The action of cytoplasmic calcium on the cGMP-activated channel in salamander rod photoreceptors

Mandeep S. Sagoo and Leon Lagnado*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

1. Truncated salamander rod photoreceptors were internally perfused to investigate the action of cytoplasmic Ca^{2+} on cGMP-activated channels in the outer segment.

2. Switching from 1 μM Ca^{2+} to 0 Ca^{2+} increased the cGMP-activated current by a factor of 7·1 ± 0·5 when measured in the first 60 s after the outer segment was opened to the bath, but only 2-fold after 5 min or more. This was attributed to the loss from the outer segment of a soluble factor required for Ca^{2+} to inhibit the cGMP-activated channel.

3. Short exposures to 0 Ca^{2+} caused an irreversible increase in the cGMP-activated current measured in 1 μM Ca^{2+}, indicating that lowering $[Ca^{2+}]$ accelerated the loss of the channel inhibitor from the outer segment.

4. Channel activation occurred with a half-time of 6·7 s on switching to 0 Ca^{2+}. Replacing 1 μM Ca^{2+} inhibited the current again with a half-time of 11·0 s.

5. The inhibition of the cGMP-activated current by Ca^{2+} could be described by a Hill curve with half-maximal suppression at 55 ± 13 nM Ca^{2+} and a Hill coefficient of 1·4 ± 0·4.

6. Addition of calmodulin (1 μM), or the calmodulin inhibitors mastoparan and calmidazolium (5 μM), did not alter the action of Ca^{2+} on the cGMP-activated current.

7. The increased affinity of the cGMP-activated channels in response to a fall in $[Ca^{2+}]$ has the magnitude, speed and Ca^{2+} dependence to suggest that it will promote recovery of the cGMP-activated current in response to the light-induced fall in $[Ca^{2+}]$ that normally occurs inside the outer segment.

The outer segments of retinal photoreceptors contain non-specific cation channels gated by cytoplasmic cGMP (Fesenko, Kolesnikov & Lyubarsky, 1985; Yau & Baylor, 1989). Absorption of light by rhodopsin acts through the G-protein transducin to activate a cGMP phosphodiesterase (PDE) and the resulting fall in cGMP leads to the closure of these channels, generating a membrane hyperpolarization (reviewed by Stryer, 1986; Lagnado & Baylor, 1992; Lamb & Pugh, 1992). The cytoplasmic $[Ca^{2+}]$ falls during this response because closure of cGMP-activated channels reduces Ca^{2+} influx while Ca^{2+} efflux through the Na^+–Ca^{2+}, K^+ exchange continues (Yau & Nakatani, 1985; McNaughton, Cervetto & Nunn, 1986; Hodgkin, McNaughton & Nunn, 1987; Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989). The fall in $[Ca^{2+}]$ promotes recovery from light and acts as a feedback signal for light adaptation, reducing the gain of the phototransduction cascade (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988*a*; Yau, 1991). Several actions of Ca^{2+} are thought to be involved in this feedback control. First, the fall in $[Ca^{2+}]$ stimulates the guanylate cyclase to synthesize cGMP (Lolley & Racz, 1982; Koch & Stryer, 1988). Second, PDE activity is reduced by two actions at the beginning of the cascade: an apparent reduction in light-triggered formation of active rhodopsin (Lagnado & Baylor, 1994) and a reduction in its active lifetime after photoisomerization (Kawamura, 1993).

It has recently been shown that Ca^{2+} also acts on the cGMP-activated channel. Hsu & Molday (1993) measured cGMP-dependent Ca^{2+} fluxes in vesicles prepared from bovine rod outer segment membrane and found that Ca^{2+}–calmodulin reduced the affinity of the channel. Chen, Illing, Molday, Hsu, Yau & Molday (1994) measured a 1·5- to 2-fold decrease in affinity when Ca^{2+}–calmodulin was added to expressed human cGMP-activated channels in excised patches. Gordon, Downing-Park & Zimmerman (1995) have found that Ca^{2+} also modulates cGMP-activated channels in excised patches of frog rod outer segment. They propose that this can occur through the action of both calmodulin and another, as yet unidentified, Ca^{2+}-binding protein.

To assess whether an action of Ca^{2+} on the cGMP-activated channels might play a role in recovery of the light response it is important to measure the magnitude, speed and Ca^{2+} dependence of channel modulation in a preparation that

* To whom correspondence should be addressed.

more closely approximates the normal rod than a patch or vesicle. We have done this using truncated salamander rod outer segments, which maintain a light response but allow the experimenter control over internal [Ca^{2+}] and other small molecules (Nakatani & Yau, 1988*b*; Kawamura & Murakami, 1989; Lagnado & Baylor, 1994). We find that Ca^{2+} regulates the affinity of the cGMP-activated channel in this preparation over a physiological range of Ca^{2+} concentrations and that a fall in Ca^{2+} increases the cGMP-activated current up to 12-fold within seconds. This action of Ca^{2+} involves a diffusible molecule that is not calmodulin. We conclude that modulation of the cGMP-activated channel by Ca^{2+} is likely to play a role in recovery of the light response and light adaptation. A preliminary account of this work has been communicated to The Physiological Society (Sagoo & Lagnado, 1995).

METHODS

Suction electrodes were used to record the cGMP-activated current in the outer segments of truncated rods from the retinae of larval tiger salamanders (*Ambystoma tigrinum*) (Nakatani & Yau, 1988*b*). The methods for obtaining rods by mechanical dissociation of the retina and electrical recording with suction electrodes were as described by Baylor, Lamb & Yau (1979) and Hodgkin, McNaughton & Nunn (1985). Retinae were prepared from salamanders dark-adapted at a temperature of 4 °C. Animals were killed by decapitation and the brain and spinal cord immediately pithed. Individual rod photoreceptors were obtained by finely chopping the retina with a razor blade, and gently teasing apart the tissue.

The truncation procedure was similar to that described by Nakatani & Yau (1988*b*) and Lagnado & Baylor (1994). Briefly, a single rod was drawn, outer segment first, into a suction pipette that collected a fraction of the current flowing across the outer segment plasma membrane. The inner segment of the rod was then amputated using a glass probe, leaving the cut end of the outer segment open to the bath. This procedure was carried out while perfusing with 0·1 mM cGMP, so that successful truncation of the outer segment could be detected as the development of an inward current flowing through cGMP-activated channels.

The suction electrode was held near to the mouth of a glass theta-tube attached to a piezoelectric stepper (PZ100, Burleigh Instruments, Fishers, New York, USA). One of up to twelve solutions, selected by a series of electric rotary valves (Omnifit, Cambridge, UK), flowed through each pipe driven by gravity. By stepping from the mouth of one pipe to the other the solution perfusing the outer segment could be changed within 20 ms. The timing of solution changes and delivery of flashes was controlled using a PulseMaster pulse generator (World Precision Instruments, Sarasota, FL, USA). Recordings were made with an Axopatch-1D amplifier (Axon Instruments) and acquired with an ITC-16 A/D board (Instrutech, Great Neck, New York, USA) using Pulse software (Heka Elektronik, Lambrecht, Germany) on an Apple Macintosh 840 AV computer. Experiments were analysed using Igor Pro software (WaveMetrics, Lake Oswego, OR, USA). Curve fitting was done by the least-squares method, with each data point weighted by the inverse of its variance so that the standard deviations for each of the parameters in the fit could be calculated from the covariance matrix. To test for a difference in the parameters between two fitted curves a confidence interval was calculated by dividing the difference between the estimates by the square root of the sum of their variances.

Cells were isolated in Ringer solution containing (mM): NaCl, 110; $CaCl_2$, 1; $MgCl_2$, 1·6; KCl, 2·5; Hepes, 10; glucose, 5; pH 7·6 with NaOH. The suction electrode contained (mM): NaCl, 112·5; Hepes, 5; $MgCl_2$, 2; EGTA, 0·1. The standard solution perfusing the inside of the outer segment contained (mM): arginine glutamate, 110; $MgCl_2$, 2; Hepes, 5; EGTA, 1; $CaCl_2$, 0·985; cGMP, 0·1; ATP, 1; GTP, 0·01; pH 7·7 with NaOH. The calculated free [Ca^{2+}] in this solution was 1 μM, assuming the K_d of EGTA under these conditions is 16·75 nM (Fabiato & Fabiato, 1979). Solutions containing nominally 0 Ca^{2+} were made by omitting $CaCl_2$. In some experiments the ATP and GTP were omitted and 0·5 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) added (Cervetto & McNaughton, 1986). All chemicals were obtained from Sigma, except EGTA (Fluka) and 1 M $CaCl_2$ stock solution (BDH).

Experiments requiring accurately buffered Ca^{2+} solutions in the submicromolar range were prepared using the 'pHmetric' method of Tsien & Pozzan (1989). In these solutions the free [EGTA] was fixed, and Ca^{2+} added as a 1:1 Ca^{2+}:EGTA complex to displace the appropriate concentration of free Ca^{2+}. The 1:1 Ca^{2+}:EGTA mixture was prepared by adding small aliquots of 1 M $CaCl_2$ solution to an EGTA stock solution and noting the resulting change in pH until this change was reduced to less than 50% of its original size. The actual free [Ca^{2+}] in solutions prepared in this way was checked using a Ca^{2+}-selective electrode (Orion), calibrated with Ca^{2+} standards (World Precision Instruments or Molecular Probes).

Light flashes lasted 20 ms and were at 500 nm wavelength light (8 nm half-height bandwidth). All experiments were performed in darkness at room temperature (18–20 °C).

RESULTS

A fall in Ca^{2+} increased the affinity of cGMP-activated channels in truncated rod outer segments

Figure 1 shows an experiment in which the solution perfusing the inside of a truncated rod outer segment contained 10 μM GTP, to allow activation of the G-protein transducin, and 1 mM ATP, to allow the phosphorylation and deactivation of photoisomerized rhodopsin. Ten micromolar GTP does not support any appreciable cGMP synthesis by the guanylate cyclase (Kawamura & Murakami, 1989; Lagnado & Baylor, 1994; Koutalos, Nakatani & Yau, 1995) so 100 μM cGMP was included to open a fraction of cGMP-activated channels in the plasma membrane. To allow control over the free [Ca^{2+}] inside the outer segment, Ca^{2+} efflux through the Na^+–Ca^{2+}, K^+ exchange was blocked by omitting K^+ from all solutions, and Ca^{2+} influx through cGMP-activated channels was prevented by filling the suction electrode in which the outer segment was held with a solution containing 0 Ca^{2+}.

Figure 1 shows that a fall in [Ca^{2+}] increased the current flowing into the outer segment. The light-sensitive current in 1 μM Ca^{2+}, measured by the delivery of a saturating flash, was 18 pA. Switching to 0 Ca^{2+} increased the current to

about 100 pA at the end of the 25 s exposure. This action of Ca^{2+} was not fully reversed by returning to 1 μM Ca^{2+}, when the dark current stabilized at 35 pA. In this experiment the rod was truncated 60 s before the start of the record, by which time the cGMP levels inside the outer segment had achieved a steady state, as indicated by the stable dark current level before removal of Ca^{2+} (dashed line). Exposure to 0 Ca^{2+} therefore appeared to cause an irreversible potentiation of the cGMP-activated current that could not be attributed to slow diffusion of cGMP into the truncated rod.

To characterize the effect of Ca^{2+} on channel activation by cGMP, 0·5 mM IBMX was included in the perfusing solution to block basal PDE activity and allow the cGMP concentration inside the outer segment to equilibrate with that outside (Nakatani & Yau, 1988*b*). ATP and GTP were omitted from these solutions. Figure 2*A* shows that channel activation could be described by the Hill equations:

$$I_1 = C^N/(C^N + K_1^N), \quad (1)$$

and

$$I_0 = C^N/(C^N + K_0^N), \quad (2)$$

where I_1 and I_0 are the normalized currents in 1 μM Ca^{2+} and 0 Ca^{2+}, respectively; C is the cGMP concentration; K_1 and K_0 are the cGMP concentrations at which current activation is half-maximal in 1 μM Ca^{2+} and 0 Ca^{2+}, respectively, and N is the Hill coefficient reflecting co-operative binding of cGMP to the channel (Yau & Baylor, 1989). A least-squares fit of eqn (1) produced estimates (mean ± S.D.) of K_1 of 37 ± 8 μM cGMP in 1 μM Ca^{2+}, falling to K_0 of 28 ± 6 μM cGMP in 0 Ca^{2+}. The apparent Hill coefficient, N, was 2·3 ± 0·5 in 1 μM Ca^{2+} and 2·2 ± 0·6 in 0 Ca^{2+}. These results are similar to those reported by Nakatani, Koutalos & Yau (1995).

The dose–response curves in Fig. 2*A* were collected from a total of seventeen cells. However, the determination of the full curve within one cell took several minutes, during which time considerable washout of the action of Ca^{2+} occurred (see below). In an attempt to minimize skewing of the results, the effects of Ca^{2+} were tested in only three cGMP concentrations in any one cell, within about 5 min of truncation. Exposures to 0 Ca^{2+} lasted 25 s, and the current was measured at the end of this time, when the relief of the inhibitory action of Ca^{2+} was usually maximal or close to maximal (see Fig. 5*A* and *B*). Nonetheless, washout of the action of Ca^{2+} was unavoidable, causing some variability in the results, especially at cGMP concentrations of 4 and 10 μM, when the small cGMP-activated currents were more difficult to measure. In fact, the differences between K_1 measured in 1 μM Ca^{2+} and K_0 measured in 0 Ca^{2+} in Fig. 2*A* were not statistically significant. However, similar relative changes in affinity were measured when IBMX was omitted and 1 mM ATP included in the perfusing solution (results not shown). In eight cells the average K_1 under these conditions was 262 ± 18 μM cGMP in 1 μM Ca^{2+}, falling to a K_0 of 176 ± 27 μM cGMP in 0 Ca^{2+}. This change was significant ($P < 0·5\%$). The apparent Hill coefficient (1·5 ± 0·4) was the same in both cases. Washout or changes in Ca^{2+} did not affect the maximum current measured in saturating cGMP concentrations.

In the intact rod in darkness, only about 2% of the cGMP-activated channels are open (Yau & Baylor, 1989), so the

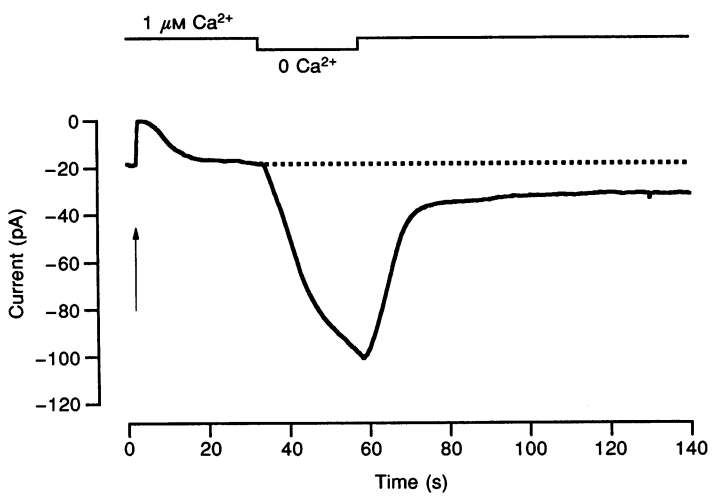

Figure 1. Removing Ca^{2+} increased the current flowing into the outer segment

Recording from a cell perfused with 100 μM cGMP, 1 mM ATP and 10 μM GTP. Stepping from 1 μM Ca^{2+} to 0 Ca^{2+} for 25 s increased the inward current 6-fold (measured at the end of the exposure to 0 Ca^{2+}). Replacing 1 μM Ca^{2+} inhibited the current again but recovery was not back to the previous level (shown by the dashed line). Flash delivered at arrow (90 s after truncation) was 17 photons μm^{-2}. The timing of the solution changes is shown by the line above the trace. IBMX was absent in this experiment.

normal range of cGMP concentrations is at the foot of the dose–response curves in Fig. 2*A*. From eqns (1) and (2), the relative activation of current when Ca^{2+} is removed is:

$$I_0/I_1 = (C^N + K_1^N)/(C^N + K_0^N), \quad (3)$$

which at low [cGMP] tends to:

$$I_0/I_1 = (K_1/K_0)^N. \quad (4)$$

However, from eqns (1) and (2),

$$(K_1/K_0)^N = I_0(1 - I_1)/I_1(1 - I_0), \quad (5)$$

so

$$I_0/(1 - I_0) = (K_1/K_0)^N(I_1/(1 - I_1)). \quad (6)$$

$I_0/(1 - I_0)$ is plotted against $I_1/(1 - I_1)$ in Fig. 2*B*. From eqns (4) and (6), the gradient of the line is equivalent to the

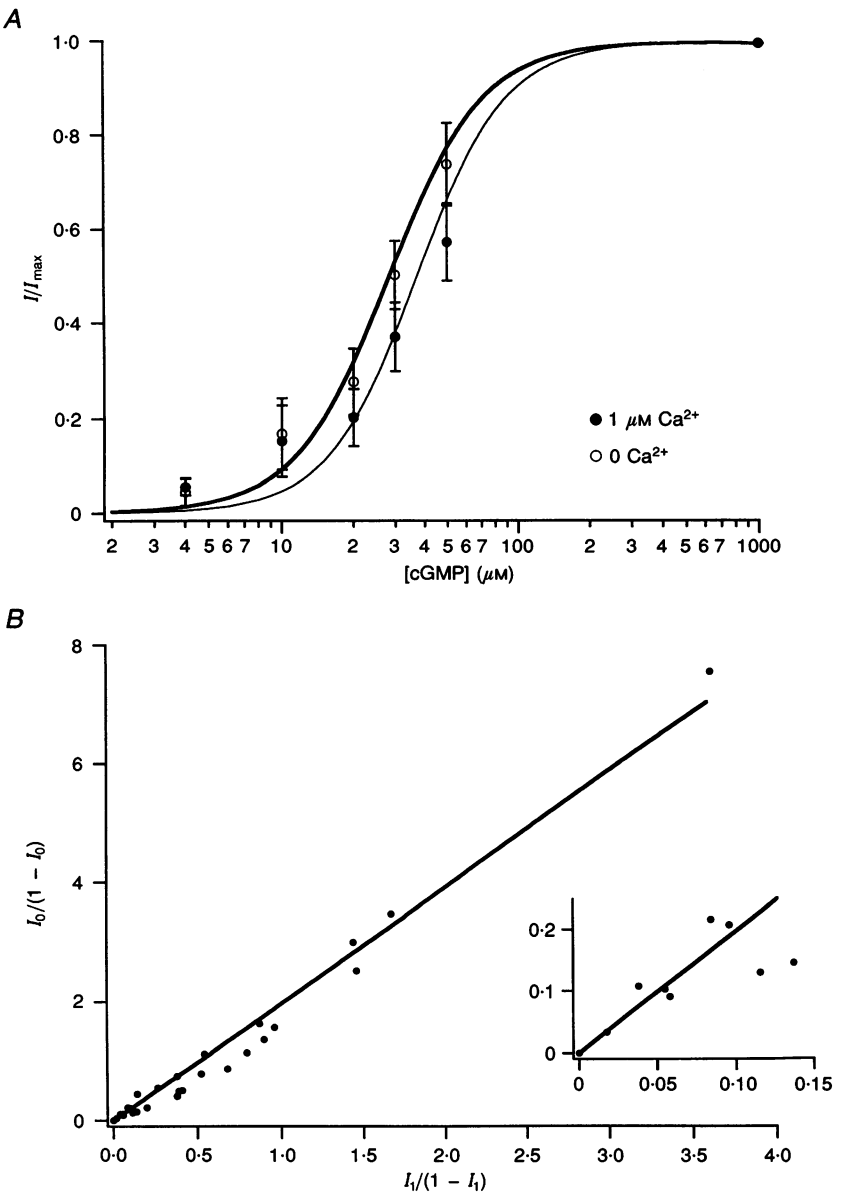

Figure 2. Effect of Ca^{2+} on the cGMP dependence of channel activation

A, the normalized current (I/I_{max}) is plotted against the [cGMP] in the perfusing solution, in 1 μM Ca^{2+} (●) and 0 Ca^{2+} (○). Each point is the mean ± S.E.M. The number of cells used were 3, 8, 6 and 4 cells for 10, 20, 30 and 50 μM cGMP in both 1 μM and 0 Ca^{2+}. For 4 μM cGMP there were 5 cells in 1 μM Ca^{2+} and 3 cells in 0 Ca^{2+}. Curves are least-squares fits of the Hill equation. In 1 μM Ca^{2+} (thin line) values of K_1 and N are 37 μM cGMP and 2·3, respectively, and in 0 Ca^{2+} (thick line) values of K_0 and N are 28 μM cGMP and 2·2, respectively. IBMX (0·5 mM) was present in all the solutions used in these experiments. *B*, the quantity $I_0/(1 - I_0)$ is plotted against $I_1/(1 - I_1)$ (see text). The straight line through the points passes through the origin and has a gradient of 1·96. Inset shows that the line is a good fit to the points at the foot of the graph.

relative activation of current at low levels of cGMP. The line was obtained by a least-squares fit constrained to pass through the origin and has a gradient of 1·96 ± 0·06. The advantage of plotting the results from Fig. 2*A* in this way is that each point is obtained from a single measurement of the relative activation of current on removing Ca^{2+}. Thus, within a given cell the action of Ca^{2+} was clear at a range of cGMP concentrations, including those activating less than 10% of channels (shown more clearly in the inset to Fig. 2*B*).

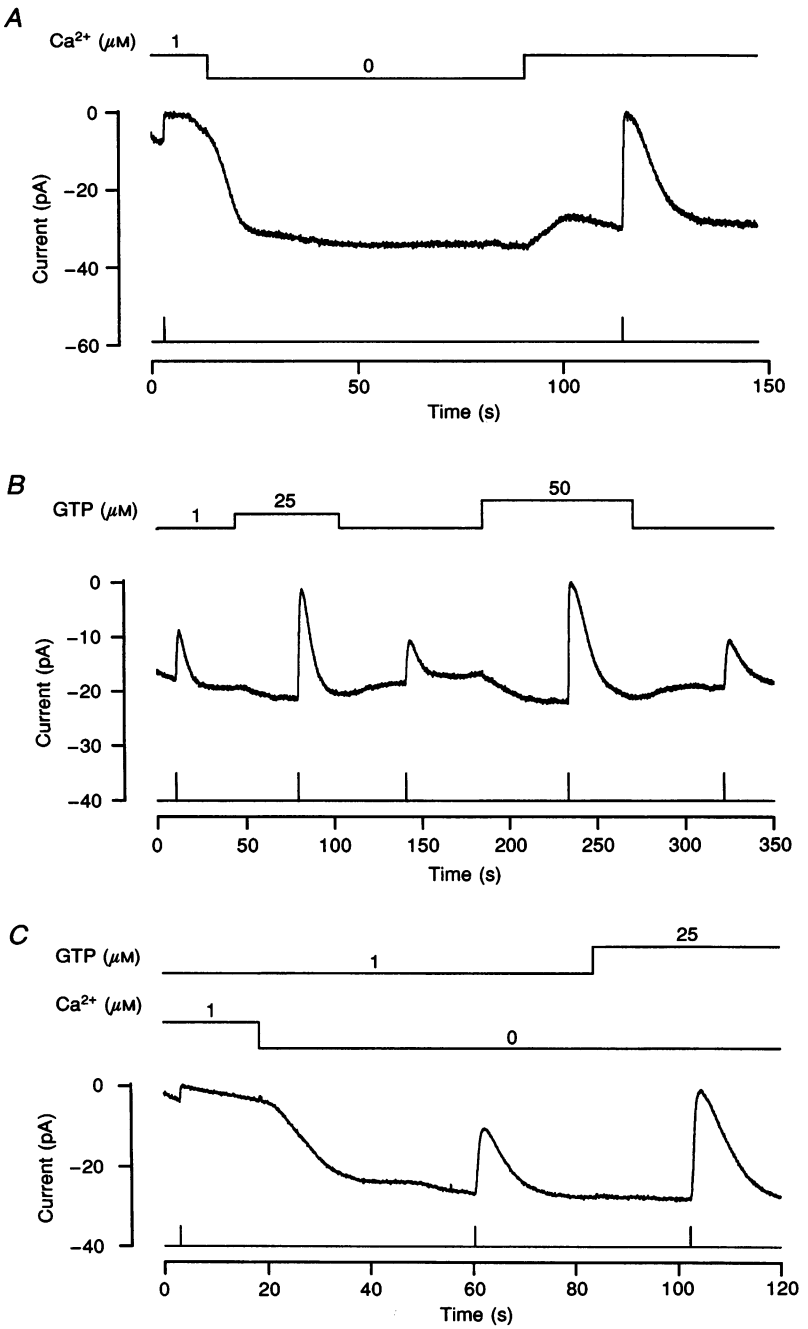

Figure 3. The GTP concentration inside the outer segment was effectively controlled

A, prolonged exposure to 0 Ca^{2+} caused a maintained increase in the cGMP-activated current. The response did not decline, as would be expected if it was dependent on endogenous GTP that slowly washed out of the outer segment after truncation. Flashes delivered 22 photons μm^{-2}. First flash 6 s after truncation (as indicated by the tick mark below the trace). *B*, changes in the GTP concentration inside the outer segment could be monitored using the amplitude of the response to a dim flash of fixed strength (12 photons μm^{-2}). Zero Ca^{2+} throughout this part of the trace. First flash 84 s after truncation. *C*, increasing GTP from 1 to 25 μM increased the amplitude of the flash response during the maintained increase in cGMP-activated current caused by exposure to 0 Ca^{2+}. Flashes delivered 12 photons μm^{-2}. First flash 19 s after truncation.

The construction of dose–response curves therefore indicated that Ca^{2+} reduced the affinity of the cGMP-activated channels without affecting the degree of co-operativity with which cGMP bound to the channel. However, this approach underestimated the true extent of the action of Ca^{2+} on channel affinity. The modulation of the cGMP-activated current was consistently greater than 2-fold when measured soon after truncation. For instance, the cell in Fig. 1, which was truncated 60 s before the start of the record, showed a 6-fold activation of current on removing Ca^{2+}. Below we show that these larger currents are unlikely to be caused by activation of the guanylate cyclase.

Was the cGMP concentration in the outer segment effectively controlled?

Although the perfusate contained only 10 μM GTP, the intact rod is expected to contain higher concentrations to support cGMP synthesis. If this endogenous GTP washed out slowly after truncation, activation of the guanylate cyclase would contribute to the increase in current on removing Ca^{2+}, although the current would then fall as GTP washed out. However, responses to prolonged exposures to 0 Ca^{2+} were maintained, as shown in Fig. 3*A*. The first flash in this record was delivered 6 s after truncation. Removing Ca^{2+} caused a 4·6-fold activation of current which was maintained for the 78 s until 1 μM Ca^{2+} was replaced. A similar observation is shown in Fig. 3*C*, where the first flash was delivered 19 s after truncation and exposure to 0 Ca^{2+} caused an 8-fold activation of current that was maintained for 100 s. Similar results were observed in all five cells tested in this way.

We made further tests of our ability to control the GTP concentration in the rod outer segment by using the amplitude of the flash response as an assay for GTP levels. Figure 3*B* shows a series of responses to a flash of fixed strength, all obtained in 0 Ca^{2+}. Increasing the GTP concentration in the perfusate from 1 to 25 to 50 μM progressively increased the amplitude of the response, and this effect rapidly reversed on returning to 1 μM GTP.

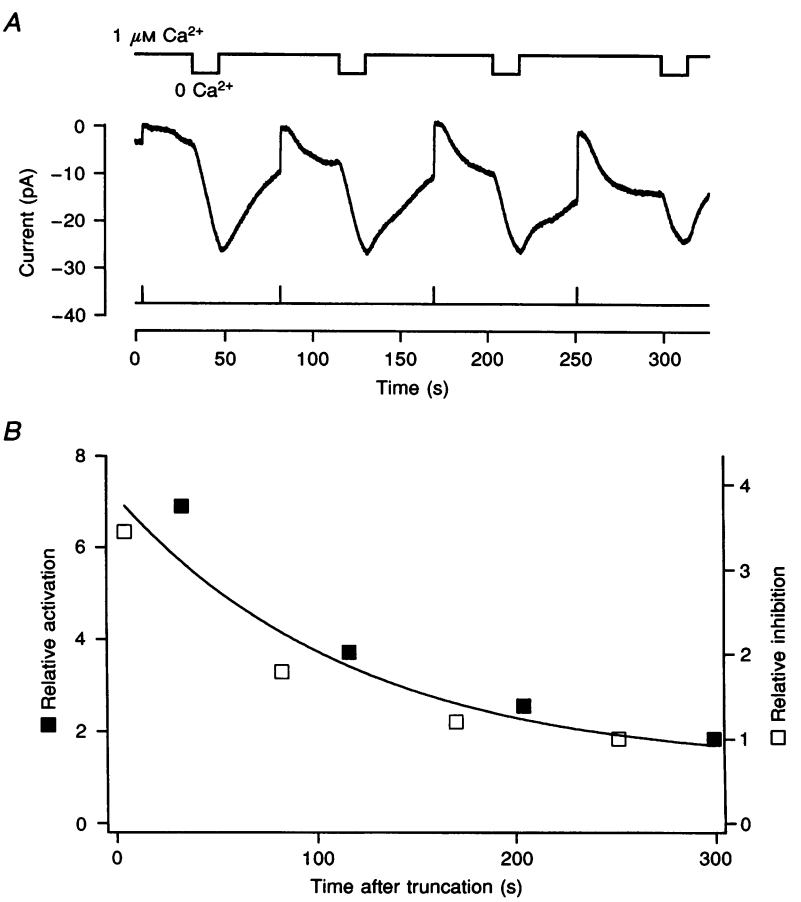

Figure 4. Washout of the action of Ca^{2+} on the cGMP-activated current

A, repeated exposures to 0 Ca^{2+} progressively increased the cGMP-activated current present in 1 μM Ca^{2+} and decreased the relative activation of current on removing Ca^{2+}. First flash 5 s after truncation (as indicated by the tick mark below the trace). All flashes 22 photons μm^{-2}. *B*, graph plotting the relative activation of current on removing Ca^{2+} at various times after truncation (■, left-hand axis). Results from cell in *A*. Also shown is the inhibition of the dark current in 1 μM Ca^{2+} normalized to the current at the end of the record (□, right-hand axis). The two quantities declined with a time constant of about 120 s, as shown by the line fitted to the two sets of points.

Similar increases in the size of the flash response were observed soon after truncation, as shown in Fig. 3*C*. In this case, the GTP concentration was increased from 1 to 25 μM during the maintained response to 0 Ca^{2+}. The increase in the size of the flash response indicates that GTP in the outer segment was effectively controlled over the concentration range 1–25 μM. There is negligible stimulation of cGMP synthesis at these GTP levels (Koutalos *et al.* 1995). For instance, in Fig. 3*B*, switching from 1 to 25 μM cGMP caused a very small increase in the cGMP-activated current, while in Fig. 3*C* it had no effect. Similar results were obtained in five cells.

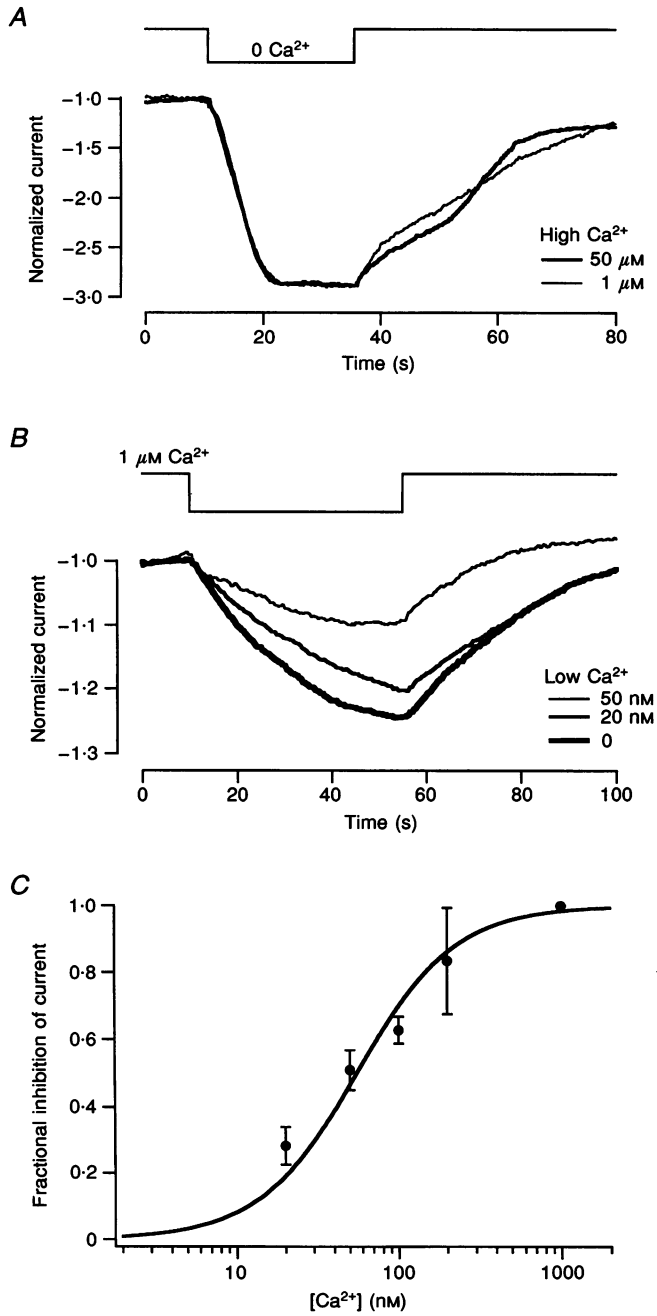

Figure 5. The Ca^{2+} dependence of the cGMP-activated current

A, the [Ca^{2+}] inside the outer segment is efficiently controlled. The activation of current on stepping to 0 Ca^{2+} followed the same time course whether starting from 1 μM Ca^{2+} (thin trace) or 50 μM Ca^{2+} (thick trace). Increasing the resting [Ca^{2+}] to 50 μM had no significant effect on the resting current. *B*, traces from 1 cell showing switches from 1 μM Ca^{2+} to 50, 20 and 0 nM Ca^{2+} solutions. Less current is activated as the [Ca^{2+}] is raised. *C*, inhibition of the cGMP-activated current by Ca^{2+}. Each point (mean ± S.E.M.) is based on at least 4 cells and is normalized to the maximal inhibition observed in 1 μM Ca^{2+}. The fit is drawn according to the Hill equation with half-maximal suppression at 55 nM Ca^{2+} and a Hill coefficient of 1·4.

Most of the experiments measuring the action of Ca^{2+} on channel activation were carried out in the presence of 10 μM GTP. The results in Fig. 3 therefore indicated that the maintained increase in the cGMP-activated current on removing Ca^{2+} was primarily due to an increase in channel affinity rather than stimulation of cGMP synthesis.

Washout of the action of Ca^{2+} on channel affinity

We often observed that a gradual loss of action of Ca^{2+} on the affinity of the cGMP-activated channel occurred in parallel with an increase in the cGMP-activated current observed in 1 μM Ca^{2+}. An example is shown in Fig. 4*A*, where 20 s exposures to 0 Ca^{2+} were repeated regularly, interspersed by saturating flashes. The maximal current during a short exposure to 0 Ca^{2+} was relatively constant, as would be expected from the stable current observed during prolonged exposures to 0 Ca^{2+} (Fig. 3). However, the current in 1 μM Ca^{2+} gradually increased. The time course over which the action of Ca^{2+} washed out from this cell is plotted in Fig. 4*B*. The filled symbols plot the relative activation of current on removing Ca^{2+} at various times after truncation, and the open symbols plot the inhibition of the dark current in 1 μM Ca^{2+} (measured relative to the current at the end of the record). The two quantities declined with a time constant of about 120 s. This is consistent with the idea that the affinity of the cGMP-activated channels is reduced by a Ca^{2+}-dependent protein that can be lost from the truncated outer segment, so that the affinity of the channels can be increased either by lowering Ca^{2+} or by loss of the protein. When the protein is lost, there is an increase in the cGMP-activated current even when Ca^{2+} is high, and a concomitant reduction in the relative increase in current when switching to 0 Ca^{2+}. Again, these results cannot be accounted for by a gradual washout of endogenous GTP, which would be expected to reduce the current observed in 0 Ca^{2+}, but leave the current in 1 μM Ca^{2+} unaffected.

Both Nakatani *et al.* (1995) and Gordon *et al.* (1995) have suggested that washout of the inhibitory action of Ca^{2+} on

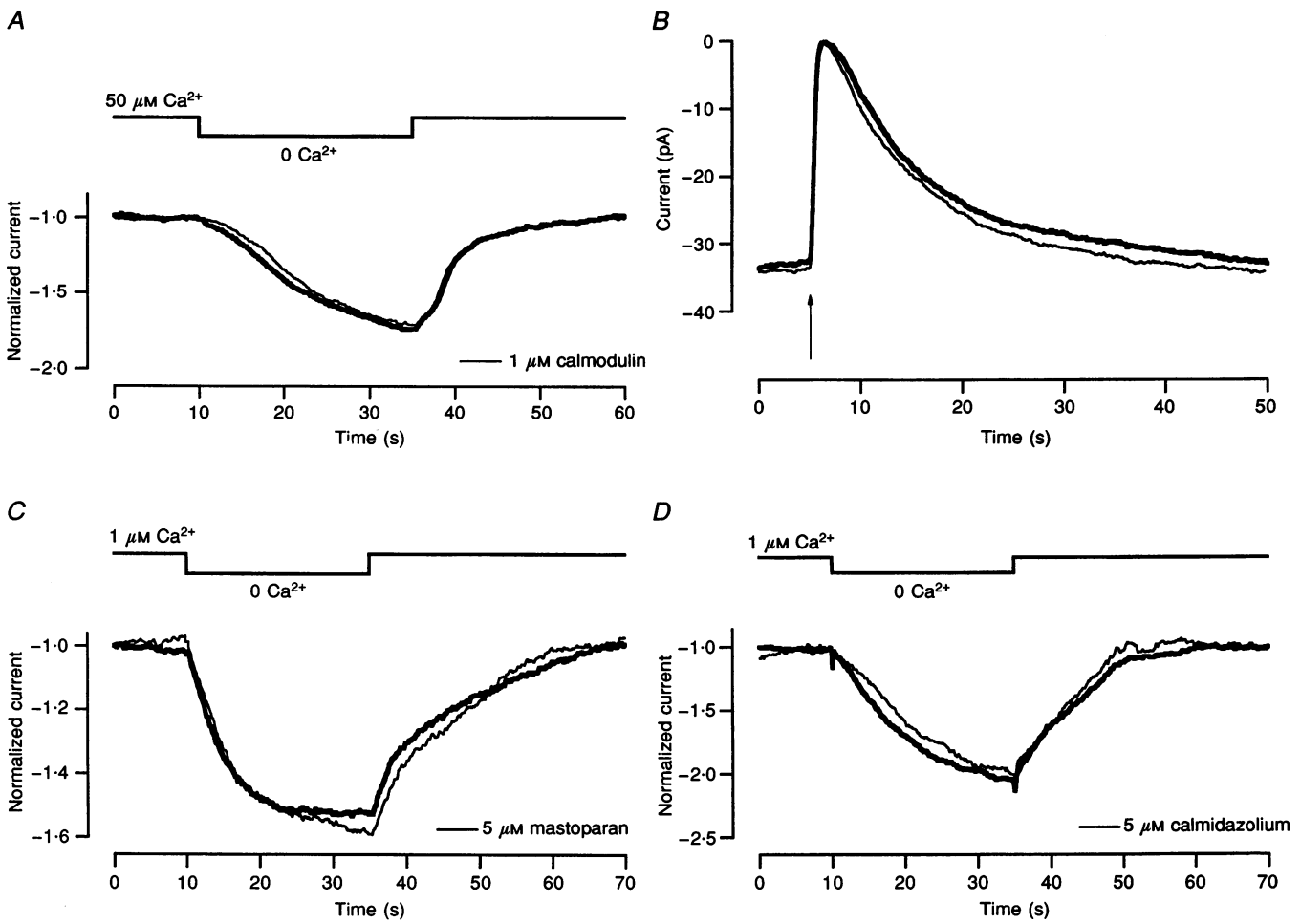

Figure 6. Calmodulin did not modulate the cGMP-activated current

A, traces show a switch from 50 μM Ca^{2+} to 0 Ca^{2+}, with (thin trace) and without (thick trace) 1 μM calmodulin. The control trace was obtained after the calmodulin trace. *B*, flash responses (24 photons μm^{-2} at arrow) in the presence and absence of 1 μM calmodulin. *C*, traces show a switch from 1 μM Ca^{2+} to 0 Ca^{2+}, with (thin trace) and without (thick trace) 5 μM mastoparan. *D*, traces show a switch from 1 μM Ca^{2+} to 0 Ca^{2+}, with (thin trace) and without (thick trace) 5 μM calmidazolium.

the cGMP-activated channel may be accelerated by exposure to low [Ca^{2+}]. This idea is supported by the experiments in Figs 1 and 3*A*, in which exposure to 0 Ca^{2+} caused a permanent increase in the cGMP-activated current present in 1 μM Ca^{2+}. The time course of washout measured in Fig. 4 may therefore partly reflect the duration and frequency of the exposures to 0 Ca^{2+} used to quantify the inhibitory action of Ca^{2+}. Varying degrees of washout were observed in over 100 cells.

The activation of current on switching from 1 μM to 0 Ca^{2+} averaged 7·1 ± 0·4-fold when measured in the first 30–60 s after truncation (17 cells). The range was 3·5- to 12-fold. These measurements were made in 100 μM cGMP in the absence of IBMX, when 30% of the channels were open in 0 Ca^{2+}. While a small contribution from the guanylate cyclase cannot be entirely excluded, the results in Fig. 3 indicate that this effect was primarily due to an increase in the affinity of the cGMP-gated channels.

The time scale of the action of Ca^{2+} on channel affinity

Recovery of the light-sensitive current after a flash occurs with a time constant of about 6 s in truncated rod outer segments perfused with 1 mM ATP (Lagnado & Baylor, 1994) and Fig. 1 shows that the increase in the cGMP-activated current when Ca^{2+} was removed occurred on a similar time scale. In seventy-nine cells the half-time ($t_{½}$) for activation was 6·7 ± 0·3 s (mean ± S.E.M.). Inhibition of current on replacing 1 μM Ca^{2+} was significantly slower, with a $t_{½}$ of 11·0 ± 0·6 s. To test whether the rate of current activation might reflect the time course of the fall in [Ca^{2+}] inside the outer segment, we compared the current activation on removing 1 μM Ca^{2+} with activation on removing 50 μM Ca^{2+}. Figure 5*A* shows that starting from a higher [Ca^{2+}] did not delay or slow current activation on stepping to 0 Ca^{2+}, indicating that the experimental technique allowed rapid control over the [Ca^{2+}] inside the outer segment. As in this experiment, the inhibition of current on raising [Ca^{2+}] often showed a sigmoidal time course.

Ca^{2+} dependence of channel modulation

Figure 5*B* shows an experiment in which the [Ca^{2+}] inside the outer segment was switched from 1 μM Ca^{2+} to 50, 20 and 0 nM Ca^{2+}. In this cell the half-maximal inhibition of the cGMP-activated current occurs between 20 and 50 nM Ca^{2+}. Figure 5*C* plots the Ca^{2+} dependence of current suppression. Results are normalized to the maximal suppression in 1 μM Ca^{2+}. The maximum current in 0 Ca^{2+} and the minimum in 1 μM Ca^{2+} were measured before and after each exposure to a test [Ca^{2+}] to minimize skewing of the results by washout. One micromolar Ca^{2+} maximally suppressed the cGMP-activated current since no further suppression could be detected in 50 μM Ca^{2+} (Fig. 5*A*). The points in Fig. 5*B* could be described by a Hill curve with half-maximal current suppression at 55 ± 13 nM Ca^{2+} and a Hill coefficient of 1·4 ± 0·4. These results indicate that modulation of the cGMP-activated channels occurs at physiological Ca^{2+} concentrations.

Calmodulin and calmodulin inhibitors did not affect the cGMP-activated current

We investigated whether the modulator of cGMP-activated channels lost from the truncated outer segments in our experiments might be calmodulin since calmodulin in the Ca^{2+}-bound state has been reported to inhibit cGMP-activated channels (Hsu & Molday, 1993; Chen *et al.* 1994). In the experiment shown in Fig. 6*A*, the endogenous factor controlling the affinity of the channel was allowed to wash out and then 1 μM calmodulin was applied in the presence of 50 μM Ca^{2+} to ensure that it was fully Ca^{2+} loaded. Calmodulin did not alter the activation of current measured on removing Ca^{2+} (Fig. 6*A*) nor the resting dark current or response to a flash (Fig. 6*B*). In eleven cells, application of calmodulin did not inhibit the cGMP-activated current early in an experiment or after washout of the endogenous inhibitor had occurred.

Calmodulin inhibitors were also without effect. In four cells, 5 μM mastoparan did not alter the current measured in 1 μM Ca^{2+} or the activation of current measured on removing Ca^{2+} (Fig. 6*C*). Similarly, 5 μM calmidazolium was without effect in two cells (Fig. 6*D*). These results do not support the idea that calmodulin normally regulates cGMP-activated channels in the rod outer segment. Rod outer segments also contain a 23 kDa Ca^{2+}-binding protein called recoverin. Bovine myristoylated recoverin (10 μM) did not inhibit the cGMP-activated current when applied in the presence of 50 μM Ca^{2+} (2 cells, not shown). Our results support the idea that there is another, as yet unidentified, Ca^{2+}-binding protein that can inhibit the cGMP-activated channel.

DISCUSSION

Inhibition by Ca^{2+} of cGMP-activated channels in truncated salamander rods was considerably greater than previously measured in truncated bullfrog rods (Nakatani *et al.* 1995), excised patches (Chen *et al.* 1994; Gordon *et al.* 1995) or vesicles of outer segment membrane (Hsu & Molday, 1993). At low levels of cGMP, the current could be modulated over a 7-fold range when measured in the first 30–60 s after truncation. After about 5 min modulation was only about 2-fold, which is similar to the value measured by Nakatani *et al.* (1995) using measurement of dose–response relations. In this study we also applied test changes in [Ca^{2+}] at low levels of cGMP to rapidly quantify channel modulation at short times after truncation.

By imposing fast changes in [Ca^{2+}] we also found that a fall in [Ca^{2+}] increased the cGMP-activated current on the time scale of seconds and with negligible delay (Fig. 5*A*). Current activation was half-maximal at about 55 nM Ca^{2+} (Fig. 5*B*). In the intact rod outer segment the free [Ca^{2+}] in the dark is about 500 nM (McNaughton *et al.* 1986; Ratto, Payne, Owen & Tsien, 1988; Lagnado, Cervetto & McNaughton, 1992; Gray-Keller & Detwiler, 1994), when we expect that the cGMP-activated channels will be maximally inhibited. When all the channels are closed by bright light the free

[Ca^{2+}] falls (McNaughton *et al.* 1986; Lagnado *et al.* 1992); estimates vary between a value close to zero (Cervetto *et al.* 1989; Younger, McCarthy & Owen, 1992; McCarthy, 1993) and 50 nM (Gray-Keller & Detwiler, 1994). The free [Ca^{2+}] falls with a time constant of 0·5 s after channel closure (Yau & Nakatani, 1985; McNaughton *et al.* 1986; Lagnado *et al.* 1992), although a second slower component with a time constant of 7 s has also been reported by Gray-Keller & Detwiler (1994). The speed with which a fall in [Ca^{2+}] increased the cGMP-activated current (Fig. 5*A*) suggests that channel inhibition will be rapidly relieved during the light-induced fall in Ca^{2+}, promoting recovery from steady lights and bright flashes lasting longer than a few seconds. Recovery from dim flashes is complete in just 3–4 s, so is less likely to involve changes in channel affinity.

The mechanism by which Ca^{2+} inhibited the cGMP-activated current is not clear. The rapid washout of this effect indicates that a small soluble mediator is involved, and it is simplest to suppose that this is also the Ca^{2+} sensor, since there are a variety of small soluble Ca^{2+}-binding proteins in the rod outer segment. The most obvious possibility was calmodulin, since this has been reported to modulate cyclic nucleotide-gated channels from both rods (Hsu & Molday, 1993; Gordon *et al.* 1995) and olfactory receptors (Chen & Yau, 1994). However, calmodulin and calmodulin inhibitors were without effect (Fig. 6). Gray-Keller, Polans, Palczewski & Detwiler (1993) have also reported that calmodulin does not affect the light response of intact rods. It may be that the mechanisms controlling the cGMP-activated channel in an intact outer segment are different to those controlling the channels in excised patches, perhaps because the process of patch excision alters channel properties (Karpen, Loney & Baylor, 1992). Interestingly, Gordon *et al.* (1995) found that cGMP-activated channels in patches of outer segment membrane from frog rods could be modulated not just by exogenous calmodulin, but also by an endogenous inhibitory factor associated with the channels. It seems that loss of this factor from truncated rods is accelerated by exposure to low [Ca^{2+}] (Fig. 1), suggesting that the protein becomes more soluble when it is in the Ca^{2+}-free state and its action on the cGMP-activated channel is relieved.

Baylor, D. A., Lamb, T. D. & Yau, K.-W. (1979). The membrane current of single rod outer segments. *Journal of Physiology* **288**, 589–611.

Cervetto, L., Lagnado, L., Perry, R. J., Robinson, D. W. & McNaughton, P. A. (1989). Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. *Nature* **337**, 740–743.

Cervetto, L. & McNaughton, P. A. (1986). The effects of phosphodiesterase inhibitors and lanthanum ions on the light-sensitive current of toad retina rods. *Journal of Physiology* **370**, 91–109.

Chen, T. Y., Illing, M., Molday, L. L., Hsu, Y. T., Yau, K.-W. & Molday, R. S. (1994). Subunit 2 (or beta) of retinal rod cGMP-gated cation channel is a component of the 240-kDa channel-associated protein and mediates Ca^{2+}–calmodulin modulation. *Proceedings of the National Academy of Sciences of the USA* **91**, 11757–11761.

Chen, T. Y. & Yau, K.-W. (1994). Direct modulation by Ca^{2+}–calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. *Nature* **368**, 545–548.

Fabiato, A. & Fabiato, J. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de Physiologie* **75**, 463–505.

Fesenko, E. E., Kolesnikov, S. S. & Lyubarsky, A. L. (1985). Induction by cyclic GMP of cationic conductance in plasma membrane of retinal rod outer segment. *Nature* **313**, 310–313.

Gordon, S. E., Downing-Park, J. & Zimmerman, A. L. (1995). Modulation of the cGMP-gated ion channel in frog rods by calmodulin and an endogenous inhibitory factor. *Journal of Physiology* **486**, 533–546.

Gray-Keller, M. P. & Detwiler, P. B. (1994). The calcium feedback signal in the phototransduction cascade of vertebrate rods. *Neuron* **13**, 849–861.

Gray-Keller, M. P., Polans, A. S., Palczewski, K. & Detwiler, P. B. (1993). The effect of recoverin-like calcium-binding proteins on the photoresponse of retinal rods. *Neuron* **10**, 523–531.

Hodgkin, A. L., McNaughton, P. A. & Nunn, B. J. (1985). The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. *Journal of Physiology* **358**, 447–468.

Hodgkin, A. L., McNaughton, P. A. & Nunn, B. J. (1987). Measurement of sodium–calcium exchange in salamander rods. *Journal of Physiology* **391**, 347–370.

Hsu, Y. T. & Molday, R. S. (1993). Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature* **361**, 76–79.

Karpen, J. W., Loney, D. A. & Baylor, D. A. (1992). Cyclic GMP-activated channels of salamander retinal rods: spatial distribution and variation of responsiveness. *Journal of Physiology* **448**, 257–274.

Kawamura, S. (1993). Rhodopsin phosphorylation as a mechanism of cyclic GMP phosphodiesterase regulation by S-modulin. *Nature* **362**, 855–857.

Kawamura, S. & Murakami, M. (1989). Regulation of cGMP levels by guanylate cyclase in truncated frog rod outer segments. *Journal of General Physiology* **94**, 649–668.

Koch, K. W. & Stryer, L. (1988). Highly cooperative feedback control of retinal rod guanylate cyclase by calcium ions. *Nature* **334**, 64–66.

Koutalos, Y., Nakatani, K. & Yau, K.-W. (1995). Characterization of guanylate cyclase activity in single retinal rod outer segments. *Journal of General Physiology* **106**, 863–890.

Lagnado, L. & Baylor, D. (1992). Signal flow in visual transduction. *Neuron* **8**, 995–1002.

Lagnado, L. & Baylor, D. A. (1994). Calcium controls light-triggered formation of catalytically active rhodopsin. *Nature* **367**, 273–277.

Lagnado, L., Cervetto, L. & McNaughton, P. A. (1992). Calcium homeostasis in the outer segments of retinal rods from the tiger salamander. *Journal of Physiology* **455**, 111–142.

Lamb, T. D. & Pugh, E. N. Jr (1992). G-protein cascades: gain and kinetics. *Trends in Neurosciences* **15**, 291–298.

LOLLEY, R. N. & RACZ, E. (1982). Calcium modulation of cyclic GMP synthesis in rat visual cells. *Vision Research* **22**, 1481–1486.

MCCARTHY, S. T. (1993). Calcium, cyclic GMP metabolism and light adaptation in rods of the bullfrog. PhD Thesis, University of California, Berkeley, USA.

MCNAUGHTON, P. A., CERVETTO, L. & NUNN, B. J. (1986). Measurement of the intracellular free calcium concentration in salamander rods. *Nature* **322**, 261–263.

MATTHEWS, H. R., MURPHY, R. L., FAIN, G. L. & LAMB, T. D. (1988). Photoreceptor light adaptation is mediated by cytoplasmic calcium concentration. *Nature* **334**, 67–69.

NAKATANI, K., KOUTALOS, Y. & YAU, K.-W. (1995). Ca^{2+} modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors. *Journal of Physiology* **484**, 69–76.

NAKATANI, K. & YAU, K.-W. (1988*a*). Calcium and light adaptation in retinal rods and cones. *Nature* **334**, 69–71.

NAKATANI, K. & YAU, K.-W. (1988*b*). Guanosine 3′-5′-cyclic monophosphate-activated conductance studied in a truncated rod outer segment of the toad. *Journal of Physiology* **395**, 731–753.

RATTO, G. M., PAYNE, R., OWEN, W. G. & TSIEN, R. Y. (1988). The concentration of cytosolic free calcium in vertebrate rod outer segments measured with fura-2. *Journal of Neuroscience* **8**, 3240–3246.

SAGOO, M. S. & LAGNADO, L. (1995). Calcium regulates the cGMP-activated current in isolated rod photoreceptors of the tiger salamander. *Journal of Physiology* **483.P**, 59*P*.

STRYER, L. (1986). The cyclic nucleotide cascade of vision. *Annual Review of Neuroscience* **9**, 87–119.

TSIEN, R. & POZZAN, T. (1989). Measurement of cytosolic free Ca^{2+} with quin-2. *Methods in Enzymology* **172**, 231–263.

YAU, K.-W. (1991). Calcium and light adaptation in retinal photoreceptors. *Current Opinion in Neurobiology* **1**, 252–257.

YAU, K.-W. & BAYLOR, D. A. (1989). Cyclic GMP-activated conductance of retinal photoreceptor cells. *Annual Review of Neuroscience* **12**, 289–327.

YAU, K.-W. & NAKATANI, K. (1985). Light-induced reduction of cytoplasmic free calcium in retinal rod outer segment. *Nature* **313**, 579–582.

YOUNGER, J. P., MCCARTHY, S. T. & OWEN, W. G. (1992). Modulation of cytosolic free calcium and changes in sensitivity and circulating current occur over the same range of steady-state adapting lights in rod photoreceptors. *Investigative Ophthalomology and Visual Science* **33**, 1104.

Acknowledgements

We are grateful to Mr Christopher Raeburn of the MRC Laboratory of Molecular Biology Instrumentation Workshop for his assistance and Professor Denis Baylor for his comments on the manuscript. This work was supported by the MRC and Human Frontiers in Science Program. M.S.S. holds a Cambridge MB, PhD Programme Scholarship.

Author's email address

L. Lagnado: ll1@mrc-lmb.cam.ac.uk

Received 28 June 1996; accepted 2 September 1996.