andard centrifugation condi-
or.
performed under a safelight
nes are separated from solu-
centrifugal pellet, are sus-
protein) by drawing the mix-
) 50 times; the suspension is
The extraction procedure is
s (soluble protein, typically
all portions at −50°.
on of sealed outer segments
er segments that have been
rifugal pellet, and the mem-
vith membrane buffer, sus-
r use the nucleotide bind-
ation procedure), bleached
°. The membranes obtained
ed outer segments may be
me specific capacity to bind
inding[3] but give a slightly

outer segment soluble pro-
membranes (an amount of
d the components of reac-
buffer). The mixture, in a
and shaken gently for 30
ayed; over 90% of the nu-
membranes and sediment.
out of a Pasteur pipet) in
nd centrifuged. This wash-
ernatants (which together
binding capacity originally
hed in the same fashion,
embrane protein) contain-
to stand for 5 min before
nen centrifuged for 30 min
nuted membranes) and as-
ith GTP may be combined
mGTPase. Typical results

ould give only two bands

PURIFICATION OF THE AmGTPase

Fraction	Protein (mg)	Nucleotide binding capacity (nmol)	Specific nucleotide binding capacity (nmol/mg protein)
Sealed rod outer segments	11[a]	5.2	0.47
Outer segment soluble protein	3.0[b]	4.1	1.4[c]
Purified AmGTPase	0.50	3.2	6.4

[a] Lowry method; this is the yield from approximately 20 retinas.
[b] Dye-binding method; for soluble protein extracted in the absence of mercaptoethanol, Lowry values are 0.9 times the dye-binding values.
[c] For comparison, values for membranes from disrupted and intact ROS (assayed without added soluble protein) were 0.08 and 0.18 nmol/mg protein, respectively.

(Coomassie Brilliant Blue staining) on SDS–polyacrylamide gel electrophoresis. These bands are of approximately equal intensity and correspond to molecular weights of 41,000 and 37,000. The purified protein is stable for 3–4 hr in reaction buffer at 0°, but 30–50% of the nucleotide binding capacity is lost on standing (or dialysis) for 18 hr. Even more capacity is lost on freezing and thawing of the purified protein (though the nucleotide binding capacity of crude soluble protein is quite stable to freezing). If mercaptoethanol is not present, the AmGTPase loses nucleotide binding capacity with a half-time of approximately 2 hr at 0°.

[78] Detection and Properties of Rapid Calcium Release from Binding Sites in Isolated Rod Outer Segments upon Photoexcitation of Rhodopsin

By U. BENJAMIN KAUPP and WOLFGANG JUNGE

Introduction

The suggestion that calcium is the transmitter between photoexcited rhodopsin and voltage transients at the plasma membrane of retinal rods has stimulated intensive research on calcium metabolism in vertebrate photoreceptors, especially under illumination conditions (for reviews, see Refs. 1, 2). After almost a decade of intensive research it is only fair to note that experimental evidence in favor of a transmitter role for calcium

[1] H. G. Smith, Jr., R. S. Fager, and B. J. Litman, *Biochemistry* **16**, 1399 (1977).
[2] U. B. Kaupp, P. P. M. Schnetkamp, and W. Junge, *in* "Detection and Measurement of Free Calcium in Cells" (C. C. Ashley and A. K. Campbell, eds.), p. 287. Elsevier/North-Holland Publ., Amsterdam, 1979.

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is only circumstantial and no unequivocal conclusions have been reached.[3]

The present article is mainly concerned with the technical aspects of time-resolved and quantitative measurements of calcium release in various preparations of cattle rod outer segments. Calcium release was followed by means of flash spectrophotometry with the calcium indicator arsenazo III.

Sample Preparation

Intact cattle rod outer segments are isolated on a continuous sucrose/Ficoll 400 density gradient[4] (see also Schnetkamp and Daemen, this volume, Article [17]). The final stock suspension of intact rod outer segments contains 150–200 μM rhodopsin and can be stored for at least three days at 4° without any detectable loss of the calcium release capacity. For release experiments in a flash spectrophotometer, the stock suspension is diluted 40-fold with standard medium, containing sucrose, 600 mM; Ficoll-400, 5% w/w; Tris-HCl buffer, 20 mM at pH 7.4. Calcium release capacity and calcium binding in cattle rod outer segments are very sensitive to the presence of monovalent cations[5,6] and therefore the sucrose/Ficoll-400 medium has been passed over a mixed-bed ion-exchange column. The final rhodopsin concentration in the cuvette is 3–6 μM. The free calcium concentration of a diluted sample is usually 2–6 μM introduced by the stock suspension of rod outer segments. In a subsequent step 30 μM arsenazo III and 10 μM of the divalent cation ionophore A23187 are added to the suspension. The A23187-induced calcium efflux from rod outer segments is monitored spectrophotometrically. Normally, cattle rod outer segments contain 1–2 calcium/rhodopsin. The calcium content depends, however, on the calcium concentration of the isolation medium.[7] On addition of A23187, endogenous calcium is released. The free calcium concentration in the cubette increases (by the A23187-mediated calcium efflux) to about 5–10 μM. When the free calcium concentration rises above the sensitivity range of arsenazo III ($\simeq 15\ \mu M$), it has to be adjusted to an appropriate level by addition of small aliquots of an EDTA or EGTA solution.

[3] W. L. Hubbell, and M. D. Bownds, *Annu. Rev. Neurosci.* **2**, 17 (1979).
[4] P. P. M. Schnetkamp, A. A. Klompmakers, and F. J. M. Daemen, *Biochim. Biophys. Acta* **552**, 379 (1979).
[5] U. B. Kaupp, P. P. M. Schnetkamp, and W. Junge, *Nature (London)* **286**, 638 (1980).
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Detection of Rapid Calcium Release

After the suspension has reached a stable absorption at 655 nm, which is indicative of complete equilibration of calcium and recovery from the pipetting step, the cuvette is placed into a kinetic flash spectrophotometer. The sample is excited by a short flash of light and transients of the free calcium concentration are followed via the absorption changes of arsenazo III at 655 nm. The methodology and instrumentation of time-resolved flash spectrophotometry have been reviewed elsewhere and the interested reader may refer to this source.[8]

Illumination of rod outer segments induces a photochemical reaction of rhodopsin and causes structural changes both of rhodopsin and of the disk membrane. This is indicated by true and apparent (because of light-scattering changes) absorption changes, which are also superimposed on calcium-indicating absorption changes of arsenazo III at 655 nm. Misinterpretations arising from apparent calcium-indicating absorption changes of arsenazo III may be caused by the following:

1. Changes of adsorption of the dye by disk membranes following excitation by light
2. Superposition of absorption changes of the dye by those resulting from rhodopsin photochemistry
3. Light-scattering transients mistaken for true absorption changes
4. The response of arsenazo III to ions other than calcium (e.g., H^+)
5. Direct photochemical reactions of arsenazo III proper

We observed that the latter three (light scattering, pH transients, and photochemistry of arsenazo III) are the most important sources of artifacts. Appropriate controls are given in the following.

Light-Scattering Transients

Apparent absorption changes due to an angular redistribution of scattered light can be minimized by placing the cuvette in front of the cathode window of the photomultiplier to capture as much scattered light as possible. Either of the following measures can be employed to evaluate possible contributions of light-scattering changes to the apparent calcium-indicating absorption changes of arsenazo III at 655 nm.

1. Recording of flash-induced absorption changes at 575 nm (isosbestic point of arsenazo III at pH 7.0) or at wavelengths > 710 nm, where the differential sensitivity of arsenazo III $dA/d\mathrm{Ca}^{2+}$ is virtually zero.

[8] W. Junge, *in* "Chemistry and Biochemistry of Plant Pigments" (T. Goodwin, ed.), Vol. 2, p. 233. Academic Press, New York, 1976.

2. Inclusion of calcium buffers [e.g., nitrilotriacetic acid (NTA) or EGTA] in the suspension. The free calcium concentration of the buffer has to be adjusted to the same value as for light-stimulated calcium release measurements.

3. Saturation of arsenazo III with calcium (only arsenazo III–calcium complex present) such that it is no longer responsive to changes in free calcium. However this procedure may fail if light-scattering signals are strongly calcium dependent.

Correction of calcium release signals for contributions from light scattering can be achieved by dual-wavelength spectrophotometry. Calcium-indicating absorption changes are recorded at 655 nm and simultaneously at a wavelength at which arsenazo III does not respond to calcium. The smooth wavelength dependence of scattering changes created by flash excitation makes the correction straightforward. According to Uhl,[9] the ratio of apparent absorption changes because of light-scattering transients at 650–700 nm is approximately 1.1. However, this procedure is not easily applied to any cell suspension. For example, chloroplast suspensions show a more complicated spectrum of light-stimulated scattering changes and quantitative assessments may not be as easy as with rod outer segments. Calcium-indicating absorption changes in intact and fragmented rod outer segment suspensions are stable and highly reproducible even when samples from different batches are compared. Thus it is even possible to record absorption changes due to calcium release and apparent absorption changes resulting from light-scattering transients in succession. If both signals are stored on a signal averager, they can be subsequently subtracted from each other by a data reduction program.

In our most intact rod outer segments, no light-initiated scattering changes were detected. However, after freeze-thawing of rod outer segments or treatment with 100 m*M* Tris buffer, some light-scattering signals could be observed. Under these conditions apparent absorption changes due to light scattering made up maximally 30% of the calcium-indicating absorption changes at 655 nm. In sonicated disk vesicles a small light-scattering transient was observed (5–10% of calcium-indicating absorption changes) that probably arises from a change of the refractive index of disk membranes associated with rhodopsin bleaching.[9]

pH Changes

Light-stimulated pH changes in rod outer segment suspensions created by rapid proton uptake of rhodopsin during the metarhodopsin I/metarhodopsin II transition[10] can give rise to a pH-indicating absorption change of arsenazo III. However, light-triggered pH changes are easily suppressed by appropriate buffering of the suspension. With bromcresol purple, a pH indicator more sensitive than arsenazo III, it was found that 2 m*M* of any buffer (e.g., MOPS, HEPES at pH 6.1–6.5) is usually sufficient to suppress the pH-indicating absorption changes of arsenazo III in a rod outer segment suspension. However, the buffering capacity has to be increased under some conditions. For example, the sensitivity to pH changes of the arsenazo III–magnesium complex is larger than that of the calcium complex.[11] When aiming at calcium release in the presence of magnesium, higher buffer concentrations are required for suppression of pH signals.

Photochemistry of Arsenazo III

Arsenazo III solutions undergo a flash-induced absorption change that has been attributed to a photochemical reaction of arsenazo III proper.[2] An example of this photochemistry is given in Fig. 1. The absorption change has a half-time of decay $\tau_{1/2} = 5$ msec and is fully reversible; even after repetitive excitation with YAG laser pulses (100 flashes, flash energy 7 mJ, excitation wavelength 530 nm, half-duration of the light pulse 10 nsec) flash-induced absorption changes were unchanged. This demonstrates that no photo damage of the dye has occurred.

In intact rod outer segments the rapid photochemical reaction of arsenazo III is not disturbing because the rate of calcium release is more than one order of magnitude slower than the decay of the photochemical signal (even at high concentrations of A23187, where the velocity of calcium release saturates[12]). This differs from the situation in sonicated disk vesicles where calcium release becomes more rapid (half-rise time $\tau_{1/2} = 10$ msec at 20° and pH 7[5]). If use is made of a signal averager, it is possible to subtract the intrinsic arsenazo III response from the response to calcium released by retinal disks. An example of this correction procedure is given in Fig. 2. The upper trace represents flash-induced absorption changes of arsenazo III from sonicated disk vesicles in the presence of A23187. This signal is a composite of a calcium signal and an intrinsic signal. The small deflection occurring immediately after the flash indicates the photochemical reaction of arsenazo III proper (Fig. 2A). Then the flash-induced absorption changes were recorded again but in the presence

[9] R. Uhl, Doctoral Thesis, Freiburg (1976).

[10] H. M. Emrich, *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol.* **26,** 352 (1971).

[11] J. T. Russell and A. N. Martonosi, *Biochim. Biophys. Acta* **544,** 418 (1978).

[12] U. B. Kaupp, P. P. M. Schnetkamp, and W. Junge, *Biochim. Biophys. Acta* **552,** 390 (1979).

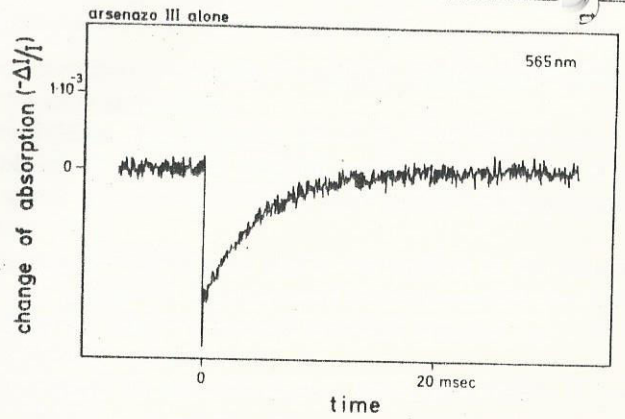

FIG. 1. Time course of absorption change at 565 nm after excitation of an anarsenazo III solution by a flash at $t = 0$. Medium:arsenazo III, 6 μM; Tris-HCl buffer, 20 mM at pH 7.4; EGTA, 1 mM. Cuvette pathlength, 20 mm; energy of flash, 7 mJ cm^{-2}; excitation wavelength, 530 nm; half-duration of flash, 10 nsec.

of 0.5 mM calcium (Fig. 2B, here the calcium indicator is saturated). The difference of both absorption changes (A − B) is also shown (Fig. 2A-B). All signals were obtained by averaging from one sample over 16 repetitions. Although the photochemistry of arsenazo III is somewhat calcium sensitive,[2] the difference can be neglected here. When the photochemistry of arsenazo III is recorded in the presence of calcium buffers or in a sample in which rhodopsin has been completely bleached, similar results are obtained. In order to obtain accurate results, the photochemistry of arsenazo III has to be recorded at the same wavelength at which calcium release is followed because the flash-induced intrinsic signal exhibits a complicated spectrum.[2]

Sensitivity and Time Resolution

Addition of calcium to a suspension of rod outer segments, yielding a change of the total calcium concentration by 1 μM, causes a change in the relative transmission (mainly arsenazo III) at 655 nm $\Delta I/I = 3 \times 10^{-2}$ (other conditions: rhodopsin, 3.5 μM; arsenazo III, 30 μM; A23187, 10 μM; pH 7; free calcium concentration 3–4 μM). The flash-induced absorption change in the lower part of Fig. 2 ($\Delta I/I = 1.4 \times 10^{-3}$) then indicates a change in the calcium concentration of $\Delta Ca = 5 \times 10^{-8}$ M. Approximately 3% of the rhodopsin molecules were bleached per flash (16 repetitions). The signal-to-noise ratio in Fig. 2A-B is 7.5 (peak-to-peak noise). The time resolution as limited by the averaging computer is

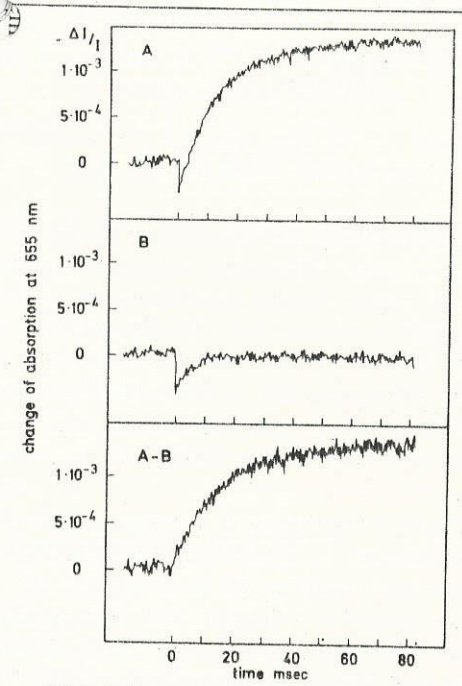

FIG. 2. Time course of light-induced absorption changes at 655 nm after excitation of sonicated rod outer segments by a flash at $t = 0$ in the presence of arsenazo III and ionophore A23187. Suspension medium: (A) sucrose, 600 mM; HEPES, 2 mM; Tris, 0.5 mM at pH 6.45; arsenazo III, 30 μM; A23187, 10 μM; free calcium concentration, 3–4 μM; and rhodopsin 3.5 μM. (B) same as in (A) but 0.5 mM calcium present. Fraction of calcium bleached per flash, 3%. Cuvette pathlength, 10 mm. Temperature, 20°C. Excitation conditions as in Fig. 1. Signals were obtained by averaging over 16 repetitions from one sample at a frequency of 1 Hz. (A − B) represents subtraction of signal (B) from signal (A) by means of a signal averager (Nicholet 1072) with a data reduction program. The trace (A − B) represents the time course of Ca^{2+} release in sonicated rod outer segments.

200 μsec. This very high sensitivity at reasonably high time resolution is obtained while the samples are exposed to rather low measuring light (intensity 50–100 μW cm^{-2}). The relative bleaching of the sample by the measuring light is less than 10^{-5}. (This is concluded from the following considerations: In the presence of arsenazo III (30 μM), a laser flash of 10 mJ cm^{-2} at 530 nm bleaches 10–20% of rhodopsin. The absorption of rhodopsin at the wavelength of the measuring light (655 nm) is approximately 0.3% of the one at 530 nm. The measuring light is applied to the sample only for 300 msec per sampling event. Since the recorded trace results from averaging over 16 repetitions with the same sample, the relative bleaching was only $\leq 10^{-5}$.)

Results Obtained with This Technique

1. On illumination calcium is released from binding sites at the lumen side of the disk membrane.

2. The release stoichiometry is 0.5 mol calcium released per mol rhodopsin bleached at a free calcium concentration of 4–5 μM. In fragmented material the stoichiometry is about 70% of that in intact rod outer segments.

3. The release kinetics are highly susceptible to the structural integrity of rod outer segments. In intact rod outer segments the half-rise time is $\tau_{1/2}$ = 300 msec, whereas in sonicated disk vesicles $\tau_{1/2}$ = 10 msec, at 20°, pH 7 and A23187, 10 μM.

4. Most likely the metarhodopsin I/metarhodopsin II transition is involved in the release mechanism.

These results were presented in Refs. 2, 5, and 12.

Conclusions

Flash spectrophotometry with the calcium-indicating dye arsenazo III is a sensitive tool for the investigation of rapid calcium fluxes in vertebrate photoreceptor preparations. Emphasis in this article was put on demonstrating that absorption changes of arsenazo III that are indicative of calcium release can be separated from those caused by other events. The calibration of absorption changes into changes of the free calcium concentration (see Refs. 2, 13) allows a quantitative assessment of the release stoichiometry. Arsenazo III is superior to calcium-sensitive electrodes with respect to time resolution, comparable with regard to sensitivity, and unaffected by electrophysiological events. However, it requires exposure of rods to an interrogating light, which may saturate the system in terms of electrophysiological responses.

Acknowledgment

The collaboration with P. Schnetkamp is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft.

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[15] W. L. Hubbell, K.-K. Fung, K. Hong, and Y. S. Chen, *in* "Vertebrate Photoreception" (H. B. Barlow and P. Fatt, eds.), p. 41. Academic Press, New York, 1977.
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[79] Light Release of ^{45}Ca Trapped in Sonicated Bovine Disk Vesicles

By ROGER S. FAGER, BURTON J. LITMAN, and H. GILBERT SMITH, JR.

Hagins and Yoshikami[1] suggested that calcium ions might serve as the principal intracellular messenger linking the visual pigment that absorbed the light with the plasma membrane sodium pores that produced the receptor potentials. More explicitly, the hypothesis stated that in rod cells that calcium was sequestered in the disk lumen and was released to the extra discal space on light absorption. For cone cells the light-induced calcium flux was from the extracellular space to the cytoplasm. This hypothesis also required a calcium pump mechanism to return the cell to the dark-adapted state.

The hypothesis has stimulated development of many experimental arrangements to demonstrate interactions of photoreceptor membranes with calcium. In the approach to be outlined here we started with extremely pure photoreceptor disk membranes prepared by the Ficoll flotation method of Smith *et al.*[2] Calcium-45 of known specific activity was trapped in disk membrane vesicles by sonication and the light-driven calcium fluxes were measured in a flow system.[3] This enabled us to observe both stoichiometry and time course of calcium fluxes, in a system free of other contaminating cellular organelles. The advantage of such a system is that it is chemically and physically well defined and homogeneous. Its potential drawback is that the cell structure has been so heavily disrupted and rod outer segment cytosol components have been lost.

Other approaches that have been used to observe calcium fluxes have employed efflux from sonicated vesicles centrifuged after light exposure,[4] efflux from reconstituted photopigment membranes,[5,6] binding differences from whole photoreceptors,[7–9] osmotic shock after light exposure,[10] and

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