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Calcium-dependent cysteine reactivities in the neuronal calcium sensor guanylate cyclase-activating protein 1

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Abstract Guanylate cyclase-activating protein 1 (GCAP-1) is a Ca^{2+}-sensing protein in vertebrate photoreceptor cells. It activates a membrane-bound guanylate cyclase. Three of four cysteines present in wild-type GCAP-1 were accessible to the thiol-modifying reagent 5,5′-dithio-bis-(2-nitrobenzoic acid) in the presence of Ca^{2+}. Only Cys106 became exposed to the solvent after Ca^{2+}-chelation. Since Cys106 is located in EF-hand 3, we could determine an apparent K_D of 2.9 μM for Ca^{2+} binding to this site with a fast off-rate ($t \sim 2$ ms). We conclude that the rapid dissociation of Ca^{2+} from EF-hand 3 in GCAP-1 triggers activation of guanylate cyclase in rod cells.

Key words: Phototransduction; Guanylate cyclase; Guanylate cyclase-activating protein; Neuronal calcium sensor

1. Introduction

Neuronal calcium sensor (NCS) proteins constitute a family of EF-hand Ca^{2+}-binding proteins [1,2]. Known members of this family among others are recoverin and guanylate cyclase-activating proteins (GCAPs) which play key roles as Ca^{2+} sensors in vertebrate phototransduction and are specifically expressed in rod and cone cells [3]. Three different GCAPs are known so far to activate the photoreceptor membrane guanylate cyclase (ROS-GC1) in isolated rod outer segments (ROS) at 100–300 nM of free [Ca^{2+}]. Activation of ROS-GC1 by GCAPs is a key step in restoration of the cGMP level in rod and cone cells after rapid hydrolysis of cGMP by illumination. Regulation of ROS-GC1 by changing Ca^{2+}-levels is one of the main mechanisms of light adaptation in photoreceptor cells [4].

GCAPs have four EF-hand motifs, but only three EF-hands are functional Ca^{2+}-binding sites. As most NCS protein GCAPs contain a consensus site for N-terminal myristoylation. The functional significance of myristoylation in GCAPs is not known and may differ among the different isoforms. For example, non-myristoylated GCAP-1 shows a different activation profile of ROS-GC1 than myristoylated GCAP-1, but no such difference was observed for GCAP-2 [4–6]. Another characteristic feature of GCAPs is their property to bind to ROS-GC1 at low and at high [Ca^{2+}]. Therefore the current concept of cyclase regulation by GCAPs favors a Ca^{2+}-induced switch of GCAP from a basal or inhibitory state to an activator state while it is bound to cyclase [7–9]. A prominent feature of the primary structure of GCAP-1 are four cysteine residues in distant parts of the protein. In order to gain more information about the functional and/or structural role of these cysteines we investigated cysteine mutants of GCAP-1. In particular, we explored the use of internal cysteines as internal monitors for Ca^{2+}-induced structural changes.

2. Materials and methods

2.1. Construction and expression of GCAP 1 cysteine mutants

The four cysteine residues in GCAP1 have been exchanged against alanines in different number and combination. Standard cloning methods [10] and oligonucleotide-directed mutagenesis were performed using cDNA of GCAP-1 [5] as a template to generate mutated GCAP-1. In a two-step PCR inner mutagenesis primers (for C18A and C29A: 5′GGG**CGC**CTCTGTCATGAACTTCTTGTACCAC-TGGTG**GGC**CTCGGTGCTGCTCAG-3′; for C106A: 5′-CGT-GGACGGCAACGGA**GCG**ATCGACCGCGACGAGCTGC-3′; for C125A: 5′-GGCGGTCATGGTCGAGTCGCT**CGC**GGGGTTAAT-GGCTCGGATGGC-3′) were used in combination with outer primers. An *Nde*I restriction site was introduced upstream of the start codon and a *Bam*HI site downstream of the stop codon. The resulting mutated GCAP1 genes were verified by sequencing and introduced in corresponding sites of pET11a (Novagen). To express the proteins, competent *Escherichia coli* BL21-CodonPlus(DE3) cells (Stratagene) were transformed. Single colonies were cultivated in dYT to an OD_{600} of 0.6 and induced with 1 mM IPTG for 4 h. Cells were harvested and resuspended in 50 mM Tris pH 8.0.

2.2. Purification of GCAP-1 and its mutants

All purification steps were performed at 4°C. Resuspended cells were disrupted by passaging through a French press (SLM Aminco). The cell lysate was then separated from cell debris by centrifugation. Subsequent purification from the supernatant was achieved as described [8]. Purified wild-type (wt) and mutant GCAP-1 (0.5–3 ml protein solution of 1–3 mg/ml) were dialyzed against 50 mM ammonium bicarbonate buffer. Aliquots of 1 mg were lyophilized by a Speedvac concentrator and then stored at −80°C until further use.

2.3. Preparation of ROS membranes and guanylate cyclase assay

ROS were prepared from freshly collected bovine eyes as before [11]. The predominant guanylate cyclase in ROS preparations is ROS-GC1 (K.-W. Koch, unpublished observation). For reconstitution experiments, ROS membranes were prepared as described [5]. Varying concentration of GCAP-1 or its mutants were added to 10 μl of washed ROS membranes to give a total volume of 20 μl. The

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Abbreviations: ROS, rod outer segments; ROS-GC1, photoreceptor membrane guanylate cyclase; GCAP, guanylate cyclase-activating protein; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid; wt, wild-type; Rh, rhodopsin

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free [Ca^{2+}] was adjusted by Ca^{2+}/EGTA buffer calculated with a Ca^{2+} buffer program CHELATOR [12]. Incubation was performed at room temperature under very dim red light. The activity of ROS-GC1 was determined by a HPLC chromatography assay as described before [5].

2.4. Counting disulfide bonds by iodoacetate derivatives

Integral numbers of disulfide bonds in GCAP-1 were counted as in [13,14]. This approach examines the charge differences that are introduced by specific chemical modifications of the cysteines using the alkylation reagents iodoacetamide (IAM) and iodoacetic acid (IAA).

2.5. Thiol reactivity of GCAP-1 and its cysteine mutants

Quantitative determination of thiol groups in solution was performed by recording the formation of 5-thio-2-nitrobenzoic acid (TNB) from 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm [15]. A fresh solution of DTNB (12.5 mM) was prepared by dissolving it in 0.1 M Tris, pH 8.0 and sonicating for 5 min at 80–100 W. Thiol reactivity of GCAP-1 wt and mutants was measured by the addition of 50–250 μM DTNB into a cuvette containing 6–12 μM of GCAP-1, 50 mM HEPES pH, 7.4, 100 mM NaCl and either 100 μM $CaCl_2$ or 2 mM EGTA. The time course of TNB formation at 412 nm was recorded after injection of DTNB using a Shimadzu UV-2101PC UV/VIS scanning spectrophotometer or using an SLM-Aminco DW-2000 UV/VIS spectrophotometer in dual wavelength mode with monochromators set at 412 nm and 750 nm. When samples were preincubated with $CaCl_2$, EGTA was injected 5 min after application of DTNB. In a second set of experiments the order of $CaCl_2$ and EGTA application was reversed ($CaCl_2$ in excess). Thiol reactivity of GCAP-1 and its cysteine mutants was also measured as a function of the free [Ca^{2+}]. All experiments were performed with non-myristoylated wt and mutants of GCAP-1. Presence of the myristoyl group changed the accessibility of cysteines in a complex matter (J.-Y. Hwang and K.-W. Koch, in preparation)

3. Results

3.1. GCAP-1 does not contain intramolecular disulfide bonds

GCAP-1 contains four cysteines and could theoretically harbor one or two disulfide bridges. We tested the presence of hypothetical disulfide bonds by treatment of GCAP-1 with IAM and iodoacetate at different concentrations of DTT according to [13,14]. Different electrophoretic mobilities of GCAP-1 would indicate the presence of intramolecular disulfide bonds, which we did not observe. Consistent with this initial observation was that all four cysteines rapidly reacted with DTNB (see below).

3.2. Monitoring the exposition of thiol groups of GCAP-1

The cysteine residues in GCAP-1 were titrated by DTNB in either the presence or absence of Ca^{2+}. The reaction of DTNB with GCAP-1 caused a time-dependent labeling by TNB. The rate of covalent modification of free sulfhydryl groups with DTNB solution was dependent on the relative accessibility and the chemical environment of the cysteines. From a cysteine standard curve the number of reactive cysteines in a protein can be calculated. The reaction of 5 μM GCAP-1 with DTNB as a function of time is shown in Fig. 1.

The absorbance at 412 nm (A_{412}) of 5 μM GCAP-1 in the presence of $CaCl_2$ was 0.205, which corresponds to three cysteines in GCAP-1. After 300 s, injection of EGTA caused an additional increase in A_{412} of 0.074, which corresponded to 5.4 μM of free thiol or one cysteine (Fig. 1A). When the injection of $CaCl_2$ and EGTA was reversed, the change in A_{412} was 0.290 with EGTA and after 300 s, injection of $CaCl_2$ caused no change (Fig. 1B). These data demonstrated that in the presence of $CaCl_2$, three cysteines were exposed to the solvent and one cysteine remains buried in wt GCAP-1. Chelating Ca^{2+} with 2 mM EGTA led to the exposure of the buried cysteine. In contrast, when GCAP-1 was preincubated with EGTA, all four cysteines were exposed and were accessible to DTNB. These results indicated that cysteines in GCAP-1 are sensitive to Ca^{2+}-induced conformational changes in their environment. Therefore, we constructed cysteine mutants of GCAP-1 to further investigate the use of cysteine residues as internal monitors. The first cysteine Cys18 is located in the α-helix of the non-functional EF-1, Cys29 in the loop of EF-1, Cys106 in the loop of EF-3, and Cys125 between EF-3 and EF-4. The single mutants were termed as C18A, C29A, C106A, and C125A. The triple mutants, where three cysteines were replaced by alanines, were termed CAAA (C29·106·125A), ACAA (C18·106·125A), AACA (C18·29·125A), and AAAC (C18·29·106A). In the quadruple mutant, termed AAAA, all four cysteines were substituted by alanines.

3.3. Activation of ROS-GC1 by cysteine mutants of GCAP-1

We tested, whether the mutations impaired the function of GCAP-1 as an activator of ROS-GC1. Activation of ROS-GC1 in washed ROS membranes was measured as a function of increasing concentrations of mutant GCAPs or as a function of free [Ca^{2+}] (see Fig. 2 for the quadruple mutant AAAA as example). Results for all mutant proteins are listed in Table 1. In comparison to the wt, single cysteine mutants C18A, C29A and C106A activated cyclase to a lower degree, but C125A reached a 50% higher maximal activation. The EC_{50} values were for all single cysteine mutants lower, which indicated that the apparent affinity of the mutants for ROS-GC1 was higher. Maximal activation of cyclase by the triple

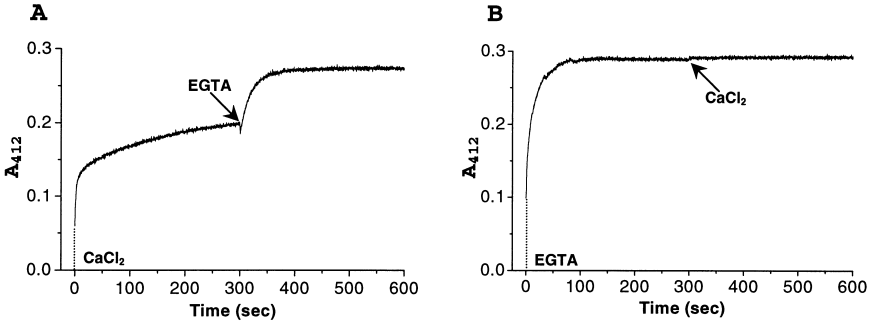

Fig. 1. Cysteine fraction of wt GCAP-1 with DTNB. A: Time course of thiol reactivity was measured at 412 nm (formation of TNB). wt GCAP-1 (5 μM) was preincubated with 100 μM $CACl_2$ and reaction was started by injection of 50 μM DTNB. After 300 s 2 mM EGTA was injected and the response was recorded for 300 s. B: Order of $CaCl_2$ and EGTA application was reversed.

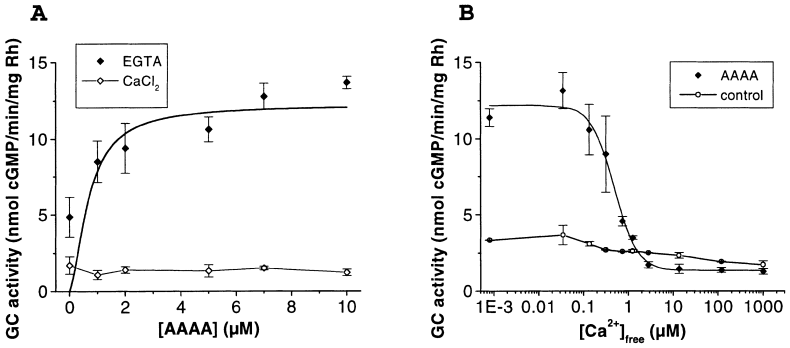

Fig. 2. Activation of ROS-GC1 by the GCAP-1 mutant AAAA. A: ROS membranes containing ROS-GC1 were incubated at increasing concentrations of mutant AAAA at 2 mM $CaCl_2$ or 2 mM EGTA (see inset) to measure guanylate cyclase activity. Halfmaximal activation (EC_{50}) was at 0.682 μM. B: Guanylate cyclase activity was measured as a function of free [Ca^{2+}]. ROS membranes were incubated with 10 μM mutant AAAA at varying [Ca^{2+}]. Activity was halfmaximal at 0.293 μM free [Ca^{2+}]. Hill coefficient was $n = 1.7$. Open circles are control determinations without added GCAP-1 mutant. Data points are from triplicates.

mutants was less variable, apparent affinity was also higher in comparison to wt. Activity at high Ca^{2+} was in most cases (C29A, C106A, all triple mutants and the quadruple mutant) lower, which indicated an inhibitory effect that was stronger than observed with the wt.

When we tested the activation profile of guanylate cyclase by GCAP-1 mutants over a range of free [Ca^{2+}] from 1 nM to 1 mM, single cysteine mutants differed also from triple cysteine mutants mainly by two aspects (Table 1): free [Ca^{2+}] at which activation is halfmaximal (IC_{50}) was always lower in the case of triple mutants as in the case of single mutants. Activation was cooperative with an Hill coefficient of $n = 1.5$–1.7 in triple mutants but no cooperativity was observed with single mutants. Thus single cysteine mutants displayed similar properties as the non-myristoylated wt (Table 1), but triple mutants were more like native myristoylated GCAP-1 in whole ROS [5]. In line with this observation were the results obtained with the quadruple mutant (Table 1 and Fig. 2), which showed almost identical properties as native myristoylated GCAP-1 in whole ROS. Additionally, the quadruple mutant showed a very pronounced inhibitory effect at high [Ca^{2+}] (Fig. 2). Thus, mutations did not impair the general function of GCAP-1, instead some aspects of its Ca^{2+}-sensitive regulation were modified.

3.4. Thiol reactivity of single cysteine mutants

The reactivity of the single cysteine mutants with DTNB is shown in Fig. 3. Three mutants, C18A, C29A and C125A showed an immediate reaction of two cysteines with DTNB in the presence of Ca^{2+} and the exposure of an additional cysteine after injection of EGTA. All three cysteines in mutant C106A reacted immediately with DTNB in the presence of Ca^{2+}. No exposure of an additional cysteine was observed after injection of EGTA. This result clearly demonstrated that the cysteine at position 106 is buried in the presence of Ca^{2+}.

3.5. Reactivity of triple cysteine mutants

To monitor the reactivity of each cysteine residue separately, triple cysteine mutants were investigated. Fig. 3 illustrates the reactivity of single cysteines in all four mutants towards DTNB. A very rapid development of the signal was recorded with the mutants CAAA and AAAC in the presence of Ca^{2+}. Injection of EGTA did not elicit a further response. A significantly slower signal in the presence of Ca^{2+} was seen with mutant ACAA, but also no signal after injection of EGTA. Only mutant AACA reacted with DTNB after injection of EGTA and in the presence of Ca^{2+} increase of absorbance was very slow. These observations agree with results that the cysteine residue at position 106 is buried in GCAP-1 in high [Ca^{2+}] and that it becomes exposed in the absence of Ca^{2+}.

3.6. Thiol reactivity of ACAA and AACA as a function of $[Ca^{2+}]_{free}$

Recordings in the presence of Ca^{2+} showed different kinetics (Fig. 3). A slower increase in A_{412} was observed when Cys29 was present, but absent when it was substituted by Ala.

Table 1
Properties of GCAP-1 mutants

Type	Activity at low $[Ca^{2+}]_{free}$	Activity at high $[Ca^{2+}]_{free}$	EC_{50} [Protein] (μM)	*x*-fold activation	IC_{50} $[Ca^{2+}]_{free}$ (μM)	*n* (Hill coefficient)
GCAP-1	12.39	2.14	0.85	5.8	2.817	0.3
C18A	10.13	2.86	0.53	3.5	10.368	0.6
C29A	8.28	1.77	0.41	4.7	5.190	0.7
C106A	6.01	1.34	0.36	4.4	2.083	1.2
C125A	17.97	2.80	0.35	6.4	4.254	1.0
CAAA	9.98	1.51	0.78	6.6	0.499	1.7
ACAA	9.47	1.71	0.82	5.5	0.721	1.5
AACA	11.84	1.12	0.49	10.6	0.551	1.5
AAAC	12.85	1.72	0.49	7.5	1.052	1.5
AAAA	12.23	1.22	0.68	10.0	0.293	1.7

Activity is expressed as nmol cGMP/min/mg rhodopsin (Rh).

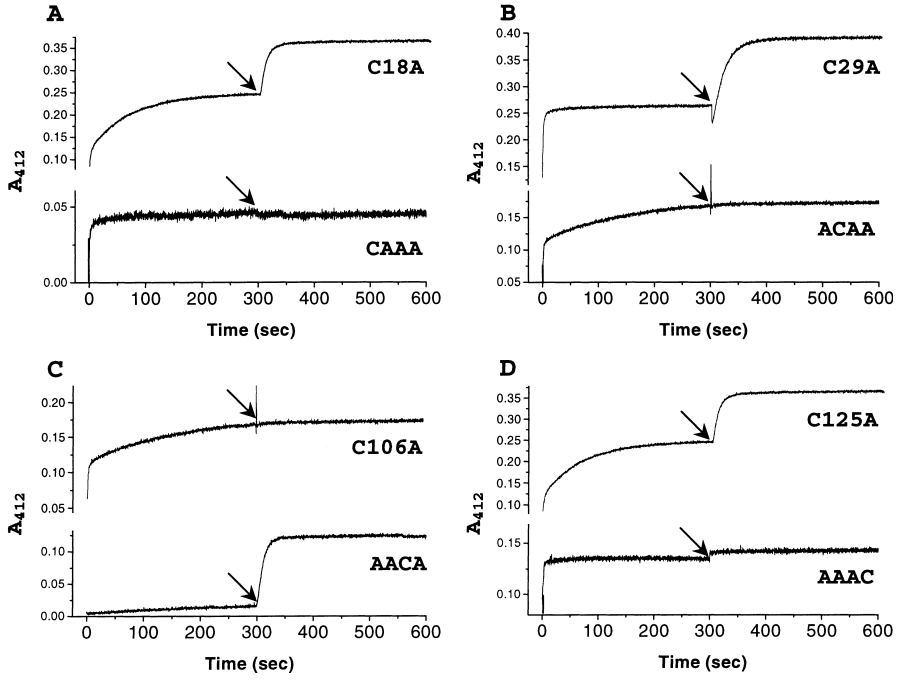

Fig. 3. Cysteine reaction of single cysteine mutants of GCAP-1 with DTNB. Time course of thiol reactivity was recorded at 412 nm according to the same protocol as in Fig. 1A (arrows indicate injection of EGTA). Upper traces show recordings with single mutants, lower traces with triple mutants. Recordings were obtained with 10 μM C18A and 6 μM CAAA (A), 12 μM C29A and 6 μM ACAA (B), 5 μM C106A and 10 μM AACA (C) and 8 μM C125A and 10 μM AAAC (D).

Results with the triple cysteine mutants (ACAA and AACA) also indicated different kinetics of DTNB reactivity as a function of $[Ca^{2+}]$. Thus we compared the reactivities of cysteines at position 29 and 106 by measuring the time-dependent change in absorbance as a function of $[Ca^{2+}]$ (Fig. 4). The reactivity (and therefore accessibility) of the cysteine in ACAA displayed different kinetics at different $[Ca^{2+}]$ (Fig. 4A). We determined different time constants by fitting the

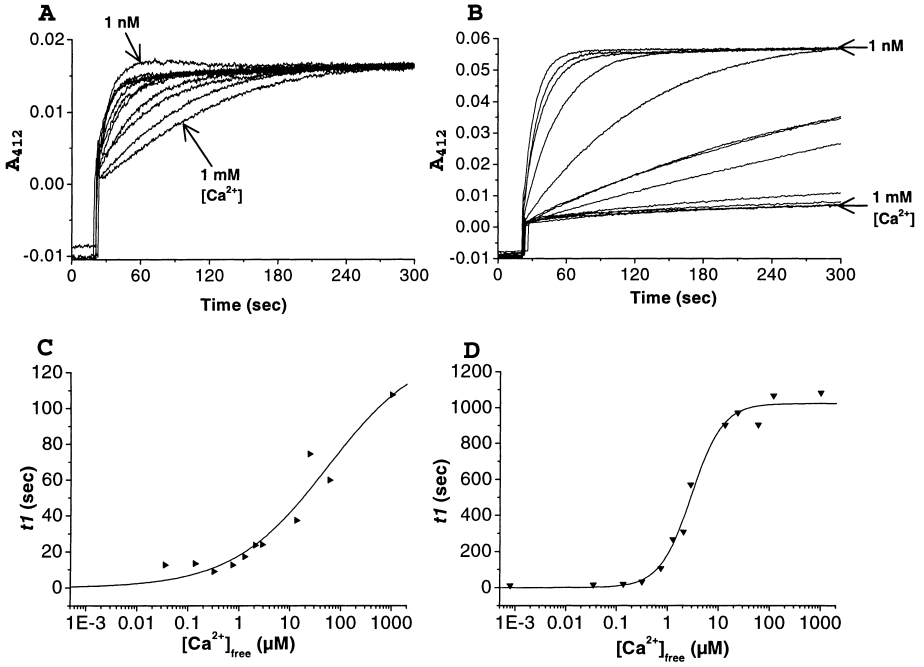

Fig. 4. Cysteine reaction of mutants ACAA (A) and AACA (B) with DTNB as a function of time and calcium concentration. Thiol reactivity was measured for various concentrations of Ca^{2+}/EGTA buffer. The reaction was started by the addition of DTNB directly to the cuvette to a final concentration of 100–250 μM and monitored using an SLM-Aminco DW-2000 UV/VIS spectrophotometer in dual wavelength mode with monochromators set at 412 nm and 750 nm. Recordings were fitted to a modified exponential growth for non-linear curve fitting; $y = y_0 - A_1 \times e^{-x/t1}$. The calculated time constants ($t1$) obtained from recordings with ACAA (C) and AACA (D) were plotted against $[Ca^{2+}]_{free}$. The solid line represents the best fit to the Hill equation.

data to a single exponential. Plotting the time constants as a function of [Ca^{2+}] yielded an apparent K_D of approximately 100 μM (Fig. 4C).

A different titration was obtained with the triple mutant AACA (Fig. 4B). Almost no reactivity was observed at high $[Ca^{2+}]_{free}$. At intermediate $[Ca^{2+}]_{free}$ (3 μM), the reaction towards DTNB occurred at a slow rate when compared with ACAA. Finally, at low [Ca^{2+}] the reaction proceeded with similar fast kinetics as in the case of ACAA. A plot of time constants as a function of [Ca^{2+}] was fitted to a Hill equation yielding an apparent $K_D = 2.9$ μM and a Hill coefficient $n = 1.4$.

4. Discussion

The Ca^{2+}-dependent activation profile of cysteine mutants exhibited some interesting differences in comparison to wt GCAP-1 (Table 1). Cysteines in position 18, 29 and 125 seem to participate in the Ca^{2+}-sensitive regulation of cyclase, because exchange of C to A increased the IC_{50} at which activation of cyclase is halfmaximal. Surprising was the result that the triple mutants and the mutant AAAA resembled more the myristoylated wt GCAP-1. For example, the IC_{50} and the degree of cooperativity we determined with mutant AAAA were like myristoylated wt GCAP-1. We conclude from these observations that the substitution of cysteines by more hydrophobic alanines in at least four positions can compensate the lack of the myristoyl group. GCAP-1 needs a hydrophobic environment close to Cys18, Cys29, Cys106, Cys125 to activate cyclase at physiologically relevant free [Ca^{2+}].

All cysteines in GCAP-1 reacted with the thiol-modifying reagent DTNB, but accessibility to C29 and C106 was dependent on the free [Ca^{2+}]. These two cysteines are located in EF-hand 1 and 3, respectively. The Ca^{2+}-dependent titration of thiol reactivities of mutants ACAA and AACA in Fig. 4 clearly exhibited different Ca^{2+}-sensitivities. We interpret these different Ca^{2+}-sensitivities as different apparent affinities of EF-hand 1 and 3 for Ca^{2+}. The low affinity of EF-hand 1 ($K_D \sim 100$ μM) is consistent with the view that EF-hand 1 does not bind Ca^{2+} in the physiologically relevant range. The apparent affinity of EF-hand 3 for Ca^{2+} was of intermediate affinity ($K_D = 2.9$ μM). However, halfmaximal activation of cyclase by the AACA mutant occurred at much lower free [Ca^{2+}] ($IC_{50} = 0.551$ μM in Table 1) and is cooperative. Thus, EF-hand 2 and/or EF-hand 4 must have a much higher affinity for Ca^{2+}.

We assume that the on-rate of Ca^{2+} to all three EF-hands is under control of diffusion ($k_1 = 2 \times 10^8$ M^{-1} s^{-1} [16]; $K_D = k_{-1}/k_1$). An apparent K_D of 2.9 μM of Ca^{2+} for EF-hand 3 in GCAP-1 would then result in an off-rate of $k_{-1} = 5.8 \times 10^{-3}$ s^{-1} with a corresponding time constant of ~ 2 ms.

This dissociation rate of Ca^{2+} from the EF-hand 3 in GCAP-1 is fast enough to trigger a conformational change in GCAP-1 within the time frame of the photoresponse (less than 200 ms). Therefore, GCAP-1 would fit perfectly into the group of Ca^{2+}-trigger proteins with rapid off kinetics (mean residence time of Ca^{2+} are a few ms) [17]. Relative affinities of Ca^{2+} among different groups of Ca^{2+}-binding proteins depend critically on the ninth position in an EF-hand [18]. If EF-hands contain a Glu at the ninth position, they release Ca^{2+} with slower dissociation rates than EF-hands that contain either an amino acid with a shorter side chain (Asp) or an uncharged side chain (Gln). Ca^{2+}-buffer proteins like parvalbumin have slower dissociation rates of Ca^{2+} than Ca^{2+}-trigger like calmodulin or troponin C (for review see [17]). EF-hand 3 in GCAP-1 contains an Asp at the ninth position, which is typical for a Ca^{2+}-trigger.

We conclude that dissociation of Ca^{2+} from EF-hand 3 in GCAP-1 is the first critical step in activating ROS-GC1. Release from this site then triggers a conformational change in the vicinity of EF-hand 3. Our hypothesis is consistent with a previous tryptophan fluorescence study [16] that reported on a Ca^{2+}-induced major conformational change around EF-hand 3.

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