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QUARTERLY

Ca^{2+} differently affects hydrophobic properties of guanylyl cyclase-activating proteins (GCAPs) and recoverin✪

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Guanylyl cyclase-activating proteins (GCAPs) and recoverin are retina-specific Ca^{2+}-binding proteins involved in phototransduction. We provide here evidence that in spite of structural similarities GCAPs and recoverin differently change their overall hydrophobic properties in response to Ca^{2+}. Using native bovine GCAP1, GCAP2 and recoverin we show that: i) the Ca^{2+}-dependent binding of recoverin to Phenyl-Sepharose is distinct from such interactions of GCAPs; ii) fluorescence intensity of 1-anilinonaphthalene-8-sulfonate (ANS) is markedly higher at high $[Ca^{2+}]_{free}$ (10 μM) than at low $[Ca^{2+}]_{free}$ (10 nM) in the presence of recoverin, while an opposing effect is observed in the presence of GCAPs; iii) fluorescence resonance energy transfer from tryptophane residues to ANS is more efficient at high $[Ca^{2+}]_{free}$ in recoverin and at low $[Ca^{2+}]_{free}$ in GCAP2. Such different changes of hydrophobicity evoked by Ca^{2+} appear to be the precondition for possible mechanisms by which GCAPs and recoverin control the activities of their target enzymes.

Guanylyl cyclase-activating proteins (GCAP1, GCAP2) and recoverin are homologous Ca^{2+}-binding proteins specifically expressed in rods and cones of vertebrate retinas where they are involved in phototransduction (Gorczyca 1999; Palczewski *et al.,* 2000). They belong to a larger family of structurally related proteins called neuronal calcium sensors

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DTT, dithiothreitol; GC, guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; mAb, monoclonal antibody; NCS, neuronal calcium sensors; pAb, polyclonal antibody; PMSF, phenylmethylsulfonyl fluoride; retGC; retina-specific guanylyl cyclase; RK, rhodopsin kinase; ROS, rod outer segment.

(NCS) (Braunewell & Gundelfinger, 1999; Burgoyne & Weiss, 2001). GCAPs have been shown to regulate in a Ca^{2+}-dependent manner the activity of retinal guanylyl cyclases (retGCs) (Gorczyca *et al.,* 1995; Dizhoor *et al.,* 1995; Frins *et al.,* 1996) while recoverin has been postulated to be a Ca^{2+}-dependent regulator of rhodopsin kinase (RK) (Chen *et al.,* 1995; Klenchin *et al.,* 1995; Senin *et al.,* 1995). Amino-acid sequences of GCAP1, GCAP2, and recoverin share about 25% identity. The identity is higher between each two proteins reaching over 40% for GCAP1 and GCAP2 (Gorczyca *et al.,* 1995; Dizhoor *et al.,* 1995; Gorczyca & Sokal, 2002). GCAPs and recoverin also share other structural features such as a similar molecular mass of about 23.5 kDa, N-terminal consensus for acylation by small fatty acids (e.g. myristic acid), and the presence of four EF-hand motifs of which the first (EF1) is functionally inactive (Dizhoor *et al.,* 1991; 1995; Ray *et al.,* 1992; Palczewski *et al.,* 1994; Gorczyca *et al.,* 1995). The three-dimensional structures of Ca^{2+}-bound recoverin and Ca^{2+}-bound GCAP2 resemble overall polypeptide chain organization (Ames *et al.,* 1997; 1999). In spite of the above similarities, significant differences between recoverin and GCAPs have also been noticed. Both GCAPs bind three Ca^{2+} ions (in EF2-4) (Dizhoor & Hurley, 1996; Rudnicka-Nawrot *et al.,* 1998; Ames *et al.,* 1999) and recoverin only two (in EF2 and EF3) (Ames *et al.,* 1997). Although all three proteins are relatively easily washed out from the rod outer segment (ROS) membranes with hypotonic buffers (Dizhoor *et al.,* 1994; Gorczyca *et al.,* 1994; Johnson *et al.,* 1997), they interact with the same membranes differently under physiological conditions. For recoverin binding important are calcium ions and a mechanism known as the Ca^{2+}-myristoyl switch (Zozulya & Stryer, 1992). This mechanism probably does not operate in the case of GCAPs (Hwang & Koch, 2002) and their binding to ROS membranes occurs also in the absence of Ca^{2+} (Gorczyca *et al.,* 1995). The interaction of recoverin with rhodopsin kinase strongly depends on Ca^{2+} concentration (Chen *et al.,* 1995; Klenchin *et al.,* 1995), while GCAPs appear to be complexed with the target enzyme independently of Ca^{2+} (Gorczyca *et al.,* 1995; Dizhoor & Hurley, 1996; Rudnicka-Nawrot *et al.,* 1998; Olshevskaya *et al.,* 1999). These differences suggest that Ca^{2+} strongly affects hydrophobic properties of recoverin but not GCAPs. In this report we present the results of experiments which support the above suggestion.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody G2 (mAbG2) was generated in BALB/c mice against truncated (GCAP180) bovine GCAP1 (Gorczyca *et al.,* 1995); rabbit polyclonal antibody 850 (pAb850) was raised against recombinant bovine GCAP2 expressed in *E. coli,* in which six N-terminal residues are replaced by a His_6 tag (Gorczyca, 2000); rabbit pAb23 was raised against recombinant bovine GCAP1 expressed in *E. coli* (Dejda *et al.,* 2002); pAb837 was raised in rabbit against native bovine recoverin.

Purification of proteins. Proteins were isolated from fresh bovine retinas dissected under dim red light from dark-adapted eyeballs obtained from the local slaughterhouse. Immediately after isolation, the retinas were homogenized at 4°C in a low ionic strength buffer (10 mM Hepes pH 7.4) containing inhibitors of proteases (0.5 mM benzamidine, 1 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml aprotinin). The homogenate was clarified by centrifugation at 30 000 × ***g*** and then at 100 000 × ***g***. The resulting supernatant (retinal extract) was further used as a source of proteins. GCAP1 and GCAP2 were separated and purified from the retinal extract by sequential immunoaffinity chromatography as described previously (Gorczyca, 2000). Briefly, a column containing pAb850 against GCAP2 coupled to Sepharose 4B (pAb850-Sepharose) was used first and then a column

containing mAbG2-Sepharose against GCAP1 was applied. The extract was loaded onto the first column and unbound material was directly loaded onto the second column connected with the first one in tandem. The columns were then washed with 10 mM Hepes, pH 7.4, containing 50 mM NaCl, disconnected, and washed with 10 mM Hepes, pH 7.4, containing 0.2 M NaCl to remove non-specifically adsorbed proteins. Bound GCAPs were eluted from each column with 0.1 M glycine/HCl, pH 2.5. Collected fractions were immediately neutralized with 1 M Tris/HCl, pH 7.4, dialyzed and used in fluorescence measurements. The extract that passed through both immunoaffinity columns was further used for the purification of recoverin. Recoverin was purified by Phenyl-Sepharose chromatography followed by ion-exchange chromatography on DEAE-Sephadex A25 according to the procedure described by Sato & Kawamura (1997) for the isolation of frog S-modulin.

Fluorescence studies. Fluorescence measurements were performed on a Perkin-Elmer LS50 spectrofluorimeter at 22°C with 300 nM concentrations of proteins in 50 mM Hepes, pH 7.4, containing 60 mM KCl, 20 mM NaCl, 1 mM DTT, and 0.4 mM EGTA. Desired concentrations of Ca^{2+} ions ($[Ca^{2+}]_{free}$) were adjusted with 50 mM $CaCl_2$ according to calculations performed using the computer program CHELATOR 1.0 (Schoenmakers *et al.,* 1992). "Low Ca^{2+}" and "high Ca^{2+}" always mean 10 nM and 10 μM $[Ca^{2+}]_{free}$, respectively. The total volume of added $CaCl_2$ did not exceed 1% of the initial sample volume. Excitation and emission bandwidths were always 5 nm. Intrinsic fluorescence emission spectra of tryptophane and tyrosine residues were recorded at 295 and 275 nm excitation wavelengths, respectively. Extrinsic fluorescence emission spectra of ANS (Fluka Chemie AG, Buchs, Switzerland) binding to proteins were recorded at 10 μM concentration of the dye and excitation wavelength of 365 nm. Fluorescence resonance energy transfer (FRET) between protein tryptophane residues and ANS was studied at dye concentrations in the range of 1–50 μM. The total volume of added ANS did not exceed 1% of the initial sample volume. The ANS (acceptor) fluorescence was induced *via* resonance energy transfer from tryptophane residues (donor) of each protein using excitation wavelengths of 295 nm. Background fluorescence of each ANS concentration was recorded similarly in the absence of protein and subtracted from the corresponding sample spectra.

Interaction of proteins with Phenyl-Sepharose. Interactions were analyzed using a Waters HPLC System and Pharmacia HR5/5 column filled with 0.5 ml of Phenyl-Sepharose CL 6B (Pharmacia LKB, Uppsala, Sweden) equilibrated with 50 mM Hepes, pH 7.5. A 1 ml portion of retinal extract (8 mg/ml) containing 2 mM $CaCl_2$ was loaded onto the column at a flow rate of 1 ml/min. The column was washed with 50 mM Hepes, pH 7.5, and then 10 mM EDTA in the same buffer was applied. Total protein content in the effluent was continuously monitored by absorbance at 280 nm, 3.0 ml fractions were collected, and 10 μl aliquots from each fraction were subjected to Western blotting analysis for the presence of GCAP1, GCAP2, and recoverin.

Western blotting analysis. Samples (10 μl) withdrawn from each fraction were resolved under reducing conditions in SDS/PAGE using 12.5% acrylamide gels and then transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were then blocked with 2% (w/v) casein and proteins were immunodetected using rabbit antibodies against GCAP1 (pAb23), GCAP2 (pAb850) or recoverin (pAb837). After washing with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20 (TBST) and subsequently three times with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl (TBS), the blots with bound primary antibodies were incubated with anti-rabbit IgG goat antibodies conjugated to alkaline phosphatase (Promega, Madison, U.S.A.) and visualized using an

NBT/BCIP-based substrate (Promega, Madison, U.S.A.).

RESULTS

Binding of Ca^{2+} differently affects intrinsic fluorescence of recoverin and GCAPs

Three tryptophan and five tyrosine residues are present in bovine recoverin (Dizhoor *et al.,* 1991; Ray *et al.,* 1992), three tryptophan and seven tyrosine residues in GCAP1 (Palczewski *et al.,* 1994), and five tryptophan and five tyrosine residues in GCAP2 (Gorczyca *et al.,* 1995; Dizhoor *et al.,* 1995). Therefore it is possible to monitor the conformational changes that occur in these proteins by measuring their intrinsic fluorescence. The fluorescence of tryptophan residues is excited with the wavelength of 295 nm while mixed fluorescence of tryptophan and tyrosine residues is excited with the wavelength of 275 nm. The observed differences between the intrinsic fluorescence spectra recorded at high and low Ca^{2+} (Fig. 1) show that each investigated protein undergoes conformational changes upon binding of Ca^{2+} and are in line with earlier reports (Dizhoor *et al.,* 1991; Ray *et al.,* 1992; Johnson *et al.,* 1997; Otto-Bruc *et al.,* 1997; Hughes *et al.,* 1998; Sokal *et al.,* 1999; 2001). The largest Ca^{2+}-dependent changes of intrinsic fluorescence excited at 275 nm occur in recoverin and the smallest in GCAP1. Similarly, the Ca^{2+}-dependent changes of fluorescence spectra excited at 295 nm are larger in the case of recoverin and GCAP2 in comparison with GCAP1. These results not only reflect the differences in the content and localization of tyrosine and tryptophan residues in the investigated proteins but also indicate that the Ca^{2+}-driven exposition of aromatic residues to the solvent is distinct in recoverin and both GCAPs. Each protein may therefore variously expose its hydrophobic surfaces. If this is the case, the proteins should reveal different ability to interact in a Ca^{2+}-dependent manner with non-polar groups of Phenyl-Sepharose.

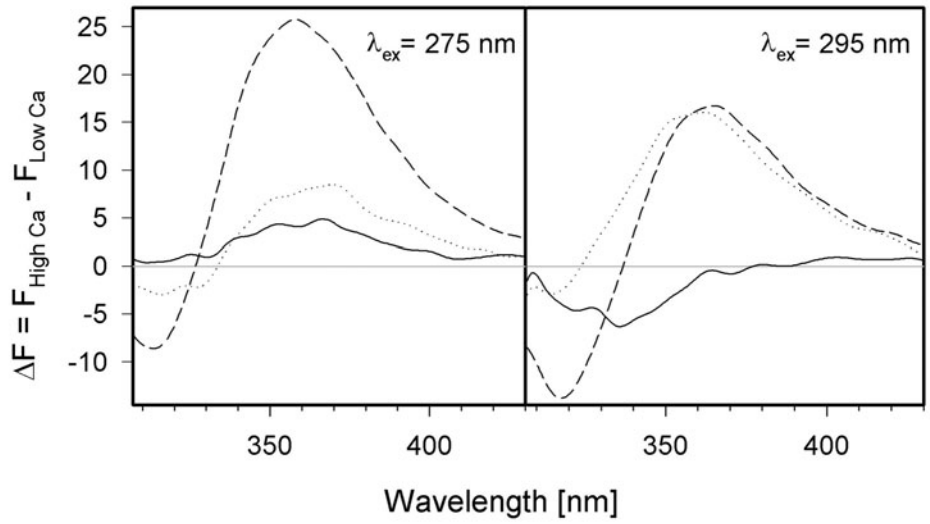

Figure 1. Conformational rearrangements induced by Ca^{2+} in GCAP1, GCAP2, and recoverin.

Difference intrinsic fluorescence spectra were obtained for each protein by subtraction of the spectra recorded at low $[Ca^{2+}]_{free}$ from those recorded at high $[Ca^{2+}]_{free}$. Thus for each emission wavelength the resulting fluorescence intensity (ΔF) is equal to the difference between the fluorescence intensities measured at high Ca^{2+} ($F_{high\ Ca}$) and low Ca^{2+} ($F_{low\ Ca}$). The excitation wavelengths were 275 nm or 295 nm as indicated. The concentration of each protein was 300 nM. The spectra obtained for GCAP1, GCAP2, and recoverin are represented by solid, dotted, and dashed lines, respectively.

Ca^{2+}-dependent interaction of recoverin with Phenyl-Sepharose differs from such interaction of GCAPs

Ca^{2+}-dependent Phenyl-Sepharose hydrophobic chromatography is a convenient method developed for purification of several Ca^{2+}-binding proteins including calmodulin, S100 proteins, neurocalcin, and recoverin (Zozulya & Stryer, 1992; Polans *et al.,* 1995; Ladant, 1995). Being more hydrophobic in a Ca^{2+}-loaded form they bind to the gel and are released from it when Ca^{2+} ions are chelated. Such an interaction of a protein with Phenyl-Sepharose reflects Ca^{2+}-dependent changes in its hydrophobicity. At 100 mM NaCl recoverin interacts with Phenyl-Sepharose in a Ca^{2+}-dependent way (Polans *et al.,* 1995), while GCAPs remain bound to the gel independently of Ca^{2+} concentration (Dizhoor *et al.,* 1994; Gorczyca, unpublished observation). This indicates that in a Ca^{2+}-free form GCAPs are more hydrophobic than recoverin. At low ionic strength, however, all recoverin still binds to Phenyl-Sepharose at 2 mM $CaCl_2$ and dissociates from the column when EDTA is applied but almost all GCAP2 and most of GCAP1 present in the retinal extract loaded are not retained on the column in the presence of $CaCl_2$ (Fig. 2). Therefore Ca^{2+}-loaded GCAPs appear to be less hydrophobic than Ca^{2+}-loaded recoverin.

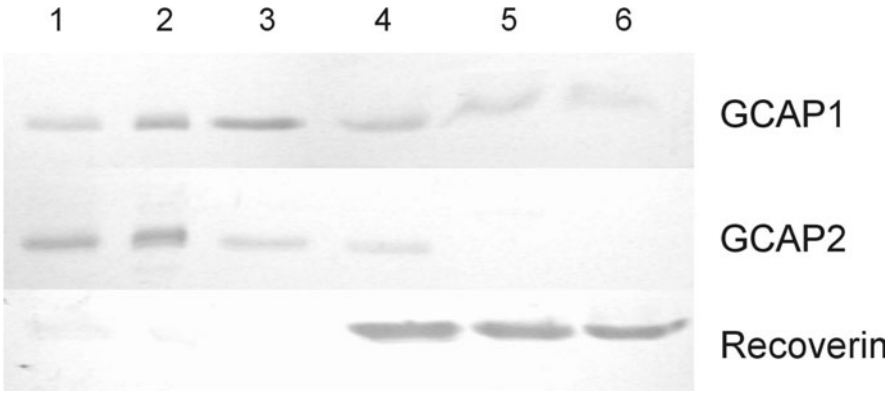

Figure 2. Ca^{2+}-dependent interaction of GCAP1, GCAP2, and recoverin with Phenyl-Sepharose.

Freshly obtained bovine retinal extract was loaded onto Phenyl-Sepharose column in the presence of 2 mM $CaCl_2$. The column was washed in the presence of 2 mM $CaCl_2$ and then 10 mM EDTA was applied. The fractions obtained in the presence of $CaCl_2$ (lanes 1–3) and EDTA (lanes 4–6) were analyzed in Western blotting for the presence of GCAP1, GCAP2, and recoverin as described in Materials and Methods.

Ca^{2+}-dependent change of ANS fluorescence is distinct in the presence of GCAPs and recoverin

To verify the above observations, we have additionally tested the Ca^{2+}-dependent changes of the hydrophobic properties of recoverin and GCAPs using ANS as a probe. Fluorescence of the dye strongly depends on the polarity of the solvent and markedly increases in a non-polar environment after binding to hydrophobic surfaces of proteins (Stryer, 1965; LaPorte *et al.,* 1980; Cardamone & Puri, 1992; Hughes *et al.,* 1995). We measured the fluorescence spectra of ANS after its binding to each protein at low and high Ca^{2+}. In aqueous solution and in the absence of proteins the fluorescence of ANS is modest, independent of Ca^{2+} concentration, and exhibits maximum intensity at a wavelength (λ_{max}) of about 510 nm (not shown). Binding of the dye to each protein at various $[Ca^{2+}]_{free}$ results in different patterns of the extrinsic fluorescence emission spectra. In the presence of recoverin the intensity of ANS fluorescence is enhanced at high Ca^{2+} with parallel shift of λ_{max} to 475 nm in comparison with the fluorescence mea-

sured at low Ca^{2+} (Fig. 3) indicating that the ANS binding sites are better accessible in the Ca^{2+}-loaded form of the protein. The opposite direction of ANS fluorescence changes is observed in the presence of GCAPs. The fluorescence intensity is then higher at low than at high Ca^{2+} and this effect is especially evident in the case of GCAP2 (Fig. 3). At low Ca^{2+} the maximal intensity of ANS fluorescence is lower in the presence of recoverin than in the presence of the same amounts of GCAP1 or GCAP2 (not shown). It is worthy of note, however, that the relative Ca^{2+}-dependent changes of ANS fluorescence are much greater in the presence of recoverin than in the presence of GCAPs (inset in Fig. 3). Hence, binding of Ca^{2+} significantly alters the hydrophobicity of recoverin and only slightly that of GCAPs.

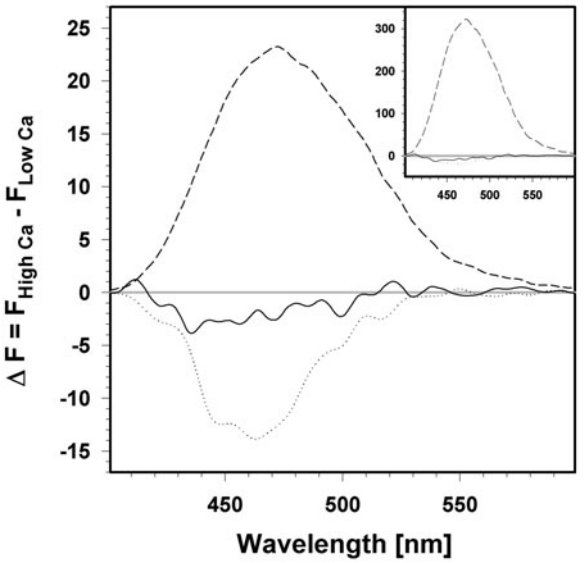

Figure 3. The effect of Ca^{2+} on ANS binding to GCAP1, GCAP2, and recoverin.

Difference fluorescence spectra of ANS binding were generated for each protein similarly as in Fig. 1 by subtraction of the spectra recorded at low $[Ca^{2+}]_{free}$ from those recorded at high $[Ca^{2+}]_{free}$. The excitation wavelength was 265 nm. The concentration of each protein was 300 nM and ANS was at 10 μM. The spectra obtained for GCAP1, GCAP2, and recoverin are represented by solid, dotted, and dashed lines, respectively. Inset: to show the relative change in fluorescence intensities, the same spectra were normalized assuming that maximum net fluorescence intensity in the spectrum recorded for each protein at low $[Ca^{2+}]_{free}$ is equal to 100.

Fluorescence resonance energy transfer between tryptophan residues and ANS depends on Ca^{2+} and is different in GCAPs and in recoverin

The accessibility of ANS binding sites on the proteins was also studied by detection of fluorescence resonance energy transfer (FRET) from tryptophan residues to ANS. Upon excitation at 295 nm aqueous solutions of ANS exhibit only background fluorescence in the absence of proteins and, conversely, only intrinsic fluorescence of proteins is observed in the absence of ANS (not shown). The intrinsic fluorescence of tryptophan residues decreases in each protein when ANS is added (Fig. 4). At the same time ANS fluorescence with λ_{max} = 470 nm appears. The quenching of the protein intrinsic fluorescence with a parallel increase of extrinsic ANS fluorescence is dose-dependent and therefore provides the evidence that FRET occurs between tryptophan residues and ANS (Stryer, 1965; Málnási-Csizmadia *et al.,* 1999). The energy transfer is more efficient at high Ca^{2+} in the case of recoverin, at low Ca^{2+} in the case of GCAP2, and there is only a slight difference in the energy transfer at high and low Ca^{2+} in the case of GCAP1. Also these results indicate that hydrophobic surfaces are better exposed at high than at low Ca^{2+} in recoverin but not in GCAPs.

DISCUSSION

Using Phenyl-Sepharose chromatography, ANS binding and FRET we demonstrate in this study that conformational changes induced by Ca^{2+} evoke substantially distinct changes in hydrophobic properties of three homologous proteins: GCAP1, GCAP2 and recoverin. Binding of Ca^{2+} markedly enhances hydrophobicity of native recoverin but

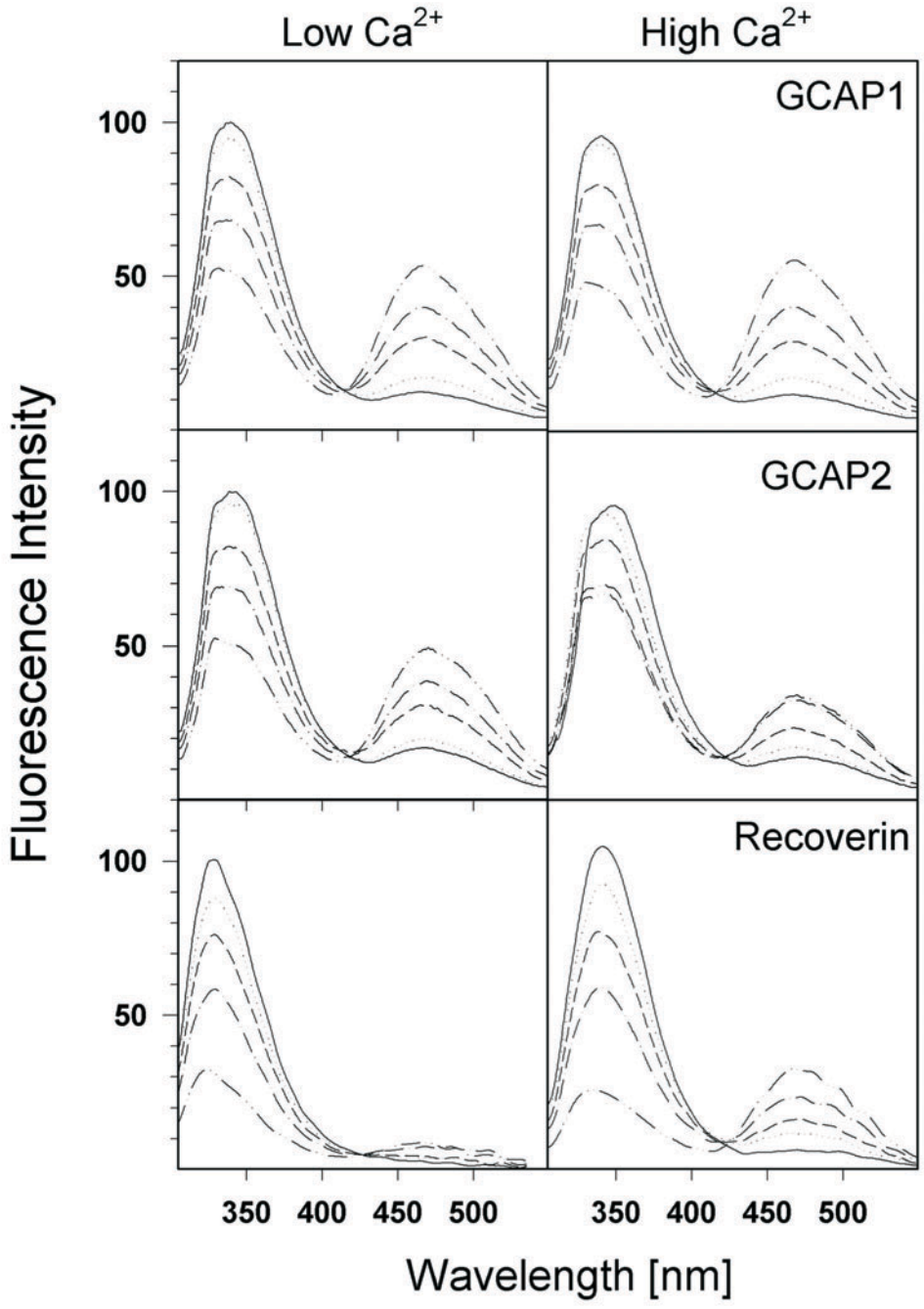

Figure 4. Fluorescence resonance energy transfer between bound ANS and Trp residues in GCAP1, GCAP2, and recoverin.

Excitation wavelength was 295 nm. Proteins were at 300 nM and ANS was added at 1 (———), 5 (· · ·), 10 (– – –), 20 (– · –), and 50 μM (– · · –). Background fluorescence obtained for each ANS concentration in the absence of proteins was subtracted from the corresponding sample spectrum. The resulting spectra were normalized, assuming that maximum fluorescence intensity in the spectrum obtained at low Ca^{2+} and 1 μM concentration of ANS is equal

exerts only a slight and rather reducing effect on the hydrophobicity of native GCAPs. Several groups have demonstrated that recoverin binds to ROS membranes and to artificial hydrophobic surfaces only in the presence of high Ca^{2+} and using the Ca^{2+}-myristoyl switch (Zozulya & Stryer, 1992; Lange & Koch, 1997). At the same time interaction of GCAPs with ROS membranes was shown to be either independent of Ca^{2+} or even weaker (as in the

case of GCAP2) in the presence of Ca^{2+} and probably independently of the Ca^{2+}-myristoyl switch (Gorczyca *et al.,* 1995; Olshevskaya *et al.,* 1997; Hwang & Koch, 2002). Recently Hwang & Koch (2002) showed by means of the surface plasmon resonance (SPR) technique that interaction of myristoylated GCAP2 with lipid membranes occurs better in the presence of EGTA than in the presence of Ca^{2+} while the opposite relationship was detected in the case of myristoylated recoverin. Showing that calcium regulates hydrophobic properties of native GCAPs and native recoverin in opposite directions, our results give an explanation of these observations in terms of hydrophobic interactions. Such different Ca^{2+}-dependent hydrophobic properties might also be a prerequisite for different mechanisms by which the investigated proteins regulate their target enzymes, RK in the case of recoverin (Chen *et al.,* 1995; Klenchin *et al.,* 1995; Senin *et al.,* 1995) and retGC in the case of GCAPs (Palczewski *et al.,* 1994; Gorczyca *et al.,* 1995; Dizhoor *et al.,* 1995). Although both effector enzymes are activated at low and inactivated at high calcium concentrations, the mechanisms of their regulation by corresponding proteins are different. In the presence of recoverin RK is inactive at high but active at low Ca^{2+}. However, the enzyme is also active at high Ca^{2+} in the absence of recoverin. This indicates that its inhibition results from direct interaction of both proteins exclusively at high Ca^{2+} (Chen *et al.,* 1995; Klenchin *et al.,* 1995). Hence, the Ca^{2+}-dependent changes in recoverin hydrophobicity directly regulate its ability to associate with RK. In contrast, retGCs in the absence of GCAPs have only basal activity while in the presence of GCAPs they are activated at low Ca^{2+} and inhibited at high Ca^{2+} (Dizhoor & Hurley, 1996; Rudnicka-Nawrot *et al.,* 1998). Since the hydrophobicity of GCAPs is high at low calcium and only slightly changes upon Ca^{2+} binding, it favors the formation of a Ca^{2+}-independent complex GCAPs–retGC in which GCAPs, by changing their conformation in response to Ca^{2+}, serve as switches between active and inactive state of the target enzymes.

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