RAPID CALCIUM RELEASE BY PASSIVELY LOADED RETINAL DISCS ON PHOTOEXCITATION

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1. Introduction

In vertebrate retinal rods, rhodopsin undergoes a conformational change upon absorption of a quantum of light. The primary photoprocess is followed by a transient depolarisation of the outer receptor membrane. It is generally accepted that the depolarisation is due to the closing of Na^+-channels [1–4]. As the rhodopsin containing inner discs and the plasma membrane of the retinal rods seem to be topographically and electrically isolated, it is unlikely that rhodopsin controls the sodium channels directly [5,6].

It has been suggested that the primary photoisomerisation and the electrical events at the outer cell membrane are mediated by a transmitter molecule, with calcium as the most likely candidate [7–9]. According to Yoshikami and Hagins [10,11], calcium is pumped into the discs and released into the cytoplasm upon photoexcitation of rhodopsin.

The reports about calcium accumulation and release respectively in vertebrate photoreceptors are conflicting. Hendricks et al. [12], Liebman [13] and Szuts and Cone [14] used atomic absorption measurements to detect calcium release. Szuts and Cone [14] reported stoichiometries ranging from 10–1000 calcium released/rhodopsin bleached. Liebman [13] and Hendricks et al. [12] reported a somewhat lower release. Sorbi and Cavaggioni [15], Smith et al. [16], Mason et al. [17], Weller et al. [18], Hemminki [19] and Bownds et al. [20] used radioactive calcium to detect calcium release. Mason et al. [17] and Smith et al. [16] reported a one-shot-carrier relation of 1:1. Hemminki [19] found a stoichiometry of 1 calcium released/6 rhodopsin bleached and Weller et al. [18] about 1 calcium released for every 100 rhodopsin bleached. Sorbi and Cavaggioni [15] and Bownds et al. [20] find no significant calcium loss upon bleaching.

Conflicting results were also obtained for the active transport of calcium into the discs. Bownds et al. [20], Weller et al. [18] and Neufeld et al. [21] reported that light has no effect on the calcium transport into the discs, whereas Mason et al. [17] and Hemminki [22] detected active accumulation of calcium with a significant light effect.

We used the calcium indicator dye Arsenazo III [23–26], together with a sensitive flash kinetic photometric technique, to detect fast calcium release from disc vesicles. To establish a calcium gradient across the disc membrane, rod outer segment (ROS) suspensions were sonicated in a high calcium medium and after resealing of the vesicles the external calcium was removed.

We could detect a fast calcium release with a half-rise time of 10–20 ms upon photoexcitation of rhodopsin. The stoichiometry is about 1 calcium released/30 rhodopsin bleached.

2. Materials and methods

2.1. *Rod outer segment preparation*

ROS were prepared according to Emrich [27]. Bovine eyes from the slaughterhouse were enucleated within 2 h after death and the retinae excised. The retinae were suspended in Ringer solution: NaCl 110 mM, KCl 5 mM, $CaCl_2$ 0.3 mM, $MgCl_2$ 0.3 mM, sucrose 50 mM and phosphate buffer 15 mM, pH 7.3. After grinding in a mortar, the solution was poured through

a nylon texture and layered on a 30% sucrose solution. After centrifugation in a swinging bucket for 30 min at 30 000 × *g*, the ROS were harvested with a syringe at the interface between the sucrose and water phases. To get the ROS suspension sucrose-free, it was washed twice in Ringer solution. The ROS suspension was stored in the dark under liquid N_2.

2.2. *Calcium trapping*

Disc vesicles with trapped $CaCl_2$ were prepared according to Smith et al. [16]. After rapid thawing, ROS suspensions were washed once with 0.1 M imidazole–HCl (pH 8.0), centrifuged, and resuspended in imidazole containing 15 mM $CaCl_2$. They were then sonicated for 15–40 s. During sonication (with a Branson Sonifier model B-12, 30 W output), the samples were cooled in an ice-water bath. For re-sealing, suspensions in the high calcium medium were put into the refrigerator overnight.

2.3. *Removal of external calcium*

The metallochromic dye Arsenazo III has its highest sensitivity at a calcium concentration of 5–15 μM (see fig.1). Therefore, the concentration of calcium in the outer volume before illumination had to be reduced to this level. Removal of external calcium was accomplished in one of two ways:

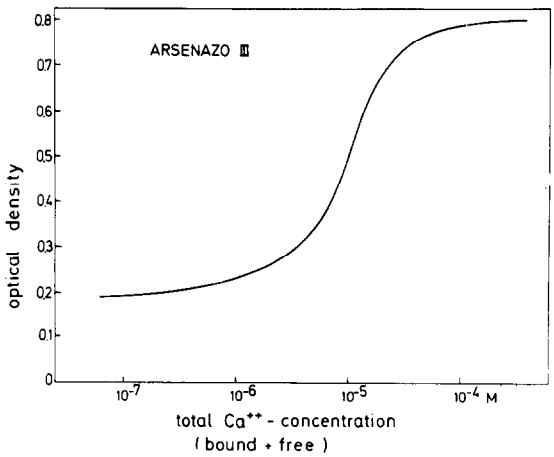

Fig.1. The dependence of the optical density of a solution of Arsenazo III on the total calcium concentration in the solution. Composition: arsenazo III, 30 μM, imidazole, 0.1 M (pH 8.0).

1. The sonicated and resealed disc vesicles were centrifuged for 35 min at 48 000 × *g*, and the pellet resuspended in 0.1 M imidazole buffer. This washing procedure was repeated twice. The final calcium concentration was always between 5 μM and 10 μM. The whole washing procedure lasted about 2 h. This method has the possible disadvantage of continuous calcium loss from the vesicles during the two hours. To minize calcium loss, we used a second procedure.

2. After the disc vesicles were resealed in the high calcium medium, Chelex-100 (Biorad, Munich) was added. As apparent from the colour change of Arsenazo III, the Chelex complexed the calcium from the outer volume within 2 min. The outer calcium concentration was titrated back to 5–10 μM before illumination. It is noteworthy that the slow and the rapid techniques for calcium removal produced similar results, which implies that the membrane of the retinal rods is relatively impermeable to calcium.

2.4. *Metallochromic dye Arsenazo III*

Arsenazo III was purchased from Sigma (Munich, M.W.776.4). Contaminating calcium was removed from the dye by passage through a Chelex-100 column, which was buffered with 0.1 M imidazole, pH 8.0. The concentration of the dye in the cuvette was always adjusted to about 50 μM. The optical density at the measuring wavelength (655 nm) was thus about 1.0 when a 20 mm cuvette was used. Figure 1 shows the titration curve of Arsenazo III.

2.5. *Flash photometric equipment*

Details of the set-up of our kinetic flash photometer have been described elsewhere [28]. As interrogating light source a 250 W tungsten lamp (Osram 55 54 40) was used. The sample in the 20 mm cuvette was excited by a Coumarin 6 liquid dye laser (Electro-Photonics Model SUA-9, Belfast). The output of the laser was about 10 mJ at 540 nm. One flash bleached about 6% of the rhodopsin in the cuvette. The photomultiplier (EMI 9556 with an S-20 characteristic) was guarded by a DAL 655 interference filter and an RG 1/4 filter (Schott and Gen., Mainz). Signals were induced repetitively and averaged on a Nicolet 1072 computer.

3. Results

The results are discussed under the probable assumption that the about 6-fold charged dye (depends on pH) Arsenazo III does not penetrate the disc membrane. The absorption changes were measured at the peak wavelength in the red band of the dye, 655 nm. At this wavelength the absorption changes of Arsenazo III are superimposed on absorption changes due to rhodopsin bleaching and to light scattering transients. The background signal is shown in fig.2B. It was obtained in the presence of Arsenazo III, with EDTA (0.1 mM) to buffer the flash-induced calcium transients in the outer volume. The composite signal (response of Arsenazo III plus background) is given in fig.2A. It was obtained in the absence of EDTA at a calcium concentration of 5 μM before firing of the flash. It is noteworthy that the extent of bleaching of rhodopsin is the same (within 10%) in the two cases (± EDTA). This is not self-evident, as the absorption of Arsenazo III is, of course, different at the two different calcium concentrations. The 'filter' action against the exciting light by Arsenazo III, however, was similar in both cases, as we excited the sample at a wavelength (540 nm) near the isosbestic point of the calcium complex of the dye (550 nm). Figure 2A–B shows the response of the Arsenazo III alone, obtained by subtracting signal B from A on the Nicolet 1072 averaging computer. The signal represents the response of Arsenazo III to calcium transients in the outer volume with no interference by any background signal. We checked for possible contributions to the signal by a direct photochemical reaction of Arsenazo III by recording signals from a solution without retinal discs. No response was seen under these conditions. Figure 3 shows the difference signal at a time resolution of 400 μs per address of the averaging computer (10 X higher time resolution than in fig.2). The signal-to-noise ratio is worse than in fig.2, and there is a considerable flash burst artefact during the first 5–10 ms. However, it is apparent that the increase in the calcium concentration in the outer volume has a half-rise time of about 15 ms. This is similar to the half-rise time of the Metal-Meta II transition (about 10 ms) under our conditions.

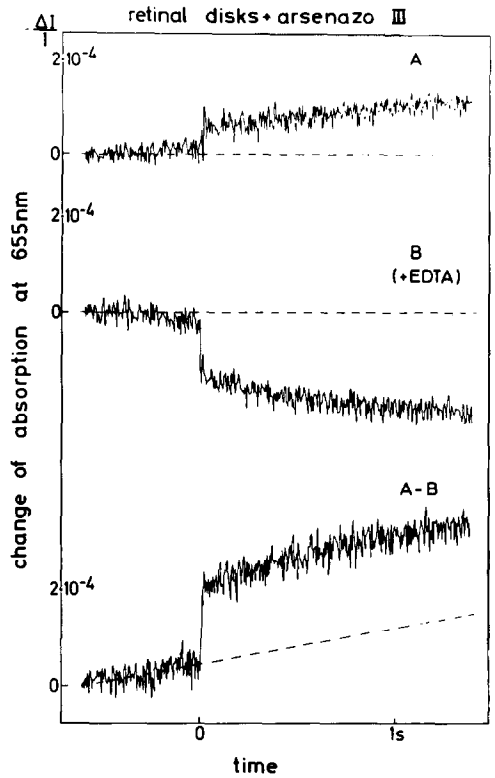

Fig.2. Time course of the changes of absorption at 655 nm on excitation of a suspension of retinal discs by a flash at $t0$ in the presence of arsenazo III (50 μM). (A) retinal discs in buffer medium (see Materials and methods). (B) retinal rods as in (A) but in the presence of EDTA (100 μM) as calcium complexing agent. (A–B) calculated difference between signals (A) and (B). Each trace represents the average of 4 signals.

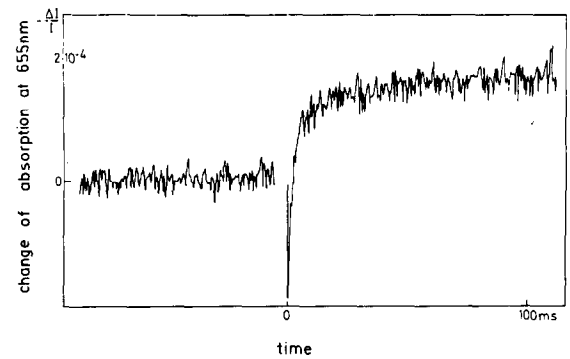

Fig.3. Time course of the changes of absorption at 655 nm on excitation of a suspension of retinal discs by a flash at $t0$ in the presence of arsenazo III (50 μM) at higher time resolution. Conditions as in fig.2(A–B) but averaged over 8 signals.

To assess the calcium/rhodopsin stoichiometry, we titrated disc suspensions with calcium under the same chemical conditions as in our flash photometric experiments. A change in the relative transmission ($\Delta I/I$) of 0.1 was observed upon increasing the total calcium concentration (bound plus free) in the sample from 5–6 μM. An observed light-induced change of absorption of Arsenazo III $\Delta I/I$ $2 \cdot 10^{-4}$ then implies a release of 2 nM calcium. Under the same conditions the absorption changes of rhodopsin at 390 nm (Meta II) were $\Delta I/I$ $1.25 \cdot 10^{-2}$. With the relation between the relative change of transmission and the absorption, $\Delta A - 1/2.3(\Delta I/I)$ [28], this implies the bleaching of about 60 nM rhodopsin (we assume a change in the molar extinction coefficient of $\Delta\epsilon$ $4 \cdot 10^4$ M^{-1} at 390 nm). Taking these two figures, we conclude that the stoichiometry of calcium released to rhodopsin bleached is about 1/30.

4. Conclusion

It has been shown that Arsenazo III can detect small changes of the calcium level in disc suspensions upon bleaching of rhodopsin. The time course of the calcium release closely resembles that of the Meta I–Meta II transition (half-rise time of about 10 ms). The stoichmetry is about 1 calcium released for 30 rhodopsins bleached. These results are in principle compatible with the suggested role of calcium as the chemical transmitter in the visual excitation process in vertebrate photoreceptors as proposed by Yoshikami and Hagins [10,11]. However, further studies, focussed on the question of whether the now too-low stoichiometry of the calcium release is higher in intact retinal rods, are required.

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