Kinetics of desensitization induced by saturating flashes in toad and salamander rods

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1. The suction pipette technique was used to examine the effect of a conditioning pre-flash on the saturation time (t_{sat}) of a bright test flash (intensity 10000–250000 isomerizations) delivered to intact salamander or toad rod outer segments. The conditioning flash was delivered 0–60 s before the test flash; its intensity was typically between six and sixty times dimmer than the test flash, and it was sufficient by itself to fully saturate the photocurrent.

2. A saturating pre-flash delivered before a saturating test flash reduced the t_{sat} of the test flash. This was equivalent to a reduction in phototransduction gain (ψ).

3. The pre-flash had little effect on τ_c, the time constant of decay of the rate-limiting species in photoresponse inactivation (activated rhodopsin or the activated G-protein–phosphodiesterase complex).

4. The t_{sat} declined exponentially as the separation time between a fixed intensity pre-flash and test flash was increased. The time constant (τ_p) of decline in t_{sat} was approximately 2·4 s. The maximum reduction in t_{sat} corresponded to a reduction in the apparent gain of phototransduction to approximately 0·10 of its original level. This exponential decline is consistent with a $[Ca^{2+}]_i$-mediated effect.

5. We conclude that the rate-limiting step in response inactivation and the step responsible for light-induced gain reduction constitute separate and distinct steps of the phototransduction cascade.

Now that the activation steps of the phototransduction cascade are well understood, the mechanisms of cascade inactivation have become an increasingly important area of research. Understanding these mechanisms also furthers understanding of the phenomenon of light adaptation, which operates by regulating some of the inactivation steps and possibly some of the activation steps as well. One method of studying the process of inactivation is to examine the recovery of rod photocurrents after bright, saturating flashes.

The duration of a photoreceptor's response to a saturating flash of light increases as the flash intensity increases. In cones, Baylor, Hodgkin & Lamb (1974) found that the saturation time increased in proportion to the logarithm of the flash intensity. To explain this relationship, they suggested a first-order decay process as the rate-limiting step in the recovery of a saturating photoresponse. A logarithmic relationship between flash intensity and saturation time was also observed in rods by Pepperberg *et al.* (1992) and Pepperberg, Jin & Jones (1994). Some published evidence suggests that the rate-limiting decay process giving rise to this relationship is the inactivation of photoactivated rhodopsin (Rh*) (Pepperberg *et al.* 1992; Corson, Cornwall & Pepperberg, 1994). However, it has recently been suggested that the rate-limiting step is the inactivation of the transducin–phosphodiesterase (G*–PDE*) complex, which occurs when the terminal phosphate of the G*–GTP is hydrolysed (Nikonov, Lyubarsky & Pugh, 1996).

The relationship between saturation time t_{sat} and flash intensity Φ (in photoisomerizations) has been given previously by Baylor *et al.* (1974) and Pepperberg *et al.* (1992), as:

$$t_{sat} = C + \tau_c \ln(\psi\Phi) = C + 2{\cdot}303\tau_c \log(\psi\Phi). \quad (1)$$

This relationship is consistent with the presence of a substance that is produced in direct proportion to light intensity and whose first-order decay is rate limiting in the recovery from bright flashes. Here C is a constant, τ_c is the time constant of decay, and ψ is a gain parameter as defined by Pepperberg *et al.* (1994). The saturation time t_{sat} may be defined as the time for recovery to an arbitrary level of circulating current; alteration of this criterion level simply alters the value of the parameter C. In this study, t_{sat} is defined as the time until the circulating current recovers to 10% of its original level (see Fig. 1*A*).

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Equation (1) predicts that a semilogarithmic plot of t_{sat} against Φ will yield a straight line; see Fig. 1*B*. When Φ is plotted in natural log (ln) co-ordinates, the slope of this line will be given by τ_c; in log co-ordinates the slope will be $2{\cdot}303\,\tau_c$. Pepperberg *et al.* (1992) showed that adapting background lights reduced the gain parameter ψ with little effect on the time constant of decay (τ_c).

Light-induced alteration of phototransduction sensitivity is known to be mediated by a change in the activity of guanylate cyclase and by some other mechanism affecting one or more steps of the cascade up to and including the inactivation of PDE* (Torre, Matthews & Lamb, 1986; Koch & Stryer, 1988; Kawamura & Murakami, 1991; for recent studies see Koutalos, Nakatani, Tamura & Yau, 1995*a*; Koutalos, Nakatani & Yau, 1995*b*). The parameter ψ provides a measure of gain change due to the latter mechanism. The time at which a response leaves saturation is determined by the balance between cGMP formation by guanylate cyclase and cGMP hydrolysis by PDE*. By the time a bright saturating flash begins to recover, typically at least 5 s after flash delivery in this type of experiment, one can assume guanylate cyclase has reached its maximal activity (Pepperberg *et al.* 1992). This assumption is further supported by the work of Matthews (1996), who showed that there was no further change in t_{sat} when $[Ca^{2+}]_i$ was clamped 5 s after delivery of a bright saturating flash. During a saturating response, the rate of hydrolysis of cGMP is very high (Lamb & Pugh, 1992), so that [cGMP] should settle very rapidly to a level determined by the ratio of the activities of the cyclase and of PDE* at any particular time. Since cyclase activity is maximal and constant after 5 s, regardless of the cell's adaptational state at the time of flash delivery, recovery occurs after PDE* activity falls below a specific constant level. A change in t_{sat} represents a change in the amount of time until PDE* activity falls below this level. For a given bright flash intensity, a change in t_{sat} thus represents a change in the gain from photon absorption to total PDE* production. The parameter ψ quantifies this gain change, and, for simplicity, ψ is defined as unity in the dark-adapted state.

Very recently two other groups have examined the contribution of PDE* factors and cyclase factors to the sensitivity of transduction under conditions of *steady-state* light adaptation. Koutalos *et al.* (1995*a*, *b*) employed the truncated rod approach to clamp $[Ca^{2+}]_i$, while Matthews (1996) employed rapid solution changes in conjunction with illumination to manipulate internal calcium levels. Our experiments complement those studies by measuring the *kinetics* of the gain change contributed by the PDE* component in response to saturating flashes.

It is now well established that, when all the light-sensitive channels are closed by a saturating light, the internal calcium concentration $[Ca^{2+}]_i$ drops quickly toward a lower level (Yau & Nakatani, 1985; McNaughton, Cervetto & Nunn, 1986; Ratto, Payne, Owen & Tsien, 1988; Gray-Keller & Detwiler, 1994; McCarthy, Younger & Owen, 1994). The absolute estimates of calcium concentrations have varied widely in the different studies, but the consensus view is that the minimum light-induced level is in the region of 10–50 nM (see Gray-Keller & Detwiler, 1994).

It is also well established that the adaptational state of rod and cone photoreceptors is controlled by changes in intracellular calcium concentration (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988; Matthews, Fain, Murphy & Lamb, 1990; Matthews, 1991; Tamura, Nakatani & Yau, 1991). Furthermore, recent experiments have indicated that the gain parameter ψ (which represents one determinant of the cell's adaptational state) is dependent on intracellular calcium concentration. Using truncated rod outer segments, Lagnado & Baylor (1994) demonstrated a calcium-dependent effect on the apparent number of activated rhodopsin molecules formed by a given flash. With calcium-clamping solutions, Matthews (1995) showed that changes in intracellular calcium are both necessary and sufficient to cause a gain change with background illumination. Also using calcium-clamping techniques, Lyubarsky, Nikonov & Pugh (1996) and Matthews (1996) demonstrated that changes in intracellular calcium cause significant changes in gain (ψ), but not in the dominant decay time constant of recovery (τ_c).

To further investigate the light-induced change in gain, we have now studied the effect of a saturating pre-flash (rather than a steady background) on t_{sat}. Beginning in darkness, we delivered a flash bright enough to saturate the photocurrent. A few seconds after the *pre-flash*, while the rod was still saturated, we delivered a brighter *test flash*, from which we measured t_{sat} (Fig. 2*A*). The test flash was typically between six and sixty times brighter than the pre-flash, and hence the number of Rh* (or G*–PDE*, if they are the rate-limiting species) remaining from the pre-flash should have been insignificant compared to the number formed by the test flash.

There are several reasons why a pre-flash experiment may give more information about gain reduction than an experiment with steady background light. (1) Internal $[Ca^{2+}]$ is known to drop to its lowest physiological level only when the cell is held in complete saturation; the pre-flash experiment allows us to deliver the test flash while the cell is in complete saturation, i.e. when a $[Ca^{2+}]_i$-dependent effect should be most pronounced. (2) Since a steady background is constantly producing new Rh*, the recovery from the saturating flash, and hence t_{sat}, may be affected in an undefined manner; the pre-flash experiment avoids this problem. (3) By altering the time interval between the pre-flash and test flash, it is possible to explore the time course of gain reduction.

METHODS

The methods used in rod isolation and suction pipette recording were similar to those described in Lamb, Matthews & Torre (1986). Adult toads (*Bufo marinus*) were dark adapted for at least 2 h and

were then killed by pithing the brain and spinal cord. The eyes were removed under dim red light and were stored in Ringer solution at 5 °C for a maximum of 8 h until used. (Ringer solution (mM): 111 NaCl, 2·5 KCl, 1 $CaCl_2$, 1·6 $MgCl_2$, 3 Hepes, 0·01 EDTA, 10 glucose; pH adjusted to 7·8 with NaOH.)

The eye was hemisected. Under infrared illumination, the retina was removed in Ringer solution, and a piece was chopped with a small section of razor blade to dissociate photoreceptor cells. Supernatant liquid (200 μl) was placed in a chamber formed between two glass microscope slides. The outer or inner segment of an intact rod was drawn into a suction pipette, and its circulating current was measured. Signals were low-pass filtered at 10 Hz and digitized at 50 Hz on a PDP-11 computer (Lamb, 1983). Data were transferred to a Macintosh computer for further analysis with Igor Pro software (WaveMetrics, Lake Oswego, OR, USA). In some experiments, rods from the larval tiger salamander (*Amblystoma tigrinum*) were prepared with a procedure identical to that described above, except that the animal was killed by decapitation followed by pithing of the brain and spinal cord. The results illustrated in all figures except Fig. 2 (salamander) are from toad rods. All experiments were performed at room temperature (22–25 °C).

The optical system was as described in Baylor, Lamb & Yau (1979*a*), with the addition that light was circularly polarized to prevent any variation in intensity due to altered orientation of the outer segment. Illumination was spatially uniform and incident transverse to the outer segment. Interference filters were used to produce narrow-band 498 nm light in one beam, and 500 nm light in a second beam, although unfiltered white light from the tungsten–iodide lamp could also be used to provide a higher effective intensity (Baylor & Hodgkin, 1973). In the experiments described in this work, all pre-flashes were 498 nm, while test flashes were either 500 nm or white light. Pre-flashes and some test flashes were attenuated with neutral density filters, and all flashes were 20 ms in duration. The incident photon flux delivered by narrow-band flashes was estimated from the unattenuated light intensity (measured with a photometer), together with the flash duration and neutral density filter calibrations. The number of photoisomerizations was then calculated assuming a standard rod outer segment collecting area of 20 μm^2 (Baylor, Lamb & Yau, 1979*b*). The effective intensity of white light was calibrated by comparing the dim-flash response of several cells to both white and 500 nm light (Baylor & Hodgkin, 1973).

Unless otherwise noted, the error estimates of all values reported in this work represent 95% confidence limits.

RESULTS

τ_c measurements in toad rods

We explored the relationship between flash intensity (Φ) and saturation time (t_{sat}) in eight toad rods. Sample recordings from one toad rod are illustrated in Fig. 1*A*, for flashes ranging from less than 1000 to more than 100 000 photoisomerizations. As the intensity increased, the saturation time t_{sat} (measured as the time until recovery of

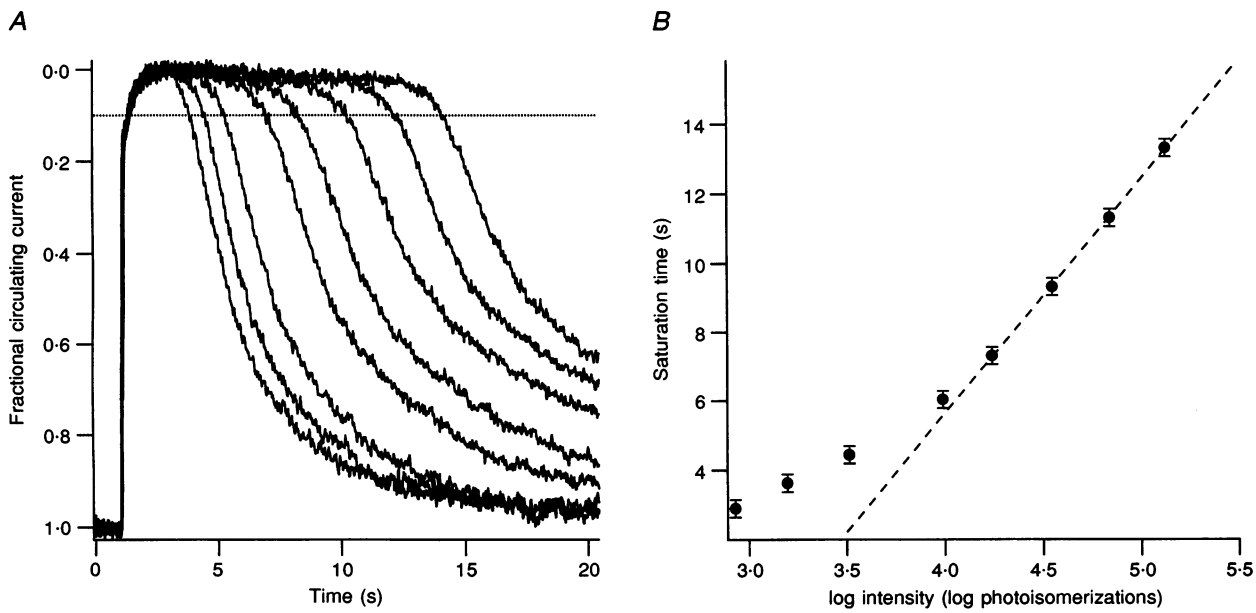

Figure 1. Measurement of τ_c

A, responses of a toad rod to increasing flash intensities delivering 850, 1600, 3300, 9700, 17 000, 35 000, 69 000 and 130 000 isomerizations. The dotted line marks photocurrent recovery to 10% of the dark level, which is used in determining t_{sat}. The dark-level circulating current of this cell was 23 pA. *B*, plot of saturation time *versus* log intensity using the data in *A*. The dashed line is a least-squares fit to the points for the four brightest intensities. Using eqn (1), a value for τ_c of 2·95 ± 0·37 s was determined from the slope. Estimates of error in t_{sat} were determined by delivering five identical bright flashes to a rod cell and measuring t_{sat}. When this protocol was followed for six cells, an S.D. for t_{sat} of approximately 0·13 s was observed in each case. This value was taken to be the S.D. of measurements of t_{sat} for all flashes in all cells, and the error bars represent a 95% confidence interval based upon this S.D. Error bars for measurements of t_{sat} in subsequent figures would be identical to these, and they have been omitted for clarity.

10% of the dark-adapted current) also increased. A plot of t_{sat} *versus* log Φ for this cell is shown in Fig. 1*B*. Note that the points fall on a straight line for Φ values greater than about 10 000. This is similar to the behaviour of salamander rods, where the log–linear range has been reported to occur for Φ values greater than 8000 (Pepperberg *et al.* 1992). For the rod in Fig. 1*B*, the slope of the line yields the dominant time constant $\tau_c = 2{\cdot}95 \pm 0{\cdot}37$ s (eqn (1)).

The value of τ_c measured in the eight toad rods ranged from 1·3 to 3·2 s, a much broader range than observed in salamander rods. A particular problem in toad rods was that the measured value of τ_c often increased markedly over the course of an experiment, sometimes by as much as 100%. Typically, we found that the fitted line not only increased in slope, but also showed an upward shift; i.e. that t_{sat} increased at all test flash intensities. Hence there was an increase both in time constant (τ_c) and in gain (ψ). Usually, the increase occurred quickly, soon after the experiment was started, and then levelled off. For eight rods, the τ_c value measured during the first half-hour of each experiment was $2{\cdot}04 \pm 0{\cdot}59$ s (mean ± S.D.), while the τ_c at later times was $2{\cdot}87 \pm 0{\cdot}25$ s (mean ± S.D.).

The cause of this increase is unclear. It may simply occur with time after the cell is drawn into the suction electrode. Perhaps the act of drawing the cell into the pipette causes some damage that gradually slows inactivation reactions. On the other hand, it may occur not with time but instead with accumulated light exposure, if, for example, the by-products of photoactivation accumulate in the rod and alter its kinetics. Alternatively, some substance influencing inactivation may be depleted with repeated light exposure and not replaced. While these possibilities were not systematically explored, our results suggested that accumulated light exposure played at least some role in the phenomenon.

Effect of a saturating pre-flash on τ_c

In all of the experiments described subsequently, a saturating pre-flash and a bright test flash were delivered at a time separation between 0·5 and 120 s. Figure 2*A* shows a typical response, when the flashes were delivered at an interval of 4 s (arrows). By analogy with the results of Pepperberg *et al.* (1994) with steady lights, it was expected that presentation of a saturating pre-flash at a fixed time before each test flash would reduce the gain parameter ψ

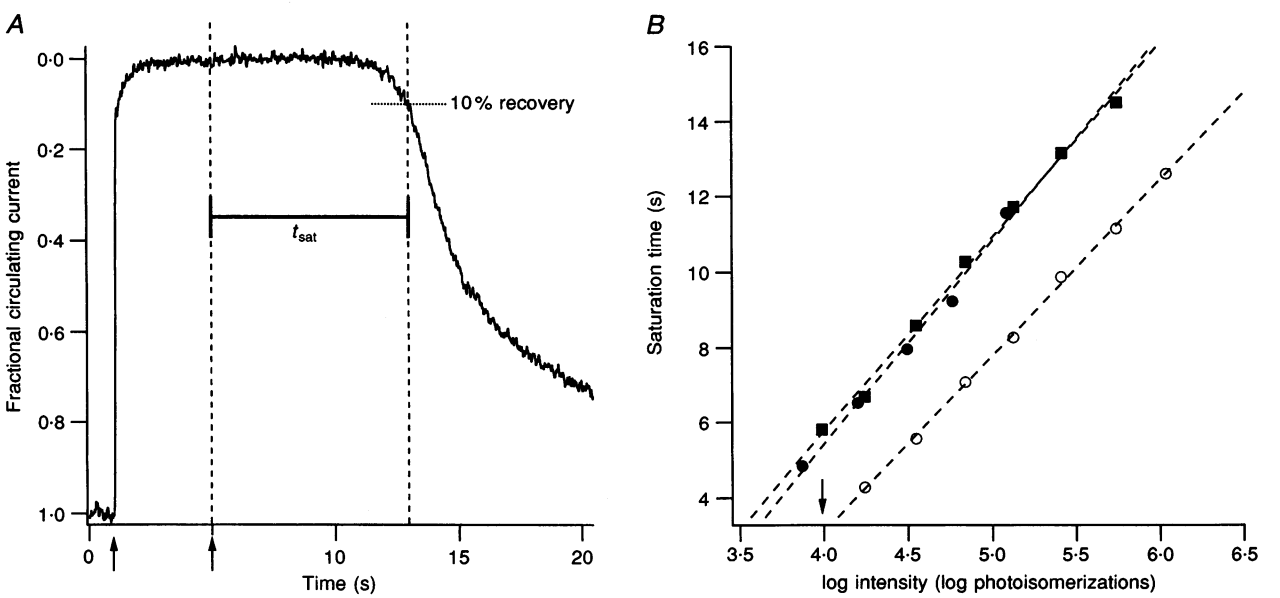

Figure 2. Measurement of τ_c with a pre-flash

A, a saturating pre-flash of 21 000 isomerizations was delivered at the time indicated by the left arrow. After 4 s, when the photocurrent was still in saturation, a test flash of 130 000 isomerizations was delivered (right arrow). The saturation time (t_{sat}) was measured from the time of test flash delivery until 10% recovery of the photocurrent. *B*, a conditioning pre-flash delivered to a salamander rod at a fixed time before each test flash decreased the saturation at every test flash intensity. ●, control family with no conditioning flash, measured before the family with a pre-flash (intensity range, 7400–120 000 isomerizations). ○, a conditioning pre-flash of 9700 isomerizations was delivered at 4 s before each test flash (test flash range, 17 000–1 100 000 isomerizations; the arrow marks the pre-flash intensity); the pre-flash held the photocurrent in saturation for the 4 s before each test flash was delivered. (As indicated by the ■ at the position of the arrow, the pre-flash by itself would have held the photocurrent in saturation for about 6 s.) ■, control family with no conditioning flash, measured after the family with a pre-flash (range, 9700–550 000 isomerizations). The τ_c calculated from the fitted slopes was: $2{\cdot}36 \pm 0{\cdot}39$ s (●), $2{\cdot}03 \pm 0{\cdot}08$ s (○) and $2{\cdot}24 \pm 0{\cdot}16$ s (■). The dark-level circulating current of this cell was 33 pA.

but have little effect on τ_c. This behaviour was verified in salamander rods, and Fig. 2*B* shows the results of one experiment. We delivered three sets of test flashes, each with an intensity range of 10^4–10^6 isomerizations. The middle set (○) was preceded by a conditioning flash producing 9700 isomerizations, delivered 4 s before each test flash. The two control sets of test flashes alone (●, ■), given in darkness before and after the set with pre-flashes, showed nearly identical τ_c of 2·36 ± 0·39 and 2·24 ± 0·16 s, respectively.

The pre-flash caused a downward shift in the line of best fit, similar to that observed by Pepperberg *et al.* (1994) using steady background illumination. The pre-flash shortened t_{sat} by approximately 3·3 s at every intensity of test flash; in addition there was a slight shortening of τ_c (with respect to the control sets of test flashes) to 2·03 ± 0·08 s. The reduction of t_{sat} by 3·3 s represented 1·4 time constants, corresponding to a ψ of about 0·25 (equal to $e^{-1.4}$).

The behaviour of toad rods exposed to saturating pre-flashes was also consistent with these results in salamander rods. Although fewer different test flash intensities were used, a reduction in the gain parameter ψ, together with a slight shortening of τ_c, was also observed.

Time course of gain reduction

By separating a conditioning flash and test flash of fixed intensities by a variable amount of time, it is possible to follow the time course of gain reduction. The seven toad rods characterized in Table 1 were used in these experiments, and typical responses obtained from one cell are shown in Figs 3 and 4. A conditioning pre-flash of 21 000 isomerizations and a test flash of 130 000 isomerizations were delivered at progressively greater separation times. Figure 3 shows eight individual responses. In each panel, the pre-flash was delivered at $t = 1$ s, and the time of test flash delivery is indicated by the arrowhead. Figure 4 shows a superimposition of five of these responses, aligned so that the test flash was delivered at time zero. This figure illustrates the reduction of the test flash saturation time, as the flash separation is increased from 0 to 8 s. So that the gradual changes in the rod's kinetics described above did not introduce artifacts into these experiments, the dominant time constant τ_c was determined before and after each set of double flashes. Thus, the slope of a t_{sat} *versus* log Φ plot (as in Fig. 1*B*) was determined before and after each experiment of the kind illustrated in Fig. 5*A*. In no case did τ_c change by more than 10% during the course of an individual experiment.

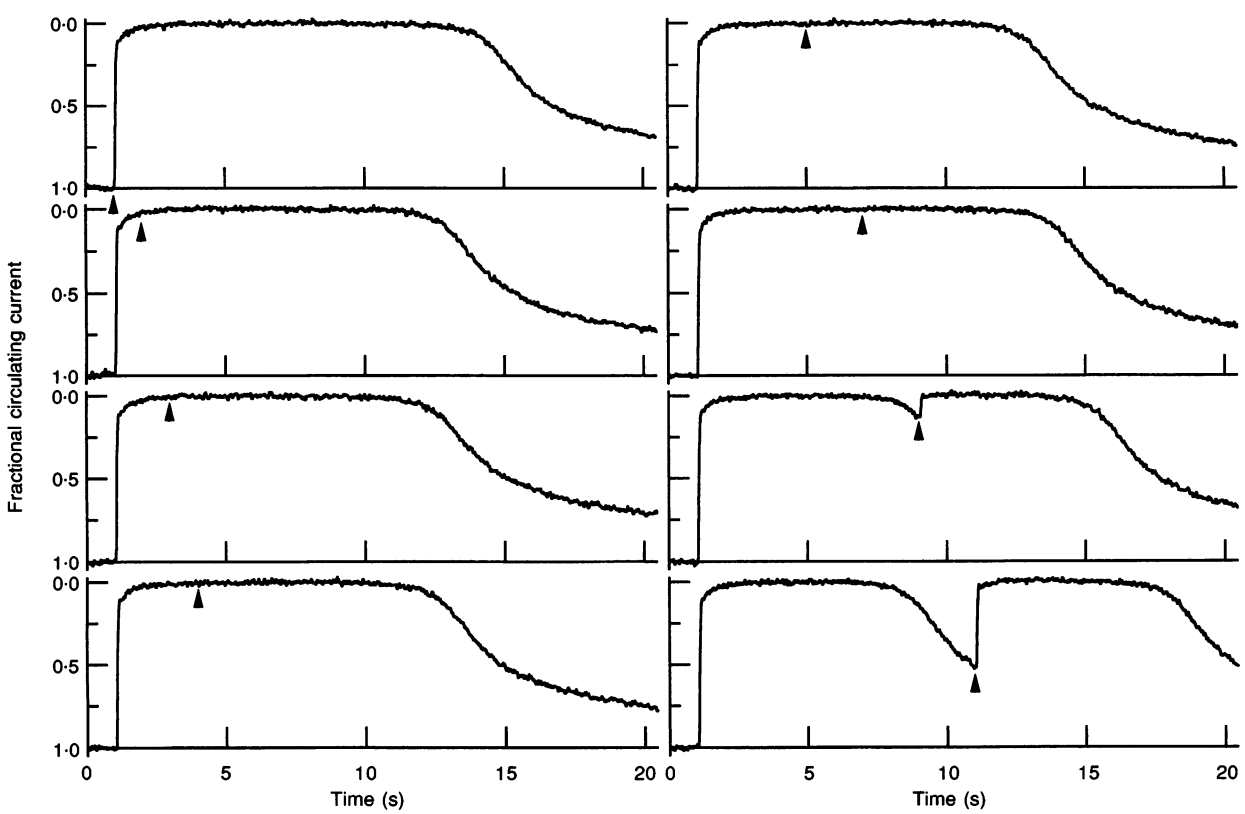

Figure 3. Responses to pre-flash and test flash of fixed intensities at different separation times

A toad rod received a conditioning pre-flash (21 000 isomerizations) and a test flash (130 000 isomerizations) at increasing separation times. In each panel, the pre-flash was delivered at $t = 1$ s. The time of test flash delivery is marked with an arrowhead. The separation time between the pre-flash and test flash was: left column (top to bottom), 0 (concurrent), 1, 2 and 3 s; right column (top to bottom), 4, 6, 8 and 10 s. The dark-level circulating current of this cell was 25 pA.

Table 1. Exponential fits to the time course of gain reduction induced by a pre-flash in toad rods

Cell no.	t_{min} (s)	Δt_{sat} (s)	τ_p (s)	τ_c (s)	ψ_{min}
1	8·35 ± 1·04	5·85 ± 1·03	2·38 ± 0·83	2·22 ± 0·13	0·072 ± 0·035
2	7·52 ± 0·60	7·10 ± 0·60	2·28 ± 0·44	2·95 ± 0·37	0·090 ± 0·033
3	6·28 ± 0·57	6·86 ± 0·56	2·66 ± 0·51	2·88 ± 0·07	0·092 ± 0·019
4	11·40 ± 0·32	6·12 ± 0·32	2·85 ± 0·40	2·75 ± 0·11	0·108 ± 0·016
5*	9·65 ± 0·39	7·92 ± 0·42	2·64 ± 0·32	3·02 ± 0·14	0·073 ± 0·013
6	9·12 ± 0·28	4·08 ± 0·33	1·38 ± 0·32	2·32 ± 0·14	0·172 ± 0·031
7*	8·16 ± 0·33	6·64 ± 0·34	2·58 ± 0·32	2·54 ± 0·07	0·073 ± 0·011
Mean (S.D.)	—	—	2·40 (0·49)	—	0·097 (0·036)

The fitted exponential is given in eqn (2). For cell 1, a 20%, rather than 10%, recovery criterion was used for measurements of saturation time (t_{sat}). * Multiple flash intensities were employed to examine kinetics at separation times up to 10 s (see text for methods). t_{min}, minimum saturation time; Δt_{sat}, maximum reduction in saturation time; τ_p, time constant of reduction in t_{sat}. τ_c, time constant of decay of the rate-limiting species in photoresponse inactivation; ψ_{min}, limiting reduction in gain.

Figure 5*A* is a plot of t_{sat} *versus* separation time for the cell of Figs 3 and 4. The pre-flash and test flash intensities were 21 000 and 130 000 photoisomerizations, respectively. It is evident that t_{sat} was reduced when the conditioning pre-flash was given before the test flash. The longer that the rod was allowed to remain in saturation before the test flash was given, the shorter t_{sat} became. If the test flash was not delivered until after the conditioning flash response had begun to recover (time marked with a dotted line), then t_{sat} continued to drop for about a second, but at longer separations t_{sat} began to recover toward its original value.

Note that the time at which a conditioning pre-flash began to recover (dotted line) differs from our definition of the saturation time, t_{sat}, which represents the time until 10% recovery of the dark-adapted circulating current. Using this precise criterion level, t_{sat} can be measured unambiguously and is highly reproducible. However, any recovery of circulating current will lead to an influx of calcium ions, and hence will tend to raise the intracellular calcium concentration above the low level that is achieved during prolonged saturation. Since the pre-flash experiments are intended to explore the effect of holding a cell fully in

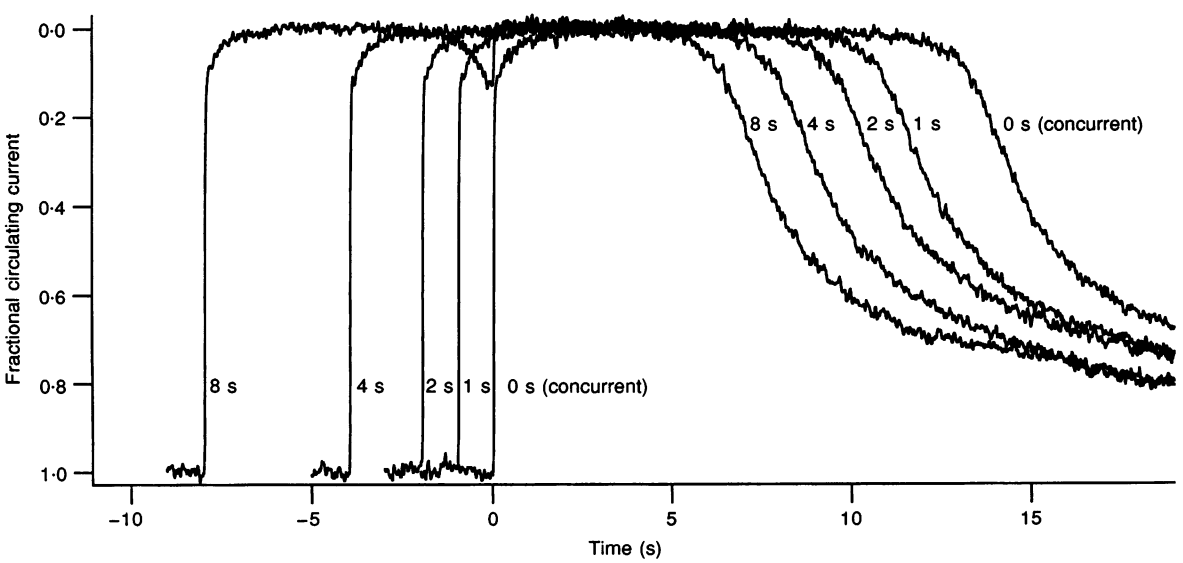

Figure 4. Superimposed responses to pre-flash and test flash of fixed intensities at 5 separation times

Five of the responses in Fig. 3 are superimposed here. The pre-flash was delivered either concurrently with, or 1, 2, 4 or 8 s before the test flash. The responses are aligned so that the test flash was delivered at time 0. The pre-flash given 8 s before the test flash had recovered by approximately 10% by the time the test flash was delivered.

saturation for defined lengths of time, the interpretation of points measured after any recovery of circulating current will be ambiguous and so they have been ignored in this study. The time at which the first hint of circulating current could be detected was generally 1–3 s earlier than the 10% recovery time, but this value is only approximate.

Points at negative separation times in Fig. 5*A* represent delivery of the test flash before the conditioning flash. The filled circle at $t = 0$ represents concurrent delivery of the conditioning and test flashes. The open circle at $t = 0$ shows the saturation time of the test flash alone, with no conditioning flash. Together, these points represent a control for determining whether the extra Rh* produced by the conditioning flash contributed to t_{sat} of the test flash. The intensity of concurrent flashes was about 115% of the test flash alone. This intensity difference had a slight but detectable effect on the saturation time, t_{sat}, which was about 0·2 s longer for the concurrent flashes than for the test flash alone. However, compared with the changes in t_{sat} that occurred when the flashes were separated in time, this change is small and can safely be ignored.

An expanded plot of t_{sat} for short flash separations is shown in Fig. 5*B*, where it is clear that t_{sat} declined steeply when the conditioning flash was shifted from concurrent with the test flash to 1 s before it. With longer separations, t_{sat} continued to decline, but progressively less steeply, until at

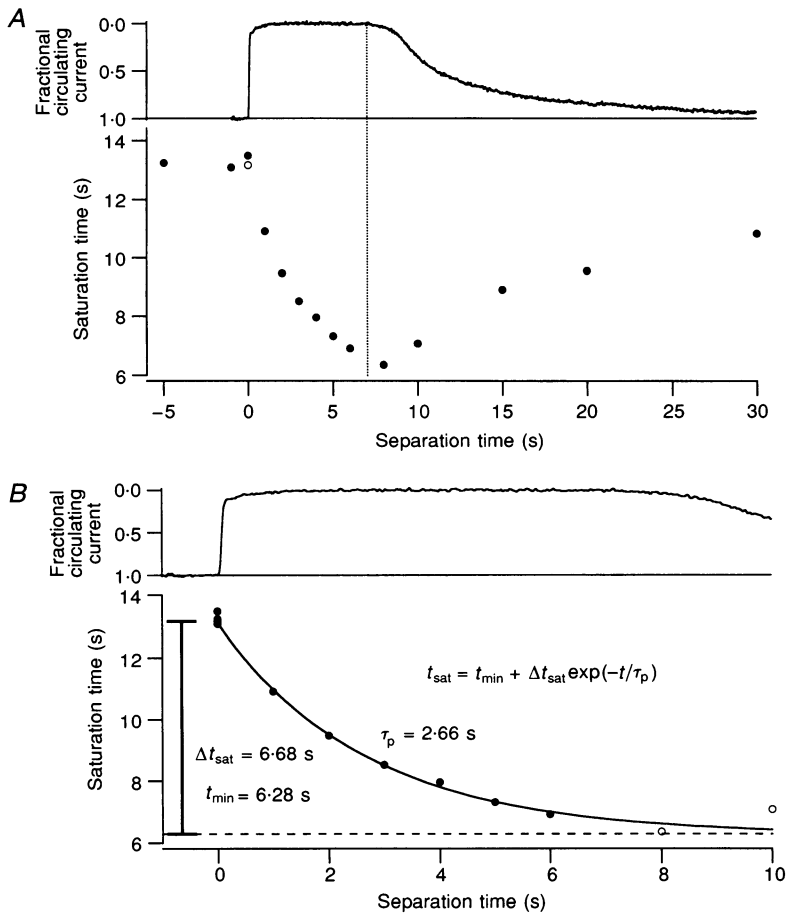

Figure 5. Exponential curve fitted to the time course of gain reduction in response to a conditioning pre-flash

A, a conditioning pre-flash delivering 21 000 isomerizations and a test flash delivering 130 000 isomerizations were given to a toad rod at a range of separation times. ●, the saturation time of the test flash is plotted *versus* the separation time. Positive separation times represent the conditioning flash's delivery before the test flash. The zero point represents concurrent delivery, and the point at −5 s represents the conditioning flash's delivery after the test flash. ○ at $t = 0$, delivery of the test flash alone, with no conditioning flash. The upper panel shows the time course of the conditioning flash when delivered alone. The dotted line marks the approximate time at which the conditioning flash leaves saturation. *B*, expansion to show only small separation times. The multiple points at $t = 0$ include points with the conditioning flash given after the test flash, the conditioning flash given concurrently with the test flash, and the test flash given alone. The upper panel shows the time course of the conditioning flash delivered alone. An exponential of the form in eqn (2) has been fitted to ●. ○, not included in the fit because the conditioning flash had already begun to recover at these separation times.

least the time at which the response to the conditioning flash began to recover (upper trace). For this cell, the maximum reduction in t_{sat} was observed at 8 s separation, which is about 1 s after the response to the pre-flash alone began to recover. The maximum reduction in t_{sat} was 6·56 s, corresponding to $\psi = 0{\cdot}10$ in eqn (1), or a tenfold reduction in this measure of gain.

Since the decline in $[Ca^{2+}]_i$ following a saturating flash has been reported to follow either an exponential time course (Yau & Nakatani, 1985; McNaughton *et al.* 1986), or the sum of two exponential components (Gray-Keller & Detwiler, 1994), it was natural to test whether the time course of gain reduction followed similar kinetics. A single exponential decline in t_{sat} can be described by the equation:

$$t_{sat} = t_{min} + \Delta t_{sat} \exp(-t/\tau_p). \quad (2)$$

The continuous trace in Fig. 5*B* shows the fit of this equation to the experimental points. The fit included all separation times up to 6 s, the longest separation tested that did not include any sign of recovery (see top panel). The results from several trials were used to obtain a good estimate of t_{sat} under dark-adapted conditions. These points (plotted at $t = 0$) comprised trials where the conditioning flash and test flash were given concurrently, trials where the

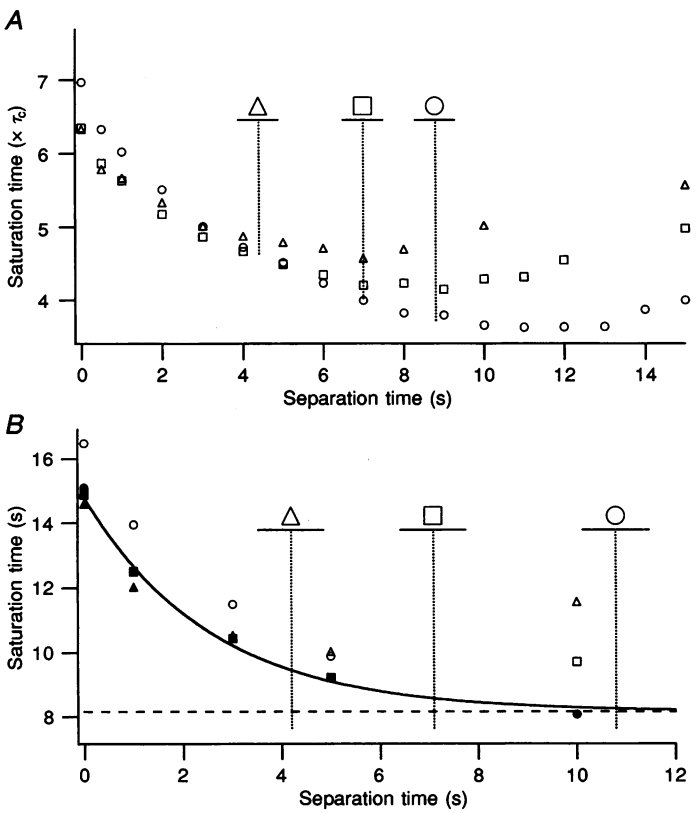

Figure 6. Reduction of saturation time after conditioning pre-flashes of different intensities

A, a toad rod was given a fixed intensity test flash (130 000 photoisomerizations), preceded by one of three different intensity pre-flashes at many different separation times. The pre-flash intensities were: △, 2100 isomerizations; □, 9700 isomerizations and ○, 43 000 isomerizations. Since τ_c for this cell was changing over the course of this experiment, t_{sat} measurements were normalized to give saturation time in number of time constants (τ_c). The dotted lines mark the approximate time that each pre-flash, when given by itself, began to recover from saturation. Thus, the 2100-isomerization pre-flash began to recover at about 4·2 s, the 9700-isomerization pre-flash at about 7·1 s, and the 43 000-isomerization pre-flash at about 8·8 s. *B*, a toad rod was given a test flash of 120 000 isomerizations, preceded by one of three different intensity pre-flashes: ▲△, 4800 isomerizations; ■□, 17 000 isomerizations; and ●○, 60 000 isomerizations. Three of the points at zero separation time indicate t_{sat} for the test flash and each pre-flash given concurrently. Three extra points included at zero separation time indicate t_{sat} for 3 trials of the test flash alone. The dotted lines show the time at which each pre-flash, when given alone, began to recover. An exponential curve was fitted to the filled symbols. Saturation times measured with the brightest pre-flash were excluded from the fit at separation times $\leqslant 5$ s (○), since these times were artificially elevated by the bright pre-flash (see text). Points measured with the two pre-flashes of lower intensity were excluded from the fit for separation times at which the pre-flash had already begun to recover when the test flash was delivered (□, △). The parameters of the fitted exponential were: $t_{min} = 8{\cdot}16 \pm 0{\cdot}33$ s, $\Delta t_{sat} = 6{\cdot}64 \pm 0{\cdot}34$ s, and $\tau_p = 2{\cdot}58 \pm 0{\cdot}32$ s. The horizontal dashed line indicates the fitted value of t_{min}.

test flash was given before the conditioning flash (the points at negative separation times in Fig. 5A), and trials where the test flash was given alone. The fitted exponential decays with a time constant (τ_p) of 2·66 ± 0·51 s toward a minimum saturation time (t_{min}) of 6·28 ± 0·51 s. The maximum reduction in t_{sat}, denoted in eqn (2) as Δt_{sat}, was 6·86 ± 0·56 s, corresponding to $\psi = 0{\cdot}09$ in eqn (1), or an elevenfold reduction in the apparent test flash brightness. In the seven toad rods studied in this way, a single exponential provided a good fit, and the parameters are listed in Table 1.

Effects of conditioning flash intensity

We were curious to know whether t_{sat} continued to drop exponentially when the cell was held in saturation for more than 6 s. To study this question, it was necessary to use brighter pre-flashes which saturated the cell for longer times. Results from a representative cell are shown in Fig. 6A, for the presentation of a constant test flash (of 130 000 isomerizations) at a range of intervals following conditioning pre-flashes of three different strengths: 2100 (△), 9700 (□), and 43 000 (○) isomerizations. Each plot was normalized to the number of time constants spent in saturation, since, as explained above, τ_c changed over the course of 3 h of recording. The dotted lines show the time at which each pre-flash, when given alone, began to recover.

For the first 4 s, the plots for the two dimmer pre-flashes nearly superimposed. This coincidence suggests that over the intensity range of these pre-flashes – fourteenfold to sixty-fivefold dimmer than the test flash – the reduction in gain at a given pre-flash separation time was independent of pre-flash strength. For each of the three pre-flash intensities, the measured gain continued to decline, as expected, for separations up to 1–2 s beyond the times at which recovery of the pre-flash responses were first detected (indicated by the vertical dotted lines).

The effect of the brightest pre-flash (○), which was only three times dimmer than the test flash, was different at small separation times. When test flashes were delivered in the first few seconds after this pre-flash, t_{sat} was noticeably larger than the common behaviour elicited by the two dimmer conditioning flashes (△, □). The likely explanation is that, in this case, the pre-flash intensity was a substantial fraction of the test flash intensity. Thus the conditioning flash itself contributed an amount of Rh* that was significant with respect to the amount formed by the test flash (an extra 33%, for example, when the two flashes were given concurrently). Therefore, for a given reduction in gain, t_{sat} was artificially elevated at short separation times. At longer separation times (approximately >5 s), most of the rate-limiting species (Rh* or G*–PDE*) formed by the conditioning pre-flash should have decayed with time constant τ_c, so that the amount left should have been insignificant with respect to the amount created by the test flash. This indeed appeared to be the case, as the curves for the brighter pre-flashes (□, ○) coincided for separation times of 3–6 s. Comparable results were obtained in three other toad rods, using a smaller number of time separations.

We interpret these results to indicate that, when a rod is held in saturation by a conditioning pre-flash, the time course of gain reduction is independent of the intensity of that flash; i.e. independent of the number of rhodopsin molecules isomerized. Instead, the gain reduction that we measure (i.e. the component of gain reduction not dependent on cyclase modulation) must be controlled by a property of the saturating response that is independent of flash intensity. One obvious candidate for this role would be the intracellular calcium concentration, $[Ca^{2+}]_i$ (Yau & Nakatani, 1985).

Gain reduction for longer pre-flash separation times

In order to study the time course of gain reduction for separation times up to 10 s, it was necessary to use a pre-flash delivering at least 50 000 photoisomerizations. However, as discussed in the previous section, the measurement of t_{sat} was then distorted at early separation times, unless the test flashes were made substantially brighter than this conditioning pre-flash. Further experiments (not shown) indicated that it was necessary to make the test flash at least six times brighter than the pre-flash to obtain an accurate measurement of the gain reduction at early separation times. Unfortunately, it was impractical to use test flashes delivering more than about 150 000 photoisomerizations, because of the excessively long time that the experiment took. Although complete recovery from a single flash at this intensity occurred in about 5 min, the need for repetition at many flash separation times meant that the cell's properties were likely to have changed before the experiment was completed.

However, given the property explored in the previous section, that gain reduction is independent of pre-flash intensity, it is possible to use conditioning flashes of different strengths to uncover the full time course of gain reduction. Dimmer pre-flashes, that recover after 3–5 s, can be used to measure t_{sat} reduction at early separation times. Brighter pre-flashes are then needed only when examining the behaviour at longer separation times. And since at long separation times the rate-limiting species formed by the pre-flash will have decayed, it will not then be necessary to employ an excessively bright test flash. Hence, by judicious choice of pre-flash and test flash intensities to suit the separation time being investigated, it should be possible to conduct the whole experiment in a reasonable length of time. All of the points obtained at different separations by this procedure would be expected to lie on a common curve, so the entire time course of gain reduction can be uncovered.

Figure 6B shows an example of this type of experiment. A test flash of constant intensity (120 000 isomerizations) was used, and pre-flashes of three intensities (2-, 7- and 25-fold dimmer) were employed. For each pre-flash intensity, filled symbols indicate those points that should most reliably measure the true saturation time, and only these points

were used for fitting the exponential curve. Open symbols indicate points that are expected to be biased: points at short separation times with the brightest pre-flash (○), when t_{sat} was artificially lengthened, and points at long separation times with the dimmer pre-flashes (△, □), when the pre-flash response had already begun to recover.

The parameters of the exponential curve fitted to the filled symbols in Fig. 6*B* were: $t_{min} = 8{\cdot}16 \pm 0{\cdot}33$ s; $\Delta t_{sat} = 6{\cdot}64 \pm 0{\cdot}34$ s; and $\tau_p = 2{\cdot}58 \pm 0{\cdot}32$ s. The limiting reduction in gain, ψ_{min}, was then calculated as:

$$\psi_{min} = \exp(-\Delta t_{sat}/\tau_c).$$

For the value of $\tau_c = 2{\cdot}54$ s obtained in this cell, ψ_{min} was calculated as 0·07, corresponding to a maximum gain reduction of fourteenfold. Alternatively, this value may be viewed as specifying the maximum change in the 'apparent intensity' of a test flash that can be elicited by a conditioning pre-flash.

For cells 5 and 7 (marked by * in Table 1) the exponential curves were fitted out to 10 s using this method of two or more different conditioning pre-flashes. The parameters obtained were very similar to those obtained for cells not marked with an asterisk, determined using only smaller separation times. These results indicate that a single exponential decay provides a good description of the decline in t_{sat}, at least for separation times up to 10 s. For seven cells, the time constant of gain reduction (τ_p) was $2{\cdot}40 \pm 0{\cdot}49$ s (mean ± S.D.), and the value of ψ_{min} was $0{\cdot}097 \pm 0{\cdot}036$ (mean ± S.D.), equivalent to a tenfold reduction in gain.

Calcium dependence of gain reduction

Given that several recent studies have suggested that reductions of t_{sat} in the presence of background light are mediated by a fall in intracellular $[Ca^{2+}]$, it is important to ask whether our observations are consistent with the idea that they are caused by changes in $[Ca^{2+}]_i$.

Using gecko rods, Gray-Keller & Detwiler (1994) found that while the photocurrent is saturated, $[Ca^{2+}]_i$ declines as the sum of two exponential components. The double exponential behaviour is thought to represent the action of two Ca^{2+} buffers – one low affinity but high capacity, and the other high affinity but low capacity. Gray-Keller & Detwiler (1994) also found that the decline of Ca^{2+}-exchange current (I_{exh}) paralleled the decline of $[Ca^{2+}]_i$ and could be described by the same double exponential:

$$I_{exh} = I_{exh}^{dark}(A\exp(-t/\tau_a) + B\exp(-t/\tau_b)), \quad (3)$$

where I_{exh}^{dark} is the dark-adapted Ca^{2+}-exchange current, and A, B, τ_a and τ_b are constants.

In several rods we studied, we were able to fit the sum of two exponentials to the exchange current. Figure 7 shows the average of seven saturating responses of a toad rod. The fitted double exponential, of the form in eqn (3), had parameters: $I_{exh}^{dark} = 3{\cdot}3$ pA; $A = 0{\cdot}72$; $\tau_a = 0{\cdot}37$ s; $B = 0{\cdot}28$; $\tau_b = 2{\cdot}0$ s. This curve fits the photocurrent well, suggesting that Gray-Keller & Detwiler's model provides a good description of the fall in $[Ca^{2+}]_i$ in the cells we studied. For the cell of Fig. 7, the time constant (τ_b) of the high-affinity component of the drop in calcium was 2·0 s. That value is similar to the time constant (τ_p) of the drop in t_{sat} which was 2·6 s in this cell. Similar results were observed in two other cells. That one component of the decline in $[Ca^{2+}]_i$ happens on a similar time scale to the decline in t_{sat}, suggests that the fall in $[Ca^{2+}]_i$ could well be responsible for the fall in t_{sat}.

Our data are also consistent with the results of Lagnado & Baylor (1994). In truncated outer segments, they observed a

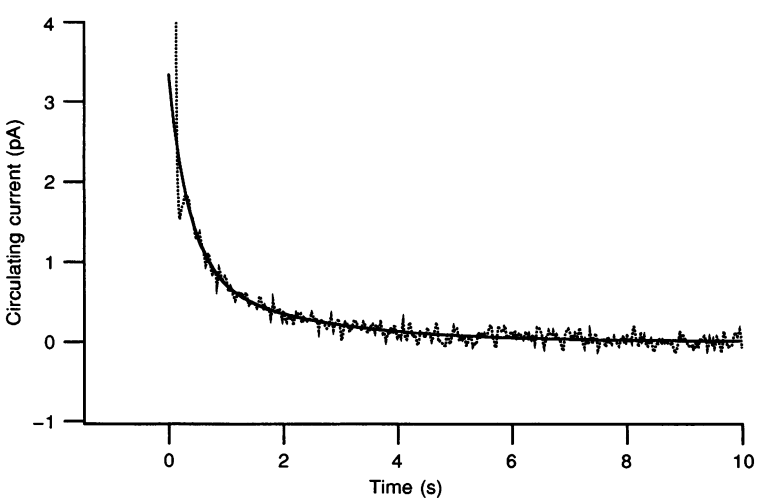

Figure 7. Double exponential fit to calcium exchange current

The trace is an average of seven saturating responses of a toad rod. The vertical scale is chosen so that the calcium exchange current is clearly visible. The fitted curve is a double exponential of the form in eqn (3). Its parameters are given in the text. The dark-level circulating current of this cell was 24 pA. The small overshoot of the photocurrent at the start of the record is an artifact of low-pass filtering.

calcium-dependent drop in phototransduction gain equivalent to dimming the light. Such a drop in gain would have the effect of reducing t_{sat} and may be partially or fully responsible for the reduction in t_{sat} that we observed. They found that the gain changed most steeply between calcium concentrations of 10–100 nM. This is precisely the range of $[Ca^{2+}]_i$ that is dominated by the slow component of decline, i.e. since $A = 0{\cdot}72$ and $B = 0{\cdot}28$, approximately the first 72% of calcium decline occurs quickly, with a time constant of approximately τ_a. This represents a decline from the dark level of around 500 to about 150 nM. The remaining 28% drop in calcium – from 150 to its minimum level of 10–50 nM – occurs more slowly with a time constant of approximately τ_b.

It is possible that the Ca^{2+}-regulated protein mediating the decline in t_{sat} is the same substance that contributes high-affinity buffering capacity responsible for the slow component of $[Ca^{2+}]_i$ decline – i.e. the substance that releases bound calcium as $[Ca^{2+}]_i$ drops the final ~100 nM from 150 to 10–50 nM. It has been estimated that for every ion of Ca^{2+} free in the cytoplasm, between 75 and 500 ions are bound to a buffer (Lagnado, Cervetto & McNaughton, 1992; Gray-Keller & Detwiler, 1994). Thus, the high-affinity buffer would need to bind ~75 × 100 nM to 500 × 100 nM, or ~10–50 μM Ca^{2+}. If each molecule of buffer can bind one Ca^{2+} ion, its concentration in the rod would need to be 10–50 μM. This concentration is reasonable for a protein that regulates transduction gain. The concentration of S-modulin, for example, has been estimated as 40 μM in frog rods (Kawamura & Murakami, 1991).

DISCUSSION

Our results can provide insight into the identity of two steps of the phototransduction cascade. The first is the rate-limiting step in the recovery from bright flashes, which occurs with first-order time constant τ_c. The second is the step that modulates the gain parameter, ψ. Are these distinct steps in the phototransduction cascade, or do they represent a single step that is both rate limiting for inactivation and modulated by intracellular calcium?

Implications of a common step

Some published evidence suggests that the single step of Rh* phosphorylation performs both these functions. Pepperberg *et al.* (1992) and Corson *et al.* (1994) have presented evidence that Rh* inactivation is the first-order decay step giving rise to τ_c, and Rh* phosphorylation is thought to be the rate-limiting step in Rh* inactivation (Pulvermüller, Palczewski & Hofmann, 1993). Additionally, the protein S-modulin, which is known to decrease phototransduction gain in low $[Ca^{2+}]$, is thought to act by regulating phosphorylation of Rh* (Kawamura & Murakami, 1991; Kawamura, 1993).

We have attempted to model such a situation, in which the rate-limiting step giving rise to τ_c is the same step regulated by $[Ca^{2+}]_i$. Our model assumes that the inactivation of Rh* is first-order, but that the first-order rate constant of Rh* removal (k_c) is calcium dependent. The rate constant k_c is small in the dark-adapted state (with high $[Ca^{2+}]_i$). During a saturating response, as $[Ca^{2+}]_i$ decreases to its minimum level, k_c increases to its maximum value. The increase in k_c must be essentially complete after about 6 s, because the decline in calcium is known to be essentially complete by then. After about 6 s, k_c will be constant at its maximum value, and Rh* will be removed at a steady, maximal rate.

Our model does not address the calcium-dependent increase in guanylate cyclase activity. It does not need to, because the gain changes we attempt to explain (i.e. changes in ψ) are due only to alternations of the gain from photon absorption to PDE* production. As discussed in the Introduction, cyclase activity is constant and maximal after 5 s, regardless of the presence or absence of a pre-flash. Therefore, the cyclase has an identical effect on the sensitivity of responses both to control flashes and to flashes given after a pre-flash. Thus it does not contribute to ψ, the pre-flash-induced change in gain.

It has been known for some time that when all of the light-sensitive channels are closed by a saturating flash, the rate of Ca^{2+} removal by the exchanger is independent of the amount of light exposure (Yau & Nakatani, 1985). Thus, in our experiments, after a pre-flash is delivered to a dark-adapted rod, the time course of $[Ca^{2+}]_i$ decline is independent of the timing of the test flash. Since the time course of $[Ca^{2+}]_i$ drop is independent of saturating flash intensity and flash separation time, so must be the time course of increase in k_c.

The eventual increase of k_c to a constant value would explain the linear relationship between t_{sat} and $\log \Phi$ observed by Pepperberg *et al.* (1992, 1994) and by us. Imagine a saturating flash delivering Φ isomerizations given in darkness. For the first 6 s, Rh* will decay with an arbitrary time course, as k_c increases to its maximal value. During this time, a fixed percentage of the Rh* originally present will have been inactivated. Because Rh* decay is assumed to be first-order, this percentage is independent of Φ. After about 6 s, the constant k_c causes an exponential decay of Rh*. Since we are assuming that the calcium-regulated step is also the rate-limiting step of inactivation, the maximum, steady-state value of k_c must correspond to τ_c. Since τ_c has been measured as approximately 2 s, the maximum value of k_c must be roughly $0{\cdot}5\ s^{-1}$.

The idea of a calcium-dependent k_c also explains the reduction in apparent gain observed with background lights and with a pre-flash. If at the time of the test flash, $[Ca^{2+}]_i$ is not at its dark-adapted level, but at some lower concentration, k_c has already begun to increase before the test flash is given. The final constant value for k_c of $0{\cdot}5\ s^{-1}$ ensures that the log–linear relationship with the same slope still holds. However, as k_c has already begun to increase, the initial rate of Rh* inactivation is faster than if the flash

were given in the dark. Thus, in the first 6 s of saturation, a higher percentage of the Rh* originally present will have been inactivated. This would result in a shortening of t_{sat} by a constant amount at every intensity Φ: exactly what is observed. Since k_c should have reached its maximal, steady-state value by approximately 6 s after a saturating flash, this model also predicts there should be little further reduction in t_{sat} as the separation time between a pre-flash and test flash is increased beyond 6 s. This behaviour is also observed.

For one crucial reason, however, this model is not consistent with our pre-flash results: according to this model, if a pre-flash and test flash are separated by time t, then the maximum possible reduction in t_{sat} as a result of the pre-flash is equal to t. This idea is best illustrated if one imagines a saturating pre-flash given at time zero and a test flash delivering n Rh* given at a certain time, say $t = 3$ s. This test flash will have a particular saturation time ($t_{sat}^{p/f}$).

Now imagine the same test flash delivered in darkness, without a pre-flash. The saturation time ($t_{sat}^{control}$) will be longer than $t_{sat}^{p/f}$, since k_c is still at its dark-adapted value, so the initial rate of Rh* inactivation will be slower. Take the most extreme case: that $k_c = 0$ for the first 3 s and then suddenly increases to its maximal value. During the first 3 s, no Rh* will be inactivated at all. So 3 s after the test flash was delivered, n Rh* are still present in the cell. But during these 3 s, $[Ca^{2+}]_i$ has dropped in the same manner as during the 3 s after the pre-flash in the example of the previous paragraph. Thus, the conditions in the cell are exactly the same as they were when the test flash was given 3 s after a pre-flash: There are n Rh* present, and $[Ca^{2+}]_i$ has had 3 s to fall. The n Rh* *must* be inactivated with the same time course as before. Recovery from saturation will occur in an additional $t_{sat}^{p/f}$ seconds. Since the n Rh* were formed 3 s earlier, $t_{sat}^{control} = t_{sat}^{p/f} + 3$ s.

This example assumed the most extreme case – that no Rh* at all were inactivated during the first 3 s. More probably, k_c does not equal zero for all of the first 3 s, but begins at a small value and increases. Therefore, there will be somewhat fewer than n Rh* present after 3 s, and the recovery will occur in fewer than $t_{sat}^{p/f}$ more seconds. In this case, $t_{sat}^{control} < t_{sat}^{p/f} + 3$ s (equivalently, $t_{sat}^{p/f} > t_{sat}^{control} - 3$ s). A similar argument can be made for any pre-flash separation time. Inescapably, this model allows that when a pre-flash and test flash are separated by time t, the maximum possible reduction in test flash saturation time is t.

Our data do not meet this criterion. For every cell we studied, a pre-flash delivered 1 s before the test flash reduced t_{sat} by at least 1·8 s. Typically, the reduction was between 2·0 and 2·6 s. Note that we made only two assumptions in constructing this model. The first is that the rate-limiting step of inactivation of the photoresponse is the same step whose regulation gives rise to the observed reduction in gain (ψ). Although the example we used in the preceding discussion was the phosphorylation of Rh*, the same argument can be made about any single step that is both rate limiting and whose regulation gives rise to the observed gain reduction. The second assumption is that the rate (k_c) of the $[Ca^{2+}]$-regulated step is dependent only upon instantaneous calcium concentration within the cell.

Possibility of separate steps

The difficulty with the preceding model leads us to consider the possibility that the rate-limiting inactivation process and the regulation of gain occur in separate steps. This idea was also proposed by Lyubarsky *et al.* (1996) and Matthews (1996), whose calcium-clamping experiments similarly suggested that the dominant time constant of recovery (τ_c) is independent of $[Ca^{2+}]_i$. One possibility is that the rate-limiting step of inactivation is indeed Rh* phosphorylation, but that the amount of Rh* initially formed by a flash changes with $[Ca^{2+}]_i$. This was suggested by Lagnado & Baylor (1994), however, a possible mechanism is not known. Another possibility, recently suggested by Nikonov *et al.* (1996), is that the dominant time constant of recovery (τ_c) is determined not by Rh* lifetime, but instead by G*–PDE* lifetime. This hypothesis requires that the time constant of Rh* inactivation (τ_{Rh*}) be shorter than τ_c.

One advantage of the latter hypothesis is that a mechanistic explanation is readily available. Since Rh* catalytically activates transducin, the amount of G* formed after a flash is dependent both upon the amount of Rh* formed and upon the lifetime of that Rh*. If Rh* lifetime (τ_{Rh*}) is reduced tenfold, ten times fewer G* – and hence ten times fewer G*–PDE* – will be formed. If the rate-limiting step in recovery is G*–PDE* decay, gain (ψ) will therefore be reduced tenfold. Any value of the gain parameter ψ can be achieved simply by a reduction of τ_{Rh*} to $\psi\tau_{Rh*}$. A possible mechanism for this reduction exists in the action of S-modulin, which is thought to increase the rate of Rh* phosphorylation in conditions of low $[Ca^{2+}]_i$. The main objection to this description is that Pepperberg *et al.* (1992) and Corson *et al.* (1994) have interpreted their results to indicate that the dominant time constant of recovery represents the lifetime of Rh*. We suggest that that interpretation may now need re-evaluation, since a parsimonious account of our experimental results can be provided by assuming Rh* lifetime to be: (a) shorter than G*–PDE* lifetime (Nikonov *et al.* 1996), and (b) calcium sensitive.

In conclusion, our results suggest that the rate-limiting step in recovery from bright flashes is not the same step as that responsible for the light-induced reduction in gain (ψ). But before we can make a secure identification of the underlying molecular mechanisms, it will be necessary to resolve definitively whether Rh* lifetime or G*–PDE* lifetime dominates recovery.

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