Domain-Specific Stabilization of Photoreceptor Membrane Guanylyl Cyclase by Adenine Nucleotides and Guanylyl Cyclase Activating Proteins (GCAPs)†

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ABSTRACT: In photoreceptor outer segments, particulate guanylyl cyclase (RetGC) is stimulated at low intracellular Ca^{2+} concentrations by guanylyl cyclase activating protein (GCAP), a Ca^{2+}-sensitive activator, to resynthesize light-depleted cGMP. In washed outer segment membranes, we find that GCAP-stimulable RetGC is rapidly inactivated at physiological temperatures (30−37 °C). Under the same conditions, RetGC remains competent for stimulation by S-100 protein preparations or Mn^{2+}/Triton X-100, indicating that the cyclase catalytic domain remains functional. GCAPs and adenine nucleotides protect against inactivation. Protection by GCAPs is independent of Ca^{2+} concentration, suggesting that there is a Ca^{2+}-independent interaction between GCAP and RetGC. Protection by ATP (EC_{50} = 150 μM) is not due to phosphorylation, since the nonhydrolyzable analogue adenylyl imidodiphosphate (AMP-PNP) protects equally well. In addition to their roles in protection, ATP and AMP-PNP also slowly stimulate cyclase activity. In parallel with the functional change in RetGC at physiological temperatures, we also observe a structural change. A 62-kDa intracellular fragment of RetGC-1 becomes more sensitive to cleavage by trypsin after preincubation at 30 °C unless ATP, AMP-PNP, or GCAP is present. Adenine nucleotides and GCAPs thus protect RetGC structurally, as well as functionally.

Photoreceptor membrane guanylyl cyclases (RetGCs)[1] play key roles in the responses of photoreceptor cells to flashes of light, continuous illumination, and darkness. Upon light excitation, cGMP is depleted in photoreceptor outer segments (OS) by a cGMP-phosphodiesterase (for reviews see refs *1* and *2*). This causes closure of cGMP-gated cation channels, which hyperpolarizes the cell, slows neurotransmitter release, and lowers intracellular Ca^{2+}. RetGC is stimulated by lowered Ca^{2+} levels to convert GTP to cGMP (*3*−*6*). The newly synthesized cGMP can bind to and reopen the cGMP-gated channels, returning the cell to the depolarized "dark" state.

Two forms of photoreceptor-specific membrane guanylyl cyclases, RetGC-1 and RetGC-2, were initially identified from a human retinal cDNA library (*7*, *8*). Subsequently, bovine and rat homologues were cloned (*9*, *10*). RetGC-1 and RetGC-2 are in the family of membrane guanylyl cyclases, which includes the natriuretic peptide receptors GC-A and GC-B (*11*−*13*), the heat-stable enterotoxin receptor GC-C (*14*), and the sea urchin sperm cell GC (*15*). These proteins each contain an extracellular domain, a single transmembrane domain, a conserved kinase homology domain (KHD), and a catalytic domain that converts GTP to cGMP (reviewed in ref *16*).

With the exception of RetGCs, the membrane GCs form a family of peptide hormone receptors that synthesize cGMP in response to binding of peptide hormone to the extracellular domain. In RetGCs, the extracellular domain is not required for stimulation (*17*, *18*). Instead, two intracellular Ca^{2+}-binding proteins, GCAP-1 and GCAP-2, stimulate RetGCs at low Ca^{2+} concentrations (*5*, *6*, *19*, *20*). Recently, it was also reported that Ca^{2+}-binding proteins of the S-100 family activate RetGC but at high Ca^{2+} concentrations (*21*, *22*).

Modulation of GC activity has been demonstrated in GC-A, GC-C, and RetGC in the form of an ATP-stimulatory effect which can be reproduced with nonhydrolyzable analogues of ATP. ATP appears to be required for activation of GC-A by hormone (*23*) and enhances, but is not required for, activation of GC-C (*24*) and RetGC (*17*, *25*−*28*). Although it is clear that ATP enhances GC activity in these *in vitro* assays, the general mechanism through which this occurs has not been well established.

In this study, we identify a discrete functional domain of RetGC that is essential for mediating activation by GCAP-2. This domain is temperature-sensitive and is separate from the catalytic domain and from the domain mediating activation by S-100. ATP, AMP-PNP, and GCAPs stabilize the temperature-sensitive domain both structurally and functionally. GCAPs stabilize this domain at both low and high Ca^{2+} concentrations, suggesting that they associate with RetGCs under both activating and nonactivating conditions. ATP or AMP-PNP, in the presence of GCAP, also causes a slow time-dependent stimulation of activity.

MATERIALS AND METHODS

Chemicals and Reagents. [α-^{32}P]GTP was purchased from NEN−Dupont, while [8-^{3}H]cGMP came from Amersham. λ phosphatase and bovine brain S100 protein were obtained from Calbiochem. For GC assays, poly(ethylenimine)-cellulose TLC plates were purchased from Merck. Myris-

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[1] Abbreviations: RetGC, photoreceptor membrane guanylyl cyclase; OS, photoreceptor outer segments; KHD, kinase homology domain; Rv, recoverin; RH, rhodopsin; CaM, calmodulin; GTPγS, guanosine 5′-*O*-3-thiotriphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, adenylyl imidodiphosphate.

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toylated GCAP-2 and myristoylated recoverin were prepared as described previously (*29*, *30*).

OS Preparation. Outer segments from frozen bovine retinas were isolated using sucrose flotation by the technique of McDowell and Kuhn (*31*), with modifications. Bovine OS membranes were washed in the dark three times in GC wash buffer [5 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.05 mM phenylmethanesulfonyl fluoride, and 5% (v/v) glycerol]. Between washes, membranes were pelleted in a TLA100.1 rotor at 80 000 rpm for 5 min at 4 °C. Membranes were resuspended in wash buffer at 2 mg/mL, aliquoted, and frozen at −70 °C until use. For preincubations and GC assays, membranes were diluted in 2× GC buffer (200 mM KCl, 100 mM MOPS, 14 mM 2-mercaptoethanol, 20 mM $MgCl_2$, 16 mM NaCl, and 2 mM EGTA) to a rhodopsin concentration of 0.48 μg/μL. Preincubations were then carried out typically at 30 °C for the indicated times. Preincubations were stopped by placing on ice and, when specified, adding ATP and/or GCAP-2.

GC Assays. Measurement of guanylyl cyclase activity was carried out as previously described (*20*). Washed OS membranes in 2× GC buffer (12.5 μL, 6 μg of rhodopsin) were combined with additional factors (detailed in figures) and 10 μM dipyridamole (to inhibit phosphodiesterase activity) to a volume of 20 μL. The reaction was initiated by the addition of 5 μL of a 5× substrate solution (5 mM GTP, 25 mM cGMP, ∼2 μCi of [α-^{32}P]GTP, and ∼100 000 dpm of [8-^{3}H]cGMP) and incubated at 30 °C for the indicated period of time (usually 10 min).

Proteolysis. Washed OS membranes (∼30 μg of Rh) in 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol were preincubated either at 30 °C or on ice. Trypsin-treated samples were incubated for 10 min at 30 °C with trypsin at a concentration of 1:7000 μg of total protein, as detailed in figure legends. Samples were boiled for 4 min and run on a 10% an SDS−polyacrylamide gel. Immunoblotting was performed using the polyclonal antibody CAT-Ab corresponding to M_{747}−S_{1052} of RetGC-1. Preparation and characterization of this antibody will be described elsewhere (Laura *et al.*, manuscript in preparation).

RESULTS

Photoreceptor GC Rapidly Loses GCAP Sensitivity at 30 °C. Incubation of washed OS membranes at 30 °C elicits a rapid and sustained loss of GCAP-stimulated guanylyl cyclase activity (Figure 1A, ●), with half of the activity lost within 5 min. The rate of inactivation is initially very rapid (0.136 ± 0.02 min^{-1}) but slows after 10 min (0.039 ± 0.002 min^{-1}). Basal activity (Figure 1A, ○) also decreases, but more slowly.

The loss of activity could represent a specific loss of sensitivity to GCAP, rather than a general loss of catalytic ability. To determine this, we examined the sensitivity of RetGC to other activators after incubations. S100 preparations appear to activate RetGC through a different mechanism than GCAP (*21*). GCs can generally be constitutively activated by Mn^{2+} and nonionic detergent (*32*). Figure 1B shows that incubating OS membranes at 30 °C causes only a relatively small loss of activity when assayed in the presence of either S100 and 1 mM Ca^{2+} (●) or Mn^{2+} and Triton X-100 (○). Taken together, these results show that RetGC remains competent for catalytic activity but loses its ability to respond to GCAP-2.

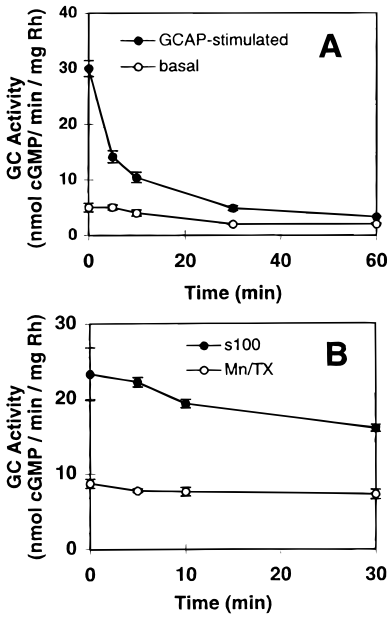

FIGURE 1: Photoreceptor GC rapidly loses GCAP sensitivity at 30 °C. (A) Time course of GC inactivation. Washed OS membranes were preincubated for various times at 30 °C in 2× GC buffer. Stimulated GC activity (●) was measured as detailed in Materials and Methods in the presence of 0.5 mM ATP, 1 mM EGTA, and saturating amounts of GCAP-2 (0.33 μM). Basal activity (○) was measured in the presence of 0.5 mM ATP, 1 mM EGTA, and 0.9 mM $CaCl_2$. (B) Time course of GC activity stimulated by S100 or Mn^{2+}/Triton X-100. Washed OS membranes were incubated in 2× GC buffer (without $MgCl_2$) for indicated times at 30 °C. GC activity was assayed for 10 min in 1 mM $CaCl_2$ and either 1% Triton X-100 and 10 mM $MnCl_2$ (Mn/Tx, ○) or 9.6 μM bovine brain S100 and 10 mM $MgCl_2$ (S100, ●). Points represent the means of duplicate determinations from a representative of several experiments.

Protection by ATP. Since ligands often stabilize proteins, we examined the stabilizing effects of factors known to influence GC activity, such as ATP, GCAPs, and S100. ATP protects against inactivation at 30 °C with an EC_{50} of approximately 150 μM (Figure 2A). ATP (1 mM) slows inactivation significantly (0.008 min^{-1}, $t_{1/2}$ = 86 min). The temperature- dependence of this effect is shown in Figure 2B. In the absence of ATP (●), GCAP-stimulated cyclase activity was fairly stable for 1 h at room temperature (20 °C), but at 30 °C, 86% of the initial activity was lost. The protective effect of ATP (○) was most significant at 30 °C (4.6-fold activity) and 37 °C (4.2-fold activity).

Protective Effect of ATP Is Not Mediated by Phosphorylation. Since previous studies have shown that ATP analogs can elicit similar effects as ATP on RetGC activity (*17*, *25*, *27*), we examined the stabilizing effects of various nucleotides and analogues (Figure 3). Washed OS membranes were preincubated at 30 °C for 30 min in the presence or absence of 1 mM nucleotide or analogue then assayed for stimulation by GCAP-2. Adenine nucleotides and analogues significantly protect GC. At 1 mM, ATPγS protects most effectively, followed by AMP-PNP, ATP, and then ADP. GTP and guanine analogs are less effective, while TTP has no effect. Since the nonhydrolyzable ATP analogue AMP-PNP stabilizes equally well as ATP, these results suggest that phosphorylation and hydrolysis are not involved in stabilization.

Protective Effects of GCAPs. We also tested activators of RetGC for their abilities to maintain RetGC in a GCAP-sensitive state. GCAP-1 and GCAP-2 are Ca^{2+}-sensitive

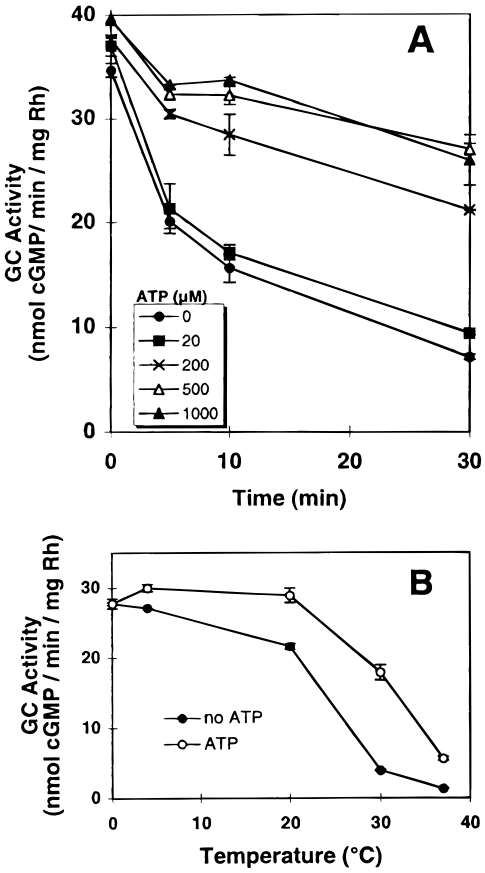

FIGURE 2: ATP protects GC from loss of GCAP sensitivity. (A) ATP titration and time course of inactivation. Washed OS membranes were preincubated for various times at 30 °C at the indicated concentrations of ATP. Subsequently, samples were brought to 0.5 mM ATP and GC activity was measured in the presence of 0.33 μM GCAP-2 and 1 mM EGTA. (B) Temperature dependence of inactivation. Washed OS membranes were preincubated at the indicated temperatures for 1 h in the presence (○) or absence (●) of 1 mM ATP. Stimulated GC activity was measured as described for panel A. Points represent the means of duplicate determinations from a representative of several experiments.

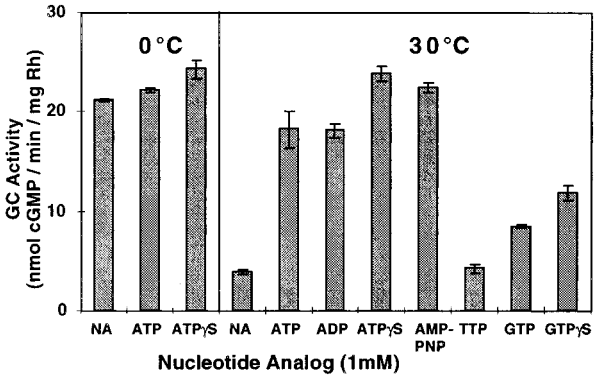

FIGURE 3: Effect of nucleotide analogs on stabilization of photoreceptor GC. Washed OS membranes were preincubated at 0 or 30 °C in the presence or absence of the indicated nucleotides and analogs. NA indicates no addition. After 30 min, samples were diluted in 1 mL of GC wash buffer containing 0.5 mM ATP and spun at 80 000 rpm in a TLA 100.3 rotor. Membranes were resuspended in 2× GC buffer containing 0.5 mM ATP. GC activity was measured with 0.33 μM GCAP-2 and 1 mM EGTA as described previously. Bars represent the means of duplicate determinations from a representative of several experiments.

proteins that stimulate RetGC-1 and RetGC-2 at low Ca^{2+} concentrations (*5*, *6*, *20*, *34*). Preparations containing S100

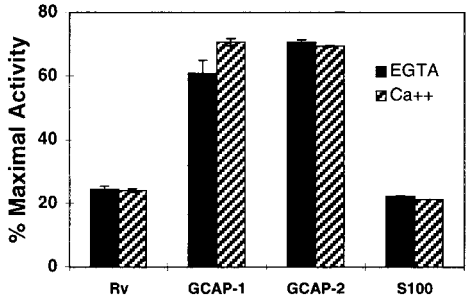

FIGURE 4: GCAPs, but not other Ca^{2+}-binding proteins, stabilize GC activity. Washed OS membranes were preincubated for 30 min at 0 or 30 °C in the presence of 5.1 μM recoverin (Rv), 5.1 μM GCAP-1, 0.84 μM GCAP-2, or 6.4 μM S100. Preincubations were done in 0.5 mM $CaCl_2$ (Ca^{2+}, hatched bars) or 3 mM EGTA (EGTA, solid bars). Subsequently, GC activity was measured in the presence of 5.1 μM recoverin, 5.1 μM GCAP-1, 0.84 μM GCAP-2, 0.5 mM $CaCl_2$, 3 mM EGTA, and 0.5 mM ATP. Maximal activity (26.09 ± 0.32 nmol of cGMP min^{-1} (mg of rhodopsin)$^{-1}$ for samples preincubated in Ca^{2+}; 24.02 ± 0.61 nmol of cGMP min^{-1} (mg of rhodopsin)$^{-1}$ for samples preincubated in EGTA) corresponds to samples incubated on ice in the presence of 5.1 μM recoverin, 5.1 μM GCAP-1, 0.84 μM GCAP-2, and 0.5 mM ATP. Bars represent the means of duplicate determinations from a representative of several experiments.

activate RetGC at high Ca^{2+} concentrations (*21*). Washed OS membranes were preincubated at 30 °C with Ca^{2+} or EGTA and in the presence of either recoverin (Rv), GCAP-1, GCAP-2, or bovine brain S100 (Figure 4). Following this preincubation, recoverin, GCAP-1, and/or GCAP-2 were added so that all samples contained equivalent amounts of proteins during the assay for cyclase activity. In addition, either Ca^{2+} or EGTA was added so the free Ca^{2+} concentration was the same for all samples (<100 nM) during the assay. GCAP-1 or GCAP-2 present during the preincubation protect against loss of GCAP sensitivity, while recoverin, S100, or BSA (not shown) do not. The specific protection by GCAPs was unaffected by the presence or absence of Ca^{2+} during the preincubation. Since recoverin, a protein that associates with membranes only in the presence of Ca^{2+} (*35*, *36*), has no effect on stability in either the presence or absence of Ca^{2+}, stabilization by GCAPs is not due merely to protein binding to the membranes.

Stimulatory Effect of ATP. During a cyclase assay, OS membranes are typically incubated for a length of time at elevated temperatures (typically 30 or 37 °C). Protection by ATP and/or GCAPs during the assay time could account for an apparent stimulatory effect. However, ATP could also have a stimulatory effect independent of a stabilizing effect. We examined GCAP-2-dependent cyclase activity after preincubating washed OS for 10 min at 0 or 30 °C (Figure 5). OS were incubated alone (no addition) or with either AMP-PNP, GCAP-2, or AMP-PNP/GCAP-2. The samples were then assayed for 4 min at 30 °C in the presence of both AMP-PNP and GCAP-2. When membranes were incubated at 0 °C, there was no difference in activity. However, at 30 °C, large differences were observed depending on which factors were present during the preincubation. With no addition, GCAP-dependent activity is lost. In the presence of either AMP-PNP or GCAP-2, activity remains stable for the preincubation time (compare with activity of samples at 0 °C). With both AMP-PNP and GCAP-2, activity increases. This increase is linear and time-dependent and gradually reaches a maximum [(1.68 ± 0.36)-fold

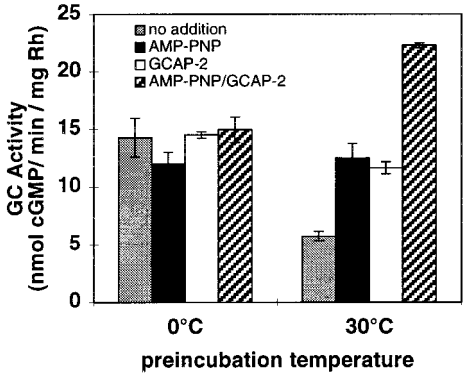

FIGURE 5: Slow time-dependent stimulation by adenine nucleotides. Washed OS membranes were resuspended in 2× GC buffer containing 1 mM EGTA. Samples were preincubated at 0 or 30 °C for 10 min with no addition, 0.75 mM AMP-PNP, 0.5 μM GCAP-2, or 0.75 mM AMP-PNP/0.5 μM GCAP-2. Following the preincubation, AMP-PNP and/or GCAP-2 was added for the assay. GC activity was measured for 4 min. Points represent the means of duplicate determinations from a representative of several experiments.

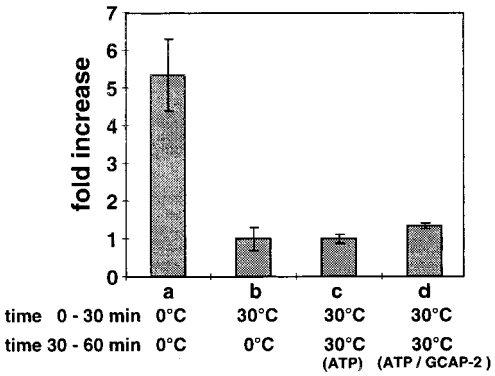

FIGURE 6: Inactivation of photoreceptor cyclase is irreversible. Washed OS membranes were preincubated for 30 min at 0 °C (a) or 30 °C (b−d). Subsequently, samples were either assayed (b) or incubated another 30 min at 30 °C in the presence of 0.75 mM ATP (c) or 0.75 mM ATP and 0.5 μM GCAP-2 (d). Stimulated GC activity was measured as previously described. Samples were normalized to *x*-fold increase over minimal activity, represented by (b). Bars represent the means of duplicate determinations from a representative of several experiments.

increase over 0 °C samples] after 10−15 min (data not shown). When ATPγS or ATP was substituted for AMP-PNP, the results were the same.

The increase in activity could represent restoration of GCAP sensitivity lost during isolation of membranes. To investigate this, we tested whether ATP and GCAP could reverse loss of GCAP sensitivity (Figure 6). We first incubated OS membranes at 30 °C for 30 min in the absence of ATP and GCAPs. We then either assayed the membranes immediately (Figure 6b), or added ATP (c) or ATP/GCAP-2 (d) and incubated 30 min further. Data are expressed as *x*-fold increase over minimal activity, represented by (b). Neither ATP nor ATP/GCAP-2 restored the activity lost during the first 30 min incubation. Samples containing ATP/GCAP-2 showed a small (1.3-fold) increase in GCAP-stimulated activity (compare b with d), but this increase was maximal after 15 min (data not shown).

Limited Proteolysis. To uncover any structural changes that might correlate with a loss of GCAP sensitivity, we performed limited proteolysis on RetGC-1. To detect proteolytic fragments, we used a polyclonal antibody, CAT-

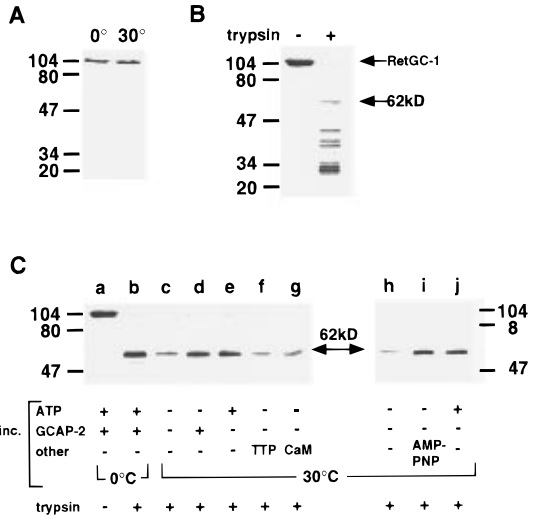

FIGURE 7: Limited proteolysis of RetGC-1. (A) Loss of GCAP sensitivity is not due to intrinsic proteolysis. Washed OS membranes (30 μg of Rh) were preincubated for 30 min at 0 or 30 °C. Samples were immunoblotted with CAT-Ab, which recognizes the RetGC-1 catalytic domain. (B) Limited proteolysis of RetGC-1. Membranes were boiled or treated with trypsin (1:7000 w/w) for 10 min at 30 °C. Samples were run on a gel that also contained lanes a−g of Figure7C. Immunoblotting was as in panel A. (C) Preincubation alters the tryptic sensitivity of a 62-kDa fragment of RetGC-1. Washed OS membranes (30 μg of Rh) were preincubated for 20 min at 0 or 30 °C. During the preincubation, samples contained indicated additional factors at concentrations of 1 mM (nucleotides and analogues) and 1 μM [GCAP-2 or calmodulin (CaM)]. For proteolysis, all samples contained 1 mM ATP and 1 μM GCAP-2. Lanes h and i contained 1 mM AMP-PNP in addition to ATP and GCAP-2 during proteolysis. Samples were boiled (lane a) or treated with trypsin (1:7000 w/w) for 10 min at 30 °C. Immunoblotting was as in panel A.

Ab, specific to the catalytic domain of RetGC-1. Intact RetGC-1 is indicated by a band of approximately 115 kDa. In the absence of protease, there was no difference in RetGC-1 immunoreactivity between OS preincubated for 30 min at 0 or at 30 °C (Figure 7A), indicating that endogenous proteases are not responsible for the temperature-dependent loss of GCAP sensitivity. Further support for this conclusion comes from our observations that the protease inhibitors leupeptin, pepstatin, and aprotinin do not prevent loss of GCAP sensitivity (data not shown).

When we partially digest RetGC-1 with trypsin, a relatively stable 62-kDa fragment appears (Figure 7B). Since the antibody we used to detect RetGC-1 recognizes only the catalytic domain, this fragment represents much of the intracellular domain of the protein including catalytic and kinase homology domains. With more trypsin, this 62-kDa band disappears and a number of smaller proteolytic products appear (not shown). Studies in our lab using domain-specific antibodies indicate that the initial proteolytic sites in the 62-kDa fragment are within the kinase homology domain (Laura *et al.*, manuscript in preparation). Thus, factors that protect the KHD would increase protease resistance of the 62-kDa band, while destabilizing factors would increase protease sensitivity.

We therefore examined the protease sensitivity of the 62-kDa band after preincubating membranes at 30 °C with or without ATP or GCAP-2 (Figure 7C). Membranes were preincubated on ice (lanes a and b) or at 30 °C (lanes c−j), in the presence or absence of ATP and/or GCAP-2. Following preincubation, ATP and/or GCAP-2 was added so

that all samples contained equal amounts of these during proteolysis. Preincubation of OS membranes at 30 °C consistently increased the protease sensitivity of the 62-kDa band over that preincubated on ice (compare lane c with lane b). This suggests that a structural rearrangement takes place at 30 °C. GCAP-2 (lane d) or ATP (lane e) present during the preincubation prevents the increase in protease sensitivity of the 62-kDa fragment. However, presence of TTP (lane g), calmodulin (lane h), or recoverin (not shown) during the preincubation does not protect this fragment. ATP and GCAP-2 therefore appear to provide structural stability in addition to the functional stability we described in the preceding sections. The stability conferred by ATP is not due to phosphorylation, since AMP-PNP protects equally well (compare lanes i and j).

DISCUSSION

In this study, we investigated the thermal stability of a specific functional domain of photoreceptor membrane guanylyl cyclase. Elevated temperatures cause RetGC to lose its ability to be activated by GCAP. Catalytic ability, however, is preserved, since basal as well as Mn^{2+}/Triton X-100 and S100-mediated activities are retained. In parallel with this loss of function, we observe a structural change in RetGC-1. The loss of GCAP sensitivity correlates with the increased trypsin cleavage of a relatively stable 62-kDa fragment of RetGC-1. This fragment contains both catalytic and kinase homology domains, and the site of cleavage appears to be within the kinase domain based on immunological evidence using domain-specific antibodies (Laura *et al*., manuscript in preparation). The increased sensitivity to trypsin is likely to arise from a change to a more unfolded state. Since the catalytic domain retains sufficient order for enzymatic activity, this structural change must be highly localized. These results point to the KHD as a thermally unstable region of RetGC that can be protected by ATP and GCAPs, and whose ordered structure is essential for activation by GCAP.

Evidence from a number of sources further implicates the KHD as the unstable domain. The KHD is structurally similar to ATP-binding domains of protein kinases and so is a likely region for ATP to bind RetGC (*11*, *37*). Studies of GC-A support this, since deleting the KHD eliminated ATP dependence (*11*) and point mutations at corresponding sites crucial for ATP binding to kinases knocked out activity (*38*). Furthermore, direct binding of ATP to RetGC at a site distinct from the catalytic GTP binding site was recently shown (*28*). Our results showing specific loss of sensitivity to GCAP but not S100 also correlate with a localized change in the KHD. S100-mediated stimulation, which does not require the KHD (*21*), is not significantly affected by incubation at 30 °C. In contrast, GCAP-mediated stimulation, which does appear to require the KHD (*18*), is greatly diminished. Finally, studies of several protein kinases show these domains to be thermally unstable regions. $pp60^{v-src}$, EGFR, and smMLCK all undergo temperature-dependent loss of activity unless protected by adenine nucleotides (*39*−*41*). Thermodynamic analysis of EGFR suggested that inactivation was due to a local reorganization of the kinase domain (*41*).

The membrane cyclase GC-C loses hormone-stimulated activity upon incubation at elevated temperatures and is protected from inactivation by adenine nucleotides (*24*, *42*). In RetGC, we also find a major role for ATP in protection. Both ATP and GCAP appear to maintain RetGC in a GCAP-sensitive state in the presence of destabilizing influences. These results may explain discrepancies in reports of ATP effects. Laura *et al.* (*17*) reported an enhancement of activity by ATP of >2-fold in a recombinant system but only 1.2-fold in OS. The assays in the recombinant cells used subsaturating levels of GCAP-2, a factor that stabilizes RetGC. Additionally, the assay time was especially lengthy (30 min). Thus, the effect of ATP would appear to be much greater than in OS. Using OS homogenates, Krishnan *et al.* (*25*), Gorczyca *et al.* (*27*), and Aparicio and Applebury (*28*) each reported an approximate 2-fold enhancement of GC activity in the presence of ATP or nonhydrolyzable ATP analogues, similar to what we report.

Ligand sensitivity of several membrane GCs correlates with phosphorylation state. GC-A and sperm cell cyclase are both desensitized to ligand upon dephosphorylation and regain activity upon rephosphorylation (*32*, *33*). RetGC can also be phosphorylated *in vitro* (*28*), but two results suggest that phosphorylation is not involved in ATP-mediated stabilization. First, both ADP and nonhydrolyzable ATP analogues are as effective as ATP (see Figure 4). Second, Mg^{2+} is not required for stabilization by ATP (data not shown), while it is required for phosphorylation (*28*).

We specifically investigated whether ATP has a regulatory role in addition to its protective role. Inclusion of both ATP and GCAP-2 during a 30 °C preincubation causes a slow increase in GCAP-stimulated RetGC activity. Direct phosphorylation is not responsible for this increase since the nonhydrolyzable analogue AMP-PNP is as effective as ATP. The effect is also unlikely to be caused by refolding of enzyme that became thermally inactivated during isolation of membranes, since ATP and GCAP cannot restore lost activity (Figure 7). The effect of ATP may be allosteric, perhaps inducing a slow change in the affinity of cyclase for GCAP.

Although GC-C also loses activity (*24*, *42*), we find notable differences between this protein and RetGC. Loss of GC-C activity is enhanced by stimulatory factors, such as peptide ligand or detergents. In contrast, RetGC is stabilized by its activator, GCAP. The variance between GC-C and RetGC may reflect fundamentally different modes of activation of these proteins: GCAP stimulates RetGC intracellularly, whereas peptide ligands stimulate GC-C extracellularly. Stabilization by GCAPs is likely due to formation of a complex between GCAP and the intracellular domain of RetGC. Since GCAPs stabilize GC at both high and low Ca^{2+} concentrations, our results suggest that GCAPs interact with RetGCs under nonactivating as well as activating conditions. Consistent with this, a recent study showed that the Ca^{2+}-loaded form of GCAP-2 competes with and inhibits stimulation of RetGC by an activating (Ca^{2+}-free) form of GCAP-2 (*29*). In another study, GCAP-1 was cross-linked to RetGC independently of Ca^{2+} levels (*18*).

In this report we have dissected out the individual roles of modulators of RetGC with respect to their contributions to maintenance of a GCAP-responsive state. Our findings reinforce the importance of examining the activity of proteins under physiological conditions. *In vivo*, the regulation and maintenance of RetGC activity appears to be a complex process in which individual effectors play multiple roles.

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REFERENCES

1. Lagnado, L., and Baylor, D. (1992) *Neuron 8*, 995−1002.
2. Yarfitz, S., and Hurley, J. B. (1994) *J. Biol. Chem. 269*, 14329−14332.
3. Lolley, R. N., and Racz, E. (1982) *Vision Res. 22*, 1481−1486.
4. Koch, K. W., and Stryer, L. (1988) *Nature 334*, 64−71.
5. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron 12*, 1345−1352.
6. Gorczyca, W. A., Gray-Keller, M. P., Detwiler, P. B., and Palczewski, K. (1994*) Proc. Natl. Acad. Sci. U.S.A. 91*, 4014−4018.
7. Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., and Lowe, D. G. (1992) *Neuron 9*, 727−737.
8. Lowe, D. G., Dizhoor, A. M., Lir, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5535−5539.
9. Goraczniak, R. M., Duda, T., Sitaramayya, A., and Sharma, R. K. (1994) *Biochem. J. 302*, 455−461.
10. Yang, R. B., Foster, D. C., Garbers, D. L., and Fulle, H. J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 602−606.
11. Chinkers, M. and Garbers, D. L. (1989) *Science 245*, 1392−1394.
12. Lowe, D. G., Chang, M. S., Hellmiss, R., Chen, E., Singh, S., Garbers, D. L., and Goeddel, D. V. (1989) *EMBO J. 8*, 1377−1384.
13. Chang, M. S., Lowe, D. G., Lewis, M., Hellmiss, R., Chen, E., and Goeddel, D. V. (1989) *Nature 341*, 68−72.
14. Schulz, S., Green, C. K., Yuen, P. S., and Garbers, D. L. (1990) *Cell 63*, 941−948.
15. Dangott, L. J., and Garbers, D. L. (1984) *J. Biol. Chem. 259*, 13712−13716.
16. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol Chem. 269*, 30741−30744.
17. Laura, R. P., Dizhoor, A. M., and Hurley, J. B. (1996) *J. Biol. Chem. 271*, 11646−11651.
18. Duda, T., Goraczniak, R., Surgucheva, I., Rudnicka-Nawrot, M., Gorczyca, W. A., Palczewski, K., Sitaramayya, A., Baehr, W., and Sharma, R. K. (1996) *Biochemistry 35*, 8478−8482.
19. Palczewski, K., Subbaraya, I., Gorczyca, W. A., Helekar, B. S., Ruiz, C. C., Ohguro, H., Huang, J., Zhao, X., Crabb, J. W., Johnson, R. S., Walsh, K. A., Gray-Keller, M. P., Detwiler, P. B., and Baehr, W. (1994) *Neuron 13*, 395−404.
20. Dizhoor, A. M., Olshevskaya, E. V., Henzel, W. J., Wong, S. C., Stults, J. T., Ankoudinova, I., and Hurley, J. B. (1995) *J. Biol. Chem. 270*, 25200−25206.
21. Duda, T., Goraczniak, R. M., and Sharma, R. K. (1996) *Biochemistry 35*, 6263−6266.
22. Pozdnyakov, N., Yoshida, A., Cooper, N. G. F., Margulis, A., Duda, T., Sharma, R. K., and Sitaramayya, A. (1995) *Biochemistry 34*, 14279−14283.
23. Chinkers, M., Singh, S., and Garbers, D. L. (1991) *J. Biol. Chem. 266*, 4088−4093.
24. Vaandrager, A. B., van der Wiel, E., and de Jonge, H. R. (1993) *J. Biol. Chem. 268*, 19598−19603.
25. Krishnan, N., Fletcher, R. T., Chader, G. J., and Krishna, G. (1978)*Biochim. Biophys. Acta 523*, 506−515.
26. Lambrecht, H. G., and Koch, K. W. (1991) *FEBS Lett. 294*, 207−209.
27. Gorczyca, W. A., Van Hooser, J. P., and Palczewski, K. (1994) *Biochemistry 33*, 3217−3222.
28. Aparicio, J. G. and Applebury, M. (1996) *J. Biol. Chem. 271*, 27083−27089.
29. Dizhoor, A. M. and Hurley, J. B. (1996) *J. Biol. Chem. 271*, 19346−19350.
30. Ray, S., Zozulya, S., Niemi, G. A., Flaherty, K. M., Brolley, D., Dizhoor, A. M., McKay, D. B., Hurley, J., and Stryer, L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5705−5709.
31. McDowell, J. H., and Kuhn, H. (1977) *Biochemistry 16*, 4054−4060.
32. Potter, L. R. and Garbers, D. L. (1992) *J. Biol. Chem. 267*, 14531−14534.
33. Bentley, K. J., Tubb, D. J., and Garbers, D. L. (1986) *J. Biol. Chem. 261*, 14859−14862.
34. Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., and Palczewski, K. (1995) *J. Biol. Chem. 270*, 22029−22036.
35. Dizhoor, A. M., Chen, C. K., Olshevskaya, E., Sinelnikova, V. V., Phillipov, P., and Hurley, J. B. (1993) *Science 259*, 829−832.
36. Zozulya, S. and Stryer, L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11569−11573.
37. Singh, S., Lowe, D. G., Thorpe, D. S., Rodriguez, H., Kuang, W. J., Dangott, L. J., Chinkers, M., Goeddel, D. V., and Garbers, D. L. (1988) *Nature 334*, 708−712.
38. Koller, K. J., Lipari, M. T., and Goeddel, D. V. (1993) *J. Biol. Chem. 268*, 5997−6003.
39. Sugimoto, Y., Erikson, E., Graziani, Y., and Erikson, R. L. (1985) *J. Biol. Chem. 260*, 13838−13843.
40. Faux, M. C., Pearson, R. B., Mitchelhill, K. I., and Kemp, B. E. (1993) *J. Biol. Chem. 268*, 12484−12491.
41. Stein, R. A., and Staros, J. V. (1996) *Biochemistry 35*, 2878−2884.
42. Vaandrager, A. B., Schulz, S., de Jonge, H. R., and Garbers, D. L. (1993a) *J. Biol. Chem. 268*, 2174−2179.

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