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Authors
Matthews, HR
Cornwall, MC
Fain, GL

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Persistent Activation of Transducin by Bleached Rhodopsin in Salamander Rods

H.R. MATTHEWS,* M.C. CORNWALL,‡ and G.L. FAIN§

From the *Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, United Kingdom; ‡Department of Physiology, Boston University Medical School, Boston, Massachusetts 02118; and §Department of Physiological Science and Ophthalmology, University of California Los Angeles, Los Angeles, California 90095

ABSTRACT The hydrolysis-resistant GTP analogue GTP-γ-S was introduced into rods isolated from the retina of the salamander *Ambystoma tigrinum* to study the origin of the persistent excitation induced by intense bleaching illumination. Dialysis of a dark-adapted rod with a whole-cell patch pipette containing 2 mM GTP-γ-S resulted in a gradual decrease in circulating current. If the rod was first bleached and its sensitivity allowed to stabilize for at least 30 min, then dialysis with GTP-γ-S produced a much faster current decay. The circulating current could be restored by superfusion with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, suggesting that the decay in current originated from persistent excitation of the phosphodiesterase by transducin bound to GTP-γ-S. We conclude that the persistent excitation which follows bleaching is likely to involve the GTP-binding protein transducin, which mediates the normal photoresponse. This observation suggests that a form of rhodopsin which persists long after bleaching can activate transducin much as does photoisomerized rhodopsin, although with considerably lower gain.

KEY WORDS: photoreceptor • light adaptation • bleaching adaptation • G protein • transducin

INTRODUCTION

Exposure of rod and cone photoreceptors to light sufficiently bright to bleach a significant fraction of the photopigment produces a decrease in sensitivity and a shortening of the time to peak of the light response, accompanied by a long-lasting suppression of the circulating current (Cornwall et al., 1989; Cornwall et al., 1990; Matthews et al., 1990). Three lines of evidence suggest that these changes, which persist indefinitely in photoreceptors isolated from the retinal pigment epithelium, may result from an "equivalent background" which adapts the photoreceptor much as does real light. First, the decrease in sensitivity induced by bleaching is much larger than can be accounted for by the decrease in the probability of photon absorption (Cornwall et al., 1990; Jones et al., 1993). Second, bleaching persistently elevates the activity both of phosphodiesterase and of guanylyl cyclase, much as does steady light (Cornwall and Fain, 1994). Third, bleach-induced changes in sensitivity and kinetics can be prevented by opposing the fall in Ca^{2+}_i which accompanies bleaching and which is believed to mediate adaptation to background light (Matthews et al., 1996).

While it has been proposed that the persistent excitation which follows bleaching may result from some form of opsin lacking retinal chromophore (Cornwall and Fain, 1994), the way in which this might interact with the normal transduction mechanism is unknown. It is well established that the normal response to light is mediated by a G protein–coupled cascade, in which the G protein transducin links the photoisomerization of rhodopsin to the hydrolysis of cyclic GMP by the phosphodiesterase. The nucleoside triphosphate analogue GTP-γ-S (guanosine 5′-*O*-[3-thiotriphosphate]) is known to be hydrolyzed only very poorly by transducin, and when introduced into the rod it produces an extended period of activation when transducin is excited by photoisomerized rhodopsin and a greatly prolonged response to light (Sather and Detwiler, 1987; Lamb and Matthews, 1988). To investigate whether the persistent excitation which follows bleaching takes place via transducin, we have introduced GTP-γ-S from a whole-cell patch pipette into isolated rods both in darkness and after bleaching.

METHODS

Preparation and Electrical Recording

Simultaneous suction pipette and patch clamp recordings were made from rod photoreceptors isolated mechanically under infrared illumination from the dark-adapted retina of the larval tiger salamander, *Ambystoma tigrinum*, after decapitation and pithing in dim red light. In most experiments the outer segment of an isolated rod was drawn into the suction pipette, leaving the in-

Preliminary results from this study have been previously reported to the Physiological Society (Matthews, H.R., M.C. Cornwall, and G.L. Fain. 1994. *J. Physiol. (Lond.).* 477:6P) and the Association for Research in Vision and Ophthalmology (Cornwall, M.C., H.R. Matthews, and G.L. Fain. 1995. *Invest. Ophthal. Vis. Sci.* 36:S382).

Address correspondence to Dr. H.R. Matthews, Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK. Fax: 01223-333840; E-mail: hrm1@cam.ac.uk

ner segment exposed. A patch pipette containing a pseudo-intracellular solution which included 2 mM GTP-γ-S was sealed onto the protruding inner segment, and the membrane patch was ruptured to yield the whole-cell recording configuration. In experiments requiring rapid exchange of the solution bathing the outer segment, the inner segment was partially drawn into the suction pipette and the patch pipette sealed onto the ellipsoid region of the inner segment. The patch pipette voltage was clamped at −40 or −50 mV, after correction for a −10 mV liquid junction potential. All experiments were carried out at room temperature (~20°C). The suction and patch pipette current signals were low-pass filtered at 20 Hz and digitized continuously for subsequent analysis at a sampling rate of 100 Hz.

Solutions and Solution Changes

Ringer solution contained 111 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.6 mM $MgCl_2$, and 3.0 mM HEPES, adjusted to pH 7.7 with NaOH, and 10 μM EDTA to chelate impurity heavy metals. The Ringer solution continuously perfusing the recording chamber also included 10 mM glucose. Ringer solution containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was prepared by adding 500 μM IBMX (Sigma Chemical Co., Poole, UK). Rapid external solution changes were effected by translating the boundary between two flowing streams of solution across the exposed outer segment using a computer-controlled stepping motor coupled to the microscope stage. Recordings were corrected by subtraction of the junction current measured when the same solution changes were carried out during intense steady illumination at the end of the experiment. The pseudo-intracellular solution filling the patch pipette contained 92 mM potassium aspartate, 7 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_2ATP, 1 mM Na_4GTP, 2 mM GTP-γ-S (tetralithium salt; Sigma Chemical Co.), 20 μM BAPTA, and 10 mM HEPES, and was adjusted to pH 7.0 with KOH. A similar solution without GTP-γ-S (but containing ATP and GTP) has been shown to support a relatively stable dark current during whole cell recordings of at least 10 min duration (Lamb et al., 1986).

Light Stimulation

Light stimuli were delivered from a dc-driven tungsten halogen source at 500 nm (bandpass interference filter, 7 nm bandwidth at 50% peak transmission; Ealing Electro-Optics, Watford, UK) and were unpolarized; flashes of 20-ms duration were controlled by electromagnetic shutters (Vincent Associates, Rochester, NY). Light intensities were adjusted with neutral density filters

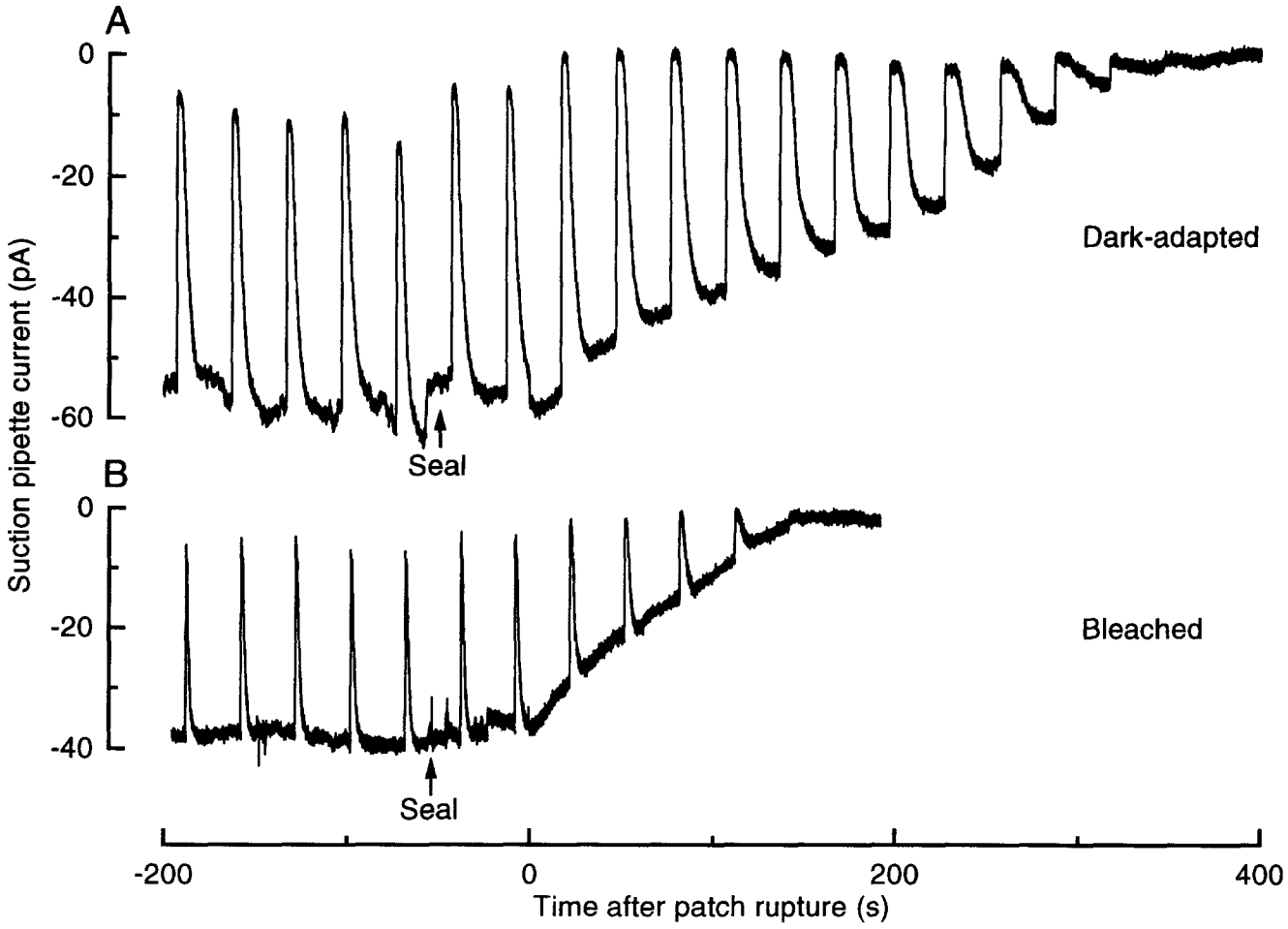

FIGURE 1. Suction pipette current recorded during the introduction of GTP-γ-S (*A*) into a dark-adapted rod or (*B*) into a rod exposed 45 min earlier to intense illumination which bleached 26% of the photopigment. The outer segment was drawn into the suction pipette, and a patch pipette containing a solution which included 2 mM GTP-γ-S sealed onto the protruding inner segment at the arrow. The patch was ruptured at time zero to establish the whole-cell recording configuration. Just-saturating flashes delivered *A*, 330 and *B*, 620 photons μm^{-2}.

(Schott, Mainz, Germany) and measured with a calibrated silicon photodiode (Graseby Optronics, Orlando, FL); they can be converted to isomerizations using a collecting area of 20 μm^2. Rods were bleached by a timed exposure to intense light, and then sensitivity and response kinetics were allowed to stabilize for at least 30 min. In most cases the bleaching light exposure took place with the rod on the base of the chamber, and the cell was only drawn into the suction pipette after a period sufficient for response stabilization. The percentage of pigment bleached was estimated from the photosensitivity for vitamin A_2-based pigments in free solution (Dartnall, 1972), corrected for the difference between the dichroism in free solution and in disk membranes (6.2×10^{-9} μm^2) (Makino et al., 1991; Jones et al., 1993).

All experiments were carried out in Cambridge.

RESULTS

To investigate the rate of G protein activation, we dialyzed isolated rod photoreceptors with the hydrolysis-resistant nucleoside triphosphate analogue GTP-γ-S from a whole-cell patch pipette. Fig. 1 *A* shows the effect of introducing GTP-γ-S into a dark-adapted rod. The outer segment of an isolated rod was drawn into the suction pipette, a patch pipette containing 2 mM GTP-γ-S was sealed onto the protruding inner segment (*arrow*), and the patch was ruptured at time zero to establish the whole-cell recording configuration and allow the pipette contents to diffuse into the cytoplasm. Repeated just-saturating flashes were delivered to monitor the zero current level during the period of whole-cell recording. As in previous experiments (Sather and Detwiler, 1987; Lamb and Matthews, 1988), incorporation of GTP-γ-S into a dark-adapted rod produced a slow decrease in circulating current, resulting in its complete suppression after ~6 min.

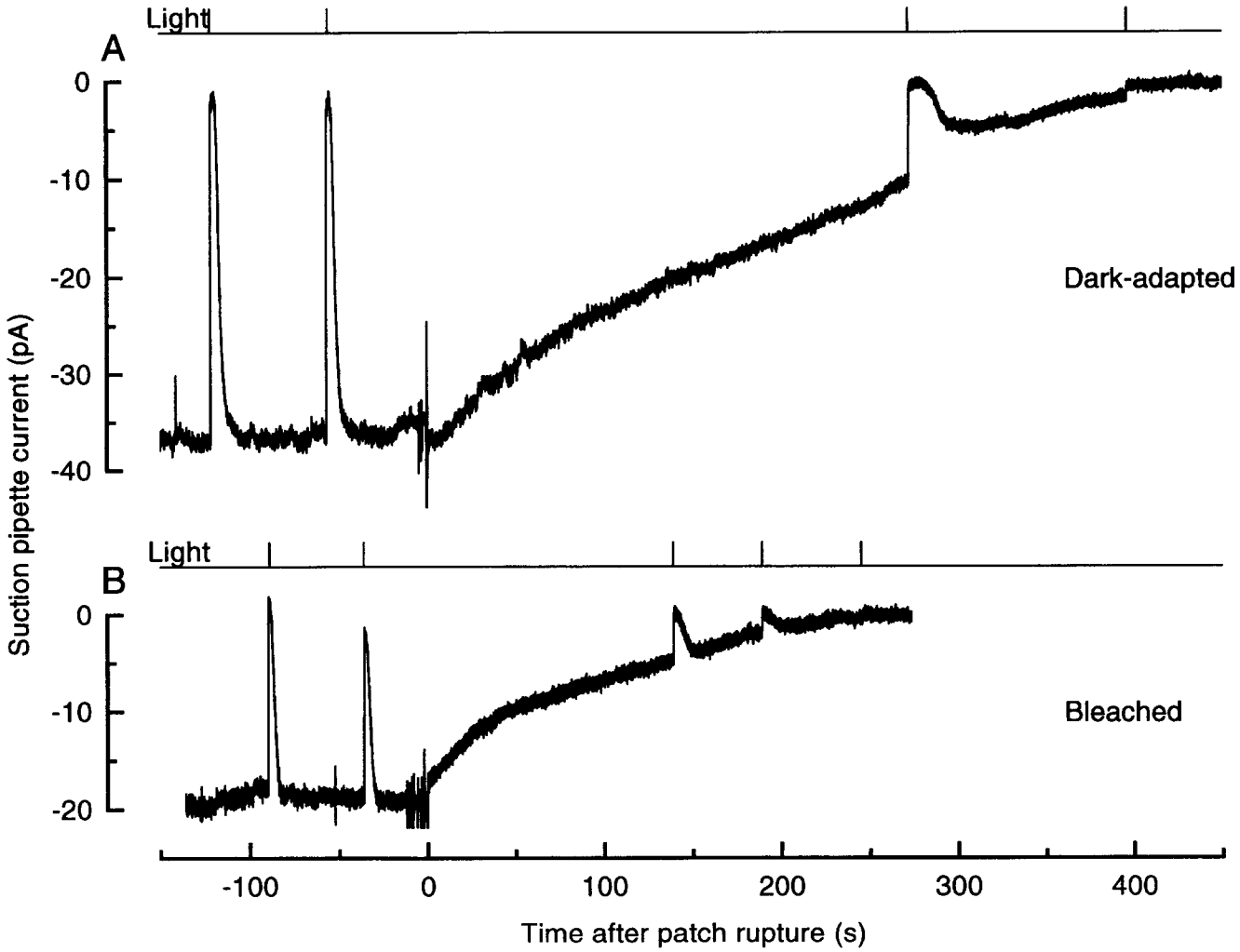

FIGURE 2. Suction pipette current recorded during the introduction of GTP-γ-S in complete darkness (*A*) into a dark-adapted rod or (*B*) into a rod exposed 70 min earlier to intense illumination which bleached 26% of the photopigment. The outer segment was drawn into the suction pipette, a patch pipette containing a solution which included 2 mM GTP-γ-S sealed onto the protruding inner segment, and the patch ruptured at time zero to establish the whole-cell recording configuration. Flash monitor indicates infrequent just-saturating flashes which delivered *A*, 150 and *B*, 310 photons μm^{-2}.

Fig. 1 *B* shows a similar experiment carried out on a bleached rod. The rod was first exposed to intense illumination sufficient to bleach 26% of the photopigment and then left for 45 min to let pigment intermediates decay (Baumann, 1972; Donner and Hemilä, 1975) and response sensitivity and kinetics to reach steady state (Cornwall et al., 1990). The outer segment was then drawn into the suction pipette so that the circulating current could be recorded. Under these conditions the rod was desensitized and the kinetics of the light response markedly accelerated (Cornwall et al., 1990; Fain and Cornwall, 1993). The introduction of GTP-γ-S now produced a much faster decay than in the dark-adapted rod, and the circulating current was abolished in a little over 2 min.

The suppression of the circulating current and disappearance of the light responses during the recordings of Fig. 1 can most simply be interpreted as representing the persistent activation of transducin which has bound GTP-γ-S. Since bleach-adapted rods show current suppression which is faster than dark-adapted rods, we infer that bleaching increases the rate of transducin activation. One difficulty with this interpretation, however, is that we have used just-saturating flashes to monitor the zero-current level in Fig. 1, and these flashes could have influenced the rate of decline of the circulating current. Fig. 2 shows comparable experiments in which GTP-γ-S was introduced without these repeated flashes. Even in complete darkness, the circulating current declined progressively during the whole-cell recording, and this decay was faster in bleached (Fig. 2 *B*) than in dark-adapted rods (Fig. 2 *A*).

Results from a number of such experiments are summarized in Fig. 3. Each point represents the circulating current measured in response to a just-saturating test flash and has been normalized according to the value before whole-cell recording commenced. In the experiments of Fig. 3 *A* flashes were delivered at 30-s intervals throughout the whole-cell recording as in Fig. 1. Immediately after patch rupture the normalized current was slightly elevated, probably because of the relatively negative patch pipette holding potential. Thereafter the circulating current progressively declined, presumably as GTP-γ-S diffused into the cytoplasm and interacted with the transduction mechanism. The time course of current decay was similar for four rods dialyzed with GTP-γ-S in darkness (*filled symbols*), although one of these cells showed a somewhat more rapid initial decline. In these dark-adapted rods the circulating current was completely suppressed some 6–7 min after the patch ruptured and GTP-γ-S started to diffuse into the cytoplasm. However, in seven rods which were first exposed to intense light sufficient to bleach 24–48% of the photopigment, the rate of current decay induced by GTP-γ-S was considerably accelerated (*open symbols*),

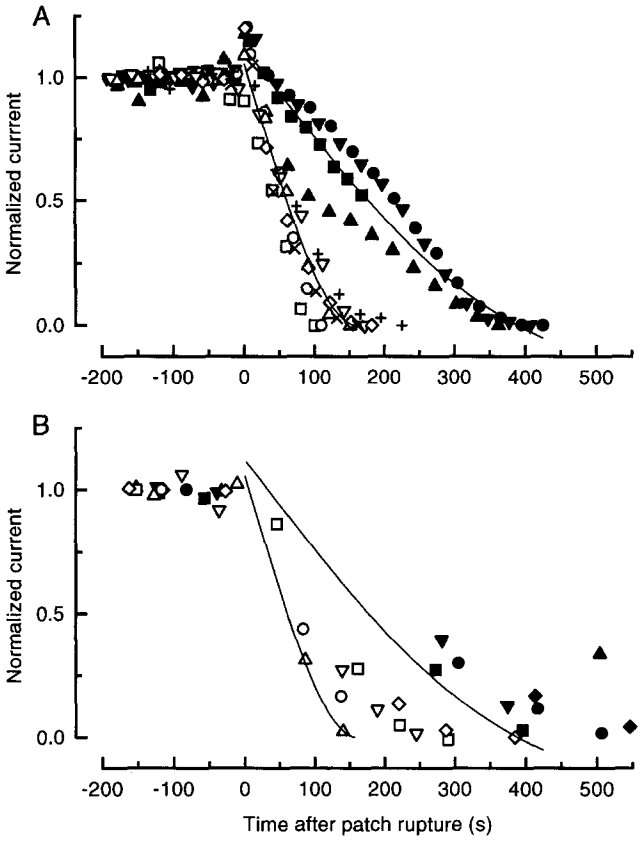

FIGURE 3. Normalized suction pipette current data collected during the incorporation of GTP-γ-S into dark-adapted and bleached rods. In each panel individual symbol types represent different cells. (*A*) Just-saturating flashes delivered at 30-s intervals during whole-cell recordings from four dark-adapted rods (*filled symbols*; *inverse filled triangles* denote cell of Fig. 1 *A*) and seven rods exposed 35–110 min earlier to intense light which bleached 24–27% (six rods, *open symbols*; *inverse open triangles* denote cell of Fig. 1 *B*) or 48% (one rod, +) of the photopigment. Solid lines are arbitrary curves fitted to the dark-adapted and bleached data after the time of patch rupture. (*B*) Flashes delayed until late in whole-cell recordings from five dark-adapted rods (*filled symbols*; *filled squares* denote cell of Fig. 2 *A*) and five rods exposed 70–115 min earlier to intense light which bleached 26% of the photopigment (*open symbols*; *open inverse triangles* denote cell of Fig. 2 *B*). Solid curves reproduced from Fig. 3 *A*. Circulating current was measured as the amplitude of the response to just-saturating flashes which delivered 80–340 photons μm^{-2} (*filled symbols*) or 300–1,300 photons μm^{-2} (*open symbols*). Patch pipette solution contained 2 mM GTP-γ-S.

leading to suppression of the circulating current after only 2–3 min.

Fig. 3 *B* collects results from experiments in which the flashes were delayed until late in the whole-cell recording as in Fig. 2. Even in complete darkness, the circulating current declined progressively during the incorporation of GTP-γ-S before the first flash was delivered. Once again, this decay was faster in bleached (*open symbols*) than in dark-adapted (*filled symbols*) rods, declining to 25% of its original value after 144 ± 22 s

(five bleached rods) and 346 ± 45 s (five dark-adapted rods; mean ± SEM), respectively. The decay of current was only marginally slower than under the same conditions in the presence of just-saturating flashes (compare with *solid curves* from Fig. 3 *A*), and bleaching resulted in an ~2.5-fold acceleration in each case. It would therefore appear that flashes of the intensity used to assess the magnitude of the remaining dark current had only a modest effect on the time course of current decline during the incorporation of GTP-γ-S, in contrast to the greatly prolonged responses to very intense flashes under these conditions (Lamb and Matthews, 1988).

The persistent activation of transducin after binding GTP-γ-S would be expected to induce a progressive increase in phosphodiesterase activity. If this interpretation is correct, then it should be possible to restore the circulating current by inhibiting the phosphodiesterase with IBMX. Previous experiments have shown this to be the case for rods dialyzed with GTP-γ-S in darkness (Lamb and Matthews, 1988). Similar results for a bleached rod are shown in Fig. 4. Before the start of the trace the rod was bleached and sensitivity and response kinetics allowed to stabilize. A period of whole-cell recording was then used to introduce GTP-γ-S, after which the patch pipette was withdrawn and the membrane allowed to reseal. Subsequent exposure of the outer segment to IBMX restored the circulating current (Fig. 4 *A*), which was unaffected by light of moderate intensity but could be suppressed by sufficiently bright flashes (Fig. 4 *B*). The amplitude of the current produced by stepping to IBMX progressively decreased, probably because exposure to such intense flashes persistently activated additional transducin through binding of GTP-γ-S (Lamb and Matthews, 1988). This seems likely to have caused a sufficiently large increase in phosphodiesterase activity even in the presence of IBMX to maintain the cyclic GMP concentration at a low level.

DISCUSSION

The decay of circulating current induced in both dark-adapted and bleached rods by the incorporation of GTP-γ-S can most simply be interpreted as resulting predominantly from the persistent activation of transducin which has bound GTP-γ-S instead of GTP. Transducin activation would lead to a maintained stimulation of the phosphodiesterase and thence to a decrease in the outer segment cyclic GMP concentration and channel closure.

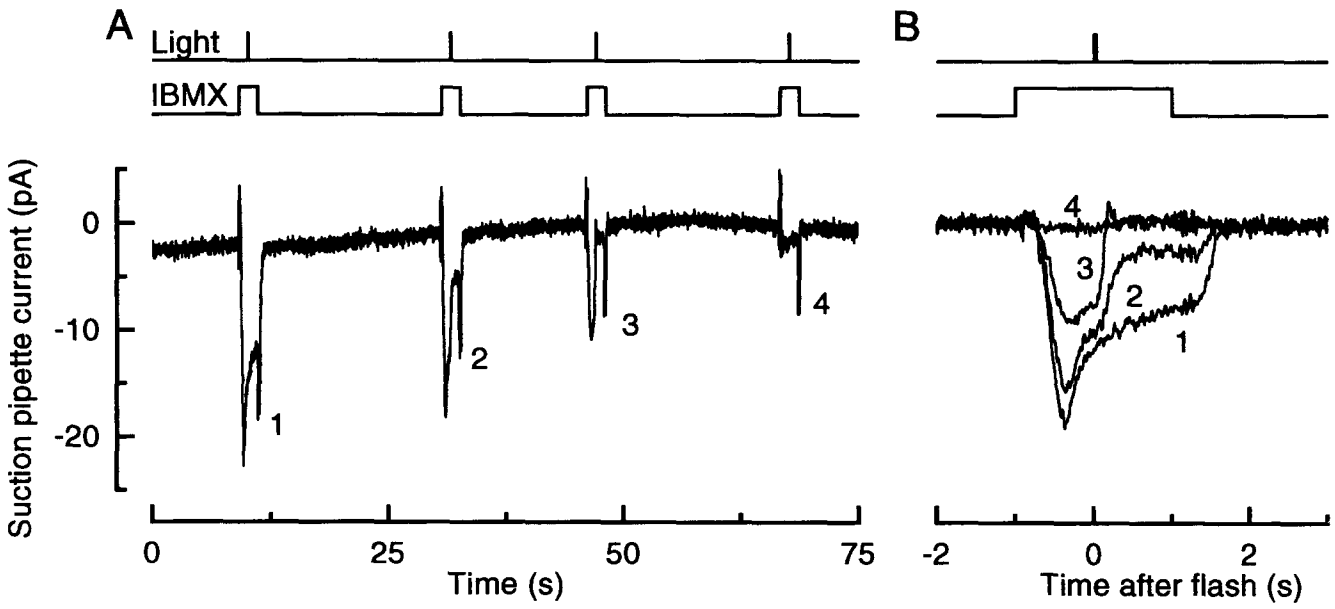

FIGURE 4. Effect of IBMX on a rod which had previously been bleached and loaded with GTP-γ-S. The rod was first exposed to intense light which bleached 24% of the photopigment, and sensitivity and response kinetics were allowed to stabilize for 200 min. Then the inner segment was partially drawn into the suction pipette, a patch pipette containing a solution which included 2 mM GTP-γ-S was sealed onto the exposed ellipsoid, and the patch ruptured. After 2 min of whole-cell recording, the patch pipette was withdrawn, and the membrane was allowed to reseal. Finally, the inner segment was drawn fully into the suction pipette, and the exposed outer segment stepped repeatedly into Ringer solution containing 500 μM IBMX. (*A*) Continuous suction pipette current recording during repeated superfusion of the bleached, GTP-γ-S–loaded rod with IBMX Ringer. A bright flash was delivered 1 s after each step into IBMX Ringer. (*B*) Superimposed responses 1–4 from *A* after subtraction of the junction current between pipette and bath. Flashes delivered (*1*) 380, (*2*) 6,400, (*3, 4*) 110,000 photons μm^{-2}. Comparable results were obtained from two other rods.

The time course for the decline in current induced by GTP-γ-S in dark-adapted rods appeared to depend rather little on whether or not just-saturating flashes were delivered to monitor the zero-current level (compare Figs. 1 *A* and 2 *A*, and the *filled symbols* in Fig. 3, *A* and *B*). As these flashes will have resulted in a mean rate of photoisomerization much greater than the rate of thermal isomerization of rhodopsin in darkness (Baylor et al., 1979; Baylor et al., 1984), it seems likely that this decline was dominated either by the spontaneous activation of transducin or by some other mechanism (Lamb and Matthews, 1988). However, it seems unlikely that the decay of current originated from an inhibitory action of GTP-γ-S on guanylyl cyclase (Sitaramayya et al., 1991), as cyclase activity remained high in the presence of GTP-γ-S even after the circulating current had been persistently suppressed by light (Lamb and Matthews, 1988) or after a bleach (see Fig. 4). Whatever the origin of this decay, it sets an upper limit for the rate of transducin activation by rhodopsin in darkness.

The more rapid decline in current in bleached rods after the introduction of GTP-γ-S is most simply interpreted as reflecting an elevated rate of transducin activation under these conditions, suggesting that bleached photopigment can activate transducin at a higher rate than can dark-adapted rhodopsin. This is consistent with other measurements showing an increased steady-state velocity of both phosphodiesterase and guanylyl cyclase in bleached rods (Cornwall and Fain, 1994) and of guanylyl cyclase in bleached cones (Cornwall et al., 1995), and with recent biochemical demonstrations of the ability of opsin to activate transducin (Cohen et al., 1992; Surya et al., 1995; Jäger et al., 1996). Our measurements suggest that bleached pigment activates transducin at a rate at least two to three times greater than dark-adapted pigment. However, this value seems likely to underestimate the actual ratio of their catalytic activities (Cornwall and Fain, 1994; Surya et al., 1995; Jäger et al., 1996) for two reasons. First, the decay of current induced by GTP-γ-S in darkness is likely to overestimate the rate of activation of transducin by dark-adapted rhodopsin for the reasons given above. Second, since diffusion of GTP-γ-S along the outer segment will have been retarded by the disk stack (Lamb et al., 1981; Olson and Pugh, 1993; Koutalos et al., 1995), the incorporation of GTP-γ-S from a patch pipette probably substantially underestimates the extent to which transducin activation is accelerated in bleached rods.

Our results suggest that the persistent excitation which follows bleaching takes place via transducin and the remainder of the normal phototransduction cascade. It seems to us likely that the bleached pigment which produces this activation is some form of opsin, i.e., pigment without chromophore (Jin et al., 1993), which may or may not be phosphorylated or bound to arrestin. It appears to be stable in an isolated photoreceptor for many hours (Jones et al., 1989; Cornwall et al., 1990), is neither inactive rhodopsin nor light-activated Rh*, and seems to activate the transduction cascade sufficiently after large bleaches to desensitize the photoreceptor through light adaptation (Lamb, 1990; Fain and Cornwall, 1993; Fain et al., 1996).

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