Recoverin: A Calcium Sensitive Activator of Retinal Rod Guanylate Cyclase

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Vertebrate retinal photoreceptors recover from photoexcitation-induced hydrolysis of guanosine 3′, 5′-monophosphate (cyclic GMP) by resynthesizing cyclic GMP, which reopens cation channels that have been closed by light. Activation of guanylate cyclase by light-induced depletion of cytosolic calcium is a key event in this recovery process. This cyclase has now been shown to be regulated by a 23-kilodalton calcium binding protein. The protein is present in both rod and cone photoreceptors and was named recoverin because it promotes recovery of the dark state. The amino acid sequence of recoverin exhibits three potential calcium binding sites (EF hands). That recoverin binds calcium was confirmed with calcium-45 and by observing calcium-induced changes in its tryptophan fluorescence. Recoverin activated guanylate cyclase when free calcium was lowered from 450 to 40 nM, an effect that was blocked by an antibody to recoverin. Thus, guanylate cyclase in retinal rods is stimulated during recovery by the calcium-free form of recoverin. A comparison of recoverin with other calcium binding proteins reveals that it may represent, along with the protein visinin, a family of proteins that are regulated by submicromolar calcium concentrations.

IN DARKNESS, CATIONIC CHANNELS IN THE PLASMA MEMbrane of rod outer segments (ROS) are kept open by bound cyclic GMP (*1*). Light activates an enzymatic cascade that stimulates cyclic GMP hydrolysis leading to channel closure (*2*). Restoration of the dark state requires cyclic GMP resynthesis by guanylate cyclase. Cyclic GMP and cytosolic Ca^{2+} concentrations are set by a feedback loop (*3*). The Ca^{2+} enters through a cyclic GMP gated channel, and this influx is matched by efflux through a Na^+–K^+, Ca^{2+} exchanger (*4, 5*). Photoexcitation blocks Ca^{2+} influx but not its efflux so that free Ca^{2+} drops detectably within 0.5 second of light onset (*6*). Guanylate cyclase activity increases when Ca^{2+} is lowered to less than 100 nM (*7*). This activation is cooperative and mediated by a protein that can be detached from the guanylate cyclase catalytic moiety in low ionic strength buffer. Here we report the purification, amino acid sequence determination, and functional characterization of this protein, named recoverin.

Recoverin was purified from bovine retinas (Fig. 1). Soluble proteins, including recoverin, were extracted with a low ionic strength buffer from isolated ROS membranes exposed to white light (*8*). Phosphodiesterase and residual transducin were separated from recoverin by DEAE-cellulose chromatography. Fractions containing recoverin were then incubated with a matrix prepared by incubating concanavalin A–Sepharose with ROS that had been solubulized with detergent (*9*). Recoverin was eluted with 1.5 percent octyl glucoside (OG). This fractionation step is efficient, but the nature of the interaction between recoverin and this column has not been determined. Recoverin was then further purified by gel filtration chromatography. Fast protein liquid chromatography (FPLC) with an ion-exchange column then produced a final product that was at least 95 percent homogeneous as indicated by SDS polyacrylamide gel electrophoresis (PAGE) (Fig. 1E). This represents a 100-fold purification from ROS. The ratio of recoverin to rhodopsin in bovine ROS, estimated from densitometric scans of gels stained with Coomassie blue, was about 1:250. The electrophoretic mobility of recoverin on SDS polyacrylamide gels corresponds to a 26-kD protein, and its gel filtration elution volume corresponds to that of a 28-kD sphere.

A rabbit polyclonal antiserum to purified recoverin was prepared, and antibodies were isolated from the serum with immobilized recoverin (*10*). The affinity purified antibody specifically recognized a 26-kD protein in retina homogenates (Fig. 2A) and in pineal homogenates. Immunoreactive proteins were not detectable in brain, heart, liver, lung, and kidney (Fig. 2A), nor were they detectable in spleen, muscle, or adrenal tissue. The antibody has also been used to purify recoverin by affinity chromatography from crude mixtures of soluble retinal proteins (*11*). The affinity purified antibody recognized the entire photoreceptor layer of frozen sections of bovine retina (Fig. 2, B and D). The outer segments, inner segments, cell bodies, and synaptic pedicles of both rods and cones were labeled with the antibody. All photoreceptor types including red, green, and blue cones were recognized (Fig. 2, B to E).

Intact recoverin could not be sequenced directly because of its modified amino terminus. Instead, the protein was digested with either cyanogen bromide (CNBr), hydroxylamine (NH_2OH), or proteases (*12*). Fragments were then purified by reversed-phase high-performance liquid chromatography (HPLC) and subjected to Edman degradation. Two partial cDNA clones were isolated from a

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λgt11 bovine retina cDNA library with the antibody as a probe (*12, 13*). A full-length cDNA was then isolated from a λgt10 bovine retina cDNA library. Aligned sequences of overlapping peptide fragments and the amino acid sequence deduced from the cDNA are shown in Fig. 3A. The complete amino acid sequence of recoverin contains 202 residues and has a calculated mass of 23.3 kD (Fig. 3B). It is 59 percent identical to visinin, a cone-specific Ca^{2+} binding protein from chicken retina (*14*). According to criteria defined by Kretsinger and coworkers (*15*), there are three potential Ca^{2+} binding sites like the EF hands in calmodulin, intestinal calcium binding protein, and other calcium binding proteins (Fig. 3C).

Fluorescence spectroscopy confirmed that recoverin binds Ca^{2+}. The tryptophan fluorescence intensity decreased and the emission spectrum shifted to the red when the Ca^{2+} was raised from less than 10 nM to 1.4 μM (Fig. 4A). In contrast, raising the concentration

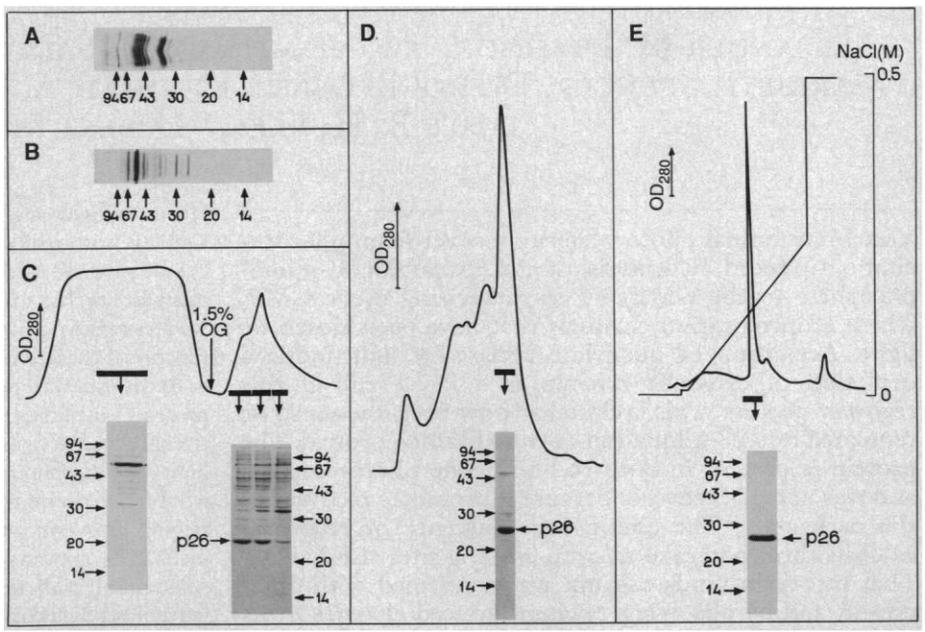


Fig. 1. Purification of recoverin. The ROS were isolated from 400 bovine retinas and bleached, and soluble proteins were extracted (*9, 20*) with 200 ml of buffer B (*20*). (**A**) ROS soluble extract. SDS-PAGE of 40 μg of protein. (**B**) DEAE cellulose chromatography. The extract (400 mg protein) was applied to 80 ml of DE52 in 10 mM tris-HCl (pH 8.0) and was eluted with 140 mM NaCl. SDS-PAGE of 26 μg of protein. (**C**) Immobilized rhodopsin column. Recoverin fractions (100 mg protein) were identified by SDS-PAGE and applied to a 20-ml column (*9*) that had been equilibrated and washed with buffer C (*20*). Recoverin was eluted with buffer C plus 1.5 percent OG. (**D**) FPLC chromatography (Superose 12 HR 10/30) in buffer C. Recoverin (4.0 mg protein) was concentrated by Amicon YM-10 filtration and applied and eluted at a rate of 1 ml/min. SDS-PAGE of 10 μg of protein. (**E**) FPLC chromatography (Mono Q HR 5/5). Recoverin (1.8 mg) was diluted threefold with 10 mM tris-HCl (pH 8.0) and was applied and eluted at 0.5 ml/min with a 0 to 0.5 M NaCl gradient in 20 mM tris-HCl (pH 8.0) plus 1 mM $MgCl_2$. SDS-PAGE of 7.5 μg of protein. Throughout, preparations were at 4°C when possible. All gels were 12.5 percent acrylamide and were stained with Coomassie blue. The yield was 0.4 mg of protein (Bradford assay with bovine serum albumin as standard).

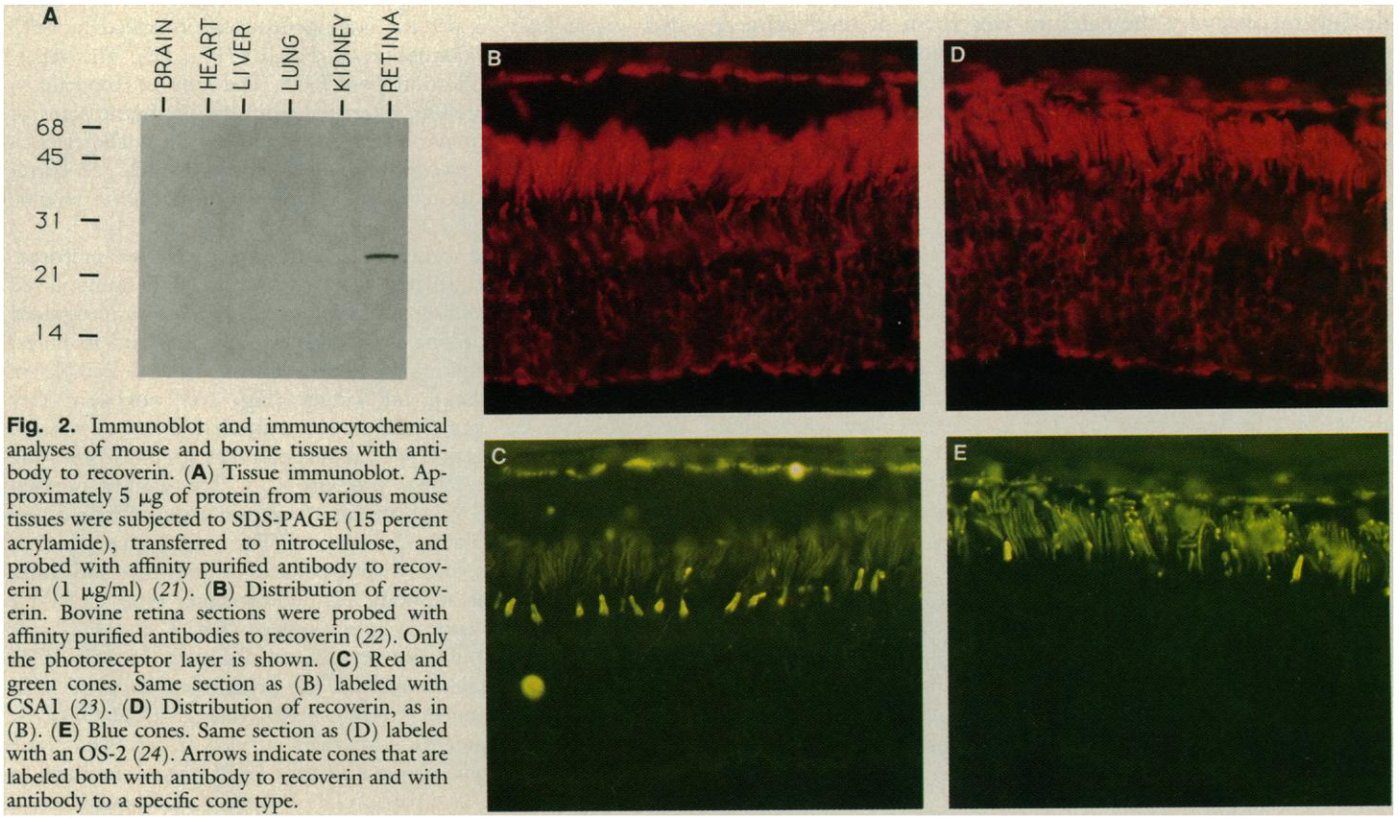


Fig. 2. Immunoblot and immunocytochemical analyses of mouse and bovine tissues with antibody to recoverin. (**A**) Tissue immunoblot. Approximately 5 μg of protein from various mouse tissues were subjected to SDS-PAGE (15 percent acrylamide), transferred to nitrocellulose, and probed with affinity purified antibody to recoverin (1 μg/ml) (*21*). (**B**) Distribution of recoverin. Bovine retina sections were probed with affinity purified antibodies to recoverin (*22*). Only the photoreceptor layer is shown. (**C**) Red and green cones. Same section as (B) labeled with CSA1 (*23*). (**D**) Distribution of recoverin, as in (B). (**E**) Blue cones. Same section as (D) labeled with an OS-2 (*24*). Arrows indicate cones that are labeled both with antibody to recoverin and with antibody to a specific cone type.

of free Mg^{2+} to 4.2 mM did not alter the fluorescence properties of recoverin. These results suggest that purified recoverin specifically binds Ca^{2+} at micromolar or lower concentrations in solution. Blots with $^{45}Ca^{2+}$ (*16*) also demonstrated that recoverin binds Ca^{2+} (Fig. 4B).

The affinity of recoverin for Ca^{2+} and its presence in rods and cones led us to investigate its relation to the putative Ca^{2+} sensitive regulator of photoreceptor guanylate cyclase (7). The ROS membranes were stripped of endogenous activator by washing with a low ionic strength buffer. As reported (7), stripped membranes exhibit a low basal guanylate cyclase activity insensitive to Ca^{2+}. Addition of purified recoverin to these membranes activated guanylate cyclase at low Ca^{2+} concentrations (Fig. 5A), but at high Ca^{2+} concentrations the cyclase returned to its basal activity. In the experiment shown, the activity of the enzyme increased nearly fourfold when Ca^{2+} was lowered from 450 to 40 nM. The maximum activation was variable in our assays, suggesting either that recoverin or the guanylate cyclase preparations are unstable or that other factors may also be involved.

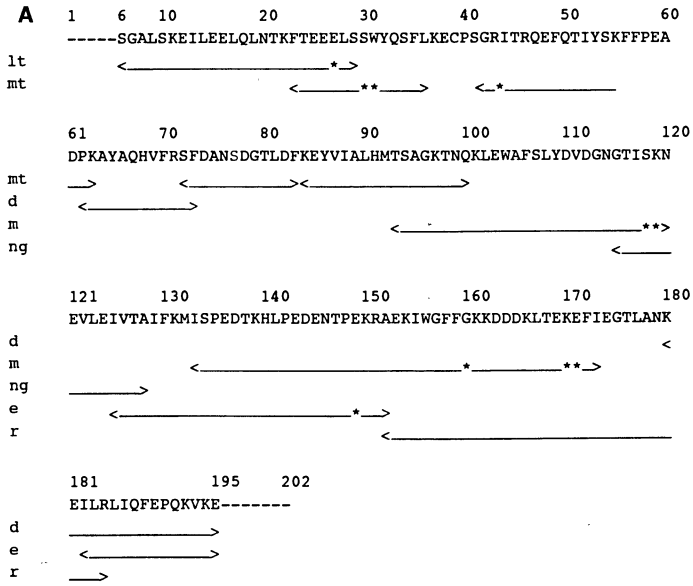
Half-maximal activation of guanylate cyclase by recoverin occurred at about 240 nM free Ca^{2+}, nearly the same concentration required in native ROS membranes (Fig. 5B). The Hill coefficient for activation of cyclase by recoverin is about 3, showing that the effect is highly cooperative, as reported for native ROS (7). Lambrecht and Koch (*18*) isolated a protein with an apparent mass of 26 kD, most likely recoverin, that activates guanylate cyclase. They observed half-maximal activation at a free Ca^{2+} concentration estimated to be between 110 and 220 nM.

If recoverin is the endogenous Ca^{2+} sensitive activator of guanylate cyclase, then it should be possible to block cyclase activation in native ROS membranes by adding antibody to recoverin. In fact, 50 μg of polyclonal antibody to recoverin added to native ROS membranes (100 μg rhodopsin) completely blocked guanylate cyclase activation at low Ca^{2+} without interfering with basal cyclase activity at high Ca^{2+}.

Recoverin differs from Ca^{2+} dependent activators such as calmodulin and troponin C, which require Ca^{2+} to activate their targets. Rather, recoverin is a Ca^{2+} sensitive activator that must be liberated from Ca^{2+} before it activates its target. Three simple models of how recoverin confers Ca^{2+} sensitivity on guanylate cyclase can be envisaged:

$$\text{(i)}\ C^* + V + Ca^{2+} \leftrightarrow Ca^{2+}\text{-}V\text{-}C$$
$$\text{(ii)}\ V\text{-}C^* + Ca^{2+} \leftrightarrow Ca^{2+}\text{-}V\text{-}C$$
$$\text{(iii)}\ V\text{-}C^* + Ca^{2+} \leftrightarrow Ca^{2+}\text{-}V + C$$

V denotes recoverin, and C^* and C are the high and low activity forms, respectively, of guanylate cyclase. In model (i), recoverin binds Ca^{2+}, forms a complex with guanylate cyclase, and inhibits its catalytic activity. This model can be ruled out because guanylate cyclase in the absence of recoverin has low catalytic activity regardless of the Ca^{2+} concentration. In models (ii) and (iii), the Ca^{2+} free form of recoverin binds to guanylate cyclase and activates it. The effect of Ca^{2+} is reversal of this activation. Both models (ii) and (iii) are consistent with the findings that both recoverin and low Ca^{2+} concentrations are required for activation and that the activity at



C

	E	n	*	*	n	n	*	*	n	X	*	Y	*	Z	G	*	I-X	*	*	-Z	n	*	*	n	n	*	*	n
Rec 1	E	L	S	S	W	Y	Q	S	F	L	K	E	C	P	S	G	R	I	T	R	Q	E	F	Q	T	I	Y	S	K	F
Vis 1	E	L	S	R	W	Y	E	G	F	Q	R	Q	C	S	D	G	R	I	R	C	D	E	F	F	R	I	Y	G	N	F
Rec 2	Y	A	Q	H	V	F	R	S	F	D	A	N	S	D	G	T	L	D	F	K	E	Y	V	I	A	L	H	M	T	
Vis 2	Y	A	R	H	V	F	R	S	F	D	T	N	D	D	G	T	L	D	F	R	E	Y	I	I	A	L	H	L	T	
Rec 3	K	L	E	W	A	F	S	L	Y	D	V	D	G	N	G	T	I	S	K	N	E	V	L	E	I	V	T	A	I	
Vis 3	K	L	E	W	A	F	S	L	F	D	V	D	R	N	G	E	V	S	K	S	K	V	L	E	I	I	T	A	I	
CaM 1	E	F	K	E	A	F	S	L	F	D	K	D	G	D	G	T	I	T	T	K	E	L	G	T	V	M	R	S	L	
ICBP 2	T	L	D	E	L	F	E	E	L	D	K	N	G	D	G	E	V	S	F	E	E	E	F	Q	V	L	V	K	K	

Fig. 3. (**A**) Partial amino acid sequence of recoverin determined by Edman degradation. The beginning and end of each sequenced region is marked by pointed brackets (< and >). Symbols on the left denote the way in which fragments were generated (*12*): lt, large tryptic fragment; mt, CNBr and then trypsin; m, CNBr; e, cleavage at Glu; d, cleavage at Asp; r, cleavage at Arg; and ng, cleavage by hydroxylamine. The cDNA sequence established the identity of residues 37 to 46 and those marked with an asterisk (*). (**B**) Complete amino acid sequence of recoverin and comparison with the sequence of chicken visinin (*14*). The sequences of the NH_2-terminal five residues and COOH-terminal seven residues of recoverin were determined from a full-length cDNA clone. Identical residues in recoverin and visinin are marked by a colon (:). The three EF hand sequences in each protein are underlined. (**C**) Comparison of the three EF hand sequences of bovine recoverin (Rec) and chicken visinin (Vis) with representative EF hands from bovine calmodulin (CaM) and bovine intestinal calcium binding protein (ICBP) (*15*). Numbers refer to the consecutive order of EF hands within the protein sequence starting from the NH_2-terminus. The consensus sequence for EF hands (*15*) is shown at the top. E, glutamate; n, nonpolar residue; *, any residue; X, Y, Z, −X, and −Z, Ca^{2+} chelating residues containing oxygen in their side chain; G, glycine; I, isoleucine, leucine, or valine. Underlined residues fit the consensus sequence. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

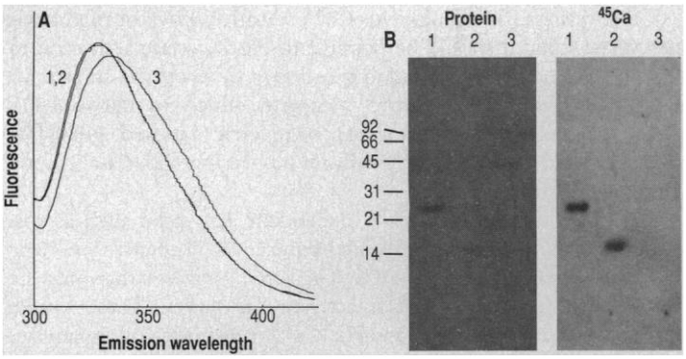


Fig. 4. (**A**) Tryptophan fluorescence. Effect of Ca^{2+} on the fluorescence emission of 0.9 μM purified recoverin excited at 291 nm (Hitachi MPF-4 fluorimeter, bandwidth 10 nm, 23°C). Buffers used were 3 mM EGTA (curve 1), 3 mM EGTA and 5 mM $MgCl_2$ (free Mg^{2+}, 4.2 mM) (curve 2), 3 mM EGTA-Ca^{2+} buffer (free Ca^{2+}, 1.4 μM) (*17*) (curve 3) in 50 mM tris, pH 8.0. Addition of Ca^{2+} or Mg^{2+} did not change the pH by more than 0.2 units. (**B**) Binding of $^{45}Ca^{2+}$ to 1 μg of purified recoverin (lane 1), 0.5 μg of purified yeast calmodulin (lane 2), 1.5 μg of purified transducin α subunit (lane 3). (Left panel) Blot stained with Ponceau S. (Right panel) Blot probed with ^{45}Ca.

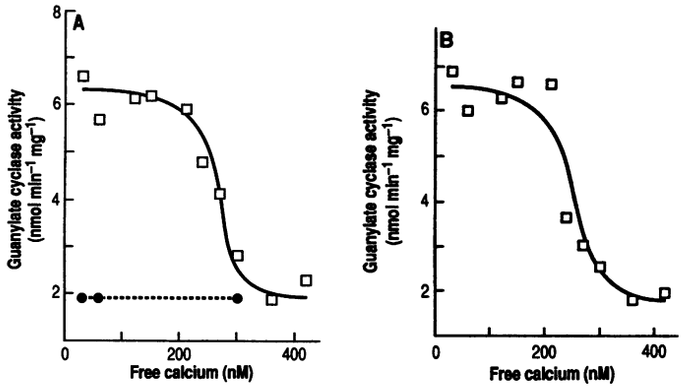


Fig. 5. The Ca^{2+} dependence of guanylate cyclase. (**A**) Restoration of Ca^{2+} sensitive activation of guanylate cyclase by addition of recoverin to stripped ROS membranes. (●) Activity of stripped ROS membranes; (□) activity of the same membranes in the presence of 1.5 μg recoverin. These experiments were performed at 12 mM Mg^{2+}. (**B**) The Ca^{2+} dependence of cyclase activity in native ROS.

high Ca^{2+} concentrations in the presence of recoverin is the same as the basal, Ca^{2+} independent activity of stripped membranes. In model (ii) the Ca^{2+}-recoverin complex remains associated with guanylate cyclase but does not activate it, whereas in model (iii) the Ca^{2+}-recoverin complex completely dissociates from the guanylate cyclase.

We have investigated the effect of recoverin only on guanylate cyclase. Recoverin may also have other regulatory roles in the retina, or it may influence the structural stability of photoreceptors. In fact, Polans and co-workers (*19*) have identified antibodies to recoverin in sera from patients with retinas that have degenerated as a result of cancer-associated retinopathy (CAR).

Our study shows that, in the photoreceptor, a 23-kD Ca^{2+}-binding protein, recoverin, activates guanylate cyclase when Ca^{2+} is lowered within the submicromolar range, a key event in the resynthesis of cyclic GMP and recovery of the dark state. The homology between recoverin and visinin suggests that these proteins are members of a family of Ca^{2+}-sensitive regulators that, through cooperative interactions, act as switches at submicromolar Ca^{2+} levels.

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10. Preparation of antibodies to recoverin: A Grey Giant rabbit was injected with 250 μg of purified recoverin in Freund's complete adjuvant and then with three 200-μg injections at 2- to 3-week intervals. Recoverin-specific antibodies were affinity purified by using recoverin coupled to CNBr-activated Sepharose CL-4B (500 μg of recoverin protein per 1 ml of gel). Antibodies were eluted with 0.1 M glycine at pH 2.8 and immediately neutralized.
11. The 23 percent sucrose supernatant from ROS isolation (*21*) was diluted with an equal volume of a mixture of 30 mM tris-HCl (pH 7.5) and 400 mM NaCl and applied at room temperature to a recoverin column [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 292 and 519]. Recoverin was eluted with 0.1 M glycine (pH 2.8) and neutralized. Sometimes this preparation was further purified by Mono Q FPLC as in Fig. 1. The final yield from 30 retinas was 100 to 200 μg of purified recoverin.
12. Recoverin was reduced and alkylated with 4-vinylpyridine, and peptide fragments were generated as described [H. Charbonneau *et al.*, *Proc. Acad. Natl. Soc. U.S.A.* **86**, 5252 (1989)]. The protein was cleaved at Met residues with CNBr, at Glu residues with *Staphylococcus aureus* V8 protease, or at Asp residues (2 percent formic acid, 6.5 hours, 110°C, at reduced pressure). Separate preparations were citraconylated and cleaved at Arg residues with trypsin, or cleaved at an Asn-Gly bond with hydroxylamine. Peptides were separated by reversed-phase HPLC and sequenced (Applied Biosystems Model 470). Native recoverin was treated for 1 hour with trypsin (1:150)(w/w) at 37°C, and a 25-kD fragment was separated on an SDS polyacrylamide gel, electroblotted onto Immobilon, and sequenced. Partial cDNA clones were isolated from a λgt11 bovine retinal cDNA library (*13*) and analyzed [C. L. Lerea, A. H. Bunt-Milam, J. B. Hurley, *Neuron* **3**, 367 (1989)]. A cDNA including the entire coding region was then isolated from a λgt10 bovine retina cDNA library [J. Nathans and D. Hogness, *Cell* **34**, 807 (1983)].
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16. The $^{45}Ca^{2+}$ blotting was performed as described [T. N. Davis, M. S. Urdea, F. R. Masiarz, J. Thorner, *Cell* **47**, 423 (1986)] except that nitrocellulose was used instead of nylon so that the amounts of protein transferred could be monitored by staining with Ponceau S.
17. Guanylate cyclase was assayed as described (7) with the following modifications. The free Ca^{2+} was varied by the addition of different volumes of an equimolar solution of Ca^{2+} and EGTA (*27*) to cyclase buffer containing EGTA. The resulting free Ca^{2+} concentration in each assay solution was calculated from the known Ca/EGTA ratio by using published Ca-EGTA and Mg-EGTA dissociation constants [R. Tsien and T. Pozzan, *Methods Enzymol.* **172**, 230 (1989)]. The calculation is based on the assumption that 7 mM of the 12 mM total Mg^{2+} is free. In reconstitution assays, 1.5 μg of recoverin was added to stripped ROS membranes containing ~75 μg of rhodopsin in a final reaction volume of 100 μl. After 10 to 20 minutes, the reactions were stopped with 100 μl of ice-cold 10 percent trifluoroacetic acid, and the precipitated proteins were removed by centrifugation. Each supernatant was dried, redissolved in distilled water, and analyzed by polyethylenimine-cellulose thin layer chromatography (developed with methanol for 5 minutes, with distilled water for 2 hours, and then with 0.2 M LiCl for 2 hours). The amount of cyclic [α-^{32}P]GMP formed was determined with an AMBIS radioanalytic imaging system. Hydrolysis of cyclic GMP by phosphodiesterase was monitored by measuring the amount of remaining cyclic [^{3}H]GMP (*7*). In antibody inhibition assays, 100 μg of ROS was mixed with 50 μg of affinity purified antibody to recoverin at 40 or 450 nM free Ca^{2+}
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20. Buffer A: 30 mM tris-HCl (pH 8.0), 25 mM NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.1 mM phenylmethyl sulfonyl fluoride (PMSF). Buffer B: 5 mM tris-HCl: (pH 8.0), 1 mM DTT, and 0.1 mM PMSF. Buffer C: 10 mM tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT.
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22. Immunocytochemical labeling of 10 μm of frozen bovine retina sections was performed as described [C. L. Lerea, A. H. Bunt-Milam, J. B. Hurley, *Neuron* **3**, 367 (1989)] except that antibody OS-2 was used at 1:200, CSA-1 was used at 1:10, and the antibody to recoverin was used at 0.2 μg/ml.
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