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An estimate of the number of G regulatory proteins activated per excited rhodopsin in living *Limulus* ventral photoreceptors

(transduction/amplification)

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ABSTRACT **Previous work by others on *Limulus* photoreceptors has shown that application of a variety of guanine nucleotide-binding regulatory protein (G protein) activators produces discrete waves of depolarization similar to those generated by single photons, but smaller in size. We investigated whether these events might originate at a site other than the G protein. Initiation of the events did not depend on the state of the visual pigment, suggesting that the events do not originate at the pigment level. The events could be blocked by the G-protein blocker guanosine 5′-[β-thio]diphosphate (GDP[βS]) and thus do not originate after the G protein. Our results thus support the conclusion that these discrete events are due to the activation of G protein itself. Quantitative measurements indicate that the average size of these events is ≈8 times smaller than that evoked by single photons under the same conditions. Given certain reasonable assumptions, these results imply that the gain of the first stage of transduction *in vivo* is ≈8, a value considerably lower than that measured *in vitro* in vertebrate rods (gain, 100–500). Furthermore, independent evidence for a low first-stage gain in *Limulus* is derived from the observation that GDP[βS] barely affects the size of the response to single photons but greatly reduces the probability that a photon evokes a response. These results can be explained if rhodopsin normally activates only a few G proteins.**

The first stage of many signal transduction systems is an amplification process in which each active receptor molecule catalytically activates many guanine nucleotide-binding regulatory protein (G protein) molecules (1–3). The gain of this stage is determined by the rate at which G proteins are activated, which can be as high as 1000 sec^{-1} (4), and by the lifetime of the active state(s) of the receptor. The factors controlling the lifetime of the active states appear to be quite complex. Termination of the active state of rhodopsin involves multiple phosphorylation of rhodopsin and the subsequent binding of a 48-kDa protein (5–8); similar reactions appear to be involved in the inactivation of the β-adrenergic receptor (9, 10). Because inactivation is a multistep process, the receptor has multiple active states and these appear to differ in the rate at which they activate G protein (11, 12). The gain of each state of the receptor is the product of the rate at which G proteins are activated and the lifetime of that state. The overall gain of the first stage of transduction is the sum of the gain of each state.

To date, information about the gain of the first stage of transduction has come exclusively from *in vitro* measurements. The gain of the β-adrenergic receptor has been estimated to be ≈10 (13, 14) and that of vertebrate rhodopsin to be 100–500 (1, 2, 15, 16). These estimates, however, are upper limits since the measurements were done under conditions in which receptor phosphorylation was slow or absent due to dilution of kinase and the absence of added ATP. The lifetime of the active receptor is thus likely to be much longer in these *in vitro* experiments than *in vivo*. However, even if efforts were made to reconstitute all the factors controlling receptor inactivation, it would be difficult to judge the adequacy of the reconstitution without *in vivo* measurements as a guide. Thus, it would be desirable to have methods for measuring the gain *in vivo*.

In this paper, we describe physiological experiments on photoreceptors of the *Limulus* ventral eye that have allowed us to estimate the first-stage gain *in vivo*. Previous work on this and other invertebrate photoreceptors indicates that phototransduction is mediated by a G protein. Biochemical studies have shown that light increases GTPase activity (17), guanine nucleotide binding (P. Robinson and J.E.L., unpublished data), and cholera toxin labeling (18). Physiological experiments have shown that phototransduction can be blocked by G-protein blockers and turned on by G-protein activators (19–22). The responses induced by these activators consist of discrete waves, which are similar in shape but smaller in size than those induced by single photons (quantum bumps). The smaller size is expected since the first stage of gain has been bypassed; indeed, a quantitative comparison of the size of the two kinds of waves is a goal of this paper since it can yield an estimate of the number of G proteins activated per rhodopsin. Based on measurements of this kind, we estimate that about eight G proteins are activated per rhodopsin. Further independent evidence for a low value of the gain of the first stage of *Limulus* phototransduction comes from a detailed examination of the effect of blocking G protein with guanosine 5′-[β-thio]diphosphate (GDP[βS]).

METHODS

Median and ventral eyes of *Limulus poliphemus* were removed under bright white light, treated with Pronase to ease electrode impalement, and perfused with artificial seawater [Tris buffer (pH 7.8)] as described (23). In the experiments with F^-, the pH was buffered with 5 mM Hepes and set at pH 7.0 (19). In experiments on the median eye, tetrodotoxin (1 μM) was included in the seawater to eliminate small action potentials (37). The ventral photoreceptors were impaled with microelectrodes filled with 10 mM GTP[γS] or GDP[βS] (Boehringer Mannheim), 300 mM potassium aspartate/10 mM Hepes, pH 7.0, with resistances of 8–15 MΩ. Pressure injection of drugs was monitored optically with an infrared video system (21).

RESULTS

Site of Action of a Putative G-Protein Activator. Our initial experiments were designed to test the possibility that puta-

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; GDP[βS], guanosine 5′-[β-thio]diphosphate.

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tive G-protein activators might be producing large discrete events by acting on the rhodopsin photoproduct, metarhodopsin (25), rather than on G protein. This possibility seemed plausible because the ventral photoreceptors used in previous experiments contained a high concentration of thermally stable inactive metarhodopsin (25). The G-protein activators might have affected the phosphorylation reactions that keep metarhodopsin inactive. If this were the case, the G-protein activators should have no effect in the absence of metarhodopsin. UV photoreceptors (24) of the median eye of *Limulus* were used to test this possibility. In these cells, the ratio of rhodopsin to metarhodopsin can be easily controlled by chromatic adaptation because the two pigment states have very different absorption maxima [metarhodopsin, 470 nm; rhodopsin, 360 nm (26)]. Furthermore, the ratio, once set, is stable because there is no thermal regeneration of metarhodopsin to rhodopsin (26). The G-protein activator used in these experiments was F^-, because it can be applied extracellularly and because its action is reversible. At the beginning of the experiments, rhodopsin was converted into metarhodopsin by illuminating the cells with a bright UV light. After the preparation dark-adapted to the maximum extent possible without pigment regeneration, it was superfused with 10 mM F^-. This produced a gradual increase in the rate of spontaneous bumps. After removal of F^-, the bump rate declined to the control level. The preparation was then illuminated with orange light to convert metarhodopsin into rhodopsin. Under these conditions, there was virtually no metarhodopsin present, but the effect of F^- was not dramatically different from that measured in the presence of metarhodopsin (Table 1). This result shows that F^- acts at a stage of the transduction cascade subsequent to the pigment molecule.

To determine whether F^- might act at a stage subsequent to the G protein, we tested whether the G-protein blocker, GDP[βS], could block the effect of F^-, as it does in other systems (29). The experiment was done on ventral photoreceptors because drugs can be easily pressure injected into these large cells. The preparation was superfused with F^- in the dark until a substantial increase in the rate of spontaneous bumps was observed (Fig. 1 *A* and *B*). Then, GDP[βS] was pressure injected into the cell. As shown in Fig. 1*C*, the F^--induced bumps were abolished. This lack of activity was not due to cell death since the cell could still respond to light (Fig. 1 *A* and *D*), although the light required was considerably brighter than before injection (Fig. 1*A*). Taken together, these experiments on F^--induced bumps strongly support the idea that F^- acts at the level of G protein, as suggested (19).

GDP[βS] Reduces the Quantum Efficiency. Previous work (20, 22) showed that GDP[βS] injection reduces the sensitivity to light but did not establish whether this reduction occurs because of a reduction in the size of the response to single photons (as occurs during light-induced desensitization) or whether the reduction is due to a lowered probability of evoking a quantum bump (the quantum efficiency). Fig. 1 shows that after injecting GDP[βS], light still evoked large bumps, but a much higher intensity was required, suggesting that the quantum efficiency is reduced. To document this effect more precisely, voltage-clamped photoreceptors were stimulated with dim light pulses of various intensities before and after GDP[βS] injections. Fig. 2 shows how the light-induced quantum bump rate varied with the intensity before injection and after one or two injections. The bump rate was linearly proportional to the stimulus intensity before and after

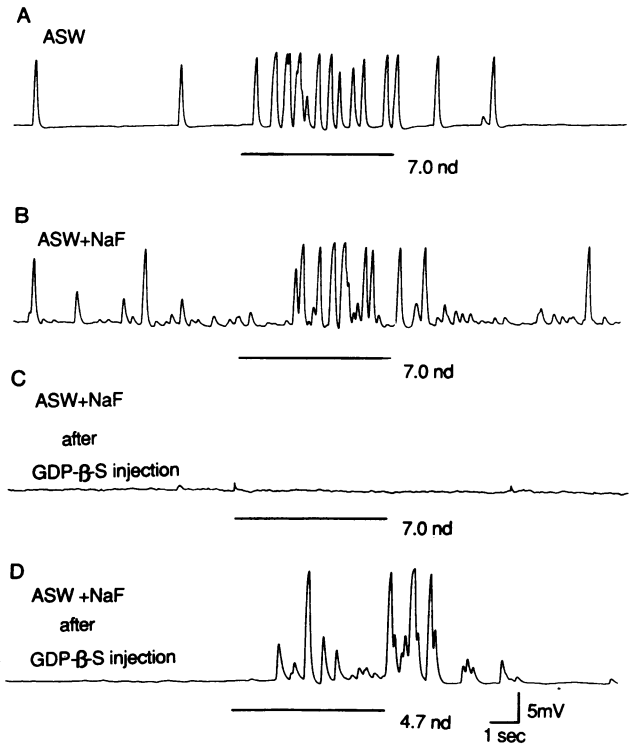

FIG. 1. Inhibition of F^--induced bumps by GDP[βS] in UV photoreceptors of *Limulus* median eye. The traces are records of the membrane potential during light (horizontal bars) and dark. (*A*) Control record. (*B*) Fifteen minutes after the start of perfusion with artificial seawater (ASW) containing 10 mM NaF. (*C*) After intracellular pressure injection of GDP[βS] in the presence of F^-. (*D*) Same as *C* but with higher light intensity. Light intensity is expressed in neutral density units (nd), indicating the log attenuation of the light beam.

Table 1. Effects of F^- on quantum bump rate in medial photoreceptors containing primarily rhodopsin or primarily metarhodopsin

	Rhodopsin		Metarhodopsin	
Cell	Spontaneous bump rate, bump·sec^{-1}	F^--induced change in bump rate, bump·sec^{-1}·min^{-1}	Spontaneous bump rate, bump·sec^{-1}	F^--induced change in bump rate, bump·sec^{-1}·min^{-1}
1	0.38 ± 0.08	0.016 ± 0.002	0.83 ± 0.17	0.024 ± 0.008
2	0.60 ± 0.20	0.112 ± 0.054	1.26 ± 0.29	0.195 ± 0.077
3	0.28 ± 0.01	0.059 ± 0.011	0.32 ± 0.07	0.039 ± 0.011
4	0.46 ± 0.06	0.019 ± 0.008	0.96 ± 0.13	0.036 ± 0.010

Application of F^- caused a linear progressive increase in quantum bump rate that never reached a steady level; the effect can thus be characterized by the increase in bump rate per unit time (Δ rate/min), where rate is number of bumps per second. In the dark there is a steady rate of discrete events similar to those evoked by single photon absorption. These discrete events, termed spontaneous quantum bumps (27), have multiple sources: spontaneous isomerization of rhodopsin (28), spontaneous reversion of inactive metarhodopsin back into an active state (26), and, possibly, spontaneous activation of subsequent components of the transduction cascade. In agreement with previous results (26), the spontaneous bump rate is usually higher when the cells contain primarily metarhodopsin, probably because of the spontaneous reversal of inactive metarhodopsin into the active state.

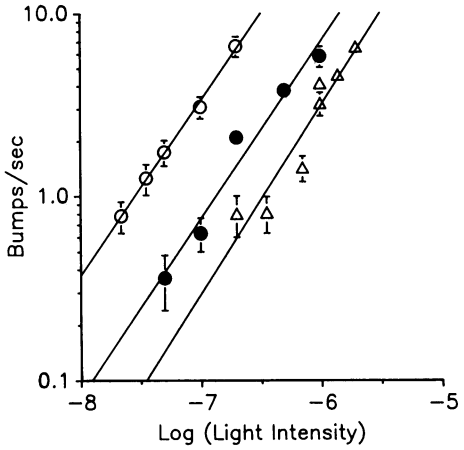

FIG. 2. Changes in quantum efficiency produced by GDP[βS] in ventral photoreceptors. The cell (cell 1 of Table 2) was voltage-clamped at resting potential (−60 mV) and injected twice with GDP[βS]. The rate of the light-induced bumps recorded before (○), after one (●), and after two (△) GDP[βS] injections is plotted vs. the stimulus intensity. The straight lines were fit to the data by eye and have a slope of 1. The vertical bars represent standard errors.

the injection; only the proportionality constant (quantum efficiency) was changed. Table 2 summarizes the effects of GDP[βS] on quantum efficiency and bump size. The results show that GDP[βS] reduced quantum efficiency by orders of magnitude but had only minor effects on the bump size (the largest reduction in bump size was to one-third of the original size). Some of the cells were given a second injection with GDP[βS]. In three cases, the quantum efficiency was further reduced, but the bump size was barely affected. In two cases, the second GDP[βS] injection produced a large further reduction in quantum efficiency (up to 10,000 times) accompanied by a modest increase in bump size. Thus, the GDP[βS] clearly acts primarily by affecting quantum efficiency, with only minor effects on bump size. These results can be accounted for on the assumption that the gain of the first step of phototransduction is low (see *Discussion*).

The Size of the GTP[γS]-Induced Bumps. Previous work has shown that G-protein activators produce bumps that are smaller than light-induced bumps (21), as would be expected since the first stage of gain has been bypassed. Given certain reasonable assumptions (see *Discussion*), the size ratio of these two kinds of bumps can be taken as a measure of the gain of the first stage of transduction. It was therefore of interest to obtain a precise value of this ratio. To make the quantitative comparison, we measured GTP[γS]-induced bumps and light-induced bumps under the same conditions—i.e., in the presence of GTP[γS]. As a measure of bump size, we used the integral of current during a bump and averaged this for many bumps. Averaging is necessary because individual bumps of the same kind vary in size due to stochastic fluctuations in the transduction cascade (30). Before injection of GTP[γS], data were collected during periods of dim illumination and during intervening periods of darkness. From the data, the average size of normal light-induced and spontaneous bumps was determined. After injection of GTP[γS], the bump rate rapidly increased, in some cases without the application of a light pulse (20). In most cases, however, it was necessary to give a single bright flash to induce an effect of GTP[γS] (20). After the flash, there was a maintained increase in the rate of bumps in the dark—i.e., a type of afterpotential. The bumps recorded in the dark after injection are a combination of true spontaneous bumps and GTP[γS]-induced bumps. The size and rate of GTP[γS]-induced bumps can be computed by subtracting out the contribution due to true spontaneous bumps as described (26). The subtractive procedure assumes that GTP[γS] does not change the size of true spontaneous bumps, an assumption supported by the observation that GTP[γS] does not change the size of light-induced bumps (see below). In any case, we restricted our analysis to cells in which GTP[γS] produced a substantial increase in the bump rate (>2-fold); thus, the correction that had applied to the contribution of spontaneous bumps was a small one.

Having measured the average size of GTP[γS]-induced bumps, we proceeded to measure the average size of light-induced bumps in the presence of GTP[γS]. The illumination required to make these measurements was itself too dim to induce a further increase in the GTP[γS]-induced bump rate. The records obtained during the light consisted of light-induced bumps, GTP[γS]-induced bumps, and spontaneous bumps. To compute the average size of the light-induced bumps, a correction was made for the contribution of spontaneous and GTP[γS]-induced bumps, which could be measured in the intervening dark periods. Recordings of bumps under different conditions are shown in Fig. 3; the average size of different kinds of bumps is given in Table 3. GTP[γS]-induced bumps were on average ≈8 times smaller than light-induced bumps (Table 3). This ratio varied from 5 to 13 in different cells.

One concern with the measurement of GTP[γS]-induced bumps is that their small size might cause many of them to go undetected; the average size of the detected bumps might thus be an overestimate of the true average size. To examine this possibility, we constructed probability distributions for the size of different types of bumps. The distribution of GTP[γS]-induced bumps could be reasonably well fit by a single exponential. The average bump size obtained by this fit can then be corrected for bumps that fall below the detection limit (31). The resulting corrected values for bump size given in parentheses in Fig. 3 and Table 3 are in reasonable

Table 2. Reduction in quantum efficiency (ΔQE) and bump size produced by GDP[βS]

	Control		After first GDP[βS] injection			After second GDP[βS] injection		
Cell	Spontaneous bump size	Light-induced bump size (S_0)	Light-induced bump size (S_1)	S_1/S_0	ΔQE	Light-induced bump size (S_2)	S_2/S_0	ΔQE
1	256.9 ± 26.0	367.6 ± 43.4	174.4 ± 19.9 (157)	0.40	2	191.4 ± 23.4 (148)	0.37	10
2	46.9 ± 14.4	71.6 ± 9.3	41.4 ± 4.9 (38)	0.63	4	49.3 ± 5.3 (42)	0.65	16
3	132.1 ± 20.0	187.9 ± 29.7	208.9 ± 15.3 (192)	1.34	500	131.1 ± 14.3 (129)	0.80	4,000
4	178.3 ± 33.3	227.5 ± 47.5	145.5 ± 16.7 (137)	0.55	50	—	—	>10,000
5	34.2 ± 3.8	48.1 ± 9.6	35.9 ± 33.5 (30)	1.05	4	—	—	>10,000
6	177.8 ± 28.2	199.8 ± 31.8	156.2 ± 12.4 (147)	0.75	500	—	—	—

The bump size was defined by the total charge flow (nA·msec) during a bump. The average size of the light-induced bumps was computed from the average size of the bumps recorded during light stimulation by subtracting the contribution of the spontaneous bumps as described (26). The size distribution of bumps recorded after GDP[βS] injection could be fit by a single exponential. In parentheses are the average bump sizes obtained by subtracting the smallest detectable bump size from the exponential constant. The average size obtained by this method is in good agreement with the size obtained by simply averaging the observed bumps, a result indicating that our measurement of size is not significantly affected by failure to detect very small bumps. —, Not measured.

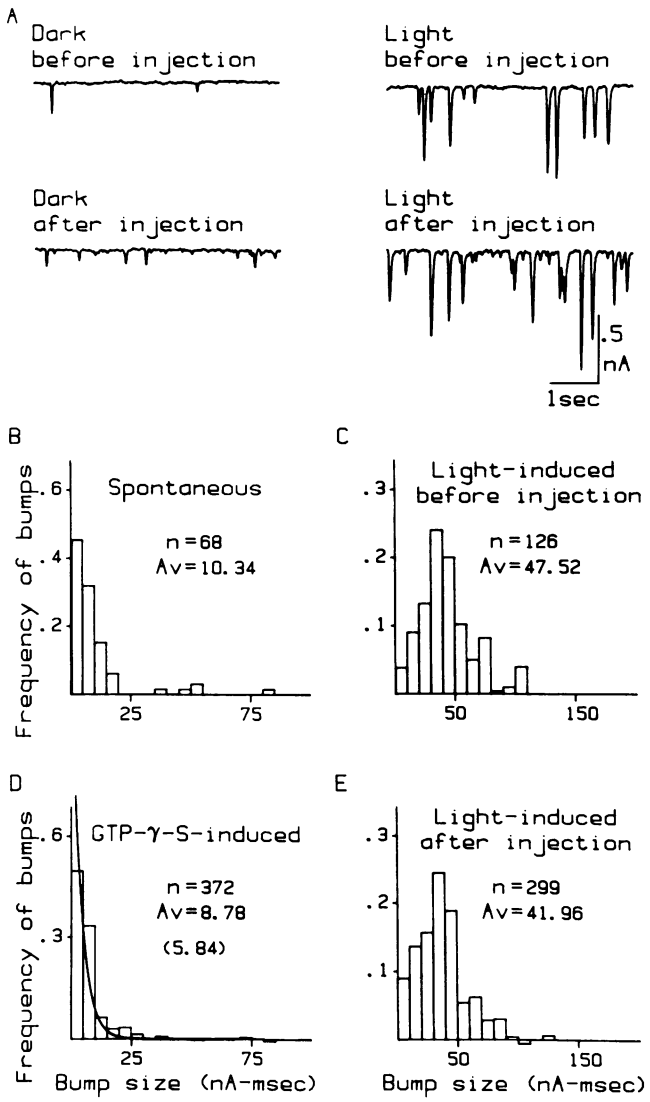

FIG. 3. Size distribution of GTP[γS]-induced bumps, light-induced bumps, and true spontaneous bumps. A ventral photoreceptor (cell 4 in Table 3) was voltage-clamped at resting potential (−65 mV) and stimulated with 10-sec pulses of dim light alternated with 20-sec dark periods. The cell was then injected with GTP[γS] and then again stimulated with light. (*A*) Current recorded under different conditions. (*B–E*) Probability distribution of bump size (in nA·msec) for true spontaneous bumps (*B*), light-induced bumps before GTP[γS] injection (*C*), GTP[γS]-induced bumps (*D*), light-induced bumps after GTP[γS] injection (*E*). Displayed in each histogram is the average size (Av) of all bumps and the number (n) of bumps used to compute the distribution. The solid line in *D* is an exponential fit by eye. The number in parentheses is the average bump size computed from the exponential decay constant after correction for undetected bumps. To obtain the distribution of the GTP[γS]-induced bumps, the scaled distribution of true spontaneous bumps was subtracted from the distribution of the bumps recorded in the dark after the GTP[γS] injection. The scaling factor was R_b/R_a, where R_b and R_a are the rates in the dark before and after the GTP[γS] injection. The resulting distribution was normalized by multiplying by $R_a/(R_a-R_b)$. The distributions of light-induced bumps were computed in a similar way by using distributions of the bumps recorded in the dark and light. The small fraction of events that were the superposition of bumps were not included in the size distribution but were considered for the computation of average size.

agreement with the values derived from measurement of the average size of the detected bumps. We thus conclude that the estimate of the size of GTP[γS]-induced bumps is not seriously affected by the presence of undetected small bumps.

DISCUSSION

Two lines of evidence presented here suggest that the gain of the first stage of invertebrate phototransduction is low. The first is the finding that the size of bumps induced by the G-protein activator GTP[γS] is ≈8 times smaller than those induced by single photons (Table 3). Given certain assumptions, this implies that during the normal response to a single photon rhodopsin activates about eight G proteins. The first assumption that we have made is that the putative G-protein activators are in fact producing the observed discrete waves by acting on G protein. We have tested this assumption by examining the alternative hypotheses that the activators are acting before or after the G protein. Our results are inconsistent with these hypotheses and support the original contention of Fein and his collaborators that the G-protein activators produce the observed waves by acting on G protein (19). The second assumption that we have made is that the bumps induced by GTP[γS] are the result of the activation of a single G protein. There is some ambiguity about this; for instance, if active G protein contains more than one α subunit, as some studies suggest (32), then it is unclear whether the bumps we observe are the result of activation of both α subunits or just one. A final assumption that we have made is that the effect of G-protein activation by GTP[γS] is similar to the activation that normally occurs when GTP binds. This assumption is supported by two lines of evidence. First, the overall gain of the transduction cascade is not affected by GTP[γS], as indicated by the observation that neither the average size of light-induced bumps nor their size distribution is affected by GTP[γS] (Fig. 3, Table 3). Second, the kinetics of bumps is not affected by GTP[γS], as indicated by lack of change of width at half height of bumps (Fig. 4). We thus conclude that our estimate of the gain of the first stage of transduction is based on reasonable assumptions.

It should be noted that if GTP hydrolysis is required for enzyme inactivation, as is widely believed, bumps ought to decay more slowly in the presence of GTP[γS], contrary to what we observe. There is, however, an alternative model of G-protein function that could account for our observations. This model, which is based on properties of vertebrate rod phosphodiesterase, posits that enzyme inactivation can occur without GTP hydrolysis (33). According to this model, activation of phosphodiesterase occurs when G protein removes an inhibitory subunit; the binding of an inhibitory subunit from a separate pool inactivates the enzyme even if the original inhibitor is still bound to the G protein.

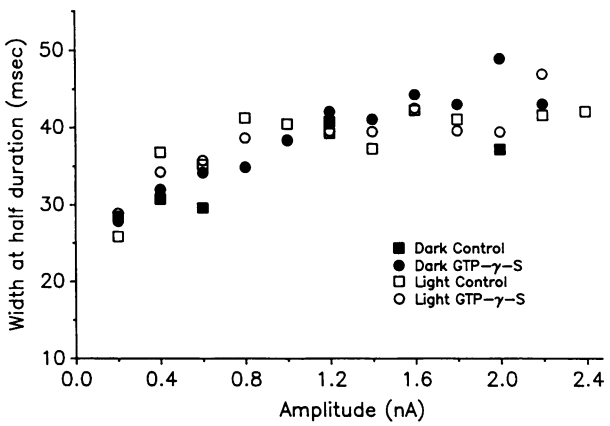

FIG. 4. Lack of effect of GTP[γS] on bump kinetics (width at half height). The width at half height was found to depend on bump amplitude; however, this relationship was not affected by injection of GTP[γS]. Cell is the same as in Fig. 3.

Table 3. Size of the spontaneous, light-induced, and GTP[γS]-induced bumps

Cell	Control: Spontaneous bump size, nA·msec	Control: Light-induced bump size, nA·msec	After GTP[γS] injection: GTP[γS]-induced bump size G_S, nA·msec	After GTP[γS] injection: Light-induced bump size (L_S), nA·msec	Size ratio, L_S/G_S
1	34.2 ± 3.3	83.3 ± 18.6	9.16 ± 1.44 (8.8)	86.8 ± 31.9	9.48 (9.83)
2	11.1 ± 0.8	49.8 ± 2.5	6.49 ± 0.60 (5.1)	58.9 ± 4.00	10.89 (11.9)
3	57.1 ± 11.3	86.9 ± 6.8	12.80 ± 7.36 (11.1)	97.3 ± 13.1	7.58 (8.8)
4	10.3 ± 1.4	47.5 ± 2.7	8.78 ± 1.32 (5.81)	42.0 ± 4.8	4.78 (7.22)
5	18.4 ± 1.3	120.3 ± 11.3	9.46 ± 1.67 (7.15)	92.20 ± 15.2	9.75 (12.9)
	Average 26.22	77.56	9.34 (7.58)	75.43	8.12 (10.2)

Numbers in parentheses are the average sizes of the GTP[γS]-induced bumps estimated from size distributions (see Table 2 for details) and the bump size ratio calculated by using these values.

The second line of evidence for a low first-stage gain in *Limulus* comes from a close examination of the effect of GDP[βS]. Our evidence clearly shows that the principal effect of GDP[βS] is to reduce quantum efficiency, although some reduction in bump size can be observed. These results are difficult to explain if the gain is high; in this case, blocking a large fraction of the G protein would lead to greatly reduced bump size with little or no change in quantum efficiency. In contrast, these results can be easily explained if the gain of the first stage of transduction were low. In this case, extensive inhibition of G protein will result in a situation in which an excited rhodopsin might fail to activate even a single G protein, thus reducing the quantum efficiency. If, however, one or more G-protein molecules were activated, the size of the resulting bump would be only moderately smaller than one by a photon in the absence of drug. If the gain of the first stage were 8, then the reduction in bump size produced by GDP[βS] should have been by a factor of 8. However, the reduction was never quite this large. This is possibly because GDP[βS] also produces secondary effects that increase bump size, especially after large injections (Table 2). Since reduction of intracellular Ca^{2+} can lead to an increase in bump size (34), one possibility is that the secondary effect of GDP[βS] is due to blocking of a tonic component of the Ca^{2+} release mechanism (35) and the consequent lowering of intracellular Ca^{2+}.

Do Different Photoreceptors Types Have Different Gains? Our estimate of 8 for the gain of the first stage of *Limulus* phototransduction is considerably smaller than the value of 100–500 estimated *in vitro* for vetebrate rod (1, 2, 15, 16). It is important to point out, however, that gain depends both on the catalytic rate at which rhodopsin activates G proteins and on the lifetime of the active state of the pigment. *In vitro*, the lifetime of the pigment is likely to be longer than *in vivo* because rhodopsin kinase and ATP, both necessary for pigment inactivation (6–8, 36), are absent or diluted in the *in vitro* assays. In fact, inclusion of physiological concentration of ATP in phosphodiesterase assays reduces the number of cGMP hydrolyzed per R^* by a factor of 10 (7). There is thus the possibility that the number of G proteins activated by vertebrate rhodopsin *in vivo* could be considerably smaller than 100–500. It would seem doubtful, however, that the gain in vertebrate rods will turn out to be as low as in *Limulus*. This is because the single photon response in rods lasts for seconds, whereas that in *Limulus* lasts for only 100 msec. Thus, the duration of the active state of rhodopsin could well be longer in rods than in *Limulus*. It is thus possible that both types of rhodopsins might activate G protein at the same rate but produce different first-stage gain merely because of differences in the lifetime of the active state(s).

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