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An unusual cGMP pathway underlying depolarizing light response of the vertebrate parietal-eye photoreceptor

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All cellular signaling pathways currently known to elevate cGMP involve the activation of a guanylyl cyclase to synthesize cGMP. Here we describe an exception to this rule. In the vertebrate parietal eye, the photoreceptors depolarize to light under dark-adapted conditions, unlike rods and cones but like most invertebrate photoreceptors. We report that the signaling pathway for this response involves a rise in intracellular cGMP resulting from an inhibition of the phosphodiesterase that hydrolyzes cGMP. Furthermore, this phosphodiesterase is driven by an active G protein in darkness. These results indicate an antagonistic control of the phosphodiesterase by two G proteins, analogous to the G_s/G_i control of adenylyl cyclase. Our findings demonstrate an unusual phototransduction mechanism and at the same time indicate that signaling involving cyclic nucleotides is more elaborate than previously known.

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In vertebrates, the rods and cones of the lateral eyes hyperpolarize in response to illumination. This response is generated by a G-protein-coupled signaling pathway that stimulates a cGMP-phosphodiesterase and thus the hydrolysis of cGMP[1–4]. In darkness, cytoplasmic cGMP binds to and opens cGMP-activated, nonselective cation channels[5] in the outer-segment membrane of the cells, sustaining an inward dark current and keeping the cell depolarized. In light, the decrease in cGMP concentration closes these channels and consequently produces a membrane hyperpolarization as the electrical response. In lizards and some other lower vertebrates, there is a parietal (third) eye[6] at the top of the head. This eye, like the lateral eyes, has a cornea, a lens and a retina, but this retina has only photoreceptors and ganglion cells, lacking bipolar, horizontal and amacrine cells. The outer segments of these photoreceptors resemble the cone outer segment in morphology, in that they have many membranous discs in continuity with the plasma membrane[6]; by analogy to cones, this is presumably where phototransduction takes place.

Under dark-adapted conditions, the parietal-eye photoreceptor depolarizes to light[7], opposite to the hyperpolarizing light responses of rods and cones. Depolarizing light responses are characteristic of most invertebrate photoreceptors and are thought to involve a phosphoinositide signaling pathway[8–10]. Thus the question arises as to whether the parietal-eye photoreceptor uses a cGMP or a phosphoinositide signaling cascade for phototransduction. We have previously found, using excised patches of plasma membrane, that a cGMP-gated cation channel is present selectively on the outer segment of this photoreceptor[11]. However, these experiments provided no direct information about the phototransduction mechanism. Here we have addressed this question by recording from intact, dissociated photoreceptors. Our findings demonstrate that although the depolarizing response of these cells is generated by a cGMP signaling pathway, the nature of this pathway is very unusual.

Results

Whole-cell recording and perforated-patch recording with nystatin[12] were made from the cell bodies of dark-adapted, single dissociated parietal-eye photoreceptors of the side-blotched lizard. In the whole-cell recording configuration, dialysis of ≤ 50 µM cGMP from the pipet into a cell invariably induced little or no membrane current in darkness (**Fig. 1a**). However, at higher cGMP concentrations, progressively larger inward currents were observed, reaching a maximum of 170–270 pA at –45 mV (mean ± standard deviation, 210 ± 37 pA at 1 mM cGMP; $n = 8$ cells; **Fig. 1a** and **d**, upper panel). The variation in the saturated current among experiments might be due to a variation in outer-segment length produced by the dissociation procedure. The hydrolysis-resistant analog 8-bromo-cGMP induced similar maximal currents but was much more effective at lower concentrations, producing a considerable inward current at concentrations as low as 5 µM (**Fig. 1b** and **d**, upper panel). Although 1 mM IP_3 or 5 mM calcium dialyzed in the same manner was also able to elicit a small current, this current was outward, opposite to that induced by light[7] (**Fig. 1c** and **d**, upper panel). For photoreceptors that had lost the outer segment during dissociation, dialysis with as much as 1 mM cGMP produced negligible inward current, whereas dialysis with 1 mM IP_3 or 5 mM calcium induced similar outward currents as before (**Fig. 1d**, lower panel). Dialysis of cAMP (2 mM) or 8-bromo-cAMP (5 mM) into intact photoreceptors produced no detectable current. Finally, dialysis of cAMP with cGMP also had little influence on the cGMP-induced current (data not shown). Hence, among the common second messengers that

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we have tested, only cGMP is able to mimic light by inducing an inward current.

To determine whether the opening of cGMP-activated channels[11] underlies the light response, we used two chemicals known to block cGMP-activated channels, L-*cis*-diltiazem[13] and dichlorobenzamil[14]. The inward current induced by exogenous cGMP dialyzed from a pipet in the whole-cell configuration, and that by a light flash in perforated-patch recordings, were both transiently suppressed by a puff of 0.1 mM L-*cis*-diltiazem directed at the recorded cells (**Fig. 2a** and **b**). Similar results were obtained with dichlorobenzamil (data not shown). Furthermore, voltage-ramp experiments indicated that both currents had identical current–voltage relations, with characteristic outward rectification[5] (**Fig. 2c**). Thus, the depolarizing light response indeed arises from the opening of cGMP-activated channels, and therefore light must elevate cGMP in the outer segment.

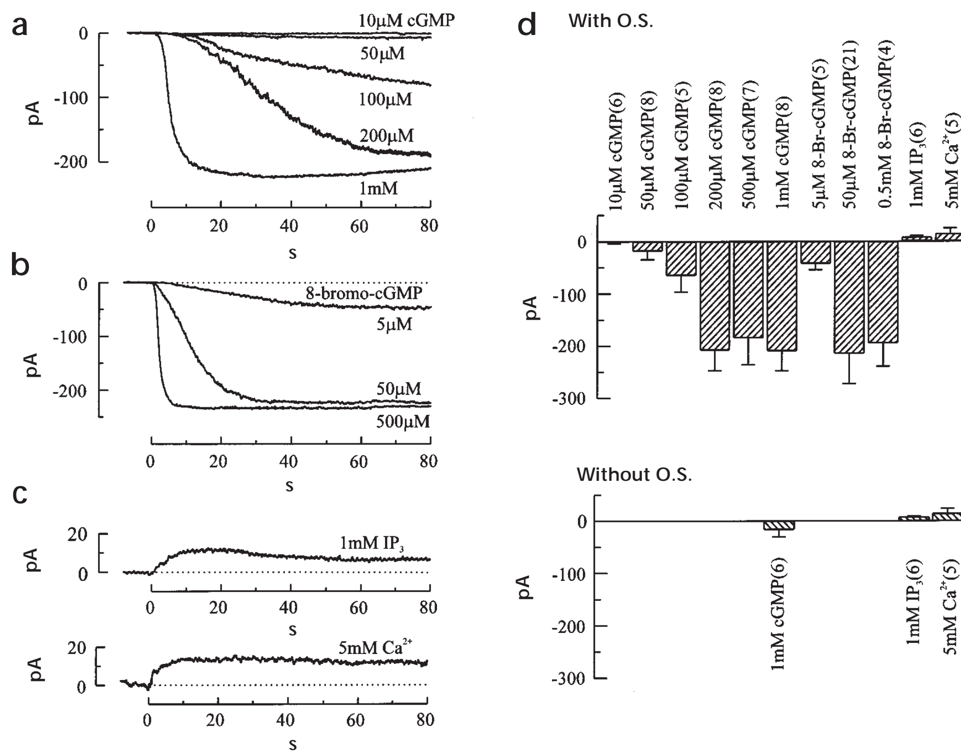

Fig. 1. Whole-cell dialysis experiments on dissociated parietal-eye photoreceptors. **(a–c)** Membrane currents induced by whole-cell dialysis of cGMP (a), 8-bromo-cGMP (b), IP_3 or Ca^{2+}(c) in darkness. Whole-cell recording began at time zero, with rupture of the membrane patch underneath the gigaseal patch electrode. Each trace represents a separate experiment. Indicated concentrations corresponded to those in the whole-cell pipet. **(d)** Upper panel, collected results from experiments (a–c); lower panel, collected results from similar experiments on cells lacking the outer segment (O.S.). Means and standard deviations of the currents are shown. Numbers in parentheses indicate number of experiments. Membrane voltage was clamped at –45 mV.

If a basal metabolic flux of cGMP exists in the photoreceptor in darkness, it should be possible to elevate intracellular cGMP with a phosphodiesterase inhibitor, such as 3-isobutyl-1-methyl-xanthine (IBMX). A brief, strong puff of this highly membrane-permeant chemical (1 mM) ejected from a pipet at a cell under perforated-patch recording indeed elicited a substantial transient inward current (**Fig. 3a**, upper panel); weaker puffs gave smaller currents (not shown). This IBMX-induced current resembled in time course the response of the same cell to a bright flash (**Fig. 3a**, lower panel); the slower rise time at the 'foot' of the light response presumably reflected the delay of the phototransduction cascade. The maximal inward current induced by a saturating puff of IBMX in Fig. 3a is very similar to that induced by a bright flash; when averaged over many experiments, the former was slightly larger, by 10% or less. The two currents also had identical current–voltage relations (**Fig. 3b** and legend). The IBMX-induced current is interpreted to reflect a transient rise in intracellular cGMP due to sustained synthesis and transiently inhibited hydrolysis.

To determine whether light elevates intracellular cGMP by activating a guanylyl cyclase, inhibiting a phosphodiesterase, or both, we applied a saturating puff of IBMX and a light flash in rapid succession. We found that light was able to elicit an inward current only when the IBMX-induced current had already declined from its peak (**Fig. 3c**). The lack of summation might have meant that all cGMP-activated channels were already opened by a saturating puff of IBMX. However, on average, the maximal IBMX-induced current (36.3 ± 15.5 pA; $n = 4$ experiments) was considerably smaller than the maximal current induced by cGMP dialyzed from a pipet (about 200 pA, see **Fig. 1**). To confirm this difference, we measured both currents from the same cell with successive perforated-patch and whole-cell recordings by including both nystatin and cGMP (50 μM 8-bromo-cGMP, which is sufficient to saturate the cGMP-induced current; **Fig. 1b** and **d**) in the patch-pipet solution. The nystatin allowed initial perforated-patch recording of the maximal IBMX-induced current as described in Fig. 3c; afterwards, the membrane was ruptured by gentle suction applied to the patch-electrode to achieve the whole-cell recording mode and to dialyze the 8-bromo-cGMP into the cell (**Fig. 4a**). The results showed that light failed to trigger any current over and above the maximal IBMX-induced current even when the latter was only a small fraction (16 ± 6%; $n = 4$ experiments) of the maximal cGMP-induced current. This observation indicates that light acts by inhibiting the phosphodiesterase activity. If light activated the guanylyl cyclase at all, the light response in Fig. 4a would have risen above the maximal IBMX-induced current. The small fraction of open channels resulting from even a strong puff of IBMX indicates that cGMP synthesis by the guanylyl cyclase was relatively slow, so that the transient inhibition of the phosphodiesterase by IBMX produced only a small elevation of cGMP. Results similar to those in Figs 3 and 4a were

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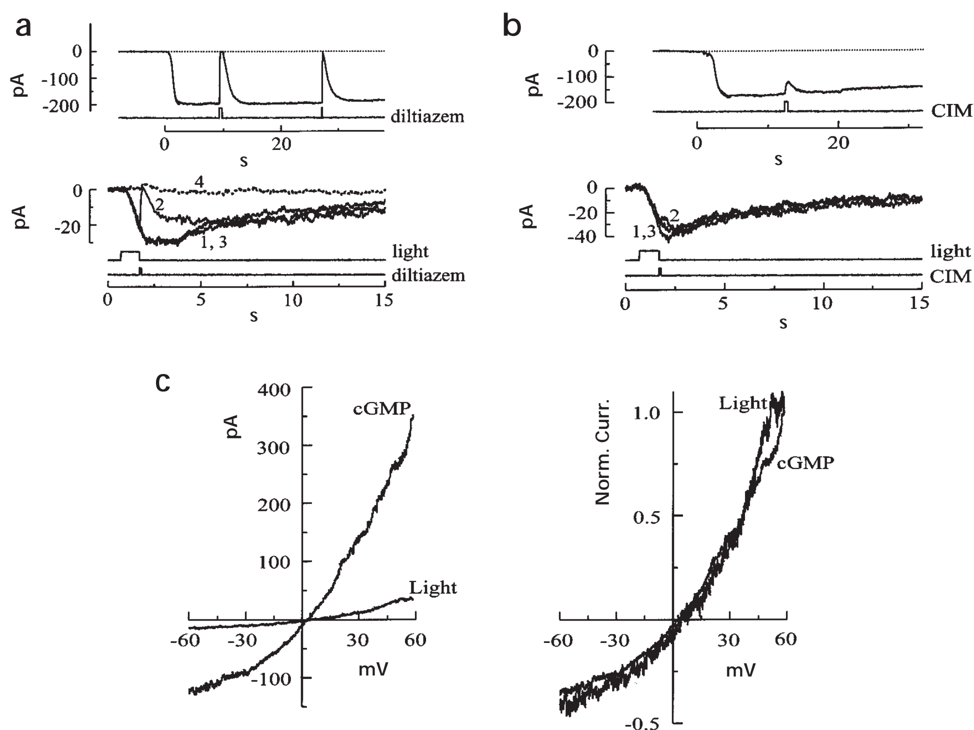

Fig. 2. Similarity between cGMP-induced and light-induced currents. **(a)** Inhibition of the cGMP- and light-induced currents by the blocker L-*cis*-diltiazem. The drug (0.1 mM) in CO_2-independent medium (CIM) was puffed at the recorded cell from a neighboring pipet. Upper panel, inward current induced by whole-cell dialysis of 0.5 mM 8-bromo-cGMP (at time zero), and L-*cis*-diltiazem puffed for 500 and 50 ms, respectively. Lower panel, inward current induced by a flash (1 s, delivering 2.2 x 10^6 photons per μm^2 at 520 nm) measured with nystatin–perforated-patch recording; the traces are in chronological order: flash only (1), flash with a 100 ms L-*cis*-diltiazem puff (2), flash only (3) and L-*cis*-diltiazem puff only (4). **(b)** Same experiments as in (a), but with puff of CIM containing no L-*cis*-diltiazem, showing the small artifact from puffing. Same 8-bromo-cGMP concentration and flash intensity as in (a), but a different cell was used. Traces in lower panel are, in chronological order: flash only (1), flash with CIM puff (2) and flash only (3). CIM puff is 500 ms, upper, and 100 ms, lower. **(c)** Current–voltage relations for the currents induced by 100 µM cGMP dialysis and by light. The relations were obtained with voltage ramps. For the cGMP experiment, the voltage ramp was run several times immediately after membrane breakthrough (and before development of the cGMP-induced current), and the resulting current–voltage relations were averaged; the same was done after the current activated by the 100 µM cGMP-dialysis had reached steady-state. The difference between the two averages gave the depicted relation. For the experiment with light, the same procedure was done in darkness and during a steady light (2.2 x 10^6 photons per μm^2 per second, at 520 nm). Left, each relation is from a different cell. Right, comparison of the two relations after normalization at +60 mV, showing their strong similarity. Membrane voltage was clamped at –45 mV in (a) and (b).

obtained with zaprinast[15,16], a specific inhibitor of cyclic nucleotide-specific phosphodiesterase (data not shown), supporting the conclusions arrived at with IBMX, which is a less specific inhibitor. To rule out any input of cAMP into cGMP metabolism[15], we also bath-applied 100 µM forskolin, a membrane-permeant activator of adenylyl cyclase, to a photoreceptor recorded in the perforated-patch configuration, but observed no effect in either darkness or light (data not shown).

Another experiment supported the conclusion that light acts by inhibiting the dark phosphodiesterase activity. With whole-cell recording, the inward current was small or undetectable in darkness when the pipet solution contained a low concentration of cGMP (see earlier), but this was substantially enhanced by a light flash (**Fig. 4b**), suggesting that light inhibited the dark phosphodiesterase activity to allow more exogenous cGMP to open the channels. The same experiments with cAMP dialysis gave no light response (data not shown), again indicating cAMP is not involved. Light responses were not observed when the pipet solution contained GTP but lacked cGMP, suggesting that the guanylyl cyclase activity was labile under whole-cell recording, perhaps because of 'washout' of a factor important for its function. However, the phosphodiesterase activity and its control by light remained intact.

The visual pigment is a G-protein-coupled receptor and is thus expected to signal through a G protein. Is the phosphodiesterase activity in darkness driven by a G protein? When GTPγS (1 mM), which permanently activates G proteins, and 50 µM cGMP were dialyzed into a cell from a whole-cell pipet, no inward dark current or light response was observed, unlike the result in Fig. 4b, although a strong puff of IBMX was still able to elicit a transient inward current (**Fig. 5a**). Our interpretation is that the dark phosphodiesterase activity was substantially increased by the presence of GTPγS, so that the light flashes (at the intensity used) were not bright enough, despite enhancement by GTPγS, to inhibit the hydrolysis sufficiently to allow the exogenous cGMP to open channels. The effectiveness of IBMX under these conditions presumably resulted from its ability to inhibit the phosphodiesterase noncompetitively by affecting the enzyme's catalytic site. With a much lower concentration of GTPγS (10 µM), flash responses were observed that were longer-lasting than control responses, as would be expected from a G protein mediating the light action (not shown). A similar result was obtained with AlF_4^- (20 µM), which is known to act like GTPγS[17,18] (**Fig. 5b**). With the low concentration of AlF_4^- used here, the progressive disappearance of the light response over time could be followed. Though not shown in Fig. 5b, IBMX was still able to elicit an inward current after the flash responses had disappeared in the pres-

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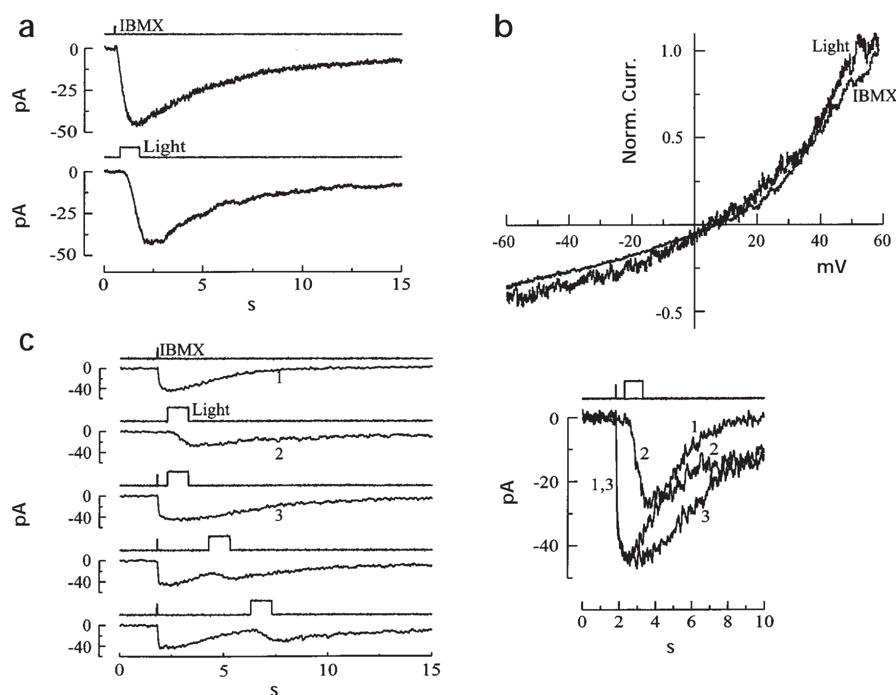

Fig. 3. Comparison of IBMX-induced and light-induced currents. **(a)** Similar inward currents induced by a saturating puff of IBMX and by a strong light flash from a photoreceptor. Nystatin–perforated-patch recording. Puff (40 ms) of 1 mM IBMX in CIM; flash (1 s) delivered 2.2×10^6 photons (520 nm) per μm^2. **(b)** Current–voltage relations for the currents induced by IBMX and light, normalized at +60 mV to show their similarity. A different cell was used for each relation. The relation for the light-induced current was identical to that in Fig. 2c. For the experiment with IBMX, the voltage ramps were run in control solution and again after the current induced by continuous bath application of 1 mM IBMX had reached steady state; the difference between the averaged current–voltage relations in the two conditions were then calculated. **(c)** Lack of summation of the currents induced by IBMX and light at the peak of the current induced by a saturating puff of IBMX. Puff (20 ms) of 1 mM IBMX in CIM; flash (1 s) delivered 2.9×10^5 photons per μm^2 at 520 nm. At right, traces 1, 2 and 3 replotted from left, scaled to the same height and superimposed. Nystatin–perforated-patch recording. Different cell from those used in (a) and (b). Membrane voltage was clamped at –45 mV in (a) and (c).

ence of 20 μM AlF_4^-, as with GTPγS. We have not used higher concentrations of AlF_4^-. When GDPβS (1 mM), which inhibits G proteins, was included in the pipet solution with 50 μM cGMP, the dark current became very large (**Fig. 5c**). This current was similar in amplitude to the maximal current induced by IBMX in the absence of GDPβS (not shown). Furthermore, in the presence of GDPβS, IBMX elicited little or no additional inward current (not shown), indicating that the dark phosphodiesterase activity was already mostly inhibited by the GDPβS, so that the exogenous cGMP became very effective in opening channels. The lack of light response in this case was not surprising, because the phosphodiesterase was already mostly suppressed and because the presence of GDPβS together with the absence of GTP should also inhibit phototrans-

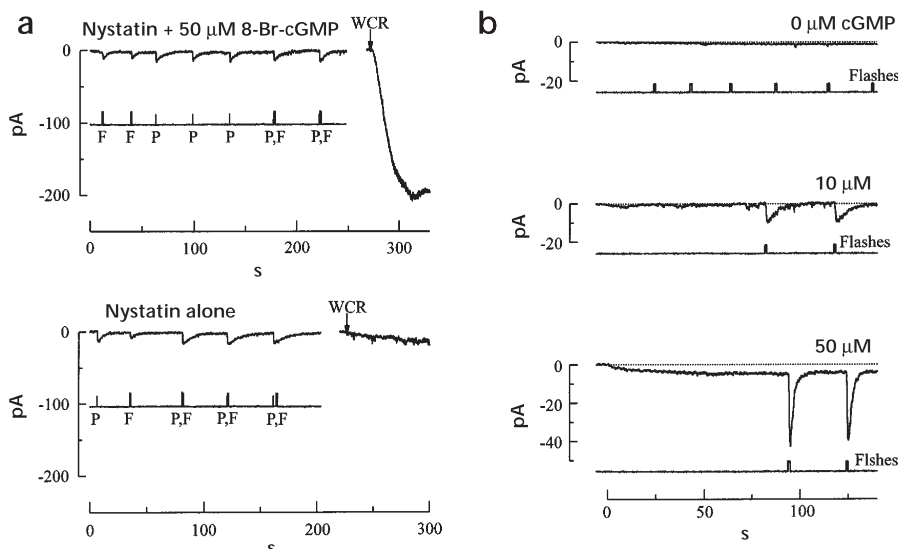

Fig. 4. Light inhibits the dark cGMP-phosphodiesterase activity in the photoreceptor. **(a)** Lack of summation of the IBMX- and light-induced currents (as in Fig. 3c), even though these currents reflected the opening of only a small percentage of the cGMP-activated channels. Top panel, nystatin–perforated-patch recording until break of trace; thereafter, whole-cell recording (WCR) with 50 μM 8-bromo-cGMP dialysis. F, flash; P, puff of IBMX; P,F, IBMX and flash in rapid succession. Bottom panel, control experiment in which 8-bromo-cGMP had been omitted from pipet. 30 ms puff of IBMX at 1 mM. **(b)** Light increased the inward current induced by whole-cell cGMP dialysis. Each trace corresponds to a different cGMP concentration in the whole-cell pipet and is from a different cell. Whole-cell dialysis began at time zero. Flash (1 s) delivered 2.2×10^6 photons per μm^2 at 520 nm. Membrane voltage was clamped at –45 mV.

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duction. In control experiments with dialysis of 1 mM GDPβS but no cGMP included, no inward current developed in darkness. Taken together, these results suggest that the phosphodiesterase activity in darkness is largely stimulated by an active G protein. Most of this enzyme activity seems to be suppressible by light, as indicated by the very similar inward currents induced by light and by IBMX (**Fig. 3a**).

Discussion

Here we have shown that the depolarizing light response of parietal-eye photoreceptors arises from an increase in cGMP concentration and the consequent activation of cGMP-gated cation channels on the outer segment of the cell. This increase in cGMP results from a decrease in phosphodiesterase activity rather than an increase in guanylyl cyclase activity. What is the underlying signaling mechanism? A cascade essentially identical, but anti-symmetric, to the phototransductioin cascade in rods and cones (**Fig. 6a**, left panel) might explain all of our findings. In this scheme, all of the photopigment molecules are active in darkness, driving a G protein and stimulating a phosphodiesterase to hydrolyze cGMP and keep the channels closed. Light inactivates the pigment and hence the cascade, causing the cGMP level to rebound and the cGMP-activated channels to open. This cascade is esthetically simple, and it embodies the key features of an active G protein and an active phosphodiesterase in darkness that are inhibited by light.

However, the above scheme cannot explain the saturating response–intensity relation observed for the parietal-eye photoreceptor[7] (also W.-H. X. and K.-W. Y., unpublished data). Based on enzyme kinetics, the increase in phosphodiesterase activity would be expected to depend on the amount of active pigment according to a function of the Michaelis-Menten type. Consequently, the number of open channels becoming closed should depend on the amount of active pigment in a similar manner (modified by the cooperativity function[13] between open channels and cGMP, which, however, does not affect our present argument). Therefore, with increasing amounts of active pigment, the dependence would approach an asymptote, corresponding to all channels being closed (**Fig. 6a**, curve 1). Thus, if an initially all-active pigment is inactivated by light in direct proportion to light intensity, the number of channels that are reopened, and hence the amplitude of the depolarizing light response, should be initially very insensitive to light because the relation is still riding on the asymptote, but the response will eventually increase steeply when the last few pigment molecules are inactivated. This prediction is considerably different from the form of the response–intensity function observed experimentally (**Fig. 6a**, curve 2), in which the response initially rises linearly with light intensity and then saturates at high intensities[7]. Even if the pigment content of the cell is so low that the phosphodiesterase activity rises linearly throughout the range of active pigment fractions, the model still cannot predict an observed saturating intensity–response relation. Thus, the cascade in Fig. 6a cannot be correct.

A more likely phototransduction cascade is one in which two signaling pathways, each associated with a distinct G protein, control the phosphodiesterase activity. One G protein (G_1) is active in darkness and stimulates the enzyme, and the other G protein (G_2) is stimulated by light and inactivates the enzyme (**Fig. 6c**). The simpler alternative in which a single G protein mediates both pathways (**Fig 6b**) is improbable because this would require the visual pigment to inhibit the G protein directly, a polarity of interaction that has not been described for any receptor coupled to a G protein. It is still not known whether the

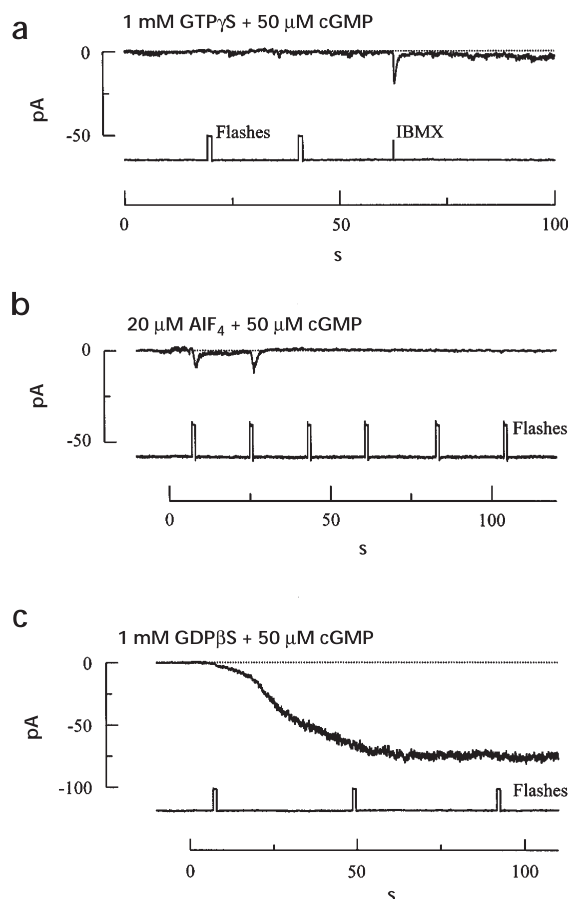

Fig. 5. A G protein is involved in the control of the cGMP-phosphodiesterase activity in darkness. cGMP (50 μM) was dialyzed with 1 mM GTPγS **(a)**, 20 μM AlF_4^- **(b)** or 1 mM GDPβS **(c)** by whole-cell recording (which began at time zero) into a cell. Note the gradual disappearance of the light response in (b). Different cells were used in (a), (b) and (c). Light flashes (1 s) delivered 2.2 x 10^6 photons per μm^2 at 520 nm. IBMX puff in (a) was at 1 mM for 30 ms. Membrane voltage was clamped at –45 mV.

stimulatory G protein is spontaneously active or is driven by an upstream signal in darkness, hence the question mark in Fig. 6c. Based on an observed chromatic antagonism[7], the photopigment in this photoreceptor may be bistable, as in invertebrates[7,19]. A candidate for driving G_1 in darkness would then be the putative second stable state of the pigment. It is also not known whether the inhibitory G_2 protein acts directly on the phosphodiesterase, or indirectly through inhibiting G_1. Possibilities include a direct inhibition of the phosphodiesterase by $G_{2\alpha}$ or $G_{2\beta\gamma}$, and an inhibition of $G_{1\alpha}$ by $G_{2\beta\gamma}$[17,20]. These questions may be answered by the molecular identification of the G proteins and the phosphodiesterase. By analogy to retinal rods and cones[1], the phosphodiesterase is probably composed of α, β and γ subunits. We have attempted to use cholera and pertussis toxins, which ADP-ribosylate specific α-subunits of G proteins and cause their permanent activation or inactivation[1,17,21–23], to investigate G_1 and G_2, but the experiments were inconclusive, perhaps because of access problems in the outer segment. Molecular cloning of the genes may be the best approach.

Our results demonstrate not only an unusual phototrans-

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duction mechanism, but also newly discovered features about signaling involving cyclic nucleotides. First, all signaling pathways known thus far to elevate cGMP invariably involve the activation of a guanylyl cyclase: either a ligand-activated membranous cyclase or a nitric-oxide-activated soluble cyclase[24–26]. However, in the parietal-eye photoreceptor, the rise in cGMP results from an inhibition of the phosphodiesterase. Because the cGMP level represents a balance between synthesis and hydrolysis, a regulation of either step can achieve the same result. By inhibiting the phosphodiesterase to elevate cGMP, a cell can allow the guanylyl cyclase activity to limit the maximal rate of cGMP increase regardless of stimulus strength in a signaling pathway. Second, the antagonistic control of the cGMP phosphodiesterase by two G proteins described here has no prior example, but it bears a striking parallel to the G_s/G_i antagonistic control of adenylyl cyclase in cAMP signaling[17,20,22,23,27]. Finally, the inhibition of phosphodiesterase activity may be exploited also by other cell types as a mechanism to elevate not just cGMP, but cAMP as well, instead of the conventional mechanism through activation of the adenylyl cyclase.

From our discoveries about the parietal-eye photoreceptor, and those by Gomez and Nasi[28] on the scallop hyperpolarizing photoreceptor—both of which are ciliary photoreceptors like retinal rods and cones—it is now clear that all ciliary photoreceptors, whether vertebrate or invertebrate, depolarizing or hyperpolarizing, use a cGMP signaling cascade for phototransduction. Nonetheless, it is still unclear from the evolutionary point of view how much variation in details exists within this common motif. For example, although light also elevates cGMP in the scallop hyperpolarizing photoreceptor, it is unknown[28] whether this involves an inhibition of the phosphodiesterase or a stimulation of the guanylyl cyclase.

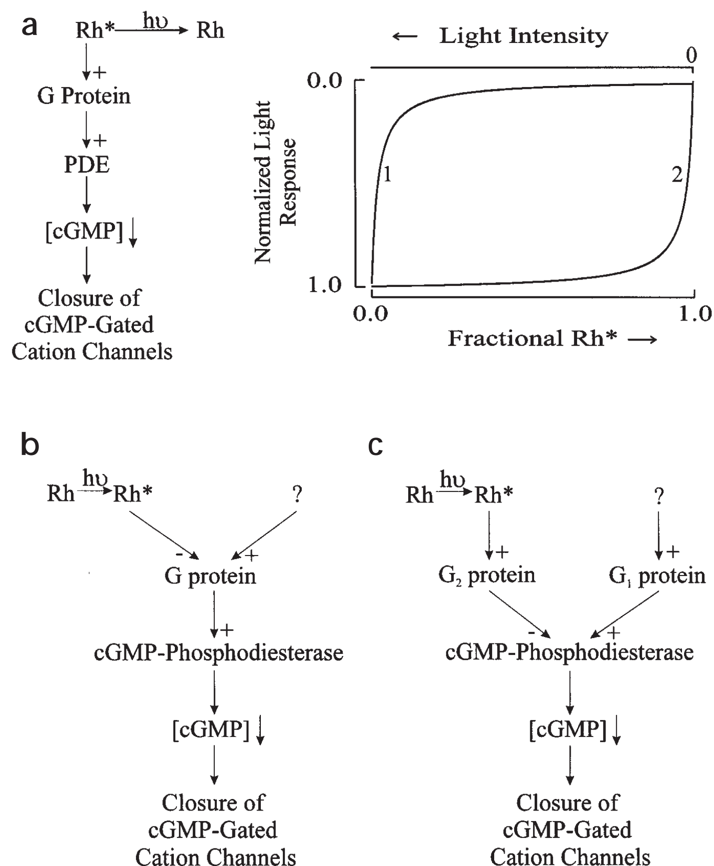

Fig. 6. Possible phototransduction schemes for the parietal-eye photoreceptor. **(a)** Left, the simplest phototransduction scheme that can explain the observations qualitatively, but not quantitatively. Right, curve 1, the response–intensity relation expected from this scheme, ignoring cooperativity in channel activation by cGMP; curve 2, the form of the response–intensity relation observed experimentally[7]. Rh, inactive pigment; Rh*, active pigment. **(b)** A more complex phototransduction scheme that involves a single G protein but is unlikely to be correct. **(c)** The probable phototransduction scheme describing the operation of the parietal-eye photoreceptor, involving an excitatory and an inhibitory G protein. Question marks (b,c) indicate an unknown; that is, whether the stimulatory G_1 protein is spontaneously active or is driven by an upstream signal in darkness.

Methods

ISOLATION OF PARIETAL-EYE PHOTORECEPTORS. Except for minor modifications in the procedure, the parietal-eye photoreceptors from the side-blotched lizard, *Uta stansburiana*, were isolated as described[7,11]. The parietal eye, still attached to the overlying skin, was dissected from the pithed lizard and immersed for 15 min in CO_2-independent medium (CIM; Life Technologies) containing collagenase (CLS1, 5 mg/ml; Worthington, Freehold, New Jersey). After washes in CIM, the eye was removed from the skin using tweezers and was incubated in CIM containing pronase (2 mg/ml; Boehringer) for 13 min so that the retina could be detached from the eye capsule. The retina was placed in a solution free of divalent cations (140 mM NaCl, 5 mM KCl, 5 mM Na-EGTA, 5 mM Na-EDTA, 10 mM Na-HEPES, pH 7.4) containing trypsin (2 mg/ml; Sigma) for 11 min, rinsed in CIM containing 10% newborn calf serum (Life Technologies) for 10 min and then in CIM for 10 min. Finally, the tissue was triturated gently with glass pipets having progressively smaller diameters (500–100 µm). The cell suspension was plated on a glass coverslip pretreated with concanavalin A (Sigma) and used for as long as six hours. The dissection and solution changes were done in room light, whereas the incubations and subsequent storage were done in complete darkness, all at room temperature (23–25°C).

ELECTROPHYSIOLOGICAL RECORDING. Whole-cell recording and perforated-patch recording with nystatin[12] were made from cell bodies of the dissociated parietal-eye photoreceptors. The initial 'gigaseal' formation was done under microscope light; immediately after, all procedures were done in the dark, except for stimulation with light flashes. For whole-cell recording, dark adaptation for about 10 min was allowed before membrane breakthrough; for perforated-patch recording, patch perforation by nystatin generally took 2–5 min, again in the dark.

The patch pipets were pulled from aluminosilicate glass capillaries (World Precision Instruments, Sarasota, Florida) to a tip lumen diameter of less than 0.5 µm. For whole-cell recording, the pipets were filled with a pseudointracellular solution (10 mM KCl, 120 mM K-gluconate, 5 mM $MgCl_2$, 1 mM Na-EGTA, 10 mM K-HEPES, 3 mM Na_2ATP, 1 mM Na_2GTP, pH 7.4), to which test chemicals were added, as needed, from stock solutions prepared daily; for GTPγS and GDPβS, they were equimolar substitutions for GTP. For perforated-patch recording, a fresh stock solution of nystatin (50 mg/ml; Sigma) in dimethylsulfoxide (DMSO) was diluted to 100–200 µg/ml in a base-pipet solution[7] (4 mM KCl, 120 mM K-gluconate, 4 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 11 mM Na-EGTA, 10 mM K-HEPES, pH 7.4). The pipet, including its tip, was filled with this nystatin-containing solution. The progress of perforation

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was assessed from the decrease in access resistance (to about 30 MΩ). The liquid-junction potential was measured to be about 7 mV for perforated-patch recording, but has not been subtracted from the holding potential indicated. In a few experiments, perforated-patch recording was intentionally changed to whole-cell recording by applying gentle suction inside the pipet to rupture the underlying plasma membrane. In both recording configurations, the membrane potential was clamped at –45 mV unless otherwise indicated. In experiments to measure the current–voltage relation, the membrane potential was first stepped to –60 mV for 10 milliseconds (ms) and then ramped from –60 mV to +60 mV at 267 mV/s. All nucleotides were obtained from Sigma.

During recordings, the chamber containing the cells was continuously perfused with CIM at 0.7–0.8 ml/min. Puffer pipets were made in the same manner as patch pipets except for their larger tip diameters (about 2–3 µm). They were filled with CIM solution, with or without a test chemical, and controlled by a picospritzer (General Valve, Fairfield, New Jersey) operating at about 10 psi. Light flashes were all at 520 nm. Because of the low light sensitivity of these cells, the flash duration was set at 1 s. The recordings were done at room temperature.

Forskolin (Sigma) was dissolved in DMSO before being diluted with CIM to the final concentration. (Final concentration of DMSO was about 0.1%.) The cholera and pertussis toxins (List Biochemicals, Campbell, California) were dialyzed, either as the holoenzyme or as the (catalytic) subunit A alone (10 µg/ml for cholera toxin and 1 µg/ml for pertussis toxin), with 50 µM cGMP, 1 mM nicotinamide adenine dinucleotide and 0.1 mM dithiothreitol from the whole-cell pipet into the cell[29,30].

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