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Time-gated fluorescence imaging of *Blepharisma* red and blue cells

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Time-gated fluorescence images of intact motile red and blue cells of the photoresponsive protozoan *Blepharisma japonicum* have been acquired, digitized and analyzed by means of a new dedicated micro-video system The spatial and spectral maps of the intracellular distribution of the endogenous fluorophores reveal that red, as well as blue cells fluoresce mainly in the blue-green with a longer lifetime, whereas the vacuoles emit in the red with a shorter decay time Upon breaking the cell membrane, the extruded pigments show a bright short-lived fluorescence in the red, attributed to blepharismin and oxyblepharismin in red and blue cells, respectively The results on fluorescence time-behavior, apparent quantum yields and fluorescence spectral distribution are discussed with respect to the photosignalling function of blepharismin and oxyblepharismin

Introduction

Blepharismin, the photoreceptor pigment responsible for triggering the step-up photophobic response of the ciliated protozoan *Blepharisma japonicum* [1,2], is located in deep-red granules homogeneously distributed beneath the cell membrane and arranged in longitudinal rows (see Refs 3–5 and references therein) The chemical structure of blepharismin chromophore seems to be quite similar to that of hypericin [6] and of the chromophore of stentorin, the photoreceptor pigment of the ciliate *Stentor coeruleus* [7–9]

When exposed in the presence of molecular oxygen to light intensities in the order of 5–10 W/m^2, *Blepharisma* changes its color from red to blue because of the photooxydation of blepharismin to oxyblepharismin [10] Whereas the absorption spectra of the two pigments are significantly different [2–4], their fluorescence emission spectra are substantially identical and consist in a single broad (about 50 nm at half height) band centered at about 605 nm, independently of the excitation wavelength (Bisi et al , data not shown)

The blue-pigmented cells too have recently been shown to exhibit light-dependent motile behavior The action spectrum of the photophobic responses of red and blue cells has a structure very similar to that of the absorption spectrum of the corresponding pigment [2] These findings suggest that the photosensing and phototransducing properties of blepharismin are not affected by photooxydation [2], even though they do not provide specific information on the molecular photoreactions occurring in blepharismin and oxyblepharismin and on the resulting photosignalling sequence

With the aim of understanding the molecular mechanisms operating in the photoreception process of *Blepharisma*, red blepharismin has been studied in suspensions of cold-extruded intact granules, as well as in solutions containing crude extracts from acetone-treated cells [1,11,12] The results of these studies and the finding that the protonophore CCCP (carbonyl-cyanide-chlorophenyl-hydrazone) inhibits photomotile responses of *Blepharisma*, without affecting cell viability and motility [13], suggested that a vectorial light-driven proton transfer from blepharismin first excited singlet state might initiate the transduction chain for *Blepharisma* photoresponses, in analogy with what suggested in the case of *Stentor* [14]

This hypothesis, however, was challenged by the results of time-resolved fluorescence measurements in ethanol and buffer solutions of acetone-extracted pigments in fact the time-gated fluorescence spectra, measured with different time windows after the excita-

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tion pulse, did not provide evidence for fast proton release from blepharismin first excited singlet state [15]

These experiments, however, were performed on crude extracts of bulk pigments, with the photoreceptor molecules in an environment quite different from the physiological state in which they are functionally operating An important contribution to clarify the nature of the primary molecular photoreactions can, therefore, come from spectroscopic studies of intact cells in vivo [16] and of isolated photoreceptor proteins [17]

This paper reports the results of a first set of measurements on red and blue intact and motile *Blepharisma* cells, accomplished by means of an innovative time-gated fluorescence imaging technique utilizing an intensified CCD video-camera [18]

Materials and Methods

Blepharisma was grown as described previously [1,19] In order to photooxidize blepharismin to oxyblepharismin in vivo, cells were kept under white cold light (about 8 W/m^2) for 96 h, in these conditions the native red form of blepharismin fully converts into the blue form, without any detectable impairment of cell viability [2]

The system for time-gated fluorescence imaging was described previously [18] A Nitrogen laser (337 nm wavelength, 1 ns pulse duration and 50 Hz repetition rate) was used as the excitation source The laser beam was coupled to a 600 μm diameter quartz fiber The distal end of the fiber was placed under the stage of a fluorescence microscope to obtain a uniform illumination of the observed field with a power density of about 40 $\mu W/cm^2$

The cells were left free to move in the liquid film (about 50–100 μm depth) between the quartz microscope slide and the coverslip

Time-gated fluorescence images were acquired by means of a time-gated intensified CCD video-camera coupled to the microscope and synchronized with the laser pulse The acquisition time-window could be suitably delayed with respect to the excitation pulse and its width could be reduced to 5 ns In the present study, either an undelayed gate, synchronous with the laser pulse, or a 10 ns delayed gate were used for the acquisition Both gates were 50 ns wide, to assure a good signal level As the video-camera is insensitive to the ultraviolet light, in the synchronous gate no laser light is detected The undelayed fluorescence image slightly enhances the short-lived components, whereas the delayed image is mainly due to the long-lived fluorescence

With each of the considered gates, the images were acquired in full spectrum, as well as in different spectral ranges, obtained with one of the following Kodak Wratten filters yellow (No 4), orange (No 16), or red (No 29) The use of these filters allows to discriminate the natural blue-greenish fluorescence due to a variety of chromophores in the cell cytoplasm from the red fluorescence due to blepharismin

The fluorescence images were captured and digitized by a frame grabber An image-processing board, driven by dedicated software, was available for image processing and enhancement A videorecorder was also used to continuously register the cell fluorescence in different time and spectral conditions

Results and Discussion

In a previous investigation [16], the spectral and time-dependent behaviour of blepharismin red fluorescence was studied in intact red cells by means of time-gated microfluorometry The time-integrated emission spectrum, an undelayed gated spectrum (500-ps width), and an 8-ns-delayed gated spectrum (3-ns width) were acquired simultaneously, together with the fluorescence decay curve, in the 580 nm to 700 nm range [20] Three molecular species, all of which fluorescing with an emission maximum at about 605 nm, were detected, with decay time constants of about 0 2 ns, 1 ns and 4 ns The short-lived component was by far the predominant species (with a fractional amplitude of about 70%)

These time-gated microfluorometry measurements were performed on intact but immobilized cells, and the fluorescence was excited focusing the laser beam on a small part of the cell To investigate non-invasively the fluorescence properties of *Blepharisma* red and blue cells, thus also dispelling any doubt about the functional state of the cells during the measurements, a time-gated fluorescence imaging study was accomplished In this case, the whole field was illuminated uniformly at a rather low power density (40 $\mu W/cm^2$) and the images were acquired at the repetition rate of 50 Hz, while the cells were free to move in their saline medium Both spectral and time-domain information was obtained by means of cut-off filters and suitable time-gates

The gate parameters were chosen on the basis of the fluorescence decay times previously measured by means of time-gated microfluorometry [16] The undelayed gate generates images very similar to the ones obtained under continuous wave excitation, since all the lifetimes are much shorter than the width of the gate (50 ns) On the contrary, a 10 ns-delay allows to strongly enhance the emission of the longest-lived component

Undelayed fluorescence images of red intact cells, collected through a yellow cut-off filter (Wratten No 4, 50% Transmittance at 470 nm) or without any filter, are qualitatively very similar They show well-defined

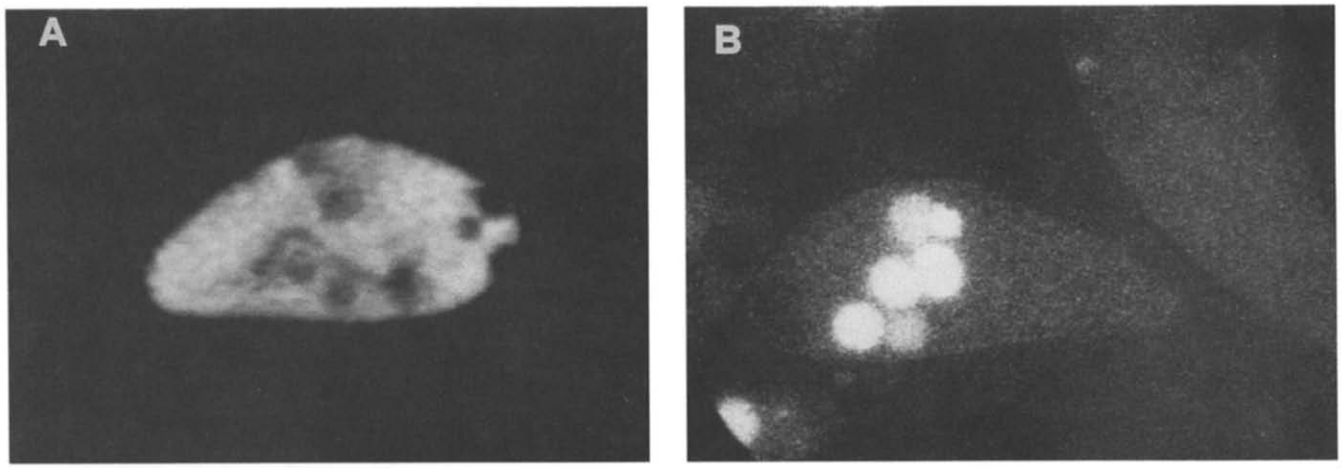

Fig 1 Undelayed fluorescence image of a red cell of *Blepharisma japonicum* (A) Acquired without any cut-off filter, (B) acquired with the red cut-off filter (Kodak Wratten No 29)

and localized dark areas, corresponding to the vacuoles, in the comparatively bright field of the cell body (Fig 1A)

The picture almost exactly inverts when using orange or red cut-off filters (Wratten No 16 and No 29, 50% transmittance at 540 nm and 620 nm, respectively) the vacuoles appear as luminous spots in the weakly luminescent background of the cell body (Fig 1B)

In intact red *Blepharisma*, therefore, the cell body seems to emit mainly in the blue-green region of the spectrum This background fluorescence is very intense and much stronger than the red emission observed in the vacuoles In fact, only a long-wavelength cut-off filter, removing the blue-green fluorescence, allows to reveal the signal coming from blepharismin-containing vacuoles, which, in these experimental conditions, is rather intense

As mentioned in the introduction, the thousands of pigment-containing granules are homogeneously distributed under the cell pellicle The vacuoles, on the other hand, contain comparatively large amounts of blepharismin, possibly also as free chromophore The relatively high concentration of blepharismin in the vacuoles can be, at least in part, responsible for the intense red emission from these localized cellular regions However, the fact that the red fluorescence from granule-embedded blepharismin is much weaker than the red fluorescence of the vacuoles could be ascribed also to a higher fluorescence quantum yield of the chromophore detached from the granule molecular framework This feature would be well in agreement with the photoreceptive function of blepharismin in the granule

10-ns-delayed images, acquired with and without cut-off filters, confirm the presence of a red fluorescence localized in the vacuoles and a remarkable diffuse fluorescence from the cell body, with a relevant component in the short wavelength range of the visible spectrum mainly due to natural fluorescence The red-filtered delayed images show a moderate decrease in

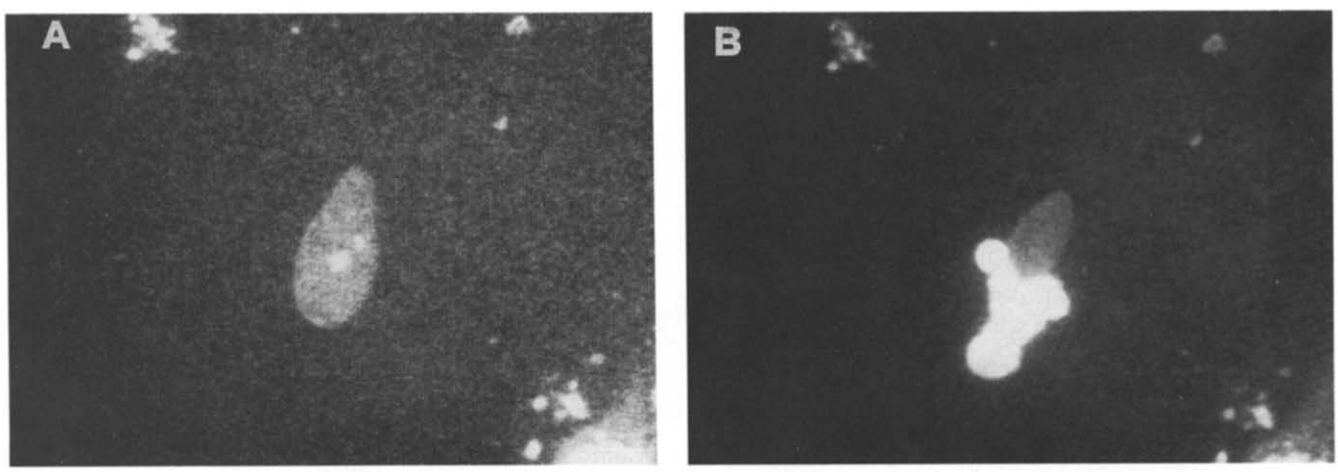

Fig 2 Undelayed fluorescence image of a red cell acquired with the red cut-off filter (Kodak Wratten No 29) (A) Intact living cell, (B) after breaking of the cell membrane

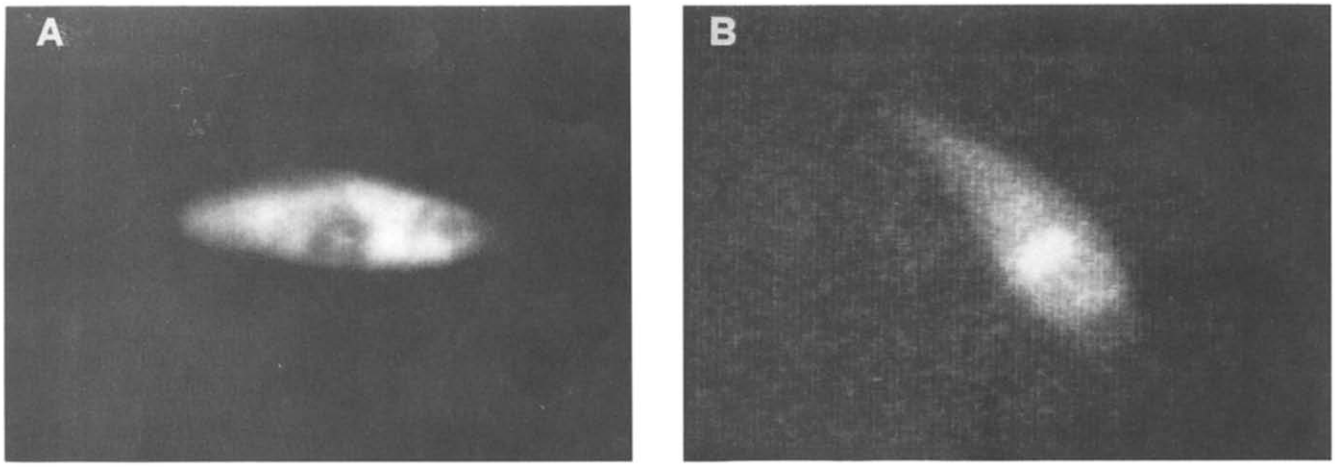

Fig 3 Undelayed fluorescence image of a blue cell of *Blepharisma japonicum* (A) Acquired without any cut-off filter, (B) acquired with the red cut-off filter (Kodak Wratten No 29)

the fluorescence intensity of the vacuoles, due to the 10-ns delay of the acquisition window, which selects the longest-lived emission On the contrary, the unfiltered image of the cell body is not significantly affected by the time delay

This observation allows to establish that the red fluorescence of blepharismin from the vacuoles is characterized by a short lifetime with respect to the blue-green emission detected in the cell body

When the cell is under uniform illumination, after a few minutes, the membrane shows the onset of a localized bright fluorescence This significant increase in the detected red emission is followed, after a short while, by the rupture of the cell membrane The fluorescence intensity rises with the degree of the cellular damage and a strong signal is detected both from the expelled cytoplasm and the cell, in the area close to the membrane breaking (Fig 2A and B) The site of the damage is fairly well-limited and differs from cell to cell The detected intensity changes neither when different cut-off filters are used nor when they are removed, whilst, of course, it decreases using a delayed gate The emitted fluorescence in the red region of the visible spectrum can be attributed to blepharismin

The above-reported observations seem to support the assumption that blepharismin in its physiological state has a low fluorescence quantum yield It is, in fact, conceivable that, upon the membrane breaking, the functional structure of the photosensing network is disrupted and the excited chromophore deactivates mainly through radiative transitions rather than through the molecular pathways which initiate the sensory transduction chain

A second series of fluorescence data was collected from intact blue cells Without filters or with the yellow cut-off, in undelayed images, again the vacuoles look dark (Fig 3A) The body is characterized by a more intense blue-green fluorescence with respect to red cells under analogous conditions However, as already noticed for the red cells, either an orange or a red cut-off filter allows the observation of bright red-emitting vacuoles on the dimly-fluorescent background of the cell body (Fig 3B)

The acquisition of delayed images confirms also for blue *Blepharisma* that the natural blue-green fluorescence of the cell body is characterized by a long lifetime with respect to the red emission detected in the vacuoles

Concluding remarks

Thanks to the fluorescence imaging technique it has been shown that the fluorescence properties of red and blue *Blepharisma* cells are definitely similar both in the time and spectral domains In particular, it has been possible to reveal that, notwithstanding the high degree of pigmentation of both red and blue *Blepharisma* cells, when exciting at 337 nm, the unfiltered bulk emission is mainly due to longer-lived blue-green fluorescing species homogeneously distributed in the cell body, rather than to the short-lived emission of blepharismin or oxyblepharismin

By means of suitable cut-off filters it has been shown that a relatively high amount of blepharismin or oxyblepharismin is clustered in the vacuoles and that the fluorescence intensity from these pigments is higher than that from granules

The dramatic increase of blepharismin and oxyblepharismin fluorescence intensity upon membrane rupture, together with the low fluorescence level of granule embedded pigments, may be due, at least in part, to a significant difference in fluorescence quantum yield between bound and unbound pigments. This assumption is in agreement with the hypothesis that blepharismin and oxyblepharismin function as photoreceptor devices when inserted in the granule structure

References

1 Scevoli, P, Bisi, F, Colombetti, G, Ghetti, F, Lenci, F and Passarelli, V (1987) J Photochem Photobiol B Biol 1, 75–84
2 Checcucci, G, Damato, G, Ghetti, F and Lenci, F (1993) Photochem Photobiol 57, in press
3 Giese, A C (1973) in *Blepharisma* The Biology of a Light-Sensitive Protozoan, Stanford University Press, Stanford
4 Giese A C (1981) in Photochemical Photobiological Reviews (Smith, K C, ed), pp 139–180, Plenum, New York
5 Ghetti, F (1991) in Biophysics of Photoreceptors and Photomovements in Microorganisms (Lenci, F, Ghetti, F, Colombetti, G, Hader, D-P and Song, P-S, eds), pp 257–265, Plenum, New York
6 Sevenants, M R (1965) J Protozool 12, 240–245
7 Kim, I-H, Rhee, J S, Huh, J W, Florell, S, Faure, B, Lee, K W, Kahsai, T, Song, P-S, Tamai, N, Yamazaki, T and Yamazaki, I (1990) Biochim Biophys Acta 1040, 43–57
8 Song, P-S, Kim, I-H, Florell, S, Tamai, N, Yamazaki, T and Yamazaki, I (1990) Biochim Biophys Acta 1040, 58–65
9 Song, P-S, Kim, I-H, Rhee, J S, Huh, J W, Florell, S, Faure, B, Lee, K W, Kahsai, T, Tamai, N, Yamazaki, T and Yamazaki, I (1991) in Biophysics of Photoreceptors and Photomovements in Microorganisms (Lenci, F, Ghetti, F, Colombetti, G, Hader, D-P and Song, P-S, eds), pp 267–279, Plenum, New York
10 Giese, A C and Zeuthen, E (1949) J Gen Physiol 32, 525–535
11 Lenci, F, Ghetti, F, Gioffrè, D, Passarelli, V, Heelis, P F, Thomas, B, Phillips, G O and Song, P-S (1989) J Photochem Photobiol B Biol 3, 449–453
12 Ghetti, F, Lenci, F, Checcucci, G and Heelis, P F (1992) J Photochem Photobiol B Biol 13, 315–321
13 Passarelli, V, Lenci, F, Colombetti, G, Barone, E and Nobili, E (1984) in Blue light effects in biological systems (Senger, H ed), pp 480–483, Springer, Berlin
14 Song, P-S (1981) Biochim Biophys Acta 639, 1–29
15 Cubeddu, R, Ghetti, F, Lenci, F, Taroni, P and Ramponi, R (1990) Photochem Photobiol 51, 56–573
16 Taroni, P, Ramponi, R, Lenci, F, Ghetti, F and Cubeddu, R (1991) Med Biol Environm 19, 61–64
17 Gioffré, D, Ghetti, F, Lenci, F, Paradiso, C, Dai, R and Song, P-S (1993) Photochem Photobiol, in press
18 Cubeddu, R, Taroni, P, Valentini, G and Canti, G (1992) J Photochem Photobiol B Biol 12, 109–113
19 Miyake, A, Harumoto, T, Salvi, B and Rivola, V (1990) Eur J Protistol 2, 310–315
20 Cubeddu, R, Docchio, F, Liu, W Q, Ramponi, R and Taroni, P (1988) Rev Sci Instrum 59, 2254–2259