Role of cytoplasmic calcium concentration in the bleaching adaptation of salamander cone photoreceptors

H. R. Matthews, G. L. Fain* and M. C. Cornwall†

*Physiological Laboratory, Downing Street, Cambridge CB2 3EG, UK, *Departments of Physiological Science and Ophthalmology, UCLA, Los Angeles, CA, USA and †Department of Physiology, Boston University Medical School, Boston, MA, USA*

1. In order to study the possible involvement of Ca^{2+} in the bleaching adaptation of cones isolated from the retina of the salamander *Ambystoma tigrinum*, changes in cytoplasmic calcium concentration ($[Ca^{2+}]_i$ were opposed by exposing the outer segment to a low-Ca^{2+}–0 Na^+ solution designed to minimize Ca^{2+} fluxes across the outer segment membrane.

2. When a cone was exposed in normal Ringer solution to bright light bleaching a significant fraction of the photopigment, the circulating current was initially suppressed completely and then recovered to a maintained value less than the value in darkness before the bleach. When the outer segment of the cone was stepped to low-Ca^{2+}–0 Na^+ solution before the bleach was delivered, the circulating current recovered more slowly or (for large bleaches) remained completely suppressed for the duration of the solution exposure.

3. If, during the period for which the current was suppressed in low-Ca^{2+}–0 Na^+ solution, the cone outer segment was exposed to the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), the circulating current was restored. The dim flash response recorded under these conditions exhibited kinetics and integration times similar to those recorded in low-Ca^{2+}–0 Na^+ solution in darkness before the bleach. If, instead, the outer segment was returned to Ringer solution after the bleach, thereby allowing $[Ca^{2+}]_i$ to fall from its dark-adapted level to the appropriate bleach-adapted level, the kinetics of the response in low-Ca^{2+}–0 Na^+ solution were greatly accelerated, and the integration time considerably reduced. This was true regardless of whether or not the low-Ca^{2+}–0 Na^+ solution included IBMX.

4. The role of Ca^{2+} in bleaching adaptation appeared to resemble its role in background adaptation, since in both cases exposure to low-Ca^{2+}–0 Na^+ solution suppressed the acceleration of response kinetics. Responses recorded from cones in low-Ca^{2+}–0 Na^+ solution were nearly identical in waveform and sensitivity during background light or after bleaches, provided that IBMX was used to restore sufficient photocurrent so that responses to flashes could be recorded, and sensitivity was corrected for loss in quantum catch.

5. These results indicate that the fall in $[Ca^{2+}]_i$ in cones after a bleach is necessary both for the acceleration of the flash response and the adaptational decrease in sensitivity, as is the case for adaptation by background light.

Exposure of rod and cone photoreceptors to light sufficiently bright to bleach a significant fraction of the photopigment produces a decrease in sensitivity and an acceleration of the time to peak of the light response (Cornwall, Ripps, Chappell & Jones, 1989; Cornwall, Fein & MacNichol, 1990; Matthews, Fain, Murphy & Lamb, 1990). These changes in response kinetics and sensitivity persist indefinitely in photoreceptors isolated from the retinal pigment epithelium and are accompanied by a long-lasting suppression of the circulating current. Furthermore, the decrease in sensitivity induced by bleaching is much larger than can be accounted for by the decrease in the probability of photon absorption (Cornwall *et al.* 1990; Jones, Fein, MacNichol & Cornwall, 1993). These

observations suggest that bleaching adaptation may result from an 'equivalent background' which excites and adapts the photoreceptor much as does real light (Cornwall & Fain, 1994).

Photoreceptor light adaptation is thought to be mediated by a decrease in cytoplasmic calcium concentration $[Ca^{2+}]_i$ induced by steady light. During steady illumination $[Ca^{2+}]_i$ falls (McNaughton, Cervetto & Nunn, 1986; Ratto, Payne, Owen & Tsien, 1987; Gray-Keller & Detwiler, 1994; McCarthy, Younger & Owen, 1994), due to the continued efflux of Ca^{2+} via the sodium–calcium exchanger following the suppression of Ca^{2+} influx through the outer segment conductance (Yau & Nakatani, 1985). If this fall in $[Ca^{2+}]_i$ is prevented, then adaptation is abolished in both rods and cones (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988; Fain, Lamb, Matthews & Murphy, 1989; Nakatani & Yau, 1989; Matthews *et al.* 1990). Furthermore, if $[Ca^{2+}]_i$ is altered in darkness, at least some of the manifestations of adaptation are produced in rods (Matthews, 1995). The light-induced fall in $[Ca^{2+}]_i$ is therefore believed to induce the changes in response sensitivity and kinetics which comprise background adaptation. The similarity of the response changes following bleaching to those during background illumination thus suggests that Ca^{2+} might be involved in bleaching adaptation also. The sustained suppression of a proportion of the dark current following bleaching may result in a steady decrease in $[Ca^{2+}]_i$, which might be expected to result in response adaptation much as takes place during steady light. We have therefore investigated the role of Ca^{2+} in bleaching adaptation by attempting to prevent the changes in $[Ca^{2+}]_i$ which accompany bleaching.

Changes in $[Ca^{2+}]_i$ were opposed by exposing the outer segment to a low-Ca^{2+}–0 Na^+ solution designed to minimize Ca^{2+} fluxes across the outer segment membrane (Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989; Matthews *et al.* 1990). The reduction of external Ca^{2+} serves to minimize Ca^{2+} influx through the outer segment conductance, while the removal of external Na^+ prevents extrusion of Ca^{2+} via the sodium–calcium exchange. Exposure of the outer segment to such a solution thus greatly slows subsequent changes in $[Ca^{2+}]_i$, holding it near its value before the solution change. By exposing the outer segment to low-Ca^{2+}–0 Na^+ solution, $[Ca^{2+}]_i$ could be held near the original dark-adapted level while the cone was bleached, and the effect on the light response investigated.

Preliminary results of this study have been presented to The Physiological Society (Matthews, Fain & Cornwall, 1993) and the Association for Research in Vision and Ophthalmology (Fain, Matthews & Cornwall, 1993).

METHODS

Preparation and solution changes

Suction pipette recordings were made from cone photoreceptors isolated mechanically from the dark-adapted retina of the larval tiger salamander, *Ambystoma tigrinum*, following decapitation and pithing (Matthews *et al.* 1990). The inner segment was drawn into the suction pipette, leaving the outer segment exposed to the superfusing solution. Rapid exchange of the solution bathing the outer segment was achieved by translating the boundary between two flowing streams of solution across the exposed outer segment of the cell (Hodgkin, McNaughton & Nunn, 1985; Lamb & Matthews, 1988). Streams of solution emerged from up to four parallel tubes built into the recording chamber. Solutions were delivered by a multichannel peristaltic pump in most experiments, or in later experiments by gravity. Solution streams were stepped across the cone held in the suction pipette using a linear stepping motor coupled hydraulically to the microscope stage, or in later experiments a rotary stepping motor and lead screw connected directly to the stage.

Solutions

Ringer solution contained (mM): 111 NaCl, 2·5 KCl, 1·0 $CaCl_2$, 1·6 $MgCl_2$ and 3·0 Hepes, was adjusted to pH 7·7 with NaOH, and included 10 μM EDTA to chelate impurity heavy metals. In addition, the Ringer solution perfusing the recording chamber (but not that in the rapid solution exchange system) included 10 mM glucose. Low-Ca^{2+}–0 Na^+ solution was made by substituting guanidinium chloride (Sigma) for NaCl, omitting EDTA, and buffering Ca^{2+} at a reduced level with EGTA (Matthews *et al.* 1990). The inclusion of 2 mM EGTA and 1·78 mM $CaCl_2$ yielded a nominal free Ca^{2+} concentration of 0·1 μM (calculated from the stability constants for EGTA; Martell & Smith, 1974), while the free Mg^{2+} concentration was maintained at 1·6 mM by increasing the concentration of $MgCl_2$ to 1·65 mM. This solution did not contain glucose and was titrated to pH 7·7 with tetramethylammonium hydroxide (Sigma). When required, 125–500 μM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; Sigma) was added to the low-Ca^{2+}–0 Na^+ solution.

Light stimulation and electrical recording

Light stimuli were delivered from a dual beam optical bench and were unpolarized. Stimulus intensities were adjusted with neutral density filters and measured with a calibrated silicon photodiode (Graseby Optronics, Orlando, FL, USA). Flash stimuli were 20 ms in duration, while intense 'bleaching' stimuli were typically 0·5 s. All experiments were carried out on red-sensitive cones (Attwell, Werblin & Wilson, 1982; Perry & McNaughton, 1991), identified by comparing the sensitivity to dim flashes at 500 and 620 nm. In most experiments illumination was with white light, and intensities are given in terms of the effective photon flux at 620 nm, calculated from the ratio of sensitivities to white and 620 nm light measured individually for each cell (Matthews *et al.* 1990). In later experiments all light stimuli were of wavelength 610 nm. These intensities can be converted to rates of isomerization using an effective collecting area of 0·7 μm^2 for circularly polarized light at 620 nm (Matthews *et al.* 1990). In experiments in which isolated cones were exposed to intense bleaching light, the percentage of pigment bleached was estimated

from the photosensitivity for vitamin A_2-based pigments in free solution (Dartnall, 1972), corrected for the difference in dichroism in free solution and in disc membranes ($6{\cdot}2 \times 10^{-9}$ μm^2; Jones *et al.* 1993).

The suction pipette current signal was filtered over the bandwidth DC-40 Hz and digitized continuously at a sampling rate of 200 Hz with an IBM-compatible microcomputer, equipped with an intelligent interface card (Cambridge Research Systems, Rochester, UK). In most experiments the timing of light stimuli and solution changes was controlled by a hard-wired digital pulse generator (DT4030, Digitimer Ltd, Welwyn Garden City, UK), while in later experiments pulses were generated under software control from the host computer.

Most protocols are identified with a two-letter code according to the general convention that the first letter represents the exposure of the cell to light, while the second indicates the superfusing solution. For example, DG (dark, guanidinium) represents a response recorded in darkness with $[Ca^{2+}]_i$ held near the dark-adapted level using low-Ca^{2+}–0 Na^+ solution, while PG (post-bleach, guanidinium) represents a response recorded after bleaching with $[Ca^{2+}]_i$ held near the bleach-adapted level. Responses recorded in low-Ca^{2+}–0 Na^+ solution which contained IBMX are denoted by SI (steady light, IBMX) and BI (bleach, IBMX) when the cell was exposed to steady light or bleached while $[Ca^{2+}]_i$ was maintained near the dark-adapted level, and by PI (post-bleach, IBMX) when $[Ca^{2+}]_i$ was held near the reduced level which follows bleaching. Further details are given in the legends of individual figures and in the text.

All experiments were carried out in Cambridge.

RESULTS

Ca^{2+} and recovery of circulating current after bleaching

Figure 1*A* illustrates the photocurrent response of an isolated cone to a 0·5 s light exposure of progressively increasing intensity, presented in Ringer solution. The dimmest background intensity caused only a partial suppression of the circulating current. As the light intensity increased, the response was driven into saturation, but thereafter recovered rapidly and completely as long as the total fraction of pigment bleached remained small. However, for the brightest intensity, which irreversibly bleached nearly half of the photopigment, the response remained in saturation for a considerable period following the light exposure and recovered only incompletely thereafter. Furthermore, it was found that the sensitivity of the cone to dim flashes was persistently reduced following the bleaching exposure. These are well-known manifestations of the bleaching adaptation which follows exposure to light sufficiently bright to bleach a significant fraction of the cone photopigment (Matthews *et al.* 1990; Jones *et al.* 1993).

Figure 1*B* shows responses obtained from another cone when the outer segment was rapidly stepped to low-Ca^{2+}–0 Na^+ solution shortly before each light exposure, thereby holding $[Ca^{2+}]_i$ near to its initial dark-adapted level throughout the response. It can be seen that when $[Ca^{2+}]_i$ was prevented from falling in this way, the response to any given intensity was of longer duration than in Ringer solution, much as for responses to brief flashes in low-Ca^{2+}–0 Na^+ solution (Matthews *et al.* 1990). In particular, following exposure to the brightest intensity the response did not recover from saturation within the duration of the trace.

Similar responses to bright bleaching light are examined in more detail in Fig. 2*A* and *B*. These show the responses of two cones to light exposures which bleached about three-quarters of the photopigment in each case. When the cell was bleached in Ringer solution (Fig. 2*A*), the circulating current did not recover completely, reflecting persistent excitation of the transduction mechanism following the bleach. The sensitivity of the bleached cone to a subsequent dim flash was greatly reduced, and the kinetics of the response accelerated in comparison to the response before bleaching (not shown). In contrast, in low-Ca^{2+}–0 Na^+ solution (Fig. 2*B*) the response did not recover following the bleach but instead remained in saturation for an extended period. It would therefore appear that the light-induced fall in $[Ca^{2+}]_i$ is necessary for the recovery of circulating current after bleaching, much as for adaptation to background light (Matthews *et al.* 1988, 1990; Nakatani & Yau, 1988).

Recovery of circulating current induced by IBMX

In Ringer solution, guanylyl cyclase velocity is known to be elevated in both rods (Cornwall & Fain, 1994) and cones (Cornwall, Matthews, Crouch & Fain, 1995) after a bleach, presumably due to a persistent reduction in $[Ca^{2+}]_i$ (Koch & Stryer, 1988). If the cone is instead bleached in low-Ca^{2+}–0 Na^+ solution, $[Ca^{2+}]_i$ will not fall but will remain near its dark-adapted level. Therefore guanylyl cyclase velocity will remain low throughout the response. The consequent imbalance between a low cyclase velocity and the persistently elevated level of phosphodiesterase (PDE) activity which follows a bleach (Cornwall & Fain, 1994) seems likely to have led to the persistent suppression of circulating current seen under these conditions. This interpretation is supported by the observation that circulating current could be restored by superfusing the outer segment with low-Ca^{2+}–0 Na^+ solution which included 125 μM of the PDE inhibitor IBMX (Fig. 2*B*, arrow). The resulting partial inhibition of the PDE led to a rapid increase in the circulating current, which could be fully suppressed by a

bright flash. It therefore presumably corresponded to an increase in the light-dependent conductance following elevation of the cyclic GMP concentration.

The ability of IBMX to restore the circulating current was investigated for another cone in Fig. 3. In this experiment the outer segment was first stepped to low-Ca^{2+}–0 Na^+ solution before the start of the trace, and the bleaching light was delivered. Then the outer segment was repeatedly exposed to low-Ca^{2+}–0 Na^+ solution containing 500 μM IBMX. Exposures to IBMX soon after the bleach had little effect on the circulating current. This observation suggests that at early times the PDE activity was so greatly elevated

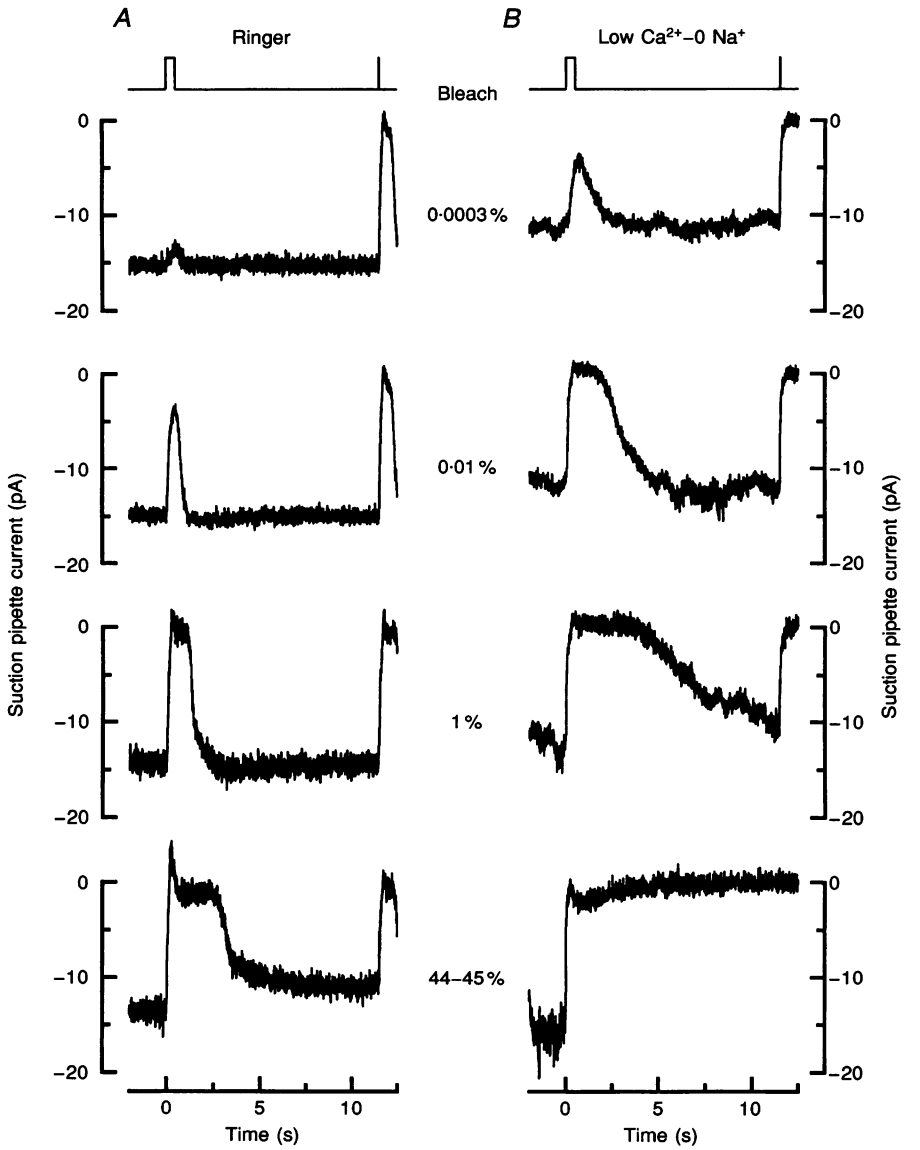

Figure 1. Effects of progressive bleaching in Ringer solution or low-Ca^{2+}–0 Na^+ solution

Responses of two cones to successive 0·5 s exposures to light of progressively increasing intensity, yielding fractional bleaches of (from top to bottom) 0·0003, 0·01, 1 and 45%, respectively, followed by a saturating flash. *A*, outer segment in Ringer solution. Bleaching light delivered $4{\cdot}15 \times 10^2$, $1{\cdot}79 \times 10^4$, $1{\cdot}63 \times 10^6$ and $9{\cdot}65 \times 10^7$ equivalent photons μm^{-2} at 620 nm; saturating flashes delivered $1{\cdot}94 \times 10^5$ (2 lowest bleaches), $2{\cdot}54 \times 10^6$ and $1{\cdot}11 \times 10^7$ equivalent photons μm^{-2} at 620 nm. *B*, outer segment stepped to low-Ca^{2+}–0 Na^+ solution 2 s before each trace. Bleaching light delivered $3{\cdot}98 \times 10^2$, $1{\cdot}72 \times 10^4$, $1{\cdot}56 \times 10^6$ and $9{\cdot}25 \times 10^7$ equivalent photons μm^{-2} at 620 nm; saturating flashes delivered $2{\cdot}19 \times 10^5$ (2 lowest bleaches), $2{\cdot}86 \times 10^6$ and $1{\cdot}25 \times 10^7$ equivalent photons μm^{-2} at 620 nm. The initial relaxation of the response seen following the onset of steady light appeared not to represent the recovery of the outer segment conductance, but may have resulted instead from the actions of ionic conductances located in the inner segment. The zero current level has therefore been set to the saturating level induced by the bright flash. Bleaching light exposures commenced at time zero. Traces for the 2 lowest bleaches are each the average of 2 responses; traces for the 2 highest bleaches are single responses.

that even in the presence of IBMX the balance between cyclase and PDE velocities could not be restored. As the time after the bleach increased, the magnitude of the current induced by IBMX became progressively larger. In each case, the current was rapidly suppressed when the outer segment was returned to low-Ca^{2+}–0 Na^+ solution without IBMX. Similar results were also obtained from two other cones. These results indicate that PDE activity declined progressively after its initial elevation by the bleach, perhaps as a result of the decay of bleaching

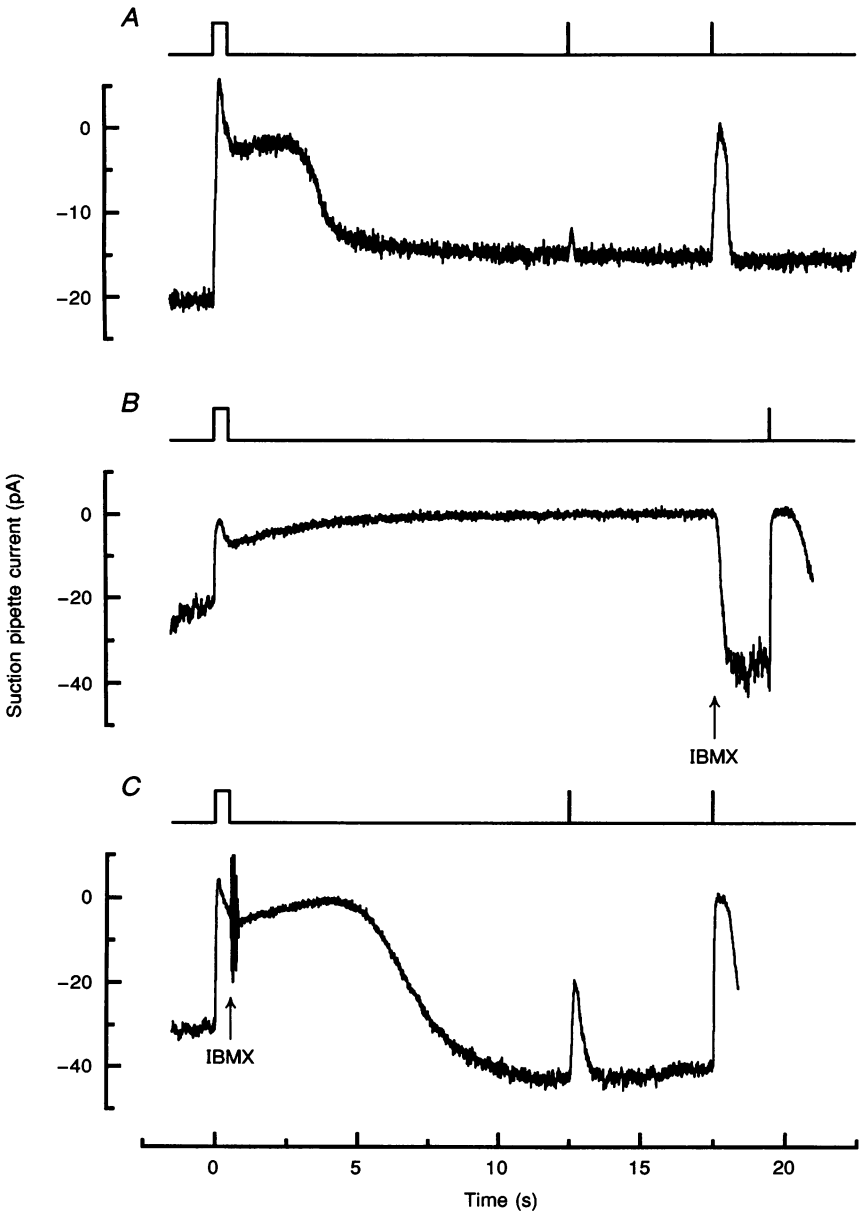

Figure 2. Restoration of circulating current in low-Ca^{2+}–0 Na^+ solution after bleaching by IBMX superfusion

Responses of 3 cones to 0·5 s exposures to intense light beginning at time zero. All stimuli delivered with white light, calibrated for each cell according to the responses to dim flashes at 620 nm. *A*, outer segment in Ringer solution. Intense light delivered $2{\cdot}58 \times 10^8$ equivalent photons μm^{-2} at 620 nm, corresponding to an 80% bleach; dim and bright flashes delivered $2{\cdot}33 \times 10^4$ and $5{\cdot}40 \times 10^6$ equivalent photons μm^{-2} at 620 nm, respectively. *B*, outer segment stepped to low-Ca^{2+}–0 Na^+ solution 2·5 s before the bleach, and then to low-Ca^{2+}–0 Na^+ solution containing 125 μM IBMX at the arrow. Intense light delivered $2{\cdot}02 \times 10^8$ equivalent photons μm^{-2} at 620 nm, corresponding to a 71% bleach; bright flash delivered $4{\cdot}71 \times 10^6$ equivalent photons μm^{-2} at 620 nm. *C*, outer segment stepped to low-Ca^{2+}–0 Na^+ solution 2·5 s before the bleach, and then to low-Ca^{2+}–0 Na^+ solution containing 125 μM IBMX at the arrow. Intense light delivered $2{\cdot}20 \times 10^8$ equivalent photons μm^{-2} at 620 nm, corresponding to a 74% bleach; dim and bright flashes delivered $2{\cdot}39 \times 10^4$ and $1{\cdot}48 \times 10^6$ equivalent photons μm^{-2} at 620 nm, respectively.

intermediates in the outer segment. Despite this decline it would appear that, even long after the bleach, the PDE activity remained at a sufficiently high enough level to completely suppress the circulating current, provided $[Ca^{2+}]_i$ was held near its dark-adapted level.

In Ringer solution much of the circulating current recovers within a few seconds after a bleach but only a rather modest and gradual recovery of current takes place thereafter (see e.g. Fig. 2*A*), despite the continued relaxation of PDE activity seen in Fig. 3. The fact that there is so little further recovery of current under these conditions suggests that the balance between PDE and cyclase velocities is so closely regulated when $[Ca^{2+}]_i$ is free to change that, a few seconds after a bleach, current returns almost to normal and changes rather little from that point on, even though neither the PDE nor cyclase rates have returned to normal. This corresponds directly to the very shallow slope of the steady-state response–intensity relation in cones (Matthews *et al.* 1990).

Ca^{2+} and response kinetics after bleaching

To investigate the importance of Ca^{2+} for the changes in response kinetics and sensitivity induced by bleaching, it is necessary to record the flash response after the bleach in low-Ca^{2+}–0 Na^+ solution. However, normally the cone circulating current is abolished by a bleach in low-Ca^{2+}–0 Na^+ solution, and responses to flashes cannot be recorded. Since the circulating current can be recovered by the addition of IBMX, it should be possible by appropriate selection of the IBMX concentration to restore the circulating current approximately to its pre-existing value in darkness, presumably corresponding to the normal dark level of cyclic GMP. This approach has been used previously to study the effect of steady light and $[Ca^{2+}]_i$ on response kinetics in rods (Matthews, 1995).

Results obtained using this approach are shown in Fig. 2*C*. The outer segment was first stepped to low-Ca^{2+}–0 Na^+ solution 1 s before the beginning of the trace. Intense light was then delivered to bleach 74% of the photopigment. Following the bleach, the outer segment was stepped to low-Ca^{2+}–0 Na^+ solution which included 125 μM IBMX (arrow). The response remained in saturation for around 5 s after the solution change, but in the presence of IBMX the circulating current progressively recovered to a new steady level close to that in low-Ca^{2+}–0 Na^+ solution before the bleach. Once the circulating current had stabilized, a dim flash was first delivered, then a bright flash to confirm stability of the saturating level. Immediately thereafter the outer segment was returned to Ringer solution, thereby allowing $[Ca^{2+}]_i$ to fall to the appropriate bleach-adapted level (not shown).

The kinetics of responses to flashes in low-Ca^{2+}–0 Na^+ solution before and after bleaching are examined for another cone in Fig. 4. Figure 4*A* compares the dim flash

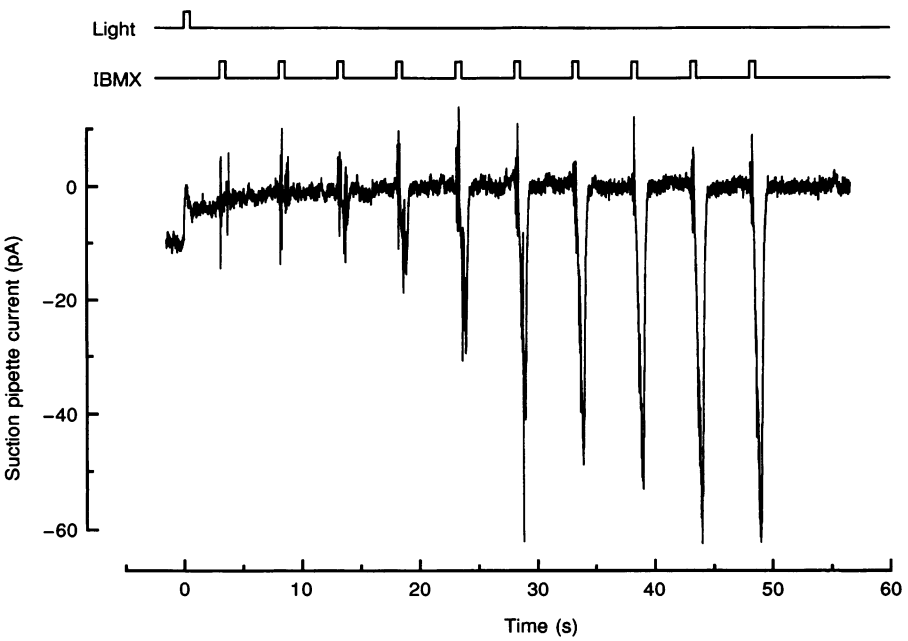

Figure 3. Repeated exposures to IBMX after bleaching in low-Ca^{2+}–0 Na^+ solution

Outer segment stepped to low-Ca^{2+}–0 Na^+ solution approximately 0·5 s before the beginning of the trace, and then exposed for 0·5 s to intense white light commencing at time zero and delivering $8{\cdot}70 \times 10^7$ equivalent photons μm^{-2} at 620 nm, corresponding to a 42% bleach. Thereafter, outer segment stepped repeatedly to low-Ca^{2+}–0 Na^+ solution containing 500 μM IBMX. Upper traces denote timings of bleaching light and solution changes.

response in low-Ca^{2+}–0 Na^+ solution in darkness before bleaching (trace DG) with the response of the same cell to a dim flash obtained after the bleach while $[Ca^{2+}]_i$ was held near its dark-adapted level (trace BI, protocol as Fig. 2*C*). It can be seen that these two normalized responses, although somewhat noisy in this cell, were very similar in waveform. Figure 4*B* shows responses following the bleach after $[Ca^{2+}]_i$ had been allowed to fall to the appropriate bleach-adapted level. Even when recorded in low-Ca^{2+}–0 Na^+ solution, the flash response (trace PG) was substantially accelerated relative to the response obtained when $[Ca^{2+}]_i$ was held near the dark-adapted level (trace DG). It might be suggested that the slower kinetics of the dark-adapted response could have resulted from the action of IBMX itself rather than from any action of $[Ca^{2+}]_i$. However, this possibility was excluded by exposing the already-bleached cell to low-Ca^{2+}–0 Na^+ solution in the presence of the same concentration of IBMX (trace PI). Although this response is slightly retarded in comparison to the response obtained under similar conditions in the absence of IBMX (trace PG), it is still considerably accelerated in comparison with the responses of Fig. 4*A*, for which $[Ca^{2+}]_i$ was held near its dark-adapted level. The slight retardation induced by IBMX under these conditions is similar to that seen in rods when the cyclic GMP concentration is elevated by IBMX in darkness while $[Ca^{2+}]_i$ is held near its dark-adapted level (Matthews, 1995).

These changes in the response waveform were quantified by calculating the integration times from responses in low-Ca^{2+}–0 Na^+ solution before and after bleaching. Integration times were obtained by dividing the integral of the drift-subtracted flash response by the peak response amplitude (Baylor & Hodgkin, 1973). Data were collected from experiments on twenty-three cones exposed to light which bleached between 38 and 85% of the photopigment, since for bleaches in this range the integration time of salamander cones varies little with percentage bleach (see Jones *et al.* 1993). We used IBMX concentrations of 125–500 μM; not all protocols were carried out on each cell. For dim flashes delivered following the bleach with $[Ca^{2+}]_i$ held near the dark-adapted level, a mean integration time of 1·03 ± 0·16 s was obtained (BI protocol, here and throughout text results are given as mean ± S.E.M., $n = 15$ cells). This can be compared with the mean integration time of 1·01 ± 0·07 s (DG protocol, $n = 37$ cells) measured in darkness from all the cells studied before bleaching. There was no significant difference between the integration times in these two cases (Student's paired t test, BI *vs.* DG, $t = -0{\cdot}09$, $n = 15$ cells). In contrast, once $[Ca^{2+}]_i$ was allowed to fall to the appropriate bleach-adapted level, the mean integration time shortened to 0·31 ± 0·04 s (PG protocol, $n = 21$ cells), which is highly significantly different from the integration time in the same cells in darkness before the bleach (Student's paired t test, PG *vs.*

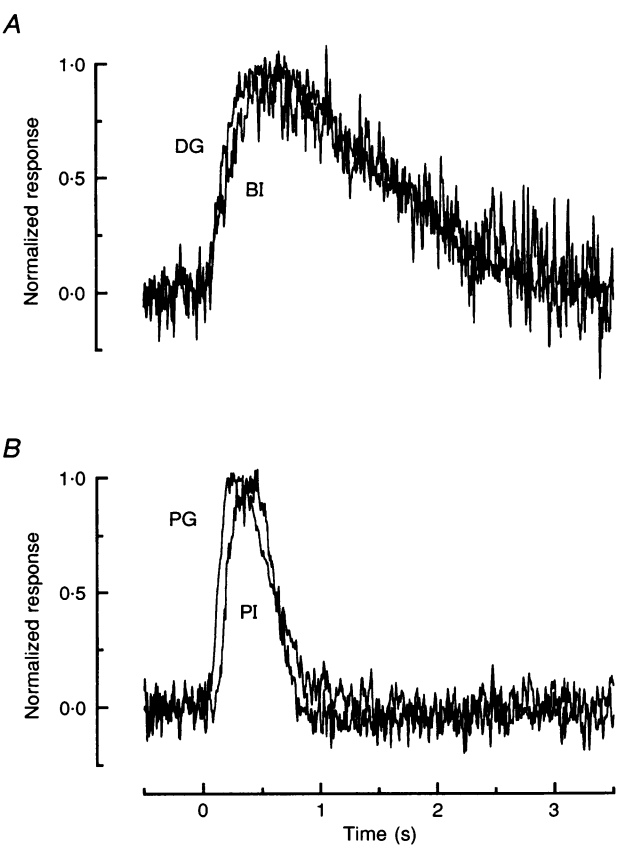

Figure 4. Comparison of dim flash response kinetics in low-Ca^{2+}–0 Na^+ solution before and after bleaching

A, responses to dim flashes delivered at time zero with $[Ca^{2+}]_i$ held near the dark-adapted level by superfusion with low-Ca^{2+}–0 Na^+ solution either in darkness (DG) or in the presence of 500 μM IBMX after a 44% bleach (BI; protocol as in Fig. 2*C*). *B*, dim flash responses from the same cone in low-Ca^{2+}–0 Na^+ solution after the bleach once $[Ca^{2+}]_i$ had been allowed to fall to the appropriate bleach-adapted level, in the presence (PI) or absence (PG) of 500 μM IBMX. Traces BI and PI are single responses, while traces DG and PG are the averages of 3 and 4 responses, respectively. Each trace has been normalized in amplitude according to the response peak, after the subtraction of linear drift in the baseline. 'Dim' flash responses refer to the responses to subsaturating flashes which typically suppressed one-third to one-half of the circulating current. Bleaching light delivered $9{\cdot}45 \times 10^7$ equivalent photons μm^{-2} at 620 nm; 'dim' flashes delivered $1{\cdot}90 \times 10^2$ (DG), $1{\cdot}26 \times 10^4$ (BI), $2{\cdot}48 \times 10^3$ (PG) and $4{\cdot}11 \times 10^5$ (PI) equivalent photons μm^{-2} at 620 nm.

DG, $t = 6{\cdot}16$). Addition of IBMX to the low-Ca^{2+}–0 Na^+ solution after bleaching had no further significant effect on the mean integration time ($0{\cdot}34 \pm 0{\cdot}05$ s, PI protocol, $n = 14$ cells; Student's paired t test, PI *vs.* PG, $t = 0{\cdot}52$). These results indicate that the flash response waveform is only accelerated following bleaching if $[Ca^{2+}]_i$ is allowed to fall. Thus $[Ca^{2+}]_i$ appears to determine response kinetics during bleaching adaptation, similar to its role in background adaptation (Matthews *et al.* 1988, 1990; Nakatani & Yau, 1988).

Effects of bright background light in low-Ca^{2+}–0 Na^+ solution

To reinforce the comparison between bleaching and background adaptation, the experiment of Fig. 4 was extended to cones exposed to low-Ca^{2+}–0 Na^+ solution in

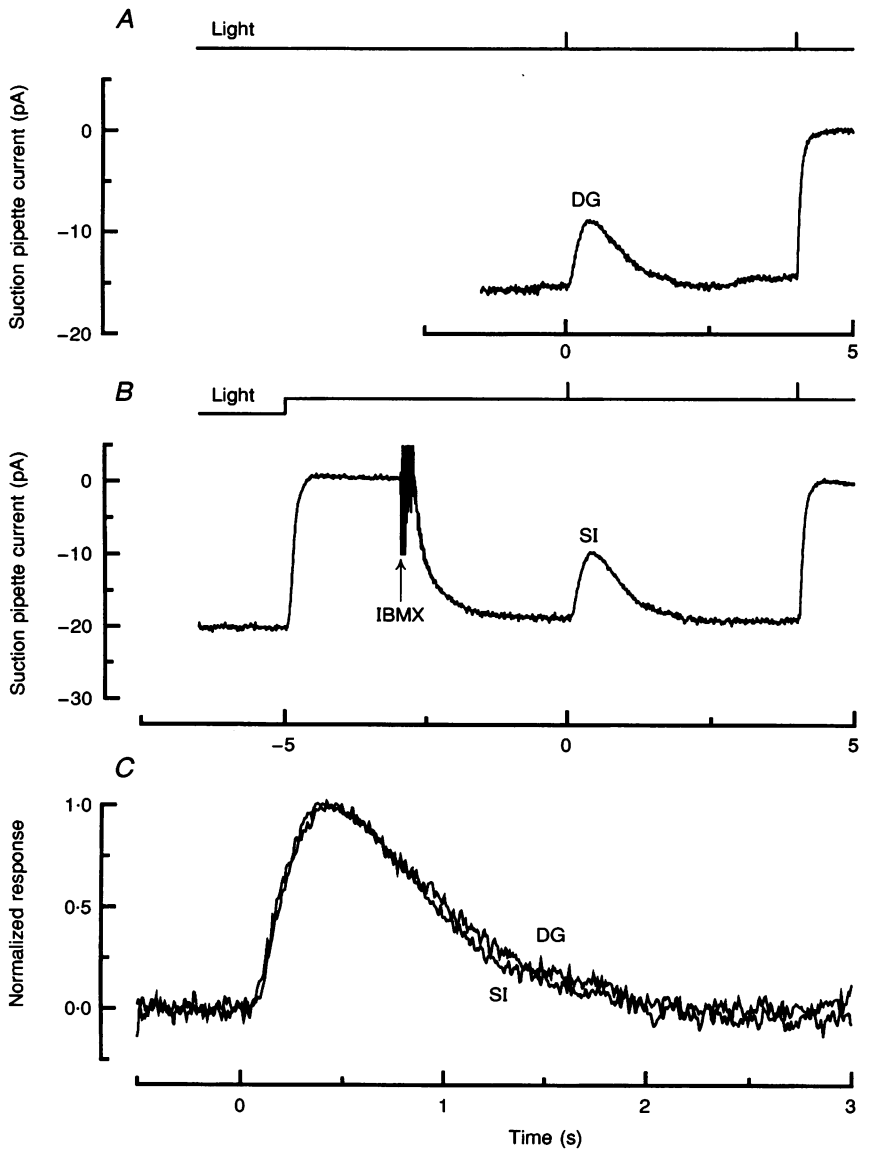

Figure 5. Effect of IBMX and steady light on the dim flash response when changes in $[Ca^{2+}]_i$ are prevented

A, dim flash response in low-Ca^{2+}–0 Na^+ solution in darkness. Outer segment stepped to low-Ca^{2+}–0 Na^+ solution 3 s before delivery of a dim flash at time zero. Dim and bright flashes delivered $2{\cdot}05 \times 10^2$ and $2{\cdot}16 \times 10^4$ photons μm^{-2}, respectively at 610 nm. *B*, dim flash response from the same cone in low-Ca^{2+}–0 Na^+ solution during background illumination. Outer segment was first stepped to low-Ca^{2+}–0 Na^+ solution, exposed 3 s later to steady light of intensity $2{\cdot}23 \times 10^4$ photons μm^{-2} s^{-1}, and then superfused with low-Ca^{2+}–0 Na^+ solution containing 500 μM IBMX. Dim and bright flashes delivered $1{\cdot}03 \times 10^4$ and $3{\cdot}71 \times 10^5$ photons μm^{-2} at 610 nm. *C*, superimposed dim flash responses from *A* and *B* with $[Ca^{2+}]_i$ held near the dark-adapted level in darkness (DG) and during steady light in the presence of 500 μM IBMX (SI). In each case the response has been normalized in amplitude according to the response peak. Each trace is the average of 4 responses.

bright background light, using a protocol previously devised for rods (Matthews, 1995). A representative example is given in Fig. 5. The waveform of the flash response was first measured in low-Ca^{2+}–0 Na^+ solution in darkness (Fig. 5*A*). The same cone was then re-exposed to low-Ca^{2+}–0 Na^+ solution and illuminated with a background light sufficient to completely suppress the circulating current. In order to record the response to a flash, a similar approach was adopted as in Fig. 2*C* for cones bleached in low-Ca^{2+}–0 Na^+ solution: the outer segment was exposed to 500 μM IBMX to restore approximately the original circulating current. Under these conditions, it was possible to record the flash response (Fig. 5*B*) and compare the waveform during background illumination with that obtained in darkness by superimposing the normalized responses (Fig. 5*C*). It can be seen that when $[Ca^{2+}]_i$ was held near the dark-adapted level in this way, the waveforms of the responses obtained in darkness (trace DG) and during steady light (trace SI) overlay each other. Measurement of the integration times from the twelve cells in which this experiment was carried out yielded values of 0·96 ± 0·11 s in darkness and in the absence of IBMX (DG protocol), and 0·86 ± 0·05 s in background light during exposure to IBMX (SI protocol). These values are not significantly different at the 5% level (Student's paired *t* test, $t = 1{\cdot}26$). This result confirms and extends to higher steady intensities previous evidence (Matthews *et al.* 1990) that background light is unable to speed the kinetics of the cone response when $[Ca^{2+}]_i$ is not allowed to change.

In the experiment of Fig. 5, the response in the presence of the background was considerably desensitized in comparison with the response in darkness. Since the response was recorded in low-Ca^{2+}–0 Na^+ solution, the desensitization seemed unlikely to have been the result of the light exposure itself (see Matthews *et al.* 1990), but it might have been caused by exposure to IBMX. IBMX inhibits the PDE competitively (Hodgkin & Nunn, 1988; Cobbs, 1991) and would be expected to reduce the modulation of PDE activity induced by a given flash, thereby reducing sensitivity by a constant factor for small responses at any given steady level of cyclic GMP. The extent of this desensitization was estimated from responses to dim flashes, after correction for response compression according to a power law relation (Matthews *et al.* 1990). In low-Ca^{2+}–0 Na^+ solution containing 500 μM IBMX and background light, sensitivity was reduced by a factor of 44 ± 9 ($n = 12$ cells) in comparison with the sensitivity in low-Ca^{2+}–0 Na^+ solution in darkness. This decrease in sensitivity can be interpreted as being due principally, if not entirely, to the competitive inhibition of the PDE by IBMX.

Bleaches also produce a large decrease in sensitivity. However, if the bleach is given in low-Ca^{2+}–0 Na^+ solution (as in Fig. 2*C*), then Ca^{2+}-dependent mechanisms of sensitivity modulation would not be expected to operate. In these circumstances, the only decrease in sensitivity produced by the bleach should be that due to loss of quantum catch and exposure to IBMX. This possibility was tested by determining the sensitivity before and after a bleach while $[Ca^{2+}]_i$ was held near the dark-adapted level (protocols DG and BI). In the seven cells for which the same concentration of 500 μM IBMX was used, sensitivity decreased by a factor of 82 ± 18 after the bleaching of 38–46% of the photopigment. To correct for loss in quantal catch, the sensitivity of each cone was divided by the fraction of pigment remaining. The resulting bleach-corrected sensitivity decreased by a factor of 47 ± 10 in comparison with the sensitivity in low-Ca^{2+}–0 Na^+ solution in darkness. This value is not significantly different from the reduction in sensitivity induced by this concentration of IBMX in background light (Student's *t* test, $t = 0{\cdot}2$). This result is consistent with the notion that when $[Ca^{2+}]_i$ is maintained at its dark-adapted level, the change in sensitivity following bleaching can be completely accounted for by the loss in quantal catch and the inhibitory actions of IBMX.

DISCUSSION

There is considerable evidence that changes in $[Ca^{2+}]_i$ are necessary for background adaptation in both rods and cones (Matthews *et al.* 1988, 1990; Nakatani & Yau, 1988; Fain *et al.* 1989). We have shown in the present study that, for the red-sensitive cones of the salamander, changes in $[Ca^{2+}]_i$ are also necessary for adaptation after bleaches. If the change in $[Ca^{2+}]_i$ is minimized by exposing the outer segment to low-Ca^{2+}–0 Na^+ solution, thereby maintaining $[Ca^{2+}]_i$ near the dark-adapted level, the adaptational changes in the kinetics and sensitivity of the response do not occur, regardless of whether the cone is subsequently exposed to backgrounds or to bleaching light. These experiments indicate that the main mechanisms of adaptation to light require a change in $[Ca^{2+}]_i$ and do not occur if this change is prevented from taking place.

This interpretation rests upon several assumptions. First, we suppose that in low-Ca^{2+}–0 Na^+ solution any remaining changes in $[Ca^{2+}]_i$ induced by illumination are likely to be small. Although actual measurements of $[Ca^{2+}]_i$ under these conditions have not yet been made, there is considerable evidence in support of the stability of $[Ca^{2+}]_i$ (Fain *et al.* 1989; Matthews *et al.* 1990). In particular, the circulating current is relatively stable in a salamander cone for periods of up to 5 s in low-Ca^{2+}–0 Na^+ solution even in darkness, and it is stable in this solution for a considerably longer period in background light or after bleaches. This would seem unlikely to be the case if $[Ca^{2+}]_i$ were changing.

We also assume that the circulating current reflects the value of the cyclic GMP concentration in the outer segment. The reason this assumption is important is that we have used IBMX to return the circulating current to near its dark level in low-Ca^{2+}–0 Na^+ solution, in order to make

explicit comparisons between the kinetics of the light response in darkness and after bleaches (Fig. 4*A*) or in background light (Fig. 5). These comparisons would be less readily interpretable if the cyclic GMP concentrations were different in the two cases, perhaps as the result of some non-specific effect of IBMX on membrane conductances.

Finally, we assume that IBMX reduced photoreceptor sensitivity by a constant factor under the conditions of these experiments. As indicated above, the restoration of approximately the normal dark current by IBMX suggests that we were successful in counteracting the background or bleach-induced increase in PDE activation, and returning the turnover of cyclic GMP to approximately its normal level in darkness. If IBMX acts as a simple competitive inhibitor and the cyclic GMP concentration was restored to about its normal value in darkness, then the modulation in PDE activity induced by a dim flash should be reduced by a constant factor. This would lead to a constant decrease in sensitivity, irrespective of the degree of activation of earlier stages in the transduction cascade.

These experiments show that adaptation produced either by background light or by bleaches is affected similarly by preventing changes in $[Ca^{2+}]_i$. In both cases, the changes in kinetics and sensitivity which normally accompany adaptation are abolished. This would suggest that the mechanism of adaptation is probably largely the same in the two cases. Both backgrounds and bleaches produce an activation of guanylyl cyclase in salamander cones (Cornwall *et al.* 1995) and the photoreceptor cyclase is known to be regulated by $[Ca^{2+}]_i$ (Koch & Stryer, 1988). Furthermore, both kinds of adaptation result in a maintained decrease in circulating current (Matthews *et al.* 1990; Jones *et al.* 1993), which would be expected to lead to a decrease in $[Ca^{2+}]_i$. If in cones as in rods (Cornwall & Fain, 1994) bleached pigment were to activate the transduction cascade to produce an equivalent background, as recent experiments suggest (Cornwall *et al.* 1995), then the mechanism of bleaching adaptation in an isolated photoreceptor may be as follows. The persistent activation of the transduction cascade may produce a maintained decrease in cyclic GMP concentration, which reduces the circulating current and leads to a decrease in $[Ca^{2+}]_i$. The decrease in $[Ca^{2+}]_i$ would then produce both an activation of guanylyl cyclase and also other effects, such as modulation of rhodopsin phosphorylation (Kawamura, 1993), a decrease in the probability of rhodopsin activation (Lagnado & Baylor, 1994) and a change in the affinity of the cyclic GMP-gated channels (Hsu & Molday, 1993), which together may be responsible for alterations in response kinetics and sensitivity. Though subtle differences in the behaviour of the receptors exist in backgrounds or after bleaching (Cornwall *et al.* 1990, 1995; Matthews *et al.* 1990; Cornwall & Fain, 1994), the basic mechanism may be the same in both cases.

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