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LIGHT-INDUCED CALCIUM RELEASE IN ISOLATED INTACT CATTLE ROD OUTER SEGMENTS UPON PHOTOEXCITATION OF RHODOPSIN

U.B. KAUPP, P.P.M. SCHNETKAMP * and W. JUNGE

Max-Volmer-Institut für Physikalische Chemie und Molekularbiologie, Technische Universität Berlin, Strasse des 17. Juni, D-1000 Berlin 12 (Germany)

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Summary

By applying flash-spectrophotometry with the calcium-indicating dye arsenazo III rapid light-triggered calcium release in various cattle rod outer segment preparations was studied. It is shown that light-induced calcium signals can be unambiguously discriminated from underlying absorption changes due to photolysis of rhodopsin and apparent absorption changes resulting from light-scattering transients.

The following results have been obtained:

1. Calcium-induced arsenazo III responses can be quantitatively and kinetically resolved within the time domain of the visual transduction process.
2. Photoexcitation of rhodopsin results in calcium release from intradiscal binding sites.
3. Calcium released does not appear in the cytoplasmic space unless the disc membrane is made permeable to calcium ions by an ionophore.
4. The shortest observed half-rise time of calcium release (300 ms) is possibly limited by the ionophore.
5. The stoichiometric ratio of calcium released/rhodopsin bleached is 0.5 at a free calcium concentration of 2 μM. The amount of calcium released is proportional to the percentage of rhodopsin bleaching (from 1—10%).
6. Upon disruption of the disc stack by lysis of intact rod outer segments the light-induced calcium release is greatly altered.

The results are discussed in relation to previous reports on a light-induced calcium release from retinal discs and in terms of the proposed role of calcium as an intracellular transmitter in vertebrate photoreceptors.

* On leave from the Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

Introduction

In 1971, Yoshikami and Hagins proposed that calcium is the primary transmitter between the absorption of a quantum of light by rhodopsin and the electrical response of the outer membrane of vertebrate rods [1]. The hypothesis was proposed because extracellular recordings of the voltage drop along the length of the outer segment suggested that raising the intracellular calcium concentration had the same inhibitory effect on the sodium current across the cell envelope as photoexciting the rhodopsin [2,3]. Intracellular recordings of the membrane potential of vertebrate photoreceptor cells in the dark and during illumination have provided additional evidence in favour of this notion, although they do not prove the hypothesis [4,5,6].

Considerable work has also been expended in attempting to detect calcium release and accumulation from a variety of photoreceptor preparations using atomic absorption spectroscopy [7—10], ^{45}Ca techniques [11—19], calcium-sensitive electrodes [20] and flash-spectrophotometry with the metallochromic dye arsenazo III [21]. These various preparations, including lysed and sonicated material, have yielded conflicting results. Some authors did not detect any light effect on the redistribution of intracellular calcium [10,11,14,37]. When a light-induced calcium release was measured the reported stoichiometric ratios of calcium released/rhodopsin bleached were highly divergent, a fact which might be attributed to the large differences between the preparations used [7,13,15,16,19,20,21]. It is noteworthy that only after previous fragmentation of the disc stack a light effect could be observed [7,13,15,19—21], but not in rod outer segments with an intact stack of discs [7,11,37], in which the outer membrane has been shown to be leaky [10,22—26].

Although it seems established that light has some effect on the redistribution of calcium in retinal rods, unequivocal evidence that calcium is released with the stoichiometry required for the transmission and especially within the time domain of the visual transduction process is still lacking.

We have recently reported a rapid calcium release from passively loaded retinal disc vesicles detected by flash-spectrophotometry with the calcium-indicating dye arsenazo III and with the above mentioned time resolution [21]. The stoichiometric ratio of 1 calcium released/30 rhodopsin molecules bleached measured in this study was far too low to be correlated with any functional role of calcium in visual transduction.

Using the same technique in this paper we have further examined the effect of light on the redistribution of intracellular calcium in rod outer segments well characterized with respect to the integrity of the plasma membrane and the disc stacking. In single flash experiments calcium-induced arsenazo III responses have been resolved with bleaching percentages down to 0.5% of the rhodopsin.

Materials and Methods

Preparations. Three types of isolated cattle rod outer segment preparations were used. They were stabilized and purified according to Schnetkamp et al. [26] and stored as a concentrated suspension (100 μM rhodopsin). The preparations differ in the permeability of the plasma membrane to small solutes

and in the integrity of the disc stacking and are referred to as 'intact', 'leaky' and 'lysed' rod outer segments. Intact rods have a plasma membrane functioning as a permeability barrier to ATP, NADPH and protons, whereas in leaky rods no permeability barrier to these solutes appears to be present [26]. Both preparations have a morphologically intact stack of discs [25,26]. Lysed rods are prepared by addition of 10 volumes of distilled water to an intact rod outer segment suspension and subsequent centrifugation and resuspension. The majority of the discs remains closed and retains calcium upon lysis, although the stack of discs is disrupted [27]. The endogenous calcium content of leaky and intact rod outer segments as determined by atomic absorption spectroscopy (1 mol calcium/mol rhodopsin and 2—3 mol calcium/mol rhodopsin [25,27]) could be confirmed by spectrophotometric determinations with arsenazo III. Intact rod outer segments, preloaded with ^{45}Ca, retain about 60—75% of their endogenous calcium content upon lysis by a hypoosmotic shock [27]. All preparations were resuspended and diluted in a standard medium, containing sucrose, 600 mM; Ficoll-400, 5% and Tris-HCl buffer, 20 mM at pH 7.4.

Experimental procedures. For the measurements rod outer segments were diluted to a final rhodopsin concentration of 5 μM. Rhodopsin concentrations were determined according to de Grip et al. [28]. The number of rhodopsin molecules bleached/flash was determined in the flash-photometer in the presence of 30 μM arsenazo III from the appearance of metarhodopsin II at 382 nm, using the same molar extinction coefficient ϵ = 40 000 mol/cm^2 as for rhodopsin at 498 nm [29]. Ionophore A23187 was a gift of Eli Lily Co. (Giessen) and was used without further purification in an ethanolic solution. The final concentration of A23187 in the cuvette was 10 μM if not otherwise indicated. The ethanol concentration introduced into the suspension by the ionophore stock solution was $\leqslant$1% v/v.

The incubation time was about 2 min. After this time all of the calcium stored within discs has been equilibrated across the disc and plasma membranes via the ionophore. The calcium efflux from rod outer segments after addition of ionophore was followed spectrophotometrically. The temperature was always 20° C.

Generally a 2 ml cuvette with 10 mm pathlength was used. For the experiments with different bleaching levels a 0.5 ml cuvette with a different geometry was used (10 mm pathlength of the measuring light but only 2 mm pathlength of the exciting light). The absorbance of the suspension in the direction of the measuring light beam was A = 1.0—1.3 at 655 nm. In the 0.5 ml cuvette the absorbance in the direction of the excitation flash was 0.2 at 530 nm (excitation wavelength). This low absorbance allows for an uniform bleaching throughout the cuvette by the laser flash. If the cuvette were 10 mm wide an exciting flash could give rise to a bleaching gradient along the light path of the flash and recordings of the flash-induced absorption changes could represent integration over a broad bleaching range. The laser beam was expanded to such an extent that uniform bleaching of the whole cross-section of the cuvette was achieved.

In all recordings shown, the sample was exposed only to the one very flash without previous illumination .

Calibration of arsenazo III. Arsenazo III was purchased from Sigma (Munich, grade I, M_r = 776.4) and used without further purification. The calcium contamination was found to be less than 5%. The arsenazo III concentration in a cuvette was always 30 μM. To calibrate the light-induced absorption changes into μM released calcium, a certain amount of calcium was added to a rod outer segment suspension and the change in absorption measured. The calibration procedure was performed under similar conditions to those used for the flash-spectrophotometric experiments, i.e. in the presence of 30 μM arsenazo III, 10 μM A23187 and rod outer segments which contained 5 μM rhodopsin. A change in the relative transmission $\Delta I/I = 3 \cdot 10^{-2}$ was observed upon addition of 1 μM calcium to a total (bound + free) calcium concentration of 15 μM. Up to a total calcium concentration of 20 μM, the absorbance of arsenazo is linearly related to the total amount of calcium present. Thus the differential sensitivity $\Delta A/\Delta Ca$ or arsenazo III is almost constant within the range of calcium concentrations used (10—20 μM total calcium concentration). The change in the relative transmission is related to the change of absorption $\Delta I/I = -2.3\ \Delta A$ if $\Delta I/I << 1$. A similar calibration procedure with arsenazo III present but in the absence of rod outer segments yielded a change in the relative transmission of $\Delta I/I = 5.5 \cdot 10^{-2}$. A similar value $\Delta I/I = 5.3 \cdot 10^{-2}$ was obtained by calculations under the assumption of a dissociation constant (K_D) of arsenazo III = 3 μM.

Flash-spectrophotometry. The principle and instrumental details of flash kinetic spectrophotometry have been reviewed elsewhere [31]. The rod outer segment suspension was excited by a flash from a liquid dye laser (SUA 9, Electro-Photonics, Belfast) operated with coumarin 6 at 540 nm. The maximum output of the laser was about 30 mJ. The half-time of duration was 1 μs. To ensure reproducibility of the excitation energy, the laser was always operated at maximum electrical energy input (20 kV). For experiments where the calcium release was studied at various bleaching levels a frequency doubled YAG-laser (Laser Associates, output 10 mJ, half-rise time of duration 20 ns, output wavelength 530 nm) was employed. For both excitation sources the output energy was attenuated by neutral density filters (Wratten, Eastman Kodak) when lower bleaching levels were desired.

The intensity of the interrogating light source was 50—100 $\mu W/cm^2$ at 655 nm. The cuvette stayed in the flash-photometer 5—30 s before application of a flash to permit recovery from the pipetting step. The recovery is indicated by a continous change of the steady scattering level until a constant value is reached. During measurement in the flash-photometer no significant bleaching of the sample by the incident measuring light was observed.

Electrical signals were recorded DC under compensation of the dark DC-level. The time resolution was limited by the time-per-address setting of the averaging computer (ranging from 40 μs in Fig. 4A to 40 ms in Fig. 5A).

Results

Separation of the calcium-indicating absorption changes from responses to other events

Rod outer segment suspensions excited by a light flash exhibit true absorp-

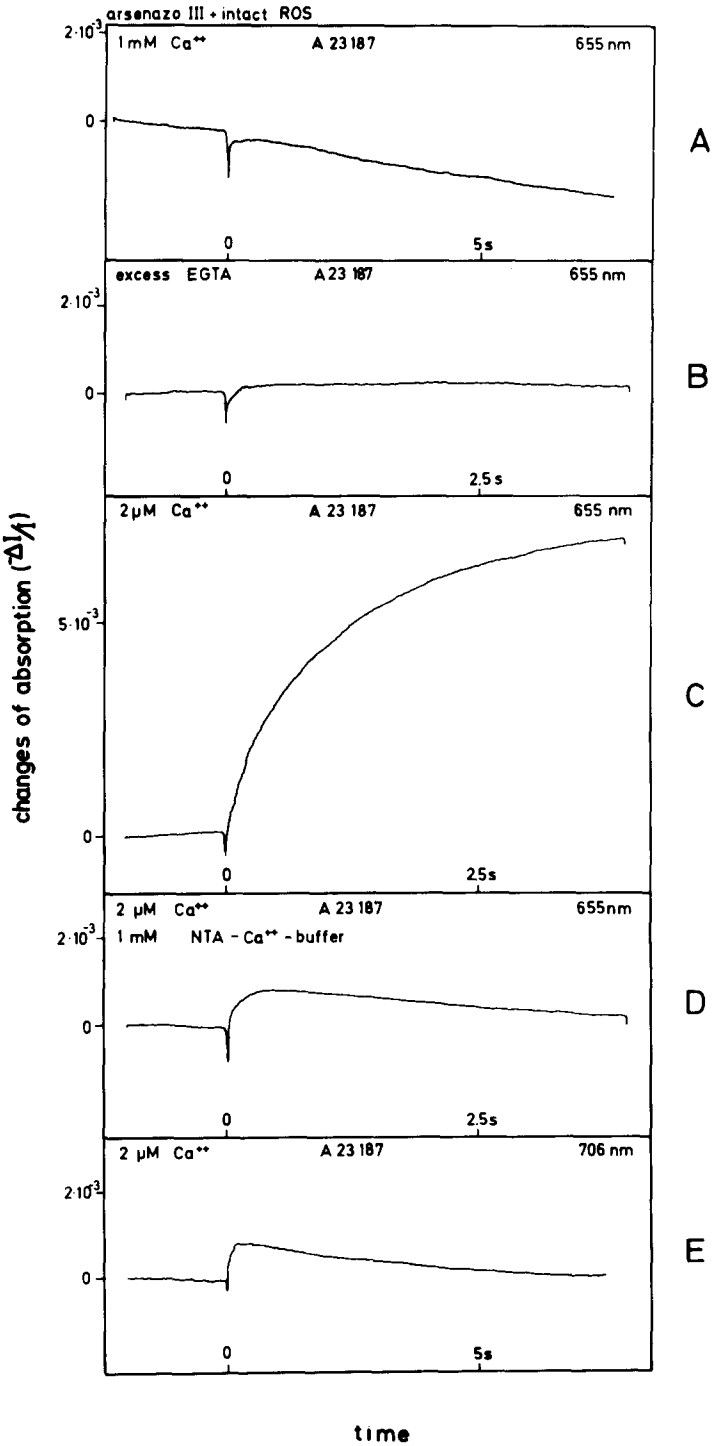

Fig. 1. Time course of the changes of absorption at 655 nm (A—D) and 706 nm (E) after excitation of a rod outer segment suspension by a flash at $t = 0$ in the presence of arsenazo III and ionophore A23187. Suspension medium: sucrose 600 mM, Ficoll 400 5%, Tris-HCl buffer 20 mM at pH 7.4, arsenazo III 30 μM, A 23187 10 μM. Rhodopsin concentration 4 μM. Bleaching level: 10% rhodopsin/flash. Flash energy 10 mJ. Excitation wavelength 530 nm. Temperature 20° C.

tion changes due to photolysis of rhodopsin and apparent absorption changes caused by transients of the light scattering properties [32]. In the presence of arsenazo III, additional absorption changes are observed with intact rod outer segments but only when the ionophore A23187 is added (Fig. 1C). These absorption changes have been monitored at 655 nm where underlying absorption changes resulting from photolysis of rhodopsin are negligible. The contribution of light-scattering transients was minimized by placing the absorption cell directly in front of the photomultiplier. Remaining apparent absorption changes due to changes of the light-scattering properties of the suspension can be isolated from the calcium-induced arsenazo III response by choosing conditions where this indicator is insensitive to small changes of the calcium concentration. The following controls were carried out:

(1) Very high (1 mM) and very low ($<10^{-7}$ M) calcium concentrations. In contrast to the rather large absorption change observed at 655 nm when the calcium concentration was matched to the maximum of the sensitivity of arsenazo III ($p\mathrm{Ca} = p\mathrm{K}_{ars}$, Fig. 1C) only minor responses were detected at very high and very low calcium concentrations (Fig. 1A and B). This clearly demonstrates that the arsenazo III response shown in Fig. 1C is sensitive to the calcium level in the suspension. Furthermore Fig. 1A and B show that at these calcium concentrations no apparent absorption change resulting from light-scattering can be detected.

(2) Measurement at the isosbestic point of arsenazo III at 706 nm. Only a small absorption change is observed (Fig. 1E) when the measuring wavelength was adjusted to 706 nm, an isosbestic point of the calcium-arsenazo III complex. The size of the apparent absorption changes resulting from light-scattering at 706 nm is comparable to that at 655 nm [32].

(3) Suppression of the changes of the free calcium concentration by a calcium buffer. In the presence of 1 mM nitrilotriacetic acid/calcium buffer adjusted to a free calcium concentration of 2 μM, a small flash-induced absorption change is observed (Fig. 1D). By comparison of Fig. 1C and D it is evident that the calcium-induced arsenazo III response is sensitive to the presence of a calcium buffer.

(4) Omission of ionophore A23187 in the reaction medium. In the absence of A23187 in the medium no light-induced arsenazo III signal is observed (Fig. 2).

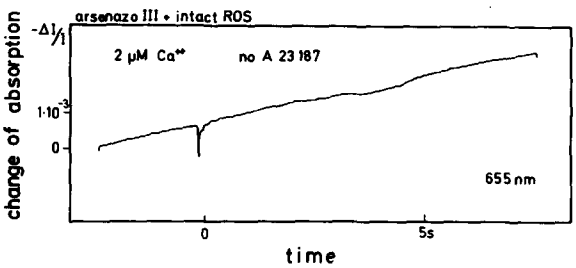

Fig. 2. Time course of the change of absorption at 655 nm after excitation of a rod outer segment suspension by a flash at $t = 0$ in the presence of arsenazo III and without ionophore present. Further conditions as in Fig. 1C.

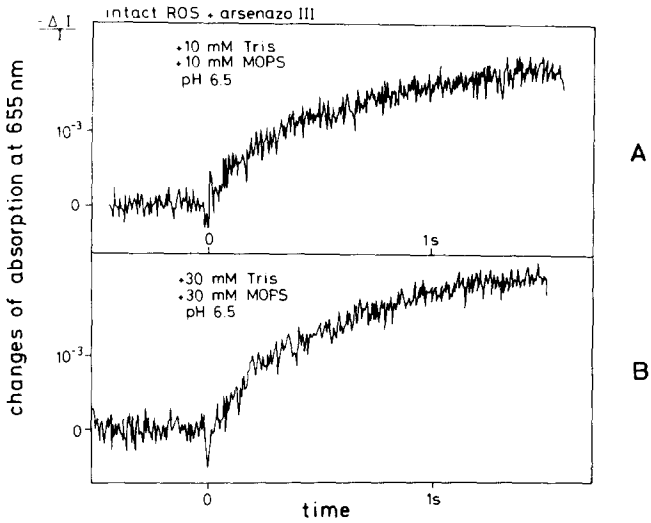

Fig. 3. Time course of the changes of absorption at 655 nm after excitation of a suspension of intact rod outer segments by a flash at $t = 0$. (a) Tris-HCl 10 mM, MOPS 10 mM at pH 6.5 (b) Tris-HCl 30 mM, MOPS 30 mM at pH 6.5. Rhodopsin concentration 5 μM. Bleaching level: 3% rhodopsin/flash. Free calcium concentration 2 μM.

It should be emphasized that for the experiments shown in Fig. 1D and E and in Fig. 2 where only minor flash-induced apparent absorption changes are observed, the calcium concentration was close to the maximum of the sensitivity of arsenazo III (pCa = pK).

The rapid net uptake of one proton/rhodopsin bleached during the metarhodopsin I/metarhodopsin II transition [33] represents another possible source of artifacts because arsenazo III is responsive to pH changes as well as to changes in calcium concentration [34]. The buffering capacity of the 20 mM Tris-HCl buffer is about 6 mM at pH 7.4, thus exceeding the maximal proton uptake of 5 μM (5 μM rhodopsin present) by a factor of thousand. Upon raising the proton buffering capacity threefold the arsenazo III response remains unchanged (Fig. 3). The adequacy of the proton buffering is not dependent on the buffering species. Imidazole, a permeating buffer, 2-(N-morpholino)-ethanesulfonic acid (MES) and morpholinopropane sulfonic acid (MOPS) gave similar results.

By choosing a higher time resolution a flash-induced absorption change of arsenazo III alone can be detected (Fig. 4A). When Fig. 4A and B are compared it can be seen that the flash-induced absorption change due to arsenazo III alone is fully reversible with a half-decay time of about 4 ms and thus does not interfere with measurements of the calcium-sensitive response shown in Fig. 1C, which was recorded at a much lower time resolution.

The foregoing experiments clearly demonstrate that calcium-indicating absorption changes of arsenazo III observed in rod outer segment suspensions are only slightly distorted by underlying absorption changes due to other events. Hence, in principle calcium fluxes in photoreceptor cells can be kinetically and quantitatively resolved.

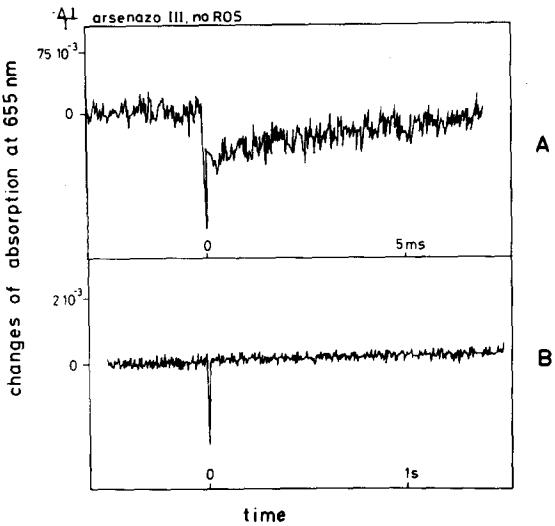

Fig. 4. Time course of the changes of absorption at 655 nm upon excitation of arsenazo III alone by a flash at $t = 0$. Arsenazo III 30 μM in standard medium at pH 7.4. Excitation wavelength 540 nm. Flash energy 4 mJ/cm^2. Free calcium concentration 2 μM.

Different rod outer segment preparations

In rod outer segments with an intact outer membrane light-evoked calcium release can be only detected in the presence of the ionophore A23187 (Fig. 5C). To permit discrimination between calcium release into the internal disc volume and calcium release into the cytoplasmic space two further preparations have been used. Leaky rods, with a plasma membrane permeable to small solutes behave in a way similar to intact rods: only in the presence of A23187 is calcium release observed (Fig. 5A). Second, in lysed rod outer segments, which have no outer membrane and where the disc stacking is disrupted, calcium release is almost completely abolished, even in the presence of A23187 (Fig. 5B).

Kinetics of light-induced calcium release

The rate at which calcium appears in the external medium (where arsenazo III is located) depends on the concentration of the carrier A23187 (Fig. 6). The rise of the calcium-indicating absorption changes at high ionophore concentrations ($\geqslant$10 μM) represents a minimal rate at which calcium is dissociated. It is of course possible that the actual dissociation rate is even faster.

Stoichiometry of the light-induced calcium release

One of the most important features of the calcium transmitter hypothesis is the high stoichiometry at which calcium must be released to give the observed electrophysiological responses [2,35,36]. In a recent estimate, 400—1000 Ca^{2+} released/rhodopsin molecule bleached are required at extremely low bleaching levels (a few photons absorbed/rod) [36]. For higher bleaching levels this high stoichiometry should decrease and finally saturate due to a depletion of calcium from discs.

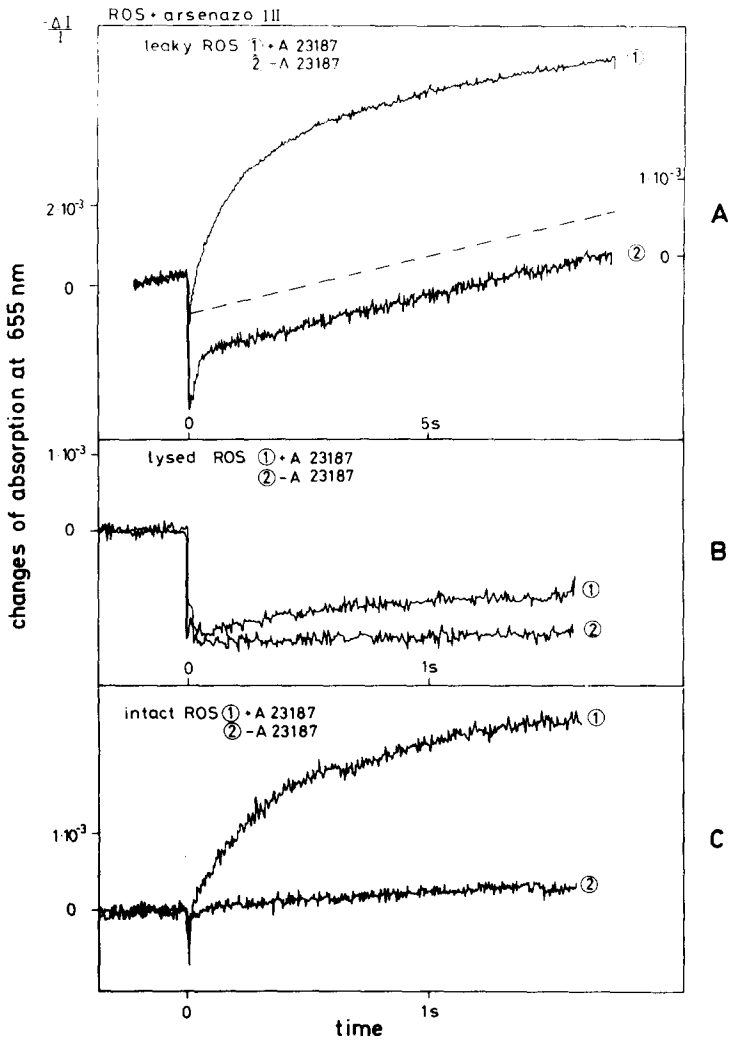

Fig. 5. Time course of the changes of absorption at 655 nm upon excitation of different suspensions of rod outer segments by a flash at $t = 0$ in the presence of arsenazo III. (a) leaky rod outer segments, bleaching level: 10% rhodopsin/flash, rhodopsin concentration 5 μM. (b) lysed rod outer segments, bleaching level: 10% rhodopsin/flash, rhodopsin concentration 5 μM. (c) intact rod outer segments, bleaching level: 3% of the rhodopsin, rhodopsin concentration 5 μM. Free calcium concentration 2 μM. Traces 2 represent absorption changes after addition of 10 μM A 23187 to the suspension. Note that in Fig. 5A trace 2 is displayed two times more sensitive than trace 1. Dashed line represents the same display as in trace 1. Further conditions as in Fig. 1C.

We therefore measured the stoichiometry of the light-evoked calcium release, observed in this study, at various bleaching levels. When 1% to 10% of the rhodopsin is bleached, the calcium-indicating absorption changes at 655 nm (arsenazo III) are proportional to those recorded at 382 nm (metarhodopsin II) (Fig. 7). The stoichiometry observed amounted to 0.5 calcium ions released/rhodopsin molecule bleached at a free calcium concentration of 2 μM. The absorption changes at 382 nm were recorded separately but under identical

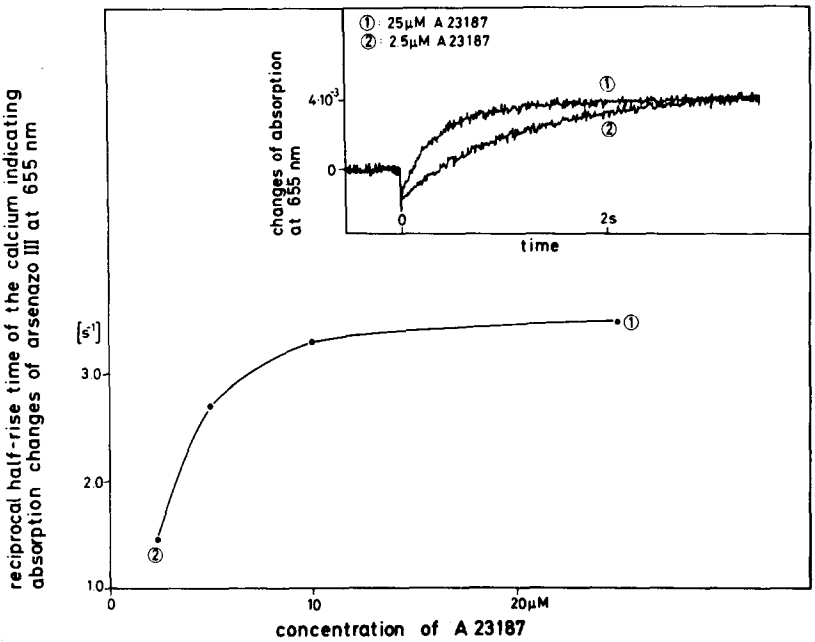

Fig. 6. Dependence of the half-rise time of the calcium-induced arsenazo III response on the concentration of ionophore A23187. Insert: time course of the changes of absorption at 655 nm upon excitation of a suspension of intact rod outer segments in the presence of arsenazo III and ionophore A23187. Concentration of A23187 as indicated. Bleaching level: 10% rhodopsin/flash. Further conditions as in Fig. 1C.

conditions to those under which the calcium-indicating absorption change of arsenazo III was measured: that is, in the presence of arsenazo III to include the filter action of the dye with respect to the exciting flash.

It should be noted that for all bleaching levels (down to 0.5%) the amplitude of the calcium-sensitive arsenazo III response was determined in single flash experiments.

Stability of the preparation with respect to the calcium release and reproducibility of the calcium-indicating arsenazo III responses

A study of the stability of the calcium storage system and a detailed analysis of the calcium fluxes in the dark have been described elsewhere for these preparations [26,27].

The light-evoked absorption changes due to release of calcium from binding sites are reproducible. Signals from different samples of the same preparation differ by at most 10%; signals from different preparations by at most 30%. In the course of our work more than 500 signals from about 12 preparations have been recorded. Storage of the stock suspension in the refrigerator for three days did not decrease the amplitude of the calcium response by more than 20%.

On the other hand, the release mechanism seems to be very sensitive to mechanical destruction of the disc stack structure. Mechanical stirring of the

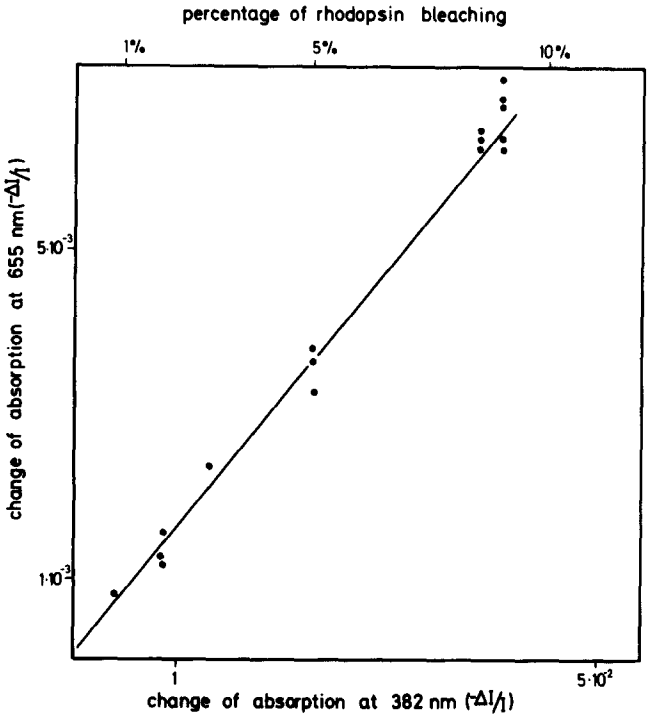

Fig. 7. Amplitudes of the absorption changes at 655 nm (arsenazo III) and at 382 nm (metarhodopsin II) for various bleaching levels. Conditions as in Fig. 1C.

suspension or the pellet during a resuspension step can abolish the release capacity almost completely. Also, after lysis of intact rod outer segments a calcium-induced arsenazo III response can no longer be observed. Finally, upon inclusion of electrolytes in the suspension medium the stability of the preparation, the calcium-indicating arsenazo III response and the calcium binding capacity are all affected [27].

Discussion

The above spectrophotometric studies with the metallochromic dye arsenazo III show that light-triggered calcium release by cattle rod outer segments can be optically detected and quantitatively and kinetically resolved. It has been demonstrated that optical signals resulting from responses of arsenazo III to other events and from intrinsic absorption changes of photoexcited rod outer segments do not interfere with the detection of the calcium release in the time range between 10 ms and 20 s. We have been able to resolve signals in single flash experiments resulting from photoexcitation of less than 1% of the rhodopsin.

In intact rod outer segments a rapid light-induced calcium release (<1 s) is only 'seen' by the external arsenazo III when the divalent cation ionophore A23187 is present. Surprisingly, in rod outer segments with a leaky outer membrane and in lysed rod outer segments also no fast light-induced calcium

release (<20 s) can be observed without ionophore. However, when the ionophore is present, leaky rod outer segments give a response similar to that observed in intact rod outer segments. Although we did not demonstrate the leakiness of the outer membrane for calcium ions directly, it seems reasonable to conclude that a membrane leaky to small solutes like ATP, NADPH and protons [26] is also leaky to calcium ions. However, the leakiness of the outer membrane for these solutes has been measured on a longer time scale than that used in our flash-spectrophotometric experiments with arsenazo III. Hence, a final conclusion on where calcium is released has to wait for kinetic data on the diffusion of calcium ions through the outer membrane of leaky rod outer segments.

The ionophore A23187 has been shown to reside in both disc and plasma membranes of intact rod outer segments [27]. This has been confirmed independently by the redistribution of calcium in intact rod outer segments upon addition of A23187 followed spectrophotometrically with arsenazo III. Therefore, the most attractive explanation of our findings in terms of the Yoshikami-Hagins hypothesis, namely that the ionophore functions exclusively in the outer membrane, thus communicating calcium, released from discs into the cytoplasma, across the envelope to the dye molecules in the external phase, is unwarranted.

Upon addition of A23187 to intact rod outer segments the free calcium concentration appears to be equilibrated between the intradiscal, cytosolic and external compartments [27]. The observation that calcium release in intact rod outer segments is seen only in the presence of the ionophore, suggests that the calcium ions were originally bound to the disc membranes rather than dissolved. The experiments with leaky and lysed rod outer segments further imply that the bound calcium pool, from which calcium is released, is exclusively located at the intradiscal membrane surface. It has been independently shown that in these preparations more than 90% of the endogenous calcium content of intact rod outer segments is stored at intradiscal binding sites [27]. From this it is concluded that the light-induced calcium-indicating arsenazo III response represents calcium release from intradiscal binding sites.

From the above it is concluded that probably no short-term control of the cytosolic calcium level by a light-evoked calcium release from the intradiscal calcium store occurs. This conclusion is consistent with those studies, which failed to detect a release from retinal discs [10,11,14,37]. However, it is difficult to compare our results to those studies which do report a light-triggered calcium release from discs [7,13,15,16,19—21]. This may be appreciated from the following aspects:

1. In the present study calcium release is abolished upon fragmentation of intact rod outer segments, whereas in other studies calcium release is observed in fragmented material [7,13,15,19,20].

2. We were unable to detect any calcium release in rod outer segments unless ionophore A23187 was added. In contrast Smith et al. [19] report that calcium release is completely abolished upon addition of A23187.

3. Smith et al. [19] only observe calcium release at high intradisc calcium concentrations (>5 mM) whereas in our experiments a comparable stoichiometry was obtained at a 1000-fold lower free calcium concentration (2 μM).

4. In most of the studies (but see Ref. 21) a half-rise time of the calcium

efflux from retinal discs on a minute scale was detected, whereas in this study it has been shown that calcium release from binding sites occurs with a half-rise time $\tau_{1/2} \leqslant 300$ ms.

Light-induced calcium release from intradiscal binding sites is communicated to the arsenazo III in the external medium by the carrier A23187. Therefore, the observed rise time of the light-induced arsenazo III response determines an upper limit for the actual time required for calcium release from intradiscal binding sites. Nevertheless, the time-course of the calcium signals, observed in our study in the presence of A23187, is compatible with the rise-time of the photoreceptor potential or photovoltage at low bleaching levels [4,6,36]. Therefore rapid calcium release from binding sites could throughout represent a trigger step in the transduction process. However, our results are not in agreement with the role of calcium as intracellular transmitter as formulated by Yoshikami and Hagins [1,2] unless two further assumptions are made: (1) Some factor, required to mediate translocation of released calcium across the disc membrane is lost in isolated rod outer segments. (2) The linear stoichiometry of calcium released/rhodopsin bleached does not hold for very low bleaching levels at which the electrophysiological response saturates ($<<1\%$).

Our further efforts are directed to meet with the technical demands necessary to check the above mentioned assumptions. Alternative functions of the described effects are also under investigation.

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