# Prolongation of Actions of $Ca^{2+}$ Early in Phototransduction by 9-Demethylretinal

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ABSTRACT During adaptation $Ca^{2+}$ acts on a step early in phototransduction, which is normally available for only a brief period after excitation. To investigate the identity of this step, we studied the effect of the light-induced decline in intracellular $Ca^{2+}$ concentration on the response to a bright flash in normal rods, and in rods bleached and regenerated with 11-cis 9-demethylretinal, which forms a photopigment with a prolonged photoactivated lifetime. Changes in cytoplasmic $Ca^{2+}$ were opposed by rapid superfusion of the outer segment with a $0Na^+/0Ca^{2+}$ solution designed to minimize $Ca^{2+}$ fluxes across the surface membrane. After regeneration of a bleached rod with 9-demethlyretinal, the response in Ringer's to a 440-nm bright flash was prolonged in comparison with the unbleached control, and the response remained in saturation for 10–15s. If the dynamic fall in $Ca^{2+}{}_i$ induced by the flash was delayed by stepping the outer segment to $0Na^+/0Ca^{2+}$ solution just before the flash and returning it to Ringer's shortly before recovery, then the response saturation was prolonged further, increasing linearly by 0.41 ± 0.01 of the time spent in this solution. In contrast, even long exposures to $0Na^+/0Ca^{2+}$ solution of rods containing native photopigment evoked only a modest response prolongation on the return to Ringer's. Furthermore, if the rod was preexposed to steady subsaturating light, thereby reducing the cytoplasmic calcium concentration, then the prolongation of the bright flash response evoked by $0Na^+/0Ca^{2+}$ solution was reduced in a graded manner with increasing background intensity. These results indicate that altering the chromophore of rhodopsin prolongs the time course of the $Ca^{2+}$-dependent step early in the transduction cascade so that it dominates response recovery, and suggest that it is associated with photopigment quenching by phosphorylation.

KEY WORDS: retinal rod • calcium • photoreceptor • rhodopsin • adaptation

## INTRODUCTION

It is well established that the cytoplasmic calcium concentration ($Ca^{2+}{}_i$) plays an important role in the modulation of transduction in vertebrate photoreceptors during light and dark adaptation (for reviews see Pugh et al., 1999; Fain et al., 2001). Both biochemical and electrophysiological evidence indicates that $Ca^{2+}$ acts on multiple stages in the transduction mechanism, including a $Ca^{2+}$-sensitive step early in the transduction cascade, which is normally only available for a brief period after light stimulation (Matthews, 1995, 1996, 1997; Murnick and Lamb, 1996; Calvert et al., 1998). These $Ca^{2+}$-dependent processes are accompanied by changes in the waveform and sensitivity of the flash response, which can largely be attributed to the acceleration in the rate of destruction of cyclic GMP during steady illumination (Nikonov et al., 2000; Fain et al., 2001).

Photoreceptor $Ca^{2+}{}_i$ is believed to be governed in darkness by the balance between $Ca^{2+}$ influx through the outer segment conductance (Yau and Nakatani, 1984a; Hodgkin et al., 1985) and $Ca^{2+}$ efflux via $Na^+/Ca^{2+},K^+$ exchange (Yau and Nakatani, 1984b; Hodgkin et al., 1987; Cervetto et al., 1989). When the outer segment conductance is suppressed during the response to a bright flash, $Ca^{2+}$ influx ceases and the level of $Ca^{2+}{}_i$ falls due to its continued extrusion by the exchanger (Yau and Nakatani, 1985; McNaughton et al., 1986; Ratto et al., 1988; Gray-Keller and Detwiler, 1994; McCarthy et al., 1994; Sampath et al., 1998). This dynamic fall in $Ca^{2+}{}_i$ is believed to overlap in time with the $Ca^{2+}$-sensitive step early in phototransduction, serving to accelerate slightly the onset of response recovery from saturation (Matthews, 1996, 1997). If the outer segment is exposed briefly to a $0Na^+/0Ca^{2+}$ solution designed to minimize simultaneously $Ca^{2+}$ influx and efflux (Matthews et al., 1988; Nakatani and Yau, 1988; Fain et al., 1989) just after a bright flash, it is possible to delay the onset of the dynamic fall in $Ca^{2+}{}_i$, thereby reducing the extent of this overlap and retarding the onset of recovery. Analysis of such data reveals that the $Ca^{2+}$-sensitive step early in transduction takes place with a time constant of ~0.5 s (Matthews, 1997), a value much faster than the longer time constant that normally dominates response recovery (Pepperberg et al., 1992, 1994; Ni-

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konov et al., 1998), which does not depend on $Ca^{2+}_i$ (Lyubarsky et al., 1996; Matthews, 1996).

The molecular basis for the actions of $Ca^{2+}$ early in the transduction cascade is still the subject of some debate (for review see Fain et al., 2001), and several possible mechanisms have been proposed. First, it has been suggested that $Ca^{2+}$ might modulate the catalytic activity of photoisomerized rhodopsin (Lagnado and Baylor, 1994; Jones, 1995). However, the invariance of the early rising phase of the flash response during light adaptation suggests that this process may not play a significant role in the intact rod (Nikonov et al., 2000). Second, $Ca^{2+}$ has been shown to modulate the phosphorylation of photoisomerized rhodopsin (Kawamura and Murakami, 1991; Kawamura, 1993) by regulation of rhodopsin kinase. Phosphorylated rhodopsin is the form that binds arrestin to terminate its ability to activate the transduction cascade (Kuhn, 1978; Wilden et al., 1986; Smith et al., 1994; Xu et al., 1997). The light-dependent fall in $Ca^{2+}_i$ is believed to act, via the withdrawal of inhibition by recoverin, to accelerate rhodopsin phosphorylation by rhodopsin kinase and thereby to decrease the effective lifetime of photoisomerized rhodopsin (Kawamura, 1993; Chen et al., 1995a; Klenchin et al., 1995; Sato and Kawamura, 1997). However, the functional significance of this process remains a matter of debate in the intact rod (Otto-Bruc et al., 1998; Hurley et al., 1999).

To investigate the identity of this $Ca^{2+}$-sensitive step, we studied the effect of delaying the light-induced decline in $Ca^{2+}_i$ after a bright flash in rods that had been bleached and regenerated with 11-cis 9-demethylretinal. This retinal analogue has been shown to form a photopigment that exhibits a prolonged photoactivated lifetime (Corson et al., 1994b) and reduced light-dependent phosphorylation (Palczewski et al., 1994; Morrison et al., 1995). The results indicate that by altering the chromophore of rhodopsin, we have prolonged the time course of this $Ca^{2+}$-dependent step involved in adaptation so that it now dominates response recovery. Preliminary results of this study have been presented to the Physiological Society (Matthews et al., 2000b) and the annual meeting of the Association for Research in Vision and Ophthalmology (Matthews et al., 2000a).

## MATERIALS AND METHODS

### *Preparation*

Details of the preparation, recording techniques, light stimuli, and solution changes have been described previously (Matthews, 1995). Suction pipette recordings were made from rod photoreceptors isolated mechanically under infrared illumination from the dark-adapted retina of the larval tiger salamander, *Ambystoma tigrinum*, which were killed by decapitation and double pithing under Home Office license. The inner segment of an isolated rod was drawn into the suction pipette, leaving the outer segment exposed to the superfusing solution. Rapid solution changes were effected by translating the boundary between two flowing streams of solution across the exposed outer segment using a computer-controlled stepping motor coupled to the microscope stage. Recordings were corrected by subtraction of the junction current measured when the same solution changes were performed during intense steady illumination at the end of the experiment, scaled for coincidence of saturating level before and after the solution change. The time of the solution change was taken as the time at which the junction current rose to 50% of its final level. Solution changes from $0Na^+/0Ca^{2+}$ solution to Ringer's were normally complete within ~80 ms, measured as the time for the junction current to rise from 10 to 90% of its final level. All experiments were performed at room temperature (~20°C).

### *External Solutions*

Ringer's solution contained the following: 111 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$ and 3.0 mM HEPES, adjusted to pH 7.7 with NaOH, and also included 10 μM EDTA to chelate impurity heavy metal ions. The Ringer's solution perfusing the recording chamber also included 10 mM glucose. $0Na^+/0Ca^{2+}$ solution was modified from this composition by the equimolar substitution of choline chloride (Sigma-Aldrich) for NaCl, the inclusion of 2 mM EGTA and omission of $CaCl_2$ and $MgCl_2$ to reduce the divalent cation concentration to extremely low levels, the titration of the HEPES buffer with tetramethylammonium hydroxide instead of NaOH, and the omission of EDTA. This solution served to oppose light-induced changes in $Ca^{2+}_i$ by simultaneously minimizing $Ca^{2+}$ influx and efflux (Matthews et al., 1988; Nakatani and Yau, 1988; Fain et al., 1989). The nearly complete removal of external permeant cations was intended to prevent substantial ionic influxes from taking place when $Ca^{2+}_i$ was held low in darkness (Matthews, 1995).

### *Light Stimuli and Electrical Recording*

Light stimuli were delivered from a dual-beam optical stimulator controlled by electromagnetic shutters. The wavelength of stimulation was selected using interference filters (bandwidth 10 nm for 440-nm and 40 nm for 650-nm center wavelength); flash stimuli were of 20 ms duration and unpolarized. Stimulus intensities were adjusted with neutral density filters and measured with a calibrated silicon photodiode (Graseby Optronics). Dark-adapted rods were stimulated with 500 nm light, to excite the native photopigment, which absorbs maximally at 516 nm (Harosi, 1975). Bleaching stimuli at 500 nm delivered $2 \times 10^7$ photons $\mu m^{-2}\ s^{-1}$ for 5 min. This intensity was estimated from the photosensitivity for a vitamin $A_2$–based pigment in solution (Dartnall, 1972), corrected for the difference in dichroism in free solution and in disk membranes (Jones et al., 1993), to be sufficient to bleach in substantial excess of 99% of the visual pigment. Exposures at this level are believed to be sufficient to reduce the initial pigment content below the level of a few percent maintained by pigment resynthesis from the small cellular stores of retinoid (Cocozza and Ostroy, 1987).

The photopigment formed upon regeneration with 9-demethylretinal absorbs maximally at 465 nm (Han et al., 1998). After bleaching and regeneration with 9-demethylretinal, rods were stimulated at 440 or 650 nm to preferentially excite either the analogue pigment or the small proportion of native pigment that remains even after extensive bleaching (Corson et al., 1994a,b). The relative efficacy of these wavelengths in stimulating these two pigments was estimated using a template curve that has been shown to provide a generic description for the α-band of photoreceptor spectral sensitivity curves (Lamb, 1995). This analysis predicts that 650-nm light would be absorbed by the native pigment ~185 times as effectively as by the analogue pigment. However, this value needs to be corrected to take account of the estimated proportion of native pigment remaining after bleaching and the relative efficacy of the two photopigments in exciting transduction. The ana-

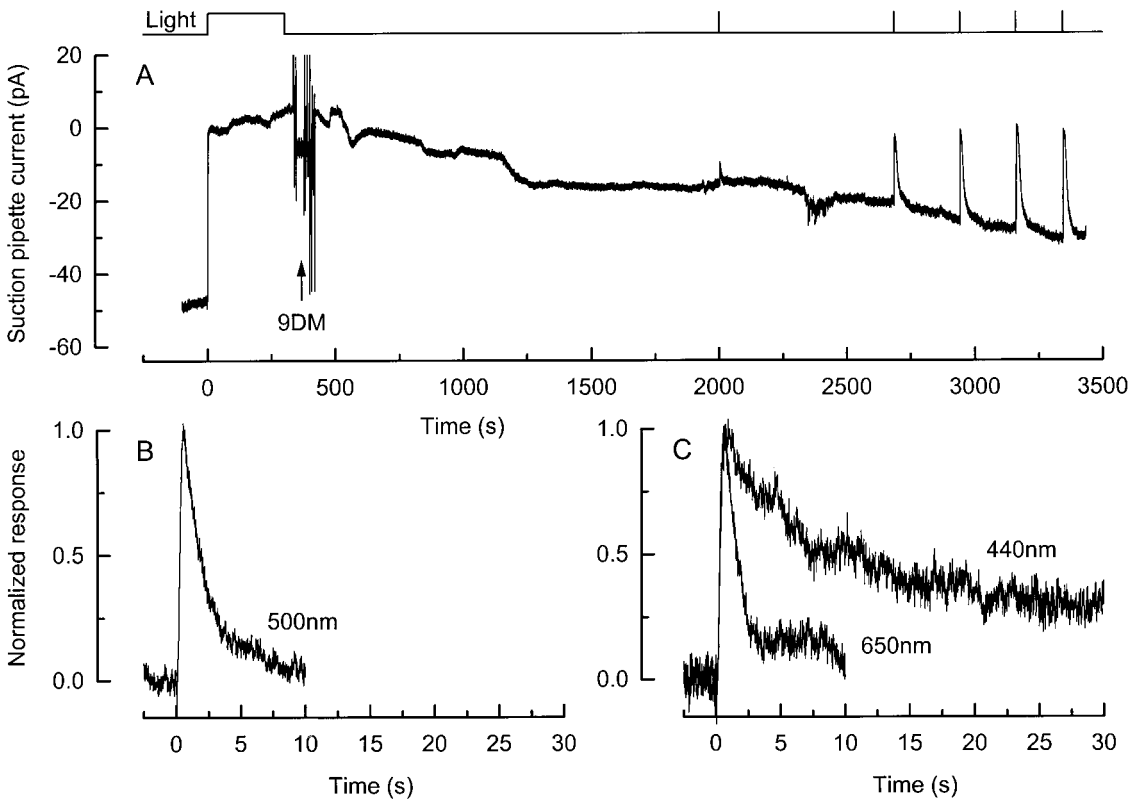


FIGURE 1. Formation of the 9-demethylretinal photopigment within an isolated salamander rod. (A) Continuous record during bleaching and regeneration with 9-demethylretinal. Top trace denotes the light monitor. Bleaching light of 5 min duration delivered $1.85 \times 10^7$ photons $\mu m^{-2} s^{-1}$ at 500 nm; bright flashes were of 20 ms duration and delivered 7,460 photons $\mu m^{-2}$ at 440 nm. Arrow denotes the injection of 200 μl of a suspension of phospholipid vesicles containing 200 μM 9-demethylretinal into the recording chamber. (B and C) Responses to dim flashes delivered before (B) and after (C) bleaching/regeneration and normalized according to their peak amplitude. (B) dim flashes delivered 1.03 photons $\mu m^{-2}$ at 500 nm; trace is the average of 10 responses. (C) Dim flashes delivered 3,780 photons $\mu m^{-2}$ at 650 nm and 24.6 photons $\mu m^{-2}$ at 440 nm; traces are the averages of 10 and 8 responses, respectively.

logue pigment appears to evoke a quantal response some 30 times smaller than does the native pigment (Corson et al., 1994a). So if even as little as 0.5% of the native pigment remained after bleaching, then 650-nm light would be expected to be ~28 times more effective at stimulating transduction via the native than via the analogue pigment. In contrast, 440-nm light would be predicted to be absorbed a little over twice as effectively by the analogue than by the native pigment. So if even as much as 0.5% of the native pigment remained after bleaching, then 440-nm light would be expected to be at least 15 times as effective at stimulating transduction via the analogue as via the native pigment.

The suction pipette current signal was filtered over the bandwidth DC-20 Hz (Bessel filter), and digitized continuously for subsequent analysis at a sampling rate of 100 Hz using an IBM-compatible microcomputer equipped with an intelligent interface card (Cambridge Research Systems).

### *Regeneration of the Photopigment after Bleaching*

After bleaching, the photopigment was regenerated by the addition to the recording chamber (volume ~200 μl) of 200 μl of a suspension of phospholipid vesicles containing either 11-cis retinal or 11-cis 9-demethylretinal. Experiments with heterologously expressed wild-type opsin (Han et al., 1998) have demonstrated that good pigment regeneration is obtained with 9-demethylretinal. In addition, optical measurements from the intact rod outer segment show that regeneration with this analogue in vivo consumes the vast majority of the available opsin (Corson et al., 1994a). Furthermore, regeneration of bleached salamander rods with 9-demethylretinal largely reverses the bleach-induced loss of quantal response amplitude when the residual native photopigment is stimulated with 640-nm light (Corson et al., 1994a). These observations suggest that little unregenerated opsin can remain to desensitize transduction after regeneration with 9-demethylretinal.

The 9-demethylretinal was synthesized as described previously (Corson et al., 1994a). The material was isolated by HPLC (μ-Porasil column, 2% ethyl acetate/hexane solvent), and the isomeric identity was confirmed by NMR. Samples were stored at −80°C under argon until use. Phospholipid vesicles were prepared from phosphatidylcholine (type V-E; Sigma-Aldrich) by sonication (model Soniprep 150; Sanyo Gallenkamp) as described previously (Cornwall et al., 2000). Vesicles were loaded with chromophore by further sonication in darkness to yield a nominal concentration of 200 μM determined spectrophotometrically. Vesicles solutions were stored in darkness in a refrigerator for up to 3 d until required. The absorption spectrum of the analogue in vesicles was measured directly before each experiment to confirm the purity of the sample.

## RESULTS

The procedure used to form the 9-demethylretinal photopigment within an isolated rod is illustrated in Fig. 1 A. First, the outer segment was exposed for 5 min to intense light calculated to bleach in excess of 99% of the

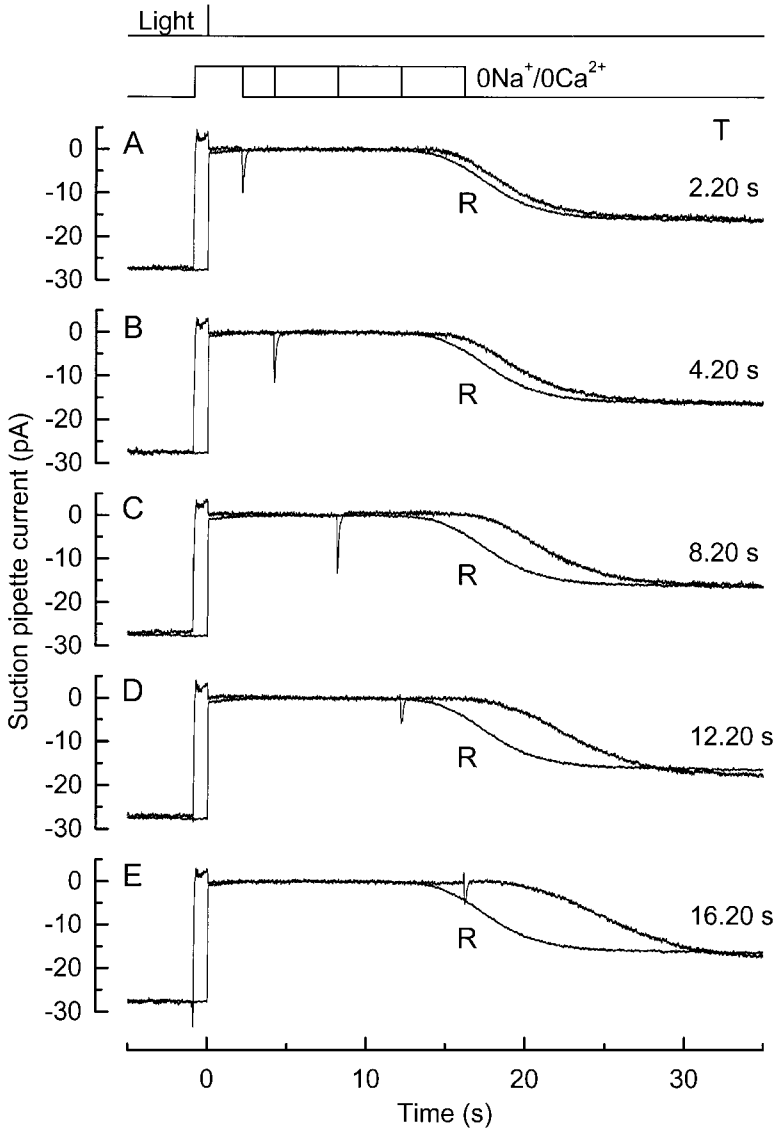


FIGURE 2. Effect on the bright flash response of superfusion with $0Na^+/0Ca^{2+}$ solution in a rod bleached and regenerated with 9-demethylretinal. Superimposed responses to 440-nm bright flashes in Ringer's solution (traces labeled R) and on exposure to $0Na^+/0Ca^{2+}$ solution from 1 s before the flash until the times (T) after the flash indicated beside each trace, measured from the half-relaxation time of the junction current. Traces are the average of five responses in Ringer's solution and two responses in $0Na^+/0Ca^{2+}$ solution; measurements were bracketed symmetrically in time. Junction currents obtained during saturating light at the end of the experiment have been subtracted from the traces in $0Na^+/0Ca^{2+}$ solution. Bright flash delivered $1.58 \times 10^5$ photons $\mu m^{-2}$ at 440 nm to stimulate preferentially the analogue photopigment.

photopigment. This resulted in the rapid and complete suppression of the circulating current recorded by the suction pipette. Next, a suspension of phospholipid vesicles containing 9-demethylretinal was introduced into the recording chamber, leading to the gradual recovery of the circulating current over the next 40 min as the analogue photopigment was formed (Corson et al., 1994a). Finally, the rod was stimulated with a sequence of dim test flashes at short or long wavelength (averaged responses shown in Fig. 1, B and C).

After regeneration with 9-demethylretinal, the light response was no longer spectrally univariant (Corson et al., 1994a,b). When stimulated with 650-nm light (Fig. 1 C, 650 nm), the kinetics of the response to a dim flash were comparable to those recorded under control conditions before the cell was bleached and regenerated (Fig. 1 B). This reflects absorption by the small fraction of native rhodopsin, which remains even after extensive bleaching, and via which this long wavelength stimulus would be expected to excite transduction considerably more effectively than via the analogue photopigment (see MATERIALS AND METHODS). In contrast, when the rod was stimulated instead at 440 nm, the kinetics of the dim flash response were greatly prolonged (Fig. 1 C, 440 nm). Since this short wavelength would be expected to excite transduction more effectively via the analogue than via the native photopigment (see MATERIALS AND METHODS), these prolonged responses can be attributed to the formation of the analogue photopigment (Corson et al., 1994b).

In cells regenerated with 9-demethylretinal, the final recovery of responses to bright flashes (as used in later figures) at 440 nm took place extremely slowly, requiring 5 min or more for the complete return to baseline and necessitating a similarly long interval between trials. Due to the considerably greater efficacy of this short wave-

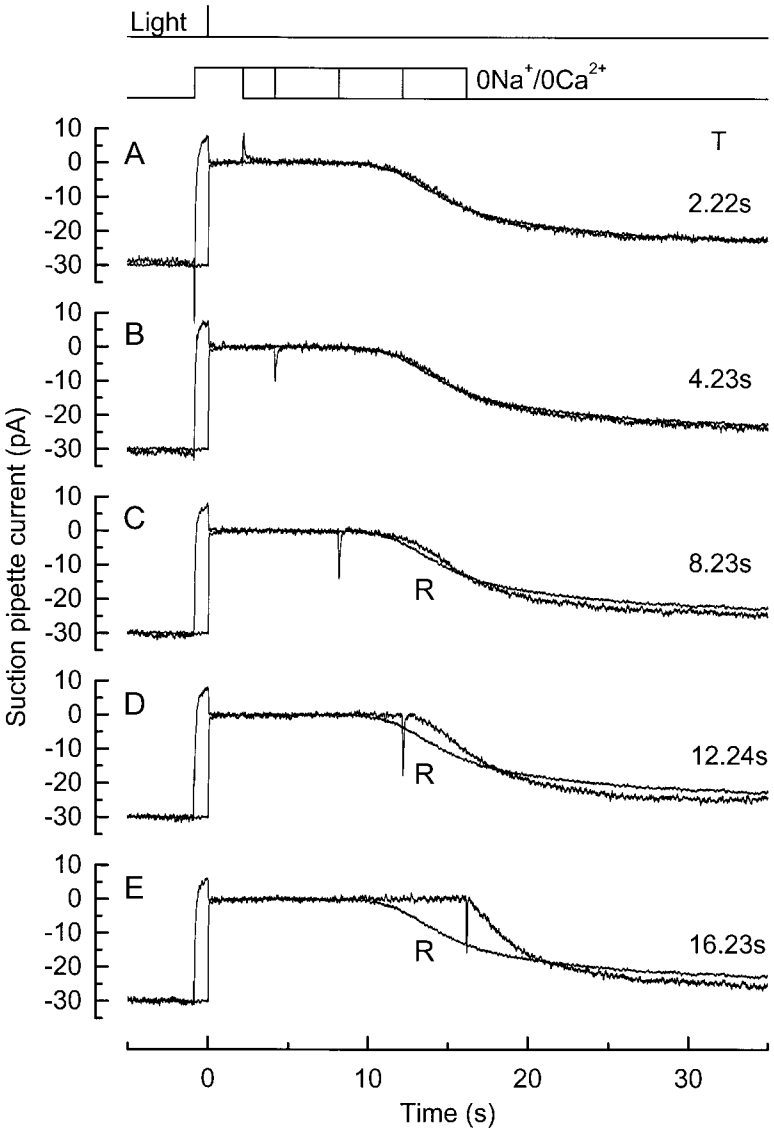


FIGURE 3. Effect on the bright flash response of superfusion with $0Na^+/0Ca^{2+}$ solution in a rod bleached and regenerated with 11-cis-retinal. Superimposed responses to 500-nm bright flashes in Ringer's solution (traces labeled R) and on exposure to $0Na^+/0Ca^{2+}$ solution from 1 s before the flash until the times (T) after the flash indicated beside each trace, measured from the half-relaxation time of the junction current. Traces are the average of five responses in Ringer's solution and two responses in $0Na^+/0Ca^{2+}$ solution; measurements were bracketed symmetrically in time. Junction currents obtained during saturating light at the end of the experiment have been subtracted from the traces in $0Na^+/0Ca^{2+}$ solution. Bright flash delivered $7.14 \times 10^4$ photons $\mu m^{-2}$ at 500 nm.

length in stimulating transduction via the analogue than via the native pigment and the much more rapid kinetics of the response evoked by the native pigment, the recovery from saturation of the response to a 440-nm bright flash seems likely to have been dominated by the quenching of the analogue photopigment. However, the responses to very bright flashes at 650 nm also often exhibited a slow final component of recovery. This observation suggests that, even at this unfavorably long wavelength, enough of the analogue photopigment could be activated by a flash of sufficiently high intensity to contribute to response recovery at later times when the native pigment would have already been quenched.

Fig. 2 examines the effect of stepping the outer segment of a cell which had been bleached and then regenerated with 9-demethylretinal into $0Na^+/0Ca^{2+}$ solution 1 s before a bright flash, and remaining in this solution for a progressively increasing period thereafter. The flash was of wavelength 440 nm, to evoke a response whose recovery from saturation will have been dominated by the excitation of the analogue photopigment. Since most permeant cations have been replaced by the impermeant cation choline, $0Na^+/0Ca^{2+}$ solution supports an inverted dark current that is carried predominantly by $K^+$ (Yau et al., 1981; Matthews, 1995) and that was suppressed completely by the bright flash. This solution is designed to minimize simultaneously both the influx and efflux of $Ca^{2+}$ (Matthews et al., 1988; Nakatani and Yau, 1988), and is believed to maintain $Ca^{2+}_i$ near to its initial value before the solution change for a period of some 10–15 s (Fain et al., 1989; Matthews and Fain, 2001). Therefore, the effect of this manipulation will be to delay the dynamic fall in $Ca^{2+}_i$, which normally commences as soon as the circulating current is suppressed, until the time (T) at which the outer segment was returned to Ringer. As the period in $0Na^+/0Ca^{2+}$ solution

was increased, the response duration became progressively longer in comparison with the response to the same flash in Ringer's (Fig. 2, traces labeled R), corresponding to a prolongation of the time spent in saturation and a displacement of the recovery phase to later times with little change in form. Similar results were obtained from a total of six rods that had been bleached and regenerated with 9-demethylretinal.

This progressive increase in response duration when the dynamic fall in $Ca^{2+}{}_i$ was delayed after regeneration with 9-demethylretinal can be contrasted with the results shown in Fig. 3, which were obtained from a bleached rod regenerated with 11-cis-retinal, using bright flashes of wavelength 500 nm to best stimulate the regenerated rhodopsin. In this case, the recovery of the flash response was little affected by the solution change unless the time spent in $0Na^+/0Ca^{2+}$ solution exceeded the normal duration of the flash response in Ringer's (Fig. 3, traces labeled R). Similarly, modest effects of exposure to $0Na^+/0Ca^{2+}$ solution were obtained from a second bleached rod regenerated with 11-cis-retinal and also from five unbleached dark-adapted rods. These results are directly comparable to those obtained previously from dark-adapted rods using a rather lower flash intensity (Matthews, 1996). Therefore, we conclude that the response prolongation observed in $0Na^+/0Ca^{2+}$ solution after bleaching and regeneration with 9-demethylretinal results from the formation of the analogue photopigment and not from the process of bleaching and regeneration per se, or from the presence of any residual all trans retinal released during bleaching within the outer segment.

These results are quantified in Fig. 4 by measuring the time taken after the flash for the response to recover 25% of the original dark current in Ringer's. The prolongation of the response after exposure to $0Na^+/0Ca^{2+}$ relative to the response to the same flash in Ringer's is plotted in Fig. 4 A as a function of the time spent in $0Na^+/0Ca^{2+}$ solution after the flash. In each case, these data have been normalized according to the time for 25% response recovery for the same cell in Ringer's. In unbleached control cells (open symbols) and in bleached cells regenerated with 11-cis-retinal (half-closed symbols), exposures to $0Na^+/0Ca^{2+}$ solution, which ended before the response in Ringer's, would normally have recovered had only a modest effect on the duration of the response. Even when the exposure extended beyond the normal time for 25% recovery in Ringer's, the response was prolonged by little more than the additional time spent in $0Na^+/0Ca^{2+}$ solution after the flash. These results are comparable to the modest slowing of the response that has been observed previously in dark-adapted rods upon delaying the dynamic fall in $Ca^{2+}{}_i$ after a dimmer but still saturating flash (see Fig. 4 B of Matthews, 1996). In contrast, in bleached cells regenerated with 9-demethylretinal (Fig. 4, closed symbols) the duration of the response increased in proportion to the time spent in $0Na^+/0Ca^{2+}$ solution after the flash.

Fig. 4 B shows mean data from the same cells plotted in absolute coordinates. In unbleached cells (open symbols) exposure to $0Na^+/0Ca^{2+}$ solution resulted in only a modest retardation of response recovery. However, in bleached cells regenerated with 9-demethylretinal (closed symbols), the mean duration of the response increased linearly with the time spent in $0Na^+/0Ca^{2+}$ solution after the flash, and could be well fitted by a regression line of slope $0.41 \pm 0.01$ over the full range of exposure durations. This observation indicates that, after regeneration with 9-demethylretinal, recovery remains $Ca^{2+}$-sensitive for the entire duration of the response, suggesting that the $Ca^{2+}$-sensitive step early in transduction has been prolonged to such an extent that it now dominates response recovery. If this were the case, then it would imply that response recovery after excitation of the analogue photopigment should vary in a graded manner with $Ca^{2+}{}_i$. This possibility was addressed by using steady light to reduce $Ca^{2+}{}_i$, and then examining the degree of response prolongation evoked by $0Na^+/0Ca^{2+}$ solution in a bleached cell regenerated with 9-demethylretinal.

Results from such an experiment are illustrated in Fig. 5. First, the cell was exposed to subsaturating steady illumination in Ringer's for 14 s to reduce $Ca^{2+}{}_i$ from its value in darkness to a light-adapted level that will have varied in a graded manner with the proportion of current suppressed (Younger et al., 1996; Sampath et al., 1999). The steady background light was of wavelength 650 nm, to stimulate maximally the residual native photopigment while evoking as little long-lived excitation as possible from the analogue photopigment. Next, the outer segment was stepped into $0Na^+/0Ca^{2+}$ solution, and 1 s later the background was extinguished and a bright flash of wavelength 440 nm was delivered simultaneously to evoke a response whose recovery from saturation will have been dominated by excitation of the analogue photopigment. Finally, after an interval of $\sim$12 s, the outer segment was returned to Ringer's before the onset of response recovery. This procedure was intended to hold $Ca^{2+}{}_i$ at a reduced level for a fixed period during the period of response saturation. These traces are compared with responses to the same illumination sequence for which the outer segment remained in Ringer's throughout. In the absence of prior background illumination (Fig. 5 A), this procedure resulted in a substantial prolongation of the bright flash response in comparison with the response in Ringer's (Fig. 5, trace labeled R) as in previous figures. However, as the background intensity was increased (Fig. 5, B and C), the response was prolonged by a progressively

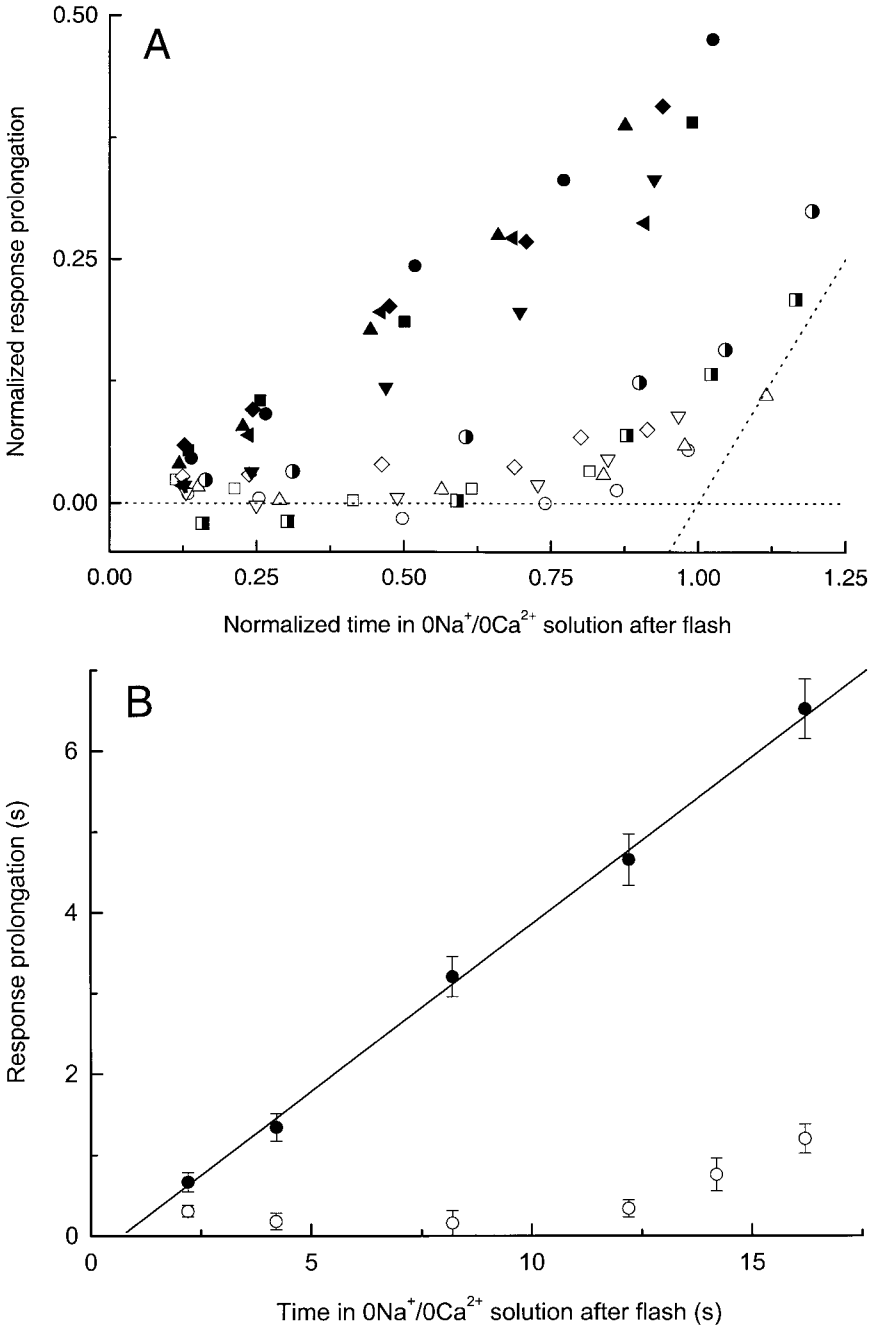


FIGURE 4. Dependence of response duration on the time spent in $0Na^+/0Ca^{2+}$ solution after a bright flash. Flashes presented in darkness as in Figs. 2 and 3. Response prolongation was calculated as the difference between the time taken after the flash for the response to recover 25% of the original dark current in Ringer's and after exposure to $0Na^+/0Ca^{2+}$ solution. (A) Closed symbols indicate cells bleached and regenerated with 9-demethylretinal (six rods; cell of Fig. 2 denoted by closed diamonds); open symbols indicate unbleached control cells (five rods); half-closed symbols indicate cells bleached and regenerated with 11-cis-retinal (two rods; cell of Fig. 3 denoted by half-closed circles). Data have been normalized for each cell according to the time for 25% recovery in Ringer's. Interrupted lines represent zero response prolongation and prolongation in direct proportion to the time spent in $0Na^+/0Ca^{2+}$ solution beyond the normal time of response recovery, respectively. (B) Mean data from six cells bleached and regenerated with 9-demethylretinal (closed symbols) and five unbleached control cells (open symbols). Error bars represent SEM. Regression line of slope 0.41 ± 0.01 fitted using a weighted least-squares algorithm.

shorter period until, for near-saturating light (Fig. 5 D), the degree of response prolongation was very small.

Results from a total of six such experiments are collected in Fig. 6, in which the degree of response prolongation is plotted as a function of the circulating current remaining for each background intensity at the time of the step to $0Na^+/0Ca^{2+}$ solution. Since $Ca^{2+}{}_i$ has been shown to vary progressively with the circulating current (Younger et al., 1996; Sampath et al., 1999), its magnitude provides an indirect estimate of the relative level of $Ca^{2+}{}_i$ in each case. The degree of response prolongation increased approximately linearly with increasing circulating current, and was well fitted by a regression line of constant slope. This observation is consistent with the notion that the time constant that dominates response recovery after excitation of the analogue photopigment varies in a graded manner with $Ca^{2+}{}_i$, in contrast to the situation under control conditions.

However, the data also admit to the alternative interpretation that, instead of being governed by the level of $Ca^{2+}{}_i$ during the initial part of the response while the outer segment remained in $0Na^+/0Ca^{2+}$ solution, the absolute prolongation of the response to this flash of fixed intensity might depend instead upon its duration,

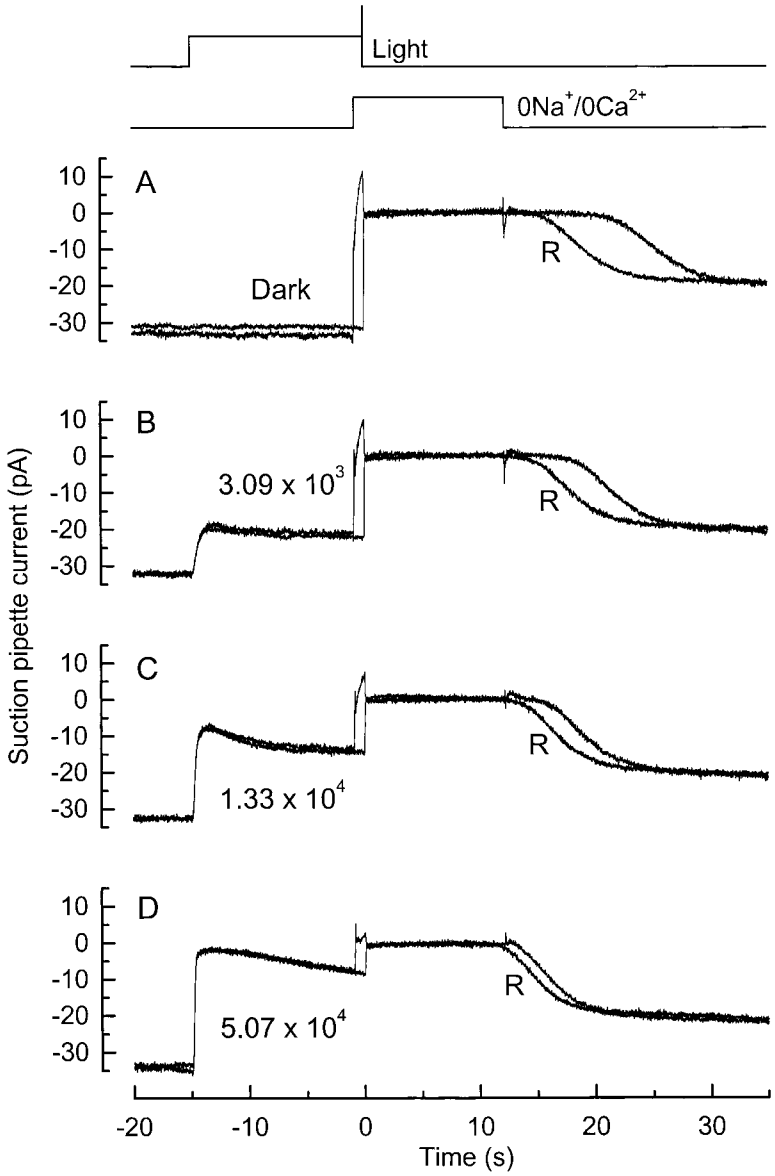


FIGURE 5. Effect of previous steady illumination in Ringer's on the prolongation of the bright flash response by exposure to $0Na^+/0Ca^{2+}$ solution in a rod bleached and regenerated with 9-demethylretinal. Superimposed responses to 440-nm bright flashes in Ringer's solution (traces labeled R) and on superfusion with $0Na^+/0Ca^{2+}$ solution. The rod was exposed for 15 s to 650-nm steady light of the intensity indicated beside each trace (photons $\mu m^{-2}$ $s^{-1}$), and then a bright flash delivering $1.37 \times 10^5$ photons $\mu m^{-2}$ at 440 nm was presented and the background was extinguished. The solution superfusing the outer segment was rapidly changed from Ringer's to $0Na^+/0Ca^{2+}$ solution 1 s before the flash, and then returned to Ringer's 12 s after the flash. Each trace is the average of two responses; measurements in Ringer's solution and $0Na^+/0Ca^{2+}$ solution were bracketed symmetrically in time. Junction currents obtained during saturating light at the end of the experiment have been subtracted from the traces in $0Na^+/0Ca^{2+}$ solution. Top traces denote light and solution change monitors.

which also was reduced progressively during adaptation as the background intensity increased. This possibility was investigated by examining (Fig. 7) the response prolongation evoked by a constant duration exposure to $0Na^+/0Ca^{2+}$ solution of a bleached cell regenerated with 9-demethylretinal for flashes of differing intensity. The almost 20-fold increase in flash intensity in Fig. 7 (A–C) led to a near doubling in the duration of the flash response, together with progressive changes in the waveform of response recovery. Nevertheless, comparison of these responses reveals that exposure to $0Na^+/0Ca^{2+}$ solution evoked a near-constant prolongation for the recovery to a criterion level relative to that in Ringer's (Fig. 7, traces labeled R) irrespective of the absolute response duration or flash intensity.

Results from three such experiments are collected in Fig. 8, in which is plotted the time for 25% recovery of the original dark current after exposure to in $0Na^+/0Ca^{2+}$ solution against the time for 25% recovery for the same flash in Ringer's. These points fall on a straight line that is nearly parallel to the line of unit slope. This observation indicates that exposure to $0Na^+/0Ca^{2+}$ solution for a fixed period evokes a near-constant delay in response recovery, irrespective of the intensity of the flash. This observation supports the notion that the graded variation in response prolongation by $0Na^+/0Ca^{2+}$ solution that was observed when $Ca^{2+}_i$ was manipulated with steady light resulted from a graded variation in the time constant governing response recovery after stimulation of the analogue photopigment. The slight deviation from unit slope may re-

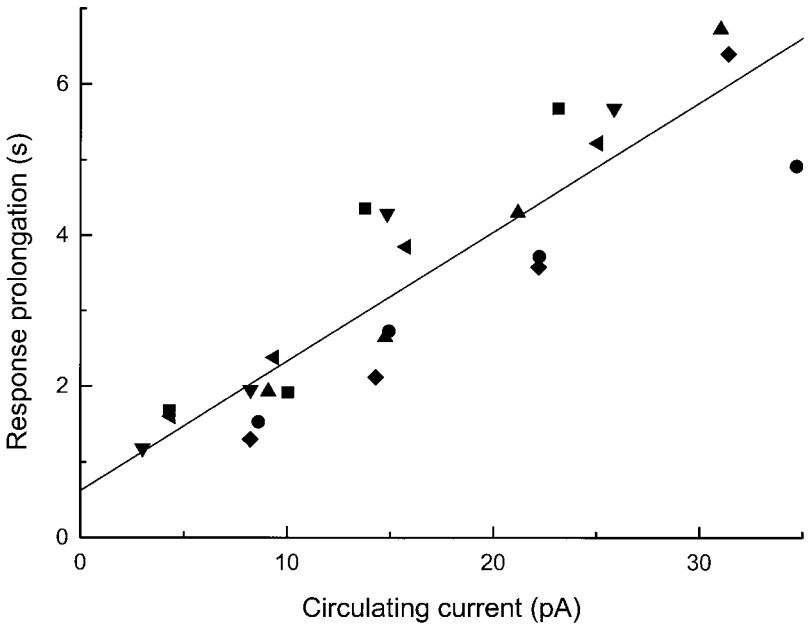


FIGURE 6. Dependence of response prolongation by $0Na^+/0Ca^{2+}$ solution on the circulating current during previous steady illumination. Data obtained according to the procedure of Fig. 5 from six rods bleached and regenerated with 9-demethylretinal. Circulating current was measured from the paired traces in Ringer's over the 1-s period immediately preceding the flash. Response prolongation was calculated as the difference between the time taken after the flash for the response to recover 25% of the original dark current in Ringer's and after exposure to $0Na^+/0Ca^{2+}$ solution. Cell of Fig. 5 denoted by closed diamonds. Regression line fitted to the data using a least-squares algorithm.

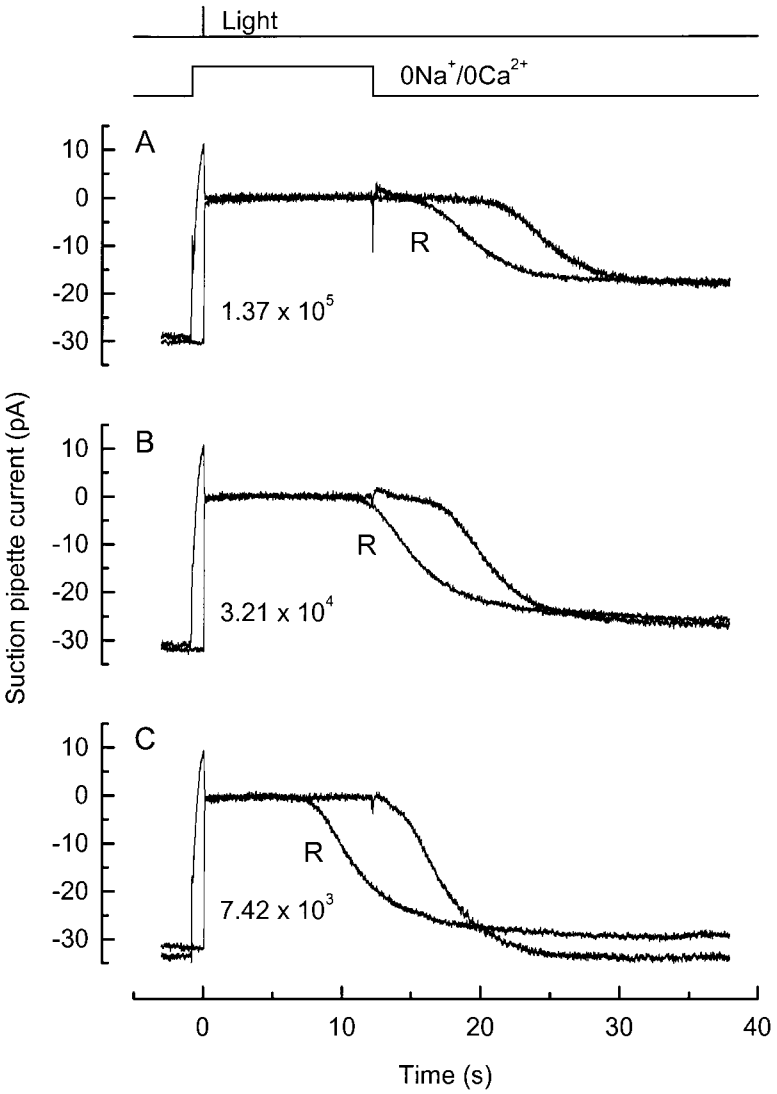


FIGURE 7. Effect of varying the intensity of the bright flash on the response prolongation evoked by superfusion with $0Na^+/0Ca^{2+}$ solution in a rod bleached and regenerated with 9-demethylretinal. Superimposed responses to bright flashes in Ringer's solution (traces labeled R) and on exposure to $0Na^+/0Ca^{2+}$ solution from 1 s before until 12 s after the flash. Each trace is the average of two responses; measurements in Ringer's solution and $0Na^+/0Ca^{2+}$ solution were bracketed symmetrically in time. Junction currents obtained during saturating light at the end of the experiment have been subtracted from the traces in $0Na^+/0Ca^{2+}$ solution. Bright flash delivered (A) $1.37 \times 10^5$, (B) $3.2 \times 10^4$, and (C) $7.42 \times 10^3$ photons $\mu m^{-2}$ at 440 nm to stimulate preferentially the analogue photopigment. Same cell as Fig. 5.

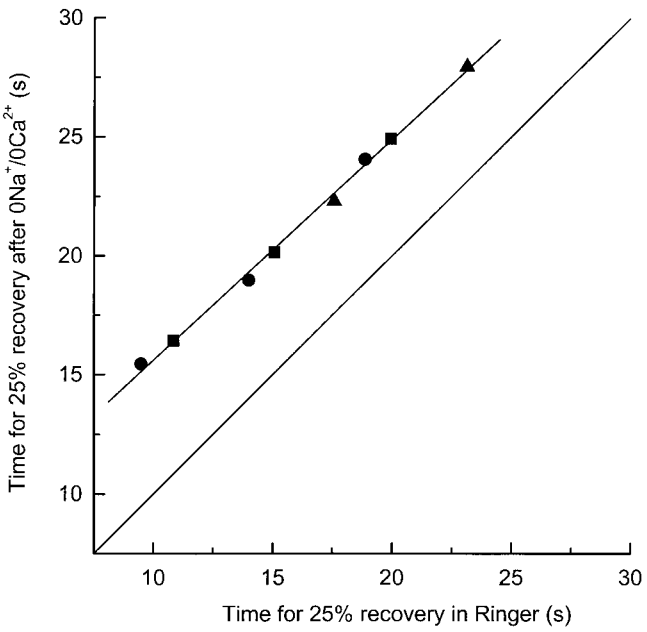


FIGURE 8. Relationship between response duration in Ringer's and after exposure to $0Na^+/0Ca^{2+}$ solution for flashes of different intensities. Data obtained according to the procedure of Fig. 7 from three rods bleached and regenerated with 9-demethylretinal. Response duration was measured as the time taken after the flash for the response to recover 25% of the original dark current in Ringer's. Cell of Fig. 7 denoted by closed circles. Regression line of slope 0.93 ± 0.02 fitted to the data using a least-squares algorithm.

flect the progressive departure from translational invariance of the recovery waveforms in Ringer's as the flash intensity was increased (Lyubarsky et al., 1996).

## DISCUSSION

### *Prolongation of $Ca^{2+}$ Sensitivity by 9-Demethylretinal*

In rods bleached and regenerated with 9-demethylretinal, the recovery of the response to a bright flash was found to depend strongly on the timing of the associated changes in $Ca^{2+}_i$. When the fall in $Ca^{2+}_i$, which accompanies the response to a bright flash, was delayed by progressively longer exposures to $0Na^+/0Ca^{2+}$ solution, the response was prolonged in a graded manner throughout its entire duration (Figs. 2 and 4). This contrasts with the much more modest effect of delaying the light-induced fall in $Ca^{2+}_i$ under control conditions (Matthews, 1996) or in cells bleached and regenerated with 11-cis-retinal (Fig. 3), for which exposure to $0Na^+/0Ca^{2+}$ solution is only effective in prolonging recovery in Ringer's during a brief period immediately after the flash (Matthews, 1997).

These results indicate that as well as retarding the recovery of the flash response (Corson et al., 1994b), excitation of the analogue photopigment formed by regeneration with 9-demethylretinal also greatly prolongs the period during which response recovery remains sensitive to $Ca^{2+}_i$ after a flash. Under control conditions, the $Ca^{2+}$-sensitive step early in phototransduction is normally removed with a time constant of ~0.5 s (Matthews, 1997). Unless one were to make the rather unlikely assumption that regeneration with 9-demethylretinal somehow introduces a novel $Ca^{2+}$-dependent process not present in the normal rod, one is forced to conclude that the $Ca^{2+}$-sensitive step early in phototransduction must remain available for an extended period after excitation of the analogue photopigment 9-demethylrhodopsin. Consequently, this normally rapid $Ca^{2+}$-sensitive step leapfrogs over the $Ca^{2+}$-insensitive time constant that normally governs response recovery (Lyubarsky et al., 1996; Matthews, 1996) so that it dominates recovery instead. This conclusion is reinforced by the ability of an earlier reduction in $Ca^{2+}_i$ by steady illumination in Ringer's to reduce the prolongation of the response by a subsequent exposure to $0Na^+/0Ca^{2+}$ solution in a graded manner (Figs. 5 and 6). This result is most simply explained if the time constant of this now dominant process is itself modulated by $Ca^{2+}$. Consequently, it would appear that regeneration with 9-demethylretinal prolongs the time constant of the $Ca^{2+}$-sensitive step itself rather than that of some other process upstream of it in the transduction cascade.

### *Quantitative Description of the Actions of $Ca^{2+}$ Early in Transduction*

The extent to which this now dominant time constant is modulated by $Ca^{2+}$ in rods regenerated with 9-demethylretinal can be estimated from the relationship between the time spent in $0Na^+/0Ca^{2+}$ solution and the prolongation of the bright flash response (Fig. 4 B). A conceptual model of this process is illustrated in Fig. 9. Suppose that the time constant that governs the exponential decay of some $Ca^{2+}$-sensitive intermediate early in the transduction cascade has been prolonged by 9-demethylretinal to such a degree that it now dominates the kinetics of response recovery. In Ringer's solution, $Ca^{2+}_i$ would fall rapidly after the flash, so that this intermediate would experience a greatly lowered $Ca^{2+}$ concentration for virtually the entire duration of the response, and, therefore, would decay rapidly (Fig. 9, heavy trace, time constant $\tau_f$). If instead the outer segment were exposed to $0Na^+/0Ca^{2+}$ solution for a period (T) after the flash, then $Ca^{2+}_i$ would remain near to its initial value in darkness and this intermediate would decay more slowly (Fig. 9, light trace, time constant $\tau_s$). However, once the outer segment was returned to Ringer's solution $Ca^{2+}_i$ would fall rapidly and the decay would accelerate to match that in the absence of the solution change. In reality, $Ca^{2+}_i$ would not fall instantaneously, but if it declined with similar kinetics in Ringer's irrespective of whether the flash was delivered in Ringer's or in $0Na^+/0Ca^{2+}$ solution, then the

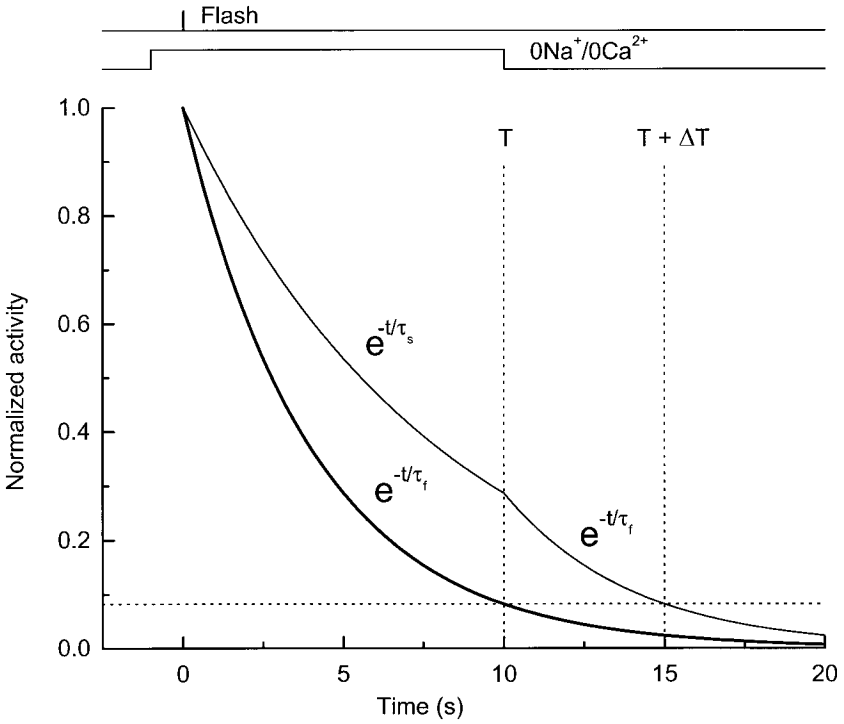


FIGURE 9. Conceptual model for the decay of a hypothetical $Ca^{2+}$-sensitive intermediate in a bleached rod regenerated with 9-demethylretinal. Protocol as in Fig. 2; top traces denote light and solution monitors. Heavy trace represents outer segment in Ringer's solution throughout; activity of intermediate decays with single exponential kinetics of time constant $\tau_f$ (taken as 4 s; Corson et al., 1994b). Light trace represents outer segment stepped to $0Na^+/0Ca^{2+}$ solution before the flash and then returned to Ringer's solution at time T. The decay of intermediate activity initially proceeds with time constant $\tau_s$ (taken as 8 s, twice the value when $Ca^{2+}_i$ is allowed to fall in Ringer's; see DISCUSSION) while in $0Na^+/0Ca^{2+}$ solution, and then accelerates to $\tau_f$ upon the return to Ringer's. Intermediate activity reaches a common criterion level at time T in Ringer's and time T + ΔT after exposure to $0Na^+/0Ca^{2+}$ solution.

time course of intermediate decay would be likely to be affected in an equivalent manner in both cases.

On the basis of this simple model, the activity of the $Ca^{2+}$-sensitive intermediate would be predicted to recover to the level at the time of the solution change according to Eq. 1 when:

$$e^{-\frac{T}{\tau_f}} = e^{-\frac{T}{\tau_s}} \cdot e^{-\frac{\Delta T}{\tau_f}}, \quad (1)$$

where T is the time spent in $0Na^+/0Ca^{2+}$ solution, and ΔT is the additional delay before the intermediate decays to the level that it would have reached at time T in Ringer's solution. Solving for ΔT:

$$\Delta T = T \cdot \left(1 - \frac{\tau_f}{\tau_s}\right). \quad (2)$$

Eq. 2 predicts that the additional delay before recovery to a criterion level should increase in direct proportion to the time spent in $0Na^+/0Ca^{2+}$ solution after the flash, as observed in Fig. 4. The precise criterion level selected is unimportant, due to the translational invariance of both the exponential curves of the model and the recovery phase of the bright flash responses in Ringer's and $0Na^+/0Ca^{2+}$ solution. The slope of 0.41 for the regression line fitted to the data of Fig. 4 B yields a value for the ratio of the fully speeded ($\tau_f$) to the resting ($\tau_s$) time constants of 0.59, implying that the dynamic reduction of $Ca^{2+}_i$ from its dark-adapted value during the flash response can speed this $Ca^{2+}$-sensitive step by a factor of 1.7 for this analogue photopigment.

*Site of Action of $Ca^{2+}$ Early in Transduction*

When a bleached rod is exposed to 9-demethylretinal, an analogue photopigment is formed with modified spectral sensitivity and a prolonged photoactivated lifetime (Corson et al., 1994a,b). It seems unlikely that 9-demethylretinal exerts any other effects on the transduction mechanism, since when the residual native pigment remaining within the outer segment is preferentially stimulated by dim flashes of long wavelength light, responses with normal recovery kinetics are observed (Fig. 1 C; Corson et al., 1994b). The prolonged period of $Ca^{2+}$ sensitivity that we observe in bleached rods regenerated with 9-demethylretinal, therefore, appears to be associated with the extended lifetime of this analogue photopigment, thereby implicating the quenching of this photoactivated state as the $Ca^{2+}$-sensitive step early in phototransduction. It is important to note that this conclusion does not depend upon the precise details of the mechanism by which this quenching process might take place, but simply upon the observation that the functional lifetime of the photopigment and the period of sensitivity to $Ca^{2+}$ are both prolonged by the analogue.

How might the extended functional lifetime of the photopigment formed with 9-demethylretinal arise? One possible solution to this question is provided by the recent demonstration that when 9-demethylrhodopsin is excited by light, the equilibrium between the meta-I and meta-II forms of the analogue photopigment overwhelmingly favors the meta-I form (Meyer et

al., 2000; Vogel et al., 2000). This imbalance would produce what is essentially a buffer system for the meta-II form. As meta-II is removed by phosphorylation and arrestin binding, it would be continually replenished from the much larger pool of meta-I. The progressive displacement of the equilibrium between the two forms would serve to buffer the level of meta-II, and thereby to prolong the activation of transduction for any given rate constant of analogue photopigment quenching. In addition, the kinetics of receptor activation have been shown to be slowed by two orders of magnitude when compared with those of the native pigment (Vogel et al., 2000). Either of these processes would prolong the period for which meta-II is available to excite the transduction cascade after a bright flash.

A second possibility is that the prolonged functional lifetime of the analogue photopigment might reflect differences in the quenching of its active form when compared with native rhodopsin. For example, the photopigment formed with 9-demethylretinal exhibits reduced light-dependent phosphorylation when compared with the native photopigment (Palczewski et al., 1994; Morrison et al., 1995). Multiple phosphorylation of photoisomerized rhodopsin is a necessary prerequisite for its quenching by arrestin (Kuhn, 1978; Wilden et al., 1986; Smith et al., 1994; Xu et al., 1997), truncation of the COOH-terminal, or deletion of the phosphorylation sites leading to prolonged light responses (Chen et al., 1995b; Mendez et al., 2000). It is also conceivable that phosphorylated 9-demethylrhodopsin might provide a poor substrate for arrestin binding, thereby prolonging the duration of excitation.

Irrespective of whether one or both of these possibilities applies, the prolongation of the period of $Ca^{2+}$ sensitivity after a flash and the modulation of the extent of this prolongation by $Ca^{2+}_i$ indicate that the functional lifetime of the form of the photoactivated analogue pigment directly preceding the $Ca^{2+}$-sensitive quenching step is prolonged. This argues against a crucial role for impaired arrestin binding to phosphorylated 9-demethylrhodopsin, since this process is not believed to be $Ca^{2+}$ dependent. In contrast, the phosphorylation of photoisomerized rhodopsin is believed to depend on $Ca^{2+}_i$ through the actions of recoverin on rhodopsin kinase (Kawamura, 1993; Chen et al., 1995a; Klenchin et al., 1995; Sato and Kawamura, 1997). Therefore rhodopsin phosphorylation constitutes a tempting candidate for the $Ca^{2+}$-sensitive step early in phototransduction (Sagoo and Lagnado, 1997). However, it is not possible from our experiments to discriminate directly between this and other photopigment quenching reactions.

It has been suggested that in addition to modulating the lifetime of an early photoproduct, the light-induced fall in $Ca^{2+}_i$ might also lead to a change in the gain with which photoisomerized rhodopsin activates the transduction cascade (Lagnado and Baylor, 1994), potentially altering the early rising phase of the response during light adaptation (Gray-Keller and Detwiler, 1994; Jones, 1995). Although our study provides a direct demonstration of the modulation of photoactivated pigment lifetime by $Ca^{2+}_i$, it does not address the question of whether the gain of transduction might also be affected. However, the recent demonstration that the rising phase of the flash response appears to be invariant during background illumination suggests that light adaptation does not alter the gain of any of the amplifying steps of phototransduction (Nikonov et al., 2000). Therefore, we conclude that the effect of $Ca^{2+}_i$ on the photoactivated lifetime that we observe here is likely to constitute the principal action of $Ca^{2+}$ early in the transduction cascade.

### *Functional Implications*

The identification of the $Ca^{2+}$-sensitive step early in phototransduction with photopigment quenching has a number of functional implications. First, it resolves the ambiguity in assigning the fast and slow time constants that shape the light response to specific processes (Nikonov et al., 1998, 2000). Our results indicate that the fast time constant can be ascribed to photopigment quenching, confirming and extending the interpretations drawn from the earlier demonstration that a site sensitive to $Ca^{2+}$ normally disappears rapidly after light stimulation (Matthews, 1997). Second, our results provide an estimate for the extent to which 9-demethylretinal photopigment quenching can be modulated by $Ca^{2+}$ in situ (Figs. 4 and 9), suggesting a 1.7-fold change in this time constant over the normal physiological range of $Ca^{2+}_i$. This value is somewhat smaller than that predicted from modeling of changes in the nondominant time constant of the bright flash response during light adaptation (Nikonov et al., 2000), and the changes in response recovery that can be obtained if $Ca^{2+}_i$ is manipulated artificially (Kawamura and Murakami, 1991; Kawamura, 1993). This relatively small modulation may reflect the rather high $K_d$ values relative to the normal $Ca^{2+}_i$ in darkness (Gray-Keller and Detwiler, 1994; McCarthy et al., 1994; Sampath et al., 1998) reported for the effects of $Ca^{2+}$ on recoverin-dependent changes in rhodopsin phosphorylation (Chen et al., 1995a) and prolongation of the bright flash response (Erickson et al., 1998). Alternatively, it is possible that the quenching of the 9-demethylretinal photopigment may be modulated by $Ca^{2+}$ to a lesser extent than is the case for native rhodopsin.

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