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INCORPORATION OF ANALOGUES OF GTP AND GDP INTO ROD PHOTORECEPTORS ISOLATED FROM THE TIGER SALAMANDER

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SUMMARY

1. Analogues of GTP and GDP were introduced into isolated rod photoreceptors using the whole-cell patch clamp technique, while simultaneously recording the photocurrent with a suction pipette. After several minutes of whole-cell recording the patch pipette was disengaged, thus trapping the analogue inside the cell.

2. During the introduction of the hydrolysis-resistant GTP analogues guanosine-5′-*O*-(3-thio-triphosphate) (GTP-γ-S) and guanylyl-imidodiphosphate (GMP-PNP) the dark current progressively declined, and the duration of responses to flashes of light which had previously been just-saturating increased slightly. The form of the rising phases of the responses to dim or bright flashes was little affected.

3. Following the incorporation of these GTP analogues the response to an intense flash was prolonged by a factor of up to 300, and the circulating current remained suppressed for up to 1 h. Ultimately the circulating current recovered and the duration of the flash response returned to near its control value.

4. Superfusion of the outer segment with the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX) during the extended period of saturation resulted in a rapid increase in the circulating current, suggesting that the analogues had their major effect on the duration of phosphodiesterase activation by light.

5. Introduction of the phosphorylation-resistant GDP analogue guanosine-5′-*O*-(2-thio-diphosphate) (GDP-β-S) resulted in a decrease in light sensitivity and a reduction in the slope of the rising phase of the flash response.

6. The response to an intense flash was also prolonged in cells containing GDP-β-S, recovery becoming progressively slower on successive presentations of the flash following the withdrawal of the patch pipette. This observation suggests that GDP-β-S may be slowly converted within the cell to form a hydrolysis-resistant product.

7. These results indicate that the presence of a hydrolysis-resistant analogue of GTP within the cell causes light activation of the transduction mechanism for an extended period. Our interpretation of this finding is that hydrolysis of the bound guanosine nucleotide is necessary for the quenching of activated GTP-binding protein.

INTRODUCTION

The process of phototransduction is believed to operate via an enzymatic cascade involving an intermediate GTP-binding protein (G-protein, also known as transducin) and has been reviewed recently by a number of authors (e.g. Stryer, 1986; Liebman, Parker & Dratz, 1987; Hurley, 1987; Pugh, 1987). A schematic summary of this cascade is shown in Fig. 1. The stages involved in excitation are shown by

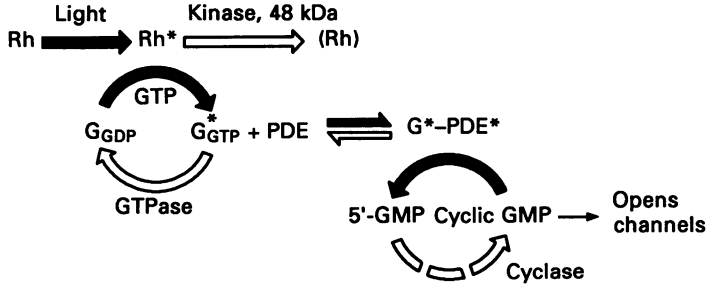

Fig. 1. Schematic summary of the transduction cascade (simplified from Pugh, 1987). Filled arrows represent events involved in excitation by light. Open arrows represent events thought to lead to recovery after a flash of light. Rh: rhodopsin; G: GTP-binding protein; Kinase: rhodopsin kinase; 48 kDa: 48 kDa protein; PDE: cyclic GMP phosphodiesterase; cyclase: guanylate cyclase. Stars denote excitation, (Rh) denotes quenched rhodopsin.

the filled arrows. Photoisomerized rhodopsin (Rh*) interacts catalytically with G-protein (G) causing it to bind GTP in exchange for GDP. The α-subunit of the G-protein then activates the phosphodiesterase (PDE), causing it to hydrolyse cyclic GMP. The ensuing reduction in cyclic GMP results in the closure of channels in the outer segment membrane, and reduction of the circulating current.

To terminate the light response and return to the initial steady state in darkness, all of the stages in the transduction cascade must be inactivated, and the free cyclic GMP concentration restored by guanylate cyclase. These processes leading to recovery are indicated by the open arrows in Fig. 1. In contrast to our knowledge of the excitatory processes, the mechanisms by which the individual stages in the cascade are quenched following illumination are comparatively poorly understood (reviewed by Pugh, 1987). Quenching of Rh* is believed to involve multiple phosphorylation by rhodopsin kinase, followed by interaction with a 48 kDa protein. In the case of the G-protein, the GTPase activity of its α-subunit (Wheeler & Bitensky, 1977), combined with the inability of the GDP-bound form to activate phosphodiesterase (Fung, Hurley & Stryer, 1981), have led to the supposition that the quenching of activated G-protein takes place via the hydrolysis of bound GTP to GDP (GTPase in Fig. 1). However, measurement of this rate *in vitro* yields a rate constant for GTP hydrolysis of 1–2 min^{-1}: much too slow to account for the falling phase of the light response (Godchaux & Zimmerman, 1979; Kühn, 1980; Baehr, Morita, Swanson & Applebury, 1982; Bennett, 1982; Lewis, Miller, Mendel-Hartvig, Schaechter, Kliger & Dratz, 1984; Yamanaka, Eckstein & Stryer, 1985; Yamazaki,

Tatsumi, Torney & Bitensky, 1987). It thus seemed possible that termination of G-protein activity in the intact rod might take place via some other mechanism, and that hydrolysis of the bound nucleotide might not, of itself, be necessary for the termination of the electrical response to light.

Hydrolysis-resistant analogues of GTP provide a powerful biochemical tool for investigating G-protein function, yielding prolonged activation of the phosphodiesterase *in vitro* (Liebman & Pugh, 1982). To investigate the quenching of G-protein under physiological conditions, hydrolysis-resistant analogues of GTP were introduced into single isolated rods. The results obtained indicate that hydrolysis of the bound guanosine nucleotide is indeed an essential mechanism in terminating the ability of G-protein to activate the phosphodiesterase under physiological conditions. A preliminary account of these results was presented to the Physiological Society (Lamb & Matthews, 1986). Similar results have also been obtained by Sather & Detwiler (1987), using a different technique.

METHODS

Recording and light stimulation

Simultaneous suction pipette and whole-cell patch pipette recordings were made from rods isolated mechanically from the retina of the tiger salamander *Ambystoma tigrinum* (Matthews, Torre & Lamb, 1985; Lamb, Matthews & Torre, 1986). During the whole-cell recording the intracellular voltage was clamped to −40 mV (after correction for a 10 mV liquid junction potential). The whole-cell access resistance was measured using 2 mV rectangular voltage pulses (Lamb *et al.* 1986); the values given were measured shortly after patch rupture. After 1–10 min in the whole-cell configuration the patch pipette was disengaged from the cell membrane (Torre, Matthews & Lamb, 1986; Lamb & Matthews, 1988*b*), thus trapping inside the cell the constituents of the patch pipette solution which had diffused into the cytoplasm. A specific advantage of this technique is that responses recorded under control conditions can be compared with those recorded from the same cell following the introduction of test substances via the patch pipette.

Rods were stimulated with brief flashes of unpolarized light of wavelength 500 nm. The majority of these flashes were of 20 ms duration, although the most intense flashes were often of 80 ms duration. Flash intensities are given in isomerizations, estimated from an assumed collecting area of 20 μm^2 (Lamb *et al.* 1986). In the text we refer to flashes delivering more than approx. 10^5 isomerizations as 'intense', to distinguish them from 'bright' flashes which only just caused the rod response to saturate.

Solutions and superfusion

Normal Ringer solution was as described previously (Lamb *et al.* 1986), with the addition, in certain experiments, of 200 μM-IBMX (3-isobutyl-1-methyl-xanthine; Sigma). In experiments with IBMX, rapid solution changes were carried out using the θ-tube method of Hodgkin, McNaughton & Nunn (1985), modified so that the hydraulic cylinder coupled to the microscope stage was driven by a solenoid, and with the perfusate delivered by peristaltic pumps (Lamb & Matthews, 1988*b*).

The pseudo-intracellular solution filling the patch pipette was based on the control solution used previously (Lamb *et al.* 1986), and contained 92 mM-potassium aspartate, 7 mM-NaCl, 5 mM-$MgCl_2$, 10 mM-HEPES–KOH and 20 μM of the calcium chelator BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetra-acetic acid (Tsien, 1980; BDH). On the day of the experiment 1 mM-Na_2ATP and 1 mM-Na_4GTP (Sigma) were added to this stock solution, together with the appropriate concentration of the desired guanosine nucleotide analogue. In the earliest experiments GTP was omitted. The pH of the solution was adjusted to around 7·2 with KOH.

Analogues of GTP and GDP

The GTP analogues used were guanosine-5′-*O*-(3-thio-triphosphate) (GTP-γ-S) and guanylyl-imidodiphosphate (GMP-PNP) (tetra-lithium salts; Boehringer Mannheim). GTP-γ-S is modified

from GTP by the replacement of an oxygen of the terminal phosphate group by sulphur. In GMP-PNP the oxygen normally linking the β- and γ-phosphates of GTP is replaced by nitrogen. These changes have been shown to render the analogues resistant to hydrolysis of the terminal phosphate group, but in certain reactions the analogues are able to substitute for GTP as the substrate (Pfeuffer & Helmreich, 1975). The GDP analogue guanosine-5'-*O*-(2-thio-diphosphate) (GDP-β-S) was also used (tri-lithium salt; Boehringer Mannheim). In the case of GDP-β-S, the substitution of sulphur for an oxygen of the terminal phosphate group renders it resistant to both hydrolysis and phosphorylation (Eckstein, Cassel, Levkovitz, Lowe & Selinger, 1979).

Assays of purity

These analogues of GTP and GDP are thermally unstable, and commercial samples are often impure (Cockcroft & Gomperts, 1985). Therefore the analogues were analysed by anion exchange high-performance liquid chromatography (HPLC), using a linear gradient of 0·15–3 M-ammonium formate (pH 4·5) on a SAX column (Partisil SAX 10, HPLC Technology), and detected by UV

TABLE 1. Purity of analogues (%): manufacturer's assay, and our assay carried out after all experiments were completed

	GTP-γ-S			
	Batch 1	Batch 2	GDP-β-S	GMP-PNP
Manufacturer's assay	81	82	86	90
Purity	63	51	71	58
GMP	3	5	8	2
GDP	18	28	—	—
GDP-NH_2	—	—	—	17
Other	16	16	21	23

absorbance at 275 nm. Table 1 gives the results of such analyses, carried out after all experiments were completed. Purities are expressed as percentages of the total peak area. Also given for each compound is the manufacturer's assay of purity. The purity at the time of the experiments is likely to fall between these two extremes. Note that all of the analogues used were significantly impure. Some of the impurities present could be identified, others could not.

Peaks due to GDP and GMP were identified by comparison with those obtained on analysing these nucleotides (from Boehringer and Sigma respectively). Small quantities of GMP were present in all of the analogue samples. GDP accounted for the major impurity in both batches of GTP-γ-S. The sample of GMP-PNP yielded a well-defined impurity peak which did not correspond to either GMP or GDP. We believe that this peak may have been due to GDP-NH_2, formed following hydrolysis of the terminal phosphate group, because its amplitude increased on successive runs when the selectively collected GMP-PNP peak was subsequently reanalysed.

The other, unidentified, impurities were mostly present as a long shoulder eluting after the major peak due to each analogue. In the sample of GDP-β-S there were also two small unidentified peaks which eluted before the analogue, accounting for some 4% of the total area. The sample of GDP-β-S also contained a very small peak, of less than 1% of the total area, which eluted with GTP-γ-S.

The stated concentrations of the analogues included in the patch pipette solutions are nominal (i.e. based on an assumed purity of 100%). In most of the experiments with GTP analogues, a concentration of 2 mM was used (batch 1 GTP-γ-S). A few experiments were carried out with concentrations of GTP-γ-S of 10 mM (batch 1), 200 μM or 50 μM (batch 2). GDP-β-S was used at 2 and 11 mM.

RESULTS

Incorporation of GTP analogues

The effect of incorporating hydrolysis-resistant analogues of GTP into rods is illustrated in Fig. 2. Figure 2*A* shows a suction pipette recording from the outer segment of an isolated salamander rod. In the top panel, under control conditions, a sequence of just-saturating (i.e. bright) flashes was delivered, together with an intense flash (arrow), which held the response in saturation for around 10 s. Over the succeeding 75 s the circulating current recovered completely to the original dark level.

A patch pipette, filled with the pseudo-intracellular solution together with 2 mM-GTP-γ-S, was then sealed onto the inner segment of the rod, which protruded from the suction pipette. About 30 s later (time zero, second panel) the patch of membrane at the pipette's tip ruptured spontaneously, allowing the contents of the patch pipette to diffuse into the cytoplasm (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). After about 2 min in this whole-cell configuration (bar) the patch pipette was gently disengaged from the cell membrane, stopping the diffusion of the analogue into the cytoplasm. During the period of whole-cell recording the circulating current steadily decreased; this decline was much more rapid than the decay seen if GTP-γ-S was not present in the solution filling the patch pipette (see Fig. 3 of Lamb *et al.* 1986). The duration of the responses to the bright flashes also increased slightly in comparison with those in control.

Following the withdrawal of the patch pipette, the same intense flash as before was delivered a second time (arrow). In dramatic contrast to the case with the first presentation, the response remained in saturation for an extended period. No response was detected to the bright flash delivered at the end of the second panel in Fig. 2*A*, or, indeed, to flashes delivered during the next hour. Following that time the circulating current gradually recovered; this recovery is investigated in more detail in a subsequent section.

Figure 2*B* shows results from a similar experiment in which the patch pipette solution contained 2 mM-GMP-PNP. Once again the circulating current declined during the period of whole-cell recording, and the duration of the response to the intense flash was dramatically prolonged following the incorporation of the analogue.

As both GTP-γ-S and GMP-PNP can interact with the G-protein *in vitro* (Yamanaka, Eckstein & Stryer, 1986), these results strongly suggest that the G-protein remains activated for an extended period when bound to a non-hydrolysable analogue of GTP.

Effect of flash intensity

The magnitude of the prolonged light-induced suppression of current following the introduction of hydrolysis-resistant GTP analogues was graded with the intensity of the flash used to elicit it. Figure 3 shows results from an experiment similar to those described above. Under control conditions an intense flash was delivered (response labelled 64, top panel), which was about 64 times brighter than the just-saturating (bright) flashes preceding and following it. As before GTP-γ-S was then introduced via the inner segment from a patch pipette containing 2 mM of the analogue (bar, panel *A*).

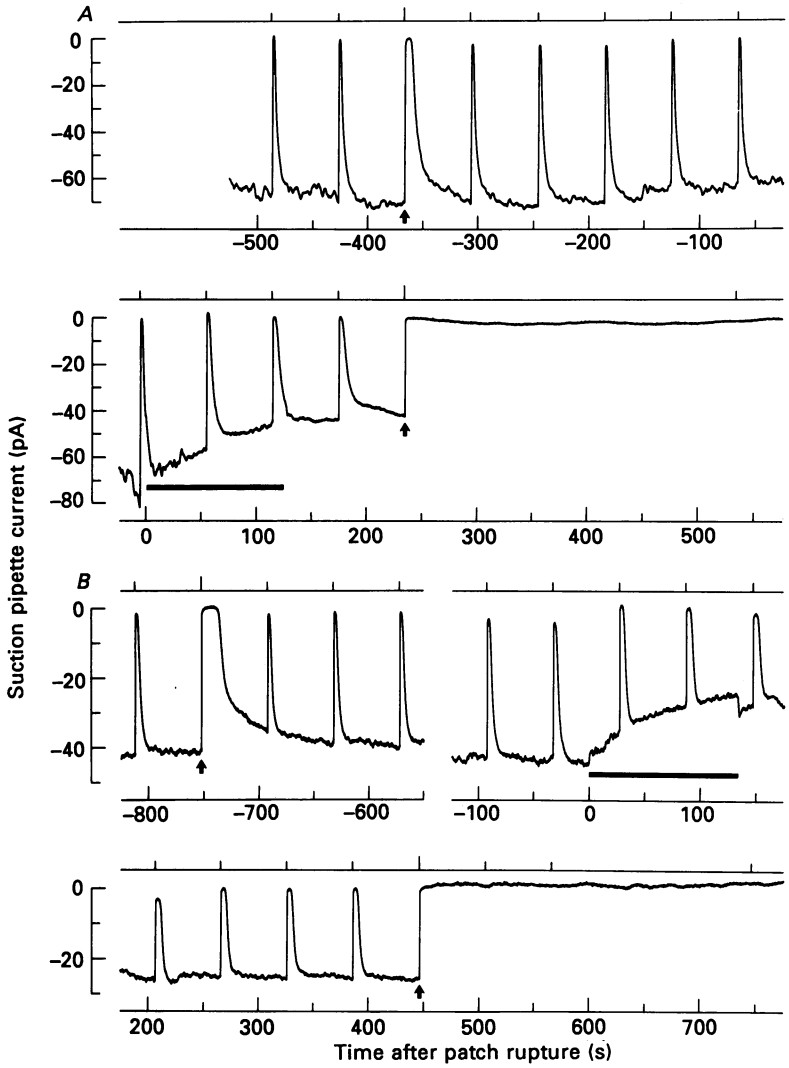

Fig. 2. Recordings from two rods illustrating the effects of introducing hydrolysis-resistant GTP analogues via a patch pipette sealed on the inner segment. *A*, patch pipette solution included 2 mM-GTP-γ-S; *B*, patch pipette solution included 2 mM-GMP-PNP. In each panel, upper trace: light monitor; lower trace: suction pipette current recorded from the outer segment. Bars denote the period of whole-cell recording, from the time of patch rupture until the patch pipette was disengaged from the cell membrane. Arrows indicate intense flashes resulting in $4{\cdot}2 \times 10^5$ isomerizations in *A* and $1{\cdot}7 \times 10^6$ isomerizations in *B*. The other bright flashes delivered $6{\cdot}7 \times 10^3$ isomerizations. Whole-cell access resistances were 17 MΩ in *A* and 13 MΩ in *B*.

For the first 300 s after the patch pipette was withdrawn the flashes remained at the original intensity. The responses to these flashes, superimposed on a slow decline in the circulating current, recovered almost completely. Then the flash intensity was increased by 4 times (responses labelled *a* and *b*, panel *B*). The responses to these brighter flashes did not recover completely: a step reduction in the circulating

current remained following the initial recovery. This reduction in circulating current was greater following the first response (labelled *a*) than for the second (labelled *b*). The flash intensity was then increased by a further factor of four (responses labelled *c* and *d*). The first of these flashes resulted in the persistent suppression of more than 40% of the circulating current (response labelled *c*). The response to the second flash

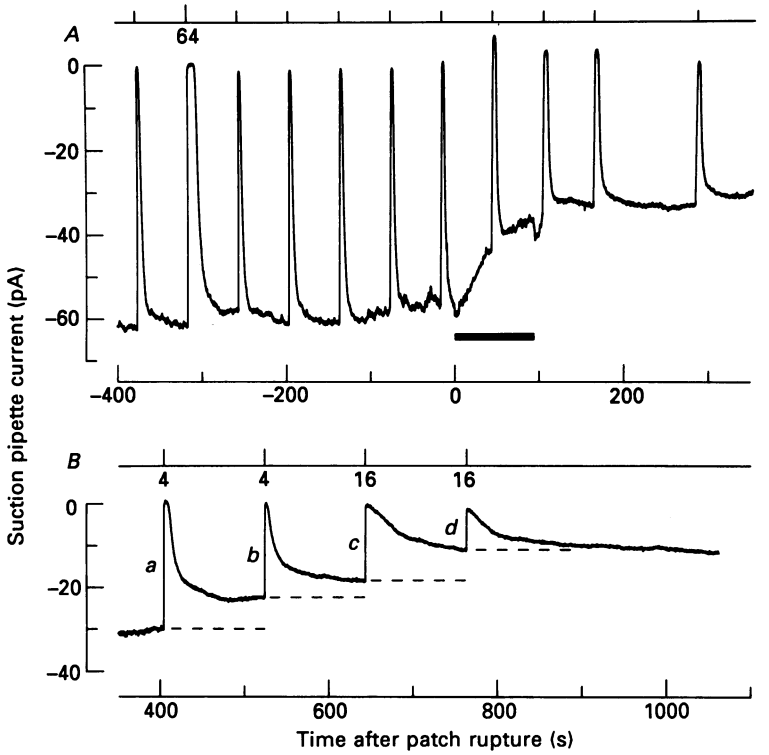

Fig. 3. Introduction of GTP-γ-S into an isolated rod via a patch pipette sealed on the inner segment. The patch pipette solution included 2 mM-GTP-γ-S. Whole-cell access resistance was 13 MΩ. In each panel, upper trace: light monitor; lower trace: suction pipette current recorded from the outer segment. The bar indicates the duration of the whole-cell recording. Numbers next to individual flashes indicate their approximate intensities relative to the unlabelled bright flashes, which delivered $6{\cdot}7 \times 10^3$ isomerizations. Flash intensities (in isomerizations) were $4{\cdot}2 \times 10^5$ for response labelled 64 (*A*); $2{\cdot}6 \times 10^4$ for responses labelled *a* and *b* (*B*); and $1{\cdot}1 \times 10^5$ for responses labelled *c* and *d* (*B*).

(labelled *d*) recovered virtually completely to this reduced steady level. The cumulative suppression of current that had taken place up to this point then gradually recovered, the circulating current reaching 55% of its value in control over the next 85 min. For comparison with these tails, note that the response to the even brighter flash (panel *A*, labelled 64) delivered in control conditions recovered within 1 min.

These results are consistent with the idea that a fairly small quantity of the analogue, introduced into the cytoplasm during the whole-cell recording, competed

with endogenous GTP for binding to the G-protein. As the flash intensity was increased, the number of G-protein molecules which bound the analogue instead of GTP would also increase. Therefore the size of the persistent decrease in circulating current would be expected to increase with flash intensity. Such binding to the G-protein, together perhaps with continual degradation of the unbound analogue,

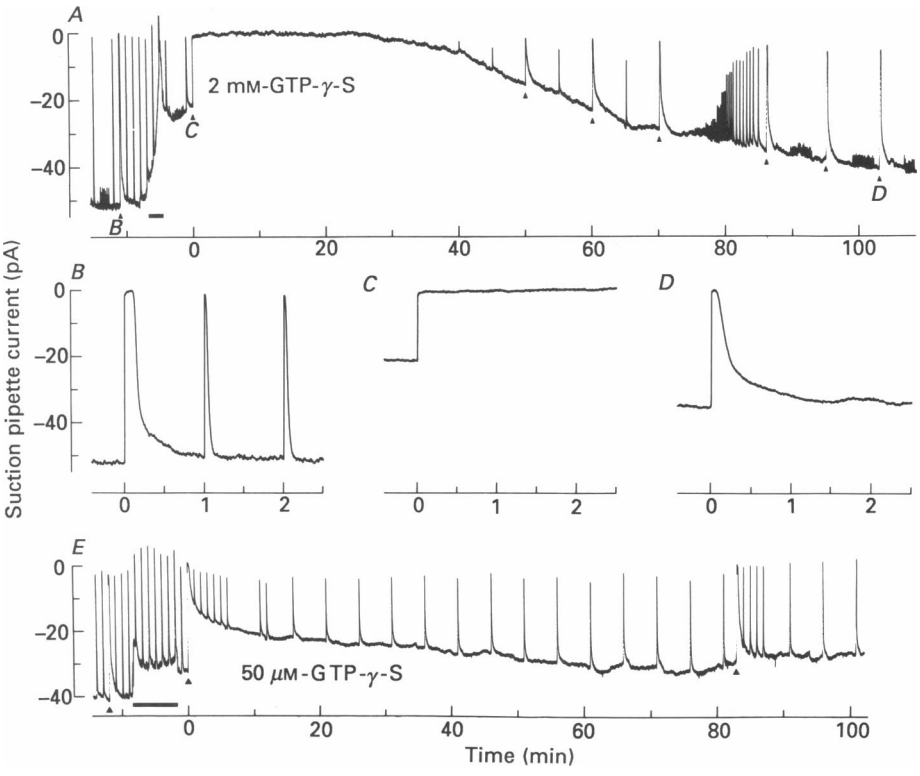

Fig. 4. Introduction of GTP-γ-S into isolated rods at two concentrations; patch pipette contained 2 mM- (*A*) or 50 μM- (*E*) GTP-γ-S. *A* and *E*, continuous suction pipette current records (outer segment drawn in) for the entire experiment. Bars indicate the duration of the whole-cell recordings. Whole-cell access resistance in *A*: 29 MΩ; in *E*: 20 MΩ. Arrows denote intense flashes delivering $4{\cdot}2 \times 10^5$ isomerizations in *A* and $2{\cdot}5 \times 10^6$ isomerizations in *E*. Panels *B*, *C* and *D* show the intense flash responses indicated in *A* on a faster time base.

would also reduce the quantity of the analogue still available for binding in response to subsequent flashes. This notion is consistent with the decreasing effectiveness of a given flash intensity at achieving further long-term circulating current suppression (compare response *a* with response *b*, and response *c* with response *d*, in Fig. 3).

Recovery from extended saturation

Results from an experiment similar to those of Fig. 2 are shown on a very slow time base in Fig. 4*A*. An intense flash was delivered in control (arrow *B*), shown on a faster time base in Fig. 4*B*. The response to this flash recovered completely within 2 min.

GTP-γ-S was then introduced from a patch pipette containing 2 mM of the analogue, sealed on the inner segment (bar), and the pipette was subsequently withdrawn. The intense flash was then delivered once more (arrow *C*, and Fig. 4*C*). The response remained near saturation for 30 min following the flash.

Over the next hour the circulating current gradually recovered, ultimately reaching 70% of its value in control. During this recovery the responses to bright flashes appeared light-adapted in comparison with those delivered before the intense flash. The responses to further presentations of the same intense flash delivered during this slow recovery (arrows) did not remain in saturation for an extended period, but recovered rapidly towards the previously existing level of circulating current. Ultimately, these responses recovered virtually as rapidly as in control, as can be seen by comparison of Fig. 4*D* with the control of Fig. 4*B*.

Qualitatively similar results could be obtained with much lower concentrations of GTP-γ-S in the patch pipette, if the duration of the period of whole-cell recording was increased, in order to maximize the degree of incorporation of the analogue. Such an experiment is shown in Fig. 4*E*; the patch pipette contained 50 μM-GTP-γ-S. The response to an intense flash after the incorporation of the analogue exhibited an initial rapid component of recovery, followed by a slowly recovering 'tail' of current suppression. Note also that the circulating current declined less during the whole-cell recording than was the case when the patch pipette contained 2 mM of the analogue.

The eventual recovery of the circulating current confirms that these cells had not simply been damaged irreversibly by the incorporation of the GTP analogue, but rather that the presence of GTP-γ-S resulted in an enormous prolongation of the falling phase of the light response. The failure of intense flashes, delivered long after a preceding intense flash, to elicit a prolonged response suggests that the free GTP analogue had by then all been destroyed or bound, making it unavailable for subsequent light-induced binding to G-protein.

Prolongation of the responses to intense flashes after the incorporation of hydrolysis-resistant GTP analogues was seen in a total of forty rods. In twenty-seven of these the circulating current ultimately regained a substantial proportion of its original magnitude under control conditions. In many of the remainder we did not wait for recovery, but there was no reason to suppose that it would not ultimately have taken place.

In most of these experiments the patch pipette contained 2 mM of the analogue (seventeen with 2 mM-GTP-γ-S, ten with 2 mM-GMP-PNP). The degree by which the responses to intense flashes were prolonged varied from cell to cell. Figure 5*A* shows representative examples of such responses spanning the observed range of behaviour. As the recording baseline was seldom as stable as for the cells of Fig. 4, the size of the circulating current was measured as the response to bright test flashes presented at regular intervals during the recovery, and these points have been plotted, expressed as a fraction of the original dark current before the introduction of the analogue. Data from the cell of Fig. 4*A* (■ in Fig. 5*A*) are replotted here to facilitate comparison.

We believe that a proportion of the variability between the different cells stemmed from differences in the quantity of the analogue successfully incorporated. In these

experiments with 2 mM of the analogue in the patch pipette the degree of prolongation of the response to an intense flash appeared to correlate to some extent with the access resistance during the 2–3 min of whole-cell recording (see legend of Fig. 5). We also had the impression that the effects of these analogues were most pronounced if the intense flash was delivered as soon as possible after the withdrawal of the patch pipette. For example, in the cell showing the greatest degree of response prolongation (▼, Fig. 5*A*), the intense flash was delivered within 80 s of withdrawing the patch pipette. Conversely, if there was a delay of many minutes before an intense flash was delivered, the effects on the duration of the response appeared to be rather smaller, or could only be elicited by flashes of even greater intensity. It is difficult to exclude the possibility that these differences stemmed from the incorporation of a smaller quantity of the analogue than might have been expected from the duration and access resistance of the whole-cell recording. However, it would be consistent with a gradual metabolic destruction of the analogue when not bound to the G-protein.

Cells into which GMP-PNP had been introduced were often affected less than was the case with GTP-γ-S, frequently exhibiting a slow tail of recovery rather than extended saturation. This tendency may correspond to differences in the affinity of the G-protein for the two analogues (see Discussion).

Figure 5*B* shows examples of experiments in which lower concentrations of GTP-γ-S were used (200 μM, filled symbols, three cells; 50 μM, open symbols, five cells; △, cell of Fig. 4*B*). In these cases the aim was to introduce as large a proportion as possible of the analogue into the cell, so as to place an upper limit on the concentration of the analogue required to exert an effect. The whole-cell recordings were therefore deliberately made longer (5–7 min) than in the earlier experiments, large patch pipettes were used to give low access resistances, and the intense flash was normally delivered soon after the withdrawal of the patch pipette.

From comparison of panels *A* and *B* in Fig. 5 it seems likely that the concentration of analogue actually incorporated into the cytoplasm during the fairly short whole-cell recordings of Fig. 5*A* was much lower than the 2 mM present in the patch pipette. The longer whole-cell durations and often lower access resistances in the experiments of Fig. 5*B* were probably more successful at equilibrating the contents of the patch pipette and the rod cytoplasm.

In a few experiments 10 mM-GTP-γ-S was used (five cells), the main aim being to investigate the effects of this analogue on the responses to dim flashes (see p. 480). The responses to intense flashes were greatly prolonged in these cells also, though the circulating current had often declined substantially by the time the flash was delivered.

IBMX perfusion during extended saturation

As well as interacting with the G-protein, GTP also acts as the substrate for guanylate cyclase, the enzyme which produces cyclic GMP and thus contributes to maintaining the steady dark current. In order to exclude the possibility that the prolongation of the intense flash response seen following the introduction of GTP analogues resulted from an inhibition of guanylate cyclase, rather than through prolonged activation of the G-protein, some means was required of investigating the

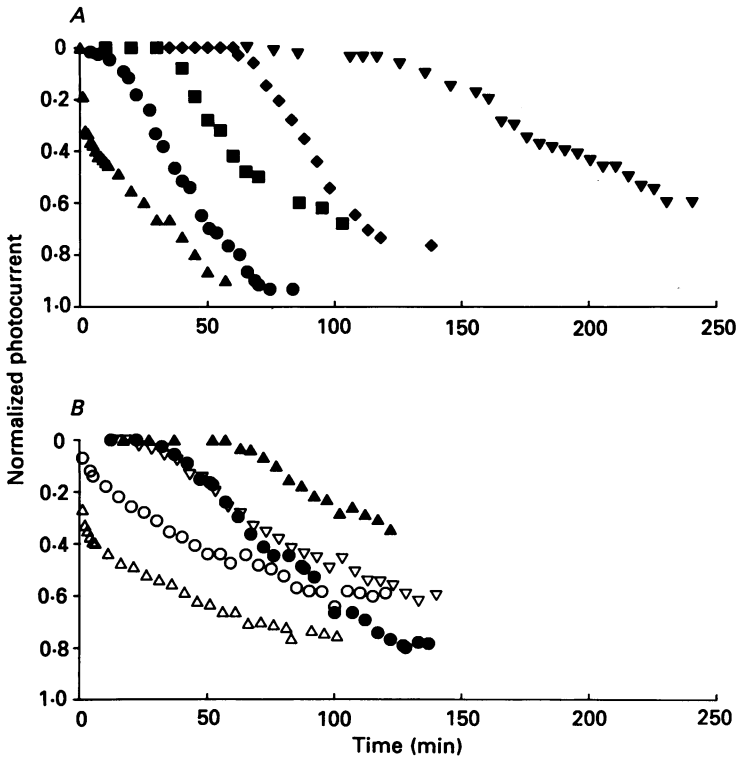

Fig. 5. Recovery of circulating current following intense flashes in ten cells containing GTP analogues. Each point was measured as the response to a bright test flash. The circulating current has been normalized in each case with respect to its value at the start of the experiment. *A*, patch pipette solution contained 2 mM-GTP-γ-S or 2 mM-GMP-PNP. *B*, patch pipette solution contained 200 μM- (filled symbols) or 50 μM- (open symbols) GTP-γ-S. The analogue concentration, flash intensity (in isomerizations), whole-cell duration, and access resistance were: *A*: ▲, 2 mM-GMP-PNP, $1{\cdot}7 \times 10^6$, 190 s, 40 MΩ; ● (same cell as Fig. 6), 2 mM-GTP-γ-S, $4{\cdot}2 \times 10^5$, 146 s, 17 MΩ; ■ (same cell as Fig. 4*A*), 2 mM-GTP-γ-S, $4{\cdot}2 \times 10^5$, 130 s, 29 MΩ; ◆ (same cell as Fig. 2*A*), 2 mM-GTP-γ-S, $4{\cdot}2 \times 10^5$, 122 s, 17 MΩ; ▼, 2 mM-GMP-PNP, $1{\cdot}7 \times 10^6$, 120 s, 12·5 MΩ. *B*: △ (same cell as Fig. 4*B*), 50 μM-GTP-γ-S, $2{\cdot}5 \times 10^6$, 404 s, 20 MΩ; ○, 50 μM-GTP-γ-S, $2{\cdot}5 \times 10^6$, 376 s, 10 MΩ; ▽, 50 μM-GTP-γ-S, $2{\cdot}5 \times 10^6$, 319 s, 11 MΩ; ●, 200 μM-GTP-γ-S, $2{\cdot}5 \times 10^6$, 348 s, 17 MΩ; ▲, 200 μM-GTP-γ-S, $2{\cdot}5 \times 10^6$, 391 s, 13 MΩ.

metabolism of cyclic GMP during the period of extended saturation. This was achieved by superfusing rods with IBMX, following the introduction of GTP-γ-S from a patch pipette. IBMX is believed to act predominantly to inhibit the rod phosphodiesterase (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970), though it may also have other effects (Weller, Virmaux & Mandel, 1975). Any rapid increase in circulating current induced by the inhibition of the phosphodiesterase by IBMX (Yau & Nakatani, 1985; Cervetto & McNaughton, 1986) would indicate continued production of cyclic GMP (Hodgkin & Nunn, 1988), and this would argue strongly against any major effect of these analogues on the cyclase.

Figure 6*A* plots on a slow time base the suction pipette current recorded in such

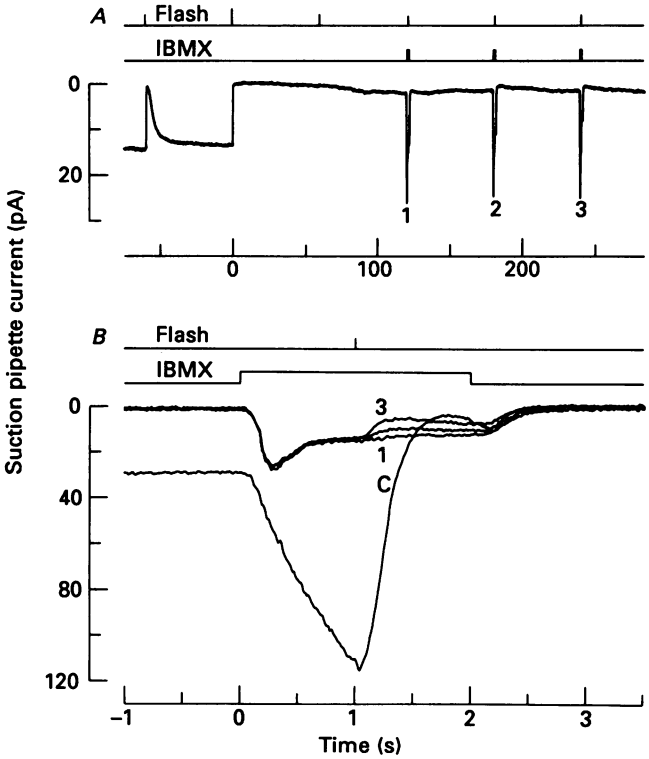

Fig. 6. *A*, effect on a rod containing GTP-γ-S of superfusion with 200 μM-IBMX soon after an intense flash. Upper trace: flash monitor; middle trace: superfusion monitor; lower trace: suction pipette current (inner segment drawn in). *B*, traces from *A* on an expanded time base for flashes of three intensities, together with a control exposure to IBMX (trace C) obtained before the introduction of GTP-γ-S. Duration of IBMX exposures 2 s, flashes delivered after 1 s. Intense flash at time zero in *A* delivered $4{\cdot}2 \times 10^5$ isomerizations; the bright flash preceding it delivered $6{\cdot}7 \times 10^3$ isomerizations. Intensities of flashes presented during the IBMX exposures were $6{\cdot}7 \times 10^3$ (traces C, 1), $2{\cdot}6 \times 10^4$ (trace 2) and $1{\cdot}1 \times 10^5$ (trace 3) isomerizations respectively. Patch pipette solution included 2 mM-GTP-γ-S; duration of whole-cell recording 146 s, access resistance 17 MΩ. The suction pipette current traces (recorded from the inner segment) have been inverted for ease of comparison with other figures. Here, and in Fig. 7, junction currents on the solution change were less than 2 pA, and therefore have not been subtracted from the suction pipette current traces.

an experiment. The inner segment of the rod was drawn into the suction pipette to allow direct superfusion of the exposed outer segment; 5 min before the start of the trace a patch pipette containing 2 mM-GTP-γ-S had been withdrawn from the outer segment. At time zero an intense flash was delivered, resulting in a prolonged response. Three exposures to 200 μM-IBMX of 2 s duration were then delivered, during which bright flashes of increasing intensity were presented. IBMX superfusion resulted in a rapid elevation of the circulating current to a level greater than that existing immediately before the intense flash. These responses to superfusion with IBMX are shown superimposed on a faster time base in Fig. 6*B*, together with the response to a similar IBMX exposure delivered in darkness before the introduction

of GTP-γ-S (trace labelled C). Note that the maximum rate of current increase due to IBMX was more rapid during the extended light response than in control.

Figure 7 shows results from a similar experiment on another cell into which it appeared that a rather smaller quantity of GTP-γ-S had been incorporated. The same intense flash as was used above resulted in the response shown in Fig. 7*A*,

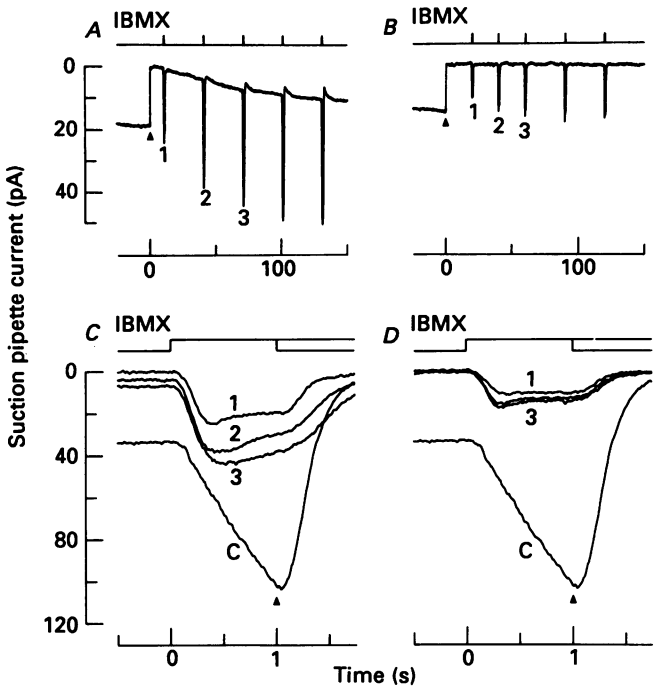

Fig. 7. Effect of superfusion with 200 μM-IBMX following intense flashes delivered after the incorporation of GTP-γ-S. Intense flashes (arrows) delivered $4{\cdot}2 \times 10^5$ (*A*) and $4{\cdot}2 \times 10^6$ (*B*) isomerizations respectively. Upper trace: superfusion monitor; lower trace: suction pipette current (inner segment drawn in). *C* and *D*, IBMX exposures from *A* and *B* respectively, on a faster time base, together with an IBMX exposure in control (trace C). Duration of IBMX exposures 1 s, except in control trace, where duration was 2 s, and the current was shut off after 1 s by a bright flash delivering $6{\cdot}7 \times 10^3$ isomerizations. Patch pipette solution included 2 mM-GTP-γ-S; duration of whole-cell recording 176 s, access resistance 25 MΩ. The suction pipette current traces have been inverted for ease of comparison with other figures.

which recovered steadily over several hundred seconds. A ten-times brighter flash was then delivered, shown in Fig. 7*B*, which held the response in saturation for the remainder of the trace. In both cases brief exposures to IBMX were delivered following the flash; these are shown superimposed in Fig. 7*C* and *D* respectively. As the response to the dimmer of the two flashes recovered (Fig. 7*C*), superfusion with IBMX elicited a progressively larger elevation of the circulating current. Following the brighter flash (Fig. 7*D*), which presumably caused greater activation of the transduction mechanism, the magnitude of the current elevation induced by IBMX was smaller, but IBMX still restored the circulating current to near its value before

the flash. In both cases, the maximum rate of increase in current was at least as fast as in the dark-adapted control (traces labelled C). Rapid elevation of the circulating current by IBMX under these conditions was seen in seven other cells.

These observations indicate that cyclic GMP is still being produced by guanylate cyclase during the prolonged light response. Indeed, the rate of rise of current elicited by IBMX was accelerated under these conditions in comparison with the preceding control. Similar speeding by light of the rate of current increase due to IBMX in normal rods has been interpreted as indicating that the cyclase is running faster than in darkness (Hodgkin & Nunn, 1988). Therefore the extended response duration seen in the presence of hydrolysis-resistant GTP analogues cannot result from a prolonged inhibition of the cyclase, but rather must stem from a greatly prolonged period of activation of the phosphodiesterase, presumably via prolonged activation of the G-protein. Note, however, that these experiments do not exclude the possibility that these analogues might exert some minor effect on the cyclase rate. This point is considered further in the Discussion.

Incorporation of GDP-β-S

The phosphorylation-resistant GDP analogue GDP-β-S has been shown to act as an inhibitor of G-protein activation *in vitro* (Eckstein *et al.* 1979) by stabilizing it in the GDP-bound form (Van Dop, Yamanaka, Steinberg, Sekura, Manclark, Stryer & Bourne, 1984). It was therefore of interest to investigate the effect of GDP-β-S on the light response of the intact rod.

Figure 8 shows the effects of introducing GDP-β-S via the inner segment from a patch pipette containing 2 mM of the analogue. First, the responses to dim, bright and intense flashes were recorded under control conditions (panel *A*, intense flash arrowed). During the period of whole-cell recording (bar, panel *B*) the circulating current declined slowly. The rate of decline varied from cell to cell, but was not as rapid as was observed with 2 mM of the GTP analogues discussed above. After the patch pipette had been withdrawn at 650 s this decline continued (bottom panel). Then the sequence of dim and bright flashes was repeated, followed by an intense flash (arrow). The introduction of GDP-β-S resulted in a decrease in the sensitivity to dim flashes; this is examined in detail in the next section. An unexpected finding was that the duration of the response to the intense flash was greatly increased, highly reminiscent of the effects of the hydrolysis-resistant analogues of GTP. Similar results were obtained from fourteen other cells in which intense flashes were delivered late after the introduction of GDP-β-S (2 mM, eight cells; 11 mM, six cells).

The development of the prolongation of the intense flash response as a function of time after the incorporation of GDP-β-S was investigated further by the experiment shown in Fig. 9. Figure 9*A* shows the response to an intense flash recorded in control, together with responses to two bright flashes. A brief period of whole-cell recording (80 s duration) was then used to introduce what we assume to have been a comparatively small amount of GDP-β-S. Shortly after the withdrawal of the patch pipette the intense flash was delivered again (Fig. 9*B*); the response was very similar to that recorded in control, apart from being somewhat reduced in amplitude. Further presentations of the intense flash at 11 and 21 min after the introduction of

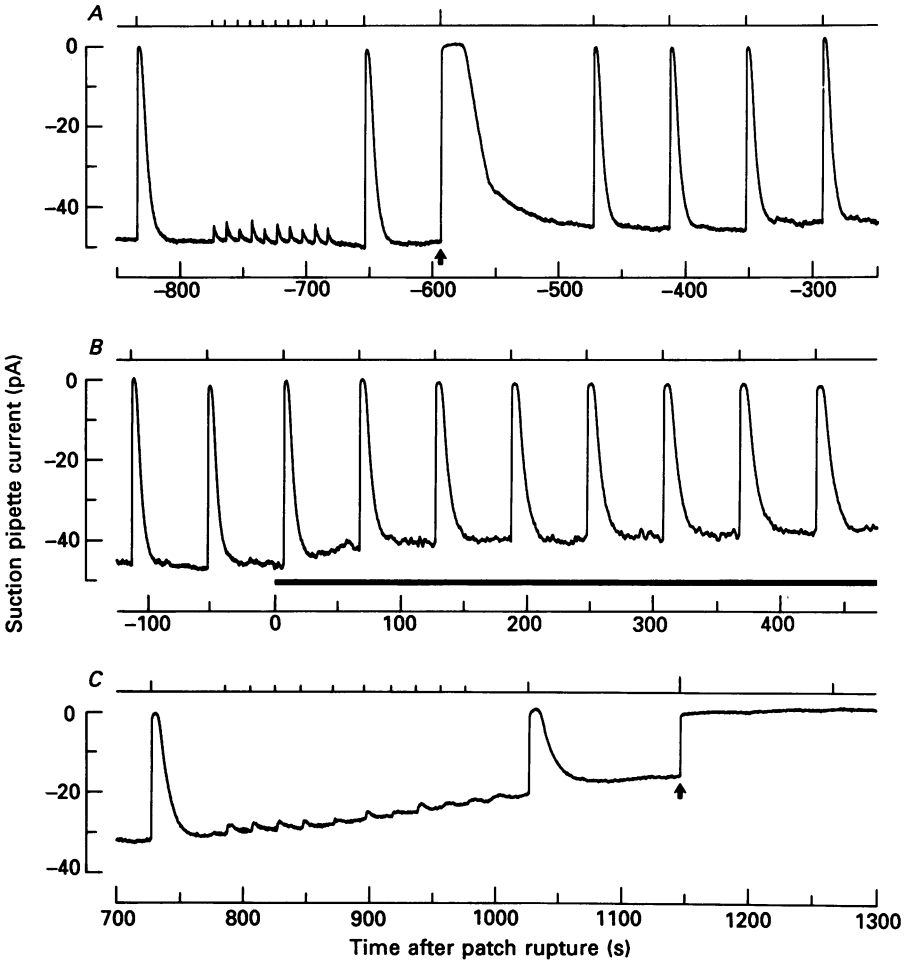

Fig. 8. Effect of introducing GDP-β-S from a patch pipette sealed on the inner segment. In each panel, upper trace: light monitor; lower trace: suction pipette current (outer segment drawn in). Patch pipette solution included 2 mM-GDP-β-S. The bar denotes the period of whole-cell recording (access resistance 20 MΩ, duration 650 s). Arrows indicate intense flashes delivering $1{\cdot}6 \times 10^6$ isomerizations. Bright flashes and dim flashes delivered $6{\cdot}2 \times 10^3$ and 16 isomerizations respectively.

the analogue elicited the responses shown in Fig. 9*C* and *D*. These responses exhibited a pronounced slow component of recovery, more pronounced in the latter case. Ultimately, after 131 min, when the circulating current had substantially recovered, the falling phase of the intense flash response had partly recovered towards its time course in control conditions. Similar results were seen in three other cells in experiments in which the duration of the whole-cell recording was deliberately kept relatively brief.

The gradual development of the slowing of the responses to intense flashes was in contrast to the effects of GTP analogues, which, as discussed above, appeared to be most pronounced if the intense flash was delivered soon after incorporation. It also

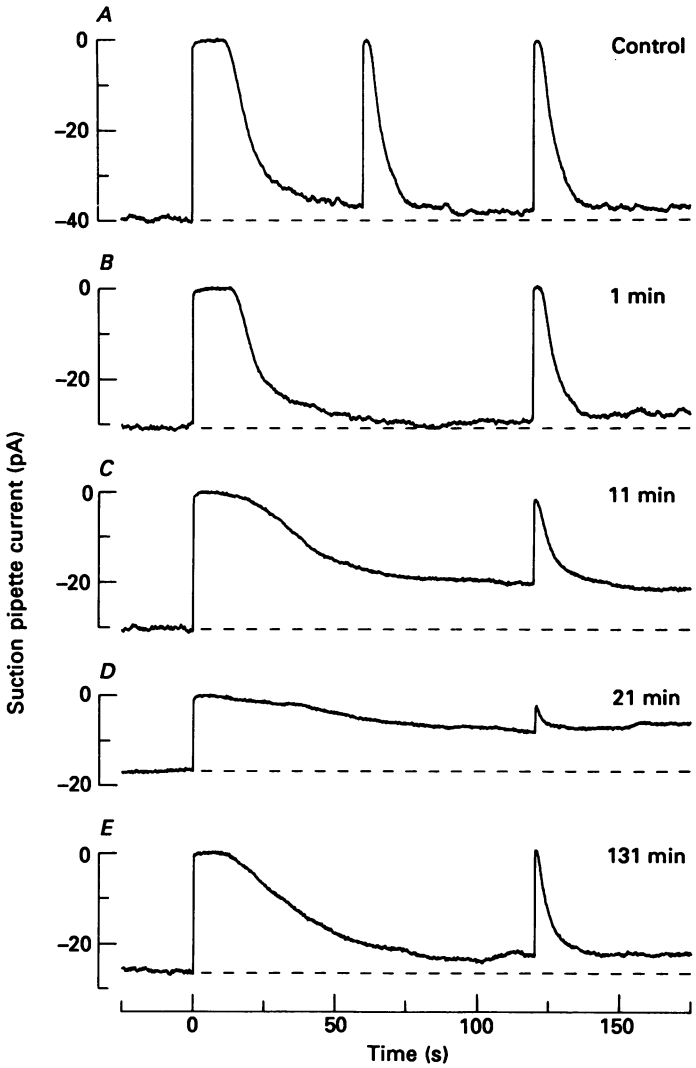

Fig. 9. Effect of the introduction of GDP-β-S on the response to intense flashes at successive times after withdrawing the patch pipette. *A*, before whole-cell recording; *B*, 48 s after withdrawing the patch pipette; and at the following times after *B*: *C*, 10 min; *D*, 20 min; *E*, 130 min. Patch pipette solution contained 2 mM-GDP-β-S; duration of whole-cell recording was 80 s; access resistance was 20 MΩ. Intense flashes delivered $4{\cdot}2 \times 10^5$ isomerizations. Bright flashes delivered $6{\cdot}7 \times 10^3$ isomerizations.

contrasts with the effects of GDP-β-S on light sensitivity, described below, which appeared rapidly. Therefore, it seems likely that the prolongation of these responses was not due directly to GDP-β-S itself. These observations are consistent with the idea that GDP-β-S may have been slowly converted within the cell into a product which could mimic GTP and was resistant to hydrolysis. This possibility is considered further in the Discussion.

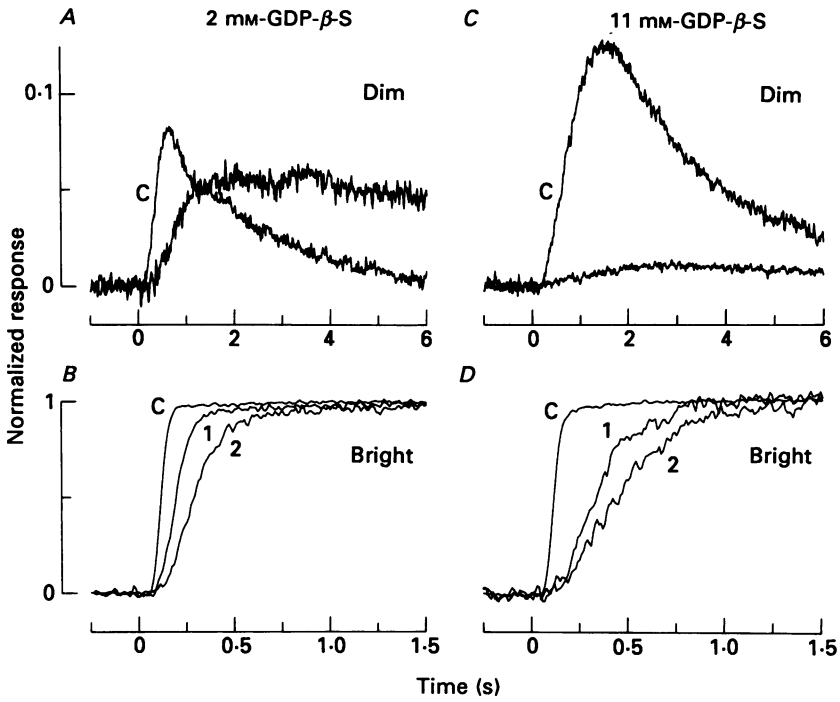

Fig. 10. Responses to dim and bright flashes in rods containing GDP-β-S. In each case the responses have been normalized with respect to the circulating current. *A* and *B*, same cell as Fig. 8; patch pipette solution included 2 mM-GDP-β-S (access resistance 20 MΩ, duration 650 s). *A*, averaged dim flash response (ten sweeps) in control (trace C; see Fig. 8, panel *A*; mean dark current 47 pA) and following withdrawal of the patch pipette (see Fig. 8, panel *C*; mean dark current 25 pA). *B*, bright flash responses in control (trace C; average of two sweeps) and following the introduction of GDP-β-S, both before (trace 1) and after (trace 2) the dim flash responses averaged in *A*. Dim and bright flashes delivered 16 and $6{\cdot}2 \times 10^3$ isomerizations respectively. *C* and *D*, similar experiment on another cell but with 11 mM-GDP-β-S in the patch pipette solution (access resistance 12·5 MΩ, duration 100 s). Dim flashes delivered 7·8 isomerizations in control (average of ten sweeps; mean dark current 45 pA) and 63 isomerizations following introduction of GDP-β-S (average of five sweeps; mean dark current 14·5 pA). The latter trace has been scaled down by the ratio of these flash intensities (7·8/63). Bright flashes delivered $6{\cdot}2 \times 10^3$ isomerizations.

Effects of GDP-β-S on the rising phase of the response

As discussed above, GDP-β-S has been found to inhibit the activation of G-proteins *in vitro* (Eckstein *et al.* 1979), by acting as a metabolically stable surrogate for GDP. Therefore, the analogue might be expected to alter the rising phase of the photoresponse, and the sensitivity of the rod to light.

Figure 10*A* plots averaged responses to dim flashes from the experiment of Fig. 8; delivered both under control conditions (trace C) and following the introduction of the analogue from the patch pipette (2 mM-GDP-β-S). In each case the responses have been normalized with respect to the circulating current. It can be seen that the effect of introducing GDP-β-S was to decrease the slope of the early rising phase of

the flash response, and also to reduce the peak amplitude of the response. The time to peak of the response was increased.

Figure 10*B* shows the rising phases of the normalized responses to bright flashes recorded from the same cell. Shortly after the introduction of the analogue (trace 1) the slope of the rising phase of the bright flash response was reduced in comparison with a control response (trace C). The rising phase of the response to a bright flash delivered just after the dim flash sequence was slowed further (trace 2). The rising phases of the responses to bright flashes delivered prior to trace 1 during the whole-cell recording were also progressively slowed (not shown), so desensitization of the light response may have taken place as rapidly as GDP-β-S entered the cell.

Results from a similar experiment in which the patch pipette contained the higher concentration of 11 mM-GDP-β-S are shown in Fig. 10*C* and *D* for dim and bright flashes respectively. It can be seen from Fig. 10*C* that the degree of desensitization was much greater than when the patch pipette contained 2 mM-GDP-β-S, and that the rising phases of the responses to both dim flashes (Fig. 10*C*) and bright flashes (Fig. 10*D*) were profoundly slowed in comparison with the control responses.

Substantial desensitization was observed in all cases in which the patch pipette contained 11 mM-GDP-β-S (seven cells), but only in seven out of thirteen cases with 2 mM-GDP-β-S. In the remaining six, we believe that incorporation was less successful, either because the duration of the whole-cell recording was deliberately kept short (80–160 s, three cells) as in Fig. 8, or because the access resistance was high (25–50 MΩ, three cells). In the latter cells the response to an intense flash was greatly prolonged, even though the responses to dimmer flashes were not desensitized. It seems possible that in these experiments the analogue was metabolized as it gradually entered the cell (as suggested above), preventing the attainment of a high concentration of GDP-β-S. In three further experiments in which GDP was incorporated from a patch pipette containing 2 mM-GDP there was little effect on the rising phase of the normalized flash response, or on the duration of the responses to intense flashes.

Effects of GTP-γ-S on the rising phase of the response

Experiments in which responses to dim and bright flashes were recorded shortly after GTP-γ-S was incorporated, before the intense flash was presented, are shown in Fig. 11. Flash responses from the experiment of Fig. 4*A*, in which the patch pipette contained 2 mM-GTP-γ-S, are replotted in Fig. 11*A* and *B* on a fast time base. Incorporation of the analogue had no appreciable effect on the normalized rising phases of the responses to either dim (Fig. 11*A*) or bright flashes (Fig. 11*B*) in comparison with the respective control responses (traces labelled C). Even when the patch pipette contained 10 mM-GTP-γ-S, as was the case in the experiment of Fig. 11*C* and *D*, the normalized rising phases of the responses were virtually unaffected by the presence of the GTP analogue, despite a substantial decrease in circulating current.

Comparable results were obtained from each of fourteen other cells into which GTP analogues were incorporated and from which both dim and bright flash responses were recorded. In some cases the peak amplitude of the normalized dim flash response was somewhat reduced, but the slope of the rising phases of the

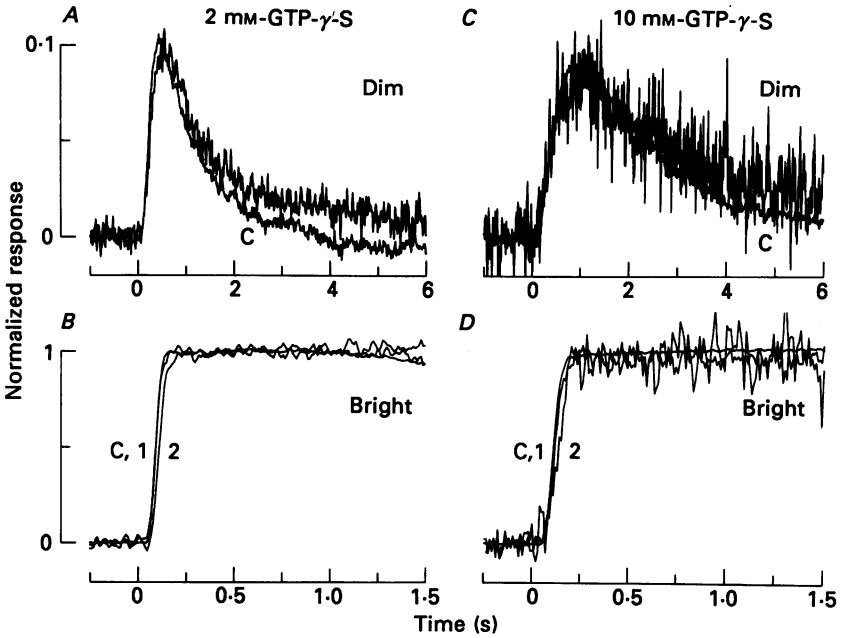

Fig. 11. Responses to dim and bright flashes recorded from rods containing GTP-γ-S. Similar protocol to Fig. 10. *A* and *B*, same cell as Fig. 4: patch pipette solution included 2 mM-GTP-γ-S (access resistance 29 MΩ, duration 130 s). *A*, averaged dim flash response (ten sweeps) in control (trace C; mean dark current 49·5 pA) and following withdrawal of the patch pipette (mean dark current 23 pA). *B*, bright flash responses from the same cell in control (trace C; average of two sweeps) and following the introduction of GTP-γ-S, both before (trace 1) and after (trace 2) the dim flash responses averaged in *A*. Dim and bright flashes delivered 34 and $6{\cdot}7 \times 10^3$ isomerizations respectively. *C* and *D*, similar experiment on another cell but with 10 mM-GTP-γ-S in the patch pipette solution (access resistance 17 MΩ, duration 92 s). Dim flashes delivered 17 isomerizations in control (average of ten sweeps; mean dark current 49 pA) and 34 isomerizations following introduction of GTP-γ-S (average of five sweeps; mean dark current 7 pA). The latter trace has been scaled down by the ratio of these flash intensities (17/34). Bright flashes delivered $6{\cdot}7 \times 10^3$ isomerizations.

responses was not significantly altered by the presence of the analogue. So it would appear that analogues of GTP cannot reproduce this effect of GDP-β-S on the rising phase of the light response.

DISCUSSION

Previously it had not been clear from biochemical measurements whether the hydrolysis of GTP by the G-protein was a necessary stage in the termination of the light response (Pugh, 1987). The results presented here indicate that the responses to intense flashes are immensely prolonged following the incorporation of hydrolysis-resistant GTP analogues into isolated rods. Experiments involving superfusion with IBMX during these extended responses show that the prolongation stems from an increased duration of phosphodiesterase activity. The only stage in the cascade leading to activation of the phosphodiesterase which is known to involve GTP is the

binding of GTP to the G-protein (Stryer, 1986; see Fig. 1), and both GTP-γ-S and GMP-PNP can substitute for GTP in this process (Yamanaka *et al.* 1986). Therefore the simplest way in which these analogues could prolong the light response would be by persistently activating the G-protein. If this were so, then it would indicate that hydrolysis of the nucleotide bound to the G-protein is indeed necessary to terminate its ability to activate the phosphodiesterase. Before this conclusion can be reached, however, it is necessary to consider several alternative possibilities.

Possible effects of impurities

It is difficult to exclude completely the possibility that our results are due to the impurities present in the commercial samples of these GTP analogues. However, there are several reasons to regard this as unlikely. First, qualitatively similar results were obtained with both GTP-γ-S and GMP-PNP, which are chemically quite distinct. So an impurity with similar effects would need to have been present in both of these compounds. Second, there was substantial prolongation of the response to an intense flash following the incorporation of a nominal concentration of 50 μM-GTP-γ-S. This corresponds to an actual concentration in the patch pipette of about 25 μM, as the analogue was only 51% pure (batch 2), and probably a rather lower concentration in the cytoplasm. As similar concentrations of GTP-γ-S are required when this analogue is microinjected into other preparations (see e.g. Turner, Jaffe & Fein, 1986) it is unnecessary to suppose that an impurity was responsible. Third, it seems unlikely that GDP, the major impurity in both batches of GTP-γ-S, could be responsible, as it is present at quite high concentrations in normal rods (Biernbaum & Bownds, 1985), and also because introduction of GDP had little effect on the light response (see p. 480). For these reasons we believe that the prolongation of the light response by GTP analogues is unlikely to stem from an impurity.

Possible effects of phosphoryl transfer

Although GTP-γ-S and GMP-PNP are resistant to metabolic modification, they can still to some extent act as substrates for many of the enzymes responsible for transfer of the terminal phosphate between nucleotides. So interconversions are likely to take place slowly within the cell, to form other nucleotide analogues. In particular, transfer of the terminal phosphate from GTP-γ-S to ADP by nucleoside diphosphate kinase would form ATP-γ-S (Sheu, Richard & Frey, 1979). ATP-γ-S might interfere with the quenching of photoisomerized rhodopsin by rhodopsin kinase (see Fig. 1), or with some other ATP-dependent quenching process, thereby prolonging the light response. However, this seems unlikely to be responsible for our results, as AMP-PNP, which competitively inhibits other ATP-dependent processes, has little effect on the duration of the response to an intense flash (Lamb & Matthews, 1988*a*). In contrast, incorporation of ATP-γ-S *does* result in the gradual development of response prolongation, but we believe this to be due to transfer of the sulphur-tagged terminal phosphate group to GDP to form GTP-γ-S (Lamb & Matthews, 1988*a*). Note that on the other hand transfer of the terminal phosphate from imidodiphosphate analogues, such as GMP-PNP and AMP-PNP, would not yield a hydrolysis-resistant product. Therefore the observed effects of GTP analogues seem unlikely to result from terminal phosphate transfer to ADP. However, such

phosphoryl transfer reactions may significantly limit the lifetime of nucleotide analogues in the intact rod. In the majority of our experiments the analogues were introduced via the inner segment, where the concentrations of enzymes catalysing phosphoryl transfer are high (Berger, DeVries, Carter, Schulz, Passonneau, Lowry & Ferrendelli, 1980). So it is possible that some breakdown, and therefore depletion, may have taken place before the analogues ever reached the outer segment. Phosphoryl transfer reactions may also account for the response prolonging effects of GDP-β-S, discussed below.

Effects of GTP analogues on the G-protein

For the reasons stated above, it seems probable that the prolongation of the light response which we observe was due to persistent activation of the G-protein following the binding of the analogues, and not to impurities, or metabolites. Therefore our results lead us to conclude that the hydrolysis of the terminal phosphate of the nucleotide bound to the G-protein is necessary to terminate the light response, and they suggest that hydrolysis of bound GTP *in vivo* must take place substantially faster than previous *in vitro* biochemical studies have shown (Godchaux & Zimmerman, 1979; Kühn, 1980; Baehr *et al.* 1982; Bennett, 1982; Lewis *et al.* 1984; Yamanaka *et al.* 1985; Yamazaki *et al.* 1987). The slower rates *in vitro* might result from the loss of a diffusible regulator substance, or from disruption of the two-dimensional diffusion geometry of the intradiscal space. However, the recovery of the circulating current is likely to be slightly faster than the deactivation of the transduction cascade, because of the acceleration of guanylate cyclase which takes place during illumination (Lolley & Racz, 1982; Hodgkin *et al.* 1985; Hodgkin & Nunn, 1988).

Intense flashes, resulting in 10^5–10^6 isomerizations, were required to elicit prolonged responses in the presence of these analogues. It seems probable that such intense flashes were necessary because of competition between the exogenously introduced GTP analogue and endogenous GTP for binding to the G-protein, as was suggested on pp. 469–470. The concentration of endogenous GTP is of the order of 2 mM (Biernbaum & Bownds, 1985), while the concentration of the analogue available to bind to the G-protein was probably much lower. From comparison of Fig. 5*A* and *B* it seems quite possible that in most experiments concentrations of only a few hundred micromolar were reached in the outer segment. Therefore less intense flashes probably resulted in the binding of too small a quantity of the analogue for the slowed final recovery to have been detected when superimposed on the slow decline in the circulating current.

Results consistent with those reported here have been obtained by Sather & Detwiler (1987), who observed that the response to light was prolonged and only recovered partially during the introduction of GTP-γ-S from a patch pipette into a single isolated outer segment. Recently these authors (G. Rispoli, W. A. Sather & P. B. Detwiler, personal communication) have observed prolongation of the responses to relatively dim flashes, as well as to intense flashes, following the introduction of GTP-γ-S into isolated outer segments which have been depleted of other guanosine nucleotides. Under these conditions the competition from GTP would be abolished.

The relatively slight slowing of the responses to flashes which were just-saturating under control conditions (see Figs 1 and 2) may have resulted in part from the binding of a small amount of the analogue to the G-protein. However, it seems entirely possible that these analogues may exert other, comparatively minor, effects within the cell which might slightly slow the responses to flashes that were not bright enough to cause significant analogue binding. One possibility would be a small effect on the rate of guanylate cyclase activity, an idea discussed in more detail below. It should also be noted that whole-cell recording *per se* can result in slight slowing of the bright flash response, presumably as a result of the wash-out of diffusible cytoplasmic constituents (Lamb *et al.* 1986).

The relative effectiveness of these GTP analogues in activating the G-protein has been shown *in vitro* to follow the sequence GTP-γ-S > GTP > GMP-PNP (Yamanaka *et al.* 1986). The lower effectiveness of GMP-PNP in activating the G-protein appears consistent with the generally smaller effect of this analogue in comparison with GTP-γ-S on response duration in our experiments.

The mechanism by which these prolonged responses ultimately recover is not known. It is conceivable that recovery represents slow hydrolysis of the analogue bound to the G-protein, though this appears improbable, as bound GTP-γ-S has been shown not to be hydrolysed *in vitro* on the time scale of several hours (Yamanaka *et al.* 1985). Alternatively, some other slow process may quench the activity of the G-protein, or of the phosphodiesterase itself, without the need for hydrolysis of the bound nucleotide. It is clear, however, from our results that any such process must be several orders of magnitude slower than the rapid quenching of the G-protein which normally occurs following a flash.

During whole-cell recording with a patch pipette containing 2 mM-GTP-γ-S or GMP-PNP the circulating current consistently declined. It is tempting to ascribe at least a part of this decline, which depended on the concentration of the analogue in the patch pipette, to spontaneous activation of the G-protein in darkness, either directly, or via thermal isomerization of rhodopsin. However, this is unlikely to be the sole explanation, as during this decline even saturating flashes cause little long-term current suppression. So it is possible that these analogues, or perhaps an impurity, have a slight effect elsewhere in the transduction mechanism. For example, even a relatively small inhibition of guanylate cyclase might significantly reduce the circulating current, which depends on the second or third power of the cyclic GMP concentration (Fesenko, Kolesnikov & Lyubarsky, 1985; Haynes, Kay & Yau, 1986; Zimmerman & Baylor, 1986).

Effects of GDP-β-S on the light response

The introduction of the hydrolysis- and phosphorylation-resistant GDP analogue GDP-β-S had two effects on the rod photoresponse. First, GDP-β-S rapidly reduced both the sensitivity, and the slope of the early rising phases, of the responses to dim and bright flashes. Second, it resulted in a gradually developing prolongation of the responses to intense flashes.

The reduction in the slope of the rising phase of the light response suggests that GDP-β-S decreases the coupling gain between photoisomerized rhodopsin and the G-protein in the transduction cascade (Stryer, 1986). This observation is consistent

with the inhibitory effects of GDP-β-S on G-protein activation in other preparations (Eckstein *et al.* 1979), and with its ability to maintain the G-protein in the GDP-bound form (Van Dop *et al.* 1984). GDP-β-S has been shown to reduce light sensitivity in *Limulus* ventral photoreceptors also (Fein, 1986). It seemed that quite high concentrations of GDP-β-S were necessary to reduce rod sensitivity, consistent with results in other preparations (e.g. Turner *et al.* 1986). Unfortunately, this makes it difficult to exclude the possibility that an impurity in the sample of GDP-β-S might have been responsible.

The effect of GDP-β-S on the duration of the responses to intense flashes was unexpected. As HPLC analysis of the sample of GDP-β-S showed a small peak of less than 1% which eluted with GTP-γ-S, it is possible that the response prolongation resulted from this trace impurity. However, this could not explain the slow onset of the prolongation, unless the diffusion of the impurity into the outer segment was very slow. This seems unlikely, as fluorescent dyes in the patch solution can be seen more or less uniformly throughout the rod within 2 min of patch rupture (Bodoia & Detwiler, 1985; Zimmerman, Yamanaka, Eckstein, Baylor & Stryer, 1985; E. N. Pugh Jr & W. H. Cobbs, personal communication).

An alternative possibility consistent with this slow development is that GDP-β-S may have been slowly metabolized to yield a phosphorylation-resistant analogue of GTP. For example, transfer of the terminal phosphate group from GDP-β-S to GDP by guanylate kinase (Schnetkamp & Daemen, 1981) would yield GTP-γ-S. Alternatively phosphorylation of GDP-β-S would yield GTP-β-S, one of whose isomers can bind weakly to G-protein (Yamanaka *et al.* 1986) and which is resistant to hydrolysis in other preparations (Eckstein *et al.* 1979).

Results consistent with ours regarding the effects of GDP-β-S on flash sensitivity have also been obtained by Sather & Detwiler (1987) in their isolated outer segment preparation. They did not, however, observe prolongation of the light response. A possible explanation for this difference might be that the suggested metabolic modification of GDP-β-S takes place in the inner segment, which was not present in their preparation. Alternatively, the continued presence of the patch pipette in their experiment may have resulted in the rapid loss of the metabolite.

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