RESEARCH ARTICLE

Characterization of Zebrafish Green Cone Photoresponse Recorded with Pressure-Polished Patch Pipettes, Yielding Efficient Intracellular Dialysis

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Abstract

The phototransduction enzymatic cascade in cones is less understood than in rods, and the zebrafish is an ideal model with which to investigate vertebrate and human vision. Therefore, here, for the first time, the zebrafish green cone photoresponse is characterized also to obtain a firm basis for evaluating how it is modulated by exogenous molecules. To this aim, a powerful method was developed to obtain long-lasting recordings with low access resistance, employing pressure-polished patch pipettes. This method also enabled fast, efficient delivery of molecules via a perfusion system coupled with pulled quartz or plastic perfusion tubes, inserted very close to the enlarged pipette tip. Sub-saturating flashes elicited responses in different cells with similar rising phase kinetics but with very different recovery kinetics, suggesting the existence of physiologically distinct cones having different Ca\textsuperscript{2+} dynamics. Theoretical considerations demonstrate that the different recovery kinetics can be modelled by simulating changes in the Ca\textsuperscript{2+}-buffering capacity of the outer segment. Importantly, the Ca\textsuperscript{2+}-buffer action preserves the fast response rising phase, when the Ca\textsuperscript{2+}-dependent negative feedback is activated by the light-induced decline in intracellular Ca\textsuperscript{2+}.

Introduction

G-protein-coupled enzyme cascades are ubiquitous signalling systems: they transduce physical or chemical stimuli, triggered by photon absorption or ligand binding (e.g. ions, hormones, neurotransmitters, or odorants), into intracellular messages that elicit adaptive responses [1, 2]. The most direct, most sensitive method for studying these cascades is to precisely control the stimulus with light (for instance, by using optogenetics or caged agonists [3–5]), and recording the stimulus response with electrophysiological methods which are suitable for those...
systems that target ion channels. The study of how changes in specific transduction proteins – naturally occurring or produced by site-directed artificial mutants (as well as by knock down or over-expressing mutants) – affect electrical response is a potent method with which to investigate their functional role in vivo or ex vivo [6–9]. However, the production of a vital transgenic animal is often expensive and time consuming; furthermore, the protein level alteration is permanent, i.e. irreversible, and often, due to the activation of compensatory pathways, the genetic manipulation does not isolate the specific function of the protein under study [10].

Vertebrate visual phototransduction (reviewed in [11–14]) is one of the most extensively studied G-protein cascades and represents a powerful model system for investigating G-protein signalling, the advantage being that it is “naturally” activated by light. It is also important to understand animal vision because it is ultimately limited by photoreceptor performance. Vertebrate vision is initiated by the light-induced, transient suppression of the current flowing into the outer segment of the photoreceptor through the cGMP-gated channels in its plasma membrane. Changes in the intracellular cGMP concentration can be monitored in real time by whole-cell or suction electrode recording, the most accurate probe of functional integrity and performance of the phototransduction cascade and of the effect specific intracellular manipulations can exert on its enzymes [15–17]. Moreover, the outer segment contains high concentrations of proteins involved in this cascade (compared with proteins of other enzymatic processes) and thus they can be purified for biochemical, protein interaction and functionality studies [12, 18]. Combined with electrophysiological experiments, these assays have provided a precise, quantitative analysis of visual enzymatic processes, also permitting the realization of functional mathematical models [19–21].

Here we present a new method for the ex vivo study of a G-protein coupled enzymatic cascade, the ultimate goal being to up- and down-regulate a photoreceptor protein and, in parallel, measure how the light response is affected. An obvious strategy for realizing this is, for instance, to deliver the specific protein itself, or its monoclonal antibody, during whole-cell recording of photoreponses, here performed for the first time on green-sensitive cones of zebrafish. This animal has attained increasing interest in recent years as a model for the investigation of vertebrate and even human vision [22]. Moreover, its embryonic development is remarkably fast [23] and huge resources for its genetic engineering are available. We narrowed our research to green cones because they can be isolated from the zebrafish retina in larger numbers than the other cone types. Finally, since phototransduction in cones is not as well understood as in rods [24], we also investigated some basic properties of cone cell responses. Whole-cell recording with intracellular perfusion was then developed for these very small cells because it circumvents many problems affecting other recording techniques. For instance, suction-pipette recordings from zebrafish cones [25, 26] do not allow the investigator to collect the entire membrane current, to clamp the membrane voltage, or to manipulate the intracellular milieu. To some extent, suction recordings from truncated photoreceptors [27] circumvent the last problem only, but they do carry a high risk of losing cytoplasmic molecules. Truncation of outer segments smaller than those from amphibians is even more demanding because it would lead to severe mechanical cell injury and poor signal-to-noise ratios. However, whole-cell recording from such minute cells as zebrafish cones requires patch pipettes with a very small tip opening; their conventional fabrication inevitably produces very long and tapered shanks, leading to high access resistance, low diffusional rate between pipette and cytosol, and makes inserting perfusion tubes in the pipette lumen infeasible.

Here, the exogenous molecules were administered during whole-cell recording by using pressure-polished patch pipettes [28] whose enlarged shank could accommodate pulled perfusion tubes very close to the pipette tip, and greatly reduced the access resistance. To provide the fast, controlled cytosolic incorporation of exogenous molecules, the intrapipette tube was
coupled to a perfusion system. In the following, the efficacy of the intrapiptide perfusion apparatus was tested by measuring the rate of accumulation of a fluorescent dye, injected in the outer segment or in the inner segment cytosol during whole-cell experiments. The whole-cell recordings of the photoresponse of green-sensitive cones, employing pressure-polished pipettes, were then analyzed in details for the first time, thus establishing the performance of these cells under control conditions. Analysis of the photoresponse recovery phase suggests the existence of physiologically distinct cones having different Ca\textsuperscript{2+} dynamics. The Ca\textsuperscript{2+}-buffering capacity of the outer segment strongly regulates this dynamics: importantly, a minimal model fitting the photoresponses demonstrates that this Ca\textsuperscript{2+}-buffer prevents the slow-down that can occur in the response rising phase when a negative feedback (whatever its nature) regulating receptor sensitivity is activated during the light response. Finally, the extremely fast rising phase of the response to supersaturating flashes is consistent with the hypothesis that rhodopsin may interact with transducin in the dark by forming preassembled complexes [29].

**Materials and Methods**

**Cone Isolation and Viewing**

All experiments on zebrafish (*Danio rerio*) were performed in compliance with the European Communities Council Directive for animal use in science (86/609/EEC) and approved by the ethical committee of the University of Ferrara named "Comitato Etico di Ateneo per la Sperimentazione Animale" (C.E.A.S.A.). Animals 4–5 cm in length were purchased from a local supplier, with no more than 30 specimens maintained in a 70-liter tank containing recirculated water continuously filtered and aerated, at 26–28°C on a 12 h light—12 h dark cycle, following the guidelines reported in [30].

A healthy zebrafish was dark-adapted for 3 h and sacrificed in dim red light (generated by LEDs with wavelength of 660 nm) by cranial concussion followed by decapitation and pithing. The head was swiftly immersed in a Petri dish containing Ringer solution of the following composition (in mM): 115 NaCl, 3 KCl, 10 HEPES free acid [N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)], 0.6 MgCl\textsubscript{2}, 0.6 MgSO\textsubscript{4}, 1.5 CaCl\textsubscript{2}, 10 glucose (buffered to pH = 7.6 with NaOH; osmolality: 260 mM mOsm/Kg). The Petri dish containing the head was transferred in a fully darkened box equipped with infrared LEDs (wavelength: 940 nm) and a high definition webcam that had its infrared filter removed and was connected to an external monitor. After inserting the hands through the two light-tight holes of the box, the cornea of each eye was focussed on the monitor screen and it was cut with a razor blade piece. Each retina was separated from the pigment epithelium by sucking it gently into the tip of a 200 μl pipette, which was also used to mechanically dissociate the photoreceptors by triturating the retina. An aliquot (~2 ml) of the Ringer solution containing the photoreceptors was transferred to the recording chamber placed on the microscope (TE 300, Nikon, Tokyo, Japan) stage. The preparation was illuminated with a cluster of ultra-bright infrared LED (900 nm) and focused on a fast digital camera (C6790-81, Hamamatsu Photonics, Tokyo, Japan) coupled to the microscope.

**Recordings from Zebrafish Cones and Intracellular Perfusion**

Light responses of zebrafish cones were recorded using the whole-cell configuration of the patch-clamp technique, under dark-adapted condition at room temperature (20–22°C). Pipettes were usually sealed on the cone outer segment, unless specified otherwise; details of the patch-clamp apparatus is described elsewhere [28, 31]. The current amplitude elicited by repetitive -10 mV pulses was used to measure the cell capacitance, and the seal, the access (R\textsubscript{a}), and the membrane resistance; the holding potential was always -40 mV. Whole-cell recordings
were low-pass filtered at 2 KHz by an eight-pole Butterworth filter (VBF/8 Kemo, Beckenham, UK), sampled at 19.2 kHz/16 bits by a A/D board (Digidata 1322A; Molecular Devices, Sunnyvale, CA, USA) controlled by Clampex software (Molecular Devices), and analyzed with Clampfit (Molecular Devices), Sigmaplot (Systat Software Inc., San Jose, CA, USA) and Mathcad (Parametric Technology Corporation, Needham, MA, USA) software. To reject as much noise as possible, each photoresponse shown in the figures was the average of three or more responses to the same flash; this average response was then further filtered by using a local smoothing routine implemented in the commercially available program Sigmaplot. The routine parameters were set to obtain low-pass filtering of 30 Hz (negative exponential was selected for smoother function, with a sampling proportion of 0.01, a polynomial degree 1, and a number of interval that was 1/10 of the total number of samples constituting each trace, i.e. 19220 samples for a 1 s trace). Traces longer than 2 s were just low-pass filtered at 20 Hz with the Gaussian filter of the pClamp routine, since they illustrate only slow aspects of the photoreponses: their huge number of points made it impractical to use the Sigmaplot smoothing routine on desktop computers.

Results are given as means±SEM.

Patch pipettes were filled with an intracellular solution containing (in mM): 40 KCl, 70 K-Asp, 5 MgCl2, 1 GTP, 5 ATP, 5 HEPES (buffered to pH = 7.6 with KOH; osmolality: 260 mM mOsm/Kg). All chemicals were purchased from Sigma (St. Louis, MO, USA). The intracellular dialysis of zebrafish cones was performed by using pressure-polished pipette and an intra-pipette perfusion system (Fig 1A). Besides improving the electrical recordings, the enlarged shank of pressure-polished pipettes [28, 31] can accommodate pulled quartz or plastic perfusion tubes close to the pipette tip (Fig 1A), allowing the fast and controlled cytosolic incorporation of exogenous molecules. The perfusion tube was filled with an intracellular solution containing the molecules under study, which was injected in the cytosol during whole-cell recording by the controlled application of pressure to the capillary lumen. The pressure was delivered by a commercially available perfusion pressure/vacuum generator (2PK+, ALA scientific instruments, New York, New York; applied pressure: ~40 PSI (~280kPa)), or with a 1 ml precision syringe coupled to a micromanipulator (Fig 1A).

Light Stimulation and Calibration

Light stimuli (flashes in the dark and flashes on a background of light) were designed using the voltage protocols of Clampex software. One of the two analogical outputs of the Digidata board was connected to a calibrated voltage-to-current converter driving an ultra-bright trichromatic LED (with center wavelength red/green/blue of 627.5±8/525±10/467.5±8 nm), that had its plastic lens replaced with a frosted filter. The LED was mounted on a miniaturized xyz micro-manipulator and coupled to the binocular port of the inverted microscope (Nikon TE-300, Tokyo, Japan). The LED was aligned so as to have its light spot automatically centered and in focus on the zebrafish cone, when the latter, illuminated with the cluster of ultra-bright infrared LEDs, was viewed in sharp focus through a 60x objective on the microscope camera (C6790-81, Hamamatsu Photonics, Tokyo, Japan). Enough space was left between the trichromatic LED and the binocular port to host neutral density filters, when required; the trichromatic LED positioning and measurement of its spot size were performed with the following procedure. First, a particular subdivision of the grid of a calibrated microscope slide was focused and centered on the inverted microscope 60x objective, by using the microscope infrared LED illumination. Then a straight microscope, mounted on a second xyz micromanipulator, was aligned to the inverted one, and it was moved so its 60x objective was focused and centered on the same grid subdivision using the trichromatic LED as a light source. The LED
spot, observed with the straight microscope, was finally focused and centered with its \(xyz\) manipulator on the grid subdivision, and its geometry measured (it resulted a uniform disk of 300 \(\mu\)m of diameter). The microscope slide was then replaced by the sensor (OP-2/LM-2 VIS, Coherent, Santa Clara, CA, USA) of a power/energy meter (Fieldmaster, Coherent), and the
light power produced by the green and the red section of the trichromatic LED was measured at all the voltages used in the experiments. Voltages, neutral density filters, and light stimulus duration were matched so as i) to have a wide range of light intensities spanning more than 5 order of magnitude, with currents always falling in the green and red LED linear range (1–50 mA) and ii) to have the same light intensity of red and green LED (the blue one was not used). The duration of all flashes up to \(1.77 \cdot 10^4\) photons/\(\mu m^2\) of intensity was 1 ms, above this intensity it was 10 ms.

To estimate the physical collecting area of the zebrafish cone outer segment (the functional collecting area cannot be estimated since cones do not detect single photons), the quantum efficiency of photoisomerization and the specific axial rhodopsin density must be known [32]. Moreover, it is difficult to have a reasonable estimate of the cone rod outer segment volume, since it is strongly invaginated, its geometry cannot be simply assimilated to a truncated cone, and its small dimension makes precisely measuring the diameter of base and tip difficult. The geometrical area of the cone outer segment was measured on digital micrographs obtained with the camera (494x674 pixels, 0.2 \(\mu m\) per pixel) using a freeware image analysis software (ImageJ, http://rsb.info.nih.gov/ij/) and was 15.3\(\pm3.9\) \(\mu m^2\) \((n = 20)\). Using the ratio between the geometrical rod outer segment area and the collecting area of \(~0.062\) calculated by Baylor et al., 1979, it can be roughly estimated that the zebrafish cone collecting area is \(~1\) \(\mu m^2\). If this line of reasoning proves to be correct, then the numerical values of the flash intensities reported throughout the paper (in photons/\(\mu m^2\)) are also the number of photons absorbed by the cone.

Fluorescence Imaging

Besides bright-field viewing of the cells, the camera and its controlling software (AquaCosmos, version 2.5.3.0; Hamamatsu Photonics) were also employed for fluorescence imaging experiments; the excitation light was generated by a monochromator (Polychrome II, Till Photonics, FEI, Hillsboro, Oregon, USA) coupled to the epifluorescence port of the microscope via an optical fiber. Solution loading in the cell was checked by using lucifer yellow (CH, dilithium salt; dissolved in intracellular solution at 40 \(\mu M\) concentration; excitation: 425 nm, emission: 528 nm; Fig 1B and 1C). Image analysis was performed by using AquaCosmos and MATLAB (MathWorks, Natick, MA, USA) software. The effectiveness of the molecular cytosolic loading during whole-cell recordings was assessed by measuring the dynamics of fluorescence rise integrated in three cellular regions (one close to the patch pipette and two far away from it, Fig 1B, panel a and Fig 1C, panel a), following the injection of lucifer yellow into the cell. In principle, many factors influence such dynamics, for example cell geometry, where the pipette is sealed on the cell, the effective pressure applied inside the pipette perfusion tube, the position of this tube inside the pipette, pipette geometry and access resistance (\(R_a\)). Despite all these factors, fluorescence grew with similar kinetics in all cells examined: in the regions close to the pipette, fluorescence dynamics were described by the sum of a fast (parameters with the subscript \(f\)) exponential component and a slower (subscript \(s\)) one:

\[
F(t) = A_f \left( 1 - e^{-\frac{t}{\tau_f}} \right) + A_s \left( 1 - e^{-\frac{t}{\tau_s}} \right)
\]

The fast component amplitude (i.e. \(A_f\)) progressively decreased (i.e. it was "low-pass filtered") as the distance between the pipette (i.e. the point of dye injection) and the integration region was larger. Eventually, the fluorescence increased in the distal regions (with a small delay that was never larger than 5 s) with a slow monoeponential kinetics (Fig 1B and 1C). Therefore, the parameters that were reproducible from cell to cell were \(\tau_f\) in proximity of the pipette and \(\tau_s\) in all points (\(\tau_f\sim0.97\pm0.18\), \(\tau_s\sim18.3\pm0.7\); \(n = 11\)). As an example, Fig 1 reports the
fluorescence dynamics and the relative fittings in the two most different recordings obtained: a large cell, where the patch pipette was sealed on the outer segment (Fig 1B), and a much smaller cell, with a very different morphology, where the patch pipette was sealed on the inner segment (Fig 1C). It can be concluded that, in all cone regions, a substantial cell loading of an exogenous molecule is achieved one minute after molecule injection.

Results

To perform whole-cell recordings from very small and fragile cells as the zebrafish cones, a particular patch pipette shape was designed (as described in [28, 31]), characterized by a very small tip and an enlarged shank, which allowed low access resistance ($R_a \approx 6.7 \pm 1.1 \, \text{M} \Omega \, \text{range: } 2.0–13.0 \, \text{M} \Omega \, n = 36$) recordings and efficient cytosolic incorporation of exogenous molecules. Long lasting recordings (Fig 2A) with pressure-polished pipettes (Fig 1) were obtained more often from isolated green cones, therefore this study was limited to this cell type only.

Since the image resolution of the bright field microscope used could not unambiguously ascertain the morphology of small cells, it seemed likely that some recording might instead be derived from the red cone counterpart of a double red and green cone [33]. To ensure that the recordings presented here were from green cones only, red and green stimuli were routinely delivered (Fig 2A and 2B). As a rationale, a cone is considered green sensitive if its response to a green (525 nm) flash $1.14 \cdot 10^3 \text{photons/\mu m}^2$ in intensity suppressed ~40% of the dark current (average suppression: 0.38±0.03, 334 flashes averaged in 25 cells) but it did not respond to a red flash (627.5 nm, Fig 2A and 2B) of same intensity. A typical recording using pressure-polished pipettes, lasting more than 20 min, is illustrated in Fig 2A on a very slow time scale: light flashes of increasing intensity (range: $1.14 \cdot 10^2$ to $3.76 \cdot 10^6 \text{photons/\mu m}^2$), were delivered in triplets in repeated sequences. The small drift in the baseline was due to small changes in the current flowing through the inner segment channels rather than in changes in the light sensitive (or dark) current flowing through the cGMP channels, because the amount of current suppressed by saturating flashes (with intensities $\geq 1.8 \cdot 10^4 \text{photons/\mu m}^2$) was the same (average: $18.7 \pm 1.8 \, \text{pA; } n = 25$) from the beginning to the end of these recordings. Responses to the same flash intensities were averaged and smoothed (see below and Methods): they were reproducible over a recording time of up to more than 20 min. This reproducibility is exemplified by the recording of Fig 3A–3C: consecutive flashes of the same intensities, delivered within ~2, ~5 ~10 and ~15 min from the beginning of whole-cell recording, did not reveal any significant differences with respect to dark current amplitude and response kinetics. Therefore these responses were all averaged, aligned, and normalized as shown in Fig 4A and 4B. Steps of light ($1.8 \cdot 10^3 \text{photons/(\mu m}^2\cdot\text{sec})$, lasting 8 s) were occasionally delivered during this recording (Fig 2A, 2C and 2D) at the following times: 227 s (black trace), 285 s (green), 583 s (red), 977 s (blue), 1100 s (pink), and 1136 s (cyan). These steps gave a constant fractional suppression of 83% (averaging the response between 2.5 and 6 s, where current was most stable) for at least 5 min of recording, and declined to 69% (Fig 2C) after 20 min, but after having delivered repetitive supersaturating flashes (Figs 2A, 4A and 4D). Light sensitive current declined from 20.6 to 19.8 pA during this 20 min recording, i.e. 3.9%, while sensitivity in the same period declined of 17.3%. The decline in sensitivity and dark current was in general not larger than 5% and 10%, respectively, in the first $12.5 \pm 1.4 \, \text{min (} n = 20; \, \text{range: } 4–23 \, \text{min})$ of recording. Responses in Fig 4A recovered from the maximum amplitude with a characteristic waveform, consisting of dual kinetic components, that was particularly evident for flash intensities in the range $2 \cdot 10^3$ to $10^4 \text{photons/\mu m}^2$. This feature was observed clearly in ~40% of the recordings ($n = 25$), while others had a faster recovery with a single kinetic component, (Fig 4B, red traces, compared with the corresponding four responses with a dual component recovery, in black, on an expanded...
time scale). In fact, a continuum of behavior in between these two recovery types (as the thick blue trace, that is the blue trace of Fig 5C) was observed.

Besides these differences in recovery phase, all green-sensitive cones had similar photore- sponse rising time and sensitivity, with a threshold of ~10^2 photons/μm^2 (the average response to this flash intensity always had recovery with a single kinetic component; Figs 4A and 6A). Therefore, all response amplitudes were collectively analyzed as a function of the light intensity \( I \) in Fig 4C. Data were poorly fitted by the Hill equation \( R(I) \):

\[
R(I) = \frac{I^m}{I^m + I^m_0}
\]

where \( I_0 \) is the half-saturating intensity and \( m \) is Hill coefficient. This equation fitted the data point at low intensities but not at higher ones (\( I_0 = 2 \cdot 10^3 \) photons/μm^2, \( m = 1 \); Fig 4C, blue curve), or vice-versa (\( I_0 = 2.3 \cdot 10^3 \) photons/μm^2, \( m = 1.4 \); green curve). The best fit was given by
the exponential saturation equation:

\[ R(I) = 1 - e^{I/I_0} \]

Where \( I_0 = 3 \times 10^3 \text{ photons/\mu m}^2 \) \textbf{(red curve)}.

In order to reduce the background noise, photoresponses were averaged and further processed with a smoothing routine \textit{(see Methods)}. This operation did not alter the entire waveform of the photoresponse to flashes \textit{−} from dim up to just saturating ones \textit{(i.e. delivering less than \( \sim 10^4 \text{ photons/\mu m}^2 \))} \textit{−} but it slightly altered the rising phase of stronger flashes at early times. Since this resulted in an underestimation of the delay between the flash delivery time and onset of the response \textit{(Fig 3D, inset), the analysis of the responses to supersaturating flashes were performed on unsmoothed traces \textit{that were then just low-pass filtered at 2 kHz by the anti-aliasing filter; Figs 3D and 4D)}. The rising phase kinetics accelerated upon increasing the flash intensity up to \( 1.85 \times 10^5 \text{ photons/\mu m}^2 \), but above this value no further acceleration was
observed (Fig 4D). The linear fit to the normalized rising phase gave a slope of 48.6±5.7 s⁻¹ 
(n = 5 responses in 5 cells to flashes delivering 1.85×10⁵ or 3.76×10⁵ photons/μm²).

Characteristic features of light adaptation in green cones were measured by delivering flashes of light in the absence or in presence of a background of light. A step of light caused a fast decline in current, followed by a much slower recovery to a steady-state level (Fig 5A). The step response amplitude slightly declined in the course of this recording (Fig 5A) because, in darkness, the time interval in between the steps was not long enough to let the cell fully recover its dark state. Despite the large amount of light absorbed by the cone, the recordings were quite stable, as exemplified by the recording of Fig 5. Indeed, the waveform of the average response
to three consecutive flashes delivered in the dark and during the step of light at the beginning (responses under the black bars, Fig 5A; black traces, Fig 5B), in the middle (green) and at the end (red) of the recording were almost identical. The average flash responses in the presence of a background of light were smaller and faster in respect to the ones recorded without any adapting light (Fig 5C and 5D). For example, the background of $1.3 \times 10^5$ photons/($\mu$m$^2$/sec) suppressed 63±9% of the dark current (10 steps averaged, $n = 3$) and reduced the sensitivity as follows: a flash delivering $4.89 \times 10^3$ photons/$\mu$m$^2$ in the dark suppressed ~70±4% of the current (274 responses averaged, $n = 25$), while a flash twice as strong delivered on the background suppressed ~45±12% of the current (27 responses averaged, $n = 3$; Fig 5C). Due to the desensitization extent, it was possible to compare only the response to saturating flashes delivered in the dark and on a background of light (Fig 5D).
Discussion

The whole-cell patch-clamp recordings from zebrafish green cones performed with pressure-polished pipettes had reproducible sensitivity, waveform photoresponse kinetics and light adaptation. We therefore conclude that the light triggered enzyme cascade was not perturbed by these pipettes which, at the same time, enabled fast loading of exogenous molecules by positioning perfusion tubes very close to the pipette tip.

A peculiar feature of the light responses to supersaturating flashes is the very fast kinetics of their rising phase produced by a burst of PDE activity able, within ~12 ms, to hydrolyze most of the intracellular cGMP pool (Fig 4D). This could arise from the small volume of the cone outer segment [34] however, the possibility that other, less well understood mechanisms could account for these fast kinetics cannot be ruled out. The frequency of encounters between the photoactivated visual pigment rhodopsin (or cone opsin) and the cognate G-protein transducin has been considered a major factor in determining the slope of the response rising phase, i.e. the cascade activation kinetics [34]. It is however possible that, in the dark, dimers of rhodopsin may interact with transducin by forming preassembled complexes [29, 35], increasing the probability of encounters between these proteins. This would obviously accelerate

Fig 6. Theoretical prediction of the effect of a Ca²⁺ buffer on the photoresponse recovery phase. A, average response (n = 9) to the same flash (1.14 \times 10^2 photons/µm²) having a slow kinetics (black dotted trace) or a fast one (grey dotted trace) fitted by x_s(t) with, respectively: A_s = 370, r = 0.052 s; A_s = 1100, r = 0.035 s; these responses are from the two cells of Fig 4B, whose responses are plotted in black and in red, respectively. B, schematic of the “delayed” feedback, the symbols are explained in the text. The gain is set to 1, so G is the fraction of the response amplitude “feedbacked” to the input. C, red trace, plot of x_o(t) with A_o = 5200 and r = 0.052 s; brown trace, plot of x_o(t) (with same A_o and r of red trace) with G = 0.6 and Δ = 0; green trace, plot of x_o(t) with the same parameters of the brown trace but Δ = 0.06 s to fit a representative experimental response with a dual kinetic component recovery (to flash of intensity 4.49 \times 10^3 photons/µm², plotted in black in Fig 4B; dotted black trace in this panel); parameters of blue trace as the green trace, but Δ = 0.1 s. D, responses with the same colour code of C: red trace, x_s(t) with A_s = 12300 and r = 0.035 s; x_o(t) is plotted in brown with G = 0.4 and Δ = 0 s, in green with Δ = 0.055 s, and in blue with Δ = 0.1 s; the green trace fits a representative experimental response with a single kinetic component recovery (to flash of intensity 4.49 \times 10^3 photons/µm², plotted in red in Fig 3B; dotted black trace in this panel).

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photoresponse kinetics, a notion consistent with the rapidity of the observed response rising phase, but the underlying hypothesis is still being debated [36–38].

Another interesting feature of the flash response waveform is the cell to cell difference of its recovery phase (single vs dual kinetic components, Fig 4B). Dual kinetic recovery was particularly evident for the responses to flashes from moderate (i.e. suppressing above 20% of dark current, Fig 4A and 4B) to just saturating intensities. On the other hand, dim flashes (Fig 6A) or supersaturating flashes (Fig 4A) always elicited response recoveries with a single kinetic component. This suggests that the dual component recovery occurs when the transient fall in intracellular Ca\(^{2+}\) elicited by the flash is significant, but at the same time the flash intensity is low enough to allow the cascade (i.e. the phosphodiesterase) to be quickly inactivated. Therefore, the difference between the two recovery phases may originate from different intracellular Ca\(^{2+}\) dynamics and this would be detected by several Ca\(^{2+}\) sensor proteins regulating the phototransduction cascade at various levels (see Introduction), collectively acting as a negative feedback. It is possible to mathematically demonstrate that the difference between single and dual kinetic component response may simply be due to different cell Ca\(^{2+}\) buffering capacity, as explained below.

Consider a system that produces a "photoresponse" \(x_s(t)\) described by the Poisson equation [39]:

\[
x_s(t) = A_s \tau e^{-t/\tau}
\]

where \(A_s\) and \(\tau\) are constants (6A, red and orange traces; 6C and D, red traces). This equation is intended to fit the smallest average response to a flash (intensity: 1.14 \(\times\) 10\(^2\) photons/\(\mu\)m\(^2\); Fig 6A, noisy traces) where the changes in intracellular Ca\(^{2+}\) are minimized, and thus the intervention of the various Ca\(^{2+}\)-dependent feedback pathways are minimized as well. The fitting (Fig 6A, orange trace) is quite accurate for the fast responses (as the one in Fig 6A, grey dotted noisy trace, produced by the same cell that generated the red responses in Fig 4B). However, no values of \(A_s\) and \(\tau\) are able to fit the entire slow response waveform: the falling phase of \(x_o(t)\) (Fig 6A, red trace) is always faster than the experimental responses (as the one in Fig 6A, black dotted noisy trace, from the same cell in Fig 4A and 4B, black traces). These slow responses can be fitted by a different expression for \(x_s(t)\) but, for the sake of simplicity, the fitting illustrated in Fig 6B (red trace) is good enough for the argument that follows. Let’s assume that the photoreponse to a strong stimulus is described by the same equation and same \(\tau\) as the response to the dimmest stimulus, but with a larger \(A_s\). Let’s also assume that this "larger" photoresponse is the "input" of a system regulated through a negative feedback mechanism (Fig 5B) aimed at reducing photoreceptor sensitivity and thus extend its dynamic range. Let \(H\) be the gain and \(G\) the amount of "regulated photoresponse" (or “output”), \(x_o(t)\), subtracted (i.e. negatively "feedbacked") from the input. The more intense the light absorbed by the photoreceptor, the larger the decline in intracellular Ca\(^{2+}\) and, consequently, the larger parameter \(G\) will be. A Ca\(^{2+}\) buffer introduces a delay, \(\Delta\), between the time in which an intracellular Ca\(^{2+}\) change takes place and the time in which the Ca\(^{2+}\) sensor proteins detect it. Let \(x_{\Delta o}(t)\) be the delayed output that is now subtracted from the input, as a consequence of the delay \(\Delta\) introduced in the feedback path by the buffer [21]. It follows that:

\[
x_o(t) = H \ast [x_s(t) - x_{\Delta o}(t) \ast G]
\]

In the absence of any delay, the amplitude of the regulated responses, \(x_o(t)\) (Fig 6C and 6D, brown traces) is just scaled down when compared to the un-regulated response, \(x_s(t)\) (Fig 6C...
and 6D, red traces), if:

\[ H(1 - G) < 1 \]

To fit two representative experimental photoresponses (of Fig 4B) to the same flash, one slow and recovering with a dual component kinetics (Fig 6C, black dotted trace), and one fast and recovering with a single component kinetics (Fig 6D, black dotted trace), it is necessary to set \( \Delta \neq 0 \). This accelerates the theoretical photoresponse recovery which exhibits a dual (Fig 6C, green trace) or single kinetics component (Fig 6D, green trace), depending upon the values of \( \tau \) and \( \Delta \). The former is constrained by the fit to the two responses to the dimmest flash (Fig 6A, noisy traces, same two cells of Fig 4B) while the latter is adjusted to optimize the fit to the experimental traces. Notably, the fitting to the fast response is still good, while the fit to the slow response (with a dual component recovery) still has a falling phase that is faster than the experimental one. Eventually, as \( \Delta \) is made larger, i.e. the cell’s buffering capacity is increased, responses exhibit damped oscillations (Fig 6C and 6D, blue traces), as observed in other cone species, where \( \Delta \) was experimentally increased by incorporating into the cone millimolar amounts of the fast Ca\(^{2+}\) buffer BAPTA [40]. The oscillations are larger and more persistent if \( G \) and/or \( \Delta \) are increased; the frequency of the oscillations become smaller as \( \Delta \) is made larger.

It is important to emphasize that the dramatic changes in the photoresponse recovery waveform, produced by \( \Delta \), is independent by the nature of the feedback (i.e. which element of the transduction system is affected by the decline in intracellular Ca\(^{2+}\)). Notably, the rising phase of the delayed regulated response is faster than the one with no delay (leaving all other parameters unchanged). In general, for a receptor to have a large operative range, its sensitivity must be progressively reduced as stimulus intensity is increased, and this requires the presence of a negative feedback. However, negative feedback compresses the entire response while, instead, a delay provides the same initial fast rising phase as the unregulated response but avoids the later response saturation. The advantage of this strategy is obvious: the survival of an animal is ultimately determined by the speed with which its photoreceptors detect a predator in dim or in strong light. It is possible that, in general, the response kinetics of any signal transduction cascade, regulated through a negative feedback by one or more intracellular messengers, is tuned by the capacity of cytosol to buffer this/these messenger/s, always ensuring a fast response onset for a wide range of stimulus intensities.

Interestingly, the damped oscillations of the recovery phase are thought to be typical of primate cones. However, as pointed out by Cao and colleagues [41], this is not the case: indeed, some primate cones showed monotonic responses, while others, during the same recording, exhibited monotonic or biphasic responses to the same flash intensity. Cao and colleagues showed that it was possible to switch from one kind of response to the other one by simply changing external Ca\(^{2+}\) concentration, strongly indicating that these two recovery types were generated by two different delays (\( \Delta \)) in Ca\(^{2+}\) feedback. On the basis of the present findings, it may be that the intrinsic Ca\(^{2+}\)-buffering capacity of the zebrafish cone was somewhat modified during these whole-cell recordings (for instance, by washing out a soluble Ca\(^{2+}\) buffer), transforming a dual component response recovery into a single component one or vice-versa. However, even in recordings lasting more than 20 min, the stability of the response waveform rules out any significant alteration in the concentration of any of the key components involved in shaping the photoresponse (besides the inevitable loss of a significant number of chromophore molecules as a consequence of very strong and repetitive light stimuli, producing the observed fall in receptor sensitivity; Fig 2). Moreover, in none of our recordings was a switch from single component to dual component response recovery and back ever observed, as in Cao and colleagues recordings. Rather, there was a large cell-to-cell variation in the kinetics of the
photoresponse falling phase (Fig 4B) to the same flash intensity, but that did not change during the course of the recordings. Because there was no correlation between $R_a$ and waveform kinetics, this cell-to-cell variation could not be due to a different $R_a$ which would, in principle, give different Ca$^{2+}$ dialysis. Indeed, in the course of five recordings, all lasting more than 7 min, $R_a$ changed up to seven-fold (the smallest change was from 4.1 to 11.5 MΩ, the largest from 3.7 to 26 MΩ) but there was no change in the response waveform within this period of time. Finally, the recordings obtained with a pipette solution from which the Ca$^{2+}$ contamination (∼1 μM) had been removed using 25 μM of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, K⁺ salt) were indistinguishable from the ones obtained in the absence of this Ca$^{2+}$ chelator. This demonstrates that the strong cooperative Ca$^{2+}$ regulation of guanylate cyclase, the influx of Ca$^{2+}$ through the light-regulated channels, and the efflux of Ca$^{2+}$ through the exchanger come together to form a powerful system to clamp the intracellular Ca$^{2+}$ concentration (and the dark current) to its physiological value. Indeed, to contrast this system and finally affect the photosresponse recovery kinetics, the photoreceptor outer segment must be dialyzed with hundreds of micromoles of BAPTA or of Ca$^{2+}$ [14, 21]. All these results suggest that there is a cell-to-cell variation in Ca$^{2+}$ dynamics and that this is primarily regulated by the Ca$^{2+}$ buffering capacity. It is then possible that the photoreceptor Ca$^{2+}$ dynamics is regulated at one or more stages by a still unknown mechanism. In different low vertebrate photoreceptor cells, the latter could be locked at different levels of activation, thus giving the observed differences in the recovery phase. With evolution, this mechanism could, in turn, be regulated in the same cell by specific physiological requirements, giving a different response to the same stimulus, as observed by Cao and colleagues.

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**Author Contributions**

Conceived and designed the experiments: GR MA MB. Performed the experiments: GR MA MB AF. Analyzed the data: GR MA MB AF. Contributed reagents/materials/analysis tools: GR. Wrote the paper: GR. Mathematical model building: GR.

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