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Purification and physiological evaluation of a guanylate cyclase activating protein from retinal rods

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ABSTRACT **In retinal rods light triggers a cascade of enzymatic reactions that increases cGMP hydrolysis and generates an electrical signal by causing closure of cGMP-gated ion channels in the photoreceptor outer segment. This leads to a decrease in internal Ca, which activates guanylate cyclase and promotes photoresponse recovery by stimulating the resynthesis of cGMP. We report here that the activation of guanylate cyclase by low Ca is mediated by an ≈20-kDa protein purified from bovine rod outer segments by using DEAE-Sepharose, hydroxylapatite, and reverse-phase chromatographies. In a reconstituted system, this protein restores the Ca-sensitive regulation of guanylate cyclase and when dialyzed into functionally intact lizard rod outer segment decreases the sensitivity, time to peak, and recovery time of the flash response.**

Vision is initiated in the outer segment of vertebrate photoreceptors by a series of chemical reactions that couple light to generation of an electrical signal (for reviews, see refs. 1 and 2). The light-triggered events activate cGMP-specific phosphodiesterase (PDE), which hydrolyzes cGMP and reduces a circulating dark current that flows into the outer segment through cGMP-gated cation channels. Closure of cGMP-gated channels during the light response reduces Ca influx but not efflux via Na/Ca,K exchange (3, 4). The resulting decrease in internal Ca stimulates guanylate cyclase (GC) and promotes recovery of the light response (5). Dark current recovers after light exposure as cGMP is resynthesized and returned to its resting dark level.

The Ca-dependent regulation of GC is mediated by a factor that is removed from rod outer segment (ROS) membranes by washing with low-salt buffer (6). The soluble activator was initially thought to be an ≈26-kDa Ca-binding protein (p26) that was named recoverin for its presumed role in photoresponse recovery (7, 8). Recoverin was reported to activate GC at submicromolar Ca concentrations in washed ROS membrane preparations. Subsequent studies with purified recoverin (9), however, did not confirm this observation. Furthermore, intracellular incorporation of exogenous recoverin and two related Ca-binding proteins into functionally intact ROS produced a delay rather than the expected acceleration in the onset of photoresponse recovery (10). These results suggested that the factor responsible for the low-Ca activation of photoreceptor GC had not been identified.

In this paper, we describe the purification and initial biochemical and electrophysiological characterization of an ≈20-kDa protein that mediates the Ca-sensitive regulation of GC. We refer to this protein as GC-activating protein (GCAP).

MATERIALS AND METHODS

Preparation of ROS Membrane Homogenate. Fresh bovine eyes were obtained from a local slaughterhouse and placed on ice in a light-tight container. The retinas from 100 eyes were dissected under dim red light and ROSs were isolated as described by Papermaster (11). This procedure yielded a pellet of ROS that was treated in one of two ways. (*i*) The ROS pellet was homogenized (six strokes with a glass/glass homogenizer) in 50 mM Hepes (pH 7.8), 60 mM KCl, 20 mM NaCl, at a final concentration of 8 mg of rhodopsin per ml and is referred to as native ROS membrane homogenate. (*ii*) The ROS pellet was homogenized in 35 ml of water containing 20 μg of leupeptin per ml at 0–5°C and centrifuged at 47,000 × *g* for 30 min. The resulting pellet was collected and resuspended in 35 ml of water and centrifuged a second time (30 min at 47,000 × *g*). The pellet was collected and resuspended in Hepes buffer (as described above) at a final concentration of 8 mg of rhodopsin per ml. This is referred to as washed ROS membrane homogenate. Rhodopsin concentration was determined by the method of Wald and Brown (12).

GC Assay. With existing assays, the apparent rate of cGMP synthesis must be corrected for continuous and rapid hydrolysis of cGMP by PDE, which is abundant and active in ROS membrane homogenates (13). Uncertainties associated with making this correction were avoided by using an assay that exploited the difference in the stereospecific requirements of GC and PDE (14). Photoreceptor GC, like many other kinds of GC, utilizes the *Sp* stereoisomer of guanosine 5′-[α-thio]triphosphate (GTP[αS]) as a substrate (≈1/10th as well as GTP) and converts it into cGMP[S] in its *Rp* configuration (15, 16). This isomer of cGMP[S] is hydrolyzed by rod PDE at ≈1/20,000th the rate that cGMP is hydrolyzed (17) and can be separated from (*Sp*) GTP[αS] either by ion-exchange HPLC or by vortex mixing reaction samples with neutral alumina gel. Under these conditions (*Sp*) GTP[αS] stays tightly bound to either the column or the gel and cGMP[S] can be recovered quantitatively from effluent or supernatant, respectively (14).

The standard GC assay was initiated by adding 10 μl of native or washed ROS membrane homogenate to 54 μl of solution that contained (final concentrations): 1.3 mM (*Sp*) GTP[α-^{35}S] (19,000–22,000 dpm/nmol; New England Nuclear), 50 mM Hepes (pH 7.8), 60 mM KCl, 20 mM NaCl, 10 mM $MgCl_2$, 0.4 mM EGTA, and 0.16 mM $CaCl_2$ (45 nM free Ca) at 30°C. Free Ca was calculated by using the computer program CHELATOR 1.00 (18) and adjusted to higher concentrations in some assay solutions by increasing the amount of added $CaCl_2$. After 8 min, the reaction was terminated by adding 15 μl of 0.4 M HCl, vortex mixed, and then centrifuged at 16,000 × *g* for 4 min. The supernatant (50 μl) was neutralized with 0.5 ml of 200 mM Tris·HCl (pH 7.4) containing 50 mM EDTA to extinguish any residual GC activity by chelating Mg. This solution (0.5 ml) was mixed with 150 mg of neutral alumina oxide (ICN alumina TSC04512), vortex mixed for 8 min, and centrifuged at 16,000 × *g* for 5 min. The

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Abbreviations: PDE, phosphodiesterase; GC, guanylate cyclase; ROS, rod outer segment; GCAP, GC-activating protein; GTP[αS], guanosine 5′-[α-thio]triphosphate; BTP, 1,3-bis [tris(hydroxymethyl)methylamino]propane.

supernatant (0.35 ml) was withdrawn and cGMP[^{35}S] formation was measured with a scintillation counter.

Purification of GCAP. A ROS supernatant containing GCAP was separated from ROS membranes by centrifugation at 47,000 × *g* for 30 min (see above). The extract was centrifuged for an additional 8 min on an ultracentrifuge at 200,000 × *g* to remove ROS membrane particles that did not sediment at 47,000 × *g*. The soluble fraction was dialyzed against water for 8 hr at 0–5°C and then loaded on a DEAE-Sepharose column (5 × 50 mm; Pharmacia LKB) in the presence of 5 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) (pH 7.5) and 50 mM NaCl. The column was then washed at a flow rate of 1 ml/min at room temperature with 5 mM BTP (pH 7.5) and 100 mM NaCl until A_{280} was <0.01. Proteins that remained on the column were eluted with a linear NaCl gradient (100–350 mM) in 5 mM BTP (pH 7.5). Elution of protein was monitored by absorption at 280 nm and aliquots from selected fractions were tested for the presence of GCAP by their ability to stimulate GC activity in a low-Ca solution (45 nM) using washed ROS membrane homogenate. Fractions that contained GCAP, which had been eluted by ≈220 mM NaCl, were combined and loaded on a hydroxylapatite column (Pentax Column SH-0710M; 7.5 × 100 mm; Asahi Optical, Tokyo), which had been equilibrated with 100 mM NaCl in 10 mM BTP (pH 7.5). One-milliliter fractions were collected from the column at a flow rate of 1.0 ml/min. The GCAP fractions were eluted with a linear gradient of KH_2PO_4 (0–60 mM) and a linearly decreasing concentration of NaCl (100–0 mM) in 10 mM BTP (pH 7.5) using a quaternary HPLC pump system (Hewlett Packard, model 1050). GCAP was eluted at ≈30 mM KH_2PO_4 and ≈50 mM NaCl. The fractions containing GCAP eluted from the hydroxylapatite column were combined and concentrated to ≈0.5 ml using a SpeedVac. Acetonitrile was then added to yield a final concentration of 15%, and the sample was loaded on a C-4 column (W-Porex 5 C4; 4.6 × 150 mm; Phenomenex, Belmont, CA) equilibrated with 30% acetonitrile in 5 mM BTP (pH 7.5). GCAP was eluted with a linear gradient of acetonitrile (30–60%) in 5 mM BTP (pH 7.5) at a flow rate of 1.5 ml/min (0.75-ml fractions were collected). Alternatively, fractions containing GCAP eluted from the DEAE-Sepharose column were applied to an octyl agarose column (Pierce, no. 20269; 5 × 50 mm) in the presence of 250 mM NaCl and 5 mM BTP (pH 7.5), using a quaternary HPLC pump system. The column was washed with 250 mM NaCl in 5 mM BTP (pH 7.5), followed by the same solution without NaCl (flow rate, 1 ml/min at room temperature). Proteins were eluted by increasing acetonitrile from 0% to 48% (vol/vol) in H_2O over 15 min (flow rate, 1.1 ml/min) and selected fractions were tested for the presence of GCAP as described above. GCAP was eluted at 30–40% acetonitrile.

Gel Filtration. Fractions containing GC-stimulating activity obtained from DEAE-Sepharose column chromatography (2 ml) were loaded onto a Superose-12 gel filtration column (Pharmacia LKB) equilibrated with 80 mM sodium phosphate (pH 7.5) containing 100 μM $CaCl_2$ at a flow rate of 0.7 ml/min. The protein profile was monitored and GC-stimulating activity was determined as described above. Fractions containing GCAP were eluted at a molecular mass of ≈20 kDa.

Protein Determinations and SDS/PAGE. Protein concentrations were determined by the Bradford method (19). SDS/PAGE was performed according to Laemmli (20) using 12% acrylamide gels.

Electrophysiology. Intracellular dialysis via whole-cell voltage clamp was used to introduce proteins into functionally intact lizard ROS as described (10). Dark-adapted ROSs were mechanically isolated from *Gecko* retina and bathed in oxygenated Ringer's solution containing 160 mM Na^+, 3.3 mM K^+, 1 mM Ca^{2+}, 1.7 mM Mg^{2+}, 168 mM Cl^-, 1.7 mM SO_4^{-2}, 2.8 mM Hepes, and 10 mM dextrose (pH 7.4 with NaOH; ≈320 mosmol/kg). Whole-cell patch electrodes (21) were filled with a standard internal solution (control solution) containing 139 mM K^+, 15 mM Na^+, 6.05 mM Mg^{2+}, 0 added Ca^{2+}, 113 mM Asp^-, 24 mM Cl^-, 5 mM Hepes, 1 mM GTP, and 5 mM ATP (pH 7.4 with KOH; ≈310 mosmol/kg with sucrose) made from either a 2- or a 4-fold concentrated stock. Purified GCAP (dialyzed against water for 8 hr at 0–5°C, and centrifuged at 47,000 × *g* for 20 min to eliminate any particles) was diluted with concentrated stocks of control solution. The final concentrations of GCAP ranged from 250 to 750 nM; low GCAP solubility prevented use of higher concentrations. Dialysis solutions containing GCAP were prepared immediately before use in a volume sufficient for filling a single pipette (300–400 μl) and centrifuged at 16,000 × *g* for 8 min at 4°C.

Breakthrough from cell-attached (seal resistance, 10–40 GΩ) to whole-cell recording (access resistance, ≈35 MΩ; V_{hold}, −29 mV) was promoted by brief suction applied to the patch electrode (pipette resistance, 10 MΩ). The calculated time constant (22) for the diffusion of GCAP into ROS with a 1-pl vol was ≈5 min. Light responses evoked by 20-ms flashes (unattenuated intensity, 1.5 × 10^6 520-nm photons·μm^{-2}·s^{-1}) were recorded after 20 min of whole-cell dialysis. Flash intensities are reported as the number of isomerized rhodopsin (Rh*) molecules per ROS using an effective collecting area of 22.8 μm^2 (23).

RESULTS AND DISCUSSION

The Ca sensitivity of GC in native ROS membrane homogenate is shown in Fig. 1 (open squares). As described (6, 25), cyclase was stimulated when free Ca was reduced from 1 μM to 45 nM. The relationship between cyclase activity and Ca was fitted by a Hill equation with a coefficient of ≈2 and half-maximal activation at ≈240 nM Ca. Raising Ca from 1 to 50 μM had no additional effect on the low level of basal cyclase activity. The Ca sensitivity of GC activity was not affected by 5 μM mastoparan (Fig. 1, solid triangles), which is a potent calmodulin antagonist in other systems (26). This is consistent with previous reports that calmodulin (6, 27) is not responsible for the calcium regulation of GC.

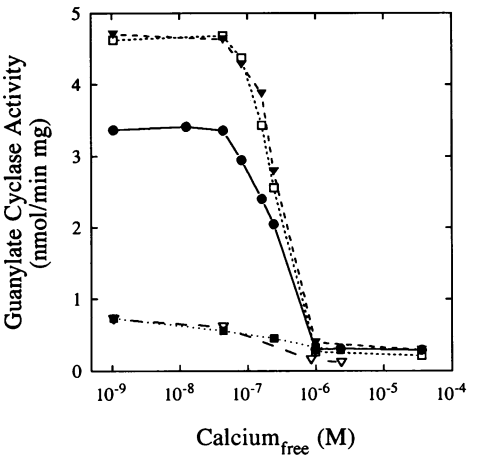

FIG. 1. Ca titration of GC activity. Ca sensitivity of GC was assayed in either native ROS membrane homogenate without (□) and with 5 μM mastoparan (▼), reconstituted washed ROS membrane homogenate with 400 nM purified GCAP (●), or washed ROS membrane homogenate without (▽) and with (■) 20 μM p26 purified as described by Polans *et al.* (24). The free Ca concentrations were adjusted by EGTA/Ca buffer as described. Data are from a single experiment that is representative of two such experiments.

The open triangles in Fig. 1 show that the factor responsible for low-Ca activation of GC is removed by washing ROS membrane homogenates in low ionic strength solution (6). In contrast to previous reports (7, 8), addition of purified p26 (recoverin) did not restore the Ca sensitivity of GC (Fig. 1, solid squares). The activity of GC was also not affected by addition of 1 mM sodium nitroprusside (data not shown). Nitroprusside stimulates soluble GC in a variety of tissues by formation of nitric oxide (28), and its lack of stimulatory effect on ROS cyclase agrees with earlier results based on electrical recordings from detached ROS (G. Rispoli and P.B.D., unpublished observations). These findings are consistent with outer segment GC being classified as a member of the family of particulate rather than soluble GCs (29, 30).

To isolate the activator (GCAP) that is removed from ROSs by washing with low-salt solution and that restored the Ca-sensitive regulation of GC in washed membrane homogenate, the soluble fraction obtained from the preparation of washed ROS membrane homogenate was partially purified on a small DEAE-Sepharose column (data not shown). Among the many proteins revealed by SDS/PAGE, the appearance of a low-intensity band at ≈20 kDa that eluted at 220 mM NaCl correlated with the GC-stimulating activity. Fractions containing GCAP eluted from the DEAE-Sepharose column were then applied to a hydroxylapatite column (Fig. 2). The column was then washed with a linear phosphate gradient (0–60 mM) and proteins eluted at different phosphate concentrations were collected as shown in Fig. 2*A*. The GCAP fractions, identified by their ability to stimulate GC activity, were eluted as a sharp peak in fractions 17–19. These fractions contained the 20-kDa protein (Fig. 2*B*). Fractions 17–19 from the hydroxylapatite column were then applied to a reverse-phase C-4 column, which had been equilibrated with 30% acetonitrile at neutral pH (Fig. 3). GCAP was eluted with a linear gradient of acetonitrile (30–60%) in low ionic strength solution (Fig. 3*A*). The activator was eluted following transducin γ subunit (fractions 9–11; the identity of the γ subunit was confirmed by protein sequence analysis; data not shown) and is apparent at ≈40% acetonitrile in fractions 10–16 as two small peaks at 228 nm and corresponding peaks of GC-stimulating activity. The protein contained in these fractions appeared as a monomer (fractions 10–12) or as a doublet in remaining fractions with a molecular mass of ≈20 and ≈19.5 kDa in the presence of Ca (Fig. 3*B*) and 23 kDa in the presence of EGTA (data not shown). Fractions containing both lower and higher molecular mass forms of GCAP were separated by SDS/PAGE and transferred on Immobilon membranes, and a partial amino acid sequence was obtained. We found that both forms of GCAP contain a tryptic peptide (7 amino acids long) with the same sequence (J. Crabb and K.P., unpublished observation). These results suggest that both forms of GCAP may be derived from the same protein. The difference in molecular mass as determined by SDS/PAGE might be explained by posttranslational modification—for example, phosphorylation or glycosylation; limited proteolysis of GCAP during isolation might also be responsible. Approximately 10–15 μg of GCAP was purified from 100 retinas. The small yield may be due to the strong hydrophobic character of GCAP. Importantly, an alternative procedure using DEAE-Sepharose and octyl agarose chromatographies yielded similar results with the purification of a 20-kDa protein having GC-stimulating activity, although with lower recovery (data not shown). Gel filtration of GCAP-containing fractions from the DEAE-Sepharose column yielded a single peak of GC-stimulating activity with an elution volume corresponding to a monomeric 17- to 20-kDa protein (data not shown), suggesting that GCAP is a monomeric protein. Support for this is provided by the recent cloning of cDNA for GCAP based on partial amino acid sequences of peptides derived from

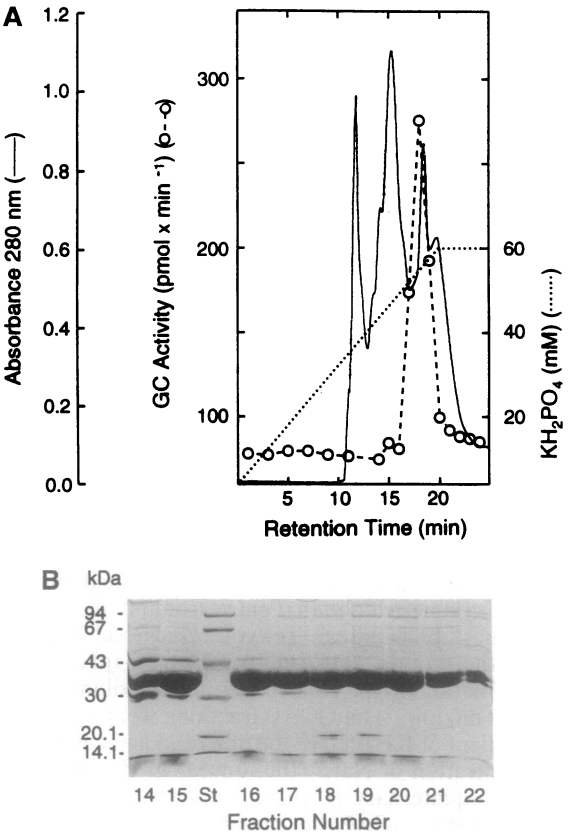

FIG. 2. Hydroxylapatite chromatography of ROS GCAP. (*A*) Combined fractions containing GCAP eluted from a DEAE-Sepharose column were loaded on a hydroxylapatite column (Pentax Column SH-0710M; 7.5 × 100 mm; Asahi Optical) in the presence of 100 mM NaCl in 10 mM BTP (pH 7.5) at a flow rate of 1.0 ml/min. GCAP fractions were eluted with a linear gradient of KH_2PO_4 (0–60 mM) and a linearly decreasing concentration of NaCl (100–0 mM) in 10 mM BTP (pH 7.5), using a quaternary HPLC pump system. One-milliliter fractions were collected and 5-μl aliquots from the indicated fractions were used to determine their stimulatory effect on GC activity by using the assay described. Retention time corresponds to the fraction number. (*B*) SDS/PAGE analysis of eluted fractions from the hydroxylapatite column. SDS/PAGE was performed by using the Laemmli system (20) with 12% acrylamide gels and stained with Coomassie blue. Twenty microliters of the indicated fractions were loaded on each lane of the gel. Lane St, standard proteins of the indicated molecular mass (Pharmacia LKB).

purified protein (31). Sequence analysis gives a predicted molecular mass of 23 kDa and indicates the presence of three putative *EF*-hand calcium binding motifs.

Purified GCAP restored low-Ca activation of GC in washed ROS membrane homogenate (Fig. 1, solid circles). While GCAP-reconstituted Ca-sensitive regulation of GC was similar to that observed in native ROS membrane homogenate, the maximum level of activity in low Ca was typically 30–50% smaller. We attribute this to the concentration of GCAP being lower in the reconstituted system than in the native preparation. The addition of GCAP to a homogenate of native membranes caused further stimulation of GC activity in low Ca (data not shown). This suggests that the concentration of activator in the unwashed homogenate is subsaturating, presumably due to losses during preparative procedures.

To further investigate the influence of the activator protein on the Ca regulation of GC, GCAP was dialyzed into functionally intact detached ROSs. In whole-cell voltage clamp, isolated ROSs dialyzed with control solution (see *Materials and Methods*) develop a sustained inward dark current that is suppressed transiently by brief flashes of light. Light

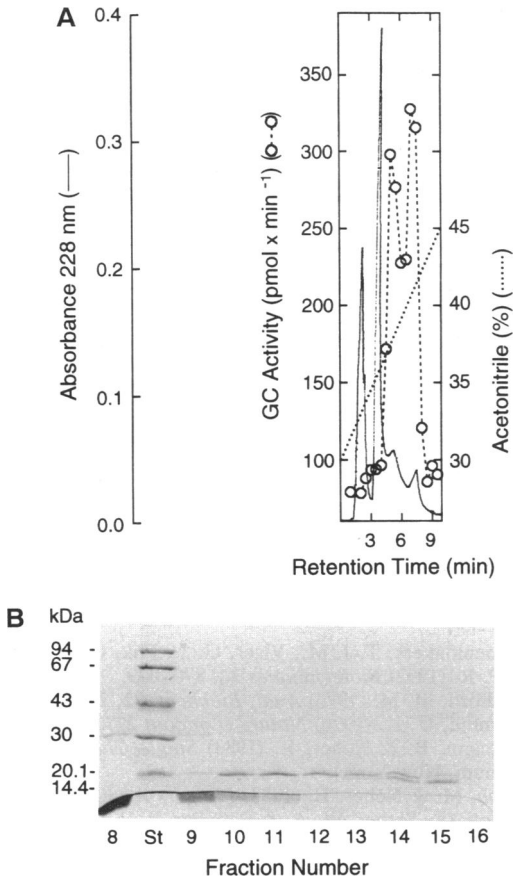

FIG. 3. Reverse-phase chromatography of ROS GCAP. (*A*) Combined fractions containing GCAP eluted from hydroxylapatite column (fractions 17–19; see Fig. 2) were concentrated to ≈0.5 ml using a SpeedVac. Acetonitrile was then added to yield a final concentration of 15%, and the sample was loaded on a C-4 column (W-Porex 5 C4; 4.6 × 150 mm; Phenomenex) equilibrated with 30% acetonitrile in 5 mM BTP (pH 7.5). GCAP was eluted with a linear gradient of acetonitrile (30–60%) in 5 mM BTP (pH 7.5) at a flow rate of 1.5 ml/min and 0.75-ml fractions were collected. From each fraction, 150 μl was lyophilized and resuspended in 30 μl of H_2O, and 10-μl aliquots from the indicated fractions were used to determine their stimulatory effect on GC activity by using the assay described. The remaining 20 μl was used for SDS/PAGE analysis. Fraction number corresponds to 2 times the retention time. (*B*) SDS/PAGE analysis of the eluted fractions from the reverse-phase column. SDS/PAGE was performed by using the Laemmli system (20) with 12% acrylamide gels and stained with Coomassie blue. Twenty microliters of the concentrated fractions were loaded on each lane of the gel. Lane St, standard proteins of the indicated molecular mass.

responses under these conditions have the same sensitivity, kinetics, and stimulus–response relationship as those recorded from intact rods (23). We assume the detached outer segment contains sufficient endogenous activator to maximally stimulate GC when internal Ca has fallen to its lowest level and essentially all the activator is in the Ca-free or active form. In the dark and during a subsaturating light response, internal Ca is considerably higher than its minimal level, and therefore only a fraction of the total pool of activator is Ca-free. Under these conditions, the addition of activator will raise the concentration of both the Ca-free and Ca-bound forms. The increase in the amount of Ca-free activator will cause greater stimulation of GC in the dark and during a light response. The resulting stimulation of cGMP synthesis would be expected to increase the resting dark current, cut short the rising phase of the light response, reduce flash sensitivity, and hasten the recovery process. Exogenous GCAP had all but one of these effects.

Responses recorded from different ROSs using control dialysis solution with (n = 12) and without (n = 13) GCAP were compared. The presence of GCAP had no significant effect on resting dark current (mean currents after 20 min of dialysis without and with GCAP were −78 ± 3 and −81 ± 3 pA, respectively), but in 10 of 12 ROSs GCAP speeded recovery onset and decreased the peak amplitude of the flash response. In these 10 ROSs the presence of GCAP reduced the time to peak of the dim flash response by a mean value of 116 ± 61 ms and reduced peak amplitude by 40% ± 1% relative to the responses recorded from the 13 ROSs without GCAP. These averages are based on results obtained by using seven different freshly prepared fractions of GCAP having nearly a 3-fold range in protein content. The superimposed traces in Fig. 4 show responses to identical flashes recorded from two different ROSs in the presence and absence of our most concentrated fraction of GCAP. The mean decrease in dim flash sensitivity (66% ± 1%; n = 2) was greater with this fraction of GCAP than with any other. The responses in Fig. 4 also show that GCAP accelerated the onset of the recovery process and reduced the total duration of the flash response. GCAP had little to no effect on the first 150–200 ms of the rising phase of dim to moderate intensity flash responses and reduced the recovery time (time for 63% recovery from the peak of the response) by ≈30%. These changes in the flash response are opposite those observed previously (10) using three different recoverin-like Ca-

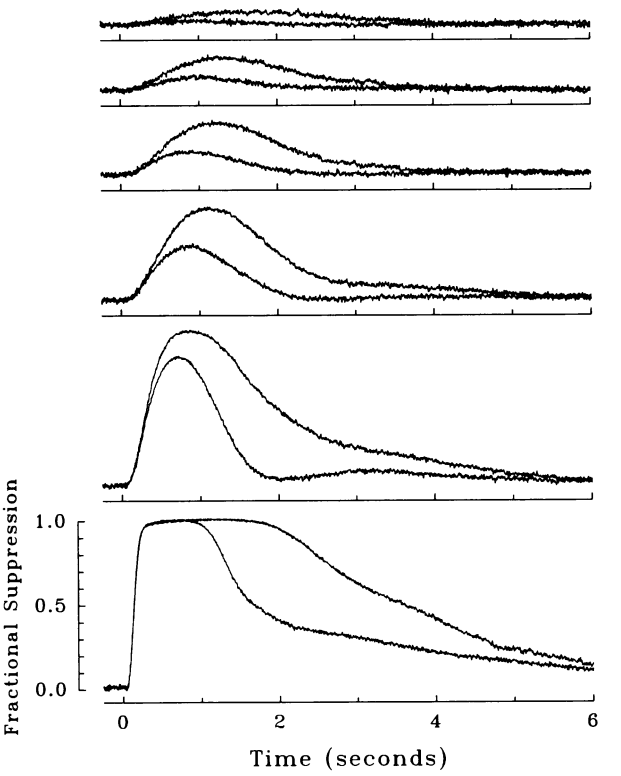

FIG. 4. GCAP decreases sensitivity, time to peak, and recovery time of the light response. Each pair of superimposed traces compares the response evoked by the same intensity flash after 20 min of whole-cell recording with control solution in the absence and presence of 750 nM GCAP; the faster response of each pair was in the presence of GCAP. Response families with and without GCAP were recorded consecutively from two different isolated ROSs from the same retina. Responses are expressed as the fractional change in the maximal light-suppressible current. Flash intensities were (from top to bottom): 5, 16, 29, 62, 266, and 2318 Rh* per flash. During the intensity series, the dark current in the absence and presence of GCAP declined from −67 to −63 and from −82 to −74 pA, respectively. Temperature was ≈16°C.

binding proteins. Intracellular incorporation of either recoverin, visinin, or *Gecko* p26 delayed the onset of response recovery and gave rise to responses that were larger and longer lasting than those recorded without added protein. In contrast to these results, in the same study calmodulin was found to have no effect on the flash response.

Since GCAP restored the Ca-sensitive regulation of GC in a reconstituted system (Fig. 1), in separate experiments we tested its effect on the low-Ca activation of GC in dialyzed ROSs by measuring the increase in dark current produced by abruptly decreasing external Ca from 1 to 0.2 mM (27). GCAP, in amounts that accelerated recovery of the light response, produced only a slight (≈5%; four experiments) increase in the peak amplitude of the low-Ca response (data not shown). The small effect of GCAP on the response to low-Ca exposure is consistent with the protein having an insignificant effect on resting dark current. Both observations may be explained by the powerful negative feedback loop (5, 32) that antagonizes an increase in dark current by coupling the increase in Ca influx and the resulting increase in internal Ca to a decrease in cGMP synthesis. It is also possible that in functionally intact rods activation by GCAP of GC may be influenced by other factors that cause the Ca sensitivity of GC in darkness and during a light response to be different. Finally, we note that GCAP reduced flash sensitivity and accelerated response kinetics in a manner that resembled the effects produced during background light adaptation. Since it is not clear at the present time if these changes can be explained solely by an effect on the Ca sensitivity of GC, it is possible that GCAP affects other elements in the transduction cascade. A full description of the mechanisms of GCAP actions in functionally intact rods will require further investigation. Such studies await the availability of recombinant GCAP (31).

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1. McNaughton, P. A. (1990) *Physiol. Rev.* **70**, 847–883.
2. Pugh, E. N., Jr. & Lamb, T. D. (1993) *Biochim. Biophys. Acta* **1141**, 111–149.
3. Yau, K.-W. & Nakatani, K. (1984) *Nature (London)* **313**, 661–663.
4. Cervetto, L., Lagnado, L., Perry, R. J., Robinson, D. W. & McNaughton, P. A. (1989) *Nature (London)* **337**, 740–743.
5. Hodgkin, A. L. & Nunn, B. J. (1988) *J. Physiol. (London)* **403**, 439–471.
6. Koch, K.-W. & Stryer, L. (1988) *Nature (London)* **334**, 64–66.
7. Dizhoor, A. M., Sanghamitra, R., Santosh, K., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B. & Stryer, L. (1991) *Science* **251**, 915–918.
8. Lambrecht, H. G. & Koch, K.-W. (1991) *EMBO J.* **10**, 793–798.
9. Hurley, J. B., Dizhoor, A. M., Ray, S. & Stryer, L. (1993) *Science* **260**, 740.
10. Gray-Keller, M. P., Polans, A. S., Palczewski, K. & Detwiler, P. B. (1993) *Neuron* **10**, 523–531.
11. Papermaster, D. S. (1982) *Methods Enzymol.* **81**, 48–52.
12. Wald, G. & Brown, P. K. (1953) *J. Gen. Physiol.* **37**, 189–200.
13. Baehr, W., Devlin, M. J. & Applebury, M. L. (1979) *J. Biol. Chem.* **254**, 11669–11677.
14. Gorczyca, W. A., Van Hooser, J. P. & Palczewski, K. (1994) *Biochemistry*, in press.
15. Senter, P. D., Eckstein, F., Mülsch, A. & Böhme, E. (1983) *J. Biol. Chem.* **258**, 6741–6745.
16. Koch, K.-W., Eckstein, F. & Stryer, L. (1990) *J. Biol. Chem.* **265**, 9659–9663.
17. Zimmerman, A. L., Yamanaka, G., Eckstein, F., Baylor, D. A. & Stryer, L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8813–8817.
18. Schoenmakers, T. J. M., Viser, G. J., Flik, G. & Theuvenet, A. P. R. (1992) *Biotechniques* **12**, 870–879.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
21. Sakmann, B. & Neher, E. (1983) *Single Channel Recording* (Plenum, New York).
22. Pusch, M. & Neher, E. (1988) *Pflügers Arch.* **411**, 204–211.
23. Rispoli, G., Sather, W. A. & Detwiler, P. B. (1993) *J. Physiol. (London)* **465**, 513–537.
24. Polans, A. S., Buczylko, J., Crabb, J. W. & Palczewski, K. (1991) *J. Cell Biol.* **112**, 981–989.
25. Lolley, R. N. & Racz, E. (1982) *Vision Res.* **22**, 1481–1486.
26. Malencik, D. A. & Anderson, S. R. (1983) *Biochem. Biophys. Res. Commun.* **114**, 50–56.
27. Rispoli, G., Sather, W. A. & Detwiler, P. B. (1988) *Biophys. J.* **53**, 388a.
28. Craven, P. A. & DeRubertis, F. R. (1988) *J. Biol. Chem.* **253**, 8433–8443.
29. Fleischman, D. & Denisevich, M. (1979) *Biochemistry* **18**, 5060–5066.
30. Garbers, D. L. (1992) *Cell* **71**, 1–4.
31. Baehr, W., Gorczyca, W., Subbaraya, I., Ohguro, H., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., Crabb, J. & Palczewski, K. (1994) *Invest. Ophthalmol. Visual Sci.* **35**, 1486.
32. Torre, V., Matthews, H. R. & Lamb, T. D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7109–7113.