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INJECTION OF GUANOSINE AND ADENOSINE NUCLEOTIDES INTO *LIMULUS* VENTRAL PHOTORECEPTOR CELLS

BY S. R. BOLSOVER AND J. E. BROWN

From the Department of Physiology and Biophysics, SUNY, Stony Brook, NY 11794, U.S.A. and The Marine Biological Laboratory, Woods Hole, MA 02543, U.S.A.

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SUMMARY

1. Several nucleotides and nucleotide analogues had striking effects when pressure-injected into *Limulus* ventral photoreceptor cells. The poorly hydrolysable GTP analogues guanosine 5′-0-(3-thiotriphosphate) (GTPγS), guanylyl imidodiphosphate (Gpp[NH]p) and guanylyl (β,γ methylene) diphosphonate (Gpp[CH_2]p) produced large increases in the frequency of 'discrete events' that were recorded from photoreceptors in darkness. This effect was only observed after the injected cell was exposed to light. Injection of the ATP analogue ATPγS had effects similar to those of the GTP analogues.

2. We conclude that GTPγS, Gpp[NH]p, Gpp[CH_2]p and ATPγS act at a common site to cause a light-dependent, long-term activation of the excitation mechanism of the photoreceptor.

3. Injection of GTP or GDP at pH 4·8 was followed by a smooth, transient depolarization that was observed neither when GTP at pH 7·5 was injected nor when ATP, 5′GMP or 2-[*N*-morpholino] ethane sulphonic acid (MES) were injected at pH 4·8. The reversal potential of the current induced by GTP injection was significantly more positive than the reversal potential of the light-induced current.

4. We conclude that GTP injection induces changes of membrane conductance either in addition to, or different from, the light-induced change of membrane conductance.

5. Injection of the ATP analogue adenylyl imidodiphosphate (App[NH]p), and the pyrophosphate analogue imidodiphosphate (p[NH]p) produced a drastic decrease in the sensitivity of photoreceptors to light. This decrease in sensitivity was partially reversed when the concentration of calcium ions in the bathing medium was reduced.

6. We suggest that App[NH]p and p[NH]p injections act by increasing the cytoplasmic concentration of calcium ions.

INTRODUCTION

Illumination of a photoreceptor cell in the ventral eye of *Limulus* elicits a positive-going change of membrane voltage called the 'receptor potential'. This receptor potential is due to an inward current (normally carried mostly by sodium ions)

resulting from a light-induced increase in the conductance of the cell membrane (Millecchia & Mauro, 1969*a*, *b*; Brown & Mote, 1974). In a *Limulus* photoreceptor cell a very dim light elicits a receptor potential composed of a series of discrete, transient depolarizations, called 'discrete events' or 'quantum bumps', that can be 10 mV or more in amplitude and about 200 ms in duration (Millecchia & Mauro, 1969*a*). It has been suggested that each discrete event arises from a change of membrane conductance induced by the absorption of one photon (Fuortes & Yeandle, 1964; Yeandle & Spiegler, 1973).

The light-induced change of membrane conductance can be interpreted as the culmination of a sequence of events triggered by light (the 'excitation sequence'). The primary event in the excitation sequence is the absorption of a photon by a rhodopsin molecule. The photochemistry of rhodopsin has been studied in detail in some invertebrates (see Goldsmith, 1972) including *Limulus* (Hubbard & Wald, 1960). However, the steps in the excitation sequence that link the photochemistry of rhodopsin to the change of membrane conductance are poorly understood.

Illumination of a photoreceptor induces a delayed reduction in the sensitivity of the photoreceptor (light-adaptation). For example, after the onset of steady illumination the change of membrane conductance declines from an early peak value to a maintained plateau; that is, the ratio of conductance change to irradiance declines (Lisman & Brown, 1975). Light-adaptation can also be interpreted as being the culmination of a sequence of events that begins with absorption of photons by rhodopsin and ends in the reduction of sensitivity. One part of this 'adaptation sequence' has been identified tentatively. Illumination causes a rise in the intracellular concentration of calcium ions; the calcium ions act as an intracellular messenger, mediating the reduction of the sensitivity (see Brown, 1977). However, both the steps that link the photochemistry of rhodopsin to the light-induced increase in the intracellular concentration of calcium ions and the steps by which calcium ions act to reduce the membrane conductance are poorly understood.

Adenosine or guanosine nucleotides have been implicated as intracellular messengers, or modulators of the metabolism of intracellular messengers, in several systems (see, for instance, Johnson, Kaslow, Farfel & Bourne, 1980). Previous studies have suggested that these nucleotides might be involved in excitation in *Limulus* photoreceptors (Bolsover & Brown, 1980; Corson & Fein, 1980; Fein & Corson, 1981). We have investigated the participation of adenosine and guanosine nucleotides in the sequences of steps between absorption of light by rhodopsin and changes of membrane conductances in *Limulus* ventral photoreceptors. The method employed was to pressure-inject naturally occurring nucleotides and their analogues into photoreceptor cells and to monitor the effects of the injection on the electrical behaviour of the cells. An abstract of part of this work as been published previously (Bolsover & Brown, 1980).

METHODS

The ventral rudimentary eye of *Limulus polyphemus* was desheathed, pinned into a silicon rubber (Sylgard 184, Dow-Corning, Midland, MI) dish and bathed with 20 mg/ml Pronase (grade B, Calbiochem, La Jolla, CA) in artificial sea water for 1–2 min. Thereafter the nerve was bathed in artificial sea water (ASW) composed of (mM): NaCl, 422; KCl, 10; $MgCl_2$, 22; $MgSO_4$, 26; $CaCl_2$, 10

and Tris Cl, 10, pH 7·8. In 0-calcium sea water and 0·5 mM-calcium sea water, $MgCl_2$ replaced $CaCl_2$. Single photoreceptor cells that appeared isolated from neighbouring photoreceptors were penetrated with a micropipette containing the solution to be injected. The compounds injected were: ATP, 5′AMP, cyclic AMP, pyrophosphate, adenosine 5′ monophosphoramidate (Ap[NH_2]) and 2-[*N*-morpholino] ethane sulphonic acid (MES) purchased from Sigma Chemical Co. (St. Louis, MO); adenylyl imidodiphosphate (App[NH]p), adenylyl (β,γ methylene) diphosphonate (App[CH_2]p), GTP, GDP, guanosine 5′-0-[3-thiotriphosphate] (GTPγS), guanylyl imidodiphosphate (Gpp[NH]p), guanylyl (β,γ methylene) diphosphonate (Gpp[CH_2]p), guanosine 5′-0-(2-thiodiphosphate) (GDPβS), and imidodiphosphate (p[NH]p) purchased from Boehringer Mannheim (Indianapolis, IN); GTP purchased from P-L Biochemicals (Milwaukee, WI). If the pH of the injection solution was outside the range 7–8, the pH was adjusted to 7·5 by adding KOH or HCl, except where indicated. Injections were made by applying pressure (2–80 Lb./sq. in) to the back of the micropipette. In most cases, the injection solution contained the dye Fast Green FCF at 9 mM. This allowed injections to be monitored visually; after successful injection the cells appeared green. In voltage-clamp experiments the cells were penetrated with a second micropipette filled with 2 M-KCl. The KCl pipette was used to pass current into the cell and the pipette containing the nucleotide analogue was used to record the membrane voltage. However, the KCl pipette was used to measure membrane voltage and the pipette containing the drug was used to pass current in the experiments with naturally occurring guanosine or adenine nucleotides. The bath was maintained at virtual ground by a current-to-voltage converter. The nerve was illuminated with light from a tungsten-iodide lamp held in an enclosed lamp housing; the onset of the stimulus beam was controlled by an electromechanical shutter (Vincent Assoc., Rochester, NY). The light passed through an I.R. absorbing filter (KG-1, Shott, Duryea, PA) and calibrated neutral density filters. Approximately 2×10^{10} photons per second absorbed by the cell corresponded to 1 W cm^{-2} on the preparation. The recording setup was enclosed in a five-sided metal box, with the front covered by opaque cloth curtains to reduce stray light. During recordings, all room lights (except for a few red indicator lamps on the electronics) were turned off. Where applicable, results are stated as the mean $\pm$ S.E. of mean.

RESULTS

Poorly hydrolysable GTP analogues

GTPγS, Gpp[NH]p and Gpp[CH_2]p, all of which are 'poorly hydrolysable' analogues of GTP (see, for instance, Pfeuffer & Helmreich, 1975) were injected into *Limulus* ventral photoreceptor cells. A striking effect of these injections was a marked increase in the frequency at which the cells produced 'discrete events' in the dark (Fein & Corson, 1981).

GTPγS

Fig. 1 illustrates the effect of the intracellular injection of a small amount of GTPγS. The frequency of discrete events was very low in the dark before injection. When the cell was illuminated with a dim light the frequency of discrete events greatly increased. Injection of a solution of GTPγS produced no change in the behaviour of the cell for up to 45 min after the injection as long as the cell was maintained in darkness. However, when an injected cell was illuminated and then returned to darkness, the frequency of discrete events observed with the cell in darkness was greatly increased and remained high for at least 2 h. In all experiments in which GTPγS was injected while the cell was in darkness, illumination of the cell was required before the frequency of discrete events became higher ($n = 6$). In Fig. 1 the discrete events that are induced by GTPγS appear to be significantly smaller than the discrete events induced by light. Such a disparity in amplitude between the (smaller) discrete events induced by drug and the (larger) discrete events induced by

light was observed for most, but not all, cells injected with GTPγS, Gpp[NH]p and Gpp[CH_2]P.

We assayed changes in the sensitivity of cells to light after small injections of GTPγS as follows. A cell was penetrated with two micropipettes. After 10 min in the dark the cell was voltage clamped and the stimulus–response curve was determined. After a small injection of GTPγS, the cell was illuminated with a bright light, then allowed to dark-adapt for 10 min. The frequency of discrete events in the dark became

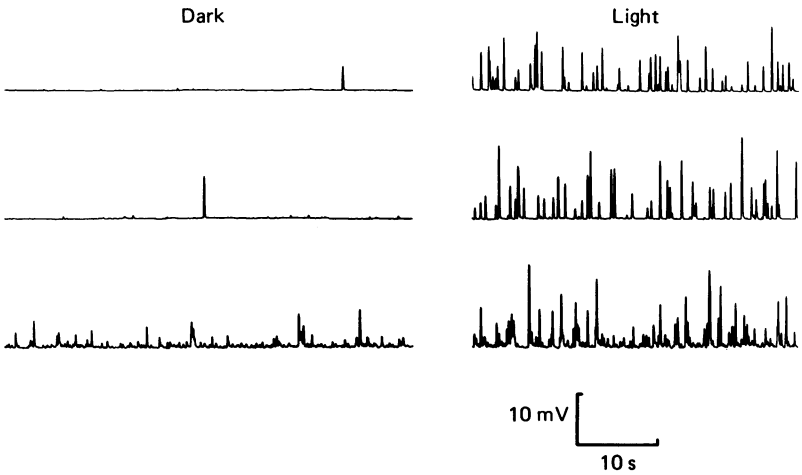

Fig. 1. Effect of an intracellular injection of GTPγS. Top: recordings of membrane potential in the dark and in a dim light ($2{\cdot}7 \times 10^{-11}$ W/cm^2) from a cell before injection of GTPγS. Middle: membrane potential in the dark and in a dim light 45 min after injection of GTPγS. The injection solution contained 0·5 mM-GTPγS, 1·8 mM-Fast Green FCF and 160 mM-KCl. The injection was made with the cell in darkness. Bottom: membrane potential 10 min after the cell was illuminated with a bright light (fifty flashes, each 30 ms long, delivered at 1 s intervals. Intensity: 26 μW/cm^2).

very high. The stimulus–response curve was determined again. The curve obtained after injection was similar to the control curve (Fig. 2).

Injections of much larger amounts of GTPγS than that necessary to increase the frequency of discrete events in the dark caused significant reductions in the sensitivity of the cells to light (Fig. 2 and Table 1). No discrete events were observed either in the dark or in dim light in such cells.

An increase of the intracellular calcium ion concentration is known to cause reduction in the sensitivity of *Limulus* photoreceptor cells to light (Lisman & Brown, 1972). Both the rise in intracellular calcium and the fall in sensitivity that follow bright illumination can be reduced by bathing the cell in saline of low calcium concentration (Brown & Blinks, 1974; Lisman, 1976). However, the sensitivity of cells injected with large amounts of GTPγS did not recover even when the extracellular calcium concentration was reduced; in many cases, the sensitivity fell further when the injected cell was bathed in sea water that contained 0·5 mM-calcium (Fig. 2 and Table 1).

Light-adaptation affects similarly the discrete events that are elicited in the dark by a small injection of GTPγS and those elicited by dim light (Fig. 3). The amplitudes

of discrete events elicited by a dim light before injection of GTPγS were attenuated by a short bright flash and recovered over 5–10 min. Similarly, the amplitudes of discrete events recorded after injection of GTPγS in the dark were attenuated by the same short bright flash and recovered with a comparable time course.

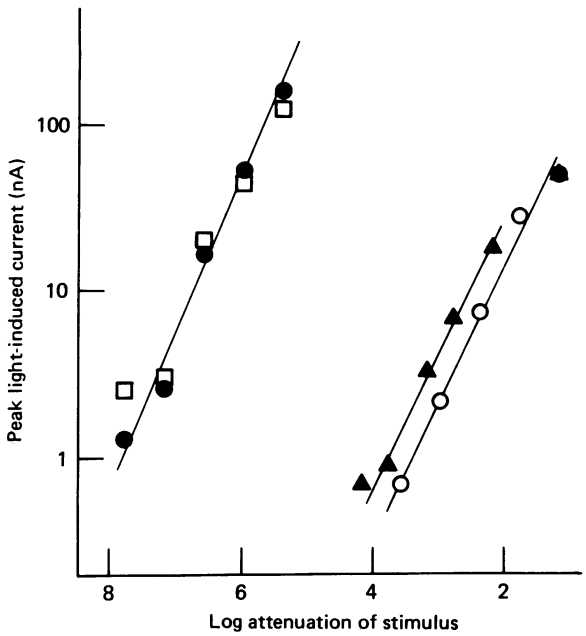

Fig. 2. Stimulus–response curves before and after injection of GTPγS. The cell was voltage clamped to its resting potential and stimulus–response curves were determined by presenting a series of flashes (4 s duration every 30 s) that had progressively increasing intensity. All stimulus–response curves were measured after 10 min dark-adaptation. Unattenuated stimulus intensity: $5{\cdot}6 \times 10^{-2}$ W/cm^2. ●, control, before injection of GTPγS. The cell was bathed in ASW. □, after a small injection of GTPγS, the frequency of discrete events in the dark was very high. The injection solution contained 2·5 mM-GTPγS, 9 mM-Fast Green FCF and 140 mM-KCl. The cell was bathed in ASW. ▲, 10 min after a larger injection of GTPγS. The cell was bathed in ASW. ○, 30 min after the larger injection of GTPγS, 10 min after changing to 0·5 mM-calcium sea water.

Gpp[NH]p and Gpp[CH$_2$]p

An injection of guanylyl imidodiphosphate (Gpp[NH]p) greatly increased the frequency of discrete events recorded with the cell in darkness ($n = 8$); however, this increase was only seen after the injected cell was illuminated. The reversal potential of the discrete events elicited by Gpp[NH]p injection was very close to the reversal potential of the light-induced current (Fig. 4). For three cells, the difference between the reversal potential of the drug-induced bumps and the reversal potential of the light-induced current was 3, 0 and 4 mV. Injections of Gpp[NH]p that caused a significant increase in the frequency of discrete events in the dark did not significantly change the sensitivity of the cells to light (Table 1). The effect of larger injections of Gpp[NH]p was not studied.

TABLE 1. Change in sensitivity of voltage-clamped cells, in log units, after pressure-injection of solution from an intracellular pipette

Compound injected	Change in sensitivity		
	Before injection: in ASW After injection: in ASW	Before injection: in low Ca^{2+} saline After injection: in low Ca^{2+} saline	Before injection: in ASW After injection: in low Ca^{2+} saline
No injection	—	—	$+0{\cdot}4 \pm 0{\cdot}1$, $n = 8$
Fast Green (9 mM)	$-0{\cdot}4 \pm 0{\cdot}3$, $n = 9$	—	—
GTPγS (50 μM–2·5 mM); small injections	$-0{\cdot}1 \pm 0{\cdot}1$ $n = 3$	—	—
GTPγS (2·5 mM–5 mM); larger injections	$-3{\cdot}5 \pm 0{\cdot}4$ $n = 6$	—	$-4{\cdot}9 \pm 0{\cdot}4$ $n = 5$
Gpp[NH]p (25 mM–50 mM)	one cell: 0·0	—	—
Gpp[NH]p (25 mM–50 mM)	$(+0{\cdot}2 \pm 0{\cdot}1, n = 6)$	—	—
Gpp[CH_2]p (25 mM)	$(-0{\cdot}4 \pm 0{\cdot}1, n = 7)$	—	—
GDPβS (5 mM); small injections	$(-0{\cdot}3 \pm 0{\cdot}2, n = 3)$	—	—
GDPβS (25 mM); larger injections	$-3{\cdot}2 \pm 0{\cdot}3$, $n = 7$	—	$-3{\cdot}0 \pm 0{\cdot}4$, $n = 5$
ATPγS (5 mM–10 mM); small injections	$(-0{\cdot}1 \pm 0{\cdot}1, n = 5)$	—	—
ATPγS (25 mM); larger injections	$-3{\cdot}9 \pm 0{\cdot}4$, $n = 6$	—	$-4{\cdot}4 \pm 0{\cdot}9$, $n = 6$
App[NH]p, Boehringer (25 mM)	$-5{\cdot}0 \pm 0{\cdot}3$, $n = 6$	$-1{\cdot}5 \pm 0{\cdot}1$, $n = 5$	$-1{\cdot}1 \pm 0{\cdot}1$, $n = 6$
App[NH]p, purified (25 mM)	$-4{\cdot}2 \pm 0{\cdot}3$, $n = 4$	—	$-2{\cdot}0 \pm 0{\cdot}4$, $n = 4$
p[NH]p (2·5 mM–25 mM)	$-4{\cdot}5 \pm 0{\cdot}2$, $n = 7$	two cells: $-2{\cdot}4$, $-1{\cdot}2$	$-1{\cdot}0 \pm 0{\cdot}1$, $n = 7$

The change in sensitivity was measured as the displacement of the linear portion of the stimulus–response relation along the stimulus irradiance axis (measured as in Fig. 2). A negative value of the sensitivity change indicates a displacement of the stimulus–response relation to higher light intensities. Figures in brackets are the shift in the stimulus–response relations of cells that were not voltage-clamped estimated from the changes in the amplitudes of receptor potentials elicited by dim lights. The classification of injections into small and large is a semi-quantitative one based on the length of time pressure was applied to the back of the pipette and on the concentration of drug in the pipette. All injection pipettes contained 9 mM-Fast Green FCF. For each pipette, the minimum pressure necessary to inject dye solution was found; that pressure was applied for variable times to vary the total amount of solution injected. After a small injection (< 2 s) cells appeared either only faintly green or no green colour was visible. After a large injection (> 20 s) cells appeared distinctly green.

Similarly, injection of (Gpp[CH_2]p) into cells ($n = 7$) significantly increased the frequency of discrete events recorded in the dark. The increase was observed only after the injected cell was illuminated.

GDPβS

Poorly hydrolysable analogues of GTP are irreversible activators of vertebrate hormone-sensitive adenylate cyclase (see for instance Pfeuffer & Helmreich, 1975). In some systems, the GDP analogue GDPβS is an inhibitor of hormone-sensitive adenylate cyclase (Eckstein, Cassel, Levkovitz, Lowe & Selinger, 1979). We therefore investigated the effect of injecting GDPβS into *Limulus* ventral photoreceptors. Small injections of GDPβS were without effect on the frequency of discrete events in the dark. Larger injections depressed the sensitivity of cells to light (Table 1).

Reducing the extracellular calcium concentration to 0·5 mM did not affect further the sensitivity of injected cells (Table 1).

Possible metabolic products of GTPγS, Gpp[NH]p and Gpp[CH_2]p

We examined the possibility that products of the metabolism of GTPγS, Gpp[NH]p and Gpp[CH_2]p were responsible for their physiological effects by injecting possible metabolic products into photoreceptors and observing the frequency of discrete events in the dark. Injections of 5′GMP, 3′:5′ cyclic GMP, GTP, GDP or methylene

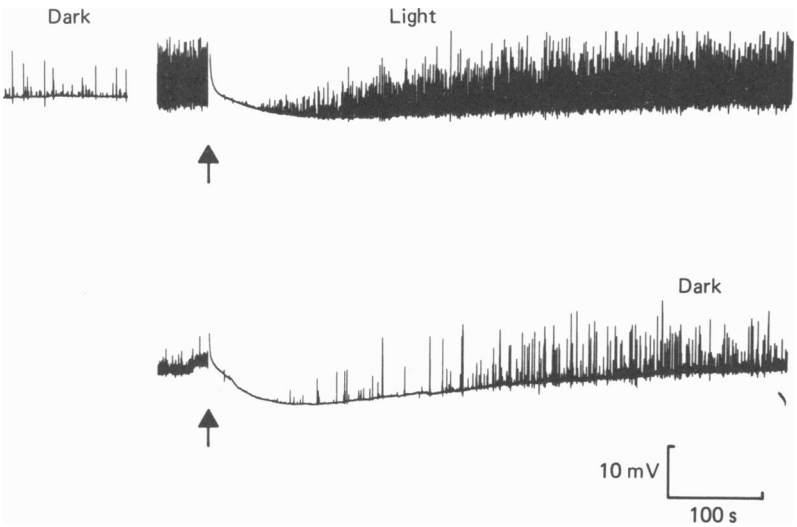

Fig. 3. Light-adaptation of discrete events induced by GTPγS. Top: membrane potential of a photoreceptor in darkness and in a constant dim light ($4{\cdot}2 \times 10^{-13}$ W/cm^2) before injection of GTPγS. Bottom: membrane potential in darkness after injection of GTPγS. The injection solution contained 2·5 mM-GTPγS, 9 mM-Fast Green FCF and 150 mM-KCl. Bright flashes, 40 ms duration, 1·3 mW/cm^2, were delivered at times indicated by the arrows on the top and bottom traces. During the responses to the bright flashes, the voltage traces were off-scale. The amplitudes of the discrete events were attenuated after the adapting flashes and recovered progressively.

diphosphonate were without effect on the frequency of discrete events recorded in the dark, and did not significantly affect the sensitivity of the cells to light. The desensitizing effect of imidodiphosphate, a possible breakdown product of Gpp[NH]p, is described later in this paper. Imidodiphosphate was never observed to increase the frequency of discrete events recorded in the dark.

GTP injection

Since injection of analogues of GTP and GDP had striking effects on the physiology of the ventral photoreceptor of *Limulus*, we examined the effect of pressure injection of GTP and GDP themselves. The GTP (or GDP), mixed with 9 mM-Fast Green FCF, was injected under visual inspection while the cell was illuminated with a relatively bright light. After 5–10 min in the dark, the membrane voltage in the dark was compared with that recorded before injection. Using this paradigm, the naturally

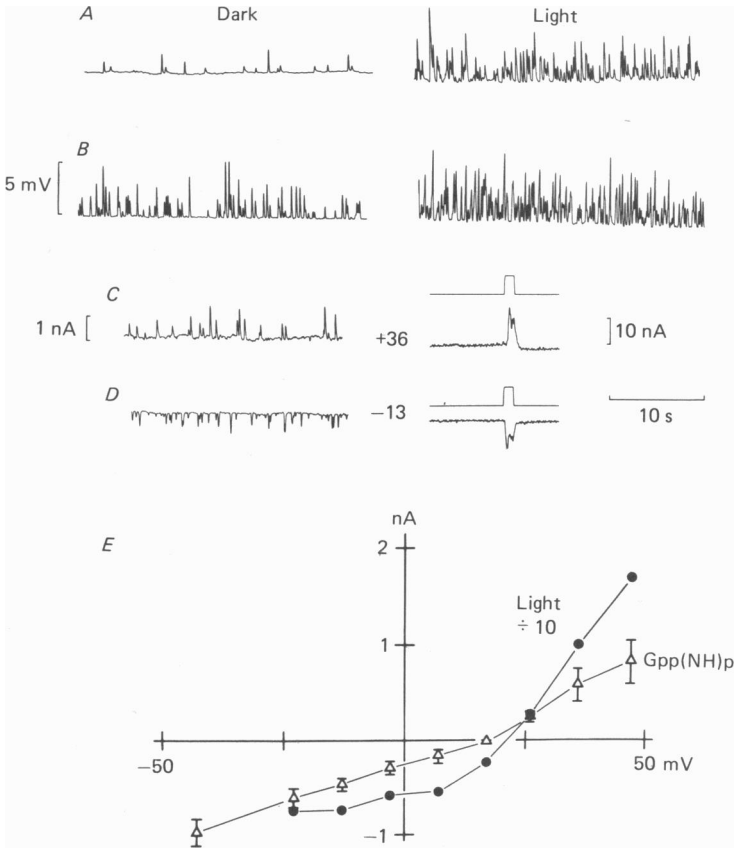

Fig. 4. Reversal potential of discrete events induced by Gpp[NH]p. *A*, membrane potential in the dark and in a dim light ($3{\cdot}8 \times 10^{-12}$ W/cm²) before injection of Gpp[NH]p. The cell was bathed in ASW. *B*, membrane potential after injection of Gpp[NH]p and after further illumination. The cell was bathed in ASW. The injection solution contained 25 mM-Gpp[NH]p, 9 mM-Fast Green FCF and 130 mM-KCl. *C*, the cell was voltage clamped at a membrane potential of +36 mV while bathed in 0·5 mM-calcium sea water. The 0·5 mM-calcium sea water was used to minimize the desensitization that occurs when cells are depolarized (Lisman & Brown, 1972). Left hand side: membrane current in the dark. Right hand side: current induced by a dim stimulus flash ($9{\cdot}5 \times 10^{-10}$ W/cm²). *D*, Same as *C* except the cell was voltage clamped at −13 mV. *E*, amplitudes of discrete events in the dark induced by injection of Gpp[NH]p and light-induced current plotted against membrane potential, both recorded under voltage clamp. The cell was bathed in 0·5 mM-calcium sea water. △, amplitude (mean ± twice the S.E. of mean) of the twenty largest discrete events in a 20 s period in the dark. ●, peak amplitude of light-induced current. Stimulus flash: $9{\cdot}5 \times 10^{-10}$ W/cm². The light-induced current values are reduced tenfold to fit on the graph.

occurring nucleotides GTP and GDP were found to be without effect on the frequency of discrete events in the dark. However when a cell was injected with a solution of GTP at pH 4·8, the cell depolarized smoothly (except sometimes the depolarization had an early rapid spike-like event (Millecchia & Mauro, 1969*a*)), and reached a maximum depolarization about 15 s after the injection (Fig. 5*A*). The membrane voltage then returned slowly to its original value. The depolarization was observed

if the cell was injected either in the dark or during repetitive light flashes. The light responses elicited by repetitive flashes were attenuated during the GTP-induced depolarization and recovered while the membrane voltage recovered. It should be noted that too large an injection of any solution can produce a depolarization due

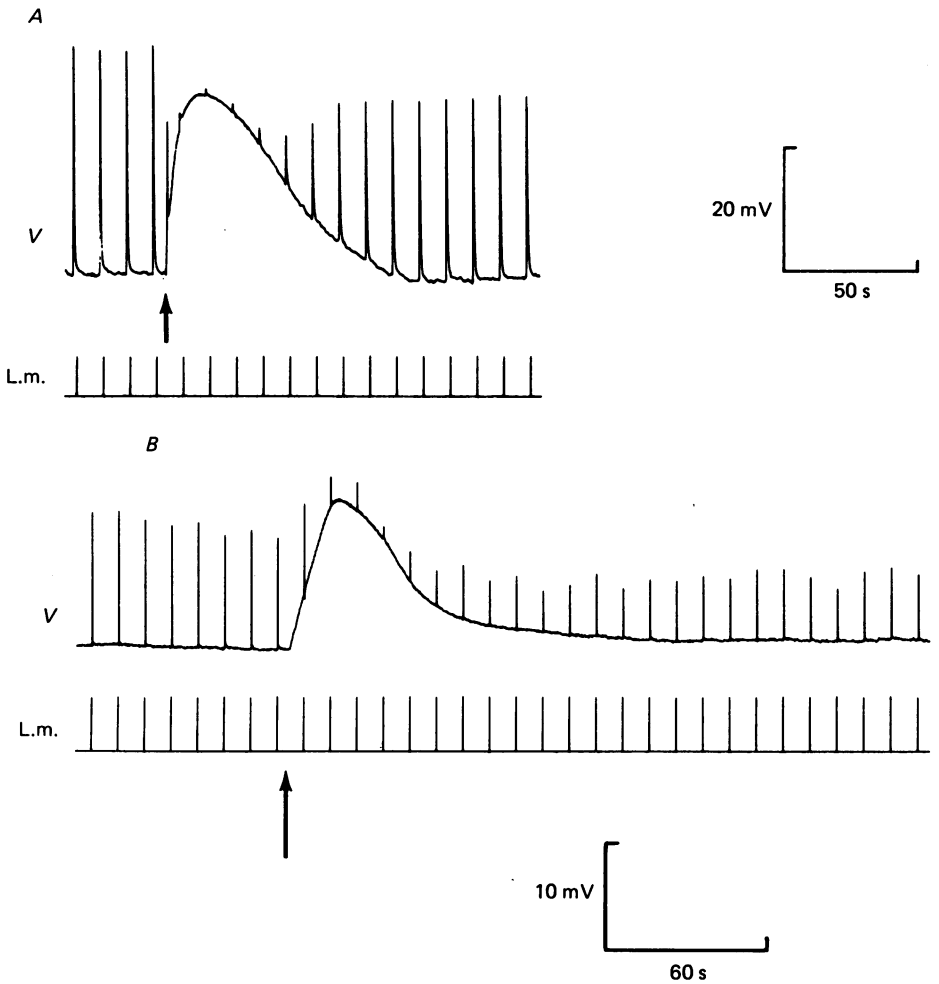

Fig. 5. Effects of intracellular injections of GTP or GDP. *V*, membrane voltage. L.m., monitor of stimulus light. *A*, cell illuminated for 40 ms every 10 s at $8{\cdot}5 \times 10^{-7}$ W/cm^2. At the arrow, a solution of GTP (100 mM at pH 4·8) was pressure-injected. *B*, different cell. Cell illuminated for 40 ms every 10 s at $2{\cdot}1 \times 10^{-6}$ W/cm^2. At the arrow, a solution of GDP (100 mM at pH 4·8) was pressure-injected.

to membrane damage. For instance, injection of the pH buffer MES at 100 mM, pH 4·8, produced only a long-lasting depolarization of rapid onset that we interpret to be due to damage caused by the injection (eleven cells). Similarly, injection of solutions of GTP at pH 7·5 (25 mM-GTP, six cells; 100 mM-GTP, seven cells), 100 mM-ATP at pH 4·8 (ten cells) or 25 mM-5′ GMP at pH 4·8 (ten cells) produced only damage phenomena, never the smooth transient depolarization seen with GTP

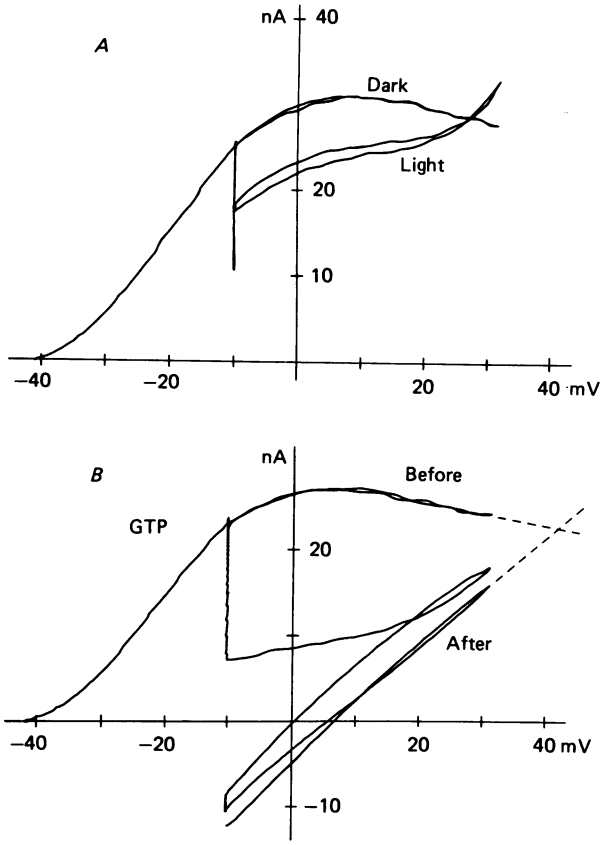

Fig. 6. Reversal voltage of GTP-induced current. *A*, current–voltage relation of a voltage-clamped photoreceptor in darkness and in a steady light. The membrane potential was slowly changed from resting voltage to −10 mV in darkness; then the command voltage was ramped to +32 mV and back to −10 mV in a total of 5 s. A steady light ($6{\cdot}0 \times 10^{-5}$ W/cm^2) was turned on and the voltage again ramped to +32 mV and back. The current–voltage curves cross at the reversal voltage for the steady-state light-induced current (28 mV). *B*, current–voltage relation in darkness before and after injection of a solution of GTP at pH 4·8. The membrane potential was slowly changed from resting voltage to −10 mV; then the command voltage was ramped to +32 mV and back to −10 mV. An injection of a solution of 35 mM-GTP at pH 4·8 was made. 12 s later the voltage was ramped to +32 mV and back. 28 s after the injection the voltage was again ramped to +32 mV and back. The extrapolated current–voltage curves cross at the reversal voltage for the GTP-induced current (~ +42 mV).

or GDP at pH 4·8. Smooth, transient depolarizations such as that shown in Fig. 5 were observed only after injection of GTP at pH 4·8 (in ten out of sixteen cells: five out of eight for 35 mM-GTP; five out of eight for 100 mM-GTP) and 100 mM-GDP at pH 4·8 (in four out of nine cells, Fig. 5*B*). In the remainder of the cells injection of GTP or GDP at pH 4·8 produced the damage phenomenon.

We compared the reversal voltage of the light-induced current with the reversal voltage of the current induced by injection of GTP at pH 4·8. In order to determine the reversal voltage of the currents induced by light, we compared the current–voltage

curve constructed for a dark-adapted cell with that constructed during steady illumination. Fig. 6 illustrates such an experiment. The cell was voltage clamped to its resting potential in the dark and then slowly depolarized to −10 mV. In order to construct a current–voltage curve in the dark (Fig. 6), the membrane voltage was swept to +33 mV and back to −10 mV (using a triangular ramp with a 5 s period). The voltage was then maintained at −10 mV and the light was turned on. The total current through the cell membrane declined, indicating that light had induced a net inward current. The voltage was again swept to +33 mV and back to construct the current–voltage curve. The current induced by light is represented by the difference between the current–voltage curves measured before and after illumination. The curves cross at the reversal voltage (+26 mV) where the light-induced current is zero.

TABLE 2. Reversal voltage of the current induced by injection of GTP at pH 4·8 compared with the reversal voltage of the light-induced current. In each experiment the membrane voltage was found to be significantly depolarized and then smoothly returned to rest when the voltage clamp was turned off after GTP injection

	Reversal voltage (mV)	
Cell no.	GTP-induced current	Light-induced current
1	+42*	+26
2	+23	+17
3	+55	+30
4	+55	+23

* By extrapolation (see Fig. 6).

In order to measure the reversal voltage for the current induced by injection of GTP at pH 4·8, we used a similar protocol. For the same cell we first constructed the current–voltage curve in the dark as described above. The voltage was then maintained at −10 mV and a solution of GTP at pH 4·8 was pressure injected. The injection induced a net inward current (Fig. 6*B*). The current–voltage curve was then constructed twice again by repetitively sweeping the voltage between −10 mV and +33 mV. The current–voltage curves constructed before and after injection of GTP at pH 4·8 did not cross, indicating that the GTP-induced current was inward over this entire voltage range. When the voltage clamp was turned off the membrane potential was found to be depolarized (to +5 mV); it smoothly returned towards its original value in the dark; we interpret this to indicate that the cell had not been damaged by the injection.

Table 2 presents the results for the four cells which satisfied our criteria for not having been injured by the pressure-injection of GTP (i.e. when the clamp was turned off after a GTP injection the membrane potential was significantly depolarized and then smoothly returned to rest). In all cells, the reversal voltage of the current induced by GTP injection was significantly different from the reversal voltage of the light-induced current ($P = 5\%$, two-tailed).

App[NH]p and p[NH]p

The sensitivity to light of *Limulus* ventral photoreceptor cells bathed in ASW became greatly reduced over a period of 10–20 min after the intracellular pressure-

injection of the poorly hydrolysable analogue of ATP, App[NH]p; however, much of the sensitivity was restored when the injected cells were bathed in 0-calcium sea water. The findings are illustrated in Fig. 7. Stimulus–response curves measured after injection of App[NH]p were shifted to higher light intensities relative to their respective controls. However, the shift of the stimulus–response curve measured in

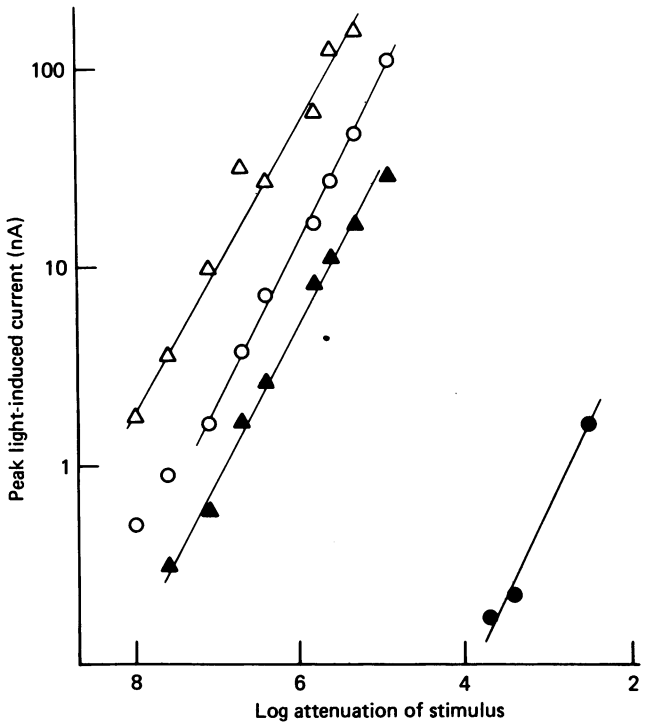

Fig. 7. Effects of App[NH]p injection on the sensitivity of a *Limulus* ventral photoreceptor. The cell was voltage-clamped to its resting potential and a stimulus–response curve was determined by presenting a series of light flashes (4 s duration, once each 30 s) that had progressively increasing intensity. Each stimulus–response curve was determined after 10 min of dark-adaptation. ○, control, before injection of App[NH]p. The cell was bathed in ASW. △, control, before injection of App[NH]p; 15 min after changing to 0 calcium sea water. ●, 13 min after injection of App[NH]p; 21 min after changing to ASW. The injection solution contained 25 mM-App[NH]p plus 9 mM-Fast Green FCF. During the injection the cell was viewed in a bright light (log attenuation 4·4). ▲, 33 min after injection of App[NH]p; 16 min after changing to 0 calcium sea water. Reduction of extracellular Ca^{2+} largely restored the sensitivity. Unattenuated stimulus: 0·16 W/cm^2.

normal ASW was considerably greater than the shift of the curve measured in 0-calcium sea water (Table 1). A large reduction in the sensitivity of cells to light could also be produced by injecting imidodiphosphate, p[NH]p, as illustrated in Fig. 8 and Table 1. The sensitivity of cells to light fell progressively during the twenty minutes after injection of p[NH]p. Such a progressive fall in sensitivity was often seen after injection of either p[NH]p or App[NH]p.

Because the effects of injecting p[NH]p were similar to those produced by App[NH]p and because the commercial samples of App[NH]p possibly were impure,

we purified App[NH]p by the procedure of Clark (1978). App[NH]p was loaded on a DEAE-52 column and eluted with 0·12 M-ammonium bicarbonate buffer. Fractions having maximum optical density at 250 nm were pooled and lyophilized. Injection of the purified App[NH]p produced effects nearly identical to those reported above for the unpurified App[NH]p (Table 1).

Intracellular injection of the methylene analogues of App[NH]p and p[NH]p,

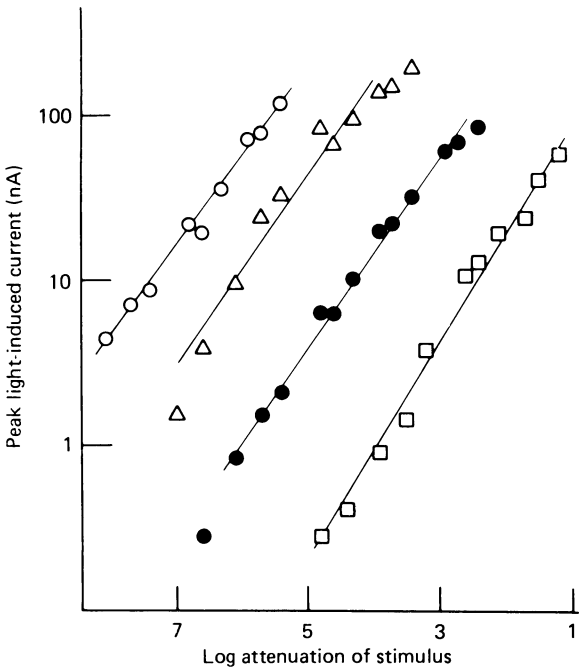

Fig. 8. Effects of p[NH]p injection on the sensitivity of a *Limulus* ventral photoreceptor. The cell was voltage clamped to its resting potential and stimulus–response curves were determined by presenting a series of light flashes (4 s duration every 30 s) that had progressively increasing intensity. Each stimulus–response curve was determined after 10 min of dark-adaptation. ○, control, before injection of p[NH]p. The cell was bathed in ASW. ●, 10 min after pressure-injection of p[NH]p. The injection solution contained 5 mM-p[NH]p, 9 mM-Fast Green FCF and 170 mM-KCl. The cell was bathed in ASW. □, 27 min after the injection of p[NH]p. The cell was bathed in ASW. △, 42 min after the injection of p[NH]p; 10 min after changing to 0·5 mM-calcium sea water. After injection of p[NH]p, the sensitivity of the cell progressively declined while it was bathed in ASW. Reduction of extracellular Ca^{2+} largely restored the sensitivity. Unattenuated stimulus intensity: 0·33 W/cm^2.

adenylyl methylene diphosphonate (App[CH_2]p) and methylene diphosphonate (p[CH_2]p), did not lead to reduction of the sensitivity of photoreceptors. Similarly, injection of ATP, 5′AMP, cyclic AMP, pyrophosphate and adenosine 5′ monophosphoramidate (Ap[NH_2]) did not reduce sensitivity.

A rise in the intracellular concentration of sodium ions causes a decrease in the sensitivity of *Limulus* ventral photoreceptor cells bathed in ASW (Lisman & Brown, 1972). Brown & Mote (1974) showed that the reversal voltage for the light-induced current was strongly dependent on the Na^+ gradient across the cell membrane.

Therefore we measured the reversal voltage of the light-induced current before and after an injection of p[NH]p to see if a large rise in intracellular sodium concentration accompanied the reduction of sensitivity caused by the injection. The reversal voltage in three cells did not change significantly after intracellular injections of p[NH]p that caused large reductions in the sensitivity of the cells (Table 3).

TABLE 3. Reversal voltage of the light-induced current before and after injection of imidodiphosphate solution (5 mM-p[NH]p; 170 mM-KCl)

Cell no.		Sensitivity relative to pre-injection level (log units)	Reversal voltage of light response (mV)
1	Control	—	+26
	Injected run no. 1	−1·6	+20
	Injected run no. 2	−5·0	+20
2	Control	—	+23
	Injected run no. 1	−0·7	+25
	Injected run no. 2	−2·2	+27
3	Control	—	+21
	Injected run no. 1	−1·4	+21
	Injected run no. 2	−5·0	+22

ATPγS

The effect of a small injection of ATPγS was very similar to that of an injection of one of the poorly hydrolysable GTP analogues (Fig. 9). The frequency of discrete events was greatly and permanently increased, but only after the cell was illuminated ($n = 6$). Injection of a larger amount of ATPγS desensitized the cells (Table 1) and reducing the extracellular calcium concentration to 0·5 mM did not affect further their sensitivity.

DISCUSSION

Poorly hydrolysable GTP analogues

Recordings from photoreceptor cells injected with one of the three poorly hydrolysable analogues of GTP studied show a striking elevation in the frequency of 'discrete events' produced when the cells were in darkness. This high frequency of discrete events is not the result of an increase in the sensitivity of the cells to light. It seems likely that one or more steps in the excitation pathway, steps that normally require light, are permanently activated in the presence of the GTP analogues so that the cell produces discrete events in the absence of continued illumination. The discrete events induced by the drugs are similar to those induced by light; their wave form is similar, their amplitude is reduced after the cell is adapted by a flash of bright light, and their reversal potential is close to that of the light-induced current. However, the amplitude of the discrete events induced by the drugs is often significantly less than the amplitude of the discrete events induced by light.

Poorly hydrolysable analogues of GTP are known to be irreversible activators of hormone-sensitive adenylate cyclase in many systems such as mouse lymphoma cells (see Johnson, Kaslow, Farfel & Bourne, 1980) and turkey erythrocytes (Pfeuffer & Helmreich, 1975). In the case of turkey erythrocytes, Gpp[NH]p does not activate adenylate cyclase until agonist is added. Once activated with Gpp[NH]p and agonist,

the cyclase then remains active even if the agonist is displaced with excess antagonist (Orly & Schramm, 1975). This behaviour is similar to the behaviour of photoreceptors injected with GTP analogues; illumination is required before an elevated frequency of discrete events is observed in the dark. However, although *Limulus* ventral photoreceptors contain an adenylate cyclase that is activated by GTP analogues, it is unlikely that this adenylate cyclase participates in the excitatory process (Brown, Kaupp & Malbon, 1981).

Mammalian adenylate cyclases (Ross & Gilman, 1980) are composed of several

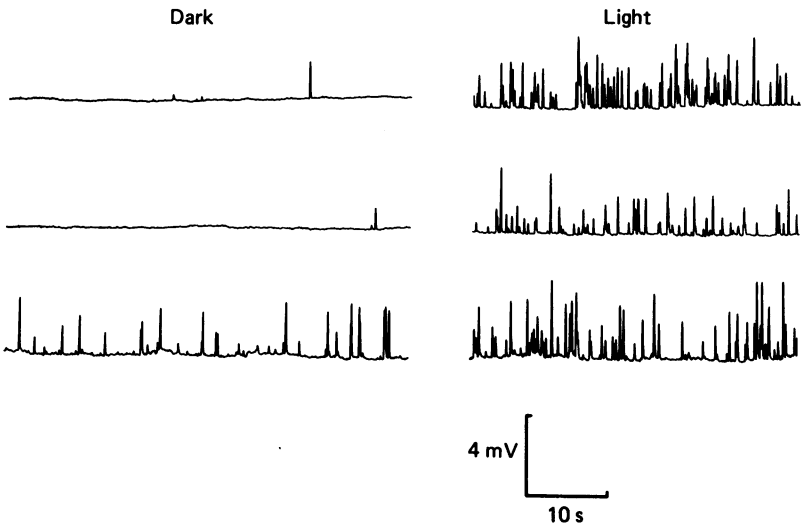

Fig. 9. Effect of intracellular injection of ATPγS. Top: recordings of membrane potential in the dark and in a dim light ($2{\cdot}7 \times 10^{-11}$ W/cm^2) before injection of ATPγS. Middle: membrane potential in the dark and in a dim light 5 min after an injection of ATPγS. The injection solution contained 10 mM-ATPγS, 9 mM-Fast Green FCF and 150 mM-KCl. The injection was made with the cell in darkness. Bottom: membrane potential 10 min after the cell was illuminated with bright light (fifty flashes, each 30 ms long, delivered at 1 s intervals. Intensity: 100 μW/cm^2).

subunits, one of which binds GTP (or GTP analogues). It has been suggested that the same GTP binding protein that forms part of vertebrate adenylate cyclase may also be a subunit of other hormone-sensitive enzymes (Johnson *et al.* 1980). Thus the effect of GTP analogues upon *Limulus* photoreceptors might be upon a GTP binding protein that is a subunit of an enzyme other than adenylate cyclase. However, injection of cholera toxin into *Limulus* ventral photoreceptors is without effect upon either the frequency of discrete events recorded with the cell in darkness, or the sensitivity of the cell to light (Brown, Bolsover & Malbon, 1981). Cholera toxin irreversibly activates adenylate cyclase in vertebrates by covalently modifying the GTP binding subunit (Gill & Meren, 1978); adenylate cyclase from the invertebrate *Aplysia* is also activated by cholera toxin (I. Levitan & S. Treistman, personal communication). Thus if the action of GTP analogues in *Limulus* photoreceptors is upon a GTP binding protein, this protein is significantly different from the GTP binding subunit of adenylate cyclase.

GTP and GDP

Injection of GTP or GDP does not induce an elevated frequency of discrete events in the dark. However, injection of GTP or GDP at pH 4·8 induces a slow, smooth depolarization that lasts 1–2 min. The depolarization does not appear to be an artifact of injection of acid solutions, because injection of ATP, 5′GMP or MES at pH 4·8 does not induce the smooth depolarization. The membrane current induced by injection of GTP at pH 4·8 is not identical to the light-induced current. It reverses in sign at significantly more positive membrane voltages than does the light-induced current. Therefore, GTP injection induces changes of membrane conductance either in addition to, or different from the light-induced change of membrane conductance.

Poorly hydrolysable ATP analogues

In contrast to the similar physiological changes produced by injections of GTPγS, Gpp[NH]p and Gpp[CH_2]p, the effects of injections of the corresponding analogues of ATP are each different. App[CH_2]p appears to be inert. The effect of an intracellular injection of ATPγS is very similar to the effect of an injection of one of the GTP analogues, such as GTPγS. It may be that ATPγS is metabolized to GTPγS in the cytoplasm. For example, beef liver nucleosidediphosphate kinase can use nucleoside thiotriphosphates as substrates (Goody, Eckstein & Schirmer, 1972). Alternatively, the ATPγS may act directly on a binding site that is not specific for guanylyl nucleotides. The present data are insufficient to decide between these possibilities.

Fein & Corson (1981) have reported that intracellular ionophoresis of either Gpp[NH]p or ATPγS into *Limulus* photoreceptor cells does not induce discrete events in the dark. Of the nucleotide analogues they tested, only GTPγS was capable of inducing discrete events. The reasons for the discrepancy between the results presented in this paper and those of Fein & Corson are not clear.

Injection of adenylyl imidodiphosphate, App[NH]p, or imidodiphosphate, p[NH]p, into *Limulus* photoreceptors causes a drastic reduction in the sensitivity of the cells to light if the cells are bathed in a normal extracellular concentration of calcium ions. The desensitization produced by an injection of App[NH]p differs from that produced by a large injection of GTPγS, GDPβS or ATPγS. When cells injected with App[NH]p are bathed in low calcium saline their sensitivity recovers. No such recovery is seen for cells that have been given large injections of GTPγS, GDPβS or ATPγS. The desensitizing effect of App [NH]p is unlikely to be due to App[NH]p acting as an ATP analogue. The other ATP analogues, App[CH_2]p and ATPγS, do not have the same effect, whereas the p[NH]p moiety alone has effects that are identical (or very similar) to those of App[NH]p. We tentatively conclude that the App[NH]p is hydrolysed inside the cell to liberate p[NH]p which then acts to desensitize the cell by causing an increase in the cytosolic concentration of calcium ions.

A rise in cytosolic concentration of calcium ions after injection of p[NH]p or App[NH]p could occur by one or more of three possible mechanisms. First, p[NH]p could increase the rate at which calcium ions enter the cell from the bathing solution or the rate at which calcium ions are released from intracellular organelles. Secondly, p[NH]p could directly inhibit the mechanisms by which calcium ions are extruded

from the cell or are taken up by intracellular organelles. Thirdly, p[NH]p could cause an increase in the cytosolic concentration of sodium ions. An increase in cytosolic sodium is known to reduce the sensitivity of *Limulus* photoreceptor cells through a mechanism that is dependent on extracellular calcium. The mechanism has been interpreted to be a sodium–calcium exchange (Lisman & Brown, 1972). We attempted to distinguish between these possibilities by measuring the reversal voltage of the light-induced current before and after p[NH]p injection. The reversal voltage is strongly dependent on the sodium gradient across the cell membrane (Brown & Mote, 1974). The reversal voltage in three cells did not change significantly after intracellular injections of p[NH]p that caused large reductions in the sensitivity of the cells (Table 3). However, an ionophoretic injection of sodium ions sufficient to desensitize significantly a *Limulus* photoreceptor cell does not measureably change the reversal voltage of the light response (Lisman & Brown, 1972). Therefore, the possibility that the imidodiphosphates act via a Na^+–Ca^{2+} exchange mechanism cannot be excluded by the results presented in Table 3.

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