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Calcium Release From Rod Outer Segments: Evidence For a cGMP-Sensitive Calcium Binding Protein

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Summary: Numerous studies investigating the cGMP-gated cation conductance in rod disk membranes have purported to measure efflux of Ca^{2+} entrapped in rod disk membrane vesicles. We have utilized sonication and osmotic shock as additional tests for sensitivity of cGMP- and A23187-induced Ca^{2+} release to elimination of the transvesicular Ca^{2+} gradient. We find that *1)* Treatment with sonication or osmotic shock in low Ca^{2+} medium does not release Ca^{2+} from either native cGMP/Ca^{2+}-loaded vesicles or solubilized, reconstituted "Ca^{2+}-loaded" vesicles, *2)* 70-100% of the cGMP-induced "flux" and 90-100% of the A23187-induced Ca^{2+} "flux" is insensitive to elimination of the Ca^{2+} gradient by sonication or osmotic shock in low Ca^{2+} medium, and *3)* total amount of releasable Ca^{2+} is related to membrane surface area rather than vesicle entrapment volume. We conclude that *1)* A23187 disrupts binding of Ca^{2+} to proteins and phospholipids as well as releasing entrapped Ca^{2+} and *2)* a large fraction of the cGMP-induced release observed in rod disk vesicles is due to release of bound Ca^{2+}.

The internal transmitter currently thought to mediate the process of phototransduction in the vertebrate retina is cyclic GMP. The work of Miller and Nicol (1) was the first to demonstrate cGMP regulation of rod membrane potential. Fesenko et al (2) established cGMP as the agent responsible for increasing the permeability of the rod plasma membrane to cations. The "dark current" conducted by the light-sensitive channel is composed primarily of Na^+ (and other monovalent cations, ≈90%), the balance (≈10%) being due to divalent cations flowing through the plasma membrane conductance (3). While cGMP is the secondary messenger responsible for phototrans-

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Abbreviations used are: DIFP, Diisopropyl Fluorophosphate; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; ROS, rod outer segments; RDM, rod disk membranes; CHAPS, (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate); RECON vesicles, reconstituted vesicles; PC, phosphatidylcholine.

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duction, Ca^{2+} is believed to have an important role in visual phototransduction and adaption. Calcium concentration in the rod decreases in response to light (3,4). Ca^{2+} is thought to mediate adaptation of the photoreceptor to background light (5,6). Retinal guanylate cyclase is thought to have a Ca^{2+}-dependent regulatory subunit (7).

Several proteins have been isolated from the rod outer segment which are thought to possess cGMP-gated ion channel activity (8,9,10). The channel activity of these proteins has been measured in a number of ways including the use of Ca^{2+}-sensitive dyes entrapped in the vesicles to measure Ca^{2+} influx (9,11), in the medium surrounding Ca^{2+}-loaded vesicles to measure Ca^{2+} efflux (12,13,14,15,16,17), or by the use of $^{45}Ca^{2+}$ loaded vesicles (18,19). Measuring Ca^{2+} release from vesicles has been widely used to assay for cyclic nucleotide-stimulated cation transport proteins. However, in a previous study Cavaggioni and Sorbi, measuring Ca^{2+} release from $^{45}Ca^{2+}$-loaded rod disk membrane (RDM) vesicles, concluded that the cGMP-induced release that they observed was due to release of bound, rather than efflux of entrapped, $^{45}Ca^{2+}$ (18).

In order to determine the mechanism of Ca^{2+} release from Ca^{2+}-loaded RDM vesicles we examined cGMP- and A23187-stimulated Ca^{2+} release using extravesicular Arsenazo III. We tested the effect of disruption of vesicles (by sonication and osmotic shock) and the resulting release of the vesicles' entrapped volume into the surrounding low Ca^{2+} medium upon cGMP- and A23187-induced release of Ca^{2+} from those vesicles. We find that *1)* disruption of Ca^{2+}-loaded vesicles in low-Ca^{2+} medium does not release any entrapped Ca^{2+}, *2)* cGMP- and A23187-induced Ca^{2+} release persists even in the absence of a transvesicular Ca^{2+} gradient, and *3)* absolute amount of releasable Ca^{2+} is related to surface area/protein amount and not to vesicle entrapment volume. This suggests that *1)* there is not a significant amount of free (unbound) Ca^{2+} entrapped within Ca^{2+}-loaded vesicles, *2)* very little of the Ca^{2+} released by cGMP and A23187 is due to efflux of free Ca^{2+} from the internal volume of the vesicles, and *3)* there is a cGMP-sensitive Ca^{2+}-binding protein present in the rod outer segment.

METHODS

Preparation of Rod Disk Membrane Vesicles- Rod disk membranes were isolated from bovine retinas according to the method of Koch and Kaupp (12). Fresh bovine eyes were obtained from a local abattoir and directly transported to the laboratory in the dark. Retinas were dissected from the eyes and rod outer segments were removed from the photoreceptor by high shear forces in 40% sucrose, 10 mM TRIS pH 7.4, 1 mM dithiothreitol (Pierce), 10 mM Ca^{2+} containing a cocktail of the following protease inhibitors: 5 µg/ml aprotinin (Boehringer-Mannheim), 5 µg/ml leupeptin (Boehringer-Mannheim), and 0.02% DIFP. Outer segments were separated from the other retinal components by ultracentrifugation at 100,000 x *g* on a 20-40% sucrose step gradient for 60 minutes at 4°C. The ROS were harvested and washed once in 20% sucrose, 10 mM Tris pH 7.4, 10 mM Ca^{2+}, 1 mM dithiothreitol with protease inhibitors present. ROS were frozen at -20°C until used. All manipulations were carried out in the dark or under dim red light. With the exception of those indicated otherwise, all chemicals were purchased from Sigma.

[^{3}H]Inulin Entrapment Experiments- For freeze-thaw loading of vesicles with [^{3}H]inulin, washed RDM were incubated in Buffer A containing 25 µCi/ml [^{3}H]inulin (Amersham) for 30 min. at 4°C followed by two freeze-thaw cycles. Sonication-loaded PC and RDM vesicles were prepared by

sonicating concentrated PC vesicles (50 mg/ml) or RDM membranes in Buffer A-[^{3}H]inulin for 45 sec using a Branson 450 probe sonifier (power 2, 50% duty cycle) at ice temperature. All [^{3}H]inulin-loaded vesicles were then dialyzed for 48 hr against Buffer A (three changes) and then 48 hr against Buffer B (three changes). Aliquots of the [^{3}H]inulin-loaded vesicles were then diluted into 3.5 ml of Buffer B in plastic cuvettes and sonicated (50% duty cycle, power setting 2). Vesicles were osmotically shocked by dilution into 3.15 ml of 10 mM HEPES, pH 7.4. KCl was then added back to 100 mM. The samples were then filtered on Millipore HA 0.45 μm or VM 0.05 μm nitrocellulose filters (for RDM vesicles) or Pharmacia/LKB Nova PES 100 kdalton filters (for PC vesicles) in a Millipore vacuum manifold and rinsed 3-fold with Buffer B. The filters were dried, solubilized with scintillation cocktail and counted in a Beckman Rackbeta scintillation counter.

Ca^{2+} Loading of Vesicles- For loading with Ca^{2+}, ROS were thawed and incubated in 10 mM Ca^{2+}, 100 mM KCl, 10 mM HEPES, pH 7.4 (Buffer A) with 140 μM cGMP present for 15 minutes at room temperature.[12] Excess Ca^{2+} was removed by dilution of the RDM in a 40x volume of 100 mM KCl, 10 mM HEPES, pH 7.4 (Buffer B), followed by centrifugation of the rod disk membranes (15 minutes, 4°C, 39,000 x *g*) and resuspension in Buffer B.

Ca^{2+} Measurements- Ca^{2+} measurements were made in a Shimadzu UV-3000 spectrophotometer in dual wavelength mode ($A_{650/730}$). Rod disk membranes were diluted into 3.5 ml of Buffer B containing 70 μM Arsenazo III in a stirred cuvette at a concentration of 7.5 μM rhodopsin. A baseline level of absorbance was determined and, if the vesicles were to be disrupted by sonication, the cuvette was removed from the spectrophotometer, placed in a room temperature water bath, and the vesicles disrupted using a Branson 450 micro-tip probe sonifier. Osmotic shock of vesicles was effected by dilution of the vesicles into 3.15 ml of 10 mM HEPES, pH 7.4, followed by addition of KCl and Arsenazo III to 100 mM and 70 μM, respectively. Experiments were begun by the addition of test substitute through a port in the spectrophotometer sample compartment. The ionophore A23187 was added (final concentration, 7.5 μM) subsequent to cGMP injections in order to measure the total releasable Ca^{2+} associated with the membranes. Ca^{2+} (1 μM final concentration) was injected into the cuvette at the end of the experimental run as a calibration standard. Spectrophotometric data were recorded digitally at a sampling rate of 10/sec using an IBM/AT computer equipped with an A/D converter. The data were corrected for baseline drift by subtraction of a linear baseline obtained from the trace before the addition of cGMP or A23187.

Preparation of Reconstituted Vesicles- Reconstituted vesicles were made by solubilization of extensively washed RDM with Buffer B containing 2 mM $CaCl_2$, 1 mM dithiothreitol, 2 mg/ml soybean phosphatidylcholine (Calbiochem), 15 mM CHAPS, 5 μg/ml aprotinin (Boehringer-Mannheim), 5 μg/ml leupeptin (Boehringer-Mannheim), 0.02% DIFP, and centrifuged for 60 min at 105,000 x *g*. The rhodopsin concentration of the supernatant was determined spectrophotometrically, the solubilized material was bleached, then dialyzed against Buffer A containing 1 mM dithiothreitol and protease inhibitors for 48 hr (three buffer changes) followed by Buffer B containing 1 mM dithiothreitol and protease inhibitors for 48 hr (two buffer changes).

RESULTS

In order to validate the methods utilized to disrupt vesicles, [^{3}H]inulin-containing vesicles were subjected to either sonication or osmotic shock. Three types of vesicles were tested: native RDM vesicles loaded with [^{3}H]inulin by 2 freeze-thaw cycles, sonication-loaded RDM vesicles and phosphatidylcholine (PC) vesicles loaded by sonication. Figure 1 shows the amount of [^{3}H]inulin remaining with the vesicles after varying amounts of disruption. Sonications of two seconds were sufficient to release ≈50% of the entrapped [^{3}H]inulin from all vesicles types tested; 8 sec sonications were sufficient to release more than 90% of the entrapped [^{3}H]inulin. Osmotic shock of the vesicles released ≈80% of the entrapped [^{3}H]inulin in each of the vesicle types. The efficacy of vesicle disruption/entrapped solute release by sonication and osmotic shock was confirmed by measuring release of horseradish peroxidase activity entrapped in PC vesicles (data not shown).

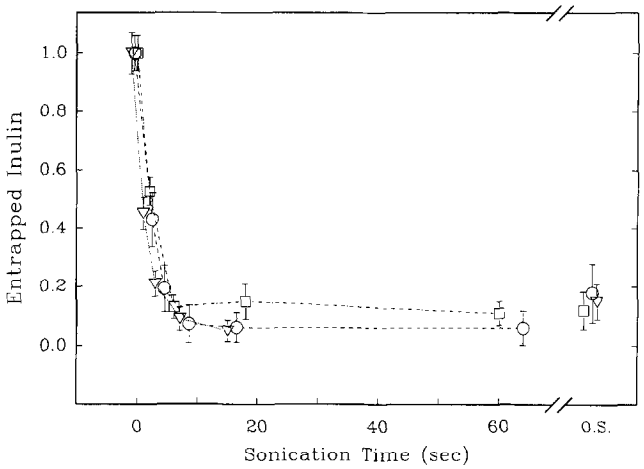


Figure 1. Effect of sonication and osmotic shock on [^{3}H]inulin entrapment. Freeze-thaw loaded RDM vesicles (open squares), sonication-loaded PC vesicles (inverted triangles) and sonication-loaded RDM vesicles (open circles) were disrupted by the indicated length of sonication or osmotically shocked (O.S.). The fraction of total entrapped [^{3}H]inulin remaining with the vesicles is plotted as a function of length of sonication and compared with osmotic shock disruption.

As shown in figure 2A, Ca^{2+} was liberated from native rod disk membrane vesicles in response to addition of cGMP. Neither cAMP, GTP, GDP or GMP caused Ca^{2+} release from the vesicles. Surprisingly, disruption of the vesicles by either sonication or osmotic shock in low Ca^{2+} buffer prior to addition of either cGMP or A23187 did not release any Ca^{2+} (data not shown). Equally surprising was the finding that vesicles which had undergone disruption in low Ca^{2+} buffer exhibited cGMP-induced Ca^{2+} release qualitatively similar to that of nondisrupted vesicles. Neither the amplitude nor the velocity of the cGMP-induced Ca^{2+} release was significantly altered by elimination of the transvesicular Ca^{2+} diffusion gradient. The Ca^{2+} ionophore A23187 was able to

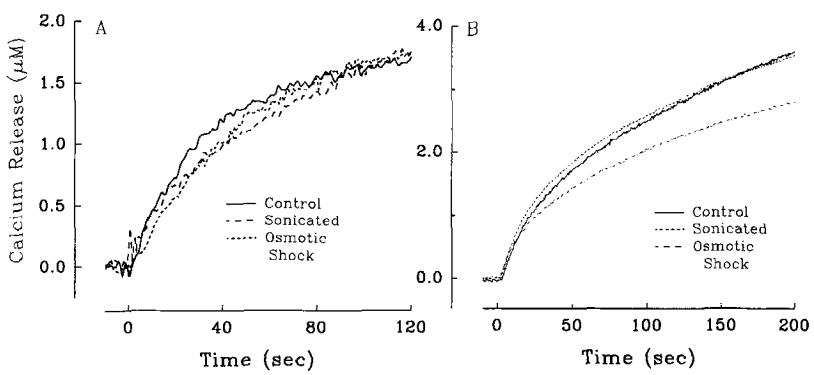


Figure 2. Effect of sonication and osmotic shock on Ca^{2+} release from RDM vesicles. ***A,*** Comparison of Ca^{2+} release in response to addition of 142 μM cGMP, control versus disrupted vesicles. Sonication duration was 10 sec. ***B,*** Comparison of Ca^{2+} release in response to addition of 7.5 μM A23187. All records were calibrated against injections of 1 μM $CaCl_2$. Baseline drift was adjusted by subtraction of a linear baseline fitted to a region of the trace prior to addition of cGMP or A23187. Sonication had no effect on the Ca^{2+} sensitivity of Arsenazo III (determined by Ca^{2+} injections).

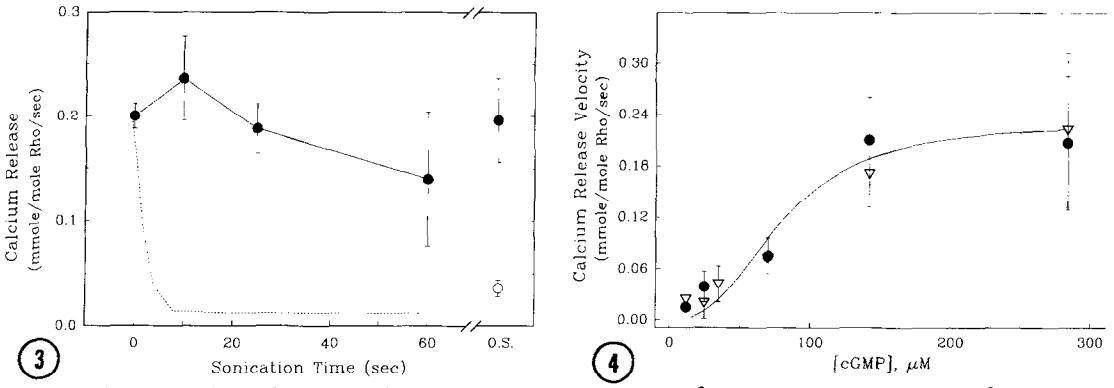


Figure 3. Effect of sonication/osmotic shock on velocity of Ca^{2+} release. cGMP-activated Ca^{2+} release velocities for several sonication durations and osmotic shock (filled circles) were calculated from traces such as those shown in figure 2. Recording, sonication and osmotic shock techniques used were the same as for the experiments shown in figure 2. cGMP was added to a final concentration of 142 μM to initiate Ca^{2+} release. Baseline-corrected initial slope values were calculated from the initial 6 sec following addition of cGMP. The data points and error bars represent mean ±SEM of three determinations at each sonication duration and for osmotic shock disruption. Inulin release data from figure 1 are superimposed (dashed line, open circle) for comparison.

Figure 4. cGMP dose-dependency of Ca^{2+} release, control versus disrupted RDM vesicles. Velocity data derived from addition of indicated concentrations of cGMP are plotted as a function of [cGMP]. Control responses, open inverted triangles. Responses obtained after 10 sec sonication, filled circles. Error bars represent SEM, n=3. The smooth curve is a hill function fit to the data with m = 2.7 and $K_{½}$= 85 μM.

release significant amounts of Ca^{2+} from vesicles which had already undergone cGMP-induced Ca^{2+} release. Figure 2B shows the ionophore-induced release of Ca^{2+} from the cGMP treated vesicles. The A23187-induced release was also insensitive to Ca^{2+}-gradient elimination by either sonication or osmotic shock in low Ca^{2+} buffer.

Figure 3 shows the effect of sonication time on absolute velocity of cGMP-activated Ca^{2+} release from RDM vesicles (filled circles). The effect of osmotic shock on Ca^{2+} release velocity is also illustrated at the right of the graph. Superimposed on the velocity measurements are the [^{3}H]inulin entrapment data for freeze-thawed RDM vesicles from figure 1 (dashed line). Velocity of Ca^{2+} release was not significantly affected by sonication durations up to 60 sec or by osmotic shock disruption. Absolute amplitude of cGMP-activated Ca^{2+} release was not affected by either the 1-60 sec range of sonication time nor by osmotic shock (data not shown). A23187-induced Ca^{2+} release velocity and amplitude were not significantly affected by osmotic shock or sonications of up to 60 sec (data not shown).

The dose dependency of the cGMP-activated Ca^{2+} release appeared unaffected by disruption by sonication. Figure 4 compares the dose-response relation of control cGMP-activated Ca^{2+} responses with cGMP-activated responses evoked after a 10 sec sonication. All Ca^{2+} release velocities plotted in figure 4 are from the same batch of RDM vesicles. They reflect the persistence of the Ca^{2+} release velocity over a range of cGMP concentrations even after elimination of the Ca^{2+} diffusion gradient by sonication. The hill function fitted to all points, control and disrupted, in the graph had a slope of 2.7 and semisaturating [cGMP] of 85 μM.

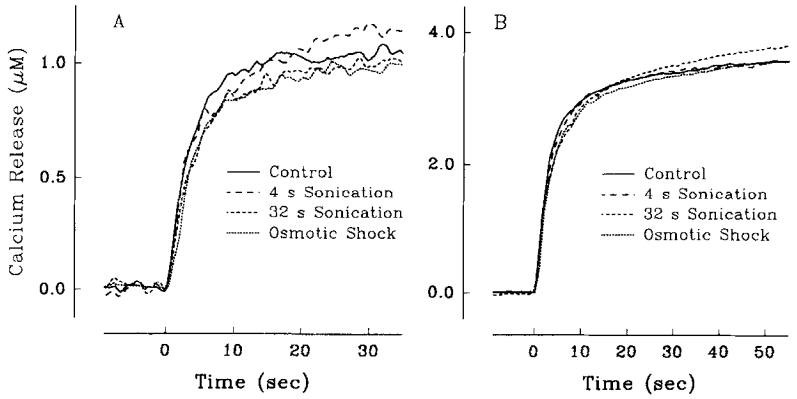
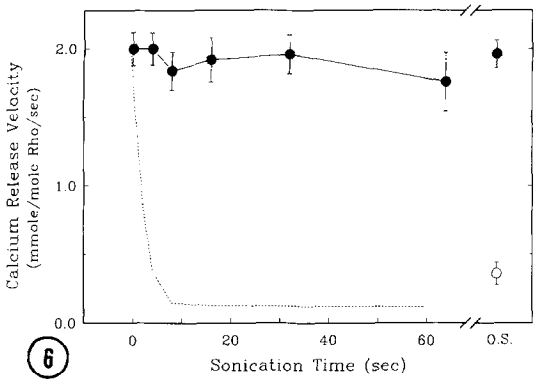


Figure 5. Effect of sonication and osmotic shock on Ca^{2+} release from RECON vesicles. ***A,*** Comparison of Ca^{2+} release in response to addition of 125 μM cGMP in control and disrupted RECON vesicles. Sonication duration was 10 sec. ***B,*** Comparison of Ca^{2+} release in response to addition of 7.5 μM A23187. All records were calibrated against additions of 1 μM $CaCl_2$. Recording conditions were identical to those described in figure 2.

Vesicles formed by CHAPS solubilization of rod disk membranes (RECON vesicles) also exhibited cGMP-induced Ca^{2+} release. Figure 5A shows the cGMP-induced release of Ca^{2+} from the RECON vesicles. As was the case for RDM vesicles, elimination of the Ca^{2+} diffusion gradient by sonication or osmotic shock had little or no effect on the release of Ca^{2+} from these vesicles. Neither sonication nor osmotic shock released any Ca^{2+} from RECON vesicles (data not shown). Addition of A23187 to cGMP treated vesicles caused release of additional Ca^{2+} from the RECON vesicles (figure 5B), release which was insensitive to elimination of the Ca^{2+} diffusion gradient.

Figure 6 shows the effect of sonication time on absolute velocity of cGMP-activated Ca^{2+} release from RECON vesicles (filled circles). The effect of osmotic shock on Ca^{2+} release velocity is also illustrated at the right of the graph. Superimposed on the velocity measurements are the [^{3}H]inulin entrapment data for sonicated RDM vesicles from figure 1 (dashed line, open circle). Velocity of Ca^{2+} release was not significantly affected by sonication durations up to 64 sec nor by osmotic shock disruption. Absolute amplitude of cGMP-activated Ca^{2+} release was not significantly affected by either sonication (up to 64 sec) or osmotic shock (data not shown). A23187-induced Ca^{2+} release velocity and amplitude were also not significantly affected by osmotic shock or sonications of up to 64 sec (data not shown).

The dose dependency of the cGMP-activated Ca^{2+} release from RECON vesicles appeared unaffected by vesicle disruption by sonication or osmotic shock. Figure 7 shows the dose-response relation of control cGMP-activated Ca^{2+} responses, cGMP-activated responses evoked after sonication, and responses evoked after disruption by osmotic shock. All Ca^{2+} release velocities plotted in figure 7 are from the same batch of RECON vesicles and represent absolute velocities; they reflect the persistence of the Ca^{2+} release velocity even after elimination of the Ca^{2+} diffusion gradient by sonication or osmotic shock. The hill function fitted to all points, control and disrupted, in the graph had a slope of 3.5 and $K_{\frac{1}{2}}$ of 45 μM.



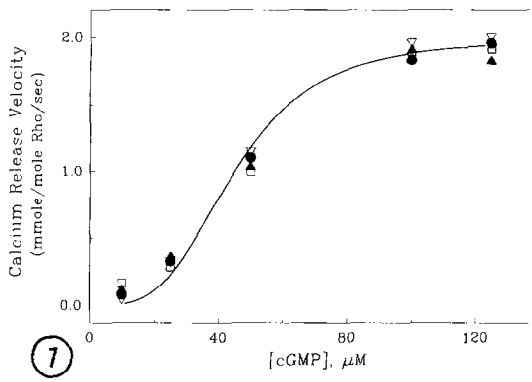


Figure 6. Effect of sonication/osmotic shock on velocity of Ca^{2+} release from RECON vesicles. cGMP-activated Ca^{2+} release velocities for several sonication durations and osmotic shock (filled circles) were calculated from traces such as those shown above. Recording, sonication and osmotic shock techniques, and calculation of slope values were the same as in figure 3.5 μM. cGMP was added to a final concentration of 125 μM to initiate Ca^{2+} release. The data points and error bars represent mean ±SEM of four determinations, respectively, at each sonication duration and for osmotic shock disruption. Inulin release data from figure 1 are superimposed (dashed line, open circle) for comparison.

Figure 7. cGMP dose-dependency of Ca^{2+} release, control versus disrupted RECON vesicles. Velocity data derived from addition of indicated concentrations of cGMP are plotted as a function of [cGMP]. Control responses, inverted triangles. Responses obtained after 8 sec sonication, filled circles. Responses obtained after osmotic shock, filled squares. The smooth curve is a hill function fitted to all the data points, m = 3.5, $K_{½}$ = 45 μM.

DISCUSSION

We interpret these results to indicate that cGMP and A23187 release Ca^{2+} bound to the surface(s) of "Ca^{2+}-loaded" vesicles and that very little Ca^{2+} associated with the vesicles is not bound to the vesicle. Approximately 15-45% of the total releasable Ca^{2+} in RDM and RECON vesicles appeared to be sensitive to cGMP. The reason as to why there is little or no free Ca^{2+} entrapped by the vesicles is not readily apparent. The RDM were isolated and stored in buffers containing 10 mM Ca^{2+}. The native RDM vesicles were calcium-loaded in the presence of 10mM Ca^{2+} and 140 μM cGMP before use in order to assure full loading with Ca^{2+}. These conditions should allow the vesicles to load Ca^{2+} through the cGMP-gated ion channel. The RECON vesicles were reconstituted in buffer containing 10 mM Ca^{2+} and should also have entrapped this concentration of Ca^{2+}. That the vesicles do entrap solutes was demonstrated by the [^{3}H]*inulin* entrapment experiments.

The lack of Ca^{2+} release due to sonication and osmotic shock and the insensitivity of release (for both cGMP and A23187) to elimination of the Ca^{2+} gradient are not consistent with a large pool of free Ca^{2+} in solution within the vesicles. The vesicles were sonicated after dilution into a low Ca^{2+} buffer, conditions which supposedly set up a diffusion gradient for the movement of free Ca^{2+}. If Ca^{2+} were in free solution within the vesicles then sonication of the vesicles should liberate this Ca^{2+}. We conclude from the sonication data that the Ca^{2+} is bound to the surfaces of the vesicles and not in solution.

Another feature of the invariance of Ca^{2+} release from RDM vesicles favoring the notion that release of Ca^{2+} by cGMP and A23187 is from a binding site and not the internal volume of the RDM

vesicles is that the diameter of RDM vesicles, hence, their volume, is markedly decreased by sonication. The mean diameter of native RDM vesicles is approximately 1 μm (16). Sonication of RDM yields homogeneous vesicles with a mean diameter of 0.15 μm (16). This reduces the volume:membrane surface area ratio of the vesicles by a factor 6.7. If the source of the Ca^{2+} were solely the internal volume of the vesicles the amount of Ca^{2+} releasable from the sonicated vesicles [at a given protein (rhodopsin) concentration] should decrease by a factor of 6.7. However, the amount of Ca^{2+} released from intact and sonicated RDM vesicles induced by cGMP or A23187 was roughly equal. Because the absolute amount of Ca^{2+} releasable from "Ca^{2+}-loaded" vesicles is unrelated to entrapment volume, but remains constant for a given amount of membrane surface area and/or protein, it is unlikely that the source of the Ca^{2+} releasable by cGMP and A23187 is free entrapped Ca^{2+}.

One important aspect of these results is that the fact that ionophore A23187 has at least two modes of action, working to release bound Ca^{2+} as well as its recognized role as a Ca^{2+} ionophore. This has been reported by other investigators, as well (18). Invoking abolition of cGMP-induced Ca^{2+} release by A23187 as proof that cGMP-induced Ca^{2+} release is "flux", therefore, is flawed because of the possibility that A23187 might release *bound* Ca^{2+} otherwise releasable by cGMP. This is applicable to other systems where abolition of a response by A23187 is solely attributed to its action as a Ca^{2+} ionophore.

At present, the identity of the cGMP-sensitive Ca^{2+} binding site(s) is unknown. Both the RDM and RECON vesicles were stripped of soluble proteins, ruling out contribution from a soluble protein component of ROS. Ca^{2+}-loaded phospholipid vesicles (containing no protein) do not liberate Ca^{2+} in response to cGMP (data not shown). It is probable, therefore, that the cGMP-induced Ca^{2+} release observed in RDM and RECON vesicles is due to a cGMP-sensitive Ca^{2+}-binding membrane protein contained in rod outer segments. Owing to the many effects of Ca^{2+} on the biochemistry and physiology of rod photoreceptors, identification of this Ca^{2+}-binding protein may prove extremely important to our understanding of phototransduction and adaptation.

REFERENCES

1. Miller, W.H., and Nicol, G.D. (1979) Nature (Lond.) **280**, 64-66.

2. Fesenko, E.E., Kolesnikov, S.S., and Lyubarsky, A.L. (1985) Nature (Lond.) **313**, 310-313.

3. Yau, K.-W., & Nakatani, K. (1984) Nature (Lond.) **311**, 299-305.

4. Ratto, G.M., Payne, R., Owen, W.G., & Tsien, R.Y. (1988) J. Neurosci. **8**, 3240-3246.

5. Matthews, H.R., Murphy, R.L.W., Fain, G.L., & Lamb, T.D. (1988) Nature (Lond.) **334**, 67-69.

6. Nakatani, K., & Yau, K.W. (1988) Nature (Lond.) **334**, 69-71.

7. Koch, K.W., & Stryer, L. (1988) Nature (Lond.) **334**, 64-66.

8. Clack, J.W., and Stein, P.J. (1988) Proc. Natl. Acad. Sci. (USA) **85**, 9806-9810.

9. Matesic, D., and Liebman, P.A. (1987) Nature (Lond.) **326**, 600-603.

10. Cook, N.J., Hanke, W., and Kaupp, U.B. (1987) Proc. Natl. Acad. Sci. (USA) **84**, 585-589.

11. Caretta, A., & Cavaggioni, A. (1983) Eur. J. Biochem. **123**, 1-8.

12. Koch, K.W., and Kaupp, U.B. (1985) J. Biol. Chem. **260**, 6788-6800.

13. Kaupp, U.B., & Koch, K.W. (1984) Vision Res. **24**, 1477-1479.

14. Cook, N.J., Zeilinger, C., Koch, K.-W., & Kaupp, U.B. (1986) J. Biol. Chem. **261**, 17033-17039

15. Koch, K.W., and Kaupp, U.B. (1987) J. Biol. Chem. **262**, 14415-21.

16. Bauer, P.J. (1988) J. Physiol. (Lond.) **401**, 309-327.

17. Cook, N.J., Molday, L.L., Reid, D., Kaupp, U.B., & Molday, R.S. (1989) J. Biol. Chem. **264**, 699 6999.

18. Cavaggioni, A., & Sorbi, R. (1981) Proc. Natl. Acad. Sci. USA **78**, 3964-3968.

19. Puckett, K.L., and Goldin, S.M. (1986) Biochemistry **25**, 1739-1746.