Guanosine 3′,5′-Cyclic Monophosphate Stimulates Release of Actively Accumulated Calcium in Purified Disks from Rod Outer Segments of Bovine Retina†

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Received August 2, 1985

ABSTRACT: Parallel lines of evidence have suggested that light initiates changes in both cGMP metabolism and calcium levels in rod outer segments (ROS). We report that cGMP stimulates release of a pool of Ca^{2+} actively accumulated within purified ROS disks. Disks were purified and actively loaded with $^{45}Ca^{2+}$ by an associated ATP-dependent calcium uptake activity as previously described [Puckett, K. L., Aronson, E. T., & Goldin, S. M. (1985) *Biochemistry 24*, 390–400]. Spikes of $^{45}Ca^{2+}$ released from disks were observed in a rapid superfusion system. The Ca^{2+} release was specifically stimulated by physiological levels of cGMP ($K_{app} \sim 20$ μM; Hill coefficient = 1.7). 8-Bromo-cGMP could also activate the release mechanism, but cAMP was ineffective. At cGMP levels of ≥100 μM, ~20% of the loaded Ca^{2+} was released. The Ca^{2+} release rate at saturating cGMP levels reached a maximum within the 10-s time resolution of the assay system. In contrast to other recent reports of cGMP activation of ROS ion conductances, the majority of the release activity terminated in a spontaneous manner, suggestive of an intrinsic inactivation process. The amount of Ca^{2+} released and the release kinetics were similar to the presence or absence of an unbleached pool of rhodopsin. Cyclic nucleotides did not stimulate release from disks passively equilibrated with $^{45}Ca^{2+}$, i.e., in the absence of ATP but otherwise under identical conditions. Preincubation of the disks with cGMP also reduced the level of ATP-dependent Ca^{2+} uptake (~30%); this apparent inhibition may be due to activation of the release mechanism, rather than direct modulation of the uptake activity. These observations demonstrate that purified disks contain two distinct Ca^{2+} transport systems: an ATP-dependent Ca^{2+} uptake system and a cGMP-stimulated Ca^{2+} release activity. The magnitude and time course of the Ca^{2+} uptake and release events suggest that this cycle of Ca^{2+} uptake and release may play a role in light-sensitive regulation of ROS cytoplasmic Ca^{2+} levels.

The rod outer segment (ROS)[1] transduces photons into electrical information, i.e., photoreceptor cell membrane potential changes [reviewed by Hubbel & Bownds (1979) and Fein & Szuts (1982)]. The ROS contains the photopigment rhodopsin, the majority of which resides in stacks of hundreds of flattened disk membranes [reviewed by Dratz & Hargrave (1983)]. Electrophysiological studies have established that absorption of photons by rhodopsin leads to a decrease in the Na^+ conductance of the ROS plasma membrane (Baylor et al., 1979). Ca^{2+} (Yoshikami & Hagins, 1971) and cGMP (Miller & Nicol, 1979; Stryer, 1983) were originally each hypothesized to be intracellular mediators of visual transduction.

Rhodopsin photolysis can initiate a well-defined sequence of events that leads to the activation of a phosphodiesterase and concomitant stimulation of cGMP hydrolysis (Bitensky et al., 1981). Cyclic GMP levels are high in the dark, and light decreases cGMP content in the ROS (Goridis et al., 1974; Woodruff & Bownds, 1979). Fesenko et al. (1985) demonstrated that cGMP increases the cation conductance of excised "inside out" patches of ROS plasma membrane. Thus, a decrease in Na^+ conductance by light-induced reduction in cGMP levels may be a critical event mediating the rod photoresponse in vivo.

Both cyclic nucleotides and Ca^{2+} play an important role in the regulation of other biological processes such as muscle contractility (Tsien, 1983; Rasmussen, 1983) and neurotransmitter release (Reichardt & Kelly, 1983). By analogy, and because of evidence that light initiates changes in both the metabolism of cGMP (Yee & Liebman, 1978) and the movement of Ca^{2+} (Gold & Korenbrot, 1980; Yoshikami et al., 1980), it is possible that both function to regulate photoreceptor electrical activity. Contrary to a previous hypothesis (Yoshikami & Hagins, 1971), it now appears unlikely that Ca^{2+} is the sole, direct modulator of the plasma membrane Na^+ channel (Fesenko et al., 1985; Matthews et al., 1985). However, indirect evidence suggests a supporting or synergistic role for Ca^{2+} in the short- and/or long-term regulation of the phototransduction process. Ca^{2+} can increase the light-induced activation of phosphodiesterase (Robinson et al., 1980) and inhibit ROS guanylate cyclase (Fleischman & Denisevich, 1979), and thus Ca^{2+} may regulate phototransduction by affecting cGMP metabolism.

Conversely, several reports have suggested that cGMP may, in turn, regulate Ca^{2+} transport in ROS. One previous report of cGMP stimulation of Ca^{2+} release from a disk-containing

† This work was supported by U.S. Public Health Service Grants NS 15236 and NS 16475 from the National Institutes of Health and awards from the McKnight Foundation and the Chicago Community Trust (S.M.G.). K.L.P. was supported by U.S. Public Health Service Training Grant GM 07306 from the National Institutes of Health.

[1] Abbreviations: ROS, rod outer segment; TCA, trichloroacetic acid; βME, 2-mercaptoethanol; Tris, tris(hydroxymethyl)aminomethane; cGMP, guanosine 3′,5′-cyclic monophosphate; cAMP, adenosine 3′,5′-cyclic monophosphate; 8-Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PDE, cGMP phosphodiesterase.

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membrane fraction of osmotically lysed ROS (Cavagionni & Sorbi, 1981) was interpreted as a reduction in Ca^{2+} binding to disk membranes. In frozen and sonicated ROS membrane vesicles filled with Ca^{2+} chelators, cGMP stimulated the rate of Ca^{2+} uptake (Carretta & Cavagionni, 1983). George and Hagins (1983) reported that a particulate fraction of ROS exhibited both light- and cGMP-stimulated Ca^{2+} uptake as determined by $^{45}Ca^{2+}$ association. Recently, cGMP-stimulated release of endogenous Ca^{2+} from both osmotically lysed ROS and ROS permeabilized by freezing and thawing was reported (Kaupp & Koch, 1984; Koch & Kaupp, 1985).

One unresolved question is that of the location of the cGMP-sensitive Ca^{2+} transport sites. These reports all cited disks as the most likely site of the observed actions of cGMP, but unambiguous localization of this cGMP-sensitive Ca^{2+} transport activity is complicated by other Ca^{2+} conductances associated with ROS preparations. These include both the dark current cation channels (Yau & Nakatani, 1984) and Na^+/Ca^{2+} exchange activity (Yau & Nakatani, 1985) of plasma membranes, and the mitochondrial Ca^{2+} transport activity (Carafoli, 1980). A second critical question is whether the in vivo effect of cGMP is to stimulate Ca^{2+} release from or Ca^{2+} uptake into the hypothesized internal storage sites. If Ca^{2+} release occurs, how is the Ca^{2+} gradient initially formed? Purification of disks, together with identification and characterization of the disk-associated Ca^{2+} transport systems, would constitute an imporant step toward answering these questions.

We have developed a new procedure for disk purification and have characterized an associated ATP-dependent Ca^{2+} uptake activity (Puckett et al., 1985a). In this report, we demonstrate that this uptake activity generates a pool of Ca^{2+} in purified disks that is discharged by physiological levels of cGMP. This demonstrates—in analogy with muscle sarcoplasmic reticulum—that photoreceptor disks contain the apparatus for *both* active accumulation and stimulated release of Ca^{2+}. The activity of these two transport systems is presumably coordinated so as to contribute to the regulation of ROS cytoplasmic Ca^{2+} levels. Preliminary accounts of these findings have appeared (Puckett & Goldin, 1985; Puckett et al., 1985b). The disk-associated Ca^{2+} release mechanism we characterize here appears to be kinetically distinguishable from the plasma membrane cGMP-sensitive cation conductance (Fesenko et al., 1985) and from the majority of the cGMP-stimulated Ca^{2+} efflux observed in lysed ROS, as described in a report that appeared as our study neared completion (Koch & Kaupp, 1985).

Experimental Procedures

All procedures were done in room light at 0–4 °C unless otherwise stated.

Disk Isolation. The entire disk isolation procedure was performed under dim red light (Kodak no. 1 safelight filter, 15 W). Osmotically intact disks were obtained from bovine retinal rod outer segments by hypoosmotic shock, floated on a 5% Ficoll 400 solution, and separated into two distinct subpopulations, the "R band" and "W band", on a linear 5–20% Ficoll density gradient as previously described (Puckett et al., 1985a). The major subpopulation, the R band, comprises isolated disks and was pooled and washed as previously described. Some control experiments were performed on the W band, as described in the text. For the experiments described here the disks could be stored for up to 3 weeks at 4 °C in the dark. The Ca^{2+} uptake activity was not stable to freezing and thawing. Exposure of the disks to room light accelerated the loss of ATP-dependent Ca^{2+} uptake activity: the $t_{1/2}$ for unbleached disks is ~3 weeks, whereas after the disks are bleached, approximately 50% of the Ca^{2+} uptake activity is lost within 24 h.

Ca^{2+} Uptake Assays. $^{45}Ca^{2+}$ uptake assays were performed as previously detailed (Puckett et al., 1985a) by separating intradisk from external $^{45}Ca^{2+}$ on a cation-exchange resin. Incubations were done in both the presence and absence of 2 mM Mg^{2+}ATP to determine ATP-dependent Ca^{2+} uptake.

Ca^{2+} Release Assays. Prior to the Ca^{2+} release assays the R-band disks (~2.5 mg/mL) were loaded with $^{45}Ca^{2+}$ by incubating with 5.5 mM $MgCl_2$ and 0.275 mM $^{45}Ca^{2+}$ (150 000 cpm/nmol) in the presence or absence of 2 mM Mg^{2+}ATP for various times at 37 °C. After the disks were loaded, external Ca^{2+} was removed by elution of the disks (550 μL) through a cation-exchange resin column (Dowex 50W-X2-200, Tris form) with a 6 mL bed volume and 6 mm i.d. Two milliliters of ion-exchange elution buffer (100 mM KH_2PO_4, 60 mM NaOH, and 10 mM Tris, pH 7.0) was used to elute the loaded disks.

All superfusion solutions and the Ca^{2+} loaded disks (2.55 mL total volume) were at room temperature during the release assays. The superfusion buffer was 100 mM KH_2PO_4, 60 mM NaOH, and 5 mM βME, pH 7.0. The disk superfusion columns were made with 0.4 mL of ultramicrofuge tubes (Sarstedt 72.700). The tubes were cut in half and the bottoms pierced with a 20-gauge needle. The columns were then packed with handcut 4 mm diameter glass-fiber filters in four layers in the following order (top to bottom of column): Whatman D, A, C, B. The disks (or control membrane fractions) were loaded onto the filters in two 150-μL aliquots by forcing the solution through the filters under pressure using a syringe. The disks were overlayed with 200 μL of superfusion buffer, and then the column was attached to a three-way stopcock. The filter column was constantly superfused with buffer (channel 1) and/or test solutions containing nucleotides as indicated (channel 2) with the flow rate maintained at 1 mL/min by a Buchler Polystaltic multichannel peristaltic pump. The disks were washed by first superfusing with 1.6 mL of buffer; then while the superfusion was maintained, two additional fractions (5 drops, ~170 μL/fraction) were collected to establish the base line for Ca^{2+} release. The time course of Ca^{2+} release from the disks was determined upon switching to the test solution reservoir (channel 2); after the switch at least 13 more fractions were collected at 10-s intervals (~170 μL/fraction).

As a control, the above procedure was repeated on a parallel aliquot of disks with both superfusion reservoirs containing only buffer to determine basal Ca^{2+} release occurring over time. The control values, determined in this manner for each fraction, were subtracted from each corresponding test solution value to determine nucleotide-stimulated Ca^{2+} release. All fractions were collected directly into 5-mL glass scintillation vials, to which 4 mL of Hydrofluor (National Diagnostics) was added. Release assays were performed in laboratory light or under dim red light.

Ca^{2+} release was expressed either as nanomoles of Ca^{2+} released per milligram of total protein or as percent of total Ca^{2+} associated with the disk membranes. Disks loaded with Ca^{2+} and ATP for 30 min could be used for release assays for up to 8 h after elution through the cation-exchange column. However, to account for the gradual leakage of $^{45}Ca^{2+}$ from disks that occurs over time after removal of external Ca^{2+}, the data were normalized by converting Ca^{2+} release to percent of total Ca^{2+} associated with the disks at the time the release assay was performed.

The total $^{45}Ca^{2+}$ initially associated with disks was determined by adding the Ca^{2+} content in each of the 10-s interval fractions collected following the initial wash, to the amount of $^{45}Ca^{2+}$ remaining associated with the disks on the filter following the superfusion. The Ca^{2+} released into each 10-s fraction was expressed as percent of this total disk-associated Ca^{2+}. The amount of $^{45}Ca^{2+}$ associated with the disks upon incubation in the absence of ATP was typically 14–20% of the total Ca^{2+} associated with the disks (incubated in the presence of ATP for 30 min at 37 °C). The ATP-independent value was not subtracted from the calculated total $^{45}Ca^{2+}$ associated with the disks.

The net nucleotide-stimulated Ca^{2+} release was determined by taking the difference between the amount of Ca^{2+} associated with the disks on the filters following superfusion in the presence or absence of nucleotide.

Ca^{2+} ionophore solutions were made by diluting a 1 mM stock solution (100% EtOH) into 100 mM KH_2PO_4–60 mM NaOH buffer to a final concentration of 5 μM (0.5% EtOH).

Rhodopsin Assays. Rhodopsin was used as a specific marker for the disk membranes. Quantitative analysis of the rhodopsin was done spectrophotometrically for each sample by bleaching and regeneration of rhodopsin in the presence of 1% digitonin (Puckett et al., 1985a). No significant rhodopsin bleaching was detected during the membrane isolation and the various assays when performed under dim red light.

Protein Assay. Protein determination was performed via the modified Lowry protein assay, with TCA precipitation of the protein in the presence of deoxycholate (Bensadoun & Weinstein, 1976).

Results

The purified disk membranes were prepared as follows: A crude preparation of disks was obtained by hypoosmotic lysis of rod outer segments and subsequent flotation on a low-density Ficoll solution. These "crude" disks were further subfractionated into two distinct components by centrifugation on a linear density gradient. The major component constitutes purified disks and is termed the R-band. The purified disks contain an ATP-dependent Ca^{2+} uptake activity, as previously identified and characterized (Puckett et al., 1985a). At physiological temperature (37 °C) the initial rate of ATP-dependent Ca^{2+} uptake activity [$\sim$0.6 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}] was 3-fold higher than the activity at 25 °C. After 30 min at 37 °C, $^{45}Ca^{2+}$ accumulation in the presence of ATP was typically 5–7-fold higher than in the absence of ATP. The Ca^{2+} uptake activity was linear for 5–10 min and plateaued by 120 min at both temperatures. Approximately 65% of the ATP-dependent accumulated $^{45}Ca^{2+}$ was released by the Ca^{2+} ionophore A23187. The remaining Ca^{2+} pool presumably includes Ca^{2+} physically bound to sites on the membrane and/or sites within the disks. Ionophore added prior to initiation of the Ca^{2+} uptake assays completely prevents Ca^{2+} accumulation, which averaged 6 ± 11% (n = 3) of that determined in the absence of ionophore.

Reduction of ATP-Dependent Ca^{2+} Uptake by cGMP. Incubating the disks with cGMP before incubating with $^{45}CaCl_2$ and Mg^{2+}ATP resulted in an apparent decrease in ATP-dependent Ca^{2+} uptake, with no effect on Ca^{2+} uptake in the absence of ATP. The effect of cGMP concentration on net Ca^{2+} uptake is shown in Figure 1. The maximum reduction of uptake occurs at cGMP concentrations >20 μM and is 28% under the incubation conditions employed. Neither Mg^{2+}GTP (1.1 mM), which activates ROS cGMP phosphodiesterase (Stryer, 1983) nor IBMX (500 μM), a phosphodiesterase inhibitor, modifies the decrease in ATP-dependent

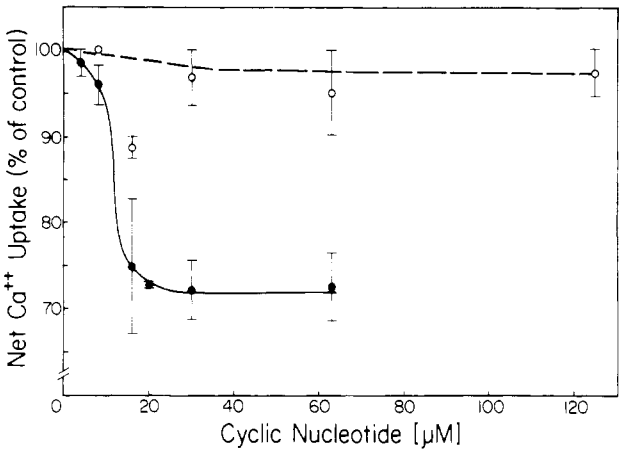

FIGURE 1: Effect of cyclic nucleotide preincubation on the ATP-dependent Ca^{2+} uptake activity in R-band disks. Disks were incubated with increasing concentrations of cGMP (●) or cAMP (○) for 2 min prior to $^{45}CaCl_2$ and Mg^{2+}ATP addition. The Ca^{2+} uptake assays were performed as described under Experimental Procedures; the incubation time with $^{45}Ca^{2+}$ ± Mg^{2+}ATP was 10 min. Data are expressed as a percent of inhibition of net Ca^{2+} uptake as a function of cyclic nucleotide concentration ($\bar{x}$ ± SEM, n = 2–13). The apparent small decrease in Ca^{2+} uptake at 16 μM cAMP was not reproducible in replicate experiments: no significant inhibition of Ca^{2+} uptake was detected at cAMP concentrations up to 1 mM.

Ca^{2+} uptake stimulated by cGMP (data not shown). Incubation with cGMP *prior* to addition of $^{45}CaCl_2$ and Mg^{2+}ATP was required to see the reduction of the ATP-dependent Ca^{2+} uptake. On the other hand, preincubation of the disks with cAMP did not cause a significant decrease in ATP-dependent Ca^{2+} uptake (Figure 1).

Stimulation of Ca^{2+} Release by cGMP. The apparent reduction of the ATP-dependent Ca^{2+} uptake by cGMP could be caused by cGMP inhibition of the Ca^{2+} uptake activity, by cGMP stimulation of Ca^{2+} release from disks, or both. The following experiments were designed to look at release directly. R-band disks were loaded with $^{45}Ca^{2+}$ via the associated ATP-dependent Ca^{2+} uptake system. The Ca^{2+}-loaded disks were retained on filters and superfused with buffers containing various nucleotides. The superfusate was collected at 10-s intervals, and the time course of Ca^{2+} release was determined.

Cyclic GMP (100 μM) caused a rapid spike of Ca^{2+} release from R-band disks actively loaded with $^{45}Ca^{2+}$ via the associated ATP-dependent Ca^{2+} uptake system (Figure 2A). cGMP released 19% of the total $^{45}Ca^{2+}$ associated with these disks. It had been previously hypothesized that cGMP or cGMP hydrolysis products may cause dissociation of Ca^{2+} from binding sites of ROS membranes (Mueller & Pugh, 1983; Cavagionni & Sorbi, 1981). To address the question of whether cGMP was affecting Ca^{2+} bound to the membranes or affecting the Ca^{2+} pool that had been transported across the membrane, we compared the magnitude of Ca^{2+} release observed with actively loaded disks (above) to that measured during cGMP superfusion of disks that were incubated with $^{45}Ca^{2+}$ in the absence of ATP but otherwise treated identically. (Disks incubated in the absence of ATP do not show any ionophore-induced Ca^{2+} release; therefore, the associated $^{45}Ca^{2+}$ is presumably bound rather than free.) These passively equilibrated disks did not show cGMP-stimulated Ca^{2+} release (Figure 2B). The lack of cGMP stimulation of release in passively equilibrated disks suggests that the ATP-dependent Ca^{2+} uptake system is responsible for generating a cGMP-sensitive pool of free Ca^{2+} in the R-band disks.

The Ca^{2+} release activity was not directly modulated by light. Both the maximum rate and the total levels of

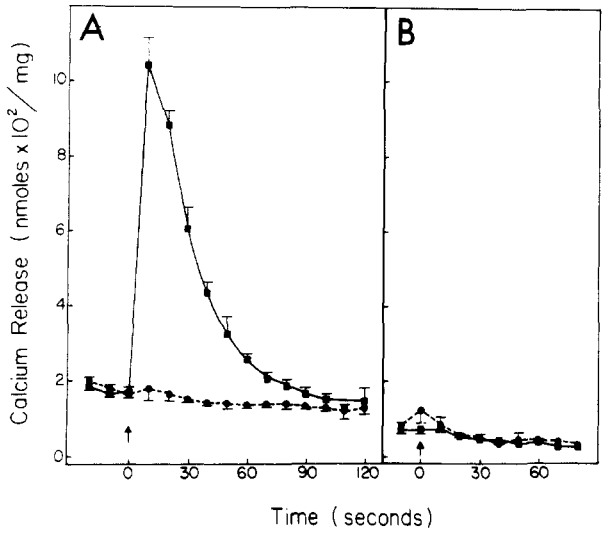

FIGURE 2: Time course of cGMP-stimulated calcium release from R-band disks. Data are expressed as Ca^{2+} release (nmol/mg of protein) vs. time of superfusion (s). (Panel A) Disks were loaded with $^{45}Ca^{2+}$ in the *presence* of 2 mM Mg^{2+}ATP for 30 min at 37 °C. Release assays were performed as described under Experimental Procedures. The arrow indicates a switch to 100 μM cGMP in the superfusion solution (■). As a control, a switch to buffer is made in parallel to determine basal levels of Ca^{2+} release (●) ($\bar{x} \pm$ SD, $n = 3$). In this experiment the peak Ca^{2+} release (10-s fraction) was 6.6 ± 0.5% of total Ca^{2+} associated with the disks, and the net cGMP-stimulated Ca^{2+} release was 19.3 ± 2.4%. The total initial level of Ca^{2+} associated with the disks was 1.7 ± 0.1 nmol/mg of protein. (Panel B) Disks were incubated with $^{45}Ca^{2+}$ in the *absence* of 2 mM Mg^{2+}ATP for 30 min at 37 °C. The arrow indicates switch to 100 μM cGMP (■) or buffer (●) in the superfusion solution ($x \pm$ SD, $n = 3$). In this experiment the peak Ca^{2+} release was −1.5 ± 1.3% of the total Ca^{2+} associated with the disks, and net Ca^{2+} release was 0.8 ± 3.9%. The total initial level of Ca^{2+} associated with the disks was 0.30 ± 0.03 nmol/mg of protein.

cGMP-stimulated Ca^{2+} release occurring over the 2-min superfusion times were compared in experiments done in dim red light vs. laboratory light (the former conditions do not photolyze rhodopsin). There was no significant difference between either the maximum observed rate of Ca^{2+} release, within statistical limits (SD) of ±0.9% ($n = 6$), or total amount of Ca^{2+} released, within statistical limits of ±5.1% ($n = 6$), in dark vs. light conditions (data not shown).

The cGMP concentration dependence of both the time course and magnitude of Ca^{2+} release in disks is shown in Figure 3A. As the cGMP concentration used during superfusion is increased from 10 to 100 μM, there is an increase in both the maximal observed rate of Ca^{2+} release and the total amount of Ca^{2+} released over the duration of the spike. The decay of Ca^{2+} release during the first 50 s following the maximum observed release rate is well approximated by exponential kinetics. Plots of the logarithm of the observed Ca^{2+} release rate as a function of time during this interval (data not shown) were linear (r^2 values for linear regression analyses exceeded 0.98). The decay constant increased as a function of cGMP concentration, ranging from 2.5×10^{-2} s^{-1} at 30 μM cGMP to 5.5×10^{-2} s^{-1} at 500 μM cGMP. These values are minimum estimates for the release kinetics and may not reflect the precise time course of the release event due to limitations imposed by the superfusion system.

To investigate the specificity of the association of the cGMP-induced Ca^{2+} release mechanism with disks, we examined the effect of cGMP on the W-band, the major membrane fraction removed from crude disks during disk purification. The W-band is separated from the R-band disks by their respective differences in sedimentation velocity on the final linear density gradient. This W-band fraction is vesicular and is depleted of rhodopsin relative to the R-band disks, but the W-band does contain an ATP-dependent Ca^{2+} uptake activity that closely resembles that of the R-band in vanadate sensitivity and other kinetic properties (Puckett et al., 1985a). Most (70%) of the ATP-dependent accumulated $^{45}Ca^{2+}$ was released by A23187 (not illustrated). However, the W-band membrane fraction did not demonstrate cGMP-induced Ca^{2+} release when loaded with $^{45}Ca^{2+}$ in the presence of Mg^{2+}ATP (Figure 3B).

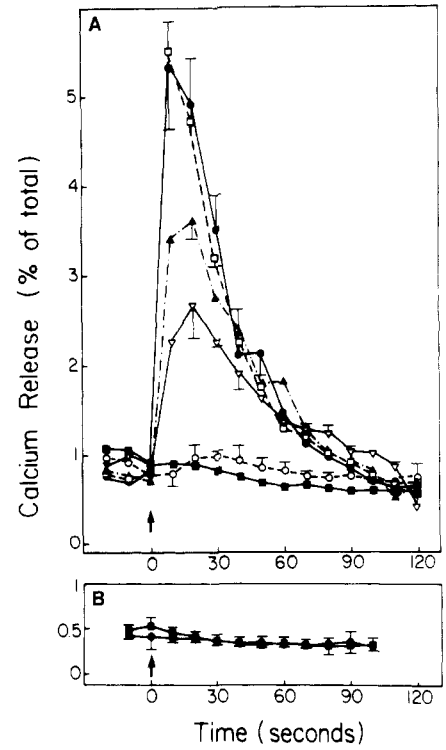

FIGURE 3: Effect of various concentrations of cGMP on the time course of Ca^{2+} release. The R-band and W-band membranes were incubated for 30 or 60 min at 37 °C, respectively, with $^{45}Ca^{2+}$ and 2 mM ATP. The release assays were then performed as described under Experimental Procedures. (Panel A) *R-band Membranes*: The arrow (at time zero) indicates a switch to superfusion solutions containing various concentrations of cGMP, as indicated. (■) 0, (○) 10, (▽) 30, (▲) 50, (□) 80, and (●) 100 μM cGMP. Data are expressed $\bar{x} \pm$ SD ($n = 3$). In this experiment the total Ca^{2+} initially associated with the R-band membranes was 1.0 nmol of Ca^{2+}/mg of protein. The maximum net cGMP-stimulated Ca^{2+} release (80 and 100 μM cGMP) was 15.7 ± 1.2% of total Ca^{2+} associated with the disks. (Panel B) *W-band Membranes*: The arrow (at time zero) indicates switch to 100 μM cGMP (●) or buffer (■) $\bar{x} \pm$ SD ($n = 3$). The total Ca^{2+} initially associated with the W-band membranes was 24.3 nmol of Ca^{2+}/mg of protein. The net cGMP-stimulated Ca^{2+} release was 0.03 ± 1.18% of the total Ca^{2+} associated with W-band membranes.

The addition of 1.1 mM GTP to the cGMP-containing superfusion buffer caused no significant change in either the maximum observed rate of Ca^{2+} release or total Ca^{2+} released from R-band disks. GTP (1.1 mM), GMP (100 μM), or cAMP (100 μM, 1 mM) in the absence of cGMP did not stimulate Ca^{2+} release (Table I).

8-Br-cGMP, a nonhydrolyzable analogue of cGMP, is a poor substrate for cyclic nucleotide phosphodiesterase in kidney (Meyer & Miller, 1974), in brain (Daly, 1977), and in ROS (Koch & Kaupp, 1985). Both cGMP and 8-Br-cGMP stimulated Ca^{2+} release in a dose-dependent manner; the K_{app} for cGMP was ~20 μM, and the K_{app} for 8-Br-cGMP was ~10-fold lower (Figure 4). Although 8-Br-cGMP appears to be more potent, the time course and magnitude of the cGMP-stimulated Ca^{2+} release–at saturating concentrations

Table I: Effect of Various Nucleotides on Calcium Release from R-Band Disks[a]

	nucleotide(s) in superfusion buffer	calcium release (% of total Ca^{2+} associated with disks)	
		initial increment of Ca^{2+} release[b]	nucleotide-stimulated net Ca^{2+} release[c]
I	cGMP	4.4 ± 0.7	15.9 ± 1.2
	cGMP + GTP[d]	5.3 ± 0.3	18.6 ± 3.1
	GTP[d]	0.1 ± 0.2	0.2 ± 1.1
	cGMP	5.2 ± 0.1	16.7 ± 0.9
	GMP	0 ± 0.1	0 ± 0.4
	cAMP[e]	0.7 ± 0.1	0 ± 2.1
II	cGMP	6.5 ± 0.3	21.1 ± 2.7
	cGMP + GTP[d]	4.3 ± 0.8	23.9 ± 3.7
	cAMP	0.1 ± 0.5	0.8 ± 1.7

[a] Disks were incubated at 37 °C with 0.275 mM $^{45}CaCl_2$ and 1.1 mM ATP prior to release assays, for the indicated time periods. (I) 30-min incubation; (II) 60-min incubation. All cyclic nucleotide and nucleotide concentrations are 100 μM unless otherwise noted. All data were run in duplicate (n = 2). Basal Ca^{2+} release, as determined by superfusion with buffer alone, has been subtracted in each of the above determinations. [b] Initial increment of Ca^{2+} release is defined as the percent of total disk-associated Ca^{2+} in the fraction collected within the first 10-s interval following the switch to nucleotide-containing superfusion buffer. [c] Net nucleotide-stimulated Ca^{2+} release is the percent of total Ca^{2+} released over the 2-min superfusion time period. See Experimental Procedures for precise definition. [d] GTP concentration = 1.1 mM. [e] cAMP concentration = 1 mM.

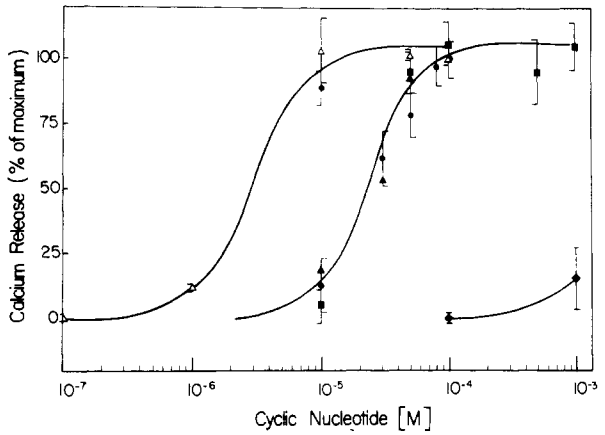

FIGURE 4: Concentration dependence of various cyclic nucleotides on stimulation of Ca^{2+} release from R-band disks. The total Ca^{2+} released over the 2-min superfusion is expressed as a percent of maximal release. Data are normalized such that "maximum" release for all three nucleotides equals the Ca^{2+} release in the presence of 100 μM cGMP. Superfusions were performed in triplicate ($\bar{x}$ ± SD), and each nucleotide was tested on up to three different R-band disk preparations. Each preparation is represented by a different symbol. All release assays were performed as described under Experimental Procedures. 8-Br-cGMP (△, ●, ■); cGMP (■, ●, ▲); cAMP (♦).

of each–were the same. In contrast, cAMP, at or exceeding physiological concentrations, did not stimulate Ca^{2+} release (Figure 4).

cGMP demonstrated positive cooperatively in stimulating Ca^{2+} release. The Hill coefficient, calculated from the data in Figure 4, was 1.7.

Definition of the Releasable Ca^{2+} Pool; Lack of cGMP Restimulation of Ca^{2+} Release. Saturating levels of cGMP elicited release of approximately 20% of the total disk-associated Ca^{2+}. In contrast, a greater fraction of the disk-associated Ca^{2+} was releasable by other means. When hypoosmotic buffer (1 mM KH_2PO_4, 0.6 mM NaOH, pH 7.0) was substituted for nucleotide-containing superfusion buffer in the release assays, approximately 40% of the total Ca^{2+} associated

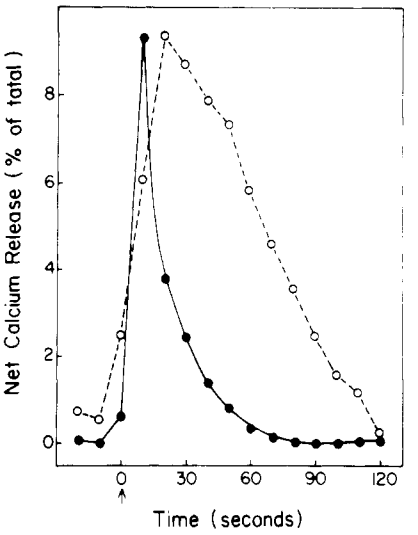

FIGURE 5: Time course of Ca^{2+} release from R-band disks stimulated by either cGMP (200 μM) (●) or the Ca^{2+} ionophore A23187 (5 μM) (○). Data are expressed as net calcium release (the percent of total Ca^{2+} associated with the disks) vs. time of superfusion (s). Disks were incubated with $^{45}Ca^{2+}$ and Mg^{2+}ATP for 30 min at 37 °C. Release assays were performed as described under Experimental Procedures. Background levels of Ca^{2+} release (superfusion with buffer alone) have been subtracted from the test solution values. The average total Ca^{2+} release over the 2-min superfusion time was 16% and 62% of the total Ca^{2+} associated with the disks for respectively the cGMP and the A23187 superfusions.

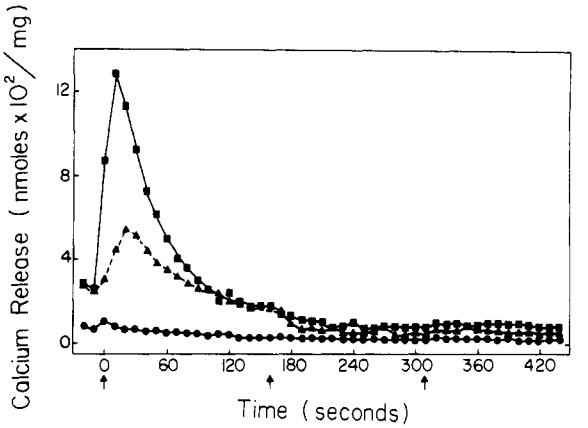

FIGURE 6: Effect of reintroduction of cGMP on Ca^{2+} release from R-band disks actively loaded with Ca^{2+}. Disks were incubated with $^{45}Ca^{2+}$ in the presence of 2 mM Mg^{2+}ATP for 30 min at 37 °C. The first arrow (at time zero) indicates a switch to 100 μM cGMP (■) or 25 μM cGMP (▲), the second arrow (at 160 s) indicates a switch back to buffer, and the third arrow (at 310 s) indicates resuperfusion with 100 μM cGMP (■) or 25 μM cGMP (▲). Also shown are control superfusions made in parallel where each arrow indicates a switch to buffer solution (●). Superfusions were run in duplicate. Disks used in control superfusions were not depleted of the cGMP-releasable Ca^{2+} pool during the extended superfusion time of the assay: when cGMP was introduced for the first time at the third arrow (310 s), the expected level of Ca^{2+} release was seen (data not shown).

with the disks was released; this release may be due to at least partial lysis of the disks. Superfusion of the disks with A23187 (5 μM) released 60% of the total Ca^{2+} associated with the disks (Figure 5).

Since saturating levels of cGMP elicit release of only one-third of the A23187-releasable Ca^{2+} pool, we investigated the effect of resuperfusion of disks with cGMP to further explore this phenomenon. Superfusion with cGMP was followed by buffer superfusion and reexposure of the disks to the same concentration of cGMP. Reexposure of disks to either saturating (100 μM) or subsaturating (25 μM) cGMP failed to elicit any further Ca^{2+} release (as shown in Figure 6). This

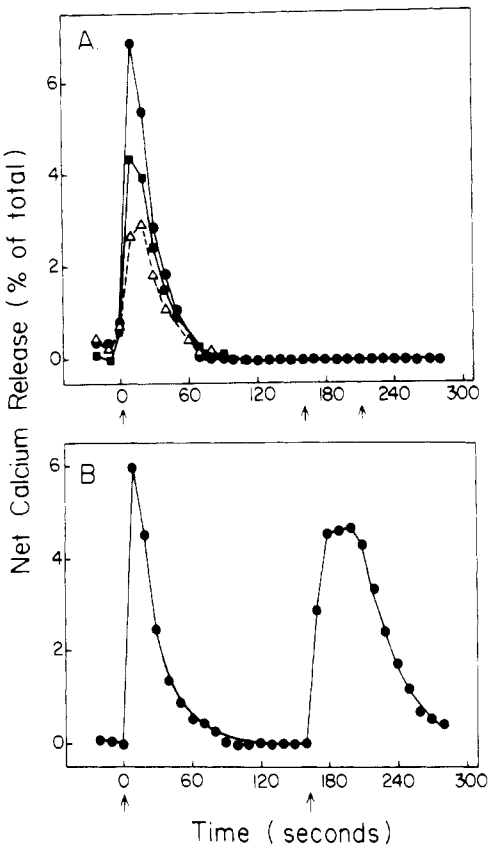

FIGURE 7: Effect of exposure of R-band disks with either saturating cGMP or the Ca^{2+} ionophore A23187 following initial exposure to cGMP. Disks were incubated with $^{45}Ca^{2+}$ and Mg^{2+}ATP for 30 min at 37 °C. Parallel superfusions with buffer alone have been subtracted from the test solution values. (Panel A) Reintroduction of saturating cGMP (200 μM) following superfusion with buffers containing various cGMP concentrations. The first arrow (at time zero) indicates a switch to 200 μM cGMP (●) 100 μM cGMP (■), or 50 μM cGMP (△), the second arrow (at time 160 s) indicates a switch back to buffer, and the third arrow (at 210 s) indicates initiation of the second superfusion (with 200 μM cGMP). Each superfusion experiment was run in duplicate, and the data were averaged. (Panel B) Introduction of the Ca^{2+} ionophore A23187 following initial exposure to saturating cGMP. The first arrow (at time zero) indicates a switch to 200 μM cGMP; the second arrow (at 160 s) indicates a switch to 5 μM A23187. Approximately 16% of the total Ca^{2+} associated with the disks was released by cGMP in the initial peak, and an additional 32% was released by the subsequent exposure to A23187.

was the case for a series of cGMP concentrations tested as described above (25 μM to 1 mM) all of which failed to generate a second peak of Ca^{2+} release (data not shown). Superfusions of the disks with *sub*saturating [cGMP] followed by buffer superfusion and then reexposure to saturating [cGMP] were also performed. Again, no second cGMP-stimulated Ca^{2+} release event was observed under these conditions (Figure 7A). On the other hand, superfusion with ionophore after saturating cGMP superfusion did elicit a second peak of Ca^{2+} release that was approximately 30% of the total Ca^{2+} originally associated with the disks (Figure 7B). These observations, taken together, show that only a fraction of the ionophore-releasable pool of Ca^{2+} is sensitive to cGMP. The lack of ability of a second cGMP superfusion to stimulate further Ca^{2+} release even when the total cGMP-sensitive Ca^{2+} pool has not been exhausted leads to the hypothesis that there is an intrinsic mechanism which shuts off the cGMP-stimulated Ca^{2+} release activity.

DISCUSSION

Two Distinct Ca^{2+} Transport Systems in the Disk Membrane: ATP-Dependent Ca^{2+} Uptake and cGMP-Stimulated Ca^{2+} Release Activities. When Ca^{2+} is actively loaded into purified ROS disks and external Ca^{2+} is removed, cGMP superfusion generates a spike of Ca^{2+} release. This release mechanism is specifically associated with the purified disk membrane fraction, the R-band, and release occurs under conditions that generate a transmembrane Ca^{2+} gradient. In contrast to previous findings employing cruder disk-containing preparations [e.g., see Cavagionni & Sorbi (1981) and Koch & Kaupp (1985)], the Ca^{2+} gradient in our studies was established by activation of a disk-associated ATP-dependent Ca^{2+} uptake activity. We are thus observing Ca^{2+} release from purified disks that have *both* the Ca^{2+} uptake and Ca^{2+} release activities.

Additional evidence that the Ca^{2+} uptake and release activities are localized to disks and are two distinct transport systems comes from our observations on the W-band—the major contaminating vesicular membrane fraction removed during density gradient purification of crude disks. No effect of cGMP on either Ca^{2+} uptake or Ca^{2+} release was detectable in the W-band. Since the W-band contains, at 10-fold higher specific activity, an ATP-dependent Ca^{2+} uptake activity of seemingly identical properties with that of the R-band (Puckett et al., 1985a), the Ca^{2+} gradient across the W-band vesicles should be at least as high as the gradient across the R-band disks. The presence of the ATP-dependent Ca^{2+} uptake activity but absence of the cGMP-stimulated release activity in the W-band is evidence that the Ca^{2+} uptake and release activities are two distinct systems.

Disks preincubated with $^{45}Ca^{2+}$ in the absence of ATP did not show cGMP-stimulated release. These disks also did not show any ionophore-induced Ca^{2+} release, indicating that under these circumstances, the associated $^{45}Ca^{2+}$ is bound to sites on the disk membrane and/or to sites within the disks. At first glance, this result seems to be in contradiction to a previous study of lysed and pelleted ROS (Cavagionni & Sorbi, 1981), where preincubation with $^{45}Ca^{2+}$ in the absence of ATP permitted detection of both cGMP-stimulated and ionophore-stimulated release of Ca^{2+}. One fundamental difference between these two studies is that our procedure for purifying disks entails exposure of the disks to Ca^{2+}-free media over a relatively long time period (>48 h). In contrast, the previous study employed a cruder membrane fraction that is more rapidly obtained, and a substantial portion of the endogenous intradisk Ca^{2+} pool may be retained under these conditions. Therefore, active preloading of the membranes may not be necessary, because exposure of the preparation to external $^{45}Ca^{2+}$ would simply radioisotopically label this endogenous Ca^{2+} pool for detection of the release event.

Kinetics and Mechanism of cGMP-Stimulated Ca^{2+} Release. The decay phase of the spike of cGMP-stimulated Ca^{2+} release could be due to depletion of the available actively accumulated pool of Ca^{2+}. However, partial hypoosmotic lysis of the disk membranes or their exposure to a Ca^{2+} ionophore while in the superfusion system releases a substantial fraction (40–60%) of the associated Ca^{2+}, whereas ~20% of the loaded Ca^{2+} is released at saturating levels of cGMP. An alternative explanation for the decay of Ca^{2+} release may be "inactivation" of the release mechanism, similar to the spontaneous inactivation of electrically excitable conductances or desensitization of chemically excitable ion channels. An observation favoring spontaneous inactivation of the release mechanism is that the total amount of $^{45}Ca^{2+}$ released during the spike is much lower at submaximal cGMP levels (Figure 3A): if the decay of the release process were due solely to dissipation of the Ca^{2+} gradient, the duration of the release event would presumably

be longer at subsaturating cGMP concentrations which generate lower initial rates of Ca^{2+} release, but the total amount of $^{45}Ca^{2+}$ released would be the same.

Bearing on the same issue is why reintroduction of cGMP into the superfusion medium fails to stimulate further Ca^{2+} release. Again, the reason could be the depletion of the cGMP-releasable pool of Ca^{2+}. However, even when submaximal levels of cGMP were used to generate the initial release event (releasing as little as 5% rather than 20% of the total disk-associated Ca^{2+}), no restimulation of Ca^{2+} release could be demonstrated, even by saturating concentrations of cGMP. This lack of restimulation is additional evidence for an inactivation process and suggests that the initial introduction of cGMP may generate a *separate* event that results in long-term inactivation of the release mechanism. It has been established that the Ca^{2+} channels of cardiac cells are modulated by a multiplicity of mechanisms [reviewed by Tsien (1983) and Reuter (1983)]; this could also be the case for the Ca^{2+} release system of photoreceptor disks.

The inability to elicit further Ca^{2+} release following exposure of the disks to subsaturating cGMP concentrations was not observed in a previous report on endogenous Ca^{2+} release from lysed ROS (Koch & Kaupp, 1985). The total Ca^{2+} release at maximal cGMP addition equaled the total Ca^{2+} release observed when cGMP was given in sequential submaximal aliquots.

The stimulation of Ca^{2+} release by cGMP occurs in the absence of external ATP during exposure to cGMP. This result suggests a possible direct effect of cGMP, perhaps analogous to the apparent direct stimulation by cGMP of the opening of the cation-selective conductance in the ROS plasma membrane (Fesenko et al., 1985). However, since ATP was present in our system during the Ca^{2+} loading of disks *prior* to addition of cGMP, the possibility that the cGMP-stimulated Ca^{2+} release mechanism is activated via a phosphokinase has not been rigorously excluded.

There is no evidence for a role of cGMP hydrolysis per se in this Ca^{2+} release process. The cGMP derivative 8-Br-cGMP was at least as effective as cGMP in stimulating Ca^{2+} release. 8-Br-cGMP is not hydrolyzed by cyclic nucleotide phosphodiesterase of ROS (Koch & Kaupp, 1985). No significant change in the kinetics or magnitude of cGMP-stimulated Ca^{2+} release occurred when GTP was added to the cGMP superfusion buffer. The experiments were performed both in the light (where PDE stimulation should be maximal) and in the dark. In addition, GMP superfusion elicited no Ca^{2+} release (GMP is a hydrolysis product of cGMP).

Possible Relationship of This Ca^{2+} Release System to ROS Plasma Membrane Cation Channels. There is a striking parallel between our observed concentration dependency of the effect of cGMP on disk Ca^{2+} release and the observed cGMP concentration profile for activation of ROS plasma membrane cation channels (Fesenko et al., 1985). They observe a half-maximal conductance increase at ~ 30 μM cGMP, and the cGMP effect exhibits positive cooperativity, with a Hill coefficient of 1.8. We observe half-maxmial stimulation of release at ~ 20 μM cGMP and positive cooperativity of the effect of cGMP on disk Ca^{2+} release with a Hill coefficient of 1.7. Thus, there may be an intimate connection between cGMP regulation of the two processes. This parallel regulation—together with the observation of Yau and Nakatani (1984) that the light-sensitive ROS plasma membrane conductance is permeable to Ca^{2+}—raises the possibility that the same ion channel responsible for the light-sensitive conductance of the plasma membrane is also found in disks. The disk-associated channel may regulate the movement of Ca^{2+}, and perhaps that of other ions as well. If this is the case, what must be reconciled is that the cGMP-stimulated plasma membrane conductance does not decay in the same time frame as does the cGMP-stimulated Ca^{2+} release from disks we have characterized here.

An alternative explanation for the difference between the two sets of observations is that there may be two distinct ion channels that are subjected to pleiotropic regulation by the same cGMP binding component. Koch and Kaupp (1985) state that all properties of the cGMP-sensitive channel mediating Na^+ conductance, as described by Fesenko et al. (1985), are virtually identical with the Ca^{2+} release system they have identified in lysed ROS. This result contrasts with our findings, in that the majority of their observed Ca^{2+} efflux did not inactivate. Nonetheless, they report that a minor component of the Ca^{2+} efflux (3–4% of the releasable Ca^{2+} pool) is more rapid and transient. This latter component more closely resembles the activity we have characterized here, which in our purified disk preparation comprises $\sim 30\%$ of the *releasable* Ca^{2+} pool.

Possible Biological Significance of cGMP Effects on Disk Ca^{2+} Transport. The magnitude and time course of Ca^{2+} release from purified disks is sufficient in principle for this release mechanism to play an important role in control of ROS Ca^{2+} levels in vivo. We find a maximal rate of cGMP-stimulated Ca^{2+} release of ~ 0.9 nmol (mg of disk protein)$^{-1}$ min^{-1}. This translates to $\sim 0.9 \times 10^5$ ca^{2+} released per ROS per second when calculated on the following basis: at least 60% of the total disk-associated protein is rhodopsin (M_r 40 000), there are 10^5 rhodopsin molecules per bovine disk (Amis et al., 1981), and there are 1000 disks per ROS (Krebs & Kuhn, 1977). This rate is comparable to that reported for permeabilized ROS (Koch & Kaupp, 1985). Approximating a bovine ROS as a cylinder 1 μm in diameter and 50 μm in length (Fein & Szuts, 1982), and assuming that the cytoplasmic space is 50% of the internal volume of the ROS, the release rate is sufficient to produce a 1 μM change in the cytoplasmic Ca^{2+} concentration within ~ 100 ms.

Although there have been no direct measurements of maximal ROS cytoplasmic Ca^{2+} levels, all of the estimates based on indirect evidence are within the 1–10 μM range (Hagins & Yoshikami, 1974, 1977; Wormington & Cone, 1978). Thus, the release rate is sufficient in principle to produce, on the subsecond time scale, changes in cytoplasmic Ca^{2+} levels of potential physiological relevance.

However, it is premature to propose that this Ca^{2+} release system plays a role in the short- as well as long-term regulation of ROS Ca^{2+} levels: the time course of activation of this Ca^{2+} release mechanism has not yet been precisely determined, although we have demonstrated that the release rate at saturating cGMP levels reaches a maximum within the first 10 s of cGMP addition. Rapid kinetic measurements are needed to define the time course of cGMP activation.

Elucidation of the possible role of this Ca^{2+} release system in controlling photoreceptor electrical activity requires a clearer picture than now exists of the function of Ca^{2+} in ROS. Vertebrate ROS reportedly contain the calcium- and phospholipid-dependent kinase, C-kinase (Kapoor et al., 1984; Inoue & Isayama, 1984); if ROS Ca^{2+} levels are, at times, submicromolar, perhaps cGMP-stimulated Ca^{2+} release from disks assists in activation of this enzyme, which in turn may play a role in regulation of photoreceptor activity. It appears that there are potent mechanisms to maintain cGMP homeostasis in ROS (Goldberg et al., 1983); since Ca^{2+} may

inhibit ROS guanylate cyclase (Fleischman & Denisevich, 1979) and may potentiate the light-induced activation of phosphodiesterase (Robinson et al., 1980), cGMP-stimulated disk Ca^{2+} release could play a role in cGMP homeostasis.

ACKNOWLEDGMENTS

We thank Arif J. Rawji for his excellent technical assistance and Elaine T. Aronson for her contributions to the early stages of this work. Timothy J. Turner designed an apparatus for measuring Ca^{2+} and neurotransmitter release from synaptosomes and plasma membrane vesicles (T. J. Turner and S. M. Goldin, unpublished results). We thank him for adapting this system to measure Ca^{2+} release from purified disks. We also thank Bruce Pearce and Kathleen Sweadner for helpful comments on the manuscript.

Registry No. cGMP, 7665-99-8; GMP, 85-32-5; cAMP, 60-92-4; 8-Br-cGMP, 31356-94-2; ATP, 56-65-5; Ca, 7440-70-2.

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