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ACTIVATION OF SINGLE ION CHANNELS FROM TOAD RETINAL ROD INNER SEGMENTS BY CYCLIC GMP: CONCENTRATION DEPENDENCE

BY GARY MATTHEWS AND SHU-ICHI WATANABE*

From the Department of Neurobiology and Behavior, The State University of New York, Stony Brook, NY 11794-5230, U.S.A.

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SUMMARY

1. Patch-clamp recordings of single cyclic GMP-activated channels from toad rod photoreceptors were made in inside-out membrane patches containing only one such channel. Patches were obtained from the inner segment, where the density of cyclic GMP-activated channels is lower than in the outer segment, making one-channel patches possible. The dependence of channel gating on cyclic GMP concentration ([cyclic GMP]) was studied. At low [cyclic GMP] (5–10 μM), channel openings were infrequent and occurred as bursts of rapid opening and closing. As [cyclic GMP] was increased, bursts became more frequent, until at 1 mM the activity fused into long bouts of rapid flicker between open and closed states.

2. The duration of brief openings and closings (flicker) within bursts was not affected by [cyclic GMP]. This suggests that the rapid flicker within bursts results from an intrinsic channel property not associated with agonist-induced receptor activation.

3. At 10 μM-cyclic GMP, the distribution of closed times was fitted by a sum of three exponential components. The briefest, with time constant averaging 0·29 ms, corresponded to the brief closings within bursts, while the two longer components, with time constants averaging 3·5 and 32 ms, corresponded to much longer closings between bursts. At 0·5 or 1 mM-cyclic GMP (saturation), the longer components disappeared, and the distribution of closed times was fitted by a single-exponential equation with the same time constant as the briefest component observed at lower concentrations.

4. Because the channel continued to flicker even at high [cyclic GMP], the maximal probability of being in the open state (P_o) did not approach 1·0, averaging $0{\cdot}30 \pm 0{\cdot}05$ ($N = 8$). The relation between P_o and [cyclic GMP] was fitted by the Hill equation with an exponent of 3, suggesting that binding of cyclic GMP to multiple sites is required to open the channel.

* Author's names are in alphabetical order. Present address of Shu-Ichi Watanabe: Department of Physiology, Keio University School of Medicine, Shinano-machi, Shinjuku-ku, Tokyo 160, Japan.

INTRODUCTION

The plasma membrane of the outer segment of vertebrate rod photoreceptors contains a cationic conductance that is activated by cyclic GMP (Fesenko, Kolesnikov & Lyubarsky, 1985). Single-channel recordings have revealed that the cyclic GMP-activated channels of the outer segment are indistinguishable in conductance and kinetics from the light-sensitive channels that mediate phototransduction in vertebrate photoreceptors (Matthews, 1987; Matthews & Watanabe, 1987). Thus, a description of the gating of these channels by cyclic GMP is important for a complete understanding of visual transduction. In this regard, it would be of interest to determine the concentration dependence of channel gating under a range of cyclic GMP concentrations. However, the density of cyclic GMP-activated channels in outer-segment plasma membrane is high: at least 100–350 channels μm^{-2} (Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986; Matthews & Watanabe, 1987). Therefore, it is impossible to discern individual channel events except in low concentrations of cyclic GMP (usually lower than 10 μM), and even at low concentrations of cyclic GMP the number of channels in a patch is uncertain, making analysis difficult.

Recently we found that the plasma membrane of rod inner segments contains cyclic GMP-activated channels (Watanabe & Matthews, 1988), in addition to voltage- and calcium-activated channels (Bader, Bertrand & Schwartz, 1982). The density of cyclic GMP-activated channels is lower in the inner segment than in the outer segment. In fact, the density is sufficiently low that it is possible to obtain inner-segment patches that contain only one cyclic GMP-activated channel. We performed experiments using these one-channel patches from the inner segment to examine the concentration dependence of channel gating at a wide range of concentrations. Openings of single cyclic GMP-activated channels in rods typically consist of bursts of rapid openings and closings (flicker). The average durations of the brief openings and closings that make up a burst of openings were unaffected by cyclic GMP concentration. The main effect of an increase in cyclic GMP concentration was to reduce the interval between successive bursts, until at high concentrations channel activity fused into long, continuous bouts of rapid flickering between open and closed states.

METHODS

Preparation

All recordings were made from isolated rod photoreceptors obtained by mechanical dissociation of toad (*Bufo marinus*) retina (Baylor, Lamb & Yau, 1979; Matthews & Watanabe, 1987); no enzyme treatment was used. Toads were killed by pithing the brain and spinal cord. Retinas were isolated and kept in Ringer solution containing (mM): NaCl, 111; KCl, 2·5; $CaCl_2$, 1·0; $MgCl_2$, 1·6; glucose, 10; Tris, 3, or HEPES, 10; pH = 7·8. A piece of isolated retina was minced with a razor blade, and an aliquot of minced bits was transferred to a recording chamber containing Ringer solution of the above composition, except that $MgCl_2$ was omitted. The recording chamber had a glass top and bottom and was equipped with a perfusion system that allowed rapid switching between control and test solutions (Brett, Dilger, Adams & Lancaster, 1986). Recordings were made from isolated rods found among the detached outer segments and bits of retina lying on the bottom of the chamber. Experiments were performed under room light and at room temperature (22–24 °C).

Electrodes and recording apparatus

Patch pipettes were made from thick-walled Pyrex tubing (o.d. = 1·2 mm; i.d. = 0·6 mm). Outer tip diameters before fire polishing were 0·7–1·4 μm. Electrodes were normally filled with 0 Ca^{2+}, 0 Mg^{2+} Ringer solution containing (mM): NaCl, 117·5; Tris, 3, or HEPES, 10; EDTA, 0·15; pH = 7·8. In some experiments, the pipette solution contained 0·15 mM EGTA, in addition. The bath electrode in the chamber was a sintered Ag–AgCl pellet, and the patch pipette electrical connection was via a chlorided silver wire. The output of the patch-clamp amplifier (List L/M-EPC7) was recorded on an FM tape-recorder (Racal Store 4DS) at bandwidth = 0–5000 Hz. Synchronizing pulses and TTL signals controlling the pinch valves, which switched the perfusion solution between control and test solutions, were also recorded. For computer analysis, selected segments were replayed through an anti-aliasing, low-pass filter and digitized in a PDP 11/73 computer. For analysis of channel events, an 8-pole Bessel filter with a cut-off frequency of 2000–4000 Hz was used, and the sampling rate was 10000–25000 s^{-1}.

Recording procedure

Under visual control, a gigaohm seal was obtained on the plasma membrane of a rod inner segment (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The seal resistance was usually 10–30 GΩ. Recordings were obtained from inner segments with or without the nucleus region. Inside-out patches were obtained by tapping the manipulator while locally superfusing the rod with 0 Ca^{2+}, 0 Mg^{2+} solution to reduce the chance of forming a vesicle. The superfusion solution also contained 100 μM-cyclic GMP, so that the presence of one or more cyclic GMP-activated channels in the excised patch could be immediately ascertained. Approximately 1/3 of inner-segment patches had no cyclic GMP-activated channel (Watanabe & Matthews, 1988); however, another 1/3 contained only one channel, and these patches are the focus of this paper.

The plasma membrane of rod inner segments possesses many kinds of voltage-activated and other types of channels (Bader *et al.* 1982; Watanabe & Matthews, 1988), and activity of these channels often interfered with analysis of cyclic GMP-activated channels. Therefore, we excluded recordings in which activity of these other channels obscured cyclic GMP-activated channel events. This substantially reduced the yield of usable patches in these experiments. Another requirement was long-term patch stability sufficient to allow testing a range of concentrations on one patch and to give long records at low concentrations, where channel openings were rare. The combination of all these requirements resulted in the relatively small number of individual channels (5–11) studied in detail in the various experiments reported here. However, the channel properties were for the most part consistent from one channel to another and, where direct comparisons with outer-segment channels were possible, were similar to those observed previously in outer segment patches containing large numbers of cyclic GMP-activated channels. This provides some indication that the properties of the small number of studied channels are general.

Perfusion

After obtaining an inside-out patch containing a cyclic GMP-activated channel, the patch pipette was transferred into the outlet portion of a Y-tube perfusion system similar to that described in detail by Brett *et al.* (1986). The perfusion solution was typically 0·2 Ca^{2+} solution containing (mM): NaCl, 117; $CaCl_2$, 0·2; Tris, 3, or HEPES, 10; pH = 7·8. Test solutions contained 5 μM-1 mM cyclic GMP dissolved in 0·2 Ca^{2+} solution.

Data analysis

To define the transition between open and closed states, a half-amplitude criterion was used (Colquhoun & Sigworth, 1983). In dose–response experiments on the relation between cyclic GMP concentration and open probability, half-saturating concentration and asymptotic open probability were estimated by fitting Hill equation templates by eye to double-logarithmic plots of dose–response data for each patch. Kinetic parameters were defined and measured as in Matthews & Watanabe (1987). A burst was defined as starting with any opening that was separated from the last previous opening by a closed time of at least 1 ms. A burst was terminated by any closure longer than 1 ms; that is, closures briefer than 1 ms were ignored. Open duration refers to the duration of individual excursions into the open state, terminated by any closing, no matter how brief. Closed duration was defined similarly for individual excursions into the closed state. Gap duration refers to the duration of brief closings that occur within a burst.

RESULTS

In an earlier study of the spatial distribution of the cyclic GMP-activated conductance within the rod photoreceptor (Watanabe & Matthews, 1988), we found that the channel density was much lower in the inner segment than in the outer segment. This low density of channels is an advantage for the study of the concentration dependence of single-channel kinetics. In the first section of this paper, we will demonstrate that, in fact, patches containing only one cyclic GMP-activated channel can be obtained from the inner segment. In the remainder of the paper, we will make use of such one-channel patches to analyse channel-gating at various concentrations of cyclic GMP.

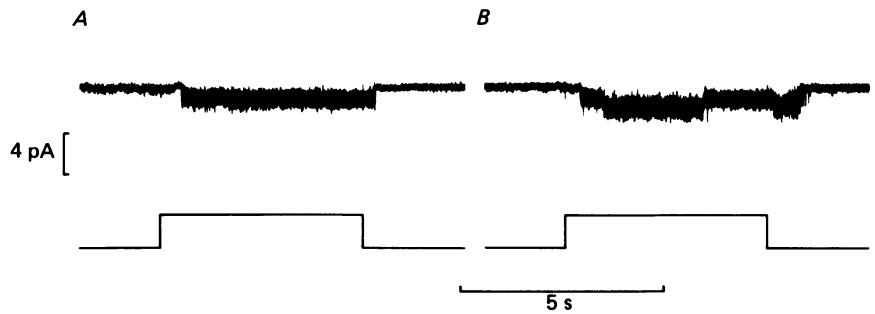

Fig. 1. Chart recordings from inner-segment patches containing one (*A*) or two (*B*) cyclic GMP-activated channels. Upper traces show membrane current at bandwidth 0–320 Hz, with inward current downward. Bottom traces show the pulse controlling the solenoid-activated valve that selected the solution flowing past the patch pipette (see Methods); during the upward deflection, cyclic GMP was applied at 1 mM (*A*) or 500 μM (*B*). The membrane potential was −76 mV in *A* and −65 mV in *B*. Openings of cyclic GMP-activated channels give a downward deflection; on this time scale, rapid openings and closings during application of cyclic GMP fuse to produce a single thick band on the chart recorder.

Patches from the inner segment can contain a single cyclic GMP-activated channel

In some patches obtained from the inner segment, a saturating concentration of cyclic GMP (e.g. 1 mM) caused the abrupt onset of long-lasting bouts of fast channel events that had the same amplitude as single cyclic GMP-activated channel observed in outer segment patches at low concentrations (e.g. 5 μm) of cyclic GMP (Haynes *et al.* 1986; Zimmerman & Baylor, 1986; Matthews & Watanabe, 1987). This behaviour, which is illustrated at low temporal resolution in the chart recorder traces of Figs 1*A* and 2 and at higher resolution in Figs 3 and 4, was very different from that of outer-segment patches at saturating concentrations, where large noisy currents consisting of superimposed activity of many channels are seen. Thus, it seemed that such patches contained only one cyclic GMP-activated channel. As shown in Fig. 1*B*, inner-segment patches with two or more cyclic GMP-activated channels could be readily distinguished from the one-channel patches on the basis of transitions to higher conductance, corresponding to two or more simultaneously open channels, in the presence of high concentrations of cyclic GMP. In Fig. 1*B*, the patch appeared

to contain two cyclic GMP-activated channels. In other inner-segment patches, three or more channels were present (Watanabe & Matthews, 1988).

Further evidence that inner-segment patches could indeed contain only a single cyclic GMP-activated channel is presented in Fig. 2, which shows examples of channel activity at a wide range of cyclic GMP concentrations. At low concentrations (e.g. 10 μM), only a few isolated openings, seen as brief downward deflections at the

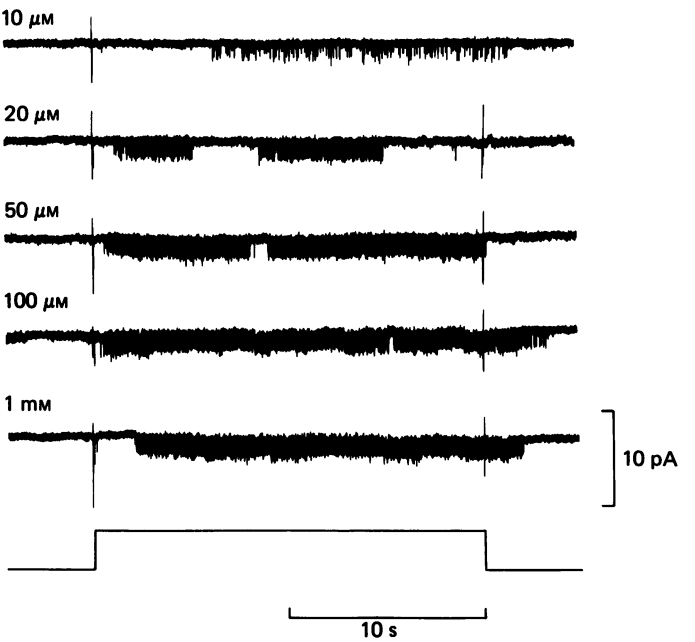

Fig. 2. Chart recorder traces showing cyclic GMP-activated channel activity at a range of cyclic GMP concentrations in an inner-segment patch containing one channel. During the upward deflection on the bottom trace, the indicated concentrations of cyclic GMP were applied to the internal face of the patch. Bandwidth, 0–320 Hz; membrane potential, −71 mV. Channel openings (inward currents) appear as downward deflections. At all but the lowest concentration, channel activity is for the most part sufficiently rapid to fuse into a single thick trace at the low temporal resolution shown here.

low temporal resolution of Fig. 2 (note time scale), were observed; as cyclic GMP concentration was raised, openings of the same amplitude occurred more frequently, until at higher concentrations (e.g. 50 or 100 μM), activity fused into long periods of rapid opening and closing. Thus, as expected of a patch with only a single channel, the amplitude of events was unchanged as agonist concentration was increased, but the frequency of opening increased dramatically. The fact that the current amplitude reached during the application of cyclic GMP was the same over a 100-fold range of concentration provides strong evidence that the patch indeed had only a single channel. This same behaviour can be seen at higher temporal resolution in Figs 3 and 4. The remainder of this paper will be concerned with the analysis of channel gating in such one-channel patches at various concentrations of cyclic GMP, in experiments like that shown in Fig. 2.

Concentration dependence of open probability

In the chart-recorder traces of Fig. 2, it is apparent that channel activity increased greatly as cyclic GMP concentration was increased from 10 μM to 1 mM; however, it is also clear that even at a high concentration, the channel continued to undergo rapid transitions between open and closed states, rather than simply spending long periods in the open configuration. This can be seen at higher temporal resolution in the wide-bandwidth traces of Figs 3 and 4. The rapid flicker observed previously during individual channel openings (Matthews, 1987; Matthews & Watanabe, 1987)

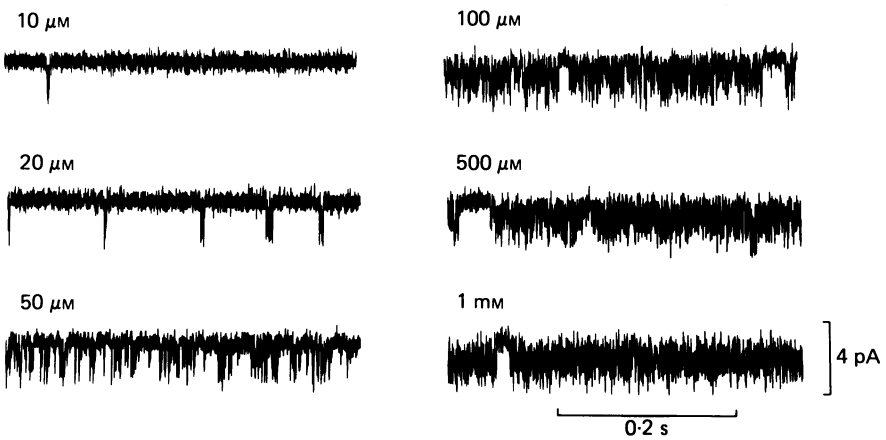

Fig. 3. Higher-resolution samples of single cyclic GMP-activated channel activity in an inner-segment patch under various concentrations of cyclic GMP (10 μM to 1 mM). Membrane potential, −75 mV; bandwidth, 0–2000 Hz. The patch contained only one cyclic GMP-activated channel.

continued unabated even during the very long periods of activity induced by 1 mM-cyclic GMP. We considered the possibility that the flicker might result from blockade of the channel by residual amounts of divalent cations, particularly Mg^{2+}. However, we think this is unlikely for the following reasons. Under our standard recording conditions, the recording pipette was filled with 0 Ca^{2+}, 0 Mg^{2+} Ringer solution (see Methods) containing 0·15 mM-EDTA, and the Ringer solution in which cells were bathed contained no Mg^{2+}. Thus, any small amount of extracellular Ringer solution sucked into the pipette during seal formation would probably have added little Mg^{2+} to the pipette solution. Additionally, some recordings were made with pipettes filled with 0 Ca^{2+}, 0 Mg^{2+} Ringer solution containing 0·3 mM-EGTA and 0·3 mM-EDTA to buffer the free divalent cation concentration to a very low level; flicker was still present in these recordings. This indicates that divalent cations within the pipette were not a likely cause of the observed flicker. Likewise, flicker was still present when the Ringer solution in which cyclic GMP was applied to the intracellular face of the membrane contained 0 Ca^{2+}, 0 Mg^{2+}, 0·3 mM-EDTA, and 0·3 mM-EGTA. It is, therefore, unlikely that the rapid flickering of the open channel can be accounted for by the blocking action of divalent cations.

Because of continuing flicker even in the presence of high concentrations of cyclic GMP, the maximal probability of channel opening that we observed was considerably less than 1. The relation between the concentration of cyclic GMP on the intracellular face of the membrane and the proportion of time in the open state (P_o) is shown in Fig. 5*A*. In this experiment, the maximum open probability was 0·42; in eight

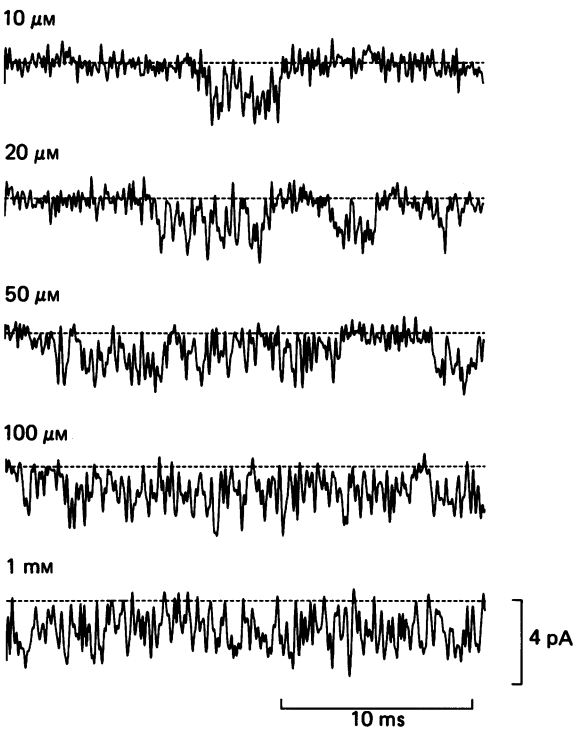

Fig. 4. Samples of cyclic GMP-activated channel activity at 10 μM to 1 mM cyclic GMP, with wide bandwidth (0–4000 Hz) to show the rapidity of the fluctuations between open and closed states during a channel event. The dashed lines indicate the baseline current in the absence of cyclic GMP. Membrane potential, −71 mV. Same experiment as Fig. 2.

experiments of this kind, the average maximum open probability was 0·30±0·05 (mean±S.E.M.).

The solid line through the data in Fig. 5*A* was drawn according to the Hill equation:

$$P_o = aG^3/(1+G^3), \quad (1)$$

where a is the asymptotic open probability at high concentration and G is cyclic GMP concentration divided by $K_{\frac{1}{2}}$, the half-saturating concentration. For the cell of Fig. 5*A*, $K_{\frac{1}{2}}$ was 50 μM. Equation (1) has been reported to fit the relation between conductance and cyclic GMP concentration in outer-segment patches that contain a large number of cyclic GMP-activated channels (Haynes *et al.* 1986; Zimmerman & Baylor, 1986). Results from eight experiments are summarized in Fig. 5*B*, in which P_o was normalized by the asymptotic P_o, $P_{o,max}$. Again, the straight line was drawn

according to eqn (1). The half-saturating concentration varied somewhat across experiments, ranging from 17 to 50 μM with an average of $32{\cdot}4 \pm 4{\cdot}6$ μM (mean $\pm$S.E.M.).

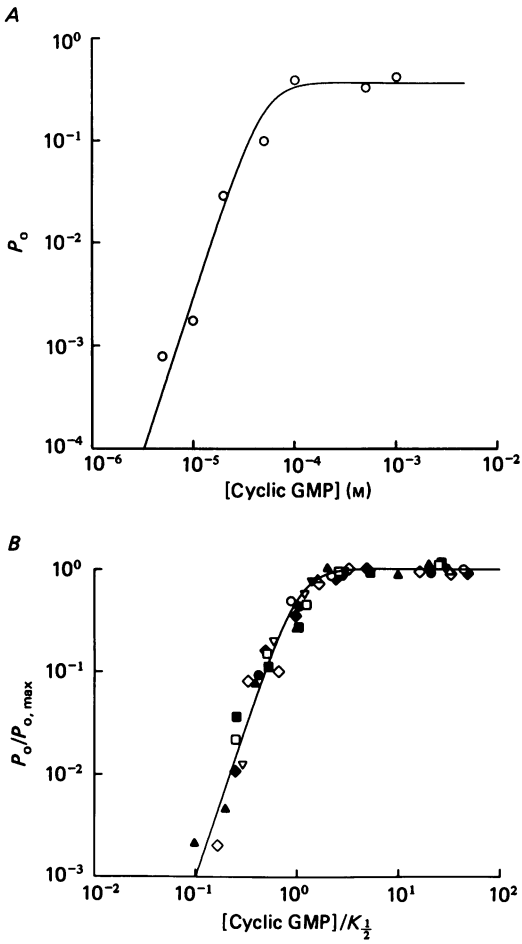

Fig. 5. Relation between cyclic GMP concentration and open probability of single inner-segment cyclic GMP-activated channels. *A*, double-logarithmic plot of results from an individual experiment. Half-saturating concentration ($K_{\frac{1}{2}}$) was 50 μM. The curve was drawn according to the equation: $P_o = 0{\cdot}42G^3/(1+G^3)$, where $G = [\text{cyclic GMP}]/K_{\frac{1}{2}}$. *B*, normalized plot of results of eight experiments on one-channel patches. To compare results across experiments, open probability (P_o) was normalized by dividing by the asymptotic value ($P_{o,max}$) for each patch, and the cyclic GMP concentration was expressed relative to the $K_{\frac{1}{2}}$. Maximum open probability ranged from 0·1 to 0·45, and $K_{\frac{1}{2}}$ ranged from 17 to 50 μM. The curve was drawn according to the relation, $P_o/P_{o,max} = G^3/(1+G^3)$.

Concentration dependence of channel kinetics

In experiments like that of Fig. 3, we examined the effect of cyclic GMP concentration on temporal parameters of channel gating in patches containing a single cyclic GMP-activated channel. Following the terminology of our earlier work on the kinetics of single light-sensitive and cyclic GMP-activated channels in the

TABLE 1. Kinetic parameters of cyclic GMP-activated channels from rod inner segments at a range of cyclic GMP concentrations (μM)

[Cyclic GMP] (μM)	5 (3)	10 (5)	20 (5)	50 (5)	100 (5)	500 (4)	1000 (4)
t_o	0·21±0·02	0·27±0·02	0·28±0·02	0·26±0·02	0·26±0·02	0·25±0·01	0·25±0·01
t_b	0·70±0·19	1·14±0·22	1·75±0·39	3·39±0·83	8·59±2·01	23·3±10·8	20·6±4·93
t_c	17·8±7·94	9·34±3·94	2·69±1·04	0·64±0·15	0·65±0·35	0·30±0·07	0·27±0·02
t_g	0·25±0·02	0·28±0·003	0·28±0·004	0·28±0·01	0·25±0·01	0·24±0·03	0·25±0·01

All results are from patches that contained one channel. Bandwidth, 0–2000 Hz. Abbreviations: t_o, average open duration; t_b, average burst duration (ignoring closures < 1 ms in duration); t_c, average closed duration, counting all closures both within and between bursts; t_g, average closed duration within a burst (average gap duration). Numbers are means±S.E.M., in ms. The number of experiments at each concentration is given in parentheses at the top of each column after the cyclic GMP concentration.

outer segment (Matthews & Watanabe, 1987), we examined the distributions of channel open time (t_o), channel closed time (t_c), and burst duration (t_b) at various concentrations of cyclic GMP. These temporal parameters were defined as in Matthews & Watanabe (1987). Closed duration refers to the duration of any excursion into the closed state, no matter how long or how brief. Open duration refers to the duration of an individual excursion into the open state; an individual opening was considered to be terminated by a closure of any duration, no matter how brief.

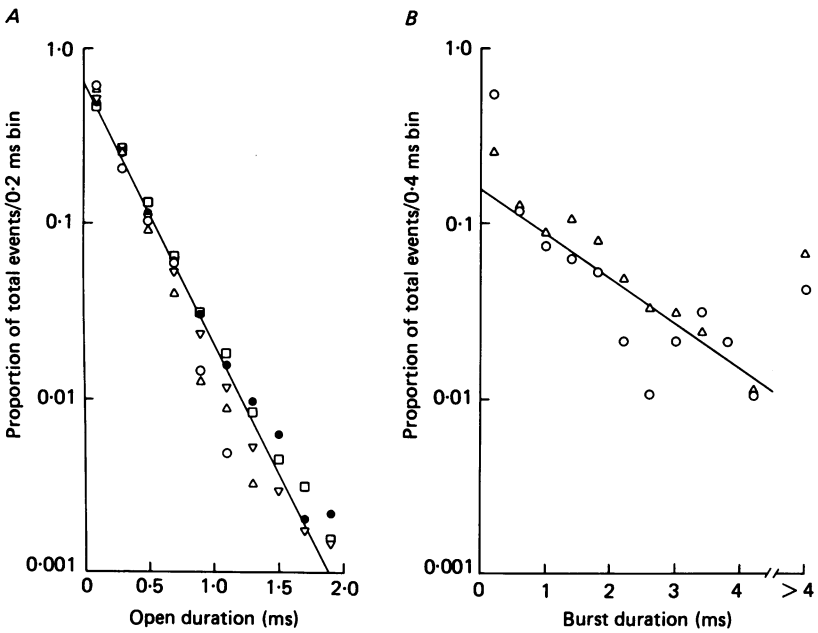

Fig. 6. *A*, distribution of open durations of a single inner-segment cyclic GMP-activated channel, under cyclic GMP concentrations of 10 μM–1 mM: ○, 10 μM; △, 20 μM; ▽, 50 μM; ●, 100 μM; □, 1 mM. The straight line corresponds to an exponential time constant of 0·29 ms. *B*, distribution of burst durations from the same single cyclic GMP-activated channel as in *A*, under 10 μM (○) and 20 μM (△) cyclic GMP. The straight line corresponds to an exponential time constant of 1·72 ms.

In measuring burst duration, however, all closings less than 1 ms in duration were ignored, so that a burst consisted of a series of one or more openings separated by closings briefer than 1 ms.

Open duration. Distributions of open duration at five concentrations of cyclic GMP are shown in Fig. 6*A*. Over the concentration range from 10 μM to 1 mM, there was no appreciable change in the distribution of open duration. At each concentration, the distribution could be well described by a single exponential, with an average time constant over all concentrations of 0·29 ms for the experiment of Fig. 6*A*. Results from five experiments like that of Fig. 6*A* are summarized in Table 1. In all cases, cyclic GMP concentration had no effect on the average duration of individual brief openings.

Burst duration. Unlike the open duration, burst duration was dramatically affected

by cyclic GMP concentration. This is apparent by eye in chart recorder records like Fig. 2, where activity changed from isolated brief events to long bursts of activity as concentration was increased from 10 μM to 1 mM. This effect is summarized in Table 1, where it can be seen that burst duration increased 18-fold, on average, as cyclic GMP concentration increased from 10 μM to 1 mM. It should be emphasized that the amount of increase in burst duration observed at higher cyclic GMP concentrations (e.g. 500 μM or 1 mM) depends strongly on the criterion closure (1 ms in Table 1) used to define the end of a burst; for example, with a longer criterion (e.g. 2 ms) the increase would be more pronounced. This arises because the distribution of brief gaps within bursts (see below) was unaffected by concentration, and therefore the average waiting time for a gap longer than the criterion used to define a burst sets a ceiling on the expected burst duration. However, because there was only one channel in each patch, the increase in burst duration nevertheless gives a convenient index of the effect of cyclic GMP concentration on the activity of the channel: although the duration of individual brief openings is unaffected by cyclic GMP concentration, the interval between long closings, and thus the burst duration, increases strongly as concentration is increased.

At low concentrations of cyclic GMP, there were other, more subtle changes in burst duration. One example is illustrated in Fig. 6*B*, which shows distributions of burst duration at 10 and 20 μM-cyclic GMP. As reported earlier for light-sensitive channels and for cyclic GMP-activated channels of the outer segment (Matthews & Watanabe, 1987), the distribution of burst duration had two components: one exponential component with a time constant of 1·72 ms in Fig. 6*B*, and a faster component of brief openings. There were more events in the first bin than would be expected from the single-exponential distribution fitted to the longer burst durations. The fast component represents bursts consisting of a single brief opening like those that occur in rapid succession during a longer burst (Matthews & Watanabe, 1987). Note in Fig. 6*B* that when concentration was decreased from 20 μM ($\triangle$) to 10 μM ($\bigcirc$), the proportion of total events accounted for by the brief bursts increased dramatically (first bin), while the remainder of the distribution was relatively unaffected. This rise in the proportion of brief events results in a decrease in the average burst duration as concentration is decreased in the low concentration range. In five experiments (three on one-channel patches and two on patches with two channels) like that of Fig. 6*B*, the average burst duration decreased from $1{\cdot}20 \pm 0{\cdot}10$ to $1{\cdot}03 \pm 0{\cdot}11$ ms (mean $\pm$ S.E.M.) when cyclic GMP concentration was reduced from 20 to 10 μM, and when cyclic GMP was further reduced to 5 μM, average burst duration declined further to $0{\cdot}67 \pm 0{\cdot}10$ ms. This suggests that the reduction in average burst duration of light-sensitive channels of intact rods during non-saturating illumination (Matthews & Watanabe, 1987) might be a simple and direct result of the fall in cytoplasmic concentration of cyclic GMP caused by light-activated phosphodiesterase activity.

Closed duration. It is apparent from inspection of Figs 2 and 3 that the closed intervals between bursts were also dramatically affected by cyclic GMP concentration. Long closings became progressively less common as cyclic GMP concentration was increased, until finally the distribution of closed durations was dominated almost entirely by the brief closings characteristic of the closures within bursts at

lower concentrations. An example of distributions of closed duration at 10 and 500 μM-cyclic GMP is shown in Fig. 7 on three different time scales to show all components of closing at the lower concentration. At 500 μM-cyclic GMP (□), the distribution was fitted by a single-exponential equation with a time constant of 0·27 ms, which is similar to the average duration of closings within a burst reported earlier for cyclic GMP-activated channels of rod outer segments by Matthews & Watanabe (1987). At 10 μM-cyclic GMP (○), however, the distribution of closed durations was fitted by a sum of three exponential components with time constants of 0·23, 1·6, and 15 ms in the experiment of Fig. 7. In five experiments at 10 μM-cyclic

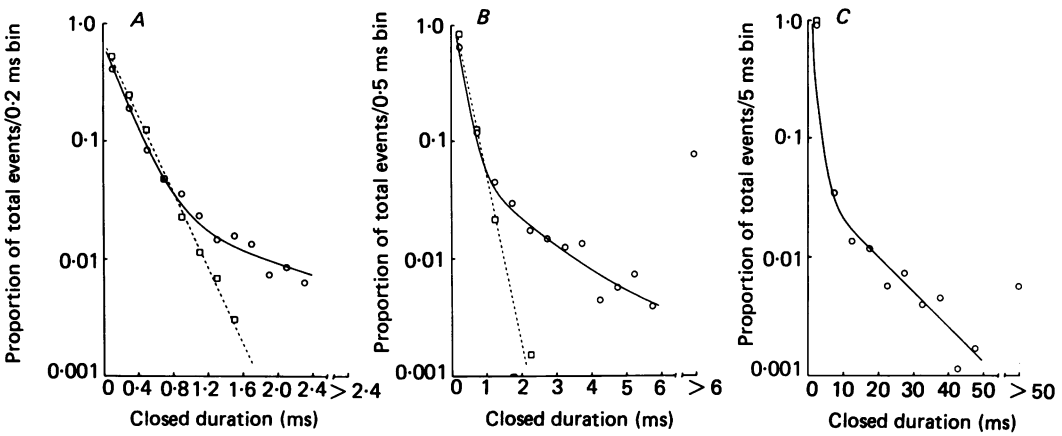

Fig. 7. Distributions of closed durations at 10 μM (○) and 500 μM-cyclic GMP (□), on three different time scales (*A*, *B* and *C*). At 500 μM-cyclic GMP, the distribution is fitted by a single-exponential equation, with a time constant of 0·27 ms (dashed line). The distribution at 10 μM-cyclic GMP is fitted by a sum of three exponential components with time constants of 0·23, 1·6, and 15·0 ms (continuous line).

GMP, these components averaged $0{\cdot}29 \pm 0{\cdot}01$, $3{\cdot}49 \pm 1{\cdot}35$, and $32{\cdot}0 \pm 8{\cdot}7$ ms (mean $\pm$S.E.M.). The briefest component represented the short closings within bursts and had about the same time constant as that of the equation fitted to the entire distribution at 500 μM-cyclic GMP; this brief component, like the brief openings within bursts (Fig. 6*A*), was not affected by cyclic GMP concentration. The lack of effect of cyclic GMP concentration on this component, labelled t_g (for gap duration), is summarized in Table 1. The two longer components represented the closings between bursts and disappeared as the concentration of cyclic GMP was increased. The progressive elimination of the long components of closing as concentration was increased resulted in a progressive decrease in average closed duration, which is also summarized in Table 1.

Pauses: a long-duration component of closing. In addition to the components of closed duration illustrated in Fig. 7, there were infrequent longer closings that occurred even at high concentrations of cyclic GMP, where the brief (approximately 0·3 ms time constant) component of closing was dominant. Examples of these long-duration pauses can be seen in Fig. 2 at 20 and 50 μM-cyclic GMP, and an example of a pause during the response to 1 mM-cyclic GMP is shown in Fig. 8. These long

closings often lasted hundreds of milliseconds even at high concentrations of cyclic GMP. However, because pauses occurred infrequently, it was difficult to make sufficiently long, stable recordings to allow compilation of dwell-time distributions that accurately reflect this very long component of closing. Thus, we have no firm information regarding their concentration dependence. The existence of these long

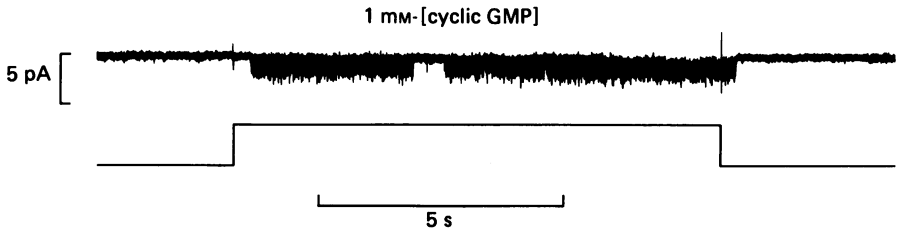

Fig. 8. Chart record of activity of a single cyclic GMP-activated channel in an inner-segment patch, showing a long closing during perfusion with 1 mM-cyclic GMP (upper trace). The bottom trace shows the pulse controlling the valve that selected the solutions. Bandwidth, 0–320 Hz.

TABLE 2. Temporal parameters and single-channel conductance for inner-segment and outer-segment cyclic GMP-activated channels at 10 μM-cyclic GMP

	n	t_b (ms)	t_o (ms)	t_g (ms)	γ (pS)
Inner-segment channel	11	1·37±0·11	0·14±0·007	0·30±0·02	25·6±0·9
Outer-segment channel	11	0·85±0·07	0·23±0·01	0·38±0·03	24·0±1·19

Temporal parameters are defined in text. Abbreviations: n, number of experiments; t_b, average burst duration; t_o, average open duration; t_g, average closed duration during burst; γ, single-channel conductance. Numbers are means±S.E.M. Bandwidth, 0–4000 Hz. Values for outer-segment channels are from Table 1 of Matthews & Watanabe (1987).

closings offers a likely explanation for the low-frequency fluctuations of cyclic GMP-activated current in outer-segment patches at high concentrations of cyclic GMP (Haynes *et al.* 1986) and possibly for part of the low-frequency, light-dependent noise observed in the dark current of intact rods (Baylor, Matthews & Yau, 1980; Zimmerman & Baylor, 1985; Matthews, 1986).

Comparison of inner- and outer-segment cyclic GMP-activated channels

The channel events activated by cyclic GMP in inner-segment patches appear to be quite similar to those observed in outer-segment patches (Haynes *et al.* 1986; Zimmerman & Baylor, 1986; Matthews, 1987; Matthews & Watanabe, 1987). There is pronounced flicker in the open state (Fig. 4) in both cases, and in both cases the distribution of open duration is fitted by a single exponential (Fig. 6*A*), while the distribution of burst duration has two components (Fig. 6*B*). The half-saturating concentrations of cyclic GMP observed by us in inner-segment patches falls within the range reported for outer-segment patches by Haynes *et al.* (1986), and the form

of the Hill equation fitting the dose–response relation is like that reported previously for the outer-segment conductance (Haynes *et al.* 1986; Zimmerman & Baylor, 1986). Also, Watanabe & Matthews (1988) showed that inner-segment and outer-segment cyclic GMP-activated channels have similar noise properties and single-channel conductance. In this paper, we have examined the kinetics of cyclic GMP-activated channels from the inner segment in more detail, and a comparison with cyclic GMP-activated channels from the outer segment is given in Table 2. Although there are some differences in detail, the kinetics are similar for inner- and outer-segment channels. Because the inner-segment channels are similar to outer-segment channels in conductance, dose–response relation, and kinetics, we conclude that the concentration dependence of channel gating observed in inner segment patches containing one cyclic GMP-activated channel should apply also to the outer-segment channels, which are normally involved in phototransduction.

DISCUSSION

Concentration independence of flicker

Openings of cyclic GMP-activated channels occur as bursts of rapid opening and closing; that is, the channel shows pronounced flicker. The experiments reported here on patches containing a single cyclic GMP-activated channel demonstrate that this flicker is unaffected by cyclic GMP concentration; both the average open duration and the average closed duration within bursts were constant in the concentration range from 5 μM to 1 mM. This independence of flicker and cyclic GMP concentration suggests that the flicker has a source other than rapid binding and unbinding of cyclic GMP. Also, flicker remained in experiments in which divalent cations were buffered to low levels with EDTA and EGTA, so residual block by Ca^{2+} or Mg^{2+} ions is not a likely source. It is probable that the rapid openings and closings within a burst result from some intrinsic property of the channel or from some blocking substance other than divalent cations. The lack of effect of cyclic GMP concentration on the duration of brief openings and closings within bursts is similar to the results reported by Dudel & Franke (1987) for glutamate-activated channels at the crayfish neuromuscular junction, where the distributions of open time and of the short gaps within bursts were also unaffected by changes in agonist concentration.

Because flicker was still present even at high concentrations of cyclic GMP, the probability of the channel being open reached an asymptotic value less than 1·0 in dose–response experiments. A maximum probability of channel opening considerably less than 1·0 had been inferred earlier by Matthews (1986) on the basis of noise analysis of cyclic GMP-activated currents; the variance of cyclic GMP-activated current increased steadily with increasing concentration up to saturation, rather than reaching a maximum at an intermediate concentration and then declining as saturation was approached, as would be expected if the asymptotic probability of opening approached 1 at saturation. Further, the power spectrum exhibited a high-frequency component, suggestive of flicker, that became progressively more prominent as cyclic GMP concentration was increased. The present experiments using single-channel recording provide direct confirmation of the earlier inference.

Because the open probability of the cyclic GMP-activated channel during a burst is low, even with agonist bound, the average current through the channel during a burst is reduced. Under physiological conditions, in addition, the open-channel current is further reduced by the blocking action of external divalent cations (Haynes *et al.* 1986; Matthews, 1986; Zimmerman & Baylor, 1986). Thus, in the normally functioning rod, the effect of closing an individual channel is small, which allows a single-photon response to consist of many channel closings (Baylor *et al.* 1980). In this manner, quantizing noise is reduced, and the waveform of the single-photon response is more stereotyped.

Dose–response relation

The dose–response curve obtained by measuring the probability of channel opening in one-channel patches (Fig. 5) is consistent with dose–response curves obtained using conductance measurement in outer-segment patches containing many channels (Haynes *et al.* 1986; Zimmerman & Baylor, 1986). In both instances, the relation between cyclic GMP concentration and channel opening was steep, with a limiting slope of about 3 on double-logarithmic co-ordinates at low concentrations. This implies that there are multiple cyclic GMP-binding sites regulating channel gating. Because of the steepness of the dose–response relation in the physiological range of concentration (i.e. below 10 μM), a small change in cyclic GMP concentration in the cytoplasm causes a large change in the probability of channel opening. This may contribute to the total amplification required within the transduction cascade to produce a detectable electrical response to a single photoisomerization.

There was considerable variation among individual channels in the present experiments in the half-saturating concentration of cyclic GMP ($K_{\frac{1}{2}}$), which ranged from 17 to 50 μM. Because of the steepness of the dose–response relation, this range of $K_{\frac{1}{2}}$ represents dramatic variation across patches in the amount of channel activity elicited by a particular low concentration of cyclic GMP. For instance, 10 μM-cyclic GMP might produce frequent channel openings in one one-channel patch, but little activity in another. Consecutive one-channel patches obtained from the same preparation could differ dramatically in sensitivity, indicating that the variation did not arise solely from differences between preparations. Thus, there appears to be individual variation in the sensitivity of single channels to cyclic GMP. Variation in $K_{\frac{1}{2}}$ like that seen here has been reported previously in experiments in which conductance–concentration relations were measured in outer-segment patches containing many cyclic GMP-activated channels (Haynes *et al.* 1986). This suggests that variation in agonist sensitivity is not unique to inner-segment channels.

Lack of desensitization

One striking feature of cyclic GMP-activated channels that sets them apart from most other agonist-activated channels is the absence of desensitization. As seen in Figs 2 and 8, for example, activity proceeds unabated for many seconds even in the presence of 1 mM-cyclic GMP. Presumably, this feature is important in a signalling system in which the removal of the agonist, and hence the closing of the agonist-activated channels, constitutes the transduction event.

Effect of cyclic GMP concentration on channel kinetics

A major action of cyclic GMP was to reduce the average closed time between bursts. This progressive reduction in interburst interval with increasing cyclic GMP concentration caused the average closed duration to decline, even though there was no effect of cyclic GMP on fast closings within bursts (Fig. 7 and Table 1). The elimination of long closings at high cyclic GMP concentrations, as seen in Fig. 7, can account also for the increase in average burst duration at high concentrations. At 1 mM-cyclic GMP, for example, the closed time distribution is fitted by a single exponential with an average time constant of 0·27 ms; the average waiting time between closings $\geqslant 1$ ms in this instance would be about 20 ms. Thus, we would expect that the average 'burst duration,' even with continuous flicker, would be about 20 ms if a closure criterion of 1 ms is adopted. This is close to the observed average burst duration of 20·6 ms at 1 mM-cyclic GMP (Table 1).

The distribution of burst durations showed two components, like the distribution of burst durations for outer-segment channels (Matthews & Watanabe, 1987). Because there appear to be multiple binding sites for cyclic GMP associated with the channel, the possibility arises that the two components represent different numbers of cyclic GMP molecules bound. One piece of evidence that bears on this question comes from the observed effect of cyclic GMP concentration on burst duration at low concentrations (5–10 μM). When the concentration of cyclic GMP was increased from 10 to 20 μM, the average burst duration increased somewhat. As shown in Fig. 6*B*, this was due to a decrease in the proportion of brief bursts at the higher concentration. This behaviour is consistent with the idea that the brief bursts occur when fewer molecules of cyclic GMP are bound to the channel.

The decline in burst duration at low concentrations of cyclic GMP also offers a simple explanation for our previous observation that the average burst duration of light-sensitive channels in the outer segment is briefer during non-saturating illumination than in darkness (Matthews & Watanabe, 1987). The intracellular concentration of cyclic GMP would be expected to decline during illumination, because of the light-induced increase in phosphodiesterase activity, and this alone would seem to be sufficient to cause the average burst duration to decrease in the intact cell.

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