Static and dynamic actions of cytoplasmic Ca^{2+} in the adaptation of responses to saturating flashes in salamander rods

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1. In order to study the relative contribution to light adaptation of the various actions of Ca^{2+} in rod photoreceptors, changes in cytoplasmic calcium concentration ($[Ca^{2+}]_i$) were opposed by manipulating the calcium fluxes across the outer segment membrane at different times during the response to a bright flash.

2. When the outer segment was superfused with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution just before a bright flash, the period of response saturation was greatly prolonged. But if instead the solution change was made at progressively increasing times after the flash, the delay before the response recovered from saturation declined exponentially towards its value in Ringer solution with a time constant of around 1 s. In contrast, recovery time was little affected by stepping to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash and returning to Ringer solution shortly before the normal time of recovery from saturation.

3. When a bright flash was delivered just before the extinction of steady light, the response recovered from saturation progressively earlier as this steady intensity was increased. If, instead, the outer segment was transferred to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution just before the bright flash then the time spent in saturation by the response was prolonged in darkness, but this additional delay progressively decreased as the steady intensity increased.

4. These results are consistent with the notion that the light-induced reduction of the time spent in saturation by the bright flash response in Ringer solution resulted from the *static* decrease in $[Ca^{2+}]_i$ induced by the background, while the additional delay in the recovery from saturation when further changes in $[Ca^{2+}]_i$ were prevented stemmed from the abolition of the *dynamic* fall in $[Ca^{2+}]_i$ during the flash response.

5. Analysis of the effects of steady light on the time spent in saturation by the bright flash response under these conditions suggests that actions of $[Ca^{2+}]_i$ at, or soon after, the time of the flash are largely responsible for the graded changes which take place in the bright flash response during light adaptation, while rapid actions of $[Ca^{2+}]_i$ at the time of response recovery also play a role in the adaptation of the steady response to background light itself.

6. These data have been interpreted in terms of differential actions of $[Ca^{2+}]_i$ on 'early' stages (e.g. events leading to phosphodiesterase activation) and 'late' stages (e.g. guanylyl cyclase) in the transduction mechanism. A quantitative model is presented which suggests that actions of $[Ca^{2+}]_i$ on 'late' stages play a proportionately larger role in background adaptation than actions on 'early' stages.

Light adaptation in rod photoreceptors is thought to be mediated by changes in cytoplasmic calcium concentration ($[Ca^{2+}]_i$). During steady illumination $[Ca^{2+}]_i$ falls (McNaughton, Cervetto & Nunn, 1986; Ratto, Payne, Owen & Tsien, 1987), due to the continued efflux of Ca^{2+} via the sodium–calcium exchange following the suppression of Ca^{2+} influx through the outer segment conductance (Yau & Nakatani, 1985). If this fall in rod $[Ca^{2+}]_i$ is prevented, then adaptation is abolished (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988; Fain, Lamb, Matthews & Murphy, 1989). Furthermore, if $[Ca^{2+}]_i$ is altered in darkness, then at least some of the manifestations of adaptation are produced (Matthews, 1995*a*). The light-induced fall in $[Ca^{2+}]_i$ is therefore believed to induce the changes in response sensitivity and kinetics which constitute light adaptation. Ca^{2+} has been

shown to inhibit the synthesis of cyclic GMP by guanylyl cyclase *in vitro* (Koch & Stryer, 1988); relief of this inhibition as $[Ca^{2+}]_i$ falls leads to the acceleration of the cyclase which takes place during the light response in the intact rod (Hodgkin & Nunn, 1988). Furthermore, Ca^{2+} appears both to prolong the activation of the transduction cascade (Kawamura & Murakami, 1991), and to enhance the formation of catalytically active rhodopsin (Lagnado & Baylor, 1994) in truncated rods, while it has been shown to modulate rhodopsin phosphorylation *in vitro* (Kawamura, 1993). In addition Ca^{2+} has a more minor effect on the affinity of the outer segment conductance for cyclic GMP (Hsu & Molday, 1993).

During steady background illumination the circulating current is reduced, giving rise to a *static* decrease in $[Ca^{2+}]_i$. The transient current suppression during the flash response leads to a further *dynamic* decrease in $[Ca^{2+}]_i$. The functional separation of these background-induced and flash-induced changes in $[Ca^{2+}]_i$ is potentially important, as the various sites of action for Ca^{2+} might be influenced to differing extents by these static and dynamic components of the light-induced fall in $[Ca^{2+}]_i$, which take place at different times during the light response. In order to investigate their relative contribution to light adaptation, changes in $[Ca^{2+}]_i$ have been opposed at different times during the bright flash response by superfusing the outer segment with a solution from which both Ca^{2+} and Na^+ have been omitted (Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989; Matthews, 1995*a*), both in darkness and following adaptation to steady light. The results obtained suggest that the static decrease in $[Ca^{2+}]_i$ induced by the background is responsible for the graded variation with steady intensity of the time at which the bright flash response begins to recover from saturation. This may occur through actions on 'early' stages in the transduction mechanism which lead to phosphodiesterase (PDE) activation. In contrast, rapid actions of Ca^{2+}, which can also be induced by the dynamic fall in $[Ca^{2+}]_i$ during the flash response, appear to play the greater role in determining the steady response to background light. This is suggested to result from actions of $[Ca^{2+}]_i$ on 'late' stages in transduction such as guanylyl cyclase. Preliminary results of this study have been presented to The Physiological Society (Matthews, 1994).

METHODS

Preparation

Details of the preparation, recording techniques, light stimuli and solution changes have been described previously (Matthews, 1995*a*). 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*, following decapitation and pithing. The inner segment 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 carried out during intense steady illumination at the end of the experiment; the junction current was scaled for coincidence of saturating level before and after the solution change. In a few cases when junction currents were not available, the circulating current was measured by reference to the zero current level in that solution evoked by saturating flashes. All experiments were carried out at room temperature (approximately 20 °C).

External solutions

Ringer solution contained (mM): 111 NaCl, 2·5 KCl, 1·0 $CaCl_2$, 1·6 $MgCl_2$ and 3·0 Hepes, adjusted to pH 7·7 with NaOH, and also included 10 μM EDTA to chelate impurity heavy metals. The Ringer solution perfusing the recording chamber also included 10 mM glucose. 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution was modified from this composition by the equimolar substitution of choline chloride (Sigma) 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 & 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*a*).

Patch pipette methods

In some experiments, whole-cell patch pipette recordings were made from the rod outer segment during suction pipette recording (Lamb, Matthews & Torre, 1986) using an L/M-EPC7 patch clamp amplifier (List Medical). During the whole-cell recording the intracellular voltage was either clamped to −40 mV (after correction for a 10 mV liquid junction potential), or allowed to vary freely with the patch pipette current held at zero. The pseudo-intracellular solution filling the patch pipette contained 92 mM potassium aspartate, 7 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_2ATP, 1 mM Na_4GTP, 20 μM BAPTA, and 10 mM Hepes, and was adjusted to pH 7·0 with KOH (Lamb & Matthews, 1988).

Light stimuli and electrical recording

Light stimuli of wavelength 500 nm were delivered from a two-beam optical stimulator controlled by electromagnetic shutters; flash stimuli were of 20 ms duration and unpolarized. Stimulus intensities were adjusted with neutral density filters and measured with a calibrated silicon photodiode (United Detector Technology, Orlando, USA). The suction pipette current signal was filtered over the bandwidth DC–20 Hz, 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, Rochester, UK).

RESULTS

Exposure of the outer segment to 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solution

During the light response $[Ca^{2+}]_i$ falls due to the continued extrusion of Ca^{2+} by the sodium–calcium exchange following the reduction of Ca^{2+} influx (Yau & Nakatani, 1985). This normal light-induced fall in $[Ca^{2+}]_i$ can be

opposed by superfusing the outer segment with a solution from which both Ca^{2+} and Na^+ have been omitted (Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989; Matthews, 1995*a*). The removal 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. Although it has not yet proved possible to measure $[Ca^{2+}]_i$ during exposure of the outer segment to such a solution, indirect evidence supports the notion that this manipulation greatly slows any subsequent changes in $[Ca^{2+}]_i$, holding it near to the value before the solution change for a period of at least 10 s (Fain *et al.* 1989). However, if such a solution is used to maintain $[Ca^{2+}]_i$ at a reduced level in darkness, then the outer segment conductance is greatly elevated. If a permeant cation such as guanidinium is used to substitute for Na^+, then the circulating current is increased considerably (Fain *et al.* 1989), leading to a substantial ionic influx which is poorly tolerated by the cell. But if Na^+ is replaced instead by the impermeant ion choline, and external Mg^{2+} removed also, then the influx of permeant cations through the greatly elevated outer segment conductance can be minimized (Matthews, 1995*a*). If this solution is to be used to control $[Ca^{2+}]_i$ in this way, then it is first necessary to characterize its effects on the photoresponse.

A family of responses to flashes of increasing intensity recorded while the outer segment was superfused with this 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution is illustrated in Fig. 1*A*. In the virtual absence of external permeant ions, the normal inward dark current was replaced by a light-suppressible outward current whose magnitude varied between cells, which presumably was carried predominantly by cytoplasmic K^+ (Hodgkin, McNaughton, Nunn & Yau, 1984; Matthews, 1995*a*). Following the initial reversal of the circulating current, whose rise may have been limited by inner segment voltage-dependent conductances, a gradual increase in current took place in darkness (trace D). This progressive increase may have resulted from a slow fall in $[Ca^{2+}]_i$ due to a residual extrusion or sequestration of Ca^{2+} following the abolition of Ca^{2+} influx (Fain *et al.* 1989), or from a net efflux of Ca^{2+} through the outer segment conductance upon the complete removal of Ca^{2+} from the external solution. However, the magnitude of this gradual decline in $[Ca^{2+}]_i$ seems likely to have been quite small in comparison with that caused by even dim steady light, as it led to a rather smaller change in circulating current in darkness (compare traces D and 1 in Fig. 2). Furthermore, in most subsequent experiments the circulating current was suppressed by light during much of the exposure to 0 Ca^{2+}, 0 Mg^{2+},0 Na^+ solution, thereby preventing any efflux of Ca^{2+} through the outer segment conductance. The inset shows flash responses recorded from the same cell in Ringer solution. Comparison of the two families of traces reveals that in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution the time to peak of the flash response was prolonged and the sensitivity increased relative to their values in Ringer solution. These changes are qualitatively very similar to those obtained using low-Ca^{2+},0 Na^+ solution in which Na^+ has been replaced with guanidinium (Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989). Normalized response–intensity relations obtained from five such experiments are shown in Fig. 1*B*. The data for each cell have been normalized in intensity according to the exponential saturation relation (Lamb, McNaughton & Yau, 1981) fitted to the response–intensity relation recorded in Ringer solution (filled symbols). The data obtained when $[Ca^{2+}]_i$ was held near the dark-adapted level using 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution (open symbols) were well fitted by this expression, but with the exponential saturation constant increased by a factor of $2{\cdot}75 \pm 0{\cdot}15$ (mean $\pm$ S.E.M.). This is comparable to the increase in normalized sensitivity which is seen when the outer segment is superfused with guanidinium-substituted low-Ca^{2+},0 Na^+ solution (Matthews *et al.* 1988; Fain *et al.* 1989). These results therefore indicate that the choline-substituted solution used here is likely to have opposed changes in $[Ca^{2+}]_i$ in a similar manner.

Figure 2*A* illustrates the results obtained when this solution was used in darkness to hold $[Ca^{2+}]_i$ near either the normal dark-adapted level (trace D) or near the reduced values corresponding to each of five different intensities of steady light (traces 1–5). The rod was first allowed to adapt to the steady background in Ringer solution. Then the outer segment was stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution and the background extinguished shortly thereafter. When steady light is extinguished in guanidinium-substituted low-Ca^{2+},0 Na^+ solution, a substantial elevation of the circulating current results, whose magnitude is graded with the intensity of the preceding background (Fain *et al.* 1989). This elevation of current when $[Ca^{2+}]_i$ is held low in darkness reflects a higher concentration of cyclic GMP resulting from an increase in guanylyl cyclase velocity (Koch & Stryer, 1988). However, when background light was extinguished in the choline-substituted solution used here only a relatively modest increase of the outward current took place. Furthermore, although this increase was graded with intensity for the two dimmest backgrounds (traces 1 and 2), following the three brightest backgrounds the current relaxed to a common level irrespective of the preceding intensity (traces 3–5).

These observations suggest that, when $[Ca^{2+}]_i$ was held at a reduced level in darkness using this choline-substituted solution, the circulating current may have been limited by purely electrical factors. It seemed possible that the large increase in outer segment conductance might have drawn the membrane potential towards the K^+ reversal potential, thereby limiting the magnitude of the current across the outer segment membrane (Matthews, 1995*a*). This possibility is investigated for another cell in Fig. 2*B* by using a whole-cell patch pipette sealed onto the outer

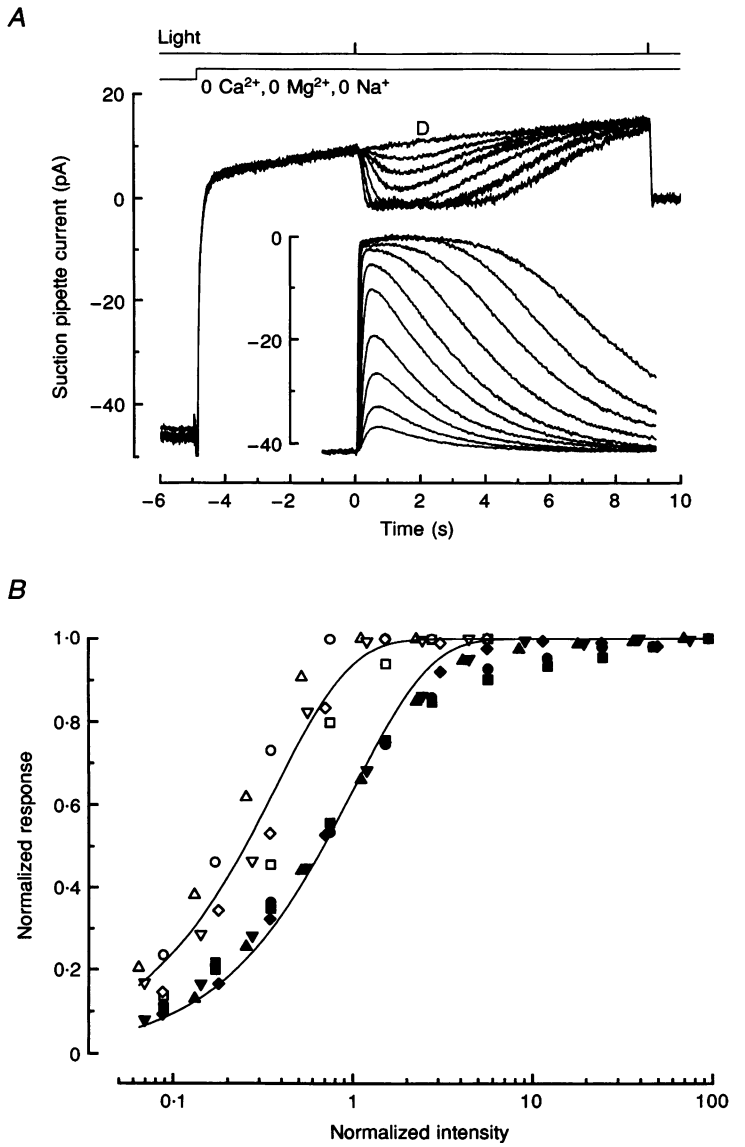

Figure 1. Light responses during exposure of the outer segment to 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^{+} solution

A, families of responses to flashes of increasing intensity delivered during exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution in darkness. Upper traces denote the times of delivery of light flashes and solution changes, respectively. First flash delivered 1·4–45 photons μm^{-2}; second flash delivered 1580 photons μm^{-2}. Trace D was obtained in darkness without the first flash. Each trace is the mean of 1–8 responses, depending on flash intensity, and has been corrected by subtraction of the junction current. Inset: responses to flashes of increasing intensity delivered in Ringer solution. Flashes delivered 1·5–1500 photons μm^{-2}. Each trace is the mean of 1–10 responses depending on flash intensity measured at both the start and the end of the experiment. *B*, normalized response–intensity relations measured at the response peak from 5 cells both in Ringer solution (filled symbols) and 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution (open symbols). Flash intensity has been normalized for each cell according to the response–intensity relation obtained in Ringer solution fitted individually to the exponential saturation equation $r/r_{max} = e^{-kI}$ (Lamb *et al.* 1981) using a least-squares algorithm. Mean exponential saturation constant in Ringer solution 0·088 ± 0·012 photons^{-1} μm^2 (mean ± S.E.M.). Response–intensity relations obtained in Ringer solution are means of data obtained at the start and end of each experiment. Continuous curves denote the exponential saturation equation fitted to the data from all cells in Ringer solution and 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution, respectively; the normalized sensitivity increased in 0 Ca^{2+}, 0 Mg^{2+},0 Na^{+} solution by a factor of 2·75 ± 0·15.

segment to control the membrane potential. If the membrane potential was allowed to vary freely by clamping the patch pipette current to zero (trace CC), then the extinction of steady light in 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution resulted in only a relatively modest increase in suction pipette current (peak current, 69 ± 11 pA; mean ± S.E.M., 6 cells). Figure 2*C* shows that this increased current was accompanied by a pronounced hyperpolarization recorded by the patch pipette (peak voltage, −70 ± 5 mV, mean ± S.E.M.; 6 cells). However, if this hyperpolarization was prevented by clamping the membrane potential at −40 mV (trace VC) then a much larger current was recorded by the patch pipette following the extinction of the background (peak current, 840 ± 160 pA, mean ± S.E.M.; 4 cells). Since the suction pipette collects only a proportion of the total circulating current (normally around a half; Lamb *et al.* 1986), the patch pipette current has been scaled down in Fig. 2*B* by the ratio of the dark current recorded by

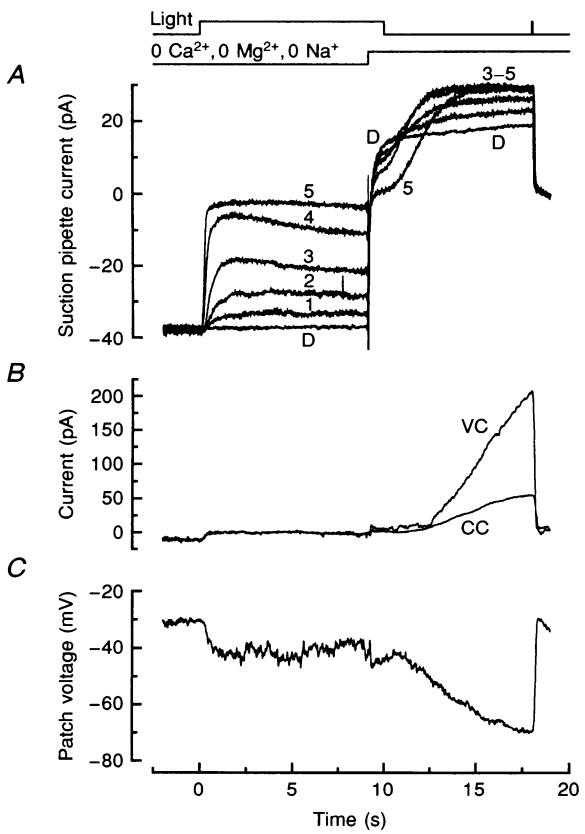

Figure 2. Extinction of steady background light during exposure to 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^{+} solution

A, responses in darkness (D) and at the extinction of background light of five different intensities (1–5) in 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution. The cell was first allowed to adapt to the background in Ringer solution, and then the outer segment stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution and the background extinguished. Upper traces denote light monitor and solution change timings, respectively. Steady background light delivered (photons μm^{-2} s^{-1}): trace 1, 1·1; trace 2, 4·6; trace 3, 18; trace 4, 75; trace 5, 285; bright flash delivered 1500 photons μm^{-2}. *B* and *C*, simultaneous suction and patch pipette recordings from another rod following extinction of steady light during exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^{+} solution. Steady background light delivered 75 photons μm^{-2} s^{-1}; bright flash delivered 400 photons μm^{-2}. *B*, trace VC: patch pipette current with the patch pipette voltage clamped to −40 mV (after correction for a 10 mV liquid junction potential); trace CC: suction pipette current with the patch pipette current clamped to zero. Patch pipette current (trace VC) has been scaled down so that the dark current in Ringer solution corresponds to the fraction collected by the suction pipette in current clamp (scaling factor 0·29). Suction pipette current (trace CC) has been corrected by subtracting the junction current recorded by the suction pipette when the patch pipette was clamped at −40 mV and the solution change carried out in darkness. Each trace is a single response; trace CC was obtained before trace VC. *C*, patch pipette voltage with the pipette current clamped to zero. Intracellular voltage has been corrected for a liquid junction potential of 10 mV. Trace is a single response recorded during trace CC above.

the two pipettes in Ringer solution. Nonetheless, it is apparent that the circulating current was considerably increased when hyperpolarization was prevented following the extinction of the background in 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solution. This observation supports the notion that when the outer segment conductance was greatly elevated in this choline-substituted solution the circulating current was normally limited by hyperpolarization towards the K^+ reversal potential.

The results of Figs 1 and 2 show that choline-substituted 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution can be used to maintain $[Ca^{2+}]_i$ at a reduced level in darkness, without allowing the substantial ionic influxes which would occur in a guanidinium-substituted solution. They also suggest that the magnitude of the circulating current faithfully the variation of the outer segment conductance during the light response unless the outer segment conductance was substantially elevated above its normal value in darkness.

Actions of $[Ca^{2+}]_i$ on the dark-adapted bright flash response

When the circulating current is completely suppressed during the response to a bright flash, Ca^{2+} influx through the outer segment conductance is abolished, and $[Ca^{2+}]_i$ consequently decreases (Yau & Nakatani, 1985). The significance of this dynamic fall in $[Ca^{2+}]_i$ can be assessed if further changes in $[Ca^{2+}]_i$ are opposed at different times during the flash response by superfusing the outer segment with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution.

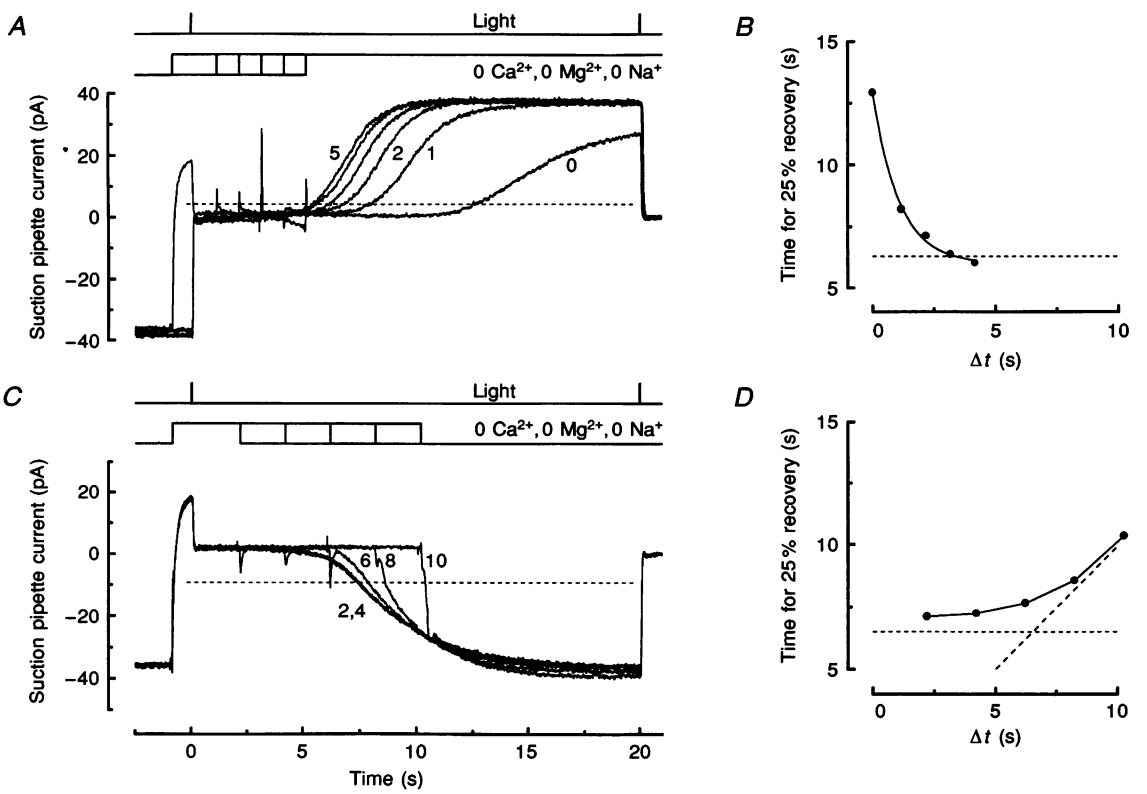

Figure 3. Exposure to 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solution before and after a bright flash in darkness

A, outer segment stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution either before, or at progressively increasing times after the flash. Numbers beside individual traces denote the nominal time interval (Δt) in seconds between the flash and the solution change. Traces above each panel denote light monitor and solution change timings, respectively. Interrupted line denotes 25% of the original dark current in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash. *B*, time for recovery of 25% of the initial dark current in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution plotted against the interval between the flash and the solution change. Continuous curve is a single exponential, of time constant 1·08 s; interrupted line indicates the time for 25% recovery of the original dark current in Ringer solution. *C*, outer segment stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash and returned to Ringer solution at progressively increasing times thereafter. Interrupted line denotes 25% of the original dark current in Ringer solution before the flash. *D*, time for recovery of 25% of the initial dark current in Ringer solution plotted against the interval between the flash and the solution change. Interrupted horizontal line indicates the time for 25% recovery of the response in Ringer solution. Bright flashes delivered 1300 photons μm^{-2}. Each current trace is the average of two responses and has been corrected by subtraction of the junction current.

Figure 3*A* shows the effect of stepping the outer segment to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution at progressively increasing times (Δt) after a bright flash in darkness, and remaining in this solution until the response had recovered from saturation. If the outer segment was stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ shortly before the flash (trace $\Delta t = 0$ s), thereby holding $[Ca^{2+}]_i$ near its original level in darkness, then the period spent in saturation was greatly prolonged (Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989). When, instead, the solution change took place at progressively later times following the flash (traces $\Delta t = 1$–5 s), the recovery of the response from saturation occurred progressively sooner. This graded reduction in the time spent in saturation was quantified by measuring the time required after the flash for the recovery of 25% of the original dark current measured shortly after stepping the outer segment to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution. This quantity is plotted in Fig. 3*B* against Δt, the delay between the flash and the time of the solution change. The time for 25% recovery decayed exponentially towards approximately the value obtained in Ringer solution, indicated by the interrupted line.

Collected data from nine such cells in which the same flash intensity was used are shown in Fig. 4*A*. To facilitate comparison, the time for 25% recovery and Δt have both been normalized for each cell according to the time for 25% recovery from the bright flash in Ringer solution (6·6 ± 0·3 s, mean ± S.E.M.). The continuous curve fitted to the data is a single exponential with a time constant of 0·173 ± 0·015 of the time for 25% recovery of the response to the same flash in Ringer solution. Data from twelve such experiments yield a mean time constant for this exponential decay of 1·15 ± 0·07 s (mean ± S.E.M.), which is of the same order as the time constant for the decline of the electrogenic current carried by the sodium–calcium exchange (Yau & Nakatani, 1985; Hodgkin, McNaughton & Nunn, 1987; Lagnado, Cervetto & McNaughton, 1992). These results are consistent with the notion that the dynamic fall in $[Ca^{2+}]_i$ which took place while the outer segment remained in Ringer solution led to the progressively earlier recovery of the flash response from saturation.

If the outer segment was instead stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution *before* the flash and then returned to Ringer solution at progressively increasing times thereafter, then the results shown in Fig. 3*C* were obtained. The corresponding times for 25% recovery of the original dark current in Ringer solution are plotted in Fig. 3*D* against the interval between the flash and the return to Ringer solution (Δt). Under these conditions, the recovery of the flash response was little affected by increasing the duration of the exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution

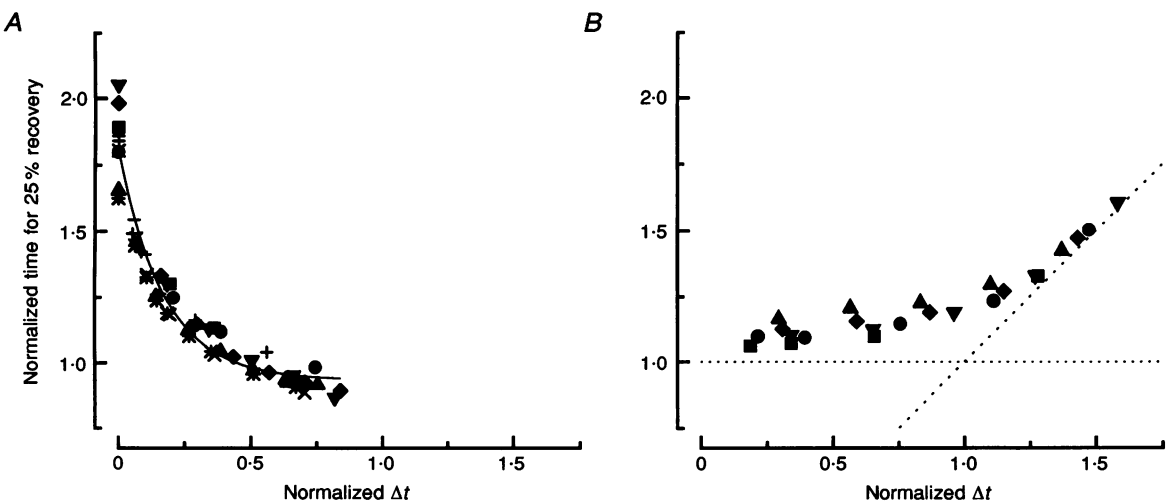

Figure 4. Normalized recovery times when changes in $[Ca^{2+}]_i$ are prevented

Collected times for 25% recovery of the original dark current when the outer segment was stepped from Ringer solution to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution at progressively increasing times after the flash (*A*), or stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash and returned to Ringer solution at progressively increasing times thereafter (*B*). In each case the time for 25% recovery and the interval (Δt) between the flash and the solution change were normalized according to the time for 25% recovery of the response to the same bright flash in Ringer solution (6·6 ± 0·3 s, mean ± S.E.M., 9 cells). Data are shown in *A* from 9 cells, while *B* shows data from 5 of these; each symbol represents an experiment on a different rod. Continuous curve in *A* is an exponential decay of time constant 0·173 ± 0·015 of the time for 25% recovery of the bright flash response in Ringer solution. Interrupted lines in *B* denote the normalized time for 25% recovery in Ringer solution, and the line of unity slope, respectively. Bright flashes delivered 1300 photons μm^{-2}. The cell of Fig. 3 is denoted by the inverted triangles (▼).

unless this extended beyond the time at which recovery from saturation would normally have commenced. Even then the response rejoined the other traces shortly thereafter. The common recovery of the responses seems likely to have resulted from the rapid fall in $[Ca^{2+}]_i$ which will have ensued on returning the outer segment to Ringer solution. It would therefore appear that this dynamic fall in $[Ca^{2+}]_i$ was able to restore the normally rapid onset of response recovery at any time during the response, suggesting that it acts at a site within the transduction mechanism which can be rapidly modulated by changes in $[Ca^{2+}]_i$ whenever they occur.

Collected data from five such cells are shown in Fig. 4*B*, again normalized according to the time for 25% recovery in Ringer solution. When the exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution ended before recovery would normally have commenced in Ringer solution, the normalized recovery time varied little with the duration of the exposure. However, even following relatively brief exposures to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution, response recovery was slightly delayed compared with that when the outer segment remained in Ringer solution throughout. When the period in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution exceeded the time at which the response would normally have begun

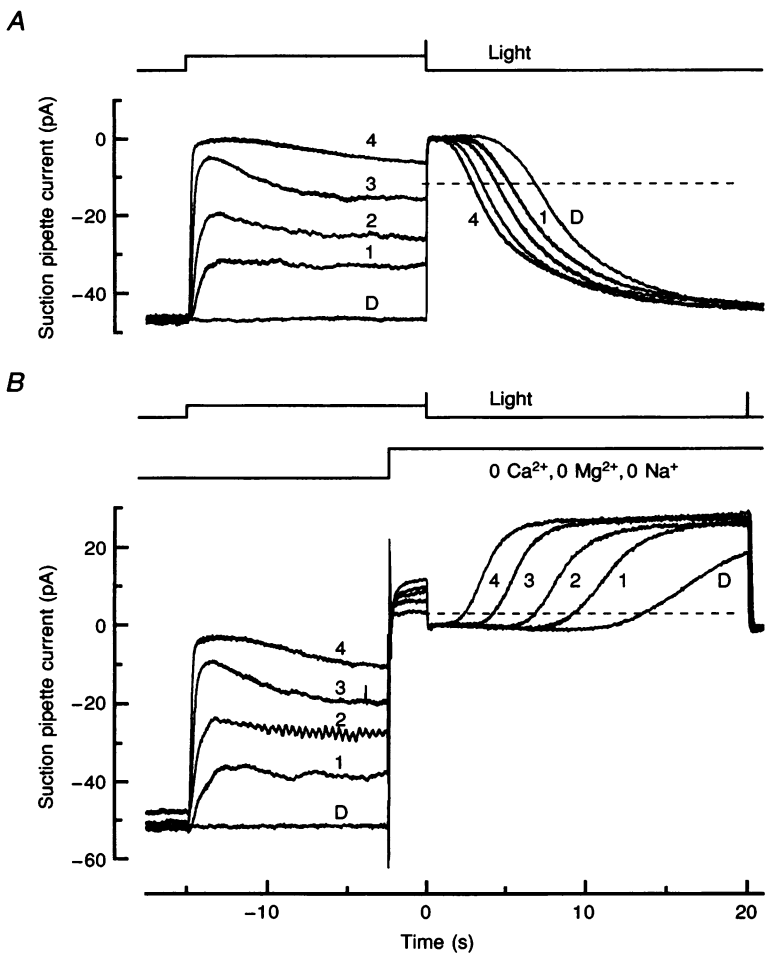

Figure 5. Effect of 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ superfusion on the light-adapted bright flash response

A, responses recorded under control conditions in Ringer solution to bright flashes in darkness (D) or at the extinction of steady light of increasing intensity (1–4). Interrupted line denotes 25% of the original dark current in Ringer solution before the flash. *B*, responses recorded with $[Ca^{2+}]_i$ held near the dark-adapted level (D) or the appropriate light-adapted level (1–4) by superfusion shortly before the flash with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution. Interrupted line denotes 25% of the original dark current in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash. Each trace is the average of two (1–4) or three (D) responses, and has been corrected by subtraction of the junction current where appropriate. Steady light delivered (in photons μm^{-2} s^{-1}): trace 1, 3·7; trace 2, 14; trace 3, 60; trace 4, 230; bright flashes delivered 1300 photons μm^{-2}.

to recover, the data fell close to the line of unity slope, corresponding to the rapid recovery seen on the return to Ringer solution. These results suggest that the processes governing the decay of PDE activity following a flash must be relatively unaffected by $[Ca^{2+}]_i$, as the time spent in saturation depended little on whether or not $[Ca^{2+}]_i$ was allowed to fall immediately after the flash.

Actions of $[Ca^{2+}]_i$ on the bright flash response during light adaptation

Exposure to steady light is known to lead to a sustained decrease in $[Ca^{2+}]_i$ (Yau & Nakatani, 1985; McNaughton *et al.* 1986; Ratto *et al.* 1987) which is necessary for adaptation to the background (Torre, Matthews & Lamb, 1986; Matthews *et al.* 1988; Nakatani & Yau, 1988; Fain *et al.* 1989). The magnitude of this *static* decrease in $[Ca^{2+}]_i$ varies with the extent to which the circulating current is suppressed by the background. When a saturating flash is delivered during background illumination, a further *dynamic* fall in $[Ca^{2+}]_i$ will ensue, provided that $[Ca^{2+}]_i$ has not already fallen to the level corresponding to a saturated response. Figure 5 investigates the interplay between the static decrease in $[Ca^{2+}]_i$ induced by steady light and the dynamic fall in $[Ca^{2+}]_i$ induced by a bright flash by examining adaptational changes in the delay before the bright flash response started to recover from saturation.

Figure 5*A* illustrates the effect of steady light on the response to a bright flash delivered just before the background was extinguished. As the background intensity increased, the response began to recover from saturation progressively earlier. This graded reduction of the time spent in saturation by the bright flash response is a well-established manifestation of light adaptation (Fain *et al.* 1989; Pepperberg *et al.* 1992). While the response remained in saturation, $[Ca^{2+}]_i$ will have fallen *dynamically* to a much lower level before the response began to recover. Therefore the variation in the time of response recovery seems likely to reflect instead the *static* decrease in $[Ca^{2+}]_i$ induced by the background, acting near the time of the flash.

In Fig. 5*B* the outer segment was stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution just before the flash, thereby holding $[Ca^{2+}]_i$ near the statically reduced level induced by the background. This manipulation was intended not only to prevent $[Ca^{2+}]_i$ from dynamically falling further during the flash response, but also to prevent it from rising again thereafter during the recovery of the circulating current. Under these conditions, the onset of response recovery was considerably retarded in darkness, much as in Fig. 3*A*. However, as the steady intensity increased, the response recovered from saturation progressively earlier. This progressive reduction in the time spent in saturation seems likely to have resulted from the graded static decrease in $[Ca^{2+}]_i$ induced by the background. Thereafter, the circulating current recovered to a level greater than the initial value in darkness, which depended little on the intensity of the preceding background, much as in Fig. 2.

These changes in the time spent in saturation by the bright flash response can be quantified by measuring again the

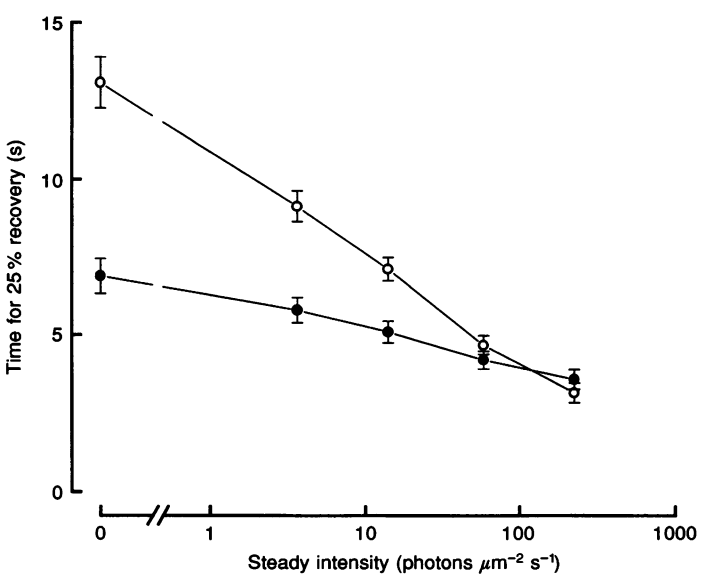

Figure 6. Effect of steady light on recovery time in Ringer and 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solutions
Time for recovery of 25% of the original dark current after a bright flash delivered at the extinction of steady light either in Ringer solution (●) or during superfusion with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution from just before the flash (○), plotted against the steady intensity. Data are the mean values from 7 cells; error bars denote standard errors of the mean.

time taken for the circulating current to recover by 25% of its original value in darkness in each solution. This quantity is plotted against background intensity in Fig. 6. As the background intensity increased, the time for 25% recovery varied over a much wider range when the response was recorded in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution (open circles) than in Ringer solution (closed circles). In 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution the response was greatly prolonged in darkness, but began to recover from saturation progressively sooner as the steady intensity was increased, presumably reflecting the graded *static* decrease in $[Ca^{2+}]_i$ induced by the background. The even earlier recovery from saturation in Ringer solution must therefore have resulted from the additional *dynamic* fall in $[Ca^{2+}]_i$ during the flash response, which was prevented in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution. However, following adaptation to the brightest steady intensity, the response began to recover from saturation after a similar time in both solutions, suggesting that this dynamic fall in $[Ca^{2+}]_i$ was of little importance in bright light, presumably because $[Ca^{2+}]_i$ had already fallen to a greatly reduced level.

DISCUSSION

Significance of dynamic changes in $[Ca^{2+}]_i$ for the dark-adapted bright flash response

The importance of the dynamic fall in $[Ca^{2+}]_i$ for the normal recovery of the flash response is supported by the experiments of Figs 3 and 4, in which the outer segment was superfused with 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solution at different times before or after a bright flash in darkness. If the outer segment was stepped to 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ solution before the flash, the time spent in saturation was greatly prolonged. But when the solution change took place progressively later after the flash, the delay before recovery commenced fell exponentially towards its value in Ringer solution (Figs 3*A* and *B* and 4*A*). This progressive shortening of the response seems likely to have resulted from the dynamic fall in $[Ca^{2+}]_i$ induced by the suppression of circulating current, which will have proceeded ever nearer to completion by the time of the solution change.

The time constant for this relaxation can be compared with estimates for the time course of the fall in $[Ca^{2+}]_i$ induced by a bright flash. The decay of the electrogenic current carried by the sodium–calcium exchange suggests that $[Ca^{2+}]_i$ falls exponentially with a time constant of 0·5–1 s in salamander rods (Yau & Nakatani, 1985; Hodgkin *et al.* 1987; Lagnado *et al.* 1992), while in isolated lizard rod outer segments a second slower component of decline is also observed (Rispoli, Sather & Detwiler, 1993). Measurements of intra- or extracellular Ca^{2+} concentration suggest that when the outer segment conductance is completely suppressed, $[Ca^{2+}]_i$ falls with a time constant of 1·5–2·5 s (Miller & Korenbrot, 1987; Ratto *et al.* 1987), and this fall in $[Ca^{2+}]_i$ has recently been shown to parallel the decline in the exchange current (Gray-Keller & Detwiler, 1994; McCarthy, Younger & Owen, 1994). These values are of the same order as the time constant of 1·15 ± 0·07 s obtained here, consistent with the notion that the earlier recovery of the response resulted from the dynamic decline in $[Ca^{2+}]_i$ which accompanies the suppression of current by a bright flash. However, the precise relationship between them is not straightforward, as it will depend on the nature and co-operativity of the interaction between Ca^{2+} and the transduction mechanism.

If the outer segment was instead stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution before the flash and returned to Ringer solution at progressively increasing times thereafter, then response recovery varied little with the duration of the exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution, unless this exceeded the time at which the response would normally have started to recover from saturation (Figs 3*C* and *D* and 4*B*). Thus, even when the dynamic fall in $[Ca^{2+}]_i$ was delayed until just before the normal time of recovery, it appeared to exert virtually the same effect on the transduction mechanism as when $[Ca^{2+}]_i$ was allowed to fall immediately after the flash. These observations are consistent with the idea that the dynamic fall in $[Ca^{2+}]_i$ induced by the flash was able to affect recovery at any time during the response. Such dynamic actions of $[Ca^{2+}]_i$ therefore seem likely to involve the modulation of stages 'late' in the transduction mechanism, which may be rapidly affected by changes in $[Ca^{2+}]_i$ whenever they occur. However, it should be noted that even a brief exposure to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution at the time of the flash resulted in a slight retardation of response recovery. This observation suggests that the dynamic fall in $[Ca^{2+}]_i$ may also have exerted a more minor effect on 'early' stages in the transduction cascade which might no longer have been able to contribute when $[Ca^{2+}]_i$ was allowed to fall later in the response.

Significance of static changes in $[Ca^{2+}]_i$ for the light-adapted bright flash response

During steady background illumination, the response to a bright flash recovers from saturation sooner than in darkness (Fig. 5*A*). This light-induced acceleration of the onset of bright flash response recovery is known to depend upon $[Ca^{2+}]_i$, as it is abolished if changes in $[Ca^{2+}]_i$ are prevented (Fain *et al.* 1989) and can be evoked if $[Ca^{2+}]_i$ is artificially reduced in darkness (Matthews, 1995*a*).

It was shown above that the decrease in the time spent in saturation induced by the dynamic fall in $[Ca^{2+}]_i$ takes place with a time constant of around 1 s. Therefore these dynamic actions of $[Ca^{2+}]_i$ seem likely to have been substantially complete by the time at which the bright flash

response started to recover (Pepperberg, Jin & Jones, 1994; Matthews, 1995*a*). Even in the presence of nearly saturating background light, the response to the bright flash in Ringer solution remained in saturation for over a second (Fig. 5*A*), a period which is sufficient in darkness for the resulting dynamic fall in $[Ca^{2+}]_i$ to speed considerably the onset of response recovery (Fig. 3*A* and *B*). The time constant with which $[Ca^{2+}]_i$ falls following the suppression of the outer segment conductance is determined by the Ca^{2+} affinity of the sodium–calcium exchange, the saturated value of the exchange current, and the Ca^{2+} buffering properties of the outer segment (Lagnado *et al.* 1992). As long as these parameters are not affected by light, $[Ca^{2+}]_i$ will have fallen with the same time constant when the bright flash was delivered during steady illumination. During the period of response saturation $[Ca^{2+}]_i$ is therefore likely to have fallen to a level which will have depended little on the intensity of the pre-existing background. Actions of Ca^{2+} 'late' in transduction which can be evoked rapidly at any time during the flash response should therefore have been largely complete by the time at which the response started to recover, so their contribution to adaptation seems unlikely to have depended much on the pre-existing background intensity. The graded variation in the time spent in saturation in Ringer solution therefore seems likely to have resulted from the different static level of $[Ca^{2+}]_i$ induced by each background intensity, acting at, or soon after, the time at which the flash was presented. This light-induced acceleration in the onset of response recovery therefore seems likely to involve the modulation by $[Ca^{2+}]_i$ of stages 'early' in the transduction cascade, which may depend little upon changes in $[Ca^{2+}]_i$ later in the response, perhaps because their activity has already been terminated.

When, instead, the outer segment was superfused with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution just before a flash in darkness, the time spent in saturation was considerably prolonged in comparison to that in Ringer solution. However, as the steady intensity was increased, the onset of response recovery took place progressively earlier. This progressive decrease in the delay before recovery in 0 Ca^{2+},0 Mg^{2+}, 0 Na^+ solution seems likely to have represented the graded actions of the static reduction in $[Ca^{2+}]_i$ induced by each background. Following adaptation to the brightest background the response started to recover after a similar time in both solutions, despite the fact that any further dynamic fall in $[Ca^{2+}]_i$ will have been prevented in 0 Ca^{2+}, 0 Mg^{2+},0 Na^+ solution. This observation suggests that the *static* decrease in $[Ca^{2+}]_i$ induced by the brightest background was also sufficient to evoke fully the effects which would normally be induced by the *dynamic* fall in $[Ca^{2+}]_i$ in Ringer solution. This *static* reduction in $[Ca^{2+}]_i$ will have acted on the transduction mechanism not only at the time of the flash, but also later in the response at the onset of recovery; a time at which $[Ca^{2+}]_i$ would normally have fallen *dynamically* in Ringer solution to an even lower level. Therefore the graded variation in the onset of response recovery observed under these conditions seems likely to have involved graded actions of this static reduction in $[Ca^{2+}]_i$ on both 'early' and 'late' stages in transduction.

Possible nature of actions of $[Ca^{2+}]_i$ 'early' and 'late' in transduction

The extent to which the functional separation between actions of $[Ca^{2+}]_i$ 'early' and 'late' in transduction corresponds to clear-cut actions of $[Ca^{2+}]_i$ at distinct sites in the transduction mechanism is difficult to establish. Indeed, the slight prolongation of the bright flash response when $[Ca^{2+}]_i$ was held at the dark-adapted level at the time of the flash (Fig. 3*B*) suggests that dynamic changes in $[Ca^{2+}]_i$ may also have a more minor effect on 'early' stages. However, it is nonetheless of interest to attempt to subdivide the biochemical actions of Ca^{2+} at different sites within the transduction mechanism according to these functional categories.

The most plausible site of action for $[Ca^{2+}]_i$ 'late' in the transduction mechanism is guanylyl cyclase, which is known to be co-operatively inhibited by Ca^{2+} *in vitro* (Koch & Stryer, 1988), and which is rapidly modulated in the intact cell both by imposed changes in $[Ca^{2+}]_i$ (Hodgkin, McNaughton & Nunn, 1985; Lamb & Matthews, 1988) and during the light response (Hodgkin & Nunn, 1988). Changes in $[Ca^{2+}]_i$ would therefore be expected to lead to a corresponding alteration in cyclase velocity whenever they occurred during the light response. Similarly, the modulation of the cyclic GMP affinity of the outer segment conductance by Ca^{2+} (Hsu & Molday, 1993) might also contribute, although the smaller magnitude reported for this effect suggests that it probably plays only a minor role.

In contrast, the identity of the putative site of action for $[Ca^{2+}]_i$ 'early' in the transduction cascade is less certain. To account for the observed behaviour, such a site should only be available for interaction with Ca^{2+} at, or shortly after, the time of the flash. One possible candidate for such an 'early' site is the modulation by Ca^{2+} of the formation of catalytically active rhodopsin (Lagnado & Baylor, 1994). However, this effect appears to be equivalent, at most, to a fourfold reduction in the number of rhodopsin molecules available for activation, and therefore may not be sufficient to account fully for the rather larger adaptational changes attributed below to 'early' actions of $[Ca^{2+}]_i$.

The significance of the proposed role for Ca^{2+} in modulating the termination of the catalytic activity of photoisomerized rhodopsin (Rh*) is less clear. The phosphorylation of Rh* is

believed to be modulated by Ca^{2+}, an effect which has been suggested to alter its effective lifetime in the catalytically active form (Kawamura, 1993). However, the time constant for the relaxation of PDE activity following a bright flash appears to vary little with increasing flash (Hodgkin & Nunn, 1988) or background intensity (Pepperberg *et al.* 1992). Furthermore, the onset of response recovery is little affected when the dynamic fall in $[Ca^{2+}]_i$ is prevented from taking place until just before the normal time of response recovery (Figs 3*C* and *D* and 4*B*), in contrast to the prolongation of the response which would be expected if Ca^{2+} affected the processes governing the decay of PDE activity. These results suggest that neither the static nor the dynamic fall in $[Ca^{2+}]_i$ has any major effect on the time constant which dominates response recovery. It has been suggested that the decay of PDE activity following a flash may be governed predominantly by the inactivation of Rh* (Pepperberg *et al.* 1994). If this is correct, then these observations suggest that Rh* lifetime is unlikely to be modulated significantly by Ca^{2+}, in contrast to the biochemical evidence (Kawamura, 1993). Alternatively, Rh* inactivation might take place significantly more rapidly than the quenching of some later stage in the cascade, which thus would dominate the relaxation of PDE activity instead. In that case, modulation of Rh* lifetime by Ca^{2+} would appear to affect the peak level of PDE activity induced by the flash, rather than the time course of its subsequent decline.

Relative importance of actions of $[Ca^{2+}]_i$ 'early' and 'late' in transduction

The relative contribution of static and dynamic changes in Ca^{2+} to the adaptation induced by steady light can be estimated if it assumed that the functional subdivision between 'early' and 'late' actions corresponds to effects of Ca^{2+} on distinct stages within the transduction mechanism. In this analysis, actions of Ca^{2+} 'late' in transduction are assumed to result from the rapid modulation of guanylyl cyclase velocity, and those 'early' in transduction from direct or indirect modulation of the peak level of PDE activity induced by the flash. PDE activity is taken to decrease exponentially after a bright flash (Hodgkin &

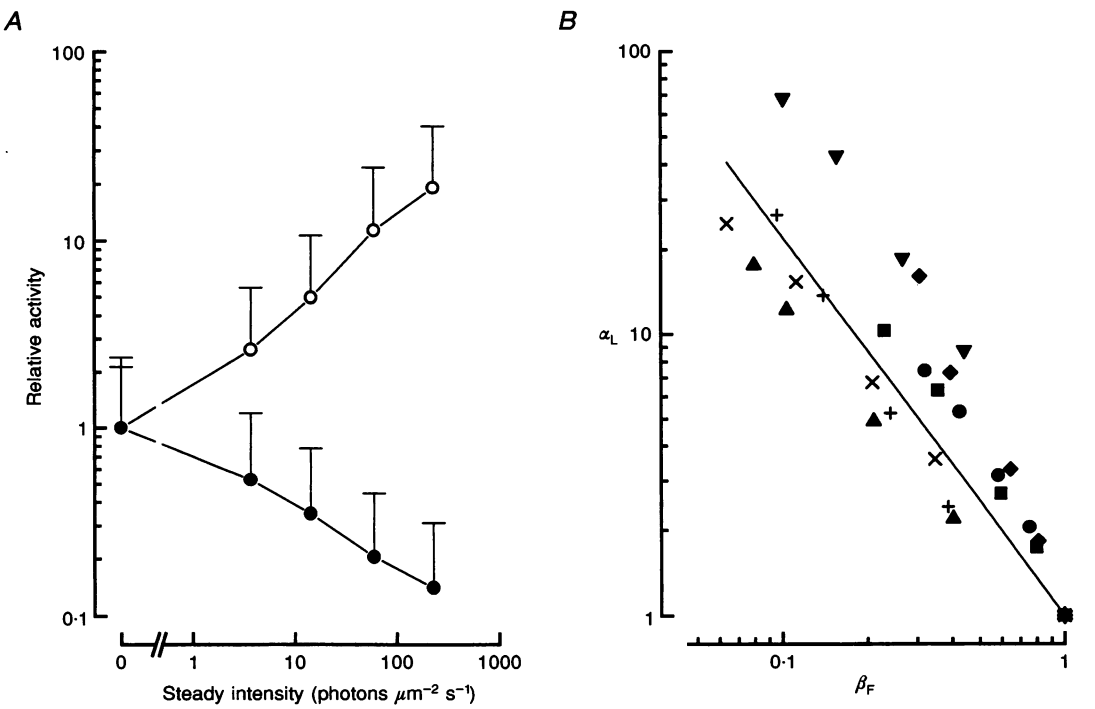

Figure 7. Predicted variation in cyclase velocity and phosphodiesterase activity during light adaptation

A, normalized variation with steady intensity of cyclase velocity (α_L, ○) and initial PDE activity induced by the flash (β_F, ●), determined according to eqns (A11) and (A14). Points were calculated from the mean recovery times of the 7 cells in Fig. 6*B*, using parameter values of $\tau = 1{\cdot}7$ s and $\kappa = 0{\cdot}63$; error bars denote positive-going standard errors. *B*, scatter plot on double logarithmic co-ordinates of predicted cyclase velocity (α_L) against the initial PDE activity (β_F) induced by the flash, calculated individually for each of 7 cells, and normalized to the values in darkness. Continuous line is a power law $y = x^n$ constrained to pass through unity and fitted according to a least-squares algorithm. Power law exponent $n = -1{\cdot}35 \pm 0{\cdot}06$. Cell of Fig. 5 denoted by the vertical crosses (+).

Nunn, 1988) with a time constant that is unaffected by light (Pepperberg *et al.* 1992). On the basis of these assumptions, the contribution of these processes to adaptation can be estimated by considering the recovery of the bright flash response when $[Ca^{2+}]_i$ is either free to change or is held near the appropriate light adapted level. Expressions are derived in the Appendix to predict guanylyl cyclase velocity and the peak PDE activity evoked by the flash from the mean recovery time data of Fig. 6.

These quantities are plotted as functions of background intensity in Fig. 7*A*. Their precise magnitudes depend upon the value of the time constant for the decline of PDE activity, which is taken here to be 1·7 s (Pepperberg *et al.* 1992). As the background became brighter the predicted velocity of guanylyl cyclase, α_L, progressively increased, reaching some 19 times its value in darkness after adaptation to the brightest steady intensity. This value can be compared with the 5- to 20-fold increase in cyclase velocity observed *in vitro* on reducing Ca^{2+} (Koch & Stryer, 1988). It is somewhat greater than the 10-fold increase seen during the bright flash response (Hodgkin & Nunn, 1988) or the 6-fold increase during bright steady light (Cornwall & Fain, 1994) measured by superfusion with IBMX, a technique which may underestimate cyclase velocity at high PDE activities. In contrast the predicted initial PDE activity induced by the flash, β_F, varied more gradually with increasing intensity, falling by a factor of 7 on the brightest background from its value in darkness.

The steeper variation of cyclase velocity with background intensity is broadly consistent with biochemical measurements of the dependence of these quantities on Ca^{2+}. Figure 7*B* plots normalized values for α_L against β_F, determined individually from each of the seven cells of Fig. 6. The continuous line fitted to the data is a power law constrained to pass through unity. The power law exponent can be interpreted as representing the ratio of the Hill coefficients for the dependence of α_L and β_F on $[Ca^{2+}]_i$. Guanylyl cyclase velocity has been shown to increase *in vitro* when Ca^{2+} is lowered, with a Hill coefficient of between 3·9 in mammalian (Koch & Stryer, 1988) and 2·0 in lizard rods (Gorczyca, Gray-Keller, Detwiler & Palczewski, 1994), while the fractional PDE activation by light decreases *in vitro* with a Hill coefficient of 1·4 (Kawamura, 1993). The value of 1·35 ± 0·06 for the power law exponent obtained here falls at the lower end of the range of 1·4–2·7 for the ratios of these biochemically determined Hill coefficients. Thus these results suggest that actions of Ca^{2+} 'late' in the transduction mechanism play a proportionately larger role in the adaptation to the background itself than do actions 'early' in the transduction mechanism.

APPENDIX

Quantitative estimation of the actions of $[Ca^{2+}]_i$ 'early' and 'late' in transduction

The cyclic GMP economy of the rod outer segment is believed to be described by:

$$\mathrm{GTP} \xrightarrow{\alpha} \mathrm{cGMP} \xrightarrow{\beta} \mathrm{GMP}, \tag{A1}$$

where α and β are the velocities of guanylyl cyclase and PDE, respectively (Kawamura & Murakami, 1986; Hodgkin & Nunn, 1988). The rate of change of the free cyclic GMP concentration, G, is given by the difference between the rate of its synthesis and destruction:

$$\eta \frac{dG}{dt} = \alpha - G\beta, \tag{A2}$$

where η is the buffering power of the cytoplasm for cyclic GMP (Hodgkin & Nunn, 1988; Cote & Brunnock, 1993). It is assumed that the GTP concentration remains constant during the response (Dawis, Graeff, Heyman, Walseth & Goldberg, 1988), so that it does not influence α, which is taken as being affected only by $[Ca^{2+}]_i$ (Koch & Stryer, 1988). Previous studies have shown that the increased PDE activity evoked by a bright flash decays exponentially with a time constant of around 2 s, which is affected little by flash intensity (Hodgkin & Nunn, 1988). This observation is consistent with the logarithmic dependence of response duration on flash intensity, which yields a similar time constant of 1·7 s (Pepperberg *et al.* 1992). Furthermore, this value appears to be unaffected by background light (Pepperberg *et al.* 1992), suggesting that the time course with which PDE activity decays following a bright flash does not depend on $[Ca^{2+}]_i$. This view is reinforced by the demonstration in Figs 3*C* and *D* and 4*B* that the time of onset of recovery depended little on when during the response the dynamic fall in $[Ca^{2+}]_i$ was allowed to take place. Therefore it is assumed that the time constant, τ, for the decay of PDE activity does not change during background illumination. If a bright flash is delivered at the extinction of steady light:

$$\beta = \beta_F e^{-t/\tau} + \beta_D, \tag{A3}$$

where β_F is the initial PDE activity following its activation by the flash, and β_D the final value following recovery in darkness. The decay of the PDE activity induced by the background is neglected, as throughout response recovery it is likely to be smaller than that evoked by the bright flash delivered as the steady light was extinguished. When the response recovers from saturation in Ringer solution, the turnover of cyclic GMP should still be sufficiently high that the cyclase and PDE rates will essentially be in equilibrium (Hodgkin & Nunn, 1988) despite a somewhat

increased buffering capacity for cyclic GMP (Cote & Brunnock, 1993), so that:

$$\alpha = G\beta. \quad \text{(A4)}$$

In 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution this is also likely to have been a reasonable assumption when $[Ca^{2+}]_i$ was held at a reduced level following suppression of the circulating current by light. However, when the outer segment was superfused with 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution in darkness (e.g. Fig. 5*B*, trace D) the cyclase velocity, and hence the turnover of cyclic GMP, will have remained near their dark-adapted values. Therefore, under these conditions it is likely that equilibrium will have been attained somewhat less rapidly, although this extra delay will probably have been quite small (Hodgkin *et al.* 1985).

Following a bright flash, the circulating current will recover to 25% of the original dark current when the PDE activity has declined sufficiently to allow guanylyl cyclase to raise the concentration of cyclic GMP, $G_{25\%}$, to some fraction, κ, of the concentration in darkness, G_D:

$$G_{25\%} = \kappa G_D. \quad \text{(A5)}$$

As the circulating current is known to depend on approximately the cube of the cyclic GMP concentration (Yau & Baylor, 1989), the recovery of 25% of the dark current will correspond to a cyclic GMP concentration of $\sqrt[3]{0{\cdot}25}$ of its value in darkness ($\kappa = 0{\cdot}63$).

When the circulating current is completely suppressed by a bright flash in Ringer solution, $[Ca^{2+}]_i$ will fall rapidly from the appropriate background-induced level towards a much lower value, thereby maximally activating guanylyl cyclase. Once the circulating current begins to recover, $[Ca^{2+}]_i$ will start to rise again. It seems likely that the corresponding decline in cyclase velocity will largely be governed by the recovery of the circulating current, and not by the intensity of the preceding background (Matthews, 1995*b*), as $[Ca^{2+}]_i$ would be expected to rise in each case from a common reduced level induced by the flash, rather than from the level corresponding to any given background intensity (see Gray-Keller & Detwiler, 1994, for a contrary view). Suppose that the velocity of the cyclase had fallen to $\alpha_{25\%}$ at t_R, the time by which 25% recovery had taken place in Ringer solution. Substituting these values into eqns (A3) and (A4):

$$\alpha_{25\%} = G_{25\%}(\beta_F e^{-t_R/\tau} + \beta_D). \quad \text{(A6)}$$

However, in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution, when $[Ca^{2+}]_i$ was unable to fall during the flash response, guanylyl cyclase velocity will have remained at the value α_L corresponding to the statically reduced value of $[Ca^{2+}]_i$ induced by steady light. Substituting this value into eqns (A3) and (A4) at t_C, the time for recovery from saturation of 25% of the original dark current in this choline-substituted solution:

$$\alpha_L = G_{25\%}(\beta_F e^{-t_C/\tau} + \beta_D). \quad \text{(A7)}$$

When, instead, the outer segment was stepped to 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution in darkness, guanylyl cyclase will have remained at its dark-adapted velocity throughout. Consequently, the final recovery of the bright flash response in darkness will have been strongly influenced by the dark-adapted PDE activity, accounting for the more gradual increase of current seen under these conditions (see Fig. 3*A*, trace $\Delta t = 0$; Fig. 5*B*, trace D). Solving eqns (A6) and (A7) for t_R and t_C and substituting from eqns (A4) and (A5) for $G_{25\%}\beta_D$:

$$t_R = \tau \ln((G_{25\%}\beta_F)/(\alpha_{25\%} - \kappa\alpha_D)), \quad \text{(A8)}$$

$$t_C = \tau \ln((G_{25\%}\beta_F)/(\alpha_L - \kappa\alpha_D)), \quad \text{(A9)}$$

where α_D and α_L are the cyclase velocities in darkness and during steady light, respectively. Subtracting the time for 25% recovery in Ringer solution following adaptation to background light, t_{RL}, from the corresponding recovery time in darkness, t_{RD}:

$$t_{RD} - t_{RL} = \tau \ln(\beta_{FD}/\beta_{FL}), \quad \text{(A10)}$$

where β_{FD} and β_{FL} are the initial PDE activities induced by the flash in darkness and during steady light, respectively. Taking anti-logs and rearranging:

$$\beta_{FL}/\beta_{FD} = e^{-(t_{RD} - t_{RL})/\tau}. \quad \text{(A11)}$$

Subtracting the time for recovery of 25% of the original dark current in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution following adaptation to background light, t_{CL}, from the 25% recovery time in this solution in darkness, t_{CD}:

$$t_{CD} - t_{CL} = \tau \ln(((\alpha_L - \kappa\alpha_D)\beta_{FD})/((1-\kappa)\alpha_D\beta_{FL})). \quad \text{(A12)}$$

Taking the difference between the dark-subtracted times for 25% recovery in 0 Ca^{2+},0 Mg^{2+},0 Na^+ solution and Ringer solution:

$$(t_{CD} - t_{CL}) - (t_{RD} - t_{RL}) = \tau \ln((\alpha_L - \kappa\alpha_D)/((1-\kappa)\alpha_D)). \quad \text{(A13)}$$

Taking anti-logs and rearranging:

$$\alpha_L/\alpha_D = (1-\kappa)e^{((t_{CD} - t_{RD}) - (t_{CL} - t_{RL}))/\tau} + \kappa. \quad \text{(A14)}$$

Equations (A11) and (A14) have been used in Fig. 7 to predict the dependence of α_L and β_F on steady background intensity from the mean recovery time data of Fig. 6.

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