Activation by PKC of the Ca^{2+}-Sensitive Guanylyl Cyclase in Bovine Retinal Rod Outer Segments Measured with an Optical Assay†

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ABSTRACT: cGMP and Ca^{2+} are intracellular messengers in vertebrate rod photoreceptors. cGMP is the excitatory messenger, while intracellular free Ca^{2+} has been implied to be (one of) the messenger(s) in the process of light adaptation in vertebrate rod photoreceptors. The enzyme guanylyl cyclase (GC, EC 4.6.1.2.) catalyzes the reaction GTP → cGMP + PP_i. Bovine retinal rod outer segments (ROS) contain a particulate GC which is inhibited by an increase in free Ca^{2+} in the submicromolar range, although the precise molecular mechanism underlying this inhibition is unclear. We have developed an optical enzyme-coupled assay to study regulation of the particulate GC endogenous to bovine ROS. The particulate GC exhibited a Ca^{2+}-inhibited (IC_{50} 83−144 nM) activity of 13−23 nmol of PP_i/(min·(mg of rhodopsin)). ATP increased the maximal velocity of GC by about 2-fold, and this increase was inhibited by the specific PKC inhibitors chelerythrine and the pseudosubstrate-based peptide inhibitor PKC R10-31N. When the factor that mediated the ATP-dependent increase in GC rate was removed by washing, the ATP-dependent increase in GC rate could be reestablished by addition of purified, constitutively active PKC.

Photoexcitation in the outer segments of vertebrate retinal rods (ROS)[1] results in the increased hydrolysis of cGMP and closure of the cGMP-gated channels (Stryer, 1986). The closure of cGMP-gated channels reduces the influx of Ca^{2+} and Na^+ across the ROS plasma membrane (Kaupp *et al.*, 1988; Yau & Baylor, 1989), whereas extrusion of Ca^{2+} via the Na^+−Ca^{2+}+K^+ exchanger is not affected (Yau & Nakatani, 1985). Therefore, light results in the reduction of the cytosolic free Ca^{2+} concentration in ROS (Ratto *et al.*, 1988). Lowering the cytosolic free Ca^{2+} concentration is thought to mediate the process of light adaptation, in part via the stimulation of ROS particulate guanylyl cyclase (GC) activity (Koch & Stryer, 1988) and in part by in activation of the cGMP-phosphodiesterase activity (Kawamura & Murakami, 1991), possibly via phosphorylation of rhodopsin (Kawamura, 1993). Inhibition by Ca^{2+} is the only regulation mechanism of ROS particulate GC described so far. Inhibition of ROS GC by Ca^{2+} was first thought to be mediated by a 26 kDa protein named recoverin (Dizhoor *et al.*, 1991) or p-26 (Lambrecht & Koch, 1991), but more recent results indicate that recoverin is not involved in the regulation of GC (Hurley *et al.*, 1993; Gray-Keller *et al.*, 1993). Instead, two other proteins have recently been reported to mediate Ca^{2+} regulation of GC (Hurley et al., 1994; Gorczyca et al., 1994). In view of the importance of the particulate GC for the phototransduction process in ROS, we have examined the possibility of regulation of ROS GC by phosphorylation. In this paper, we report that ATP increased the maximal GC rate in ROS that were purified with an intact plasma membrane and were only permeabilized with saponin or streptolysin S immediately prior to the GC assay. Selective inhibitors and reconstitution with purified kinases suggests strongly that phosphorylation by protein kinase C (PKC) mediates the observed ATP-dependent increase in GC. We have developed an optical assay to obtain real-time recordings of GC activity as low as 1 nmol of cGMP/(min·(mg of rhodopsin)). Real-time recordings enabled us to measure initial rates of GC activity and, for example, to measure its Ca^{2+} dependency with much greater precision. Our assay proved particularly useful in the case of nonlinear GC kinetics such as observed for the ATP-dependent increase in the GC rate. Our optical assay, unlike other GC assays, is not affected by the very high, light-stimulated cGMP phosphodiesterase activity endogenous to ROS and, hence, can be carried out in the light when applied to the study of ROS GC.

MATERIALS AND METHODS

Pyrophosphate reagent, GTP, ATP, and NADH were obtained from Sigma Chemical Co. (St. Louis, MO); GDP $\beta\gamma$-methylene, GDP-β-S, GTP-γ-S, ATP-$\beta\gamma$-methylene, and ATP-γ-S were obtained from Boehringer (Mannheim, Germany); Pefabloc SC was obtained from Pentapharm AG (Basel, Switzerland). Chelerythrine was obtained from Calbiochem (La Jolla, CA). The PKC inhibitory peptide (R19-31N) and the constitutively active PKC were generous gifts of Dr. M. P. Walsh and Dr. B. Allen (University of Calgary).

Purification of Bovine ROS. Bovine eyeballs were purchased from a local slaughterhouse, and the retinas were dissected as soon as possible. Ca^{2+}-depleted bovine ROS with an intact plasma membrane were purified from freshly

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[1] Abbreviations: ROS, rod outer segments; GC, guanylyl cyclase; PP_iase, inorganic pyrophosphatase BAPTA, 1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid; PKC, protein kinase C.

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dissected retinas as described before (Schnetkamp *et al.*, 1979; Schnetkamp, 1986) with an additional sucrose gradient purification step: 20−50% (w/w) sucrose, 20 mM Hepes (pH 7.4), 10 mM D-glucose, and 500 μM EDTA. Intact bovine ROS were stored at 4 °C as a concentrated suspension containing 200−300 μM rhodopsin in 600 mM sucrose, 5% (w/v) Ficoll 400, 100 μM EDTA, and 20 mM Hepes (the pH of this and all other solutions was adjusted to pH 7.4 with arginine).

Washed ROS for Reconstitution Experiments. Intact ROS (containing 2 μM rhodopsin) were incubated for 10 min at room temperature in 1 mL of washing medium (50 units/mL streptolysin S, 1 mM $MnCl_2$, 100 mM NaCl, 25 mM Hepes/Arg, pH 7.4) and sedimented in a table-top centrifuge (14 000 rpm). ROS were resuspended in the GC assay medium containing ATP, GTP, and kinases as indicated.

Principle of GC Assay. The GC assay measures the formation of PP_i (O'Brian, 1976) and is based on a commercially available kit (P 7275, Sigma Chemical Co., St. Louis, MO). The key enzyme, PP_i-dependent bacterial fructose-6-phosphate kinase (EC 2.7.1.90), uses fructose 6-phosphate as a substrate and couples PP_i formation to an enzymatic cascade involving the enzymes aldolase, glycerophosphate dehydrogenase, and triosephosphate isomerase and leads to the oxidation of 2 mol β-NADH/mol of PP_i formed. The bacterial PP_i-dependent fructose-6-phosphate kinase utilizes PP_i rather than GTP or ATP, whereas the mammalian ROS fructose-6-phosphate kinase uses GTP or ATP but not PP_i.

The PP_i-based assay was carried out in a volume of 2 mL in 400 μM BAPTA, 15 mM sodium fluoride, 75 mM sodium chloride, 1 mM citrate, 100 μM Pefabloc SC, and the PP_i detection kit [15 mM imidazole-HCl, pH 7.4, 0.03 mM EDTA, 0.066 mM Mn^{2+}, 0.006 mM Co^{2+}, 2 mM Mg^{2+}, 0.26 mM β-NADH, 4 mM fructose 6-phosphate, 1.0 mg of bovine serum albumin, 1.0 mg of sugar stabilizer, 0.33 units of PP_i-dependent fructose-6-phosphate kinase, 4.95 units of aldolase (EC 4.1.2.13), 3.33 units of glycerophosphate dehydrogenase (EC 1.1.1.8.), and 33.3 units of triosephosphate isomerase (EC 5.3.1.1.)].

Spectrophotometric Recordings. The optical recordings of β-NADH oxidation in the GC assay were performed in an SLM-Aminco DW2C dual-wavelength spectrophotometer in the dual-wavelength mode with the wavelength pair of 340/410 nm and slit width of 6 nm. The temperature (26 °C) was controlled with a circulating waterbath, and the suspension was mixed with a magnetic spinbar.

In order to establish the GTP-independent background, intact ROS containing 50−100 μg of rhodopsin were added to the cuvette, followed by the addition of saponin to a final concentration of 0.0025% to permeabilize the plasma membrane. Finally, GTP (500 μM) was added to initiate GTP-induced changes in the optical readings caused by the activity of the particulate GC.

Validation of GC Assay. ROS enzymatic activities other than GC can interfere in our assay in two ways. First, they can increase the background signal by utilizing the substrate present, and, second, they can compete with the cascade for the product of the reaction catalyzed by GC. In the first category, the mammalian fructose-6-phosphate kinase (EC 2.7.1.11) present in the ROS cytosol proved to be the main interfering enzyme. The bacterial PP_i-dependent fructose-6-phosphate kinase depends on PP_i as a high energy

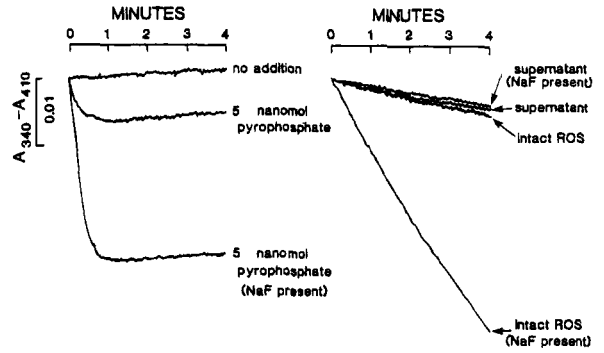

FIGURE 1: Fluoride inhibits inorganic pyrophosphatase present in ROS. Dual-wavelength recordings of the PP_i-based assay are shown that illustrate the effect of 15 mM sodium fluoride (as indicated) on the signal observed (1) upon addition of 5 nmol of PP_i to the cuvette at time zero (left panel) and (2) upon addition of 500 μM GTP at time zero (right panel). The standard PP_i-based assay medium contained saponin-permeabilized ROS at a final opsin concentration of 1.5 μM. "Intact ROS" indicates saponin-permeabilized ROS; "supernatant" indicates supernatant obtained after centrifugation of saponin-permeabilized ROS.

phosphate source and is not able to utilize GTP or ATP, whereas the mammalian cytosolic ROS fructose-6-phosphate kinase uses GTP or ATP but not PP_i. Both enzymes compete for the fructose 6-phosphate present in the assay medium. We used 1 mM citrate as a selective inhibitor of ROS fructose-6-phosphate kinase (the PP_i-dependent bacterial fructose-6-phosphate kinase was not inhibited by citrate) (data not illustrated). In the second category, the inorganic pyrophosphatase (PP_iase, EC 3.6.1.1; PP_iase catalyzes the reaction $PP_i \rightarrow 2P_i$) present in ROS (Hakki & Sitaramayya, 1990; Yang & Wensel, 1992) was an effective competitor with the PP_i-dependent fructose-6-phosphate kinase for PP_i produced by GC (Figure 1). We used 15 mM fluoride as a strong inhibitor of the ROS PP_iase (Yang & Wensel, 1992) and observed that inhibition of the ROS PP_iase caused a large increase in the amplitude of the signal caused by addition of 5 nmol of PP_i to the cuvette (Figure 1, left panel). Therefore, addition of fluoride is expected to cause a large and selective increase in the rate of a PP_i producing enzyme, i.e., GC. Figure 1 (right panel) shows that the rate of GTP-induced β-NADH oxidation was greatly stimulated by fluoride, but only for the particulate fraction of ROS. We believe that the above results present very strong evidence that the GTP-induced signals observed in the presence of both fluoride and citrate represent the particulate GC in ROS.

RESULTS

Under Materials and Methods we have described in detail a new optical assay for measuring GC activity. Briefly, the assay is based on an enzymatic cascade that links the initial product (PP_i) to the oxidation of two molecules of β-NADH. The assay is based on a commercially available kit to measure PP_i. We have used citrate and fluoride to inhibit other ROS enzyme activities that may feed the enzymatic cascades (i.e., GTP/ATP-dependent ROS phosphofructokinase) or may interfere with the product formed (i.e., degradation of PP_i by inorganic pyrophosphatase endogenous to ROS). Under our experimental conditions, other enzyme activities accounted for <5% of the maximal GC activity (Figure 1).

Inhibition of ROS GC by Ca^{2+}. The particulate GC from bovine ROS is inhibited by an increase in the free Ca^{2+}

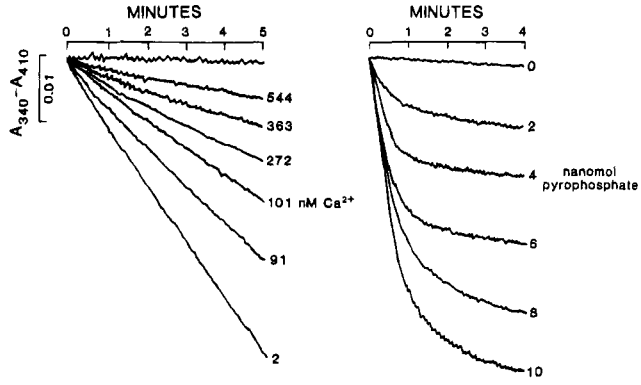

FIGURE 2: Inhibition of GC by Ca^{2+}. The inhibition of the GC activity by Ca^{2+} is illustrated by real-time dual-wavelength recordings (left-hand panel). GC activity was inititated at time zero by the addition of 500 μM GTP to a cuvette containing 2 mL of a suspension of saponin-permeabilized ROS (in the PP_i assay medium described under Materials and Methods) with 400 μM EGTA present (free Ca^{2+}, 2 nM). The GC activity was recorded for 5 min, CaEGTA was added to increase the free Ca^{2+} concentration to 91 nM, and the GC activity was again recorded for 5 minutes. Subsequent additions of CaEGTA increased free Ca^{2+} and the traces illustrated were recorded at the indicated free Ca^{2+} concentrations. Saponin-permeabilized ROS were present at a final opsin concentration of 2 μM. The free Ca^{2+} concentrations in the assay medium following the addition of different concentrations of CaEGTA were measured with the fluorescent Ca^{2+} indicator Fluo 3. Dual-wavelength recordings are shown of the calibration of the PP_i-based assay (right-hand panel). Traces were started at time zero by addition of the indicated amounts of PP_i. The standard assay media (see Material and Methods) was used including saponin-permeabilized ROS at a final opsin concentration of 1.5 μM (no GTP present).

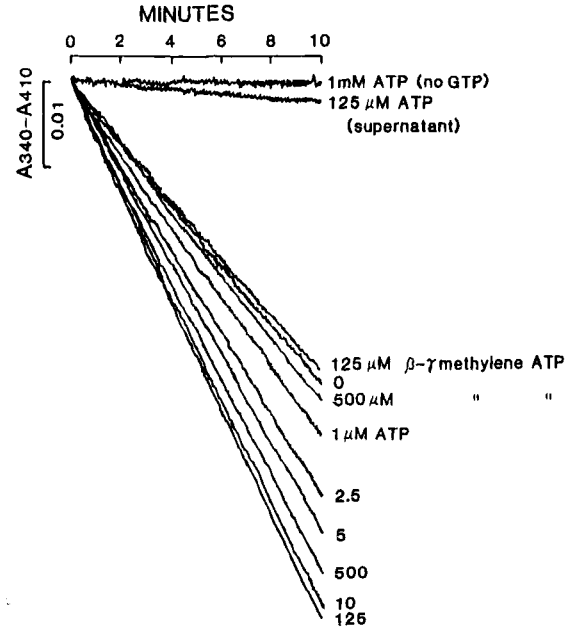

FIGURE 3: Stimulation of ROS GC by ATP. Dual-wavelength recordings are shown of GTP-induced GC activity in the presence of different concentrations of ATP and ATP$\beta\gamma$methylene. The standard assay media (see Material and Methods) was used including saponin-permeabilized ROS at a final opsin concentration of 1.5 μM. The GC rate was initiated by addition of 0.5 mM GTP (except in the trace labeled no GTP) and the indicated concentrations of ATP and ATP-β,γ-methylene. The linear GC rates that developed 15 min after addition of ATP and GTP are illustrated. In the trace labeled "supernatant" the ATP- and GTP-dependent PP_i production of the soluble fraction of saponin-permeabilized ROS is shown.

concentration in the assay medium (Dizhoor *et al.*, 1991; Koch & Stryer, 1988; Lambrecht & Koch, 1991) although the molecular mechanism underlying this inhibition is not fully understood. In order to test our GC assay, we examined the effect of submicromolar Ca^{2+} concentrations on the ROS particulate GC. Figure 2 illustrates the real-time recordings observed in one representative experiment. ROS GC activity was progressively inhibited as the free Ca^{2+} concentration in the assay medium was raised from 2 nM to 1 μM. The IC_{50} for Ca^{2+} inhibition of ROS GC in this experiment was 170 nM. The free Ca^{2+} concentrations of the assay mixtures were measured with the fluorescent Ca^{2+} indicator Fluo 3 as described in Schnetkamp *et al.*, (1991) with a K_d of 400 nM (Minta *et al.*, 1989). In the right-hand panel (Figure 2) a series of calibration traces is shown that represent additions of different amounts of PP_i. In five fresh ROS preparations the average IC_{50} for Ca^{2+} inhibition was 144 nM (SD 6), while in nine preparations of ROS that had been stored frozen an IC_{50} of 83 (SD 7) was observed. In 30 preparations of intact bovine ROS the particulate GC exhibited a GTP-dependent and Ca^{2+}-sensitive activity of 18.5 $\pm$ 5.6 (mean $\pm$ SD) nmol of PP_i/(min·(mg of rhodopsin)). If not stated otherwise, all experiments were performed with freshly purified ROS with an intact plasma membrane that was permeabilized with saponin immediately before use in the GC assay.

Modulation of GC by ATP: Maximal Rate. Our preparation of intact bovine ROS purified on Ficoll gradients has previously been shown to optimally retain certain cytosolic proteins such as rhodopsin kinase (Sitarayyama & Liebman, 1983). In order to examine the possible effect of phosphorylation on ROS CG activity, intact ROS were permeabilized in the presence of both ATP and GTP. The GC rate was initially not affected by the presence of ATP, but, after a brief lag period, the traces recorded in the presence of ATP showed a downward curvature indicating an increase in the GC rate. After 5−15 min a new linear rate was established, and its dependence on the ATP concentration is illustrated in Figure 3. The effect of ATP on GC rate became prominent between 1 and 10 μM ATP, whereas the nonhydrolyzable ATP analogues ATP-β,γ-methylene (illustrated in Figure 3) and β,γ-imidoadenosine 5′-triphosphate (not shown) did not increase the GC rate. ATP-γ-S increased the GC rate about half as effectively as ATP (both at 125 μM), although it took considerably more time for the increase in GC rate to develop (not illustrated). Higher concentrations of ATP caused inhibition of GC rate that, in part, could be countered by increasing the GTP concentration. For example, at 1 mM ATP (and 0.5 mM GTP) the GC rate was 68% of the optimal rate observed at 0.125 mM ATP (and 0.5 mM GTP); in the presence of 1 mM GTP and 1 mM ATP the GC rate was 83% of the optimal rate [note that ROS contain equimolar amounts of ATP and GTP (Schnetkamp, 1986, and references therein)]. Two control traces are also illustrated in Figure 3. First, addition of 1 mM ATP did not lead to any PP_i formation when no GTP was present. Second, the soluble fraction of saponin-permeabilized ROS (supernatant obtained after sedimentation of the particulate fraction) showed only a minor rate of PP_i production in the presence of 0.125 mM ATP and 0.5 mM GTP. In 14 different ROS preparations, application of an optimal dose of 125 μM ATP increased the GC rate to 197% $\pm$ 40% (mean $\pm$ SD).

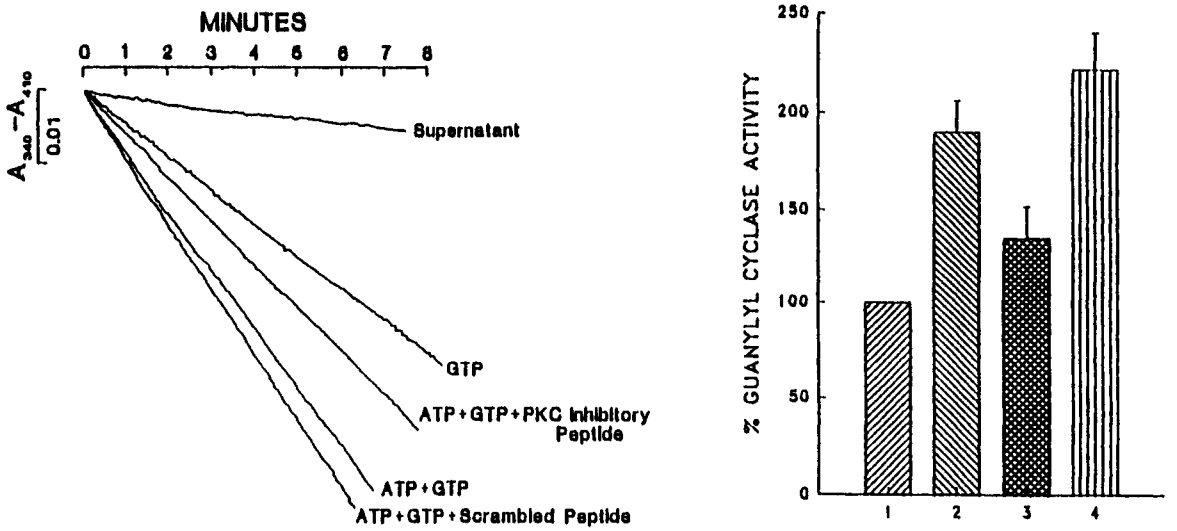

FIGURE 4: ATP-dependent increase in ROS GC rate and its inhibition by the PKC inhibitory peptide. (Left panel) Dual-wavelength recordings are shown of the inhibition of the ATP-dependent increase in ROS GC rate by the PKC inhibitory peptide (see text for details). GC assays were performed with the PP_i-based assay as described in Material and Methods with 1 mM citrate present. GC activities were measured in the presence of 500 μM GTP (all traces), 125 μM ATP (as indicated), and 900 nM PKC inhibitory peptide or scrambled peptide (as indicated). Saponin-permeabilized ROS (final opsin concentration 2 μM, or the supernatant after centrifugation of ROS in the trace labeled "supernatant") were preincubated for 15 min before the illustrated traces were started. (Right panel) (1) GC rate in the absence of ATP normalized to 100%; (2) GC rate in the presence of 125 μM ATP; (3) GC rate in the presence of 125 μM ATP and 900 nM PKC inhibitory peptide; (4) GC rate in the presence of 125 μM ATP and 900 nM scrambled peptide. The bars represent mean ± "sd of six experiments with six different ROS preparations.

The ATP concentration dependence of the above ATP stimulation of GC rate is consistent with the involvement of protein phosphorylation by a kinase. The presence of PKC in bovine ROS has been reported (Kelleher & Johnson, 1985; Wolbring & Cook, 1991), and PKC-mediated phosphorylation reactions have been reported to activate the enterotoxin-dependent GC from *Escherichia coli* (Crane *et al.*, 1993) and the soluble GC in PC12 cells (Claude *et al.*, 1993). The primary amino acid sequence of ROS GC shows 12 possible PKC phosphorylation sites (serine 122, 143, 177, 324, 441, 530, 535, 626, and 1010 and threonine 2267, 400, and 557) [for PKC consensus phosphorylation site motifs, see Hunter (1991)]. Therefore, we examined the possible involvement of PKC in mediating the observed ATP-dependent increase in ROS GC activity. We used two selective PKC inhibitors, the PKC pseudosubstrate-based peptide inhibitor PKC R19-31N (RFARKGALRQKNV) (Kemp *et al.*, 1991; House & Kemp, 1990) and the PKC inhibitor chelerythrine, a benzophenanthridine alkaloid (Herbert *et al.*, 1990; Ko *et al.*, 1990) (Figures 4 and 5). Both inhibitors are competitive toward the substrate and not toward ATP. Both inhibited the ATP-dependent increase in GC rate (Figures 4 and 5). Real-time recordings of the effect of the PKC inhibitory peptide on ATP-stimulated GC activity are illustrated in Figure 4 (left panel): 900 nM PKC inhibitory peptide inhibited the ATP-stimulated increase in GC rate, whereas a control peptide (same amino acid composition, but with a scrambled sequence KVQRRNGAKLRAF) had no effect or slightly increased GC rate. The PKC inhibitory peptide had no effect on GC activity in the absence of ATP and the ATP-dependent increase in the GC rate was exclusively associated with the particulate fraction of ROS (data not shown). Figure 4 illustrates the ATP-dependent increase in GC rate after a 15 min preincubation with ATP. Figure 4 (right panel) summarizes six experiments with different ROS preparations. Maximal inhibition of the ATP-dependent increase in GC rate was achieved with 900 nM PKC inhibitory peptide. We

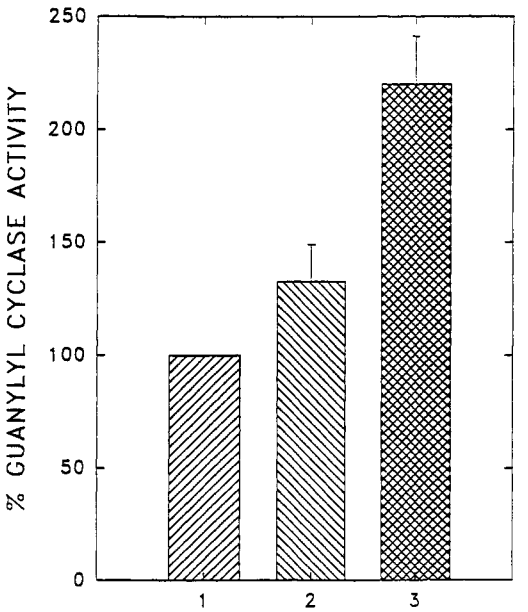

FIGURE 5: Inhibition of the ATP-dependent increase in ROS GC rate by chelerythrine. GC activity was measured in saponin-permeabilized ROS (final opsin concentration of 1−2 μM) with the PP_i-based assay as described in Material and Methods. (1) GC rate in the absence of ATP normalized to 100%; (2) GC rate in the presence of 10 μM chelerythrine and 125 μM ATP; (3) GC rate in the presence of 125 μM ATP. The bars represent mean ± SD of four independent experiments.

observed for the PKC inhibitory peptide an IC_{50} of 230 nM, which is in good agreement with values published by others [130 nM by House and Kemp (1990); 1 μM by Meier *et al.* (1991)]. The IC_{50} of the PKC inhibitory peptide toward other protein kinases is reported to be 1 mM for S6-kinase and myelin basic protein kinase (Meier *et al.*, 1991) and 450 μM for protein kinase A (House & Kemp, 1987). To support the notion that PKC mediated phosphorylation increases the ROS GC rate, we applied another selective PKC inhibitor,

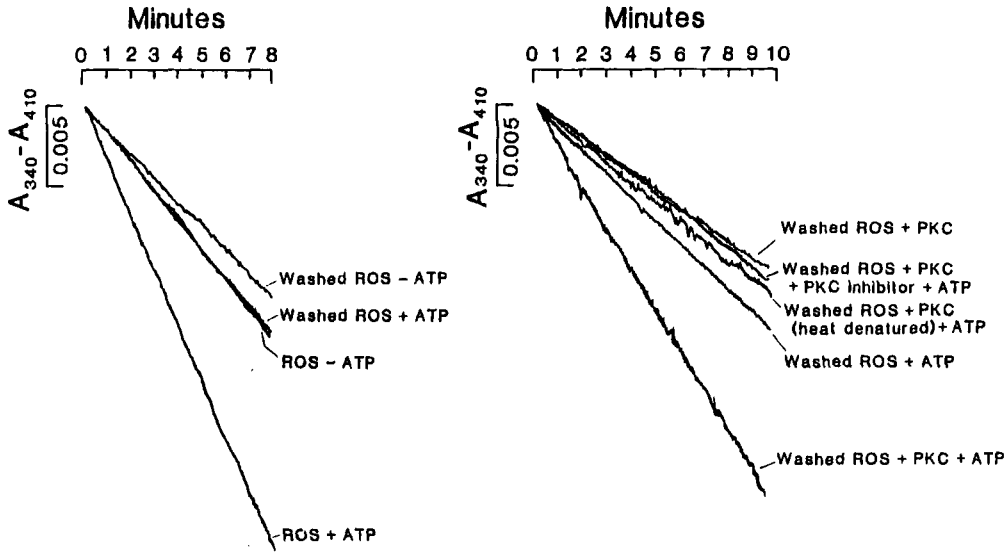

FIGURE 6: Reconstitution of the PKC-mediated, ATP-dependent increase in ROS GC rate. (Left panel) Dual-wavelength recordings are shown of the effect of ATP on the increase of ROS GC rate from unwashed and washed ROS. GC assays were performed with the PP_i-based assay as described in Material and Methods. GC activities were measured in the presence of 500 μM GTP (all traces) and 125 μM ATP (when indicated). Saponin-permeabilized ROS and washed saponin-permeabilized ROS (as described in the text) (final opsin concentration 2 μM) were preincubated for 15 min with or without ATP before the illustrated traces were started. Right panel: Dual-wavelength recordings are shown of the effect of preincubation with ATP and exogenous PKC on the GC rate observed in washed ROS. GC assays were performed with the PP_i-based assay as described in Material and Methods. Washed ROS were preincubated for 15 min in a volume of 20 μL containing 250 μM BAPTA, 10 mM $MgCl_2$, 125 μM ATP (when indicated), constitutively active PKC (Allen et al., 1994) (150 ng/mL) (active or heat-denatured when indicated), and 900 nM PKC pseudosubstrate-based peptide inhibitor PKC R19-31N (when indicated). Subsequently, preincubated ROS were diluted to 2 mL (final opsin concentration, 2 μM) with the GC assay medium containing 500 μM GTP, and the illustrated traces of GC activity were started.

chelerythrine (Figure 5). Maximal inhibition of the ATP-dependent increase in GC rate was achieved with 10 μM chelerythrine. This concentration is in good agreement with concentrations used by others [5 μM by Montero *et al.* (1993); 25 μM by Ko *et al.* (1990)].

Reconstitution of the ATP-Dependent Increase in GC Rate by External PKC. We sought to substantiate the involvement of PKC in mediating the ATP-dependent increase of GC rate observed in ROS by means of reconstitution experiments. Intact ROS were permeabilized with streptolysin S and incubated in the presence of 1 mM Mn^{2+} (see Materials and Methods). Subsequently, ROS were sedimented, the supernatant was removed, and ROS were preincubated with ATP and constitutively active PKC (Allen *et al.*, 1994). The left panel of Figure 6 illustrates that washed ROS had lost most of the factor that mediates the ATP-dependent increase in GC rate, while the ATP-independent and Ca^{2+}-sensitive GC was retained (GC rate was inhibited by Ca^{2+}, not illustrated). The right panel of Figure 6 illustrates that the ATP-dependent increase in GC rate was reestablished when washed ROS were preincubated with ATP and constitutively activated PKC. In control experiments, no increase in GC rate was observed when preincubations were carried out with ATP alone, PKC alone, or ATP plus heat-denatured PKC. As observed with PKC endogenous to ROS (Figure 4), the ATP-dependent increase in GC rate, which was mediated by added constitutively active PKC, was inhibited by the peptide inhibitor (Figure 6, right panel).

DISCUSSION

In this paper, we have described and validated a new optical assay to measure GC activity, and we have applied this assay to study the particulate GC of bovine retinal ROS. We have confirmed the well-established inhibition of ROS GC when the free Ca^{2+} concentration in the assay medium was increased in the submicromolar range (Figure 2). In addition, we report here a new modulator of the maximal GC velocity (ATP-dependent phosphorylation, Figures 3–6). Our GC assay offers a continuous real-time monitor of GC activity in contrast to single time points obtained after fixed time intervals in GC assays used in other studies. The latter require radiolabeled guanine nucleotides: one assay is based on the detection of cGMP via a commercially available radioimmunoassay, whereas other assays use the separation of nucleotides on either thin layer chromatography (Koch & Stryer, 1988; Dizhoor *et al.*, 1991) or high performance liquid chromatography columns (Lambrecht & Koch, 1991). To study ROS GC, these assays must be carried out in complete darkness or under infrared illumination due to the extremely high activity of the light-activated cGMP phosphodiesterase endogenous to ROS. In contrast, the optical enzyme-coupled assay developed in this study can be carried out either under normal room light or in darkness. We believe our new assay will be very helpful for further functional analysis of GC as it greatly improves the resolution, precision, and convenience of measurements of GC activity. For example, we recently used this assay to show that tyrphostins, a class of tyrosine kinase inhibitors, inhibit ROS GC as well other GTP-utilizing proteins (Wolbring *et al.*, 1994). In this study, we screened a large number of different tyrphostins in their ability to affect both GC activity and transducin-associated GTPase activity, which would have been impractical to do without the convenience of real-time, enzyme-coupled optical assays.

With the use of our assay, we observed a GTP-dependent and Ca^{2+}-sensitive GC activity of 13–23 nmol of cGMP/(min·(mg of rhodopsin)). This value is significantly higher when compared with values published elsewhere, 6–8 nmol

of cGMP/(min·(mg of rhodopsin)) (Dizhoor *et al.*, 1991) or 12−15 nmol of cGMP/(min·(mg of rhodopsin)) (Koch & Stryer, 1988; Lambrecht & Koch, 1991). We contribute our higher value to two factors. First, we measured initial rates of GC activity. Second, we used freshly isolated intact ROS that were solubilized by saponin immediately before use as opposed to ROS that had been stored frozen and subjected to hypotonic shocks. A value of 25 nmol of cGMP and PP_i/(min·(mg of rhodopsin)) at 26 °C is equivalent to a production of 40 μM cGMP/s on the assumption that the rhodopsin concentration in ROS is 3 mM (Rodieck, 1973). Such high GC activities are consistent with the observed cGMP flux of 67 μM cGMP/s in illuminated ROS in the isolated rabbit retina [as measured by ^{18}O incorporation into guanine nucleotides at 37 °C (Goldberg *et al.*, 1983; Ames *et al.*, 1986)].

We confirmed the inhibition of ROS GC by a rise in free Ca^{2+} in the submicromolar range with an IC_{50} for Ca^{2+} inhibition of 144 nM when freshly prepared intact ROS were used or of 83 nM when ROS were used that had been stored frozen. The above values can be compared with reported values of 240 nM (Dizhoor *et al.*, 1991), 90 nM (Koch & Stryer, 1988), or 80−185 nM [dependent on the Ca^{2+}-indicating dye used to calibrate the Ca^{2+} buffer solutions (Lambrecht & Koch, 1991)], all of which studies used ROS that had been stored frozen.

Regulation of ROS GC by PKC-Mediated Phosphorylation. In addition to the well-established inhibition by Ca^{2+}, we observed in this study that ATP increased the maximal velocity of the particulate GC in bovine ROS. We believe that the ATP-dependent stimulation of ROS GC is mediated by PKC-dependent phosphorylation on the basis of the following observations: (1) A nonhydrolyzable ATP analogue could not replace ATP (Figure 3). (2) Chelerythrine and the PKC pseudosubstrate-based peptide inhibitor PKC R19-31N inhibited the ATP-dependent increase in GC rate (Figures 4 and 5). (3) The observed ATP concentration dependence (EC_{50} between 1 and 10 μM, Figure 3) is consistent with the EC_{50}'s reported for different purified PKC isozymes (5−8 μM; Burns *et al.*, 1990). (4) The ATP-dependent increase in GC rate could be abolished by washing permeabilized ROS and, subsequently, could be reconstituted with externally added, constitutively active PKC (Figure 6).

Numerous reports have described the effect of ATP on the particulate, ANF, or CNP-receptor type GC (Duda *et al.*, 1993; Chinkers *et al.*, 1991; Marala *et al.*, 1991, 1992; Goraczniak *et al.*, 1992; Chang *et al.*, 1990; Kurose *et al.*, 1987; Song *et al.*, 1988; Jewett *et al.*, 1993). These authors found that 500 μM−1 mM ATP potentiated the peptide-dependent activation of the GC up to 10-fold. Although significant sequence similarity exists between one of the cloned photoreceptor GC's and the above members of the particulate GC family (Shyjan *et al.*, 1992), no functional similarity with respect to regulation by ATP appears to exist. In an earlier study on the Ca^{2+}-independent basal GC activity in ROS membranes, ATP was reported to inhibit this basal activity with an IC_{50} of 0.25 mM (Sitaramayya *et al.*, 1991), which may explain our observation that higher ATP concentrations (>0.2 mM) caused a slight inhibition of GC after the initial stimulation by low ATP. Gorczyca *et al.* (1994) observed that ATP stimulated the Ca^{2+}-sensitive GC in bovine ROS about 2-fold with some inhibition at higher ATP concentrations, very similar to our observations. Two significant differences between the two sets of observations should be noted. Gorczyca *et al.* (1994) report an EC_{50} of 150 μM for ATP, while a nonhydrolyzable ATP analogue could replace ATP. In contrast, in our study an EC_{50} of <10 μM for ATP was observed, while nonhydrolyzable ATP analogues could not replace ATP. As a result, Gorczyca *et al.* (1994) suggest that it is unlikely that a protein kinase is involved, whereas we believe that PKC-mediated phosphorylation is involved. Our conclusion is based on our observation that the ATP effect could be reconstituted by addition of exogenous, purified PKC to washed ROS (see above). Our experiments are carried out with Ca^{2+}-depleted ROS [intracellular free Ca^{2+} <10 nM (Schnetkamp *et al.*, 1991)], in an incubation medium with a free Ca^{2+} concentration of <10 nM and in the absence of externally added diacylglycerol. Although PKCα is the only isoform described so far for ROS (Wolbring & Cook, 1991; Kelleher & Johnson, 1985), our results are more suggestive for the involvement of a Ca^{2+}- and diacylglycerol-independent isoform of PKC. Experiments are in progress to elucidate the details of regulation of ROS GC by PKC. We speculate that regulation of GC may be important during the different stages of light adaptation. For example, down-regulation of the maximal GC rate by dephosphorylation may prevent a futile cycle of cGMP synthesis and immediate degradation by the phosphodiesterase when rods are saturated during continuous bright light for a prolonged period of time (e.g., bright daylight).

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