Ca^{2+} modulation of the cGMP-gated channel of bullfrog retinal rod photoreceptors

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1. The outer segment of an isolated rod photoreceptor from the bullfrog retina was drawn into a pipette containing choline solution for recording membrane current. The rest of the cell was sheared off with a glass probe to allow internal dialysis of the outer segment with a bath potassium solution ('truncated rod outer segment' preparation). The potential between the inside and the outside of the pipette was held at 0 mV.

2. Application of bath cGMP, in the presence of 3-isobutyl-1-methylxanthine (IBMX), gave rise to an outward membrane current. At saturating cGMP concentrations, this current was insensitive to intracellular Ca^{2+} at concentrations between 0 and 10 μM. At sub-saturating cGMP concentrations, however, this current was inhibited by intracellular Ca^{2+}. This sensitivity to Ca^{2+} declined after dialysis with a low-Ca^{2+} solution, suggesting the involvement of a soluble factor.

3. At low (nominally 0) Ca^{2+}, the half-maximal activation constant and Hill coefficient for the activation of the cGMP-gated current by cGMP were 27 μM and 2·0, respectively. At high (*ca* 10 μM) Ca^{2+}, the corresponding values were 40 μM cGMP and 2·4.

4. The inhibition of the current by Ca^{2+} was characterized at 20 μM cGMP. Ca^{2+} inhibited the current by up to 60%, with half-maximal inhibition at 48 nM Ca^{2+} and a Hill coefficient of 1·6.

Visual transduction takes place in the outer segments of retinal rod and cone photoreceptor cells. In this process, photoisomerization of the visual pigment triggers a biochemical cascade that stimulates a cGMP-phosphodiesterase and thus the hydrolysis of cGMP (for recent reviews, see Detwiler & Gray-Keller, 1992; Lagnado & Baylor, 1992; Pugh & Lamb, 1993; Koutalos & Yau, 1993; Yau, 1994). In darkness, cytoplasmic cGMP binds to and opens cGMP-activated cation channels on the plasma membrane of the outer segment (see Yau & Baylor, 1989, for review). These open channels sustain an inward dark current, which keeps the cell partially depolarized and causes a steady release of neurotransmitter from the synaptic terminal of the photoreceptor. In the light, the hydrolysis of cGMP leads to the closure of the channels, producing a membrane hyperpolarization as the electrical response. This hyperpolarization reduces synaptic transmitter release from the cell.

An important property of photoreceptor cells is their ability to adapt to background light. This adaptation involves a negative feedback regulation on phototransduction, mediated by a change in internal Ca^{2+}. In the dark, Ca^{2+} enters the outer segment through the cGMP-gated channels and is continuously extruded by a Na^+–Ca^{2+}, K^+ exchanger (Yau & Nakatani, 1984; Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989). When the channels close as a result of illumination, the Ca^{2+} influx through the channels stops, but the efflux through the exchanger continues, thus resulting in a decrease in the cytoplasmic Ca^{2+} concentration in the outer segment. This Ca^{2+} reduction triggers a negative feedback to produce light adaptation. When Ca^{2+} is experimentally prevented from falling in the light, the photoreceptors essentially fail to adapt to background light (Matthews, Murphy, Fain & Lamb, 1988; Nakatani & Yau, 1988*a*).

Ca^{2+} is now known to exert its effect through several pathways, all of which lead to negative feedback control on phototransduction. One of these is an inhibition by Ca^{2+} of the guanylate cyclase enzyme which synthesizes cGMP (see, for example, Koch & Stryer, 1988). Another is an enhancement by Ca^{2+} of the light stimulation of the cGMP-phosphodiesterase, with the sites of action at the

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inactivation step of rhodopsin and possibly also another target (Kawamura & Murakami, 1991; Kawamura, 1993; Gray-Keller, Polans, Palczewski & Detwiler, 1993; Lagnado & Baylor, 1994). Finally, Ca^{2+} also reduces the apparent affinity of the cGMP-gated channel for cGMP through a Ca^{2+}-binding protein, perhaps calmodulin (Hsu & Molday, 1993; Gordon & Zimmerman, 1994; Chen, Illing, Molday, Hsu, Yau & Molday, 1994; see also Gordon, Downing-Park & Zimmerman, 1995).

With the ultimate aim of understanding the relative importance of each of the above Ca^{2+}-mediated pathways in phototransduction, we have undertaken their quantitative characterizations. In this paper we describe experiments on the Ca^{2+} modulation of the cGMP-gated channel. In previous studies on this subject, the experiments were carried out either at low ionic strength (Hsu & Molday, 1993) or in the absence of divalent cations (Gordon & Zimmerman, 1994), conditions that could affect the Ca^{2+} dependence of the channel modulation (see, for example, Kilhoffer, Haiech & Demaille, 1983). Accordingly, our experiments were carried out under intracellular ionic conditions close to the physiological situation. At the same time, we have tried to avoid perturbing the concentrations of the soluble Ca^{2+}-binding proteins in the intact rod outer segment as much as possible. For these purposes, we have adopted the truncated rod outer segment preparation (Yau & Nakatani, 1985; Nakatani & Yau, 1988*c*), which allows recording of membrane current from the outer segment and also manipulation of the internal Ca^{2+} concentration.

METHODS

The experiments were carried out primarily on bullfrogs (*Rana catesbeiana*), but a few larval tiger salamanders (*Ambystoma tigrinum*) were also used. Animals were decapitated and pithed under dim red light. All subsequent procedures were performed in infrared light using image converters. After enucleation, the eyes were hemisected, and the retinas isolated into amphibian Ringer solution which contained (mM): 110 NaCl, 2·5 KCl, 1·6 $MgCl_2$, 1·0 $CaCl_2$, 5·0 tetramethyl ammonium (TMA)-Hepes and 5·0 glucose, pH 7·55. The retinas were stored in Ringer solution at room temperature (20–23 °C) for up to several hours until use. Isolated rod photoreceptor cells were obtained by chopping a piece of retina (receptor side up) under Ringer solution with a razor blade in a Petri dish coated with Sylgard elastomer (Dow Corning, Midland, MI, USA).

The suction pipette for recording membrane current from a single rod outer segment and the glass probe for truncating the outer segment were both made from Corning 7740 borosilicate glass capillaries (A-M Systems, Everett, WA, USA). Details of making the suction pipettes and the probes, as well as the recording and truncation procedures, have been described previously (Nakatani & Yau, 1988*b*, *c*). The suction pipette was filled with a choline solution containing (mM): 110 choline chloride, 0·5 (0·5 free Mg^{2+}) $MgCl_2$, 2·0 BAPTA, 1·79 (0·001 free Ca^{2+}) $CaCl_2$, 5·0 TMA-Hepes, 5·0 glucose, pH 7·55). The Ca^{2+} concentration was kept low to avoid any significant Ca^{2+} influx when the cGMP-gated channels opened. A potassium gluconate bath solution was used for dialysis, containing (mM): 110 potassium gluconate, 2·0 BAPTA; 5·0 TMA-Hepes and 5·0 glucose, pH 7·55, together with 0·5 mM 3-isobutyl-1-methylxanthine (IBMX), 0·5 mM free Mg^{2+} and appropriate cGMP and free Ca^{2+} concentrations. For both pipette and bath solutions, the total concentrations of Ca^{2+} and Mg^{2+} to be added in order to give the desired free concentrations were calculated according to the formulae:

$$[Ca]_{total} = [Ca]_{free} + \frac{0{\cdot}0093 \times [Ca]_{free} \times [BAPTA]}{1{\cdot}084 + 0{\cdot}059 \times [Mg]_{free} + 0{\cdot}0093 \times [Ca]_{free}},$$

$$[Mg]_{total} = [Mg]_{free} + \frac{0{\cdot}059 \times [Mg]_{free} \times [BAPTA]}{1{\cdot}084 + 0{\cdot}059 \times [Mg]_{free} + 0{\cdot}0093 \times [Ca]_{free}},$$

where $[Ca]_{total}$, $[Mg]_{total}$ and $[Mg]_{free}$ are in millimolar, and $[Ca]_{free}$ is in nanomolar. The total BAPTA concentration, [BAPTA], was 2·0 mM. The formulae were derived based on the published association constants of BAPTA for Ca^{2+}, Mg^{2+} and H^+ (Tsien, 1980), and for a solution pH of 7·55 (corresponding to a H^+ concentration of $10^{-7{\cdot}44}$ M, see Martell & Smith, 1974). Since the dissociation constant (K_d) of BAPTA for Ca^{2+} is 107 nM (Tsien, 1980), the buffer is not very effective for free Ca^{2+} concentrations above 1 μM, so the free Ca^{2+} concentration of 10 μM is only approximate. Since the full inhibitory effect of Ca^{2+} on the current was already attained at *ca* 1 μM, however, this point is not critical. Also, with a Ca^{2+} concentration in the pipette of 1 μM, the Ca^{2+} concentration inside the cell was set by BAPTA, without any interference due to Ca^{2+} influx. With the above solution arrangement, the membrane current was outward, being carried by K^+, and is indicated as positive in all of the figures. Contamination of the current from electrogenic Na^+–Ca^{2+}, K^+ exchange activity can be neglected, because there was practically no Na^+ in the preparation (except for the sub-millimolar concentration from the cGMP salt). The switching between solutions containing different Ca^{2+} concentrations gave rise to small currents (see, for example, Fig. 1), which we have interpreted as junction currents (see Hodgkin, McNaughton, Nunn & Yau, 1984; Nakatani & Yau, 1988*b*). These currents were already subtracted for the cGMP-activated currents shown in Figs 2 and 4. The seal resistance did not depend on the Ca^{2+} concentration, at least over the concentration range we employed.

All reagents were of analytical grade and all experiments were carried out in the dark at room temperature.

RESULTS

Figure 1 demonstrates the effect of Ca^{2+} on the cGMP-activated current from a truncated bullfrog rod outer segment. In this experiment, the current elicited by 20 μM cGMP was first measured with *ca* 10 μM free Ca^{2+} in the dialysing bath solution. The measurement was then repeated at 10 nM Ca^{2+}, with the current becoming three times that at 10 μM Ca^{2+}. The effect of low Ca^{2+} was only partially reversible, in that upon return to high Ca^{2+} the current elicited by 20 μM cGMP remained larger than at the beginning. After an exposure to nominally 0 Ca^{2+}, the cGMP-induced current in 10 μM Ca^{2+} increased still

further. The small current change upon switching the bath solution to, and from, nominally 0 Ca^{2+} reflects a junction current, which is also obvious at the switch to 10 nM Ca^{2+}. Since this experiment was carried out in the presence of the phosphodiesterase inhibitor IBMX and in the absence of ATP and GTP, i.e. conditions that rendered the metabolism of cGMP inactive, the current inhibition should reflect an effect of Ca^{2+} on the cGMP-gated channel, as first reported by Hsu & Molday (1993) and Gordon & Zimmerman (1994). In Fig. 1, the current recovery rates from the 20 μM cGMP steps at high and low Ca^{2+} were the same (0·2 s^{-1}), consistent with the absence of any Ca^{2+}-dependent residual phosphodiesterase activity, which would otherwise have given rise to different recovery rates at different Ca^{2+} concentrations. The washout of the Ca^{2+} inhibition at low Ca^{2+} suggests the involvement of a soluble factor in this action. The time course of this washout was quite fast. In the experiment of Fig. 1, for example, most of the Ca^{2+} inhibition disappeared after dialysis with low Ca^{2+} for a few minutes. The washout was slower at higher Ca^{2+} concentrations. In 10 μM Ca^{2+}, for example, no significant washout was observed over a 4 min period (see the first exposure to 10 μM Ca^{2+} in Fig. 2*A*).

Figure 2*A* shows recordings from another bullfrog rod outer segment, in which the membrane currents elicited by different concentrations of cGMP were compared at two Ca^{2+} concentrations, 10 μM and nominally 0. The saturated current, obtained with 1 mM cGMP, was the same at high and low Ca^{2+}. After exposure to low Ca^{2+}, the cGMP dose–response relation was redetermined at high Ca^{2+}; as in the experiment of Fig. 1, there was a

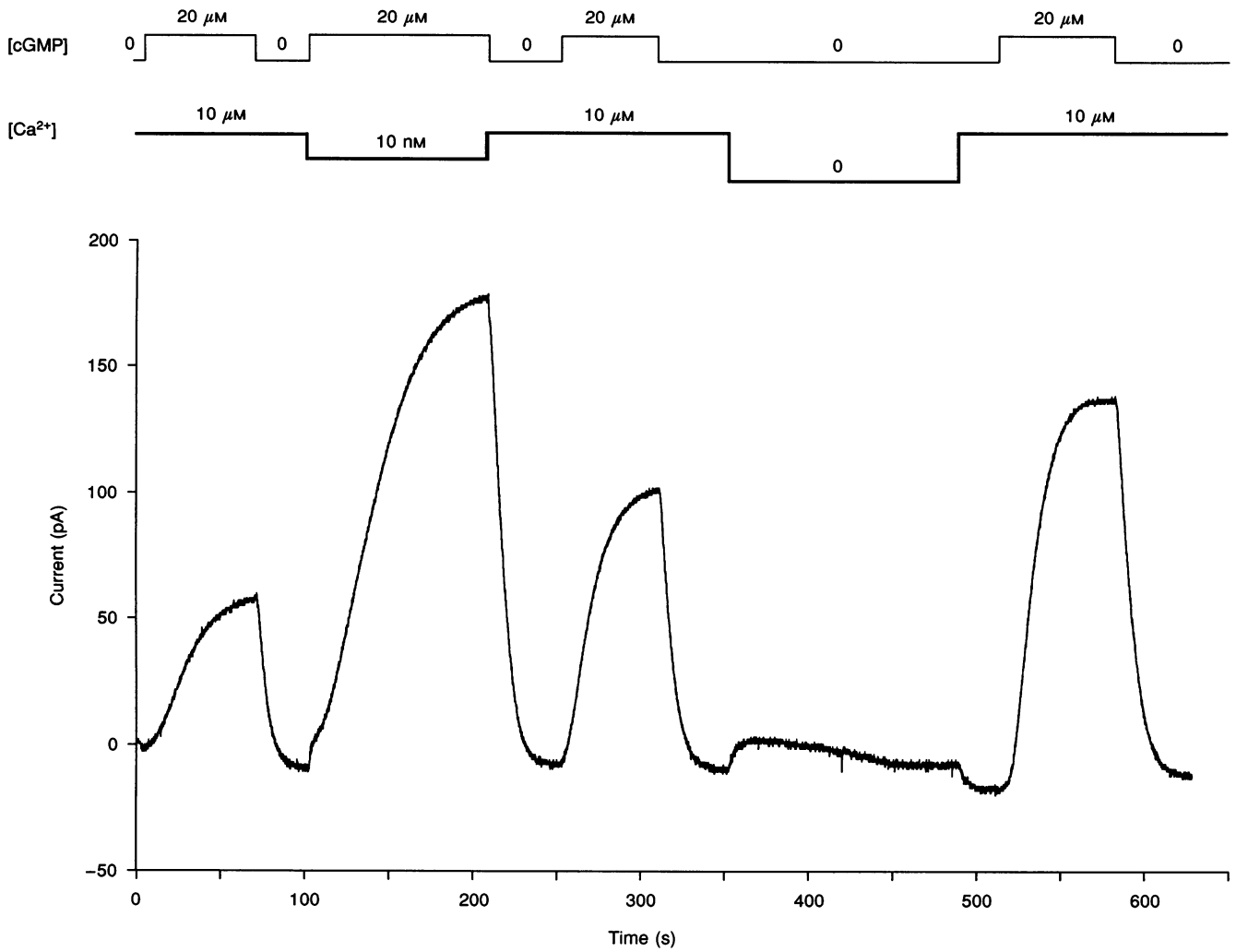

Figure 1. Effect of cytoplasmic Ca^{2+} on the cGMP-activated current recorded from a truncated bullfrog rod outer segment

The current elicited by 20 μM cGMP was measured at 10 μM or 10 nM Ca^{2+}. On the time scale indicated here, the solution changes were virtually instantaneous. The inhibitory effect of Ca^{2+} was partially lost after exposure to 10 nM or nominally 0 Ca^{2+}. Junction currents have not been subtracted. The length of the truncated rod outer segment inside the pipette was 52 μm. Truncation occurred 2·5 min before the beginning of the record.

partial loss of the inhibitory effect of Ca^{2+}. During the initial 10 μM Ca^{2+} exposure, however, the currents elicited by the first and the second 20 μM cGMP steps were essentially the same, indicating that there was no significant washout of the Ca^{2+} inhibition over several minutes. The currents from Fig. 2*A*, normalized against the saturated current at 1 mM cGMP, are plotted against cGMP concentration in Fig. 2*B*. It can be seen that the dose–response relation is shifted to lower cGMP concentrations at low Ca^{2+} (●). Figure 2*C* shows collected

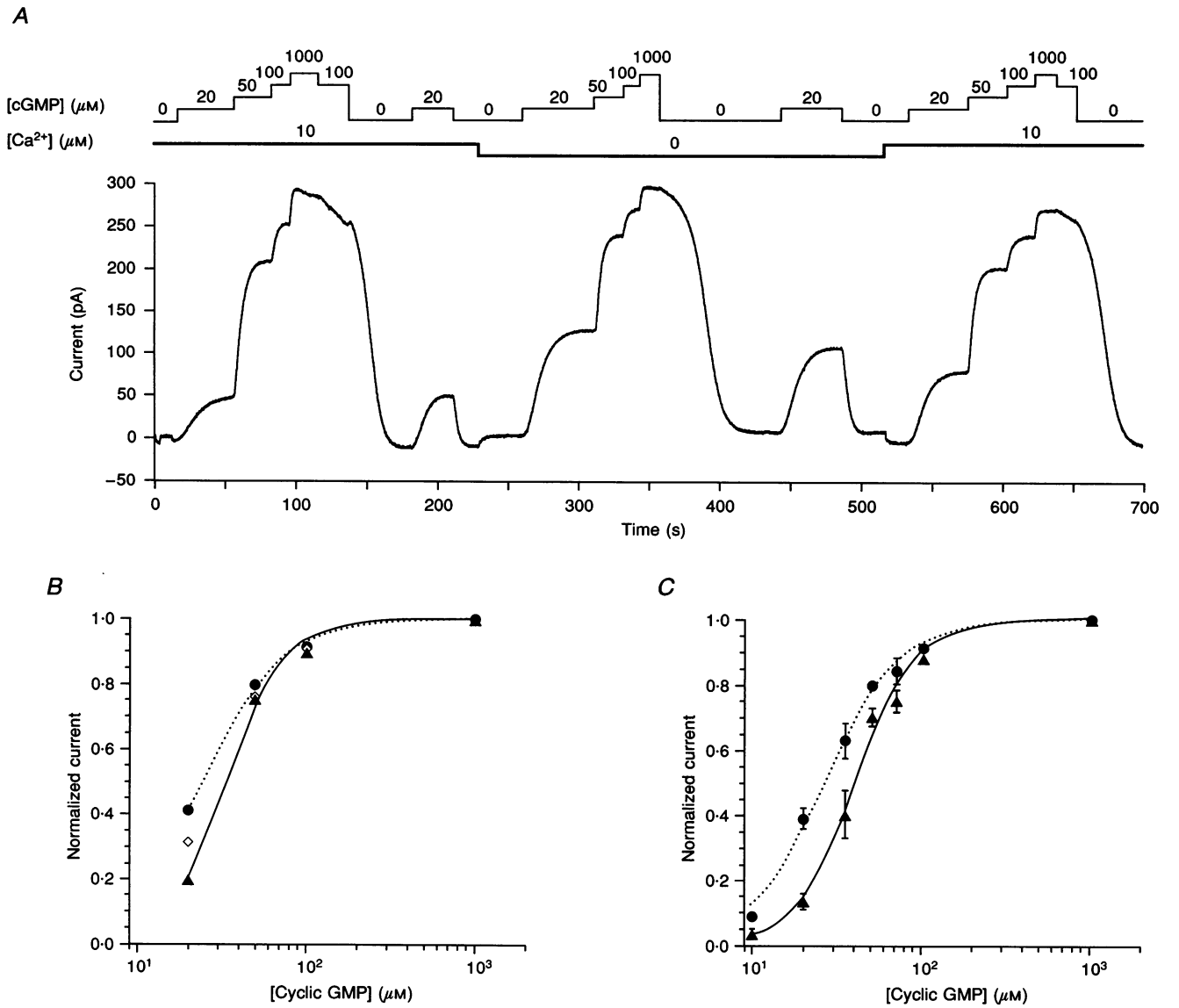

Figure 2. Effect of Ca^{2+} on the cGMP dose–response relation for the cGMP-gated channel

A, relation between cGMP-activated current and cGMP concentration at high (10 μM) and low (nominally 0) Ca^{2+} measured from a bullfrog rod outer segment. The junction currents have not been subtracted. The length of the truncated rod outer segment was 40 μm. Truncation took place 1 min before the beginning of the record. *B*, plot of the data from panel *A*. Junction currents have already been removed. The current amplitude is normalized against the saturated value elicited by 1 mM cGMP. Curves are Hill equation fits. ▲: currents obtained initially at 10 μM Ca^{2+}, fitted with a half-maximal activation constant, $K_{½}$, of 34 μM and a Hill coefficient, n_H, of 2·5. ●: currents obtained at nominally 0 Ca^{2+}, fitted with a $K_{½}$ of 24 μM and a n_H of 1·8. ◇: currents obtained at 10 μM Ca^{2+} after the exposure to nominally 0 Ca^{2+}. *C*, collected results from a total of eight bullfrog rod outer segments. Truncated rod outer segment lengths from 32 to 50 μm. Each point represents the average measurement from some of the cells only (5 cells for each of 20, 50 and 100 μM cGMP; 3 cells for each of 10, 35 and 70 μM cGMP). Vertical bars are S.E.M. ▲: 10 μM Ca^{2+}. The continuous curve represents the Hill equation with a $K_{½}$ of 40 μM and a n_H of 2·4. ●: nominally 0 Ca^{2+}. The dotted curve corresponds to the Hill equation with a $K_{½}$ of 27 μM and a n_H of 2·0.

results from eight bullfrog rod outer segments. In all of these experiments, measurements were initially made at 10 μM Ca^{2+}, then repeated at nominally 0 Ca^{2+}. The curves are least-squares fits of the Hill equation to the experimental points. At 10 μM Ca^{2+}, the fit gave a half-maximal activation constant, $K_{½}$, of 40 μM and a Hill coefficient, n_H, of 2·4. At nominally 0 Ca^{2+}, $K_{½}$ was 27 μM and n_H, 2·0. With these $K_{½}$ values, the sensitivity of the channel activation to cGMP would change by a maximum of $(40/27)^2 = 2·2$ to $(40/27)^{2·4} = 2·6$ times at low cGMP concentrations.

Figure 3 shows an experiment in which the dependence of the cGMP-activated current on Ca^{2+} concentration was examined. Cyclic GMP at 20 μM was used throughout. There was no significant inhibition at 10 nM Ca^{2+}, in that the current was close to that at nominally 0 Ca^{2+}. Also, the inhibitory effect was nearly complete at 1 μM Ca^{2+}, in that the current was close to that at 10 μM Ca^{2+}. The current obtained in 10 μM Ca^{2+} after exposure to 10 nM was a little larger than before due to washout, and was not used. The washout in 100 nM Ca^{2+}, on the other hand, was negligible because the ratio of currents at 10 μM and 10 nM Ca^{2+} (*ca* 1:3) measured afterwards had the expected value (cf. Figs 1 and 2*C*). To minimize washout, we tried to avoid unnecessarily long solution exposures. For this reason, the solution was sometimes changed before the current had fully reached steady state, as is evident for the 10 nM and nominally 0 Ca^{2+} concentrations in Fig. 3. In these cases, we had to adopt the current amplitude attained just before removal of cGMP. Nonetheless, from comparing the time courses of development of these currents with those that did attain steady state, the errors are unlikely to be more than 10%.

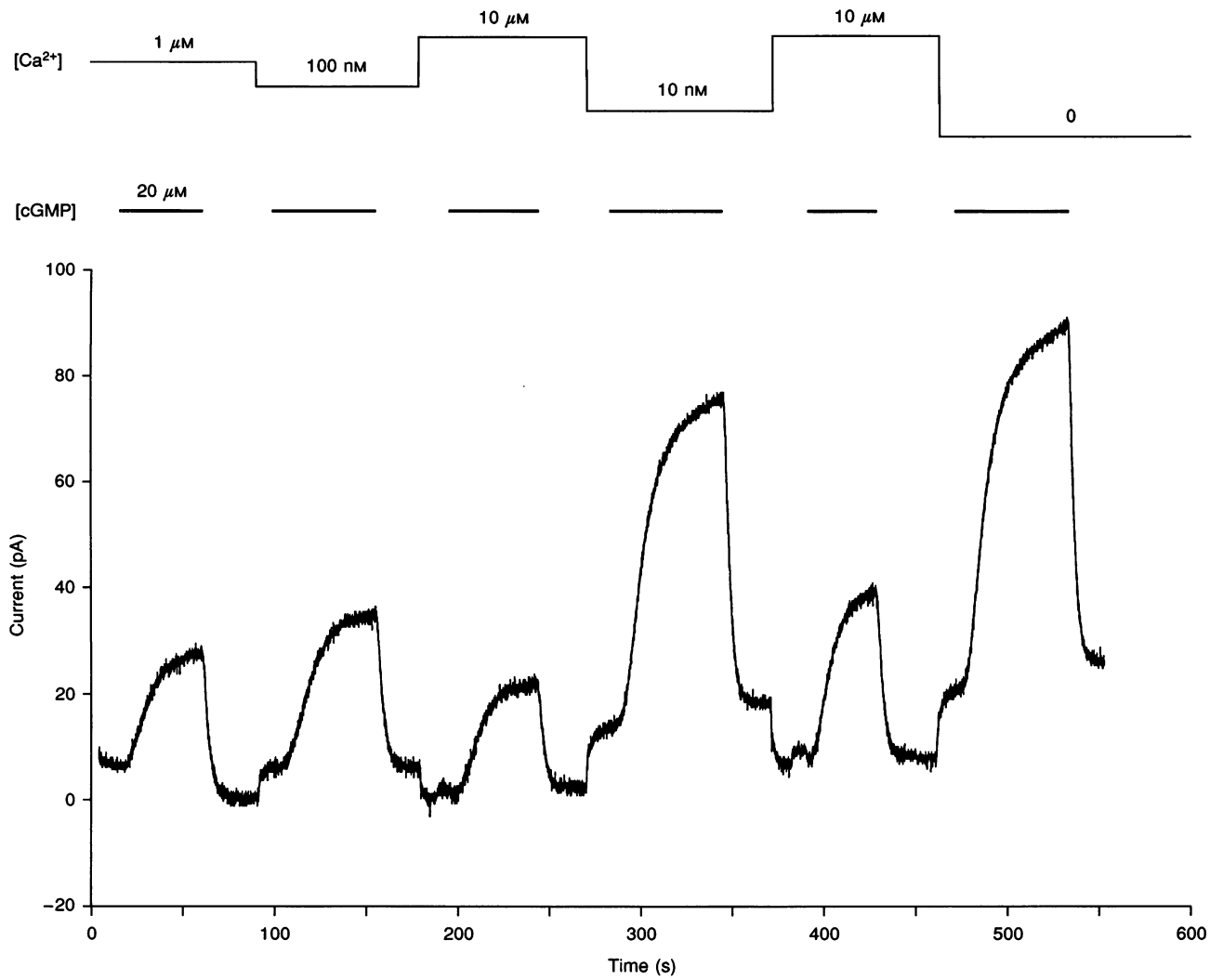

Figure 3. Dependence of cGMP-elicited current on cytoplasmic Ca^{2+} concentration in a truncated bullfrog rod outer segment

The current was activated by 20 μM cGMP. Junction currents have not been subtracted. The length of the truncated outer segment was 34 μm. The cell was truncated 3 min before the beginning of the record.

The cGMP-induced currents from Fig. 3 are normalized against that at nominally 0 Ca^{2+} and plotted against free Ca^{2+} concentration in Fig. 4*A*. The maximum inhibition is about 70%. The curve is a least-squares fit based on the Hill equation, with a half-maximal inhibition at 39 nM Ca^{2+} and a n_H of 2·0. Collected results from five bullfrog rod outer segments are shown in Fig. 4*B*. In all of these experiments, we have minimized washout of the mediating factor by switching to 10 nM or nominally 0 Ca^{2+} only at the end of the experiment, as in Fig. 3, and by shortening the exposure time to low Ca^{2+} as much as possible. The maximum inhibition of the current is 60% (i.e. 40% residual current), in good agreement with the results in Fig. 2*C*. The curve is a Hill equation with half-maximal inhibition at 48 nM Ca^{2+} and a n_H of 1·6.

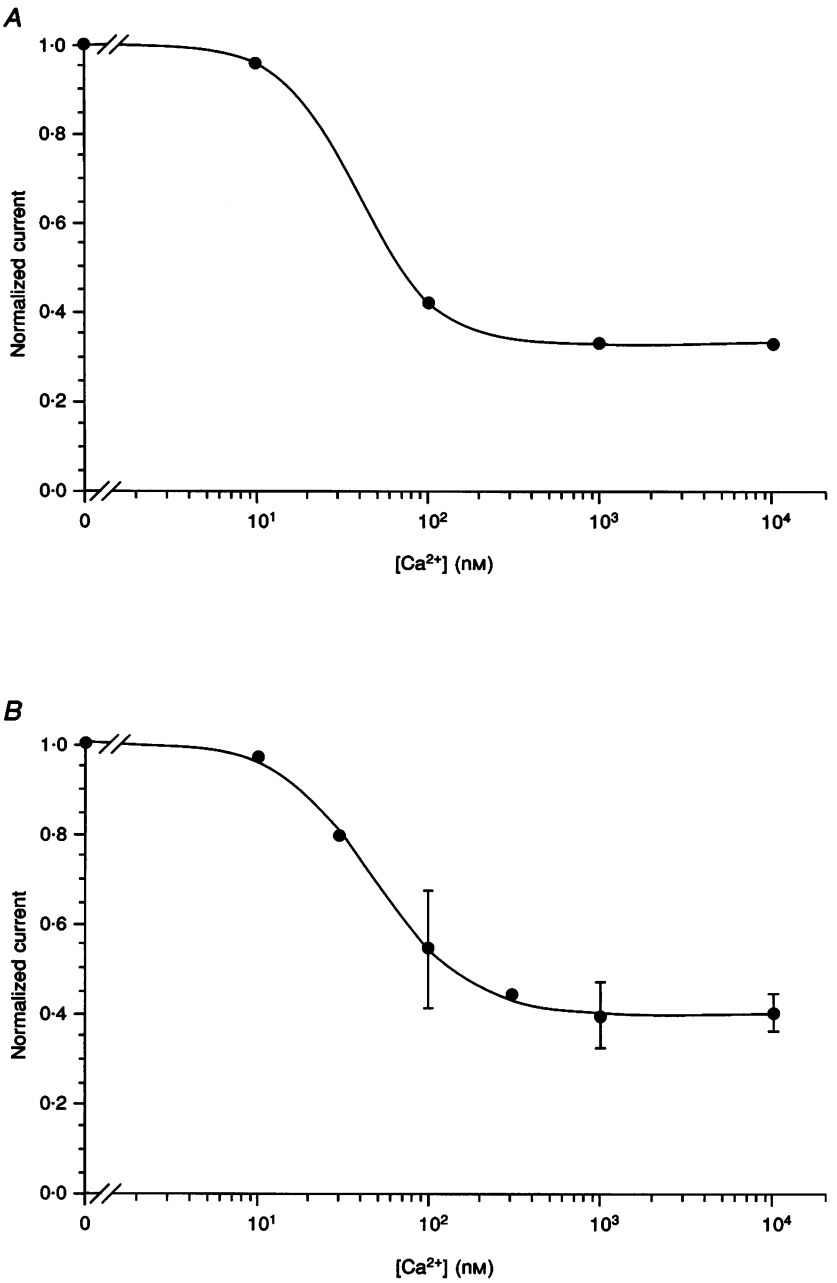

Figure 4. Ca^{2+} dependence of cGMP-activated current

A, data from Fig. 3. Current normalized against that at nominally 0 Ca^{2+}. The curve is a least-squares fit according to the Hill equation, giving a half-maximal inhibition at 38 nM Ca^{2+} and a n_H of 2·0. *B*, collected results from five bullfrog rod outer segments. Length of truncated rod outer segment from 34 to 40 μm. Each point represents the average data from 5 (10 nM Ca^{2+}), 3 (30 nM), 2 (100 nM), 3 (300 nM), 2 (1 μM) and 4 cells (10 μM), respectively. Vertical bars are S.E.M. The least-squares fit by the Hill equation gave a $K_{½}$ of 48 nM and a n_H of 1·6.

A similar effect of Ca^{2+} on the cGMP-activated current was observed in truncated tiger salamander rod outer segments. However, we have not characterized it quantitatively because the phenomenon was not consistently observed in these experiments. The reason for this is unclear. It may be due to the shorter length of the salamander rod outer segment which results in a faster washout of the mediating factor, or to a difference in the soluble factor affinity for the channel.

DISCUSSION

The experiments described here have confirmed that Ca^{2+} reduces the apparent affinity of the rod cGMP-gated channel for cGMP. Since the effect required only cGMP and Ca^{2+}, the involvement of guanylate cyclase or a kinase can be excluded (see also Chen *et al.* 1994). At the same time, interference from basal cGMP-phosphodiesterase activity in darkness can be ruled out because of the presence of 0·5 mM IBMX in our experiments, which is enough to inhibit this enzymatic activity (Koutalos, Nakatani & Yau, 1995). We conclude that Ca^{2+} directly modulates the cGMP-gated channel complex.

The washout phenomenon we observed at low Ca^{2+} concentrations suggests that a soluble factor mediates the Ca^{2+} effect, though our experiments have not addressed the identity of this factor. Previous biochemical experiments on bovine rod outer segment membranes by Hsu & Molday (1993) have demonstrated a similar effect of Ca^{2+} on the cGMP-gated channel in the presence of exogenously added calmodulin, which is known to be present in the rod outer segment (Kohnken, Chafouleas, Eadie, Means & McConnell, 1981; Nagao, Yamazaki & Bitensky, 1987). They observed a maximal shift in the cGMP activation constant due to Ca^{2+}–calmodulin by about 1·5-fold (see also Chen *et al.* 1994), which is close to our present findings. Likewise, the relation they measured between channel activity and Ca^{2+} concentration was broadly the same as what we determined; in both cases, the half-inhibition point occurs at 50–70 nM Ca^{2+}. This may be a coincidence, however, because in their experiments a somewhat arbitrary concentration of calmodulin (400 nM) and solutions of low ionic strength were used, conditions that might affect the Ca^{2+} dependence (see Kilhoffer *et al.* 1983, for the dependence of the Ca^{2+}–calmodulin dissociation constants on ionic concentrations). More recently, Gordon & Zimmerman (1994) reported that in excised patches both calmodulin and an apparently distinct endogenous factor mediate the Ca^{2+} effect on the channel.

The Ca^{2+} modulation of the rod cGMP-gated channel, while interesting as a form of ion channel modulation, is nonetheless weak in magnitude. From Fig. 4, the maximum change in current at 20 μM cGMP would be only by a factor of 2·5. Since 20 μM cGMP is less than the $K_{1/2}$ of the cGMP dose–response curve for the channel (see Fig. 2*C* and associated text), the same maximum change should apply to lower, physiological cGMP concentrations (a few micromolar, see Nakatani & Yau, 1988*c*). Since the sensitivity of amphibian rods changes well over a 100-fold range in the presence of background light, the contribution of this channel modulation to the overall light adaptation should be rather small.

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