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*Articles*

# Modulation of the Calcium Sensitivity of Bovine Retinal Rod Outer Segment Guanylyl Cyclase by Sodium Ions and Protein Kinase A[†]

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ABSTRACT: Guanylyl cyclases (GC, EC 4.6.1.2) serve as receptors that produce cGMP in response to ligand binding. The production of cGMP is essential for the ability of retinal photoreceptor cells to restore the dark state after photoexcitation. GC activity is enhanced in rod outer segments (ROS) by a decrease in the cytosolic free $Ca^{2+}$ concentration. We recently developed a new real-time assay to measure initial rates of ROS GC activity with much improved precision [Wolbring, G. & P. P. M. Schnetkamp (1995) *Biochemistry 34*, 4689−4695]. With this assay we examined the $Ca^{2+}$ sensitivity of ROS GC, and we report here that protein kinase A-mediated phosphorylation and $Na^+$ cause significant shifts in the $IC_{50}$ for $Ca^{2+}$ of the particulate guanylyl cyclase from bovine retinal rod outer segments. The $IC_{50}$ for $Ca^{2+}$ ranged between 30 and 270 nM $Ca^{2+}$ dependent on the presence of $Na^+$, choline, cAMP, cGMP, 8-bromo-cAMP, 8-bromo-cGMP, or the catalytic subunit of protein kinase A.

The phototransduction cascade in the outer segments of retinal rod photoreceptors (ROS)[1] results in the increased hydrolysis of cGMP and subsequent closure of cGMP-gated channels. Resynthesis of cGMP results in the reopening of cGMP-gated channels and is essential for the recovery of photoreceptors to the dark state. Guanylyl cyclases (GC) serve as receptors that produce cGMP in response to ligand binding. Members of the GC family are currently grouped into those found in the particulate fraction of tissue homogenates and stimulated by extracellular peptides, and those found in the soluble fraction and stimulated by nitric oxide (Garbers & Lowe, 1994). GC has been purified from the particulate fraction of bovine ROS as a 112 kDa (Koch, 1991) or a 60 kDa protein (Horio & Murad, 1991), and from frog ROS as a 115 kDa protein (Hayashi & Yamazaki, 1991). Five different clones of members of the particulate GC family have been isolated from retinal cDNA libraries (Shyjan et al., 1992; Ahmad & Barnstable, 1993; Duda et al., 1993; Lowe et al., 1995; Goraczniak et al., 1994), each of which has been reported to be expressed in photoreceptors, although the exact localization to rod or cone outer segments or to other parts of photoreceptor cells has not been determined yet. A soluble factor is required for full ROS GC activity and confers $Ca^{2+}$ sensitivity to it (Koch & Stryer, 1988; Dizhoor et al., 1991; Lambrecht & Koch, 1991a). GCAP is a small soluble protein purified from bovine ROS which stimulates basal ROS GC activity in a $Ca^{2+}$-sensitive fashion (Gorczyca et al., 1994; Palczewski et al., 1994), while a different soluble protein (p24; Dizhoor et al., 1994) has been purified from bovine retinas which acts in very similar

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[1] Abbreviations: ROS, rod outer segments; GC, guanylyl cyclase; $PP_i$ase, inorganic pyrophosphatase; BAPTA, 1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKA, cAMP-dependent protein kinase.

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fashion as GCAP. The latter protein has also been shown to activate two of the above retinal GC clones when heterologously expressed in cell lines, and this activation was abolished by submicromolar $Ca^{2+}$ concentrations (Lowe et al., 1995) as observed in ROS. Subsequently, GCAP and p24 were shown to be structural homologs, and they are now referred to as GCAP1 and GCAP2, respectively (Gorczyca et al., 1995). A third small soluble factor was recently purified from bovine retinas, which stimulates basal ROS GC activity, but this time in the presence of $Ca^{2+}$ (Pozdnyakov et al., 1995) rather than in the absence of $Ca^{2+}$ as observed for GCAP and p24. The above findings may suggest a hitherto unappreciated complexity of total ROS GC activity. Most previous biochemical studies have focused on reconstitution of the $Ca^{2+}$ sensitivity of ROS GC from washed ROS membranes and purified soluble factors. ROS GC is probably the least understood major component of the visual transduction cascade, in large part due to the absence of a simple and precise GC assay. We recently developed an optical and nonradioactive assay to measure GC activity in real-time recordings in intact ROS with a full complement of putative regulatory factors (Wolbring & Schnetkamp, 1995). Real-time recordings enable us to measure initial rates of GC activity and their $Ca^{2+}$ dependency with much improved precision. We used our GC assay to describe regulation of the maximal activity of ROS GC by endogenous protein kinase C (Wolbring & Schnetkamp, 1995). The $Ca^{2+}$ sensitivity of ROS GC activity plays a central role in a negative feedback loop: light-induced lowering of cytosol free $Ca^{2+}$ results in increased GC activity and accelerates the recovery to the dark state. This feedback loop is thought to mediate, at least in part, the process of light adaptation (Matthews et al., 1988; Nakatani & Yau, 1988). cGMP and sodium ions are two other constituents in the ROS cytosol whose concentration decreases upon illumination. ROS contain cAMP-dependent protein kinase A (PKA), and cGMP can activate PKA at the high concentrations known to exist in ROS in darkness (Walter, 1984). We report here that $Na^+$ and cyclic nucleotides can modulate the $Ca^{2+}$ sensitivity of ROS GC activity. The $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC can be modulated in a range between 30 and 270 nM by $Na^+$ and by PKA-mediated phosphorylation, whereas these conditions did not alter the maximal rate of ROS GC.

## MATERIALS AND METHODS

Pyrophosphate reagent, GTP, ATP, cGMP, cAMP, 8-bromo-cAMP, 8-bromo-cGMP, and the catalytic subunit of PKA were obtained from Sigma (St. Louis, MO); Pefabloc SC was obtained from Pentapharm AG, Basel, Switzerland, while PKI (5−24-amide) was obtained from Peninsula.

*Preparation of Bovine ROS.* Bovine eyeballs were purchased from a local slaughterhouse, and the retinas were dissected as soon as possible. $Ca^{2+}$-depleted intact bovine ROS were purified from freshly dissected retinas as described before (Schnetkamp et al., 1979; Schnetkamp, 1986) with an additional sucrose gradient purification step (20−50% (w/v) sucrose, 20 mM Hepes/arginine, pH 7.4, 10 mM D-glucose, 500 $\mu$M EDTA). Intact bovine ROS were stored at 4°C as a concentrated suspension containing 200−300 $\mu$M rhodopsin in 600 mM sucrose, 5% (w/v) Ficoll 400, 100 $\mu$M EDTA, and 20 mM Hepes/arginine, pH 7.4 (the pH of this and all other solutions was adjusted to 7.4 with arginine).

*Principle of GC Assays.* The GC assay measures the formation of $PP_i$ (O'Brien, 1976) and is based on a commercially available kit (P 7275, Sigma Chemical Co., St. Louis, MO). The key enzyme is $PP_i$-dependent bacterial fructose-6-phosphate kinase (EC 2.7.1.90), which utilizes $PP_i$ rather than GTP or ATP as an energy source; it 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 of $\beta$-NADH/mol of $PP_i$ formed. 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 (fructose 6-phosphate) present, and second, they can compete with the cascade for the product of the reaction catalyzed by GC ($PP_i$). 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 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) (Wolbring & Schnetkamp, 1995). In the second category, the inorganic pyrophosphatase ($PP_i$ase, EC 3.6.1.1; $PP_i$ase 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 (Wolbring & Schnetkamp, 1995). We used 30 mM fluoride to fully inhibit $PP_i$ase endogenous to ROS (Yang & Wensel, 1992).

The $PP_i$-based GC assay was carried out in a volume of 2 mL in the following solution: 75 mM NaCl, 4 mM $MgCl_2$, 30 mM NaF, 400 $\mu$M BAPTA, 1 mM trisodium citrate, 100 $\mu$M Pefabloc SC, 125 $\mu$M ATP, and the $PP_i$ detection kit containing (final concentrations) 13.5 mM imidazole hydrochloride, pH 7.4, 0.03 mM EDTA, 0.06 mM $Mn^{2+}$, 0.006 mM $Co^{2+}$, 0.6 mM $Mg^{2+}$, 0.24 mM $\beta$-NADH, 3.6 mM fructose 6-phosphate, 1.5 mg of bovine serum albumin, 1.5 mg of sugar stabilizer, 0.15 units of $PP_i$-dependent fructose-6-phosphate kinase, 2.25 units of aldolase (EC 4.1.2.13), 1.5 units of glycerophosphate dehydrogenase (EC 1.1.1.8), and 15 units of triosephosphate isomerase (EC 5.3.1.1). In some experiments NaF was replaced by KF and NaCl was substituted with choline chloride or KCl. Earlier we observed that addition of ATP leads to a higher maximal GC velocity, a process we attributed to PKC-mediated phosphorylation. Hence, in this study ATP was always included in the incubation media to maximize GC activity (Wolbring & Schnetkamp, 1995).

*Spectrophotometric Recordings.* The optical recordings of $\beta$-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 water bath, and the suspension was stirred with a magnetic spinbar.

## RESULTS

*$Ca^{2+}$ Sensitivity of ROS GC.* GC activity from bovine ROS is inhibited by an increase in the free $Ca^{2+}$ concentration

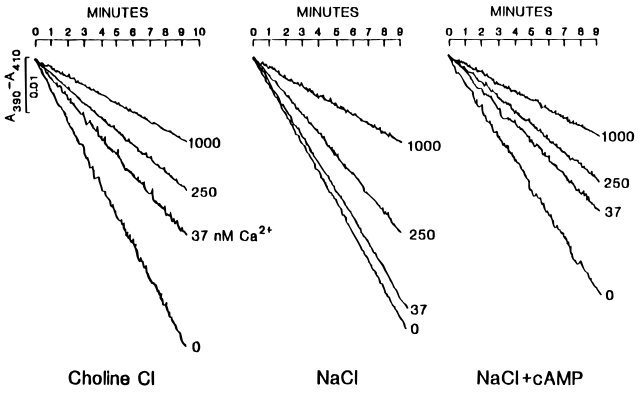


FIGURE 1: Effect of $Na^+$ and 8BrcAMP on the $Ca^{2+}$ sensitivity of ROS GC. The inhibition of the GC activity by $Ca^{2+}$ was examined with the $PP_i$-based assay as described in Wolbring and Schnetkamp (1995) and in Materials and Methods with the following modifications. In the left panel, 30 mM NaF and 150 mM choline chloride were present. In the middle panel, 150 mM NaCl and 30 mM NaF were present, and in the right panel, 150 mM NaCl, 30 mM NaF, and 2.5 $\mu$M 8-bromo-cAMP were present. Real-time traces of dual-wavelength recordings are shown with the wavelemgth pair of 340 and 410 nm. The first trace was started at time zero by the addition of 500 $\mu$M GTP to the cuvette in the presence of 400 $\mu$M BAPTA (zero free $Ca^{2+}$). The GC activity was monitored for 10 min, CaBAPTA was added to increase the free $Ca^{2+}$, and the GC activity was again followed for 10 min. Subsequent additions of CaBAPTA further increased free $Ca^{2+}$ and resulted in the illustrated traces. A typical experiment of a $Ca^{2+}$ dependency covered the following free $Ca^{2+}$ concentrations: 0, 12.5, 25, 37.5, 50, 75, 125, 250, 500, and 1000 nM. Traces representing the four indicated $Ca^{2+}$ concentrations are shown here. Saponin-permeabilized ROS were present at a final opsin concentration of 2 $\mu$M. Maximal GC activity at zero $Ca^{2+}$ amounted to 19 nmol of cGMP/(min·mg of rhodopsin). The free $Ca^{2+}$ concentrations of the assay media 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).

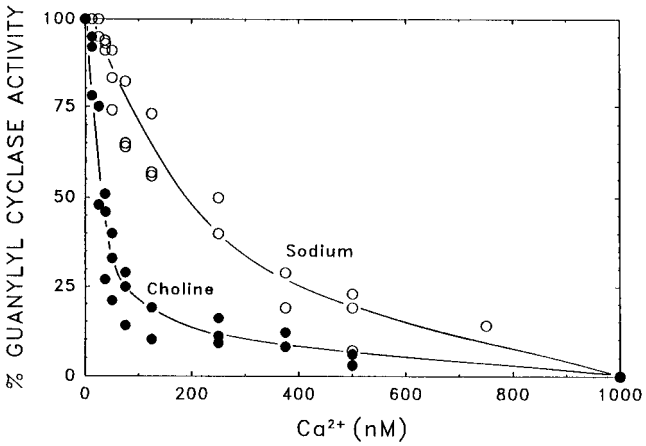


FIGURE 2: Decrease of the ROS GC $Ca^{2+}$ sensitivity by $Na^+$. Results of 3 separate experiments with different ROS preparations are shown. The incubation medium contained either 150 mM NaCl (open circles) or 150 mM choline chloride (filled circles) in addition to the components of the $PP_i$ assay kit (see Materials and Methods). The $Ca^{2+}$ dependency of GC activity was obtained as described in Figure 1. Saponin-permeabilized ROS were present at a final opsin concentration of 1.5−2 $\mu$M. Data from 3 experiments with different ROS preparations were used with maximal GC activities at zero $Ca^{2+}$ of 19, 18.8, and 20.3 nmol of cGMP/(min·mg of rhodopsin), respectively.

(Koch & Stryer, 1988; Dizhoor et al., 1991; Lambrecht & Koch, 1991a). The values reported for the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC range from 80−185 nM (Koch & Stryer, 1988; Lambrecht & Koch, 1991a), 80−150 nM (Wolbring & Schnetkamp, 1995), and 240 nM (Dizhoor et al., 1991), to as high as 400−500 nM (Gorczyca et al., 1994). We investigated the $Ca^{2+}$ sensitivity of ROS GC in detail and observed a pattern of systematic shifts in $Ca^{2+}$ sensitivity dependent on the composition of the incubation medium. Figure 1 illustrates the two key findings of this study with real-time traces of ROS GC activity observed at four different $Ca^{2+}$ concentrations: replacing choline chloride (Figure 1, left panel) or KCl (not shown) with NaCl (Figure 1, middle panel) in the incubation medium led to a marked increase in the $Ca^{2+}$ concentration required to inhibit ROS GC activity (e.g., compare the GC traces observed at 37 and 250 nM free calcium, respectively). In our experiments we used the $Ca^{2+}$ buffer BAPTA to stabilize free $Ca^{2+}$ concentrations, and the apparent $Ca^{2+}$ dissociation constant for BAPTA was very similar whether choline, potassium, or sodium was the major cation in the medium [as judged from $Ca^{2+}$ titration curves monitored with the fluorescent $Ca^{2+}$-indicating dye Fluo-3 (Minta et al., 1988)]. Addition of 8-bromo-cAMP to the NaCl medium (Figure 1, right panel) led to an increase in the $Ca^{2+}$ sensitivity of ROS GC activity comparable to that observed in choline medium, while 8-bromo-cAMP addition to the choline or potassium medium was without effect (not illustrated). The $PP_i$ assay was not affected by the addition of 8-bromo-cAMP in the above media as judged by the identical responses to a test pulse of 10 nmol of $PP_i$ (not illustrated). Based on the above observations, we suggest that NaCl causes an increase in the $IC_{50}$ observed for $Ca^{2+}$ inhibition of ROS GC activity and that cAMP can reverse this effect. The remainder of this paper describes experiments to corroborate these suggestions and to define the molecular mechanism by which cAMP acts.

*Effect of Sodium Ions.* Figure 2 illustrates a more extensive analysis of the effect of $Na^+$ on the $Ca^{2+}$ sensitivity of ROS GC activity. The $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC was increased from 20−30 nM (observed in choline medium) to a value around 200 nM in $Na^+$ medium. In the experiments illustrated in Figures 1 and 2, 30 mM NaF was included in the incubation medium in order to inhibit ROS $PP_i$ase (see Materials and Methods). In order to evaluate the $Na^+$ dependence more precisely, NaF was substituted with KF in the experiment shown in Figure 3. The $IC_{50}$ of $Ca^{2+}$ inhibition of ROS GC activity as a function of the $Na^+$ concentration is illustrated in Figure 3: the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC increased from 25 to 260 nM when the $Na^+$ concentration was raised from 0 to 200 mM. The increase in $IC_{50}$ showed a sigmoidal dependence on the $Na^+$ concentration, suggesting the involvement of more than one sodium binding site.

Inhibition of maximal ROS GC activity by NaCl ($IC_{50}$ 120 mM) has been decribed earlier (Gorczyca et al., 1994). Under our experimental conditions, we observed half-maximal inhibition of ROS GC activity at a concentration of 300 mM NaCl, and this effect was quite distinct from the $Na^+$-induced shifts in the $Ca^{2+}$ sensitivity of ROS GC described above. Figure 3 again illustrates that 8-bromo-cAMP can reverse the effect of $Na^+$ on the $IC_{50}$ of $Ca^{2+}$ inhibition of ROS GC. An increase of $Na^+$ from 0 to 200 mM in the presence of 2.5 $\mu$M 8-bromo-cAMP led only to an increase in the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC from 25 to 95 nM, much smaller when compared with the shift from 25 to 260 nM observed in the absence of 8-bromo-cAMP.

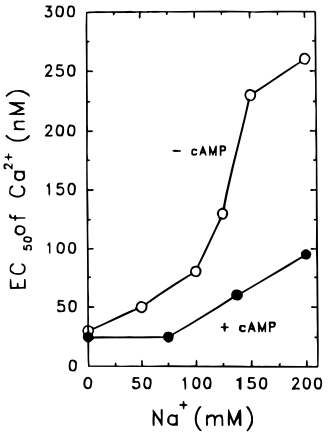


FIGURE 3: $Na^+$-dependent increase of the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC. The $IC_{50}$ of $Ca^{2+}$ inhibition of ROS GC activity was measured as a function of the $Na^+$ concentrations in the incubation medium in the presence (filled circles) or in the absence (open circles) of 2.5 $\mu$M 8-bromo-cAMP. The $PP_i$ assay was used as described in the Materials and Methods section, and the $Ca^{2+}$ dependency of GC activity was obtained as described in Figure 1. Saponin-permeabilized ROS were present at a final opsin concentration of 2 $\mu$M. Maximal GC activity at zero $Ca^{2+}$ amounted to 21.3 nmol of cGMP/(min·mg of rhodopsin).

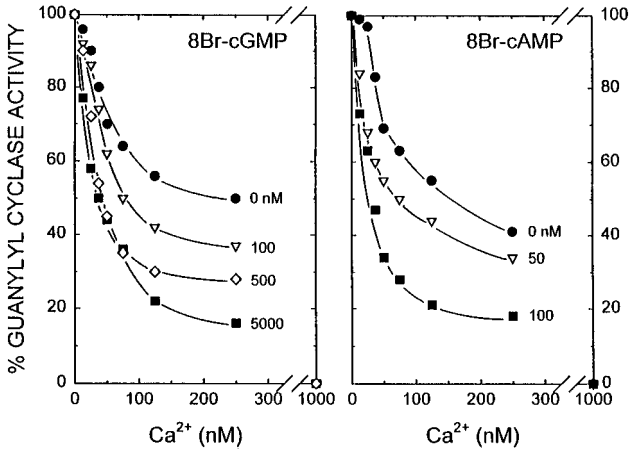


FIGURE 4: Effect of cyclic nucleotides on the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC. $Ca^{2+}$ sensitivity of ROS GC activity was measured as a function of the concentration of 8-bromo-cAMP or 8-bromo-cGMP as indicated. The incubation medium contained 180 mM NaCl in addition to the components of the $PP_i$ assay kit (as detailed under Materials and Methods). Saponin-permeabilized ROS were present at a final opsin concentration of 2 $\mu$M. Maximal GC activity at zero $Ca^{2+}$ amounted to 16.9 nmol of cGMP/(min·mg of rhodopsin).

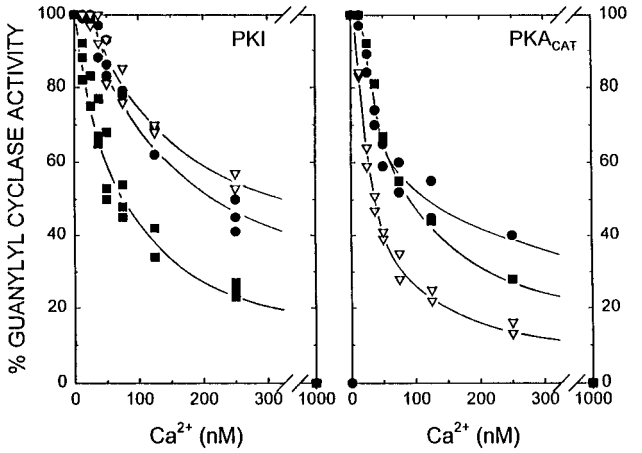


FIGURE 5: Effect of 8BrcAMP, PKI, and $PKA_{cat}$ on the $Ca^{2+}$ sensitivity of ROS GC. In the left panel, the effect of PKI on the 8-bromo-cAMP-induced shift in $Ca^{2+}$ sensitivity of GC is illustrated. Results of 3 separate experiments with different ROS preparations are shown. The incubation medium contained in addition to the components of the $PP_i$ assay kit (as detailed under Materials and Methods) 180 mM $Na^+$ (closed circles); 180 mM $Na^+$ and 2.5 $\mu$M 8-bromo-cAMP (filled squares); and 180 mM $Na^+$, 2.5 $\mu$M 8-bromo-cAMP, and 3 $\mu$M PKI (inverted triangles). Saponin-permeabilized ROS were present at a final opsin concentration of 1.5−2 $\mu$M. Maximal GC activities at zero $Ca^{2+}$ amounted to 17.4, 20, and 18.2 nmol of cGMP/(min·mg of rhodopsin), respectively. The effect of PKI on the $PKA_{cat}$-induced shift in $Ca^{2+}$ sensitivity of GC is illustrated is shown in the right panel. Results of 2 separate experiments with different ROS preparations are shown. ROS were preincubated for 10 min with a medium containing in addition to the components of the $PP_i$ assay kit (as detailed under Materials and Methods) 180 mM $Na^+$ (filled circles); 180 mM $Na^+$ and 1 unit of $PKA_{cat}$ from bovine heart (inverted triangles); and 180 mM $Na^+$, 1 unit of $PKA_{cat}$ from bovine heart, and 3 $\mu$M PKI (filled squares). Saponin-permeabilized ROS were present at a final opsin concentration of 1.5−2 $\mu$M.

*Effect of cAMP and cGMP.* Bovine ROS have been reported to contain PKA, but not a cGMP-dependent protein kinase (Walter, 1984). cAMP and cGMP can activate PKA (Walter, 1984), suggesting that the micromolar levels of cGMP that exist in ROS in darkness are sufficient to activate PKA in ROS. We approached the question whether PKA is involved in mediating the effect of 8-bromo-cAMP on the $Ca^{2+}$sensitivity of ROS GC activity with three tools. (A) We compared cGMP with cAMP (in both cases we used the 8-bromo derivatives to avoid hydrolysis by the cyclic nucleotide-dependent phosphodiesterase present in ROS); (B) we used the PKA peptide inhibitor PKI (Cheng et al., 1986); and (C) we used the purified catalytic subunit of PKA which is active in the absence of cyclic nucleotides.

First, we tested the involvement of PKA by examining the ability of submicromolar concentrations of 8-bromo-cAMP or 8-bromo-cGMP to cause shifts in $Ca^{2+}$ sensitivity of ROS GC. We measured the $Ca^{2+}$ sensitivity of ROS GC activity at a constant concentration of 180 mM NaCl in the presence of different concentrations of 8-bromo-cGMP (Figure 4, left panel) or 8-bromo-cAMP (Figure 4, right panel). We observed an increase in the $Ca^{2+}$ sensitivity of ROS GC as the concentration of 8-bromo-cAMP increased from 0 to 100 nM (Figure 4, right panel), while a further increase to 2.5 $\mu$M 8-bromo-cAMP did not cause any further increase in the $Ca^{2+}$ sensitivity of ROS GC (data not shown). We observed similar changes in the $Ca^{2+}$ sensitivity of ROS GC when the concentration of 8-bromo-cGMP was increased from 0 to 5 $\mu$M (Figure 4, left panel). In all our experiments, 8-bromo-cAMP acted at concentrations 2- to 5-fold lower than 8-bromo-cGMP. No effect of 8-bromo-cAMP or 8-bromo-cGMP was detected on the maximal ROS GC rate (data not shown).

In the second test to corroborate the involvement of PKA in modulating the $Ca^{2+}$ sensitivity of ROS GC, we examined the ability of the PKA peptide inhibitor PKI to prevent the shift in $Ca^{2+}$ sensitivity observed with 8-bromo-cAMP. Figure 5 (left panel) combines data of three experiments with different ROS preparations: the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC was 230−250 nM in the presence of 180 mM $Na^+$ (filled circles). Addition of 8-bromo-cAMP caused a shift to 50−70 nM (filled squares), whereas addition of 8-bromo-cAMP in the presence of 3 $\mu$M PKI resulted in a $Ca^{2+}$ sensitivity very similar to that observed in the absence of 8-bromo-cAMP (inverted triangles). Thus, the PKA peptide

inhibitor PKI is able to counteract the effect of 8-bromo-cAMP, and this is another strong indication that PKA is involved in the regulation of the $Ca^{2+}$ sensitivity of ROS GC activity observed here.

In the third test for PKA-mediated shifts in the $Ca^{2+}$ sensitivity of ROS GC, we looked for the ability of the catalytic subunit of PKA to change the $Ca^{2+}$ sensitivity of ROS GC in the absence of cyclic nucleotides. PKA consists of a cAMP binding regulatory subunit and a catalytic subunit; binding of cAMP to the regulatory subunit causes it to dissociate from the catalytic subunit, which is then active and catalyzes the phosphorylation of proteins. We preincubated saponin-permeabilized ROS with 1 unit of the commercially available catalytic subunit of PKA from heart ($PKA_{cat}$) and subsequently measured the calcium sensitivity of ROS GC. Preincubation with $PKA_{cat}$ mimicked the effect of preincubation with 8-bromo-cAMP and caused a shift in the calcium sensitivity of ROS GC. Figure 5 (right panel) combines data of two experiments with different ROS preparations: the $IC_{50}$ for $Ca^{2+}$ inhibition of ROS GC was 140−160 nM in the absence of $PKA_{cat}$ or cyclic nucleotides (filled circles). Preincubation with $PKA_{cat}$ caused a shift to 30−40 nM (inverted triangles), whereas preincubation with $PKA_{cat}$ in the presence of 3 $\mu$M PKI resulted in a $Ca^{2+}$ sensitivity very similar to that observed in the absence of $PKA_{cat}$ (filled squares). Thus, $PKA_{cat}$ caused very similar shifts in $Ca^{2+}$ sensitivity of ROS GC as observed with cyclic nucleotides, and the peptide inhibitor PKI counteracted the effect of $PKA_{cat}$.

## DISCUSSION

Inhibition of ROS GC by free $Ca^{2+}$ in the submicromolar range is perhaps the physiologically most important characteristic of this key enzyme in the visual transduction cascade of retinal rod and cone photoreceptors (Koch & Stryer, 1988; Dizhoor et al., 1991; Lambrecht & Koch, 1991b), since it mediates the negative feedback loop initiated by the light-induced lowering of cytosolic free $Ca^{2+}$. The increase in GC activity upon lowering $Ca^{2+}$ is thought to be an important mechanism that underlies the process of light adaptation (Matthews et al., 1988; Nakatani & Yau, 1988). $Ca^{2+}$ sensitivity of total ROS GC activity is mediated by small soluble proteins, and three such proteins have been reported: GCAP and p24 increase GC activity when added to washed ROS membranes, and this increase in GC activity is inhibited by $Ca^{2+}$ (Palczewski et al., 1994; Dizhoor et al., 1994), while another soluble factor increases GC activity in the presence of $Ca^{2+}$ (Pozdnyakov et al., 1995). Light not only causes a lowering of cytosolic free $Ca^{2+}$, but also of cytosolic cGMP and $Na^+$ concentrations. In this study we report results which suggest that both cGMP and $Na^+$ may participate as well in feedback loops that regulate total ROS GC activity; more specifically, both cGMP and $Na^+$ concentration were found to modulate the $Ca^{2+}$ sensitivity of ROS GC without affecting maximal GC activity. In contrast, we reported earlier that PKC-mediated phosphorylation increased the maximal rate of total ROS GC activity (Wolbring & Schnetkamp, 1995). Five different members of the particulate GC family have been cloned from retinal cDNA libraries and are thought to be expressed in photoreceptors (Ahmad & Barnstable, 1993; Duda et al., 1993; Shyjan et al., 1992; Chang et al., 1990; Goraczniak et al., 1994). Therefore, the possibility must be considered that different regulatory features may reflect the properties of different GC's and/or those of different $Ca^{2+}$-sensitive and GC-activating proteins. Most previous studies have focused on reconstitution of $Ca^{2+}$-sensitive GC activity from washed ROS membranes and purified soluble factors. It should be emphasized that our studies used bovine ROS with an intact plasma membrane and a full complement of putative regulatory proteins and solutes. To examine the $Ca^{2+}$ sensitivity of total ROS GC activity in detail, we also used a new optical and light-insensitive GC assay, which permits real-time registrations of GC activity with much improved precision (Wolbring & Schnetkamp, 1995).

*Effect of $Na^+$ on the $Ca^{2+}$ Sensitivity of ROS GC.* Sodium ions play an essential role in two aspects of the vertebrate phototransduction process. First, $Na^+$ carries most of the depolarizing inward current via the cGMP-gated and light-sensitive channels in the ROS plasma membrane (Yau & Baylor, 1989). Second, $Na^+$ is essential for regulating cytosolic free $Ca^{2+}$ in ROS via the Na-Ca/K exchanger in the plasma membrane (Schnetkamp, 1989). Light is likely to cause significant changes in cytosolic $Na^+$ concentration by attenuating the persistent influx of $Na^+$ via the cGMP-gated channels, although no values have been published for the $Na^+$ concentration in mammalian ROS, either in darkness or in bright light. Our results suggest that a light-induced lowering in cytosolic $Na^+$ concentration may be used to fine-tune the $Ca^{2+}$ sensitivity of ROS GC. Prolonged bright light is likely to lower cytosolic $Na^+$ in ROS to levels observed in most other mammalian cells, i.e., 5−8 mM. Under these conditions, we observed that ROS GC activity was most sensitive to $Ca^{2+}$, with an $IC_{50}$ of 30 nM (Figures 1−3). This may indicate that ROS GC activity will be inhibited at low $Ca^{2+}$ when cytosolic $Na^+$ is lowered in continued bright light. As a result, a futile cycle of continued cGMP synthesis and immediate hydrolysis by the light-activated phosphodiesterase may be prevented.

*Role of PKA in Regulating the $Ca^{2+}$ Sensitivity of ROS GC.* The presence of cAMP-dependent protein kinase (PKA) in ROS is well-established while no cGMP-dependent protein kinase has been found (Walter, 1984). Dark levels of cGMP in ROS are sufficient to activate PKA, and it has been shown that PKA can phosphorylate ROS proteins such as phosducin; phosducin is phosphorylated by PKA in the dark whereas dephosphorylation of phosducin is observed in the light (Lee et al., 1984). In our study, we observed that both 8-bromo-cGMP and 8-bromo-cAMP could reverse the $Na^+$-induced shift in $Ca^{2+}$ sensitivity of ROS GC (Figures 1−3). Three lines of evidence suggest that the effect of 8-bromo-cAMP or 8-bromo-cGMP is mediated by PKA-dependent phosphorylation: (1) 8-bromo-cAMP was 5-fold more potent compared with 8-bromo-cGMP (Figure 4). (2) The catalytic subunit of PKA ($PKA_{cat}$) could replace 8-bromo-cAMP and cause a shift in $Ca^{2+}$ sensitivity of ROS GC (Figure 5, right panel). (3) The PKA peptide inhibitor PKI prevented the effect of 8-bromo-cAMP (Figure 5, left panel) and the effect of $PKA_{cat}$ (Figure 5, right panel) when present at a concentration of 3 $\mu$M, at which PKI is reported to be a selective inhibitor of PKA (Cheng et al., 1986). Our experiments reported here do not directly demonstrate PKA-mediated phosphorylation of target proteins in ROS. ROS GC and GCAP1 would be prime candidates as substrates for PKA-mediated phosphorylation, although other components may be involved as well. The predicted primary amino

acid sequences of the various retinal GC clones contain many possible PKA phosphorylation sites, while GCAP1 shows one possible PKA phosphorylation site at serine 201 (for PKA consensus phosphorylation site motifs, see Hunter, 1991). Inspection of autoradiograms of $^{32}$P-labeled phosphoproteins of saponin-permeabilized ROS did not permit a clear conclusion in this matter. The best way to address this question is to use a reconstituted system with washed ROS membranes and recombinant GCAP1, and such experiments are currently in progress.

*Physiological Relevance.* At first glance it would appear that the effects of $Na^+$ and cyclic nucleotides on total ROS GC activity cancel each other out under physiological conditions. In darkness, high cytosolic $Na^+$ causes a desensitization of ROS GC toward $Ca^{2+}$, which effect is reversed by PKA-mediated phosphorylation in the presence of micromolar cGMP concentrations known to exist in darkness. Three possibilities could be considered. First, the $Na^+$-induced desensitization of ROS GC to $Ca^{2+}$ is undesirable and dark cGMP levels merely prevent this from occurring. Second, the kinetics and/or light dependence of dephosphorylation of the substrate protein of PKA-mediated phosphorylation is quite different (e.g., much faster or occurring at lower light levels) compared with the light-induced lowering of cytosolic $Na^+$ concentration. This would result in a dependence of the $Ca^{2+}$ sensitivity of ROS GC on either the light level or the duration of a light stimulus. Third, our experiments were performed in the presence of 30 mM fluoride (necessary to inhibit $PP_i$ase) which will result in a nearly complete inhibition of protein phosphatases, and this may significantly alter the cyclic nucleotide dependence and/or the time dependence of the effects observed here.

Although the physiological significance of our findings is unclear, largely in view of the absence of any reports on cytosolic $Na^+$ concentrations in mammalian ROS in darkness or bright light, we believe that our results suggest a subtle regulation of ROS GC with feedback loops involving not only cytosolic $Ca^{2+}$, but also cytosolic $Na^+$ and cGMP levels. We believe we have found a novel role for cytosolic $Na^+$ to act as an internal messenger and regulate enzymatic activities as we are not aware of any similar reported observations in ROS or any other mammalian cell type.

## REFERENCES


Ahmad, I., & Barnstable, C. J. (1993) *Exp. Eye Res. 56*, 51.

Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., & Walsh, D. A. (1986) *J. Biol. Chem. 261*, 989.

Dizhoor, A. M., Ray, S., Kumar, S., Niemi, G., Spencer, M., Brolley, D., Walsh, K. A., Philipov, P. P., Hurley, J. B., & Stryer, L. (1991) *Science 251*, 915.

Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., & Hurley, J. B. (1994) *Neuron 12*, 1345.

Duda, T., Goraczniak, R. M., Sitaramayya, A., & Sharma, R. K. (1993) *Biochemistry 32*, 1391.

Garbers, D. L., & Lowe, D. G. (1994) *J. Biol. Chem. 269*, 30741.

Goraczniak, R. M., Duda, T., Sitaramayya, A., & Sharma, R. K. (1994) *Biochem. J. 302*, 455.

Gorczyca, W. A., Van Hooser, J. P., & Palczewski, K. (1994) *Biochemistry 33*, 3217.

Gorczyca, W. A., Polans, A. S., Surgucheva, I. G., Subbaraya, I., Baehr, W., & Palczewski, K. (1995) *J. Biol. Chem. 270*, 22029.

Hakki, S., & Sitaramayya, A. (1990) *Biochemistry 29*, 1088.

Hayashi, F., & Yamazaki, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4746.

Horio, Y., & Murad, F. (1991) *Biochim. Biophys. Acta 1133*, 81.

Hunter, T. (1991) *Methods Enzymol. 200*, 3.

Koch, K. W. (1991) *J. Biol. Chem. 266*, 8634.

Koch, K. W., & Stryer, L. (1988) *Nature 334*, 64.

Lambrecht, H. G., & Koch, K. W. (1991a) *EMBO J. 10*, 793.

Lambrecht, H. G., & Koch, K. W. (1991b) *FEBS Lett. 294*, 207.

Lee, R. H., Brown, B. M., & Lolley, R. N. (1984) *Biochemistry 23*, 1972.

Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spenser, M., Laura, R., Lu, L., & Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5535.

Matthews, H. R., Murphy, R. L. W., Fain, G. L., & Lamb, T. D. (1988) *Nature 334*, 67.

Minta, A., Kao, J. P. Y., & Tsien, R. Y. (1988) *J. Biol. Chem. 264*, 8171.

Nakatani, K., & Yau, K.-W. (1988) *Nature 334*, 69.

O'Brien, W. O. (1976) *Anal. Biochem. 76*, 423.

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., & Baehr, W. (1994) *Neuron 13*, 395.

Pozdnyakov, N., Yoshida, A., Cooper, N. G. F., Margulis, A., Duda, T., Sharma, R. K., & Sitaramaya, A. (1995) *Biochemistry 34*, 14279.

Schnetkamp, P. P. M. (1986) *J. Physiol. 373*, 25.

Schnetkamp, P. P. M. (1989) *Prog. Biophys. Mol. Biol. 54*, 1.

Schnetkamp, P. P. M., Klompmakers, A. A., & Daemen, F. J. M. (1979) *Biochim. Biophys. Acta 552*, 379.

Schnetkamp, P. P. M., Basu, D. K., Li, X.-B., & Szerencsei, R. T. (1991) *J. Biol. Chem. 266*, 22983.

Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., & Lowe, D. G. (1992) *Neuron 9*, 727.

Walter, U. (1984) *Adv. Cyclic Nucleotide Protein Phosphorylation Res. 17*, 249.

Wolbring, G., & Schnetkamp, P. P. M. (1995) *Biochemistry 34*, 4689.

Yang, Z., & Wensel, T. G. (1992) *J. Biol. Chem. 267*, 24634.

Yarfitz, S., & Hurley, J. (1994) *J. Biol. Chem. 269*, 14329.

Yau, K.-W., & Baylor, D. A. (1989) *Annu. Rev. Neurosci. 12*, 289.


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