Regulation of cGMP Levels by Guanylate Cyclase in Truncated Frog Rod Outer Segments

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ABSTRACT Cyclic GMP is the second messenger in phototransduction and regulates the photoreceptor current. In the present work, we tried to understand the regulation mechanism of cytoplasmic cGMP levels in frog photoreceptors by measuring the photoreceptor current using a truncated rod outer segment (tROS) preparation. Since exogenously applied substance diffuses into tROS from the truncated end, we could examine the biochemical reactions relating to the cGMP metabolism by manipulating the cytoplasmic chemical condition.

In tROS, exogenously applied GTP produced a dark current whose amplitude was half-maximal at ~0.4 mM GTP. The conductance for this current was suppressed by light in a fashion similar to when it is activated by cGMP. In addition, no current was produced in the absence of Mg~2+, which is known to be necessary for the guanylate cyclase activity. These results indicate that guanylate cyclase was present in tROS and synthesized cGMP from exogenously applied GTP. The enzyme activity was distributed throughout the rod outer segment.

The amount of synthesized cGMP increased as the cytoplasmic Ca~2+ concentration of tROS decreased, which indicated the activation of guanylate cyclase at low Ca~2+ concentrations. Half-maximal effect of Ca~2+ was observed at ~100 nM. tROS contained the proteins involved in the phototransduction mechanism and therefore, we could examine the regulation of the light response waveform by Ca~2+. At low Ca~2+ concentrations, the time course of the light response was speeded up probably because cGMP recovery was facilitated by activation of the cyclase. Then, if the cytoplasmic Ca~2+ concentration of a photoreceptor decreases during light stimulation, the Ca~2+ decrease may explain the acceleration of the light response during light adaptation. In tROS, however, we did observe an acceleration during repetitive light flashes when the cytoplasmic Ca~2+ concentration increased during the stimulation. This result suggests the presence of an additional light-dependent mechanism that is responsible for the acceleration of the light response during light adaptation.

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INTRODUCTION

In vertebrate photoreceptors, photon absorption by visual pigments initiates a series of biochemical reactions and reduces cytoplasmic cGMP levels (for review see Pugh and Cobbs, 1986; Stryer, 1986). Since cGMP induces a current that flows continuously in the dark (dark current) by directly activating a cationic conductance (Fesenko et al., 1985), reduction in cGMP levels causes a hyperpolarizing light response. The photoreceptor not only responds to ON-OFF of the light stimulation but also changes its response characteristics under various light conditions. For example, the time course of the light response is accelerated when the cell is light-adapted (Baylor and Hodgkin, 1974). In the present work, we try to understand this mechanism by assuming that the changes in the light response characteristics are attained by modifying the cGMP metabolism.

To study the modification mechanism of the cGMP metabolism in rod outer segments, it is desirable to use a preparation in which we can manipulate the intracellular chemical composition and measure the resultant changes in the cGMP-induced current. Such a preparation has been introduced and was originally called a truncated rod outer segment (tROS) (Yau and Nakatani, 1985b; Nakatani and Yau, 1988b). The preparation is an open-ended rod outer segment which is held in a suction electrode. With this preparation, membrane current can be measured while the cell interior is being perfused with exogenous chemicals. tROS, therefore, may be called an inside-out rod outer segment. The preparation retains not only the cGMP-activated channel but also the proteins such as cGMP phosphodiesterase (PDE) and transducin that are required for the light-induced hydrolysis of cGMP (Yau and Nakatani, 1985b; Kawamura and Murakami, 1988). The light response, therefore, can be observed in this preparation when cGMP and other necessary chemicals are supplied.

Cytoplasmic cGMP levels are regulated by its synthesis and hydrolysis. Though its hydrolysis has been investigated rather extensively, its synthesis has not been thoroughly worked out yet despite its equal importance for the cGMP regulation. The presence of the synthesizing enzyme guanylate cyclase in rod outer segments has been suggested by a whole-cell patch-clamp experiment on detached rod outer segments (Sather and Detwiler, 1987). In this study, first we will show conclusive evidence for the presence of this enzyme. Then, by measuring the cGMP-induced current in tROS under various intracellular chemical conditions, we will demonstrate that reduction in the cytoplasmic Ca\(^{2+}\) concentration induces guanylate cyclase activation and that this reaction possibly operates for modulation of the cGMP metabolism during light adaptation. Finally, we will show that, in addition to Ca\(^{2+}\), an additional reaction is involved in the modulation of the cGMP metabolism.

A preliminary report has been published elsewhere (Kawamura and Murakami, 1988).

MATERIALS AND METHODS

Preparation of tROS

Bullfrogs (*Rana catesbeiana*) were dark-adapted and killed by decapitation in accordance with "Guiding Principles in the Care and Use of Animals." Retinas were removed from eyeballs
and photoreceptors were mechanically dissociated in Ringer solution (115 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 0.2 mM taurine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.5, supplemented with bovine albumin (1.5 mg/ml). The rod outer segment was sucked into an electrode according to the method of Baylor et al. (1979). We always used the cell that showed a light response at this stage. The exposed part outside of the electrode (inner segment plus basal part of outer segment) was truncated with a fine glass rod under visual control with the aid of an IR TV monitor. A light microscopic view of a tROS preparation is shown in the inset of Fig. 3.

The perfusion system is schematically illustrated in Fig. 1. The inside of the chamber was continuously perfused with a pseudo-intracellular solution (115 mM potassium pyruvate, 2.5 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 0.2 mM ATP, 0.2 mM taurine, 0.2 mM (ethylenebis(oxyethylenenitriilo)tetraacetic acid (EGTA), 0.1 mM CaCl₂, pH 7.5). The chamber was equipped with five outlets from which test solutions were ejected. To perfuse the tROS interior, the electrode was placed in the stream of a test solution. The position of the electrode was controlled mechanically.

The test solutions were made by adding different levels of nucleotides and calcium buffers to the pseudo-intracellular solution. In most of these experiments (except Fig. 2), 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX, a PDE inhibitor) was added to the test solutions. IBMX was necessary to reduce the dark PDE activity (Yau and Nakatani, 1985b; Hestrin and Korenbrot, 1987), probably because tROS lost intrinsic cGMP phosphodiesterase inhibitors during internal perfusion. Our previous biochemical experiment (Kawamura and Murakami, 1986a) showed that the dark PDE activity increased after the intact disk membranes were washed. Since 0.5 mM IBMX only partially suppresses the light PDE activity (Robinson et al., 1980), a light stimulation elicited a light response in tROS.

The composition of the solution in the electrode (electrode solution) was varied depending on the type of experiment and is specified in the text or the legend. When the ionic composition of the electrode solution was different from that of the Ringer solution, the electrode solution was possibly contaminated during the suction of the rod outer segment that was bathed in Ringer solution. To avoid contamination, therefore, the electrode was not immersed in the chamber’s solution until Ringer solution was replaced by the electrode solution.

Using Ca²⁺-EGTA system, Ca²⁺ concentrations were buffered in test solutions and also in the electrode solution when necessary. The concentrations of CaCl₂ and EGTA used for buff-
ering were: 0.1 mM CaCl₂/5.24 mM EGTA for 0.5 nM Ca²⁺, 0.1 mM CaCl₂/2.78 mM EGTA for 1 nM Ca²⁺, 0.1 mM CaCl₂/0.386 mM EGTA for 10 nM Ca²⁺, 0.1 mM CaCl₂/0.2 mM EGTA for 30 nM Ca²⁺, 0.152 mM CaCl₂/0.2 mM EGTA for 100 nM Ca²⁺, and 0.195 mM CaCl₂/0.2 mM EGTA for 1 μM Ca²⁺.

**Measurement of Membrane Current**

The membrane current of tROS was measured by a patch-clamp amplifier (EPC-7; List Co., FRG). The voltage in the pipette was continuously clamped at 0 mV. Because there was no potential difference across the tROS plasma membrane, the driving force of the current, if it flowed, was the ion gradient across the membrane. In the experiment in which the cytoplasmic Ca²⁺ concentration was varied (Figs. 7-9), reduction in the Ca²⁺ concentration caused a decrease in the seal resistance that probably reduced the measured membrane current. This effect was significant when the seal resistance was high (30 MΩ or higher), but was negligible at 10-20 MΩ, which was the range of the resistance of the preparation used in this experiment. The current records were low-pass filtered at 30 Hz.

We used rod photoreceptors that showed morphological intactness: straight outer segment and tapered inner segment. When the size and the shape of the suction electrode fitted well with the outer segment, the tROS preparation functioned for >1.5 h even after truncation. Occasionally, the dark current gradually decreased during the course of experiment, and then we discarded the data. In some cases, the amplitude of the dark current suddenly changed when the electrode was moved, probably because the seal resistance changed. For this reason, it was inevitable that the quantitative estimation of the dark current (Fig. 8) would be slightly rough (~±10% deviation, judging from the current amplitude measurement with repetitive application of cGMP of the same concentration).

ATP, cGMP, GDP, GMP, guanylylimidodiphosphate (GMPPNHP), IBMX, and bovine albumin were obtained from Sigma Chemical Co. (St. Louis, MO), organically synthesized GTP (trisodium salt) was purchased from Yamasa (Choshi, Japan), and the other chemicals were from Nakarai (Kyoto, Japan).

**Light Stimulation**

Light stimulation was a flash whose duration was 1 s. The unattenuated intensity of the light was 5.7 × 10⁵ rhodopsin molecules bleached per outer segment per second.

**RESULTS**

**Exogenously Applied GTP Produces a Dark Current in tROS**

The outer segment of a dissociated rod photoreceptor was sucked into an electrode that contained Ringer solution and the light response before truncation is shown in Fig. 2A. The exposed part of the cell was then perfused with the pseudo-intracellular solution which contained high potassium. The cell still responded to light (Fig. 2B, first two upward deflections indicated by arrowheads). At the point indicated by a downward arrow (Fig. 2B), the cell was truncated, which produced an inward membrane current (downward deflection in the figure). The amplitude of this current differed in different preparations depending on the seal resistance and the length of the outer segment in the electrode. Even after truncation, tROS still responded to light flashes (Fig. 2B, starting from asterisk) and the light response survived for ~1.5 min. The result indicated that, up to 1.5 min after the start of the internal perfusion, cGMP was present, hydrolyzed by PDE on light illumination, and
supplied internally after the illumination. The period of the light response survival varied from 1 to 3 min in different preparations.

Then, by applying exogenous GTP, we examined whether GTP alone could induce an inward membrane current. In Fig. 2B, after the light response was lost, tROS interior was perfused with a test solution containing 1.8 mM GTP. With a short delay (2–3 s), an inward current began to flow. The current could be suppressed by light and, therefore, it was regarded as the dark current. After removal of GTP, the dark current gradually decayed. The current could be repetitively produced when GTP was applied (Fig. 2C). In the same preparation, 1 mM cGMP also produced an inward current of larger amplitude that was also suppressed by light (Fig. 2D). These observations suggested that tROS contained guanylate cyclase that synthesized cGMP from exogenously applied GTP. Conclusive evidence will be shown later (Fig. 4). ATP, GDP, GMP, or GMPPNHP did not produce the dark current at 0.4 mM concentration, whereas GTP did at the same concentration.

The inward direction of the dark current could be explained as follows. Since

The decay of the dark current after removal of exogenous cGMP (Fig. 2D) was much faster than that of the current produced by GTP application (Fig. 2, B and C). This observation would explain why tROS continued responding to light after the truncation (Fig. 2B): the survival of the light response was probably due to newly synthesized cGMP from GTP, and not due to the remaining store of free cGMP in tROS. The gradual decrease of the current after removal of GTP would suggest the presence of a GTP-binding component(s) in rod outer segments.

In this experiment, we did not add IBMX to the test solutions to make clearer the difference between the decay time courses after removal of the two chemicals. The decay of the current after removal of cGMP was slowed in the presence of IBMX probably because of inhibition of PDE, but it was always faster than that observed after removal of GTP.

The inward direction of the dark current could be explained as follows. Since
there was no voltage difference across the tROS plasma membrane (see Materials and Methods), the ion gradients were solely the driving force of the current. Though the concentration of external Na⁺ was almost the same as that of cytoplasmic K⁺, Na⁺ permeates the cGMP-activated channel more easily than K⁺ by 1.25–1.35 times (Yau and Nakatani, 1984a; Hodgkin et al., 1985). For this reason, an inward current should be produced.

**Dark Current Production by GTP Application in Modified Electrode Solution**

In the experiments shown later (Figs. 7 and 8), the regulation of internal cGMP levels was studied as a function of the cytoplasmic Ca²⁺ concentration. In intact photoreceptors, the cytoplasmic Ca²⁺ concentration is probably regulated by two mechanisms: Ca²⁺ efflux brought about by the Na⁺-Ca²⁺ exchanger (Yau and Nakatani, 1984b; Hodgkin et al., 1987; Miller and Korenbrot, 1987) and Ca²⁺ influx through the cGMP-activated channel. Since these mechanisms seem to function even in tROS, local Ca²⁺ concentration in tROS might not equal the Ca²⁺ concentration in the test solution. To obtain a reliable value of the cytoplasmic Ca²⁺ concentration, we tried to suppress the Ca²⁺ fluxes across the plasma membrane. To inhibit the exchanger, Na⁺ in the electrode solution (outside of tROS) was replaced with choline, since external choline cannot support the Ca²⁺ efflux in place of Na⁺ (Schnetkamp, 1986). Then, to reduce the Ca²⁺ influx, Ca²⁺ concentration in the electrode solution was reduced.

Even under this condition, exogenously applied GTP could produce an outward membrane current that was suppressed by light (Fig. 5). Application of cGMP also produced an outward current (Fig. 4, see below). In the absence of Na⁺ and Ca²⁺, K⁺ in the test solution was the major ion species that permeated the cGMP-activated channel, so that the direction of the current was necessarily outward. Since the dark current was larger and more stable in this modified electrode solution than in Ringer solution, we used this condition in the following experiments.
Evidence for cGMP Synthesis from GTP in tROS

To obtain conclusive evidence that shows the synthesis of cGMP from GTP in tROS, we used two approaches. In one type of experiment, we will show that the conductance activated by GTP application is the same as that directly activated by cGMP. When the two conductances are the same, one substance can no more increase the dark current once it is fully activated by the other. First, we applied a nearly saturating dose of GTP (2 mM, see below) and the resultant outward dark current was measured by giving an intense light flash (Fig. 4A, first trace). After the light response, GTP was washed out and 4 mM cGMP was applied (second trace). Its application also induced an outward dark current whose amplitude was almost the same as that produced by an application of 2 mM GTP%. After cGMP was washed out, a mixture of 2 mM GTP and 4 mM cGMP were applied (third trace). The amplitude of the dark current was almost the same as that obtained by application of either 2 mM GTP or 4 mM cGMP alone, which indicated that the conductance activated by GTP application was the same as that activated by cGMP.

In excised patches of the plasma membrane, cGMP effect saturates at ~0.2 mM (Fesenko et al., 1985). In tROS, on the other hand, 4 mM cGMP did not seem to saturate the reaction (Fig. 4A). It is probable that, at the tip of tROS, the cGMP concentration did not reach the saturating level because cGMP was hydrolyzed by PDE during diffusion toward the tip. The light response waveform differed when...
different chemicals were used in Fig. 4 A. A possible explanation will appear in the Discussion.

The second type of experiment was based on the biochemical experiment showing that guanylate cyclase requires Mg$^{2+}$ to exert its activity (Fleischman and Denisevich, 1979). On the basis of this finding, we could expect that no current flowed on application of GTP when Mg$^{2+}$ was absent. Firstly, endogenous Mg$^{2+}$ in tROS was washed out for 5 min with a Mg$^{2+}$-free solution that was obtained by adding 3 mM EDTA to the pseudo-intracellular solution. Then, as a control, GTP was applied together with 2 mM Mg$^{2+}$ (no EDTA), which produced the dark current (Fig. 4 B, first application). When GTP was applied in the absence of Mg$^{2+}$, no dark current was produced as expected (second application). Since cGMP itself induced the dark current without Mg$^{2+}$ (third application), the above result clearly demonstrated that the current induced by GTP application was due to the synthesis of cGMP from GTP by guanylate cyclase in tROS.

The suppression of cGMP synthesis was observed only when Mg$^{2+}$ was removed completely: when 2 mM Mg$^{2+}$ was present in the electrode solution, the suppression of cGMP synthesis was partial. This result could be explained by the entry of external Mg$^{2+}$ into tROS, since Mg$^{2+}$ has been shown to permeate the cGMP-activated channel (Torre et al., 1987; Nakatani and Yau, 1988a).

**Dose Dependence of the Dark Current on GTP Concentration**

At various GTP concentrations, we measured the dark current amplitude. The current measured, however, contained an outward Na$^+$ current, because the GTP used was a 3Na$^+$ salt. Since the amplitude of the Na$^+$ current should be different at different GTP concentrations, we made a correction as shown in the following.

When 8 mM GTP was applied, for example, the Na$^+$ concentration inside of tROS was 24 mM. At low Ca$^{2+}$ concentrations, the relative permeability of Na$^+$ to K$^+$ is 1.25 (Hodgkin et al., 1985), which led us to estimate that 24 mM Na$^+$ was equivalent to 30 mM K$^+$. As the test solution contained 115 mM K$^+$, 30 out of 145 (=115 + 30) of the dark current should have been carried by Na$^+$. This current was subtracted from the measured dark current.

These corrections were made at various GTP concentrations, and the dose-response curve thus obtained (Fig. 5) indicated that the current half saturated at ~0.4 mM GTP and fully saturated at 4–8 mM GTP.

**Distribution of Guanylate Cyclase in the Rod Outer Segment**

At the base of the rod outer segment, some biochemical reactions preferentially take place (for example, phosphorylation; Shichi and Williams, 1979). Also, the light response is faster (Baylor et al., 1979). Therefore, it was interesting to examine the longitudinal distribution of the guanylate cyclase activity. Since the dark current amplitude reflects the cytoplasmic cGMP concentration, which is an index of the cyclase activity, we measured the relation between the length of the sucked tROS in the electrode and the amplitude of the dark current induced by GTP application. In this experiment, if a saturating concentration of GTP is used, all of the cGMP-activated channels are open and the length-current amplitude relation represents the longitudinal distribution of the channel, but not that of the cyclase. For this reason,
GTP concentration must be under saturating, and 1 mM GTP was used in this experiment (see Fig. 5).

The entire rod outer segment was sucked into an electrode, and the truncation was made at the basement of the outer segment. GTP was applied, and both the dark current and the length of tROS were measured. Then the outer segment was slightly protruded stepwise from the electrode by giving a positive pressure and the protruded portion was truncated each time in order to eliminate the contribution of the outer segment outside of the electrode. In this experiment, we selected electrodes so that protrusion could proceed smoothly.

The relation between the sucked tROS length and the dark current amplitude is shown in Fig. 6. The result showed that the current was almost linear to the length of tROS. The cGMP-activated channel distributes almost uniformly in the outer segment plasma membrane (Yau and Nakatani, 1985b), and we used 1 mM GTP so that the cGMP-activated channels were not saturated. Therefore, the linear relationship between the tROS length and the dark current amplitude suggests that the cytoplasmic cGMP concentration in tROS was almost constant along the long axis of the outer segment.

Nine out of twelve cells showed a linear relationship between the sucked tROS length and the current amplitude. Moreover, the extrapolated line seemed to cross the zero-zero point as in Fig. 6. In the rest of the cells examined, the length-amplitude relation also showed linearity, but the extrapolated line seemed to cross the zero-current value even when a significant length of tROS (~20 μm) still remained. Though we do not know the reason for this, it is possible that in these preparations the loss of synthesized cGMP by diffusion from the truncated end was high.

**Figure 5.** Dose dependence of the dark current on GTP concentration. tROS interior was perfused with test solutions containing GTP of various concentrations. For each GTP concentration, the amplitude of the dark current was measured by giving an intense light and was expressed as the percentage of the current obtained at 8 mM GTP. The contribution of Na⁺ to the current cannot be neglected at high GTP concentrations (see text) and it was subtracted from the total current by assuming that the relative permeability of Na⁺ to K⁺ is 1.25 (Hodgkin et al., 1985). The result shown is a mean of three experiments done in three different tROS preparations. Ca²⁺ concentration was 30 nM both inside and outside of tROS.
Cytoplasmic cGMP levels are determined by the balance between its formation by the cyclase and its hydrolysis by PDE. Unfortunately, the distribution of PDE has not yet been discovered, and quantitative estimation of the cyclase distribution was not possible. However, we could safely conclude that the cyclase distributes throughout the rod outer segment.

**Effect of Ca²⁺ on Cytoplasmic cGMP Levels**

Recently it has been suggested that light causes a decrease in the cytoplasmic Ca²⁺ concentration ([Ca²⁺]i) in intact photoreceptors (Bownds, 1980; Yau and Nakatani, 1985a; McNaughton et al., 1986; Miller and Korenbrot, 1987). Therefore, it was of interest to examine the effect of Ca²⁺ on the regulation mechanism of cGMP levels in tROS. The Ca²⁺ concentration in the electrode solution, namely the Ca²⁺ concentration outside of the tROS ([Ca²⁺]o) was kept constant and tROS was internally perfused with test solutions of which Ca²⁺ concentrations ([Ca²⁺]int) were varied. The concentration of GTP was kept constant at 2 mM throughout this experiment. As will be shown in the following, the dark current amplitude, and thus the cytoplasmic cGMP concentration, was increased by lowering [Ca²⁺]i, and the effect was reversible. However, the interpretation of the data was slightly complicated.

In Fig. 7 A, where [Ca²⁺]o = 1 mM, the dark current was small at [Ca²⁺]int = 100 or 10 nM, but when [Ca²⁺]int decreased to 1 nM, the current abruptly increased with a remarkable oscillation (see below). When [Ca²⁺]int was further reduced to 0.5...
nM, the current increased. Fig. 7B shows that the profile of the dark current–
[Ca$^{2+}$]_{test} relation was quite different when [Ca$^{2+}$]_{o} = 1 μM: a large dark current
started to flow at a concentration that was ten times higher ([Ca$^{2+}$]_{test} = 10 nM). At a
much lower [Ca$^{2+}$]_{o} (0.5 nM, Fig. 7C), the current flowed in all the ranges of
[Ca$^{2+}$]_{test} (1 μM–0.5 nM).

![Diagram of Figure 7](image)

**FIGURE 7.** Effect of cytoplasmic Ca$^{2+}$ on the dark current amplitude in tROS at three
different external Ca$^{2+}$ concentrations. (A) Ca$^{2+}$ concentration outside of tROS ([Ca$^{2+}$]_{o}) was 1
mM. tROS interior was perfused with test solutions containing 2 mM GTP plus various concentra-
tions of Ca$^{2+}$ ([Ca$^{2+}$]_{test}). To measure the dark current amplitude, an intense light flash
(log attenuation, 1.8) was given. (B) The same type of experiment as in A except that [Ca$^{2+}$]_{o}
was 1 μM. Log light attenuation was 1.5. (C) [Ca$^{2+}$]_{o} was 0.5 nM. At the region indicated as
30 nM Ca$^{2+}$ the cell was perfused with the intracellular solution that did not contain GTP.
Log light attenuation was 1.8. To suppress the Na$^{+}$-Ca$^{2+}$ exchange, Na$^{+}$ was eliminated in the
electrode solution (see text).

The above result was interpreted as follows. In general, [Ca$^{2+}$]_{o} could possibly be
affected by both the Na$^{+}$-Ca$^{2+}$ exchange and the Ca$^{2+}$ influx through the cGMP-
activated channel. In the present experiment, however, the exchanger was sup-
pressed by eliminating Na$^{+}$ in the electrode solution (see explanation for Fig. 3 in
text). On the other hand, Ca$^{2+}$ could easily permeate the cGMP-activated channel
(Torre et al., 1987; Nakatani and Yau, 1988a) and could affect the Ca~+ buffering in tROS to result in the modification of the cGMP metabolism. For example, at the beginning of the experiment in Fig. 7 A, [Ca~+] o was 1 mM and [Ca~+]t~t was 100 nM. With such an inward gradient of Ca~+, even if cGMP was once synthesized, cGMP levels were reduced by a feedback mechanism: when synthesized cGMP opened the cGMP-activated channel, Ca~+ entered tROS and the resultant increase in [Ca~+], reduced cGMP levels by reducing the cyclase activity (see below) as well as by increasing the PDE activity (Robinson et al., 1980; Kawamura and Bownds, 1981). Since [Ca~+]t~t was not low enough to compensate for the Ca~+ entry and because [Ca~+], became relatively high, no dark current flowed at [Ca~+]t~t = 100 and 10 nM. When [Ca~+]t~t decreased to 1 nM, cGMP synthesis was increased by lowering [Ca~+], and a large current started to flow. With the production of the dark current, Ca~+ entered tROS and the current decreased to some extent as considered above. However, at [Ca~+]t~t = 1 nM, [Ca~+], did not increase so much that a significant amplitude of the current remained. With the reduction of the current, less Ca~+ entered tROS and, therefore, [Ca~+], decreased to the value of [Ca~+]t~t because of the Ca~+ buffering. As a result, the current increased again. Since the successive reactions described above proceeded gradually, oscillation took place. The above consideration also explains the rebound observed after light stimulation in Fig. 7 A.

When the Ca~+ concentration gradient was reversed, e.g., [Ca~+] o = 0.5 nM and [Ca~+]t~t = 1 nM ~ 1 μM (Fig. 7 C), cGMP synthesis caused Ca~+ efflux from tROS to decrease [Ca~+] t~t in all the ranges of [Ca~+]t~t. As a result, the cytoplasmic cGMP concentration, and hence the dark current, were maintained at a high level. When [Ca~+] o was in an intermediate range (1 μM, Fig. 7 B), the Ca~+ fluxes were not large and the result was intermediate between those in Figs. 7, A and C.

If the above speculation was actually the case, [Ca~+] t~t equaled [Ca~+]t~t only when [Ca~+] o = [Ca~+]t~t. Keeping this condition in mind, we examined the relation between the dark current and [Ca~+],. [Ca~+] o was changed from 1 μM to 0.5 nM using different cells at each [Ca~+] o. The dark current amplitude was measured at various [Ca~+]t~t at each [Ca~+] o. In this range of [Ca~+] o (1 μM~0.5 nM), the dark current was saturated at almost the same level whether [Ca~+]t~t was 0.5 or 1 nM irrespective of [Ca~+] o. Taking the dark current amplitude at [Ca~+]t~t = 0.5 nM as 100%, the current amplitude at [Ca~+]t~t = [Ca~+] o ([Ca~+] o ~ [Ca~+] o) was plotted as a function of [Ca~+]t~t in Fig. 8 (filled circles). The result indicated that cytoplasmic cGMP levels were increased by reducing [Ca~+], and the half effect was observed at ~100 nM. The large variability of the data at [Ca~+] o ~ 100 and 30 nM suggested that this range of [Ca~+] is critical for the regulation of cGMP. In the figure, open symbols represent the relations between the dark current amplitude and [Ca~+]t~t at [Ca~+] o = 1 mM, 1 μM, and 0.5 nM shown in Fig. 7.

Cytoplasmic cGMP levels in tROS are determined by the balance between the activities of guanylate cyclase and PDE. Therefore, the result in Fig. 8 indicated that high [Ca~+], caused guanylate cyclase inhibition (Pepe et al., 1986; Koch and Stryer, 1988) and/or PDE activation (Robinson et al., 1980; Kawamura and Bownds, 1981). To examine which was dominant, cGMP, but not GTP, was applied to tROS, since on application of cGMP, only the effect of Ca~+ on PDE should be observed. The
concentration of cGMP was made 1 mM so that, at $[Ca^{2+}]_o = 1 \mu M$ and $[Ca^{2+}]_{test} = 0.5 \text{ nM}$, the current amplitude induced by cGMP was similar to or slightly less than that induced by 2 mM GTP application. The experiment therefore simulated the condition that the cyclase constantly synthesized cGMP irrespective of $[Ca^{2+}]_i$. We took the current amplitude induced by 1 mM cGMP under this condition as 100%. If the PDE activation was dominant, we should observe a current of small amplitude when $[Ca^{2+}]_{test}$ increased to 1 $\mu M$. However, this was not the case.

The horizontal broken line indicated by an arrow in Fig. 8 shows the current amplitude obtained at $[Ca^{2+}]_{test} = 1 \mu M$ relative to that obtained at $[Ca^{2+}]_{test} = 0.5 \text{ nM}$. At $[Ca^{2+}]_{test} = 1 \mu M$, 2 mM GTP application produced a dark current <30% of the maximum (Fig. 8) while 1 mM cGMP produced ~70% of the maximum (horizontal broken line). From this result, it is reasonable to conclude that PDE activa-

![Figure 8](image)

**Figure 8.** Dependence of the dark current on the cytoplasmic Ca$^{2+}$ concentration. (Filled circles) The amplitude of the dark current induced by addition of 2 mM GTP was determined at various Ca$^{2+}$ concentrations in test solutions ($[Ca^{2+}]_{test}$). For details, see text. The amplitude at 0.5 nM internal Ca$^{2+}$ was taken as 100% and each value was expressed as the percentage. Each datum shown is a mean of 3–14 experiments ± SE. The number of the experiments are 14 (1 $\mu M$ Ca$^{2+}$), 9 (100 nM), 11 (30 nM), 4 (10 nM), and 3 (1 nM). The horizontal broken line indicated by an arrow shows the relative dark current amplitude when 1 mM cGMP was applied at $[Ca^{2+}]_o = [Ca^{2+}]_{test} = 1 \mu M$ (see text). The mean of four experiments was 71 ± 7%. (Open symbols) The relation between $[Ca^{2+}]_{test}$ and the dark current amplitude is calculated from the data in Fig. 7. Squares, $[Ca^{2+}]_o = 1 \mu M$; triangles, 1 $\mu M$; circles, 0.5 nM. The current amplitudes were measured at the point of light responses in Fig. 7.

... tion was not the primary determinant of the small dark current at higher $[Ca^{2+}]_i$, in Fig. 8. This, in turn, indicated that the effect of $[Ca^{2+}]_i$ on cytoplasmic cGMP levels (dotted line) reflects mainly the Ca$^{2+}$ dependence of guanylate cyclase.

In the present experiment, we estimated the cytoplasmic cGMP level but not the cyclase activity itself. It might be possible to obtain a direct relation between $[Ca^{2+}]_i$ and the cyclase activity from the result in Fig. 8. However, variability of the data is rather large in Fig. 8. Furthermore, we cannot estimate the loss of cGMP due to diffusion from the truncated end as well as the amounts of cGMP hydrolyzed by PDE at various Ca$^{2+}$ concentrations. For these reasons, further analysis was not conducted.

Concerning the waveform of the light response, it should be emphasized that, in
each record in Fig. 7, the light response became more transient as \([\text{Ca}^{2+}]_{\text{int}}\) decreased. The result indicated that the recovery was accelerated at low \(\text{Ca}^{2+}\) concentrations because cGMP synthesis was facilitated. Therefore, if \([\text{Ca}^{2+}]_{\text{int}}\) is constantly reduced during light adaptation in intact photoreceptors, the time course of a light response should be speeded up with this mechanism.

**Rapid Effect of Lowering \([\text{Ca}^{2+}]_{\text{int}}\), on the Light Response Waveform**

\([\text{Ca}^{2+}]_{\text{int}}\) has been suggested to decline quickly after a light flash (Yau and Nakatani, 1985a; Miller and Korenbrot, 1987), and it was interesting to investigate how rapidly the cyclase is activated by \([\text{Ca}^{2+}]_{\text{int}}\), decline.

![Graph showing the effect of lowering \([\text{Ca}^{2+}]_{\text{int}}\) on the light response waveform.](image)

In Fig. 9, first the tROS interior was perfused with a test solution containing 100 nM \(\text{Ca}^{2+}\) and 2 mM GTP, and a light response was recorded (top trace in Fig. 9). \([\text{Ca}^{2+}]_{\text{int}}\) was 30 nM. After the light response recovered, a second light flash was given (second trace). In this case, to reduce \([\text{Ca}^{2+}]_{\text{int}}\), tROS interior was pulse-perfused for 0.7 s with a 1 nM \(\text{Ca}^{2+}\) solution at the time indicated by an arrow. As can be seen in the trace, the effect was detected within several seconds. When the 1 nM \(\text{Ca}^{2+}\) solution was applied for a longer period of time (1.3 s), the acceleration was more prominent and an overshoot was observed (third trace).

The result in Fig. 9 demonstrated that \([\text{Ca}^{2+}]_{\text{int}}\) reduction immediately increases the cyclase activity and speeds up the light response recovery.
An Additional Mechanism for the Acceleration of the Light Response Time Course

At present, $[Ca^{2+}]$, decline during a light response is explained by the Na$^+$-Ca$^{2+}$ exchanger as follows (Yau and Nakatani, 1985a). In the dark, Ca$^{2+}$ enters the cell through the cGMP-activated channel. When the cell absorbs light, the cGMP-activated channel closes so that Ca$^{2+}$ cannot enter the cell. Since the exchanger operates constantly irrespective of light conditions, $[Ca^{2+}]_i$ decreases after a light stimulation. Consequently, we wanted to examine whether the light itself affects the cGMP metabolism through a mechanism other than the Na$^+$-Ca$^{2+}$ exchange. The following experiment showed that it is possible.

To test this possibility, $[Ca^{2+}]$ should not decrease during light stimulation. To suppress the exchanger and hence the $[Ca^{2+}]$, decline during a light response, Na$^+$ in the electrode solution was replaced with choline since the exchanger requires external Na$^+$ to operate (Yau and Nakatani, 1984b; Schnetkamp, 1986; Hodgkin et al., 1987). In addition, $[Ca^{2+}]_o$ was made lower (1 nM) than $[Ca^{2+}]_e$ (100 nM) to ensure that the Ca$^{2+}$ influx in the dark did not occur. Under this condition, when cGMP is synthesized from GTP in the dark, Ca$^{2+}$ goes out from the tROS interior through the cGMP-activated channel according to the Ca$^{2+}$ gradient so that the local $[Ca^{2+}]_i$ is lower than 100 nM. When a light stimulation is applied, the channel closes and Ca$^{2+}$ cannot go out from the cell. As a result, $[Ca^{2+}]_i$ is expected to increase during light stimulation.

In this experiment, repetitive light flashes were given. Even with this stimulation, $[Ca^{2+}]_i$ is expected to increase. In Fig. 10, after a light flash was given, a train of light flashes of the same intensity were given every 7 s. It was evident from the figure that each light response became more transient as the stimulation proceeded. In addition, a rebound was observed after an intense light stimulation. Since $[Ca^{2+}]_i$ did not decrease during the stimulation, the experiment indicated that repetitive light stimulation facilitated the cGMP recovery with a mechanism different from the $[Ca^{2+}]$, decline attained by the exchanger. In other words,
there is an additional light-dependent mechanism responsible for the acceleration of the light response time course during light adaptation.

One may suspect that during the repetitive light stimulation in Fig. 10, GTP in tROS were depleted because of the increased consumption of GTP. However, this does not explain the acceleration of the time course; if it happens, the cGMP supply would decrease to slow the recovery time course, contrary to the result in Fig. 10.

**DISCUSSION**

The present study shows that tROS synthesizes cGMP from GTP (Figs. 2–4) and the enzyme responsible for the reaction, guanylate cyclase, distributes throughout the rod outer segment (Fig. 6). The enzyme is activated by lowering the cytoplasmic Ca$^{2+}$ concentration (Figs. 7 and 8), and with this mechanism, the cyclase contributes to shape the light response waveform (Figs. 7 and 9). Therefore, if the cytoplasmic Ca$^{2+}$ concentration decreases in intact photoreceptors during light adaptation as suggested, this mechanism seems to explain the acceleration of the time course of the light response. However, there is an additional light-dependent mechanism that facilitates the cGMP recovery during light adaptation (Fig. 10).

**Guanylate Cyclase in Rod Outer Segments**

Recently, Sather and Detwiler (1987) reported that in the rod outer segment attached with a whole-cell patch electrode the dark current was produced when GTP was supplied from the electrode. They concluded that rod outer segments contain guanylate cyclase. Our present study supports their view and provides conclusive evidence on it: the current produced by GTP application flows through the cGMP-activated channel (Fig. 4 A) and GTP does not induce the current without Mg$^{2+}$, which is known to be necessary for the guanylate cyclase activity (Fig. 4 B).

The guanylate cyclase activity has been shown to be located in an axoneme-basal apparatus complex which includes the outer segment microtubule doublets, the basal body, etc. (Fleischman and Denisevich, 1979). The microtubule doublets extend only a few μm from the basement of the outer segment toward the distal part (Yamada, 1984). However, this experiment shows that guanylate cyclase distributes throughout the rod outer segment (Fig. 6), which indicates that the microtubule doublets are not the only site of guanylate cyclase. Besides the doublets, singlet microtubule structure was observed as occupying up to two-thirds of the total length from the base of the rod outer segment (J. Usukura, personal communication). It is possible that the singlet microtubule is also the site of the cyclase. Alternatively, the enzyme might be present on another structure in the outer segment. Histochemical work suggested that the cyclase is located on the disk membrane (Toibana et al., 1982).

As shown in Fig. 9, application of a low-Ca$^{2+}$ solution during a light response facilitates the recovery time course of the light response most probably because of the increase in cytoplasmic cGMP levels. The effect was observed within several seconds after the application. When the diffusion process of the applied solution is taken into account, the time necessary to activate the cyclase by lowering [Ca$^{2+}$], is rapid and less than a few seconds. The published data indicate that, on bright light
stimulation to intact rods, \([\text{Ca}^{2+}]_i\) decreases in the effective range of the cyclase regulation (1 \(\mu\text{M} - 10 \text{nM}\), Fig. 8) from \(~0.5 \mu\text{M}\) in the dark to a much lower level (McNaughton et al., 1986). The time constant of the \([\text{Ca}^{2+}]_i\) decline in rods has been reported to be \(<1 \text{s}\) (Nakatani and Yau, 1988a). Therefore, the closure of the cGMP-activated channel after a light stimulation immediately reduces \([\text{Ca}^{2+}]_i\), and immediately activates the cyclase so that the waveform shows relaxation from its initial peak. It has been recently shown that without external \(\text{Na}^+\), and therefore \(\text{Ca}^{2+}\) extrusion, the light response does not show relaxation (Nakatani and Yau, 1988c).

After a light stimulation in intact photoreceptors, the activation of the cyclase seems to continue for a while since the photoreceptor membrane potential recovers more rapidly than the PDE activity (Kawamura and Murakami, 1986b). In agreement with the finding of Goldberg et al. (1983) that light increases the cGMP metabolic flux, it appears that the sustained hydrolysis of cGMP by PDE after a light stimulation is compensated for by the cyclase.

In the present experiment, cytoplasmic cGMP levels are determined mainly by the guanylate cyclase activity (Fig. 8). On this basis, we estimated the cyclase activity in tROS from the initial rate of rise of the dark current. In excised patches of the rod outer segment, the cGMP-activated channel is fully activated at 0.2 mM cGMP (Fesenko et al., 1985; Yau and Nakatani, 1985b). In our experiment, the dark current was almost maximum at \([\text{Ca}^{2+}]_o = [\text{Ca}^{2+}]_{\text{rest}} = 0.5 \text{nM}\) (i.e., \([\text{Ca}^{2+}]_i = 0.5 \text{nM}\)) (Figs. 7C and 8). It would be reasonable to assume that under this condition, the cGMP concentration in tROS was close to 0.2 mM. From the initial rise of the dark current induced by GTP application at \([\text{Ca}^{2+}]_i = 0.5 \text{nM}\), we obtained the value of \(~20 \mu\text{M}\) cGMP synthesized per s. This value, however, would be underestimated, because the loss of the synthesized cGMP due to diffusion from tROS interior as well as hydrolysis by PDE could not be taken into account.

In intact rods, \([\text{Ca}^{2+}]_i\) is possibly below 0.5 \(\mu\text{M}\) (McNaughton et al., 1986). In this physiological range of \([\text{Ca}^{2+}]_i\), the cyclase activity would be reduced several fold from the maximum value obtained in tROS according to the result in Fig. 8, and there would be several micromolar cGMP synthesized per second. In intact rod photoreceptors, the cGMP concentration in the dark is thought to be several micromolar (Yau and Nakatani, 1985b; Fesenko et al., 1986). Therefore, even if cGMP is entirely hydrolyzed by PDE that was activated by photon absorption, the cyclase would be able to restore the cGMP concentration within seconds after PDE was inactivated. Recently, using biochemical measurement, Koch and Stryer (1988) reported that the \(\text{Ca}^{2+}\) effect on the cyclase is similar to the result shown in this work. Their estimated value of the cyclase activity is in the same range as ours.

"Soluble" Proteins in Rod Outer Segments

Biochemical experiments have revealed that several protein components are involved in the phototransduction mechanism. Some of them are thought to be soluble proteins (for a review, see Kühn, 1984). However, even though the tROS interior was continuously perfused in our experiment, the light response could be observed for \(>1.5 \text{h}\) when GTP, ATP, and IBMX were supplied. Our result there-
fore suggests that the protein components supposed to be “soluble” are not always “solubilized” under in situ conditions. It is possible that those proteins loosely bind to the membrane under in situ conditions but become soluble after some biochemical manipulations.

Waveform of the Light Response in tROS

In Fig. 4 A, we recorded the light responses in the presence of 2 mM GTP, 4 mM cGMP, or a mixture of both. The recovery of the light response was fastest with both GTP and cGMP present, and slowest with cGMP alone. Though this result may appear to indicate that GTP itself facilitates the response recovery, we should be cautious of this conclusion because the rate at which cGMP is supplied greatly influences the recovery phase as shown in Figs. 7 and 9.

When cGMP is exogenously applied to tROS, cGMP is supplied by diffusion from the perfusion solution. In the case of GTP application, on the other hand, cGMP supply is attained locally by guanylate cyclase and it is probably supplied faster than when the diffusion is from the outside. Since in this experiment the concentration of synthesized cGMP was <0.2 mM and lower than that of the applied GTP (2 mM) as discussed above, some amount of GTP was always present in tROS and could be readily supplied to the cyclase. If this was the case in the experiment in Fig. 4 A, the fastest recovery in the presence of both GTP and cGMP could be explained: cGMP supply in this case was largest because both synthesis and diffusion took place.

The above consideration raises the possibility that GTP is preferable to cGMP as the exogenous chemical when the light response waveform is studied in tROS or comparable preparations.

Regulation of Light Response Waveform without Ca\textsuperscript{2+} Changes

In Fig. 10, the acceleration of the time course of the light response is observed during repetitive light flashes even when [Ca\textsuperscript{2+}] increases by light stimulation. The result indicates that a train of light flashes induce gradual facilitation of cGMP supply after each flash. At present, we speculate that a membrane-bound process(es) induces the activation of the cyclase and/or the facilitation of the PDE inactivation. One of the bases of this speculation is the light activation of guanylate cyclase observed biochemically in intact frog retina (de Azeredo et al., 1981). However, as an alternative, it is also possible that [Ca\textsuperscript{2+}], decreases during a repetitive stimulation through an unknown mechanism(s) associated with the disk membrane and/or the plasma membrane.

Dowling and Ripps (1972) reported that the light adaptation of the skate retina developed in the range of minutes rather than seconds when an intense background illumination was given. As discussed above, the [Ca\textsuperscript{2+}], decline after a light stimulation immediately affects the light response waveform. On the other hand, the data in Fig. 10 suggests that the waveform control by the unknown mechanism is a relatively slow process. It is possible that this mechanism starts to function after a prolonged and/or an intense illumination. The mechanism and the physiological significance of this control certainly deserves further study.
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