Calcium-Sensitive Downregulation of the Transduction Chain in Rod Photoreceptors of the Rat Retina

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ABSTRACT In vertebrate rod outer segments phototransduction is suggested to be modulated by intracellular Ca. We aimed at verifying this hypothesis by recording saturated photosignals in the rat retina after single and double flashes of light and determining the time t_c to the beginning of the signal recovery. The time course of Ca_i after a flash was calculated from a change of the spatial Ca^{2+} concentration profile recorded in the space between the rods. After single flashes t_c increased linearly with the logarithm of flash intensity, confirming the assumption that t_c is determined by deactivation of a single species X^* in the phototransduction cascade. The photoresponse was shortened up to 45% if the test flash was preceded by a conditioning preflash. The shortening depended on the reduction of Ca_i induced by the preflash. The data suggest that the phototransduction gain determining the amount of activated X^* is regulated by a Ca_i-dependent mechanism in a short time period (<800 ms) after the test flash. Lowering of Ca_i by a preflash reduced the gain up to 20% compared to its value in a dark-adapted rod. The relation between phototransduction gain and Ca_i revealed a $K_{1/2}$ value close to the dark level of Ca_i.

INTRODUCTION

In vertebrate rod outer segments (ROS), light activates a cascade of enzymatic reactions leading to a reduction of ion current flowing across the outer membrane (1–3). In a first step, light-activated rhodopsin Rho* initiates a guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange in the G-protein transducin and a liberation of the α-subunit $T_\alpha GTP$. Binding of $T_\alpha GTP$ to cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase (PDE) abolishes the inhibitory function of the two PDE_γ subunits, resulting in accelerated cGMP hydrolysis. Finally, lowering the cGMP concentration causes a closure of cGMP-dependent channels. Activation of one rhodopsin interrupts the inflow of $>10^5$ Na^+ and Ca^{2+} ions into the cell. By reducing the Ca^{2+} influx through the cGMP-dependent channels (4–6), illumination reduces the Ca^{2+} concentration Ca_i in the ROS (7–14). Reduction of Ca_i is suggested to play a prominent role in regulating sensitivity and light adaptation: A desensitization of the rod during exposure to steady light and an accelerated recovery of the light-regulated conductance after flashes of light are both mediated by light-induced lowering of the Ca_i (15–17).

The reduction of Ca_i activates cGMP synthesis by guanylyl cyclase (18), which is thought to play a major role in light adaptation during background light (19). After saturating flashes, the reopening of the light-dependent channels is dramatically accelerated if guanylyl cyclase is allowed to be stimulated by a reduction of Ca_i (20). In addition to affecting guanylyl cyclase, a reduction of Ca_i was suggested to 1), reduce the amplification within the transduction cascade (21–23); 2), reduce the lifetime of activated PDE (24); or 3), increase the affinity of cGMP-gated ion channels to cGMP (25). Each of these effects would represent a negative feedback mechanism attenuating the process of phototransduction.

After a saturating flash, the light-dependent channels are kept close despite guanylyl cyclase being rapidly activated by a fall of Ca_i. For reopening the channels, PDE needs to be deactivated to a distinct level at which cGMP production and cGMP hydrolysis are balanced again. Deactivation of PDE requires a preceding inactivation of Rho* and $T_\alpha GTP$. Indeed, the time between flash and channel reopening is proportional to the logarithm of the saturating-flash intensity (26,27). This finding strongly suggests that the time of channel reopening is determined by the deactivation of only a single component X^* of the phototransduction chain, which is activated proportional to the flash intensity and deactivated by first-order kinetics. Recent experiments suggest that this species is $T_\alpha GTP$ (23,28–30). A reduction of the number of X^* activated per Rho* is suggested to contribute to light adaptation during steady background illumination and to desensitization after preflashes (22,31).

Herein, we studied an effect of Ca_i on the sensitivity of the phototransduction chain. Preflashes were applied to reduce Ca_i to a variable degree and the desensitization was tested by subsequent test flashes. For determination of Ca_i, we used our method of calculating Ca_i from the spatial profile of the extracellular Ca^{2+} concentration, which was determined by use of microelectrodes inserted into the photoreceptor layer of the isolated retina (8). Calcium-sensitive fluorescence probes used in amphibian rods are not applicable for recordings of preflash-induced Ca_i in rats because mammalian rods are much smaller and fluorescence itself affects the phototransduction.

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In our recordings, the cGMP channels reopened earlier when a preflash was given before the test flash. Our results strongly suggest that the phototransduction gain is reduced upstream to X^* as a consequence of a lowered Ca_i induced by the preflash.

MATERIAL AND METHODS

Materials

Albino rats ''Wistar'' were purchased from Schering AG (Berlin, Germany). Ca-Ionophor Cocktail A, carbontetrachloride (CCl_4), and trimethylchlorosilane (Me_3SiCl) were obtained from Fluka (Neu Ulm, Germany). The Ca^{2+} ionophor A23187 was obtained from Sigma Chemie (Deisenhofen, Germany).

Retinal preparation

Albino rats were kept in complete darkness for 2 h or more before they were sacrified by peritoneal injection of 2 ml of the Na^+ pentobarbiturat Nembutal (Ceva, Bad Segeberg, Germany). After cardial arrest the eyes were enucleated and the bulbus was meridially cut and transferred into Ringer solution. After ~10 min the retina was gently removed from the pigment epithelium and stored in Ringer solution at room temperature in darkness. For recordings, a piece of retina (~1 mm^2) was placed receptor side up on a cellulose acetate filter (SM 11104 Satorius, Göttingen, Germany) and taken into the recording chamber. The preparation was carried out under dim red light.

Solutions

Ringer solution was prepared after Hagins et al. (32), consisting of (in mM) 130 NaCl, 2.2 KCl, 0.18 Mg·6 H_2O, 11 Glucose, 1.3 KH_2PO_4, 5.4 Na_2HPO_4, 10 HEPES, pH 7.4 (NaOH). If not indicated otherwise, 0.25 mM $CaCl_2$ was added to the Ringer solution. Ca^{2+} concentrations <10 μM were adjusted by 10 mM EGTA.

Recording chamber and photostimulation

The recording chamber consisted of a glass cuvette perfused with Ringer solution. The cuvette was thermostated by a Peltier element. From above the recording chamber a recording microelectrode was moved vertically between the photoreceptor cells by a stepper motor (AM2 M2, Bachofer, Reutlingen, Germany) with a step precision of ± 0.1 μm.

The piece of retina in the recording chamber was illuminated by a wave guide from below the recording chamber. Light flashes were produced by an LED which could be pulsed for generation of short flashes. Light intensity was attenuated by neutral density filters (Kodak No. 96, Eastman Kodak Co., Rochester, NY). We denoted the flash intensity I_f as the number of photoisomerizations Rho* produced in an ROS per flash. The scaling is based on the finding that in a dark-adapted retina, flash activation of 30 Rho*/ROS produces 50% reduction of the dark current (33).

Electrophysiology

Microelectrodes were pulled from borosilicate glass tubing. Single-barrel microelectrodes were used if only the field potential was recorded. They consisted of a resistance of 4–8 MΩ after filling with Ringer solution. The field potential was considered to be proportional to the circulating photoreceptor current (8). Light flashes reduce the field potential (photosignal) concomitantly to the receptor current. Photosignal amplitudes reached a maximum of 30–150 μV as measured after bright saturating flashes. Double-barreled microelectrodes (tip diameter 1.5–3.0 μm) were used for simultaneous recordings of field potential and extracellular Ca^{2+} concentration. The barrel designated for voltage measurement was filled with Ringer solution. The second barrel was prepared as a Ca^{2+}-selective microelectrode. It was filled with a solution of 10 mM $CaCl_2$ and 135 mM KCl and silanized by repetitive suction of Me_3SiCl/CCl_4 solution (5 vol %) into the tip. Finally, the silanized tip was equipped with a Ca-selective membrane by filling with an organic matrix containing 10% of the Ca ionophore ETH 1001 (Ca-Ionophore Cocktail A). The Ca^{2+}-selective barrels of microelectrodes selected for measurements had a resistance of 5–10 GΩ, a risetime of 50–80 ms, and a steepness of 27–30 mV per decade determined with test solutions containing 0.1–1 mM $CaCl_2$ and 150 mM NaCl. A reference electrode of 4–8 MΩ filled with Ringer solution was situated ~100 μm above the retina. Changes of field potential (photosignal) and extracellular Ca^{2+} concentration (Ca signals) were recorded with a voltage difference amplifier of high-input impedance (Neuro Hel IRIS, Meyer, München, Germany) and further amplified and RC lowpass-filtered with a cascade of difference amplifiers. The overall transmission band was 13 Hz. The amplifier assembly enabled a simultaneous recording of Ca^{2+} concentration and field potential.

Determination of the net Ca^{2+} flux and calculation of Ca_i

The time course of the free intracellular Ca^{2+} concentration Ca_i in the ROS after a preflash was determined as described previously (8,34). A double-barreled electrode was moved into the photoreceptor layer by steps of $\Delta z = 8$ μm. At each step position three flash stimuli were applied. Photo- and Ca signals were recorded and averaged. Ca signals were obtained at any step position, whereas photosignals were obtained only after the electrode tip had passed the photoreceptor tips at $z = 0$ (Fig. 1, *A* and *B*). The series of Ca signals obtained by this procedure represent the flash-induced change of the extracellular Ca^{2+} concentration as a function of penetration depth z and time t ($c = c(z,t)$). Ca signals were smoothed three times alternately in time and penetration depth (bandwidth after smoothing 0.4 Hz). By considering that Ca^{2+} ions diffuse in the extracellular space only along gradients parallel to the z axis of the rods (8–10), the one-dimensional diffusion equation

$$q(z,t) = \frac{\partial c}{\partial t} - D \times \left(\frac{\partial^2 c}{\partial z^2} + \frac{1}{F} \times \frac{dF}{dz} \times \frac{\partial c}{\partial z} \right) \qquad (1)$$

was used to calculate the source function $q(z,t)$, which describes at a position z the Ca^{2+} flux across the rod outer membrane. $F = F(z)$ is the cross-section area of the space between the rods, which was estimated by assuming that the electrode tip is surrounded by four ROS that at $z = 0$ have a radius of 0.9 μm (32). At $z > 0$, $F(z)$ was derived from the longitudinal resistance in the extracellular space per unit length (32), which is inversely proportional to $F(z)$.

The value of q determines whether a change of the Ca^{2+} concentration at a position z is due to diffusion ($q = 0$) or if sources ($q > 0$) or sinks ($q < 0$) in the rod outer membrane do contribute. From $q(z,t)$ the time course of the Ca^{2+} efflux ΔQ_z after a flash of light was determined at different positions along a photoreceptor cell (Fig. 1 *C*). The net Ca^{2+} flux J_{OS} from the whole ROS was calculated by integration of q over the length of an ROS, i.e., from $z = 0$ to $z = 25$ μm

$$J_{OS}(t) = \int_0^{25\mu} q(z,t) \times N_A^{-1} \times F(z) dz, \qquad (2)$$

where N_A is Avogadro's constant (Fig. 1 *D*). From $J_{OS}(t)$, $Ca_i(t)$ was calculated by using a model of Miller and Korenbrot (35). In this model $J_{OS}(t)$ represents the sum of the Ca^{2+} influx J_{in} through the light-dependent channels and the Ca^{2+} efflux J_{eff} caused by the Na^+/K^+-Ca^{2+} exchanger (35–37):

$$J_{OS}(t) = J_{eff}(t) + J_{in}(t). \qquad (3)$$

In the dark, the Ca influx $J_{d,in}$ and the efflux $J_{d,eff}$ are balanced and J_{OS} is zero. J_{in} is assumed to be a constant fraction of the light-sensitive current.

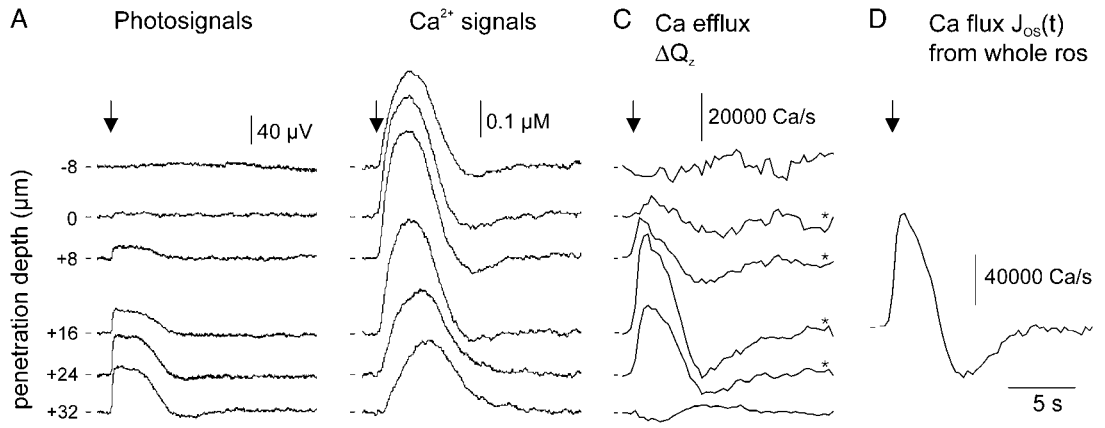

FIGURE 1 Determination of Ca flux J_{OS} from the outer segment. Temperature 30°C; Ca^{2+} concentration in the Ringer solution 0.1 mM. (*A*) Photosignals in response to a saturating flash of light (indicated by *arrow*). Recordings were done by positioning the tip of a double- barreled electrode in different depths z above (< 0 μm) and within (>0 μm) the photoreceptor layer. At 0 μm the electrode tip passed the tips of the outer segments. Each trace is an average of three single recordings. (*B*) Ca signals recorded at different positions z simultaneously with the photosignals. (*C*) Ca efflux: extrusion of Ca^{2+} ions $\Delta Q_z(t) = q(z,t) \times N_A \times F \times \Delta z$ from an ROS into the volume $V_z = F \times \Delta z$ at the position z of the recording electrode (F is the cross-section area between rods and Δz is the step width of the electrode). The source function $q(z,t)$ was calculated from Ca signals in *B* by use of Eq. 1. (*D*) Ca^{2+} flux J_{OS} from the outer segment obtained by integration of q from $z = 0$–24 μm (Eq. 2).

Hence, when $A(t)$ is the amplitude and A_{max} is the maximum amplitude of the photosignal, it follows that

$$J_{eff}(t) = J_{OS}(t) - J_{d,in} \times \frac{A_{max} - A(t)}{A_{max}}. \quad (4)$$

After a saturating photoresponse $J_{in}(t)$ is abruptly blocked and $J_{OS}(t)$ raises to a maximum value $J_{OS,max}$ (Figs. 1 *D* and 2 *C*) that represents the Ca^{2+} efflux when Ca_i is close to its dark level Ca_d. By taking into account that after the flash a maximum hyperpolarization of −25 mV activates the Na^+/K^+-Ca^{2+} exchanger by 37% (35,38,39), it follows that $J_{d,in} = J_{d,eff} = J_{OS,max}/1.37$. Finally, Ca_i/Ca_d was calculated supposing that J_{eff} is proportional to Ca_i. As an approximation, we assumed that hyperpolarization follows the time course of the photosignal (35,40). Then, it is

$$Ca_i/Ca_d = J_{eff}(t)/J_{d,eff} \times \frac{1}{1 + 0.37 \times A(t)/A_{max}}. \quad (5)$$

Recordings of the spatial Ca^{2+} concentration were time consuming. Therefore, if not stated otherwise, the experiments were carried out at 23°C instead of at physiological temperature to improve the long-term stability of the retinal tissue.

RESULTS

Deactivation time τ_X determined from photosignals evoked by single flashes

Photosignals were recorded in response to flashes of increasing light intensity (Fig. 2 *A*). From signals reaching maximum amplitude, the signal length t_c was determined. We defined t_c as the time between the flash given in the dark-adapted state and the return of the photosignal to the half-maximum amplitude (see Fig. 2 *B*, *upper trace*). Between 60 and 3000 Rho*/ROS induced per flash, t_c increases linearly with $\log(I_f)$ (Fig. 2 *C*, *upper trace*). The slope τ_X was considered to represent the lifetime of one species X^* of the transduction cascade (Appendix 1). The average value of τ_X was 1.8 ± 0.5 s ($n = 5$) at 23°C, agreeing well with τ_X = 1.7–2.4 s obtained from tiger salamander and toad rods (20,27,28,31). Like in rods of tiger salamander, t_c deviated from linearity at bright flashes: at $I_f > 3000$ Rho*/ROS, t_c was longer than expected from linear extrapolation (Fig. 2 *C*, *upper trace*).

Photoresponses that were just saturating showed an exponential recovery exhibiting a time constant τ_{rec}equal to τ_X (Fig. 2, *C* and *D*). At increasing flash intensity the recovery of the photosignals was slowed down (Fig. 2 *A*). Accordingly, τ_{rec} increased (Fig. 2 *C*, *lower trace*). Here, a difference between τ_X and τ_{rec} was apparent: At highest flash intensity, τ_{rec} increased sixfold, whereas τ_X increased only by a factor of 2.5. Occasionally, τ_{rec} began to increase at flash intensities slightly above saturation level, whereas higher flash intensities were necessary to cause a deviation of t_c from linearity (see, e.g., Fig. 6 *B*). Therefore, we suggest that τ_{rec} and τ_X may be affected by different mechanisms. Herein, we focused upon τ_X, because it is assumed to represent the lifetime of X^* (see Discussion).

Flash responses are shortened after a preceding conditioning flash

In amphibian rods a test flash produces a shorter photoresponse if it is preceded by a preflash (6,31). We obtained similar results in rat rods (34). In double-flash experiments, we illuminated the retina by a saturating conditioning preflash before applying the test flash. Representative photosignals in response to a saturating test flash with and without a preflash are shown in Fig. 2 *B*. The signal length t_c' was defined as the length of the test-flash-induced photosignal produced after a

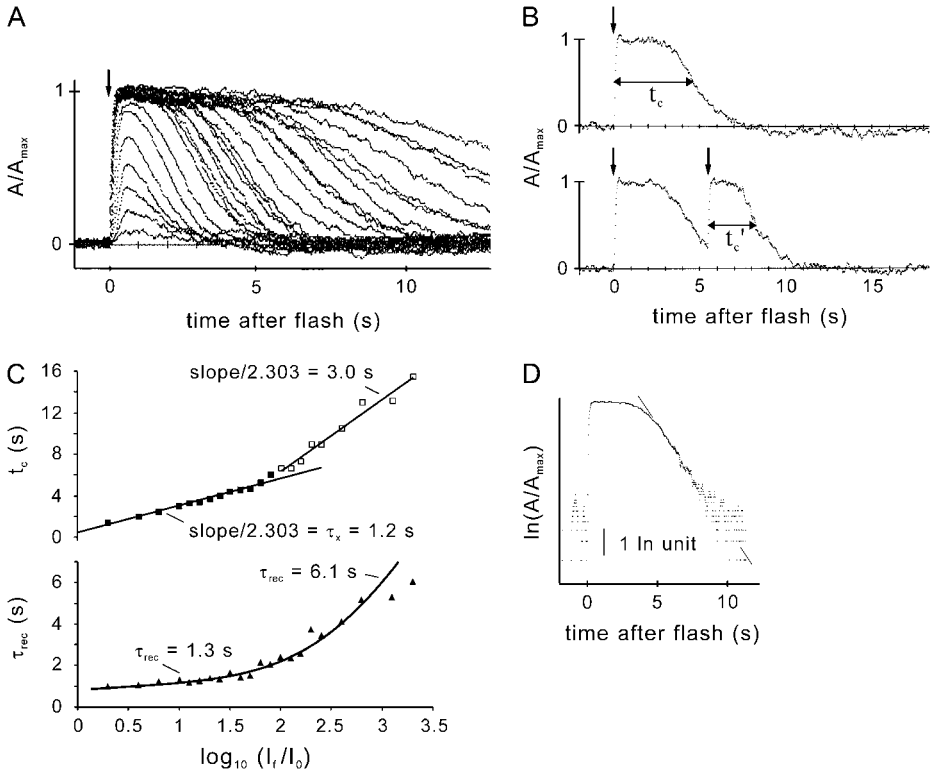

FIGURE 2 Photosignals in response to flashes of light (indicated by *arrows*) given to dark- or flash-adapted retina. Traces are averages of two to three single responses. (*A*) Dark-adapted retina was exposed to flashes of light that excited 3.8, 7.5, 15, 30, 60, 120, 190, 300, 380, 475, 600, 750, 950, 1200, 1500, 1900, 2400, 3000, 3800, 4750, 6000, 7500, 12,000, 19,000, 38,000, and 60,000 Rho*/ROS). (*B*) Different retina from that shown in *A*. (*Upper trace*) A saturating single test flash was applied to a dark-adapted retina. The signal length t_c is determined as time between the flash and the recovery of the photosignal to 50% of the maximum amplitude. The signal recovers with a time constant $\tau_{rec} = 1.4$ s. (*Lower trace*) Two flashes of the same intensity as used in the upper trace, separated by 5.4 s. The first conditioning flash produces a photosignal of identical time course to that indicated in the upper trace. The test flash produces a photosignal with reduced length t_c' and steeper falling phase ($\tau_{rec}' = 0.9$ s). In this trace, t_c' is reduced to ~50% compared with t_c. (*C*, *upper trace*) Signal lengths t_c obtained after single flashes as a function of the logarithm of the flash intensity. The arbitrary unit I_0 was taken as 30 Rho*/ROS. The relation $t_c = \tau_X \times \ln(I_f/I_0)$ is valid over a range of 60–3000 Rho*/ROS. From the slope, τ_X was determined to be 1.2 s in this experiment. At higher intensities the slope increases to 3.0 s. (*Lower trace*) τ_{rec} plotted versus log (I/I_0). τ_{rec} increases from 1.3 s to 6.1 s. (*D*) Logarithmic plot of a photosignal. The recovery phase of the signal is exponential. Time constant $\tau_{rec} = 1.3$ s.

preflash (Fig. 2 *B*, *lower trace*). After the preflash, the test flash produces a remarkably shortened photosignal: t_c' is only half as long as t_c which is the signal length obtained without a preflash.

In a first series of experiments, we applied a test flash at variable times Δt_f after the preflash. The dependence of t_c'/t_c on Δt_f is shown in Fig. 3 *A* and the time course of the preflash-induced photoresponse $A(t)/A_{max}$ is shown in Fig. 3 *B*. The quantity $1 - A(t)/A_{max}$ represents the receptor current flowing at the moment when the test flash was given. At $\Delta t_f = 0$, it was $t_c'/t_c > 1$ because test and preflash coincide. This is equivalent to exposing the dark-adapted retina by a flash consisting of the sum of pre- and test-flash intensity. The effect is predicted by Eqs. A3 and A5 (Appendixes 1 and 2, respectively) if the intensity of the preflash is of similar order to or larger than the test-flash intensity. When the test flash was applied during the period of completely interrupted dark current, t_c'/t_c was reduced with increasing Δt_f to a minimum of $t_c'/t_c = 0.6$. Fitting to a monoexponential decay yielded a decay with a constant $\Delta t_c = 4.4$ s to a minimum limit value $t_c'/t_c = 0.46$. The minimum of t_c'/t_c appeared when the test flash was given during the process of reopening of light-dependent channels. Further increase of Δt_f leads to a recovery of t_c'/t_c before having reached the minimum limit value. At 14 ± 1 s after the preflash, the receptor current recovered to 95% of the dark level. However, a test flash given at that time still produced a photosignal with a reduced signal length t_c'. Recovery to 95% of t_c'/t_c was observed when the test flash was applied at $\Delta t_f = 17 \pm 1$ s after the preflash. Hence, it was suggested that after a preflash, the recovery of t_c' shortening was delayed with respect to recovery of the light-dependent conductance. Average values for Δt_c and the minimum limit value of t_c'/t_c were 2.3 ± 0.7 s and 0.44 ± 0.14 ($n = 11$), respectively.

The decrease of t_c'/t_c to values <1 is in contrast to the concept that the intermediate *X** of the transduction chain that dominates the duration of phototransduction is activated with a constant gain. Calculation by use of this concept (Eq. A5, Appendix 2) yielded that by increasing Δt_f, t_c'/t_c should decay steadily from the maximum value at $\Delta t_f = 0$ and converge against 1 (Fig. 3 *A*, *upper trace*). For any value of Δt_f, it is $t_c'/t_c \geq 1$. This discrepancy between calculation and measured data demands a modification of this concept (see Discussion).

Shortening of t_c' depends on a reduction of the Ca^{2+} concentration in the ROS

It is feasible that the effect of the preflash on t_c' is mediated by Ca_i. To test this hypothesis we determined how the preflash reduced Ca_i before the test flash was applied (briefly described in Materials and Methods; see also Knopp and Rüppel (8)). In a first step, we determined the time course of the net Ca^{2+}

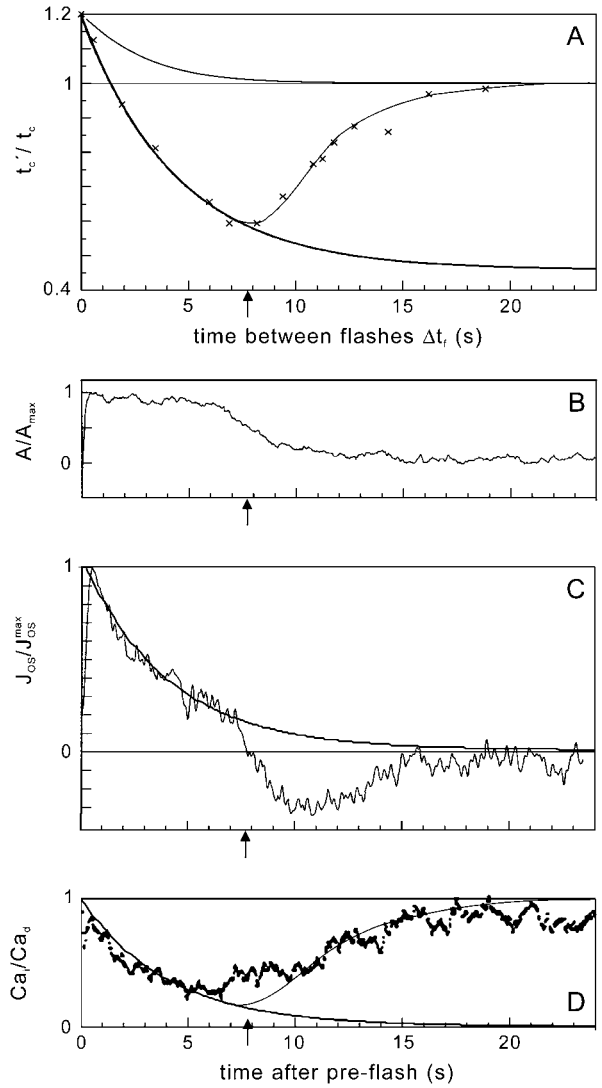

FIGURE 3 Shortening of t_c' induced by a preflash and correlation to $Ca_i(\Delta t_f)$ present at the moment of the test flash. It was $\tau_X = 2.3$ s, and $t_c = 7.5$ s. Intensities of pre- and test flash were identical. (*A*) t_c'/t_c versus time Δt_f by which the test flash followed after the preflash. (*Upper trace*) Calculation according to Eq. A5. It is $t_c' = 1.2 \times t_c$ at $\Delta t_f = 0$, i.e., when pre- and test flash coincide. Upon increasing Δt_f, t_c'/t_c is steadily reduced approaching 1. (*Lower trace*) Recorded values of t_c'/t_c plotted against Δt_f. From the maximum value of 1.2 at $\Delta t_f = 0$ s, t_c'/t_c is reduced to a minimum value 0.6 at $\Delta t_f = 7.8$ s, which occurs when the test flash is applied during recovery of the dark current. At the same time, Ca_i has reached a minimum level (see *arrows* in *A*, *B*, and *D*). At $\Delta t_f \geq 20$ s, t_c' has returned to the dark value t_c. Here and in the following figures the recovery of t_c'/t_c to the dark state has been fitted to data by eye (see *solid line*). The reduction of t_c'/t_c with growing Δt_f was fitted by an exponential decline with $\tau_{tc} = 4.4$ s to a minimum limit value $t_c'/t_c = 0.46$ (*bold line*). (*B*) Photosignal caused by a preflash alone. Plateau length ~6 s. The photosignal represents the time course of Ca^{2+} influx J_{in} before applying the test flash (see Materials and Methods). (*C*) J_{OS} representing net Ca^{2+} flux in response to the preflash. From the maximum value (1×10^5 $Ca^{2+}s^{-1}$), J_{OS} declines exponentially with a time constant of 4 s during completely interrupted dark current. (*D*) Time course of Ca_i in response to the preflash normalized to the dark concentration Ca_d. Ca_i is exponent-reduced with $\tau_{Ca} = 4$ s. The Ca^{2+} concentration Ca_i (t) at a time t after the preflash is equivalent to the Ca^{2+} concentration Ca_i (Δt_f) present at the moment of the test flash applied at a time Δt_f after the preflash: $Ca_i(t) = Ca_i(\Delta t_f)$.

efflux from the ROS, $J_{OS}(t)$, during the preflash-induced photosignal (Fig. 3 *B*). When the Ca^{2+} influx J_{in} through light-dependent channels was abruptly blocked (Fig. 3 *B*) $J_{OS}(t)$ steeply increased to a maximum followed by an exponential decay with a time constant of 4 s (Fig. 3 *C*). This exponential decay lasted as long as the plateau phase of the photosignal. When the photoreceptor current recovered to ~50%, $J_{OS}(t)$ reversed to negative values, indicating that Ca^{2+} flowed back into the Ca^{2+}-depleted ROS; ~20 s after the preflash, $J_{OS}(t)$ was zero again, indicating that J_{eff}, J_{in}, and Ca_i recovered to the dark value.

During the whole plateau phase of a photosignal J_{in} is completely blocked so that during this time interval $J_{OS}(t)$ exclusively represents J_{eff}, which during the plateau phase is proportional to Ca_i. Necessarily, Ca_i must decay with the same time constant as $J_{OS}(t)$. The time course of Ca_i is shown in Fig. 3 *D*. It shows an exponential decay during the plateau phase of the preflash-induced photosignal. The time constant was $\tau_{ca} = 4$ s. Similar results are obtained in five out of five experiments, showing a strong correlation between $t_c'(\Delta t_f)$ and $Ca_i(\Delta t_f)$: 1), $Ca_i(\Delta t_f)$ and $t_c'(\Delta t_f)$ decrease exponentially with equal time constants $\tau_{Ca} = \tau_{tc}$; 2), the minimum of t_c'/t_c coincides with the minimum of Ca_i (Δt_f); and 3), the shortening of t_c'/t_c is abolished as soon as the test flash is applied after Ca_i has recovered to the dark level.

We next examined whether the shortening of t_c' is affected when the reduction of Ca_i is prevented. We fixed Ca_i to the Ca^{2+} concentration of the external medium by adding 20 μM of the Ca^{2+} ionophore A23187 to the Ringer solution. The result of a typical experiment is shown in Fig. 4. The presence of A23187 prevented a preflash-induced shortening of t_c' below the value of t_c. Instead, the dependence of t_c' on Δt_f now obeyed Eq. 5A (Fig. 4, *upper curve*). The same result was obtained with three other retinae, giving strong

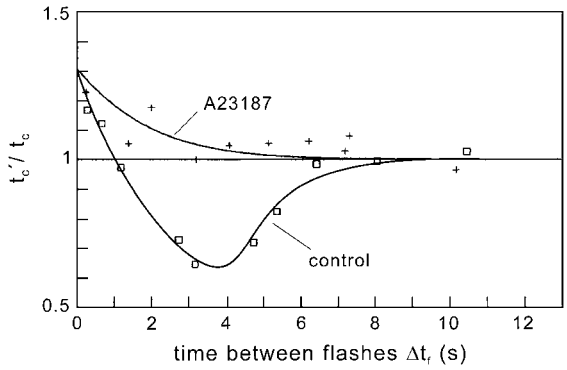

FIGURE 4 Proof that the shortening of t_c' is due to the fall of Ca_i induced by the preflash. Test and preflash had the same intensity. (*Lower trace*) Control recording, with $t_c = 3.8$ s and τ_X determined to be 1.7 s. (*Upper trace*) Twenty micromoles of A23187 was applied to shortcut Na^+/K^+-Ca^{2+} exchanger. Ringer solution contained 100 nM Ca^{2+}. The extensive reduction of t_c'/t_c is abolished. The solid line represents a data fit of Eq. A5, using the experimental parameters $R_{0,pre}/R_0 = 1$ and $t_c = 3.4$ s. The fit yielded $\tau_X = 1.51$ s.

evidence that the shortening of t_c' below the value of t_c (Fig. 4, *lower curve*) is caused by a reduction of Ca_i.

We also tested whether the congruence of τ_{Ca} and τ_{tc} is preserved if the temperature is changed (Fig. 5). After raising the temperature from 23 to 30°C, the preflash produced a photosignal with an accelerated recovery and a shortened plateau phase (*lower traces*). Similar effects of temperature have already been studied in rat and amphibian rods (41–43). In correspondence to the faster reopening of the light-regulated channels at 30°C, J_{OS} indicated an earlier reentry of Ca^{2+} ions (*middle traces*). Moreover, J_{OS} decayed faster, suggesting an accelerated depletion of Ca_i after the preflash. Concomitantly, in double-flash experiments the reduction of the signal length t_c' was accelerated (*upper traces*). During the plateau phase of the photosignal, J_{OS} decays with a time constant equal to τ_{Ca} (see above). Determination of the time constants yielded that the rise of temperature reduced τ_{Ca} from 3.4 to 0.7 s and τ_{tc} from 3.1 to 0.9 s. A similar result was obtained in one additional experiment. Hence, within an error limit of 10%, independent of the temperature, it was $\tau_{Ca} = \tau_{tc}$, i.e., t_c' was reduced proportional to the Ca_i present in the moment of the test flash. Averaging the differences, $\tau_{Ca} - \tau_{tc}$, obtained in all experiments at 23° and 30°C yields 0.04 ± 0.30 s (n =5).

Variation of the preflash intensity

In our next approach, we tested whether the preflash-induced shortening of t_c' depends on the intensity of the preflash. We created a data set of t_c'/t_c values (Fig. 6 *A*) by successively increasing the preflash intensity from 150 to 2400 Rho*/ROS (traces *a–d*). The intensity of the test flash was kept constant at 2400 Rho*/ROS. All traces *a–d* show an exponential reduction of t_c'/t_c with growing Δt_f independent of the preflash intensity with the same time constant $\tau_{tc} = 2.9 \pm 0.1$ s. At each preflash intensity, t_c'/t_c showed a minimum when the test flash was applied during the recovery phase of the preflash-induced photosignal. The minimum was more pronounced the longer the preceding saturation lasted.

For traces *a–c* in Fig. 6 *A*, the extrapolated initial value of (t_c'/t_c) at $\Delta t_f = 0$ was ~1 because the preflash intensity was less than the test flash intensity (Eq. A5). At increasing Δt_f, t_c' was reduced independent of the intensity of the preflash (Fig. 6 *A*, *bold trace*). Trace *d*, however, showed a remarkable deviation from traces *a–c*. This deviation is explained by Eq. A3, which is valid for single-flash experiments (Appendix 1), and by Eq. A5, which was derived for double flashes without considering a desensitization (Appendix 2). According to Eqs. A5 and A3, at $\Delta t_f = 0$, when pre- and test flash coincide, it is $t_c'/t_c \approx 1$ if the preflash is weaker than the test flash (traces *a–c*). In the case of the same intensity for pre- and test flash (trace *d*), t_c'/t_c was calculated to be 1.16. Hence, the calculation was in good agreement with the measured data at $\Delta t_f = 0$. The further deviation of trace *d* at $\Delta t_f > 0$ was produced by residual X^* originating from the preflash, which was still active when the test flash was given. This residual X^* prolonged the t_c'/t_c of trace *d* with respect to traces *a–c*. With increasing time between pre- and test flash, the influence of residual X^* on t_c' lessened, and all traces converged exponentially against the same minimum value. Therefore, the deviation of trace *d* minimizes with Δt_f.

We also tested whether τ_X is affected by a preflash. First, a series of single flashes was given to the dark-adapted retina. The plot of t_c against the $\log(I_f)$ yielded $\tau_X = 1.31 \pm 0.2$ s (Fig. 6 *B*, lower). The corresponding time constant τ_X' in the flash-adapted retina was determined by giving a preflash of constant intensity before the test flash. The test flashes were given at 50% recovery of the preflash-induced photosignal, i.e., when t_c' and Ca_i were at a minimum (cf. Fig. 3). Plotting t_c' against $\log(I_f)$ yielded $\tau_X' = 1.52 \pm 0.3$ s and 1.32 ± 0.3 s at a preflash intensity exciting 75.5 and 600 Rho*/ROS, respectively (Fig. 6 *B*, *lower*). Thus, a preflash produced a parallel shift but did not change the slope of the semilogarithmic plot, indicating that the preflashes did not affect τ_X. Rather, the shift in the semilogarithmic plot points to a preflash-induced reduction of the transduction gain γ. In Fig. 6 *C* the relative decrease of γ versus Δt_f has been determined from the data of the signal-length reduction (see Discussion for details).

If in this retina a single flash just reached saturation level, the photosignal recovered with $\tau_{rec} = 1.1$ s (Fig. 6 *B*, *upper* and *middle*). Hence, τ_{rec} was similar to τ_X at this flash intensity. By further increasing the flash intensity, τ_{rec} increased considerably. In contrast, τ_X was constant over the whole range of tested flash intensities. Interestingly, preflashes

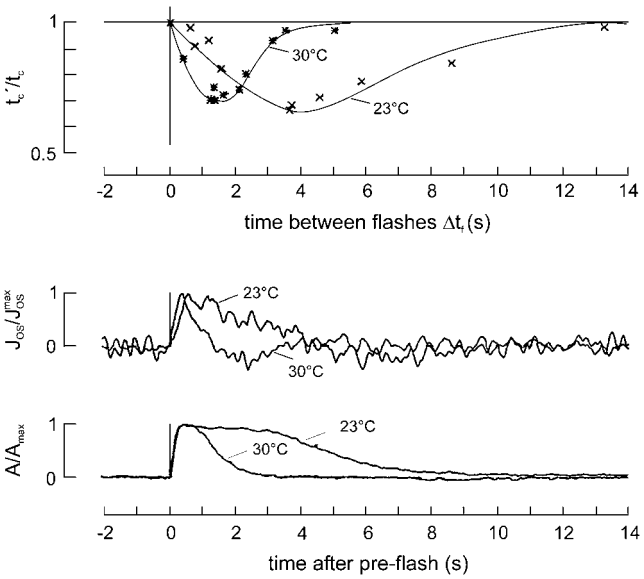

FIGURE 5 Effect of temperature on the shortening of t_c'. Recordings were conducted at one piece of retina at 30° and 23°C. Preflash excited 600 and test flash 3000 Rho*/ROS. (*Upper*) t_c'/t_c plotted versus time Δt_f by which the test flash followed after the preflash. Note that at $\Delta t_f = 0$, it is $t_c' \approx t_c$. (*Middle*) Time course of normalized net Ca^{2+} flux J_{OS} in response to the preflash. Maximum values are 6 and 1 × 10^5 Ca^{2+}/s at 23 and 30°C, respectively. (*Lower*) Normalized photosignals in response to the preflash.

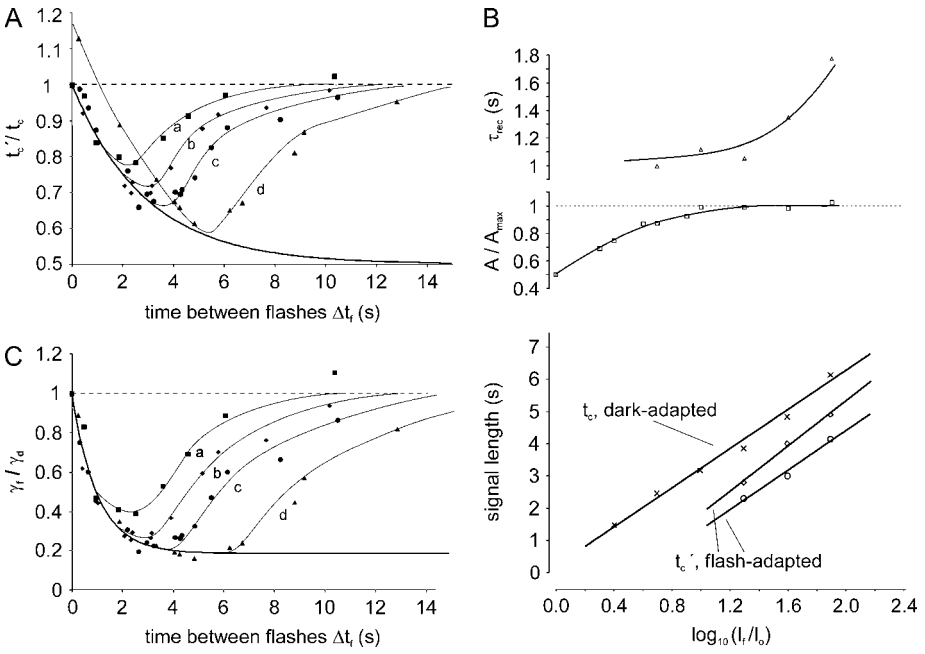

FIGURE 6 Double-flash recordings with variation of the preflash intensity. Preflash intensity I_{pre} = 150 (a), 300 (b), 600 (c) and 2400 (d) Rho*/ROS. The test-flash-activated I_f = 2400 Rho*/ROS throughout. The test flash produced a photosignal with $t_c = 6.13$ s. (*A*) t_c'/t_c as a function of time Δt_f between pre- and test flash. For each of traces *a–d*, t_c'/t_c is reduced exponentially with $\tau_{fc} = 2.9$ s to a minimum limit value of 0.5 by increasing Δt_f as long as the test flash was given during completely reduced dark current. If $I_{pre} \ll I_f$ (as in traces *a–c*), t_c'/t_c starts near 1 and shares a common decrease with growing Δt_f (*bold line*). With growing values for I_{pre} an increased deviation from the common trace is observed (most obviously for trace *d*, with $I_{pre} = I_f$). This deviation depends on the relation $R_{0,pre}/R_0$ in Eq. A5. (*B*, *upper* and *middle*) Time constant of the photosignal recovery τ_{rec} and normalized photosignal amplitude in dependence of log(I_f). Flashes were applied to the dark-adapted retina. (*Lower*) Signal lengths plotted against log(I_f) (I_0 = 30 Rho*/ROS). The slope is independent of whether a preflash was given before the test flash. (*C*) Gain γ_f of the transduction chain after a preflash versus Δt_f. The gain is normalized to its dark value γ_d. The values of γ_f/γ_d were calculated using Eq. A7 from the measured values of t_c'/t_c shown in *A*.

reduced τ_{rec} back to or even below the value of τ_X (Fig. 2, *B* and *C*). Moreover, a photosignal with a long plateau phase of several seconds showed a slow recovery ($\tau_{rec} \gg \tau_X$) if it was evoked by one strong single flash, whereas it was followed by a fast recovery ($\tau_{rec} \approx \tau_X$) if it was evoked by successive application of two or more saturating flashes of weak intensity (not shown). Therefore, the increase of τ_{rec} does not depend on the plateau length or the level of Ca_i at the time when the channels reopen. Rather, unlike τ_X, τ_{rec} seems to be sensitive to the conditions at which the rod is illuminated. Illumination favors a slow recovery if the rod is dark-adapted or the light intensity of flashes or constant light is high. Moderate light intensity and light adaptation of a rod seem to favor a fast recovery of the photosignal.

DISCUSSION

Isolated retinae of the albino rat were exposed to saturating test flashes with or without preceding preflashes. Photosignals, as well as the Ca^{2+} signal, were concomitantly recorded in the extracellular space between the rods. From this data, Ca_i was determined. The length of the photosignal, which is the time interval between the test flash and the return to the half-maximum signal amplitude, is remarkably reduced if the test flash is preceded by a conditioning preflash. This preflash-induced signal shortening runs parallel to the reduction of Ca_i present at the moment of the test flash. The signal shortening is completely abolished if reduction of Ca_i induced by the preflash is prevented. The data suggest that a test flash applied at reduced Ca_i activates the transduction cascade with reduced gain. The gain is defined as the amount of an intermediate *X** of the transduction chain activated per absorbed photon. The $K_{1/2}$ value of the gain reduction by means of Ca_i is estimated to be either similar to or above the dark level of Ca_i.

Photoresponses evoked by single flashes

If single saturating flashes are given to the dark-adapted retina, the length t_c of the photosignal rises linear with log(I_f) over a range of flash intensities of at least 1 log unit (Figs. 2 *C* and 6 *B*; cf. Pepperberg et al. (27)). Moreover, the recovery of the photosignals is approximately monoexponential, exhibiting a time constant τ_{rec}. At low saturating-flash intensities, within the linear range of the semilogarithmic plot, the slope τ_X is largely similar to τ_{rec}. Comparable results were reported for amphibian rods (20,27,31). These results can be explained based on the idea that the recovery of these photosignals is dominated by the deactivation of one component *X** of the transduction cascade, with a lifetime τ_X (27; see Appendix 1).

At brighter flash intensities the slope of the relation between t_c and log(I_f) rises (Fig. 2 *C*). It is not clear yet whether the slope obtained at these flash intensities still represents the lifetime τ_X. In amphibian rods, this deviation has been attributed to an unknown component of the transduction cascade with slow deactivation kinetics (27,44). It may also be explained by multiple photoisomerizations per disc leading to a $T_\alpha GTP$ concentration that exceeds that of PDE_γ. This could decrease the rate at which $T_\alpha GTP$ deactivates (45). Compared to τ_X, τ_{rec} is more sensitive to flash intensity (Figs. 2 *C* and 6 *B*). It seems to be affected by

additional processes. Especially reentering Ca^{2+} ions may affect the time course of recovery by having an influence on guanylyl cyclase activity, membrane conductance, and the Hill coefficient of cGMP binding to the light-dependent channels. A Ca^{2+}-dependent GTPase accelerating factor may also affect τ_{rec} (30). Double-flash experiments were performed within the linear range of the t_c versus $\log(I_f)$ relation. Within this range, the variability of τ_{rec} was estimated to affect t_c by $<10\%$. It was therefore considered negligible.

It was shown earlier that τ_X is not influenced by guanylyl cyclase (20,28), suggesting that guanylyl cyclase is maximally activated during the plateau phase of a photosignal due to rapidly declining Ca_i. We found that after a saturating flash Ca_i is reduced with a time constant $\tau_{Ca} = 2.3$ s at 23°C (8). A typical saturated photosignal shows a signal length of $t_c = 2.5$–10 s. Hence, assuming a dark level of $Ca_i = 300$ nM (7,35,46), Ca_i is reduced to 4–100 nM, which should indeed result in a maximal activation of guanylyl cyclase when the channels reopen ($K_{1/2} = 200$ nM Ca_i) (47).

Ca_i-dependent gain reduction in double-flash experiments

We found that a preflash reduces the signal length in rat rods (Fig. 2 *B*; see also Knopp (34). Similar results were obtained from amphibian rods (31). The effect of signal shortening depends on the time period of channel closure that has prevailed when the test flash is applied. The shortening is maximal when the test flash is applied during the reopening of the channels and gradually disappears by further increasing the flash separation. This preflash-induced signal shortening does not agree with the concept of an intermediate X^* being light-activated with a constant gain (Appendix 1, *ii*).

We observed that after a preflash, the process of signal shortening is still active after the photoreceptor current has fully recovered (Fig. 3, *A* and *B*). This finding suggests that the shortening of t_c' is not due to the cGMP concentration or the conductance state of the light-dependent channels. However, determination of the preflash-induced reduction of Ca_i showed that the shortening of t_c' and Ca_i present in the moment of the test-flash are strictly related (Fig. 3). In particular, preventing a decrease of Ca_i completely abolishes the shortening of t_c'. We therefore conclude that a preflash-induced shortening of t_c' is mainly based on the reduction of Ca_i. Hence, the dependence of t_c' shortening on the flash separation reflects the time course of Ca_i after the preflash.

In principle, the photosignal shortening may be due to either a reduced X^* lifetime τ_X (24) or a reduced phototransduction gain (21–23,27). A reduced lifetime τ_X was concluded from experiments with isolated enzyme preparations showing an accelerated PDE inhibition after a reduction of Ca_i (24). In contrast, in intact rods of the tiger salamander, background light shortens t_c without affecting τ_X (22,27). We found that τ_X was not affected by a preflash (Fig. 6 *B*). A similar result was reported for isolated amphibian rods (31). In truncated rods, a reduction of Ca_i was shown to reduce the amplification of the transduction cascade (21). All of these findings give evidence for a reduced transduction gain instead of a reduced X^* lifetime τ_X.

Model for describing signal shortening in double flash experiments

The concept of an exponentially decaying intermediate X^* that dominates the recovery of phototransduction (Appendix 1) supposes a constant transduction gain. It describes the dependence between signal length and flash intensity only if single flashes are applied. According to our results of signal shortening in double-flash experiments, a variable transduction gain has to be introduced (Appendix 3), which is reduced by a depletion of Ca_i after a preflash.

After a flash, the reduction and reincrease of Ca_i is a dynamic process continuously changing the activity of Ca_i-regulated enzymes. It is unlikely that enzymatic activity controlling the transduction gain downstream of X^* changes in parallel to Ca_i because a gain reduction in this part of the transduction chain would lead to a nonlinear t_c relation versus $\log(I_f/I_o)$. In fact, linearity was observed to be independent of whether the test flash was applied to the dark-adapted retina or after a preflash (Fig. 6 *B*). Therefore, we suggest that Ca_i acts only on this part of the transduction chain that determines the number of X^* initially activated by the test flash, i.e., the overall gain γ upstream of X^* (see Appendix 1, Eq. A1a). Furthermore, to establish linearity of the t_c relation versus $\log(I_f/I_o)$, γ must be fixed at the beginning of a photoresponse. Therefore, in accordance with others (23,28), our data suggest that generation of X^* with gain γ is completed in a short time period after the flash. Hence, the value of γ depends on Ca_i present in this time window. Then, in the dark-adapted rod, the test flash produces an initial amount X_0^* of the intermediate X^* with a gain γ_d. After a preflash, Ca_i is reduced and the test flash produces a lower number of $X_0^{*\prime}$ due to a reduced gain γ_f. Consequently, constant background illumination should reduce γ to a constant value γ_b due to a reduced steady-state concentration of Ca_i. Regulation of γ is roughly estimated to be completed in a time window of <800 ms after a flash by considering that just saturated photosignals start to recover 700–800 ms after the flash, with a time constant τ_{rec} similar to τ_X.

By use of Eq. A7 (Appendix 3), γ_f/γ_d was calculated from the data shown in Fig. 6 *A* and plotted as a function of Δt_f (Fig. 6 *C*). As a result, γ_f/γ_d is reduced with a time constant of 1.1 s. The preflash can reduce γ_f maximally to 20% of its dark value. During the plateau phase of the preflash-induced photosignal γ_f is reduced independent of the flash intensity. This is in agreement with the idea that Ca_i regulates γ_f. Based on our result that after a preflash Ca_i is reduced with a time constant $\tau_{Ca} = 2.3$ s at 23°C (8), we obtained a relation between Ca_i and γ_f/γ_d as shown in Fig. 7. Within the range in which Ca_i can be regulated by light the gain γ_f increases in a

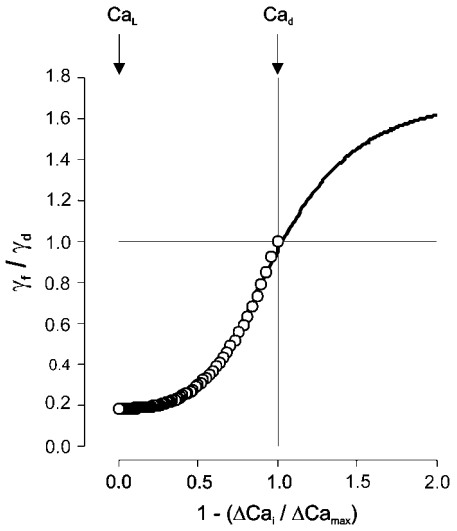

FIGURE 7 Transduction gain γ_f normalized to the dark value γ_d in dependence of the relative change of the Ca^{2+} concentration $1 - \Delta Ca_i/\Delta Ca_{max}$, where ΔCa_{max} is the maximum reduction of Ca_i achieved by intense illumination of an ROS. The data points were obtained by assuming that γ_f is reduced, with a time constant of 1.1 s after the preflash, whereas Ca_i is exponentially reduced from the dark level Ca_d to a minimum concentration Ca_L by a time constant $\tau_{Ca} = 2.3$ s. Data points were fitted to a Michaelis-Menten equation assuming that $K_m = Ca_d$. The fit yielded a Hill coefficient $n = 3.87$.

sigmoidal shape from a basic light-adapted level at minimum Ca_i (at Ca_L) to the dark-adapted value γ_d (at Ca_d). Unfortunately this dose-response curve is incomplete, because data are available only for Ca_i values $<Ca_d$. However, the curve suggests that γ_f is about half maximally at $Ca_i = Ca_d$. Given $K_m = Ca_d$, we obtained a Hill coefficient $n = 3.87$. According to these results, γ_f is most sensitive to changes of Ca_i at conditions of dark adaptation. Fig. 7 suggests that a half-maximal reduction of Cai, which is achieved approximately by 50% reduction of the dark current, is sufficient to produce a 90% reduction of γ_f. Thus, the control of γ_f should be most distinct at light intensities below saturation of the photoresponse, i.e., at physiological levels of illumination.

Mechanism for Ca_i-dependent gain control

The enzyme representing X^* and the mechanism underlying the gain control are hitherto unknown. Originally, Pepperberg (27) suggested that X^* is catalytically active rhodopsin, Rho*, which deactivates with a τ_X of 1–2 s. A similar Rho* lifetime was reported for dialyzed ROS (48). Measurements of light scattering, which was attributed to the rhodopsin-transducin interaction, yielded a Rho* lifetime of 3–5 s (27). Actually, a Ca_i regulation of the gain α (see Appendix 1), which controls the number of Rho* generated by a flash (21), has never been measured directly. Moreover, much shorter Rho* lifetimes of ~0.4–0.5 s were found in truncated rods at 1 mM Ca^{2+} (29) and in dark-adapted rods (at Ca_d) of tiger salamander. In the latter report, the lifetime of Rho* was assumed to be further reduced by exposure to light (23). According to these reports, Rho* deactivation is a Ca^{2+}-sensitive step that controls the amplitude of the photosignal without being rate-limiting.

Recent results suggest that X^* is T_αGTP (29,30) and the rate-limiting step that terminates PDE activation is hydrolysis of GTP bound to T_α. The GTP_{ase} activity of T_α depends on GTPase accelerating proteins as RGS9 (30,49) and PDE_γ (50). In intact photoreceptors, the lifetime of T_αGTP may range from 0.6 (51) up to several seconds (52), which is in the same order of magnitude as the measured values for τ_X.

In principle, a decreased Ca_i may reduce the gain γ of T_αGTP formation by 1), reducing the number of Rho* activated per photon (21); 2), reducing Rho* lifetime (23,28,53); 3), reducing the rate of Rho*-mediated transducin activation; and 4), reducing the effective transducin level in the discs. No indication is given that Ca_i affects the gain α of Rho* formation (case 1). However, significant experimental evidence is given for a Ca_i regulation of the gain β, which determines the fraction of T_αGTP/Rho* (cases 2–4). The enzyme recoverin may play a major role in this respect. After Ca^{2+} unbinding, recoverin accelerates the phosphorylation of Rho* through rhodopsin kinase (24). This may reduce the effectiveness of the Rho*-transducin interaction and shorten the time of T_αGTP formation (cases 2 and 3). Indeed, by dialysis of recoverin into an ROS (54,55) and in knockout mice Ca, recoverin increases the duration of light-induced PDE activity (56). Our estimation yields that the transduction gain has a $K_{1/2} \geq Ca_d$ (Fig. 7), which is in good agreement with the $K_{1/2}$ value of recoverin. Whereas $K_{1/2}$ of recoverin in truncated rods perfused with the recombinant enzyme is in the Ca_i range of several micromolar, it is estimated to be lower, close to Ca_d in the intact cell (55). An additional mechanism (57) in accordance with the observed signal shortening is a reduction of the effective transducin level in the discs (case 4). It is known that after unphosphorylation the enzyme phosducin binds effectively to $T_{\beta\gamma}$ which has been shown to block the interaction of transducin and Rho* and to facilitate the translocation of transducin from the outer segments (58). Phosphorylation of phosducin is established by Ca/calmodulin-dependent protein kinase II (59).

APPENDIX 1

Model 1, for single-flash measurements

During the plateau phase of a saturated photosignal, all cGMP-dependent membrane channels are closed. The channels reopen during the subsequent recovery phase. According to Pepperberg et al. (27), the plateau length t_c can be derived as a function of flash intensity if the following assumptions are met:

1. The number of excited rhodopsin Rho* per rod is proportional to the intensity of the test flash I_f: $R_0 = \alpha \times I_f$. An initial amount X_0 of an intermediate X^* of the transduction cascade is activated proportional to R_0: $X_0 = \beta \times R_0$, where β is the system gain. This connection is demonstrated by the following simplified scheme

$$I_f \xrightarrow{\alpha} \text{Rho}^* \xrightarrow{\beta} X^* \rightarrow \rightarrow \text{cGMP hydrolysis},$$
$$\downarrow$$
$$\text{Rho}_i,$$

where Rho_i is deactivated Rho*. Thus, the overall gain of X^* formation is $\gamma = \alpha \times \beta$ and

$$X_0 = \gamma \times I_f. \tag{A1a}$$

The deactivation of X^*, proceeding by first-order kinetics with a time constant τ_X is the rate-limiting step in the deactivation of the transduction cascade.

$$X^* = X_0 \times e^{-t/\tau_X}. \tag{A1b}$$

2. The guanylyl cyclase is maximally activated at the end of the plateau phase of a saturated photosignal. Hence, its effect on the duration of the signal length is the same for all saturating-flash intensities.
3. When the cGMP-dependent channels start to reopen, cGMP production is balanced by a distinct rate of cGMP hydrolysis. The rate of hydrolysis at the end of the plateau phase is determined by the remainder of X* activity, Xc*.

In practice, we determined the signal length t_c as the time between the flash and the return of the membrane current to a certain criterion level, i.e. a fixed fraction of its dark value. This level was recognized by a recovery of the photosignal to 50% of its maximum amplitude (cf. Fig. 2 *B*). X_c* is the amount of X^* still active at this criterion level. It is

$$X_c^* = X_0 \times e^{-t_c/\tau_X}. \tag{A2}$$

Since τ_X and X_c* are supposed to be constant, the signal length t_c is a function of X_0 only:

$$t_c = \tau_X \times \ln(X_0/X_c^*).$$

According to Eq. A1a, a simple but fundamental relation follows as

$$t_c = \tau_X \times \ln(I_f/I_c), \tag{A3}$$

where I_c is the flash intensity producing an amount of X_c* of the intermediate X^*.

APPENDIX 2

Model 2, for double-flash recordings

A first conditioning preflash and a second test-flash are assumed to activate an amount of $X_{0,pre} = \gamma \times I_{pre}$ and $X_0{}' = \gamma \times I_f$ of the intermediate X^*, respectively. Then, if the preflash and the test-flash flash are separated with a time Δt_f, and if t_c' is the signal length of the second photosignal, according to Eq. A2, X_c* is given by

$$X_c* = (X_{0,pre} \times e^{-\Delta t_f/\tau_X} + X_0') \times e^{-t_c'/\tau_X}. \tag{A4}$$

Here, $X_{0,pre} \times e - \Delta t_f/\tau_X$ is the residual part of X^* remaining from the activation by the preflash at the time the second flash is fired. On the other hand, the signal length t_c of the photosignal of the same test flash applied to a dark-adapted retina is provided by Eq. A2. Supposing that always the same amount X_c* of intermediate is active when the photosignal surpasses the criterion level, it is possible to relate t_c' (flash-adapted) and t_c (dark-adapted) by combining Eqs. A2 and A4:

$$(X_{0,pre} \times e^{-\Delta t_f/\tau_X} + X_o') \cdot e^{-t_c'/\tau_X} = X_0 \times e^{-t_c/\tau_X}.$$

Transformation gives the ratio of the signal lengths

$$t_c'/t_c = 1 + (\tau_X/t_c) \times \ln(X_{0,pre}/X_0 \times e^{-\Delta t_f/\tau_X} + (X_0{}'/X_0)). \tag{A5}$$

With $\Delta t_c = t_c' - t_c$, it follows that

$$\Delta t_c = \tau_X \cdot \ln(X_{0,pre}/X_0 \times e^{-\Delta t_f/\tau_X} + (X_0'/X_0)). \tag{A5a}$$

From Eq. A5, it can be concluded that upon variation of Δt_f, t_c'/t_c should always be >1, i.e., the signal length should be prolonged as a result of a preflash.

APPENDIX 3

Model 3, for double flashes with variation of the system gain

Our data, as well as those of others (21,22,27,31), suggest that light adaptation causes a reduced transduction gain. We suppose a reduction of the overall gain γ of X^* formation, whereas the deactivation time τ_X of the intermediate remains unchanged. Hence, if a test flash after a preflash produces an initial amount of intermediate $X_0' = \gamma_f \times I_f$, and if γ_f and γ_d are the overall gains in a flash- and in a dark-adapted rod, respectively, then, applying Eq. A1a, it follows that

$$X_0'/X_0 = \gamma_f/\gamma_d. \tag{A6}$$

As a result, Eq. A5a can be converted to describe the change Δt_c of the signal length in dependence of the reduced gain γ_f:

$$\Delta t_c = \tau_X \times \ln(X_{0,pre}/X_0 \times e^{-\Delta t_f/\tau_X} + (\gamma_f/\gamma_d)). \tag{A5b}$$

Finally, it follows that

$$\gamma_f/\gamma_d = (e^{+\Delta t_c/\tau_X} - X_{0,pre}/X_0 \cdot e^{-\Delta t_f/\tau_X}). \tag{A7}$$

Assuming that the initial amount of the flash-induced intermediate is smaller after a preflash than without a preflash ($X_0' < X_0$), it follows from Eq. A 5b, with $\gamma_f/\gamma_d < 1$ and $\Delta t_f \rightarrow \infty$, that Δt_c can be negative and correspondingly $t_c'/t_c < 1$.

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