Archives Italiennes de Biologie, 149 (Suppl.): 161-166, 2011.

The toxic effect of light on retinal photoreceptors, its mechanism and the protection by endogenous indolamines

P.L. MARCHIAFAVA

Dipartimento di Fisiologia Umana, Università di Pisa, Italy

ABSTRACT

Light absorption by the outer segment of retinal rod photoreceptors initiates visual sensory stimulation, but it may as well induce, once these cell segments are detached from the main cell body, a less well known but fatally toxic oxidative stress which we monitored by the use of the oxidation-sensitive probe Rhodamine 123. The molecular mechanism of the light induced oxidative damage is initiated by an intermediate of activated rhodopsin without requiring the presence of GTP-dependent proteins.

Since no oxidative damage is produced when light is falling on outer segments as part of intact cells, we postulated the existence, within the photoreceptors inner segment of a molecular mechanism contrasting the toxic prerogative of light, thus protecting photoreceptors.

It is known that photoreceptors synthesize indoleamines capable of antioxidant activity, and we investigated whether such endogenous substances would actually provide a protective role. This possibility was suggested by our earlier finding that the application of exogenous melatonin and its precursor N-acetyl serotonin effectively protected the photoreceptors outer segment from the light-induced damage. Subsequently, by either inhibiting or stimulating the synthesis of endogenous melatonin within rod receptor cells of adult Rana pipiens, we verify whether endogenous indoleamines could afford protection of the whole photoreceptor against the visible light-induced damage. It was found that by inhibiting melatonin production, a dose-dependent increase in oxidant generation and membrane damage was observed in intact rods. On the contrary, the stimulation of photoreceptor' synthesis of melatonin almost completely abolishes the oxidative stress and damage induced by visible light. Conclusions: Our results provide the first direct evidence that endogenous indoleamines protect photoreceptors from oxidative stress and damage induced by visible light.

Key words

Rod photoreceptor • Frog • Light damage • Endogenous indolamine

Introduction

The damaging effect of prolonged illumination upon retinal cells, and more specifically upon photoreceptors has been the subject of many investigations in the past (see: Organisciak and Vaughan, 2010). The damages were mostly assessed by structural morphometric criteria, or by biochemical analyses which showed progressive membrane fatty acid oxidation (Wiegand et al., 1983; De La Paz and Anderson, 1992). The molecular mechanism of the light induced damage however, which is of prime interest for developing potential protective strategies, was largely unknown except that it resided in the photoreceptors outer segment.

In this article a cellular model will be described

Corresponding Author: P.L. Marchiafava, Dipartimento di Fisiologia Umana, Università di Pisa, via San Zeno 29, Pisa, Italy

B. Longoni and GC. Demontis critically participated to most of the experiments described in the present paper. The collaboration of C. Kusmic and D. Lapi during the early stages which led to the initial identification of the light damage in other animal species, beside the frog, was invaluable.

which reproduces the light dependent oxidative damage to retinal rods and which we used for the analysis of the molecular steps involved. Furthermore, we tried to identify the nature of the necessary defence mechanism that intact photoreceptors should be equipped with to contrast the light induced damage in order to preserve their integrity.

Methods

Intact rod receptors cells and their detached outer segments were isolated by manual dissociation of retinas excised from adult frogs (*Rana pipiens*), dark-adapted for 24 hours as already described (Marchiafava and Longoni, 1999). Cells were bathed in Ringer solution containing 10 μM of Dehydrorhodamin (DHR) 125, a membrane permeant probe that upon oxidation converts to florescent Rhodamine (RHO) 125 (Henderson and Chappell, 1993). Reactive oxygen species produced by photoreceptors during illumination were monitored by a corresponding level of RHO125 fluorescence. A T.I.L.L. Polychrome III Monochromator (T.I.L.L. Photonics GMBH) coupled to the microscope via an epi-fluorescence condenser provided the photoxidative stimulus consisting of long-wavelength blue light in the form of a 4μm spot (23 x 10^3 photons μm^{-2} s^{-1}, 485 nm peak emission and 8 nm bandwidth). Fluorescence microscopy was performed on a Nikon inverted microscope supporting a small chamber where cells were bathed and initially observed under infrared light, and their images recorded. The light induced oxidative processes in the outer segment were studied after blocking the phototransductive reactions downstream of activated rhodopsin through the use of patch pipette with ATP- and GTP-free solutions (see Demontis et al., 2002). The methods used in the experiments where the synthesis of endogenous melatonin was inhibited or enhanced by the use of p-CPA or 8-Br-cAMP, respectively, are extensively described in Demontis et al. (2011, *submitted for publication*). Also the statistical analysis of these treatments as well as the use of an ELISA kit (MP Biomedicals - Solon, Ohio) to measure the level of endogenous melatonin in photoreceptors, are described in detail in the same paper.

Results

The rod outer segment as a model for light induced oxidative damage

An initial difficulty in reproducing the light induced damage described by previous authors consisted in the long time of illumination required, that is a few days. This problem was overcome in the present

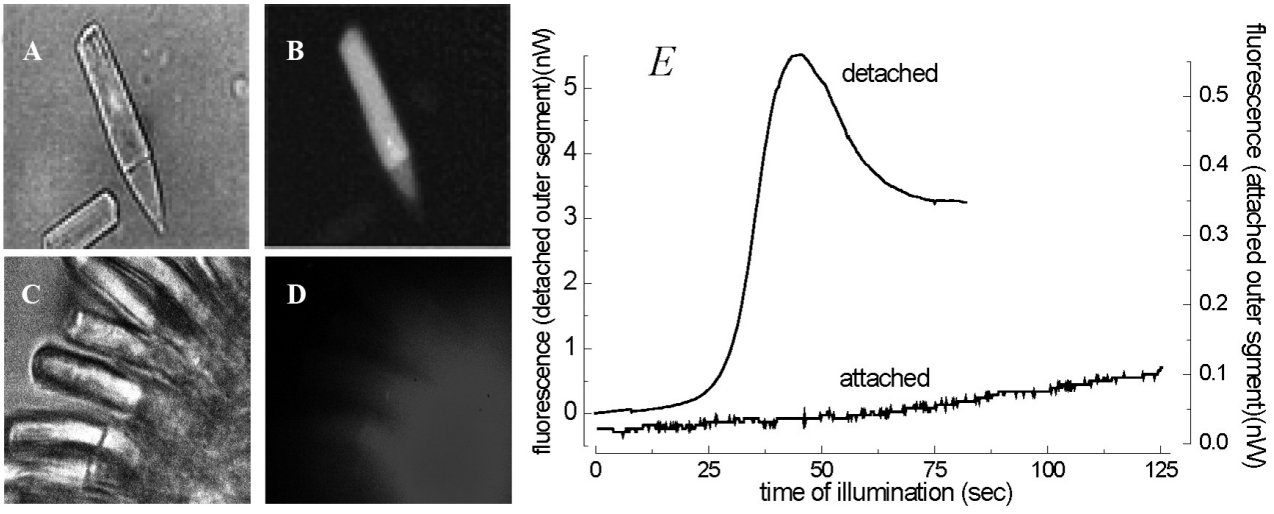

Fig. 1. - Light induced fluorescence responses from outer segments of retinal rod receptor cell. *A* and *C*, infrared images of detached and attached outer segment, respectively. *B* and *D*, after 50 seconds of illumination with a 4 μm spot (λ 485 nm, 23x10^3 photons μm^{-2} sec^{-1},). *E*, typical photometric recording of the light induced fluorescence during illumination. Note that ordinate at right refer to the lower trace (attached).

study, by means of a satisfactory experimental protocol whereby a damage to isolated, single photoreceptors was obtained by increasing the stimulation light intensity and by shortening the illumination time down to a few minutes. This comparatively brief time of about two minutes was compatible both with the electrophysiological methods we used and with a satisfactory cell viability.

The illumination with a 4μm diameter light spot ($2.3x10^4$ ph $sec^{-1}\mu m^{-2}$ at λ_{max} 485 nm) of the outer segment of intact, isolated rod receptor cells produced a saturating membrane voltage and current response which recovered after a few minutes, without the generation of any significant fluorescence increase. However, when the light spot was directed upon the cell region where light is absorbed, i.e. an outer segments detached from the inner portion of the photoreceptor, it was observed that this cell fragment underwent a rapid deterioration and death. Also, a strong fluorescence increase occurred during illumination of the detached outer segment, indicative of an underlying oxidative process, with a time course indicated in Fig. 1. The oxidative nature of the light damage was confirmed by its dependence on the oxygen supply, as well as by a fluorescence increase of Bodipy, a membrane permeable dye, selective for lipid peroxidation.

By lowering the illumination intensity by 0.2 log units, the fluorescence amplitude decreased significantly, indicating that the light intensity used for stimulation was at threshold for damaging the outer segment. Electrophysiological patch clamp recording during illumination revealed a dramatic transmembrane current increase starting at the time of the fluorescence peak (Fig. 2). Such sudden membrane breakdown could be responsible for the dye diffusion out the cell, which characterized the exponential fluorescence decay.

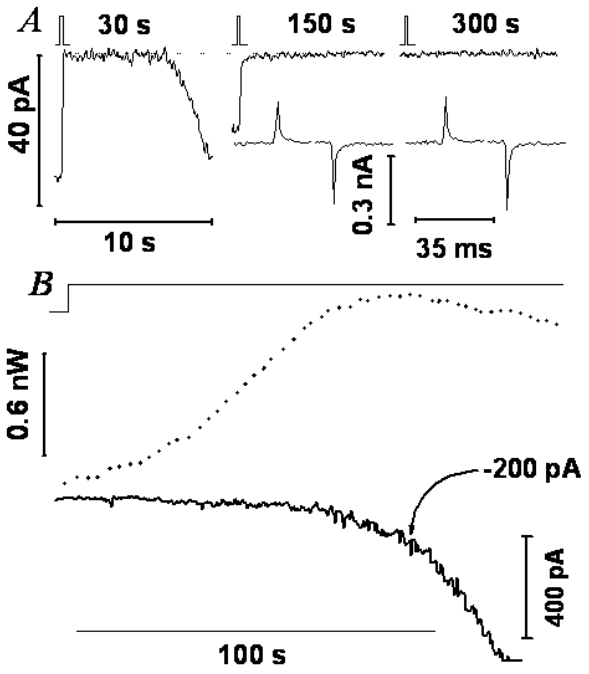

Fig. 2. - Light-induced oxidative stress evoked with ATP- and GTP-free intracellular solutions. *A*, light responses evoked after 30, 150 and 300 seconds of whole-cell recording with. Light stimulus (λ 576 nm, $3x10^2$ photons sec^{-1} cm^{-2}, 200 ms duration) is indicated above the current records. The dotted line corresponds to the holding current of -47 pA, measured at -50 mV in bright light, which provides an input resistance of 1.05 GOhms. Records in the insets are voltage-clamp responses to 5 mV voltage steps of 200 ms duration, taken before light stimulation at 150 and 300 ms from the start of internal dialysis. Note the break in the recordings. *B*, simultaneous measurements of membrane current (lower trace) and RHO123 fluorescence (upper trace) in response to test light, as indicated by the upper line. Arrow indicate that the peak of fluorescence occurs after the exponential increase in membrane current. Note the different calibration of membrane current in *A* and *B*. (rearranged from Demontis et al., 2002).

The molecular mechanism of the oxidative damage

From this first series of experiments it appeared that the isolated rod external segment represented a convenient model to investigate the molecular mechanism of the light induced damage.

Previous studies showed that rhodopsin is primarily involved in such mechanism, but the intermediate events leading to the lipid peroxidation, were not yet identified. The following results indicate that the oxidative process develops independently of the phototransduction pathways downstream of active rhodopsin. We showed that after washing out the ATP- and GTP-dependent steps of the phototransduction, thus interrupting the visual cascade (Lamb and Pugh, 1992; Arshavsky et al., 2002), still the oxidative process fully developed while the photoresponse had completely disappeared (see also Fig. 3 in Demontis et al., 2002).

Accordingly, a conditioning illumination strongly influenced the intensity of the oxidative event, indicating that the level of retinal pigment at the time of illumination regulates the extent of lipid peroxida-

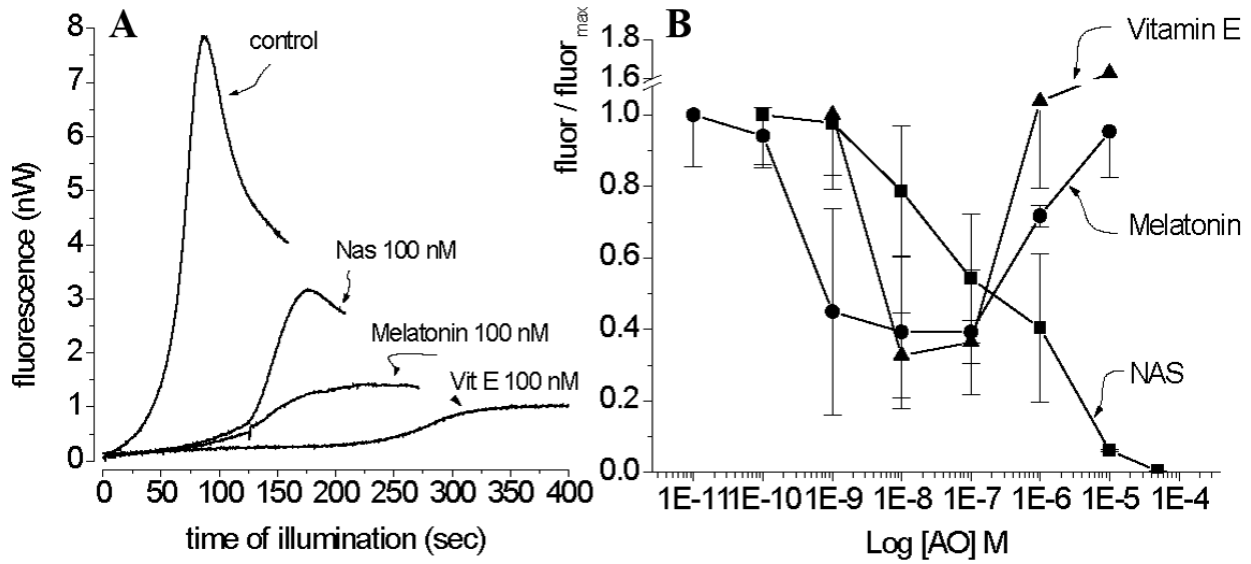

Fig. 3. - The effect of exogenous antioxidants upon the light induced fluorescence response from detached outer segments. *A,* the traces represent typical photometric recordings from a detached outer segment (control), and the attenuation produced by the application of N-acetylserotonin (Nas 100 nM), melatonin (100 nM) and Vitamin E (Vit E 100 nM) on the fluorescence responses. The effects of Nas and Vit E are illustrated to stress the predominant effect of melatonin over other antioxidants. *B,* superimposed graphs of the effects of melatonin (filled dots), Vitamin E (filled triangles) and Nas (filled squares) on the light induced fluorescence recorded from 56 detached outer segments. Vertical bars are standard errors.

tion. Based on kinetic consideration (see Demontis et al., 2002), MII appeared as a good candidate for the retinale intermediate initiating the observed lipid peroxidation of outer segment.

The protection of photoreceptors against the oxidative stress

The evidence that equal intensity of illumination applied to the outer segment of intact rod cell, at difference with the isolated outer segment, did not produce oxidative reaction nor a fluorescence increase, indicated a protective system as a prerogative of the cell integrity, and specifically confined to the inner photoreceptor segment. Here it is known that vertebrate photoreceptors contains the molecular apparatus for the synthesis of indolamines, which behave as potent antioxidant in various substrates (Longoni et al., 1997; Longoni et al., 1998), but their physiological role within photoreceptors was still unknown. To clarify this point, we applied *exogenous* melatonin to a solution of isolated cells, at the physiological concentration of 0.2 nM, obtaining an almost complete protection of the isolated outer segment from the light induced oxidative damage (Marchiafava and Longoni, 1999) (Fig. 3).

To identify the physiological role of *endogenous* indoleamines we investigated the effects of either increasing or decreasing their synthesis on the level of oxidants during illumination. The original results from these experiments will be extensively described in a forthcoming paper (Demontis et al., 2011, *submitted for publication*). Briefly, isolated photoreceptors obtained from fragmentation of dark adapted retinas incubated for 45 minutes with 1 mM 8-Br-cAMP, a membrane permeable analogue of cAMP, showed an increased synthesis of endogenous MLT while in isolated outer segments the light induced oxidants generation, was strongly inhibited. Conversely, 4-Chloro-D,L-phenylalanine (p-CPA), an inhibitor of indoleamines synthesis (Koe and Weissman, 1966; Jequier et al., 1967), dose-dependently decreased MLT while favouring the production of oxidant species during intense illumination of intact rods (Fig. 4).

Furthermore, the inhibitory effects of p-CPA on the production of melatonin were subsequently abolished by the application of exogenous MLT.

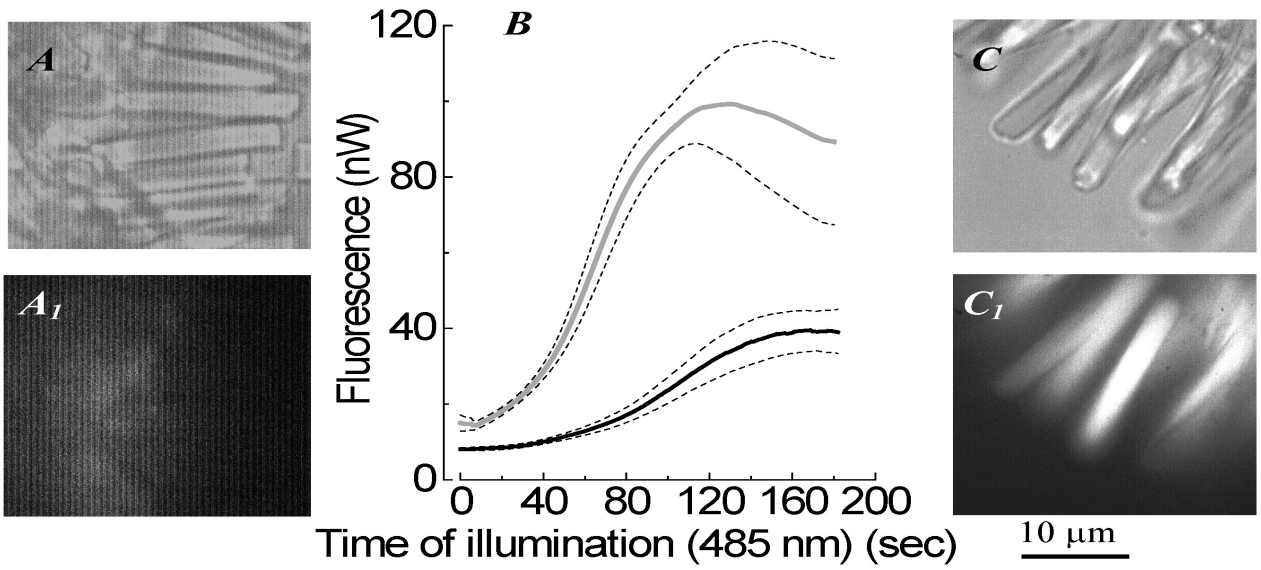

Fig. 4. - Light induced oxidative stress in bundles of entire photoreceptors and the effect of p-CPA. *A,* shows the infrared micrographs of a rod bundle before diffuse illumination with strong blue light. A_1, the same cells at the peak of the fluorescent response to the strong blue light. *B, lower curve*: *solid trace:* averaged fluorescence of eight intact cells without p-CPA. *B, upper curve*: illustrates the average fluorescence photometrically recorded from eight cells of a correspondent number of bundles treated with p-CPA. *Dashed curves* (close to *solid traces*): SEM. *C,* IR micrograph of a bundle of photoreceptors, treated with p-CPA before strong illumination, and C_1, the same bundle at the peak of the fluorescence emission.

Discussion

These data provide the first direct evidence that endogenous melatonin, synthesized within the inner segment of photoreceptors, plays a critical protective role against light-induced oxidative stress initiated in the outer segment. In the future, these studies may help defining the best antioxidant therapy for specific forms of neurodegenerative diseases, where oxidative stress is an ordinary contributor.

The peak melatonin synthesis at the end of the night seems particularly appropriate to eliminate the conspicuous dark-current oxidant production by rod photoreceptors (Ames et al., 1992), and at the night-day transition, when dark-adapted photoreceptors are especially sensitive to light-induced damage.

This conclusion should also be evaluated with respect to the increase sensitivity to light damage after intravitreal administration of the melatonin receptor-antagonist luzindole which activates specific membrane receptors, particularly numerous in inner retinal neurones (Sugawara et al., 1998; Wiechmann et al., 1992). Rather than contrasting the direct protective effect of melatonin against the light induced oxidative stress, it indicates that melatonin is also involved in paracrine signalling among retinal cells. Such dual melatonin effects, directly protecting and/or increasing retinal susceptibility to light damage may become dissociated in mice lacking melatonin inner retinal receptors (Demontis, personal communication).

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