versus inhibitor states [26,30]. This change includes shifting between more polar versus non-polar environment of a conserved Trp residue located in the non-metal binding EF-hand 1 of GCAPs [26,30]. Based on its Trp fluorescence alterations between apo form of GCAP1 and its Mg^{2+} bound form, in GCAP1 the conformation of EF1 domain is directly sensitive to Mg^{2+} binding in EF-hand 2 [26,30]. Interestingly, the EF-hand 1 domain has been implicated as an element of GCAP structure required for its interaction with RetGC [38,39]. Since cation binding in EF-hand 2 is both necessary for efficient docking of GCAP1 with retGC and strongly affects the conformation of EF-hand 1 as its neighboring domain, it seems reasonable to suggest that both EF-hand domains together create the “docking” structure in GCAP1 required for its primary binding with RetGC1 [35].

In a striking contrast to EF-hands 2 and 3, whose inactivation affects GCAP1 interaction with RetGC1, prevention of Mg^{2+} binding in EF-hand 4 has no detectable effect on RetGC activation. When Ca^{2+} binding is inactivated in EF-hand 4, GCAP1 becomes a constitutive activator of RetGC, regardless of whether or not EF-4 binds Mg^{2+} [26,30] (Fig. 4a). Therefore, only EF-hands 2 and 3 are likely required to maintain their cation-bound state in the light to preserve the GCAP complex with RetGC, while Mg^{2+} binding in the EF-hand 4 is unlikely to have any structural role in the cyclase activation. What is then the function of the metal binding in EF-hand 4? Evidently, Ca^{2+} binding in this EF-hand is critical for deceleration of RetGC in the dark, when the Ca^{2+} concentrations rise. Inactivation of Ca^{2+} binding in EF-hand 2 does not produce any major effect on RetGC regulation as long as Mg^{2+} binding is permitted in that EF-hand, but the Ca^{2+} -dependent RetGC turn-off in the dark does require that both EF-hands 3 and 4 assume their Ca^{2+} -bound conformations [26,30,31]. An important property of GCAPs is that the two halves of the protein molecule are each formed by two pairs of EF-hands connected by a flexible hinge region (Fig. 2) [40-43]. In GCAP1, EF-hand 3 and 4 operate together as a single Ca^{2+} binding semi-globule “module” converting GCAP1 into its inhibitor conformation, such that binding of Ca^{2+} in EF-3 domain promotes the subsequent Ca^{2+} binding in the neighboring EF-hand 4, but not vice versa [30,32]. Mutations suppressing Ca^{2+} binding

in EF-3 and EF-4 domains of GCAP1 even associate with congenital cone degenerations in humans and result in photoreceptor death in transgenic mice expressing mutant GCAP1 through the abnormal elevation of cGMP and free Ca^{2+} in photoreceptors in the dark [25, 44-48] (the position for the first two such mutations in GCAP1 that were found in human patients, Y99C and E155G [44,45], are shown by arrows in Fig. 2). Even though Mg^{2+} binding in EF-4 of GCAP1 in the light is not required for creating the proper “RetGC activator” conformation per se, it nevertheless could be physiologically significant, because Mg^{2+} binding would accelerate the transition of the inhibitor state into the activator state in the light by competing with Ca^{2+} in this EF-hand and thus expediting the release of GCAP1 from its inhibitor conformation.

The sensitivity of $\text{Ca}^{2+}/\text{Mg}^{2+}$ exchange in two different GCAP isoforms

There are two homologous isozymes of RetGC in the retina, RetGC1 and RetGC2 [9], also known as GC-E and GC-F [10] or ROS-GC1 and ROS-GC2 [49]. RetGC1, the first identified GCAP-regulated isozyme [8], seems to provide most of cGMP synthetic activity in cones and is critical for their survival [50]. The exact contribution of RetGC1 versus RetGC2 in rods remains to be determined. Both RetGC1 and 2 are present in outer segments [8,51,52] and both accelerate rod recovery from light exposure [52]. In addition to that there are several isoforms of GCAPs [29] in photoreceptors, of which GCAP1 and GCAP2 are ubiquitously present among vertebrates. Some explanations for such a complexity can be drawn from the $\text{Ca}^{2+}/\text{Mg}^{2+}$ exchange sensitivity in different GCAPs (Fig. 5). Competition between Mg^{2+} and Ca^{2+} ions for the same binding sites in EF-hands has a strong effect on the regulatory properties of GCAP1 and 2, and the efficiency of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ exchange in different isoforms may explain the need in multiple sensor proteins being involved in fine RetGC regulation in photoreceptors to provide the proper recovery kinetics and light adaptation. GCAP1 has lower and GCAP2 higher apparent affinity for Ca^{2+} in vitro and in vivo [25,47,53,54], and they both decrease proportionally with the increase in Mg^{2+} concentrations [33] (Fig. 5). As a result, under physiologically relevant free intracellular Mg^{2+} (~1 mM; [34]), GCAP1 can more and GCAP2 less readily exchange bound Ca^{2+} for Mg^{2+} in response to small decrease in free Ca^{2+} concentrations (Fig. 5). The difference in metal sensitivity between the two sensor proteins creates a possibility of the cyclase regulation being a sequential process where each of the two GCAPs is responsible for a different stage of boosting the cGMP synthesis during photoresponse [27,53,54] (Fig. 6a): GCAP1 would turn into a RetGC activator earlier in the course of photoresponse, as soon as the free Ca^{2+} concentrations start to fall after rod CNG channel closure, while GCAP2 would require deeper fall in free Ca^{2+} levels in order to bind Mg^{2+} , which may occur at later stage of the photoresponse and/or at brighter illumination levels. This hypothesis was recently directly addressed in vivo using disruption of the GCAP2 gene in mouse [54]. The Ca^{2+} -sensitive RetGC activity measured in rods lacking GCAP2 was reduced, and its Ca^{2+} -sensitivity was shifted toward higher than normal range of Ca^{2+} concentrations, and both response kinetics and light sensitivity observed in those rods in single-cell recordings were generally consistent with the predictions from the model. GCAP2^{-/-} rods displayed slowed recovery kinetics, increased light sensitivity, and faster saturation by background light, but the GCAP2 knockout did not substantially affect the amplitude of a single-photon response, which is all what one would expect if GCAP2 only contributed to RetGC activation at lower Ca^{2+} concentrations developed later in the excitation phase (Fig. 6). When the entire tail-to-tail pair of genes coding for GCAP1 and 2 [55] is disrupted with a single (GCAP1?GCAP2) knockout construct, there is drastic increase in the rod single-photon response amplitude [4,27]. Virtually no increase of the amplitude was observed in the individual GCAP2 knockout rods, despite a delay in their recovery phase [50], therefore, it is reasonable to expect that it is a function of GCAP1 rather than GCAP2 to restrain the single-photon response amplitude by accelerating cGMP production earlier in the course of the

response. In order to verify this suggestion, it would require testing a genetic model lacking individual GCAP1.

Conclusion

GCAPs have long been regarded as merely Ca^{2+} binding proteins, but evidence accumulated during recent years demonstrate that GCAPs are $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding proteins, whose physiological function is controlled by the exchange of their bound Ca^{2+} for Mg^{2+} in the light versus dark, rather than merely release and binding of Ca^{2+} . Both Ca^{2+} - and Mg^{2+} -bound states of GCAPs have distinct conformational and functional features and both are required for normal regulation of retinal guanylyl cyclase in photoreceptors upon changes between light and dark.

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Fig. 1.
 Ca^{2+} feedback and cGMP synthesis in photoreceptors. Explanations are provided in the text

Fig. 2.

Three-dimensional structure of GCAP1 [41] and GCAP2 [40]. GCAPs comprise two semi-globular halves formed by two pairs of EF-hand structures (EF-1+EF-2 and EF-3+EF-4), numbered beginning from the N-terminus in primary structure. Spheres represent divalent cation bound in EF-hand loops. The loop in the first EF-hand domain lacks some key consensus side chain residues required for coordinating the metal ions. The *arrows* indicate the positions of two mutations associated with congenital blindness, Y99C and E155G. Other explanations are given in the text

Fig. 3. Mg^{2+} binding makes GCAP1 into RetGC activator in the conditions of light adaptation whereas Ca^{2+} binding is required to decelerate the cyclase at high Ca^{2+} concentrations typical for dark-adapted rods (from [26,30]). **a** Mutations in 12-amino acid EF-hand loops of each of EF-hands 2, 3, and 4 that can disable either Ca^{2+} or both Ca^{2+} and Mg^{2+} binding [26] in EF-hand loops of bovine GCAP1. **b** Activation of recombinant RetGC1 by mutant forms GCAP1 was assayed in vitro at different free Ca^{2+} concentrations in the presence of Ca/Mg/EGTA buffer maintaining Mg^{2+} at near 5 mM level. Wild-type GCAP1 (*open circle*) produces maximal retGC activity below 200 nM free Ca^{2+} but decelerates it as Ca^{2+} rises; inactivation of only Ca^{2+} binding turns GCAP1 into a Ca^{2+} -insensitive Mg^{2+} -bound activator of RetGC (*filled square*), whereas mutations preventing both Ca^{2+} and Mg^{2+} binding turn GCAP1 into an apo-form and completely block activation of RetGC (*filled triangle*). **c** Cation binding cycle in GCAP1 as it undergoes transitions between the RetGC activator and inhibitor states, driven by light versus dark exposure of photoreceptors. Other explanations are given in the text

Fig. 4. Effects of Mg^{2+} binding prevention in individual EF-hands (reproduced from [35] with minor modifications). **a** Inactivation of Mg^{2+} binding in EF-hands 2 and 3, but not in EF-hand 4 inhibits RetGC1 activation by GCAP1. Mutations blocking only Ca^{2+} binding in all three EF-hands (*open circle*) or inactivation of both Ca^{2+} and Mg^{2+} binding in EF-hand 4 (*filled triangle*) do not alter RetGC activation by GCAP1 (*wt*, *open square*). Mutation blocking both Ca^{2+} and Mg^{2+} binding in EF-hand 3 (*open triangle*) and especially EF-hand 2 (*filled inverted triangle*) suppress activation of RetGC1; measured at constant 5 mM free Mg^{2+} and <20 nM free Ca^{2+} . For other details see reference [35]. **b** Inactivation of Mg^{2+} binding in EF-hand 2 suppresses docking of GCAP1-GFP with RetGC1 in cultured cells (modified from [35]). RetGC1 was co-expressed in transfected HEK293 cells with GFP-tagged GCAP1, either wild type (*left*) or its D64N mutant (*right*), incapable of binding Mg^{2+} in EF-hand 2. Distribution of the GFP tag green fluorescence and anti-RetGC1 red immunofluorescence was recorded across each cell. For other details see [35]

Fig. 5.

Ca^{2+} and Mg^{2+} sensitivity of RetGC regulation by GCAP1 and GCAP2 in rod outer segment (ROS) membranes (reproduced from [33]). **a, b** ROS membranes were depleted from the endogenous GCAPs by multiple hypotonic extractions, reconstituted with recombinant GCAP1 (**a**) or GCAP2 (**b**), and total RetGC activity in ROS was assayed as a function of free Ca^{2+} in the presence of 0.5 mM (open square), 1 mM (filled triangle), 2 mM (filled circle), and 5 mM (inverted filled triangle) free Mg^{2+} ; basal RetGC activity is shown at 0.5, 1, 2, and 5 mM Mg^{2+} (filled square, filled diamond, open circle, and open diamond, respectively) in the ROS membranes without GCAPs added. **c** The $[Ca]_{1/2}$ values for RetGC inhibition by GCAP1 and GCAP2 increase proportionally with the free Mg^{2+} concentration, but their relative difference remains unchanged

Fig. 6.

Two-step activation model of retGC by GCAP1 and GCAP2 (reproduced from [54]). **a** Neither GCAP1 nor GCAP2 activate retGC in the dark when the Ca²⁺ concentration in rods is high. After illumination, as soon as Ca²⁺ starts to fall, GCAP1, which has the lower affinity for Ca²⁺ of the two GCAPs, converts into a Mg²⁺-bound state and activates retGC. This activation alone is not fully sufficient to achieve the normal, physiological kinetics of photoresponse recovery and light adaptation. Any fall in Ca²⁺ below 100 nM stimulates Ca²⁺/Mg²⁺ exchange to occur in GCAP2, which then provides additional activation of retGC. At present, it remains unclear whether or not each GCAP acts through a particular retGC isozyme or whether both GCAPs can directly or via an allosteric mechanisms compete for the same isozyme(s). **b** Slowed recovery of the GCAP2^{-/-} single-photon response. The mean, fractional single-photon response from the knockout rods (gray trace) was similar in size to that from the wild-type rods (black trace), but the recovery was slower. The divergence of the two traces late in the rising phase suggests that GCAP2 normally activates retGC at this time. See [54], for the details