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Light-dependent effects of a hydrolysis-resistant analog of GTP on rod photoresponses in the toad retina

(rod photoreceptors/visual adaptation/phototransduction/GTP-binding protein)

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ABSTRACT **Responses to 100-ms flashes were recorded intracellularly from dark- and light-adapted rod photoreceptors in the isolated retina of the toad, *Bufo marinus*. Properties of photoresponses were analyzed under each condition of adaptation when retinas were superfused with 1.0 mM guanosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppG), a hydrolysis-resistant analog of GTP. When applied to retinas that previously had been subjected to intense light (≈30% bleach), p[CH_2]ppG increased both the amplitude and duration of photoresponses. By contrast, treatment of dark-adapted retinas with p[CH_2]ppG did not alter these response parameters. When similarly applied to either dark- or light-adapted retinas, GTP had no effect on amplitude or duration of photoresponses. These results are discussed in terms of GTP-dependent mechanisms for rod adaptation.**

Exposure of the vertebrate retina to intense light markedly alters visual responses of rod photoreceptors. Numerous studies have emphasized the contribution to this adaptational process of a mechanism linked to the state of visual pigment in the receptor outer segments. For example, partial bleaching of visual pigment in the isolated retina permanently decreases the amplitude and duration of rod photoresponses; the sustained loss of sensitivity is correlated with the extent of bleaching and greatly exceeds the loss expected through reduction in quantum catch (1–4). These observations suggest that bleached visual pigment, through a mechanism as yet undetermined, acts to sustain a condition of light adaptation in rods.

Within rod photoreceptors, GTP participates in several reactions stimulated by the bleaching of visual pigment. The activation of cyclic GMP phosphodiesterase (PDEase) in outer segments requires both photoactivated visual pigment and GTP (5, 6). The pigment molecule is believed to function in this process by catalyzing the binding of GTP to another protein ("transducin"), which then interacts with the PDEase (7–10). In this process, analogs of GTP resistant to hydrolysis between the β and γ phosphates readily substitute for GTP (6, 11, 12). Through events possibly linked to activation of the PDEase, bleaching of the visual pigment also is associated with an increase of GTPase activity within the rod (11–16).

The indications of a close relationship between the bleaching of visual pigment and the utilization of GTP suggest the possibility that one or more GTP-dependent processes may regulate light adaptation in rods. For example, as a consequence of intense irradiation, free GTP or the activated form of a GTP-binding protein might become limiting for a step in some visual process. To explore such a possibility, we examined whether dark- and light-adapted rods in an isolated retina respond differently to treatments designed to influence GTP-dependent mechanisms. We report here that toad rods exhibit such a differential response to superfusion with guanosine 5′-(β,γ-methylene)triphosphate (p[CH_2]ppG), a hydrolysis-resistant analog of GTP. Results of this study were presented at the 1981 meeting of the Association for Research in Vision and Ophthalmology (17).

METHODS

Electrophysiology. All experiments used the isolated retina preparation of the toad, *Bufo marinus* (18). Animals were dark-adapted for 12–18 hr and then sacrificed by double-pithing. With the use of infrared illumination and image converters, an eye was excised and a section of retina (≈5 × 5 mm) was dissected free. The isolated retina was placed receptor-side up in a recording chamber (volume, 0.3 ml) that had a transparent bottom. The chamber was placed on the stage of an inverted compound microscope (Zeiss Invertoscope, model D), and the retina was viewed from below with infrared illumination and an image converter (Varo, model 6914).

Under visual control, a rod outer segment was impaled with a glass micropipette electrode. The electrodes contained 1.0 M potassium acetate (pH 7.1) and had resistances ranging from 200 to 400 MΩ. The impaled cells were likely the larger and more numerous red rods, based on their response waveform and sensitivity to 500-nm stimuli (18). The microelectrode voltage was measured with respect to a Ag/AgCl electrode connected to the fluid surrounding the retina through a Ringer's solution/agar bridge. The microelectrode voltage was amplified, displayed on an oscilloscope, and stored on frequency-modulation (FM) tape (Hewlett–Packard recorder, model 3960) for off-line analysis. Selected recordings were digitized by a computer (Digital Equipment, LSI 11/03) and redisplayed with a digital plotter (Hewlett–Packard, model 7225A). In several figures, certain waveforms are averages of two or more responses.

In all experiments, stimuli were 100-ms flashes of 500-nm light. The stimuli, which were 2.0 mm in diameter and centered upon the impaled rod, more than covered the receptive field of the rod (19–21). The stimulus irradiance was varied with calibrated neutral density filters. The irradiance of the unattenuated stimulus in the plane of the retina, measured with a calibrated photodiode (United Detector Technology, model 10DP), was 1.0×10^5 quanta $\mu m^{-2} s^{-1}$. Because the effective collecting area of the toad rod is ≈29.5 μm^2 (22, 23), an unattenuated 100-ms stimulus would cause 3.0×10^5 photoisomerizing events per rod.

Solutions. Throughout each experiment, the retina was superfused (2.0 ml/min) with oxygenated Ringer's solution. The control solution, modified from that used by Brown and Pinto

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Abbreviations: p[CH_2]ppG, guanosine 5′-[β,γ-methylene]triphosphate; PDEase, cyclic GMP phosphodiesterase.

(18), was 111 mM NaCl/2.5 mM KCl/0.86 mM $CaCl_2$/1.6 mM $MgCl_2$/3.0 mM Hepes/5.6 mM glucose/0.014 mM phenol red. The pH was adjusted to 7.8 with NaOH. The solution flowing over the retina could be switched to various test solutions by using a three-way valve; the 1.5-ml dead space in the superfusion system introduced a delay of about 45 s in the arrival of a test solution at the retina. Test solutions were prepared by dissolving p[CH_2]ppG, GTP, or ATP (Sigma) to a final concentration of 1.0 mM in the control Ringer's solution. Because each of these compounds chelates Ca^{2+} (24), the activity of Ca^{2+} (a_{Ca}) was measured in each solution with a Ca^{2+}-selective electrode (Corning, model 476041). The concentration of Ca^{2+} in each test solution was equated to within 0.01 mM of that of the control solution by the addition of $CaCl_2$; in addition, the pH of each test solution was equated to the pH of the control solution.

Light Adaptation. A dark-adapted rod was impaled and characterized by the presentation of test flashes of varying irradiance. The retina was then exposed for 60 s to intense, full-field, white light ("standard" adapting irradiation). Invariably, the microelectrode became dislodged from the impaled rod within 15 min after this adapting irradiation, necessitating the impalement of another rod. Data from all rods referred to as light-adapted were obtained at least 20 min after termination of the adapting irradiation. This 20-min period was found to be sufficient for completion of the rapid, "neural" adaptation exhibited by rods in the isolated retina (1–4).

Spectrophotometry. The bleaching efficiency of the adapting light was measured by recording transretinal absorbance from 350 to 650 nm (Perkin–Elmer spectrophotometer, model 320, modified for use with horizontally positioned retinas). For each measurement, an initially dark-adapted retina was subjected to the standard adapting irradiation, and the reduction in absorbance was compared with the overall reduction in absorbance exhibited after exhaustive bleaching in the presence of hydroxylamine (4). In four experiments, the fractional decrease in absorbance at 525 nm induced by the standard irradiation was 0.29 ± 0.07 (SD). This value was taken to represent the fractional extent of bleaching of rhodopsin in the red rods (4, 25).

RESULTS

Light-Adaptation of the Rods. The standard adapting irradiation induced significant and sustained changes in the amplitude and time course of rod photoresponses. The wave-forms in Fig. 1 were obtained from a dark-adapted and a light-adapted rod in the same retina; these data show the sustained effects of the adapting exposure on the response to a stimulus of fixed irradiance. The data in Fig. 2, obtained from a total of 16 rods in 8 retinas, describe photoresponses of dark- and light-adapted rods to stimuli of varying irradiance. As a consequence of light adaptation, the function representing response amplitude is shifted to the right and reduced in maximum value; in addition, the duration of the photoresponse (defined as the interval between stimulus onset and the time at which the response decays to 10% of its peak amplitude) is decreased markedly. These persisting effects of strong light adaptation closely resemble those observed previously in rods of the isolated retina of axolotl (3).

Treatment with Test Substances. Superfusion of light-adapted retinas with 1.0 mM p[CH_2]ppG increased the amplitude and duration of rod photoresponses. Fig. 3 *Upper* shows a record obtained from a light-adapted rod before and during treatment with p[CH_2]ppG. Within 3 min after the start of treatment, the photoresponses began to increase in amplitude and duration; by 16 min, the response amplitude had increased by 55% and the response duration had increased by 150%. Spectrophotometric measurements showed that treatment of the light-adapted retina with 1.0 mM p[CH_2]ppG had no detectable effect on the level of visual pigment in the rods (data not illustrated). Fig. 3 *Lower* shows a record obtained from a dark-adapted rod in another retina. Treatment of this retina with p[CH_2]ppG did not significantly affect response amplitude or duration. In all rods examined, treatment with p[CH_2]ppG had

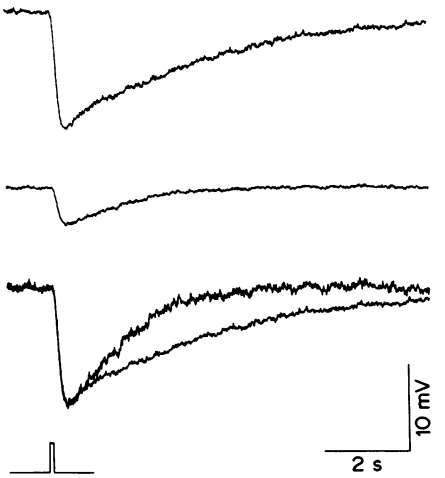

FIG. 1. Responses recorded from dark- and light-adapted rods. (*Top*) Average of two responses obtained from a dark-adapted rod. In this and every other figure illustrating response waveforms, the stimulus was a 100-ms flash having an irradiance of 25 quanta $\mu m^{-2} s^{-1}$. (*Middle*) Average of five responses obtained from a second rod in the same retina, 50–52 min after extinction of the standard adapting light. (*Bottom*) Records (*Top* and *Middle*) scaled for equal peak voltages (normalized).

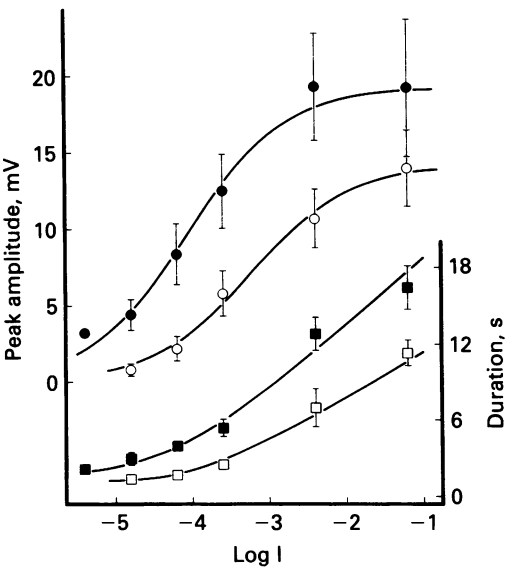

FIG. 2. Effects of the standard adapting irradiation on peak amplitude (●, ○) and duration (■, □) of photoresponses recorded from dark-adapted (●, ■) and light-adapted (○, □) rods. Each set of data represents measurements from at least six rods (in at least six retinas); each data point represents the mean ± 1 SD for measurements obtained from at least three rods. The curve representing the response amplitudes is of the form $V/V_{max} = I^n/(I^n + \sigma^n)$, where V is the amplitude, V_{max} is the maximum amplitude, I is the stimulus irradiance, and σ and n are constants (3, 22, 26). The curve was fit to the mean values of the data using a nonlinear, least-squares regression. For the dark-adaptation data, σ = 22 quanta/rod-flash, n = 0.68, and V_{max} = 19.3 mV. For the light-adaptation data, σ = 150 quanta/rod-flash, n = 0.67, and V_{max} = 14.3 mV.

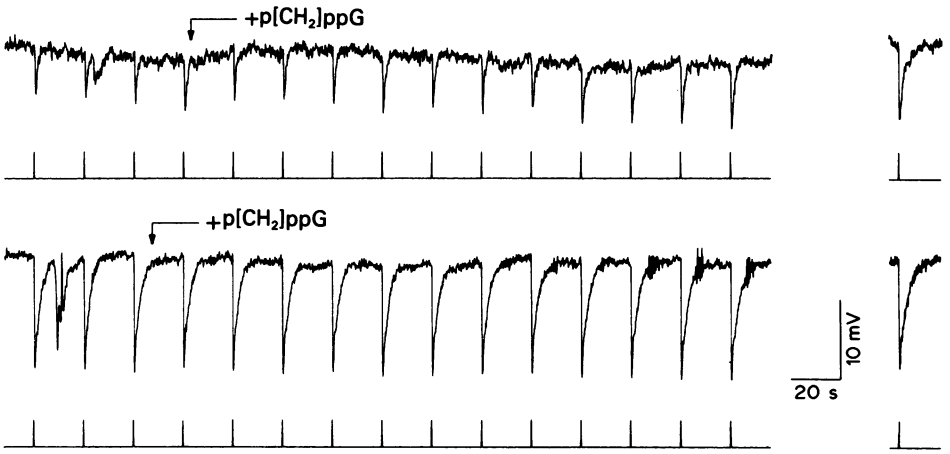

FIG. 3. Responses of a light-adapted (*Upper*) and a dark-adapted (*Lower*) rod to p[CH_2]ppG. Stimuli were presented every 20 s. Arrows indicate the times of arrival of Ringer's solution containing 1.0 mM p[CH_2]ppG in the perfusion chamber.

no appreciable effect on baseline membrane potential; when variations were observed, they did not appear to be correlated with changes in the photoresponse.

Fig. 4 shows data from a light-adapted rod in another retina that was treated with p[CH_2]ppG. Responses to stimuli of varying irradiance were obtained both before and during exposure to p[CH_2]ppG. As a consequence of this treatment, the curves describing amplitude and duration of photoresponses shifted in the direction of dark adaptation. A return to superfusion with control Ringer's solution, carried out after 10 min of treatment with p[CH_2]ppG, did not reverse the effects of treatment.

Fig. 5 shows photoresponses from three retinas; these experiments involved the application of p[CH_2]ppG to a light-adapted retina (Fig. 5 *Left*), of p[CH_2]ppG to a dark-adapted retina (Fig. 5 *Middle*), and of GTP to a light-adapted retina (Fig. 5 *Right*). Responses shown in row A were recorded from a dark-adapted rod in each of the two retinas that later were light-adapted. Responses shown in rows B and C of each column were recorded from a single rod in each retina, before and during treatment with the test substance. Shown in row D are superimposed responses from the corresponding column, scaled in Fig. 5 *Left* and *Right* to have equal peak amplitudes. Data from Fig. 5 *Left* further illustrate the effects of p[CH_2]ppG on light-adapted rods. In this rod, as in rods from three other light-adapted retinas treated with p[CH_2]ppG, the photoresponse recorded after treatment with p[CH_2]ppG had a time course virtually indistinguishable from that of a dark-adapted rod in the same retina. Neither treatment of the dark-adapted retina with p[CH_2]ppG nor treatment of the light-adapted retina with GTP significantly altered the amplitude or the duration of the photoresponses. In similar experiments (not illustrated), GTP was inactive in dark-adapted retinas; ATP was inactive in both dark- and light-adapted retinas.

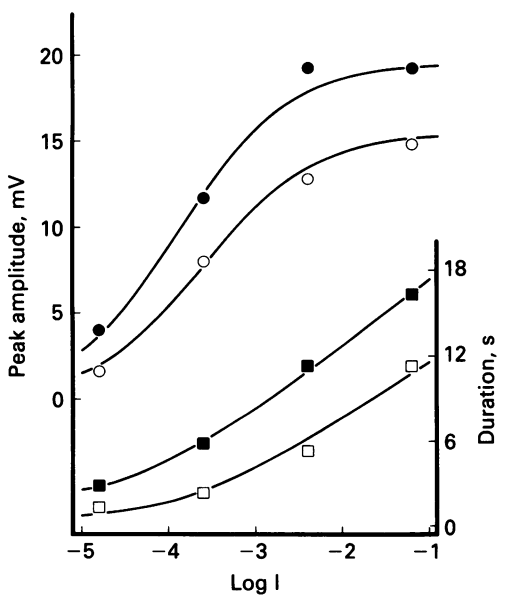

FIG. 4. Effects of p[CH_2]ppG on response properties of a light-adapted rod: peak amplitude (○) and duration of photoresponses (□) recorded prior to treatment with p[CH_2]ppG (these data were obtained 23–26 min after extinction of the adapting light); peak amplitude (●) and duration of photoresponses (■) recorded after 4–9 min of treatment with p[CH_2]ppG. The solid curves describing the peak amplitudes are as described for Fig. 2. Prior to treatment, σ = 63 quanta/rod-flash, n = 0.69, and V_{max} = 15.5 mV. After treatment, σ = 33 quanta/rod-flash, n = 0.70, and V_{max} = 19.5 mV.

Changes in the amplitude and duration of the photoresponse might be expected if p[CH_2]ppG had a direct effect on one of the voltage-dependent conductances in the plasma membrane of the rod. Evidence that p[CH_2]ppG does not alter these conductances in light-adapted retinas came from an experiment in which effects of p[CH_2]ppG were examined in the presence of pharmacological agents that block voltage-dependent conductances in rods (28, 30, 31). Under such experimental conditions, photoresponses recorded from different rods in the same retina, before and during treatment with p[CH_2]ppG, displayed substantial differences in amplitude and time course (Fig. 6), analogous to those observed in control solutions. Thus, it is unlikely that the mechanism of action of p[CH_2]ppG involves direct effects on voltage-dependent conductances.

DISCUSSION

The principal observation of this study is that p[CH_2]ppG stimulates significant changes in photoresponses of light-adapted rods in the isolated retina of the toad. An influence of p[CH_2]ppG on extracellularly-recorded, aspartate-isolated photoreceptor potentials, similarly dependent on state of adaptation, has been observed in the isolated retina of the skate (32).

In view of the particular conditions used in our experiments, certain mechanisms are not likely to underlie the observed effects of p[CH_2]ppG on response waveform. It is unlikely that the action of p[CH_2]ppG upon light-adapted rods involved

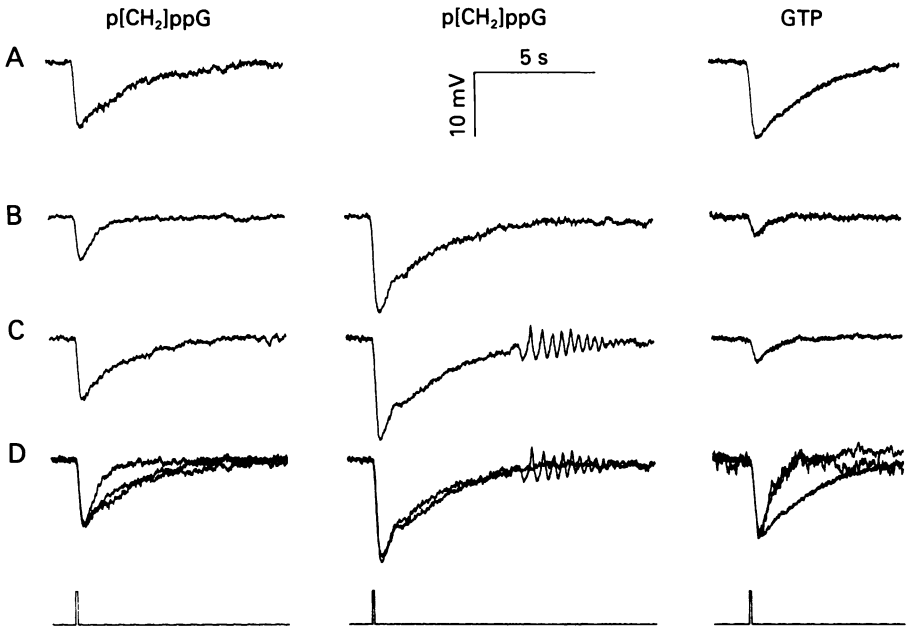

FIG. 5. Effects of p[CH_2]ppG and GTP on rod photoresponses. Responses in row A were obtained from dark-adapted rods. Responses in rows B and C were obtained under the following conditions. (*Left*) Light-adapted rod before (row B) and after (row C) 7 min of superfusion with p[CH_2]ppG. (*Middle*) Dark-adapted rod before (row B) and after (row C) 7 min of superfusion with p[CH_2]ppG. (*Right*) Light-adapted rod before (row B) and after (row C) 6 min of superfusion with GTP. Shown in row D are superimposed responses from the corresponding column; the responses (*Left* and *Right*) have been scaled to have equal peak amplitudes. Oscillations appearing in one waveform represent a transient phenomenon observed in several experiments after introduction of a test substance. The oscillations appeared to be independent of either the test substance or the state of adaptation; as yet, the cause of these oscillations is unclear. A similar oscillatory component in the photoresponse of toad rods has been observed under other experimental conditions (27–29).

changes in electrical coupling between receptors because the areas illuminated by the stimulating and adapting beams greatly exceeded the domain over which electrical coupling mediates interactions between red rods (19–21). Furthermore, all rods presumably were exposed to equal concentrations of p[CH_2]ppG. The similar stimulation and chemical treatment of all rods suggest that all rods should have similar response waveforms; thus, it is difficult to see how p[CH_2]ppG could have its effect simply by altering lateral interactions between rods. A selective effect of p[CH_2]ppG in cone photoreceptors, transmitted to rods by electrical or chemical synaptic interaction, also appears unlikely. In previous studies, cone input was observed to dominate the rod responses only when intense stimuli were superimposed on rod-saturating backgrounds (22, 33). The fact that p[CH_2]ppG still has its effect in the presence of 0.5 mM Co^{2+} (Fig. 6) argues against the possibility that the action of p[CH_2]ppG involves input to rods from horizontal cells (21).

Three findings argue against the possibility that some direct effect on the conductances of the rod plasma membrane accounts for the observed action of p[CH_2]ppG. First, when applied to either dark- or light-adapted retinas, p[CH_2]ppG does not significantly alter resting membrane potential. Second, in dark-adapted rods, p[CH_2]ppG does not significantly affect the light- and voltage-dependent conductances that shape the waveform of the photoresponse. Third, in light-adapted retinas, the blockage of voltage-dependent conductances in the rods appears not to suppress the action of p[CH_2]ppG on the rod photoresponse.

On the basis of the above arguments, it is likely that p[CH_2]ppG acts within the rods themselves. What might be the nature of the activity of p[CH_2]ppG? In view of the known properties of transducin and other GTP-binding regulatory proteins (10, 12, 34, 35), we suggest the following hypothesis for the effects of p[CH_2]ppG in relation to rod adaptation. (*i*) An intracellular protein, activated by the binding of GTP, influences the photic responsiveness of rods. However, in dark-adapted rods, generation of the photoresponse is not limited by the availability (i.e., maximal level or lifetime) of the protein in its activated, GTP-charged form. (*ii*) Strong light adaptation causes the availability of the activated protein to become limiting for generation of the photoresponse [perhaps as a consequence of increased GTPase activity (11–16)]. This condition is characterized by reduced amplitude and duration of responses to dim flashes. (*iii*) Introduction of hydrolysis-resistant p[CH_2]ppG into the light-adapted rod, possibly in concert with repeated photic stimulation (10, 12), increases the availability of activated (p[CH_2]ppG-charged) protein. This increase promotes changes in the photoresponse (increased amplitude and duration) that mimic those occurring naturally during dark adaptation. GTP, when similarly introduced, is hydrolyzed rapidly and, thus, has no significant effect on availability of the activated protein.

Viewed according to this hypothesis, our data suggest that the desensitizing effect of bleaching on vertebrate rods (1–4) derives in significant part from the reduced availability, in activated form, of a GTP-binding protein. Furthermore, the regeneration of visual pigment, an event which appears to promote dark adaptation of rods (4), may function to restore maximal availability of the activated GTP-binding protein.

Could the role of GTP in rod adaptation, as hypothesized above, be mediated (exclusively) by transducin, which, in its GTP-charged form, activates PDEase (7–10)? Evidence that PDEase controls generation of the photoresponse recently has been reported by Nicol and Miller (36, 37); these investigators found that an injection of cyclic GMP into toad rods increases the amplitude and duration of the subsequent photoresponse. The similarities of these results to those that we obtained with p[CH_2]ppG raise the possibility that, in our light-adapted preparation, p[CH_2]ppG binds to transducin (through a light-stim-

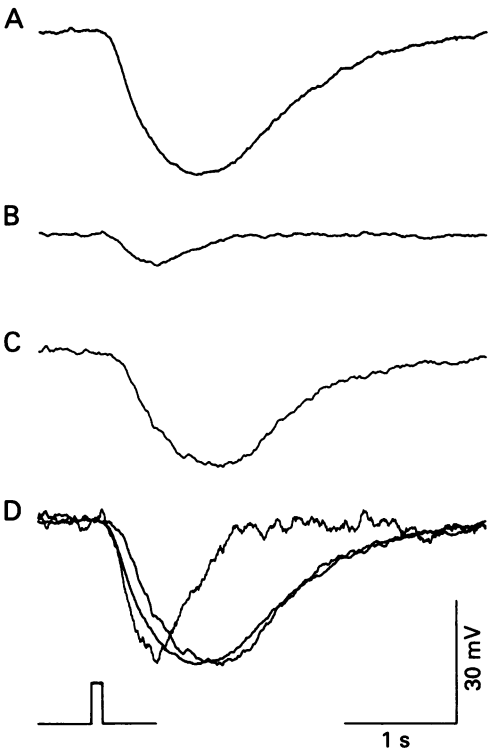

FIG. 6. Effects of p[CH_2]ppG on rod photoresponses during suppression of voltage-dependent conductances. Voltage-dependent conductances were blocked throughout this experiment; this was accomplished by inclusion of 10 mM tetraethylammonium chloride/10 mM cesium chloride/1.0 mM 4-aminopyridine/0.5 mM cobalt chloride in all solutions (28, 30, 31). (*A*) Record from a dark-adapted rod. (*B*) Record from a different rod, 21 min after extinction of the adapting light. (*C*) Record from yet another rod, 15 min after initiation of treatment with p[CH_2]ppG. (*D*) The three records just described, scaled for equal peak amplitudes (normalized).

ulated exchange with GDP) and, thereby, potentiates the photic stimulation of PDEase. However, compatibility of our findings with the notion that cyclic GMP level determines the photoresponse would appear to require the assumption that either (*i*) the binding of p[CH_2]ppG activates transducin only transiently (e.g., bound p[CH_2]ppG is slowly hydrolyzed) or (*ii*) an incremental, sustained increase in PDEase activity (due to the formation of p[CH_2]ppG-charged transducin) only transiently depresses the level of cyclic GMP. In the absence of a compensating synthesis of cyclic GMP, a flash-dependent, permanent increase in PDEase activity would progressively decrease the level of cyclic GMP and, thus, progressively hyperpolarize the rod even in darkness; we observed no significant change in the dark membrane potential of rods treated with p[CH_2]ppG. Indications that GTP-binding proteins regulate a variety of cellular processes (35, 38, 39) emphasize the possibility that p[CH_2]ppG may act at multiple sites within the light-adapted rod.

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