Cyclic GMP releases calcium from disc membranes of vertebrate photoreceptors

(phototransduction/ionophore/calcium loading and releasing)

ANDREA CAVAGGIONI AND ROBERT T. SORBI

Instituto di Fisiologia Umana, Università di Parma, 43100 Parma, Italy

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ABSTRACT Physiological concentrations of cyclic guanosine 3',5'-monophosphate (cGMP) inhibit ^{45}Ca uptake and increase ^{45}Ca release from vertebrate photoreceptor rod outer segment disc membranes. These effects are specific for cGMP. Several facts, including the independence of these effects from added triphosphates, suggest that cGMP diminishes the Ca-binding capacity of the disc membranes. Preliminary data show that the apparent affinity constant of the cGMP-dependent Ca-binding sites of the disc membranes is of the same (or even higher) order of magnitude as that of ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid. As expected, the observed cGMP effects are not dependent on the light or dark conditions of the disc membranes.

Light decreases plasma membrane Na conductance of vertebrate photoreceptor rod outer segments (ROS) (1, 2). Both 3',5'-cyclic GMP (cGMP) and Ca have been proposed as candidates for the internal messenger to transduce the bleaching events in the disc membranes into a conductance change of the ROS plasma membrane (3–7).

Superfusing intact rods with solutions containing cGMP (8) or injecting cGMP directly into the rods (9) produces results that are consistent with a cGMP-induced increase in the plasma membrane permeability to Na. In isolated ROS it has been possible to show a correlation between plasma membrane permeability and endogenous cGMP content (10). Finally, cGMP has been shown to modulate the membrane permeability of isolated discs to Na within the physiological range of cGMP concentrations (11). However, some controversies persist concerning the time course of the light-induced decrease of endogenous cGMP (4, 12–14).

On the other hand, electrophysiological experiments have demonstrated that Ca can control the light-dependent Na conductance of the rod plasma membrane (7, 15). Even if it is true that in the ROS the internal cGMP concentration is Ca dependent (16), Ca seems to modulate the sodium conductance directly and rapidly enough to rule out the possibility that its action occurs via cGMP metabolism (17). For Ca to be the internal messenger, the Ca activity in the cytoplasm should increase after rhodopsin has been bleached, in order to block the Na channels (6). This increase has been proposed to occur mainly via a light-induced release of Ca from the discs or leakage from the external medium through the plasma membrane (6). Although many experiments have attempted to show a light-induced Ca release from discs (reviewed in refs. 18 and 19), this has never been clearly demonstrated. Recently, a light-induced increase of Ca activity has been observed in the medium surrounding the rods by workers in two different laboratories (7, 20). These workers propose that the increased external activity reflects an increased Ca activity in the cytoplasm. However,

these data still fail to prove unequivocally that the released Ca is coming from the cytoplasmic space of the ROS.

In the present work, using either a perfusion or a filtration system, we have been studying whether cGMP could influence both ^{45}Ca loading and ^{45}Ca release from disc membranes. The results obtained lead us to suggest that disc membranes have binding sites for Ca that are affected by cGMP.

MATERIALS AND METHODS

Materials. Fresh bovine retinas were obtained from a local slaughterhouse (A.C.M., Reggio Emilia, Italy). All the nucleotides used were purchased from Boehringer Mannheim, and 3-isobutyl-1-methylxanthine (iBuMeXan) from Sigma. Celite was obtained from Fisher. The glass fiber prefilters were AP25 and the filters in the microcolumn were SSWP, Millipore. $^{45}\text{CaCl}_2$ was obtained from Amersham with a specific activity of 0.78 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). The Ca ionophore A23187 was a generous gift from Eli Lilly. All the other reagents, obtained from Merck, were of the best grade available and were used without any further purification. The water used was distilled twice.

Preparations. Dim red light (Kodak safety filter 1) was used throughout the whole experimental procedure.

Frog disc membranes were obtained from bullfrogs (*Rana catesbeiana*) as described (11).

Bovine disc membranes were obtained by the following procedure. Retinas from fresh cattle eyes were rinsed (3 ml per retina) with a 35% sucrose solution in NaCl buffer (100 mM NaCl/20 mM Tris-HCl, pH 7.4) and vigorously shaken for 1 min. The resulting suspension was spun at $1600 \times g$ for 5 min; the supernatant was filtered through a 400-mesh nylon gauze and, after dilution with 2 vol of NaCl buffer, pelleted by centrifugation at $1600 \times g$ for 5 min. The pellet was collected with 125 μl per retina of 28% sucrose in NaCl buffer and layered on a sucrose gradient (33 ml of 28–38% sucrose in NaCl buffer) with 25 retinas per gradient. After centrifugation for 90 min at $100,000 \times g$, the top band containing ROS was collected, diluted with 4 vol of distilled water, and centrifuged 10 min at $4500 \times g$. The pellet was resuspended in KCl buffer (150 mM KCl/2.3 mM MgCl_2 /8.5 mM Tris-HCl, pH 7.4) to a concentration of 200–250 μM rhodopsin. The absorbance ratio of 280 nm to 500 nm was 2.0 ± 0.1 . The suspension was used within 4 days of preparation.

For the experiments with the mitochondria-rich membranes, the white band underlying the ROS-containing bands in the gradient was collected and treated as was the ROS-containing band. The final membrane suspension was obtained by adding

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Abbreviations: cGMP, 3',5'-cyclic GMP; EGTA, ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid; ROS, rod outer segments of photoreceptors; iBuMeXan, 3-isobutyl-1-methylxanthine.

FIG. 1. (A) Effect of cGMP on ^{45}Ca release from preloaded frog disc membranes. The arrows indicate when the perfusing solutions were changed to test solutions containing 0.1 mM (upper curve) or 0 mM cGMP (lower curve). (B) Relationship of the magnitude of Ca release by frog disc membranes to the cGMP concentration in the perfusing solutions. The points indicate the mean value of two sets of experiments, each made with disc membranes from the same preparation.

the same amount of KCl buffer as was added to the disc membranes.

Perfusion Experiments. Disc suspension (60 μl) was incubated with 2 μCi of $^{45}\text{CaCl}_2$ for 60 min. Afterwards, the discs were collected with 800 μl of KCl/iBuMeXan buffer (0.5 mM iBuMeXan in KCl buffer) and injected into a microcolumn (80 μl) filled with Celite (11). The discs were then perfused with KCl buffer. The initial 4 ml of perfusate was discarded and then the perfusate was collected by means of an automatic drop collector (Gilson) at a rate of 10 drops per sample (15 sec per sample) with a flux of 1.04 ml/min. The solution was changed (shown by arrows in the figures) to a test solution containing the given nucleotide at 100 μM . Sometimes the perfusing solutions were made with NaCl buffer. At the end of the perfusion, the Celite containing the discs was collected. Three milliliters of a scintillation cocktail (Lumagel) was added to all samples, and their radioactivities were measured in a Packard liquid scintillation spectrometer. In the figures, the ordinates show the amount of ^{45}Ca released as percentage of the total ^{45}Ca content in the discs.

The magnitude of the cGMP effect was computed as the ratio of the peak to the base line release.

Loading Experiments. A suspension of discs (usually 7.5 nmol of rhodopsin per filter) was mixed at time $t = 0$ with the incubation mixture to have 150 mM KCl, 2.3 mM MgCl_2 , 1 mM

iBuMeXan, 10–20 μM CaCl_2 , 6 μCi of ^{45}Ca , and 8.5 mM Tris-HCl, pH 7.4. In tests of the effect of cGMP, the incubation mixture also contained 0.5 mM cGMP. At given times, samples were taken, filtered on glass fiber prefilters previously loaded with 100 mg of Celite, and washed twice with 5 ml of KCl buffer containing 0.5 mM ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA). The complete filtration procedure took about 20 sec. The filters together with the Celite were collected and mixed with 10 ml of the scintillation cocktail, and radioactivities were measured.

In some experiments (see Fig. 4) the incubation began without any cGMP. After 7 min of incubation, the incubation mixture was split into two equal parts: cGMP in concentrated solution (20 mM in KCl buffer) was added to one to make the incubation mixture 0.4 mM in cGMP; to the other, the same amount of buffer was added in order to equalize the dilution.

In two experiments, the incubation mixture also contained 0.5 mM EGTA in order to reduce the free Ca^{2+} concentration to less than 20 nM.

As a control experiment to test the possibility that cGMP directly effects Ca concentration, we found that the addition of 0.2 mM cGMP to a solution containing 1 μM CaCl_2 did not change the response of a Ca^{2+} -sensitive electrode (Nuestro Research, Salt Lake City, UT), which was sensitive to as little as 1 nM Ca.

RESULTS

Effect of cGMP on ^{45}Ca Release. The presence of 0.1 mM cGMP in the solution perfusing bovine or frog disc membranes increases the release of ^{45}Ca by a factor of 2.38 ± 0.95 (mean $\pm$ SD, 9 experiments) and 2.13 ± 0.63 (12 experiments), respectively (see Fig. 1A for an experiment with frog disc membranes).

The effect of cGMP on the ^{45}Ca release is roughly proportional to the logarithm of the cGMP concentration in the test solution, in the range of 5–150 μM (Fig. 1B).

Effect of cGMP on the ^{45}Ca Uptake. cGMP in the incubation mixture diminishes the ^{45}Ca uptake by the disc membranes as shown in Fig. 2. Without cGMP in the incubation mixture, the uptake reaches a plateau of 0.09 ± 0.02 Ca per rhodopsin (mol/mol) (11 experiments) within 30 min. The presence of 500 μM cGMP in the incubation mixture decreases the uptake capacity

FIG. 2. ^{45}Ca uptake by bovine disc membranes in the presence (○) and absence (●) of 0.5 mM cGMP in the incubation mixture. The values are normalized so that the control samples at 16 min, from different experiments, are set equal to 100. Thus comparison between different experiments is possible. The symbols are the mean of the number (shown) of experiments; bars indicate + or -SD.

by about 25%, apparently without affecting the uptake rate. This decrease of the uptake capacity is consistent with the increased ^{45}Ca release observed in the perfusion experiments.

Specificity of the cGMP Effect. The effects of other nucleotides on ^{45}Ca release were compared with those of cGMP, using the same membrane preparations. As shown in Fig. 3, neither cyclic AMP nor 5'-GMP affected ^{45}Ca release from the disc membranes in two experiments.

cGMP Effect Is Independent of Light, Triphosphates, and Ionic Composition of the Solution. The above-mentioned effects of cGMP are present in dark-adapted membranes as well as in fully bleached discs.

The ^{45}Ca uptake by the disc membranes and the cGMP effect on the uptake are unaffected by the presence of 1 mM ATP in the incubation mixture (Fig. 4A). In contrast, ^{45}Ca uptake by the mitochondria-rich membranes is dramatically enhanced by 1 mM ATP (Fig. 4B). However, cGMP is completely without effect on the ^{45}Ca uptake by these membranes. GTP is as ineffective as ATP on disc membranes.

The effect of cGMP is present in either 150 mM KCl or 100 mM NaCl.

FIG. 3. Effect of different nucleotides on ^{45}Ca release from preloaded frog disc membranes. The arrows indicate when the perfusing solutions were changed to test solutions. All perfusions were made with disc membranes from the same preparation and in each case the nucleotide concentration was 0.1 mM. cAMP, cyclic AMP.

FIG. 4. Effect of ATP (1 mM) and cGMP (0.4 mM) on the ^{45}Ca uptake by bovine disc membranes (A) and by mitochondria-rich membranes (B). ●, Absence of both ATP and cGMP; ○, absence of ATP and presence of cGMP; ▲, presence of ATP and absence of cGMP; △, presence of both ATP and cGMP.

Effect of cGMP in the Presence of EGTA or of the Ionophore A23187. The presence of 0.5 mM EGTA in the incubation mixture decreases the ^{45}Ca uptake by about 80% when compared with uptake in the absence of EGTA (two experiments). When ROS membranes, loaded in the presence of EGTA, are perfused with 0.5 mM EGTA, 0.1 mM cGMP is still able to increase the ^{45}Ca release (Fig. 5).

The presence of the ionophore A23187 in the incubation mixture (8.6 mg/liter) also inhibits the ^{45}Ca uptake by about 80% (two experiments), but when the membranes loaded in the presence of the ionophore are perfused with the ionophore still present in the perfusing solution, cGMP is unable to elicit any increase in the ^{45}Ca release (Fig. 5).

DISCUSSION

cGMP increases the release and decreases the uptake of ^{45}Ca by the disc membranes. However, cGMP does not affect the ^{45}Ca uptake and release from the mitochondria-rich membranes obtained from the retinas during the ROS purification procedure. This observation excludes the most probable contaminant of the disc membrane preparation as a target of cGMP.

These effects of cGMP on Ca differ from the effect cGMP has on disc membrane ^{22}Na fluxes (11). cGMP increases both the influx and the efflux of ^{22}Na from the disc membranes, suggesting an effect on the membrane permeability to sodium ions (11). From the present study, a permeability change to Ca may be involved only if the disc vesicles build up Ca inside. However, several lines of evidence argue against this possibility: (i) Exogenous GTP and ATP do not affect either calcium uptake or the cGMP effect on Ca uptake. Endogenous ATP and GTP

FIG. 5. Effect of 0.1 mM cGMP on ⁴⁵Ca release from bovine disc membranes loaded and perfused in the presence of 0.5 mM EGTA (▲), of the Ca ionophore A23187 (8.6 mg/liter) (■), or in their absence (●) as control. The numbers indicate the ⁴⁵Ca radioactivity (cpm) of the membranes before collection of the samples. All perfusions are made with equal amount of disc membranes from the same preparation.

have been reported to be hydrolyzed within seconds after isolation of the ROS (14, 21). Therefore, it is difficult to imagine the development of an energy-dependent Ca concentration gradient under the present experimental conditions (without added triphosphates). From the above considerations, it seems possible to rule out any active transport of calcium toward the inside of the disc vesicles. Others have also reported that no active transport is involved (18, 19). (ii) Sonicated disc vesicles, with a lower volume-to-surface ratio, show a cGMP effect similar to that obtained with nonsonicated discs (data not shown). This result is consistent with a cGMP effect on some membrane surface properties because the membrane surface area remains unchanged after sonication. On the other hand, if the effect of cGMP were on the membrane permeability, a smaller cGMP effect would be expected with the sonicated vesicles, because the sonicated vesicles can sequester less ⁴⁵Ca than the larger native membrane vesicles. (iii) cGMP affects the calcium release and uptake from repeatedly frozen and thawed disc membranes as well as it does from native discs (data not shown). It has been reported that freezing and thawing disc membranes generates inside-out vesicles (22). Thus it would seem to be possible to rule out any directional properties of the disc membrane from

the cGMP effect. One can argue, however, that the vesicles obtained by the freezing and thawing procedure may have only part of the membrane surface turned inside-out and the rest in the normal configuration. (iv) The observation that cGMP seems to influence only the plateau value of the calcium uptake (but not the rate) suggests that cGMP is most likely acting on the pool of calcium taken up and not on the membrane permeability.

Thus, instead of affecting membrane permeability to calcium, it seems that cGMP induces a decrease in the binding capacity of the disc membranes for calcium. A similar result has been reported for cardiac microsomal membranes (23).

The results obtained with the ionophore seem to show some discrepancies with a cGMP effect on binding sites. However, it has been proposed that the action of the A23187 is not just to increase the membrane permeability to calcium ions but also to release any calcium that is bound to the membranes (24, 25). This is consistent with our observations that the ⁴⁵Ca uptake by the disc membranes is lowered by the presence of the ionophore in the incubation mixture, and that the appearance of A23187 in the perfusing solution greatly increases the ⁴⁵Ca efflux from the disc membranes (data not shown). Therefore, in the presence of the ionophore, cGMP cannot release any calcium because it has already been released by A23187. A preliminary estimate of the apparent affinity constant of the cGMP-influenced binding sites from the loading and the perfusion experiments in the presence of EGTA is that it is of the same, or even higher, order of magnitude as that of EGTA, $4.9 \times 10^6 \text{ M}^{-1}$ (26).

It might seem surprising that the effects of cGMP observed in these experiments appear independent of light or dark conditions. However, the effect of light on the cGMP cascade is to control the cGMP concentration via the activation of the cGMP phosphodiesterase (27). Therefore, it is not surprising that in our system the disc membranes respond to cGMP regardless of light or dark conditions, because phosphodiesterase activity has been greatly reduced by washing the disc membranes with low ionic solutions (28) and by using iBuMeXan in the solutions (27), and because cGMP concentration is kept constant.

Finally, because physiological concentrations of cGMP (10, 14) are able to release calcium from disc membranes, the simplest inference for rod physiology is that the binding of calcium to the disc membrane sites is enhanced when the cytoplasmic cGMP concentration is reduced by the light activation of phosphodiesterase. Assuming that bovine ROS contain 50×10^7 molecules of rhodopsin and have a cytoplasmic volume of 15 fl (29), then the cGMP-induced release of 25% of the calcium molecules bound (0.09 Ca per rhodopsin) implies that the light-induced hydrolysis of cGMP can maximally decrease the cytoplasmic free calcium concentration by 130 μM .

Although the mechanism by which cGMP increases the Na permeability and causes the Ca release is unknown, it is tempting to speculate that both Ca and cGMP may intervene in regulating the Na permeability of the plasma membrane of the ROS (30).

Note Added in Proof. Since this manuscript was sent in for publication, it has been reported that cGMP decreases Ca activity in a suspension of disc membranes in 70 mM potassium isethionate/40 mM NaCl/5 mM ATP/5 mM GTP/20 mM creatine phosphate. This observation suggests a cGMP-induced Ca binding to the disc membranes (31). We repeated an efflux experiment with the same buffer and we obtained a result similar to that shown in Fig. 1A.

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1. Penn, R. D. & Hagins, W. A. (1969) *Nature (London)* **223**, 201–205.
2. Tomita, T. (1970) *Q. Rev. Biophys.* **3**, 179–222.
3. Bitensky, M. W., Gorman, R. E. & Miller, W. H. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 561–562.
4. Yee, R. & Liebman, P. A. (1978) *J. Biol. Chem.* **253**, 8902–8909.
5. Polans, A. S., Hermolin, J. & Bownds, M. D. (1979) *J. Gen. Physiol.* **74**, 595–613.
6. Hagins, W. A. (1972) *Annu. Rev. Bioeng.* **1**, 131–159.
7. Yoshikami, S., George, J. S. & Hagins, W. A. (1980) *Nature (London)* **286**, 395–398.
8. Lipton, S. A., Rasmussen, H. & Dowling, J. E. (1977) *J. Gen. Physiol.* **70**, 771–791.
9. Miller, W. H. & Nicol, G. (1979) *Nature (London)* **280**, 64–66.
10. Woodruff, M. L., Bownds, M. D., Green, S. H., Morrissey, J. L. & Shedlovsky, A. (1977) *J. Gen. Physiol.* **69**, 667–679.
11. Caretta, A., Cavaggioni, A. & Sorbi, R. T. (1979) *J. Physiol.* **295**, 171–178.
12. Kilbride, P. & Ebrey, T. G. (1979) *J. Gen. Physiol.* **74**, 415–426.
13. Kilbride, P. (1980) *J. Gen. Physiol.* **75**, 457–465.
14. Robinson, W. E. & Hagins, W. A. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 2067 (abstr.).
15. Oakely, B., III & Pinto, L. H. (1980) *Invest. Ophthalmol. Vis. Sci.*, Suppl. **19**, 102.
16. Cohen, A. I., Hall, I. A. & Ferrendelli, J. A. (1978) *J. Gen. Physiol.* **71**, 595–612.
17. Hagins, W. A. & Yoshikami, S. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1814 (abstr.).
18. Schnetkamp, P. P. M. (1980) *Biochim. Biophys. Acta* **598**, 66–90.
19. Szuts, E. Z. (1980) *J. Gen. Physiol.* **76**, 253–286.
20. Gold, G. H. & Korenbrot, J. I. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5557–5561.
21. Caretta, A. & Cavaggioni, A. (1976) *J. Physiol.* **257**, 687–697.
22. Adams, A. J., Tanaka, M. & Shichi, H. (1978) *Exp. Eye Res.* **27**, 595–605.
23. Weller, A. M. & Laing, D. (1979) *Biochim. Biophys. Acta* **551**, 406–419.
24. Brown, E. M., Gardner, D. G. & Auerbach, G. D. (1980) *Endocrinology* **106**, 133–138.
25. Borle, A. B. & Studer, R. J. (1978) *J. Membr. Biol.* **38**, 51–68.
26. Portzehl, H., Caldwell, P. C. & Rungg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591.
27. Miki, N., Keirns, J. J., Marcus, F. R., Freeman, J. & Bitensky, M. W. (1973) *Proc. Natl. Acad. Sci. USA* **76**, 3820–3824.
28. Bignetti, E., Cavaggioni, A. & Sorbi, R. T. (1978) *J. Physiol.* **279**, 55–69.
29. Mason, W. T. (1973) in *Biochemistry and Physiology of Visual Pigments*, ed. Langer, H. (Springer, New York), pp. 295–298.
30. Sorbi, R. T. (1981) in *Molecular Mechanisms of Photoreceptor Transduction*, ed. Miller, W. H. (Academic, New York), in press.
31. George, J. S. & Hagins, W. A. (1981) *Biophys. J.* **33**, 288a (abstr.).