

# The Effect of Nucleotides on the Rate of Spontaneous Quantum Bumps in *Limulus* Ventral Photoreceptors

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**ABSTRACT** The effect of intracellular nucleotides on the rate of spontaneous quantum bumps in *Limulus* ventral photoreceptors has been examined. Internal dialysis of photoreceptors with solutions lacking nucleotide leads to an elevation of the quantum bump rate that can be reversed by introduction of nucleotide. Similarly, elevation occurs after treating intact cells with the metabolic inhibitor 2-deoxyglucose. This effect can be reversed by intracellular injection of ATP. The rate of spontaneous quantum bumps in unpoisoned cells can be reduced to below normal levels by injection of ATP. These results support the hypothesis that high-energy nucleotides suppress the rate of spontaneous quantum bumps.

## INTRODUCTION

High-energy nucleotides have been implicated in a variety of light-dependent biochemical reactions in photoreceptors. For example, in both vertebrate and invertebrate photoreceptors, photoactivated rhodopsin undergoes nucleotide-dependent phosphorylation (Kuhn and Dryer, 1972; Bownds et al., 1972; Paulson and Hoppe, 1978). The role of this phosphorylation appears to be that of terminating the active state of rhodopsin, at least as judged by the ability of rhodopsin to activate phosphodiesterase (Leibman and Pugh, 1980; Sitaramayya and Liebman, 1983). A related but distinct reaction found in both vertebrates and invertebrate photoreceptors is a light-activated GTPase (Wheeler and Bitensky, 1977; Calhoon et al., 1980). In addition to the involvement of nucleotides in light-activated biochemical reactions, nucleotides serve as an energy source in the maintenance of ion gradients.

The role of nucleotides in photoreceptors has been studied physiologically by examining the effects of metabolic inhibitors. In both vertebrates and invertebrates, the receptor potential is reduced or abolished by such agents (Noell, 1959; Bauman and Mauro, 1973; Lantz and Mauro, 1978). In experiments on *Limulus* ventral photoreceptors, it was shown that metabolic inhibition leads to a rise in the intracellular free  $\text{Ca}^{2+}$  concentration ( $\text{Ca}_i^{2+}$ ) (Lo et al., 1980), a rise

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that can itself reduce the receptor potential (Lisman and Brown, 1972), and that injection of a calcium chelator during metabolic inhibition can restore the receptor potential (Wong et al., 1979). Thus, the abolition of the response to light by metabolic inhibitors in *Limulus* appears to be a secondary result that follows from inhibition of ion pumps.

In the results reported here, we have studied the role of nucleotides in *Limulus* photoreceptors using the internal dialysis method (Lee et al., 1978; Stern and Lisman, 1982*b*). In this method, a small region of photoreceptor membrane is sucked into a pipette and disrupted. The solution filling the pipette is then able to exchange with the soluble cytoplasm. The method allows rapid, direct, and reversible addition of molecules to the cytoplasm of a functioning cell under conditions where the membrane can be voltage-clamped. Because the dialysis method relieves the dependence of intracellular ion concentration on membrane pumps, it has been possible to study the physiological effects of lowered nucleotide concentration without the confounding effects that follow secondarily from changes in ion concentrations. Using this method, we have examined the effect of nucleotide concentration on the rate of spontaneous quantum bumps. Spontaneous quantum bumps closely resemble the quantum bumps evoked by single photons, but they occur in complete darkness (Adolph, 1964). Our principal finding is that the rate of spontaneous bumps depends on the concentration of nucleotides in the dialysis solution. Furthermore, we have been able to confirm the relationship between nucleotides and bump rate using several independent methods. Preliminary accounts of these results have been presented elsewhere (Stern et al., 1983; Stern and Lisman, 1982*b*).

#### METHODS

*Limulus* ventral nerves were excised and dissected under bright white light and bathed in artificial seawater (ASW) as described by Lisman and Brown (1972). Individual photoreceptors were denuded by mechanically removing their encasement of glial cells and connective tissue while viewing the cells through a compound microscope (Stern et al., 1982). After several cells on the same nerve were denuded, the preparation was dark-adapted for at least 30 min. All subsequent manipulations were done using infrared illumination and an infrared-sensitive television system.

The internal dialysis and recording procedures were as follows. A patch of membrane on the arhabdomeric lobe (Stern et al., 1982) of a dark-adapted, denuded photoreceptor was sucked onto the tip of a glass suction pipette (20  $\mu\text{m}$  i.d.). Seals between the cell and pipette were usually 10–20  $\text{M}\Omega$ . The patch was disrupted using a large, transient current pulse, thereby providing a pathway for dialysis solution to enter the cell. The resistance of the disrupted patch (measured as the resistance in series with the cell membrane) was typically  $<500$   $\text{k}\Omega$ . The voltage-clamp circuitry was as described by Stern and Lisman (1982*b*). Using an array of inport tubes, the solution within the pipette could be changed within 30 s. The time required for nonmetabolized small molecules to reach steady state levels in the cytoplasm is 3–5 min (Stern and Lisman, 1982*b*).

The standard internal dialysis solution contained 300 mM K-aspartate, 200 mM sucrose, 15 mM glutathione, 5 mM  $\text{K}_2\text{PO}_4$  with  $\text{Na}_2\text{ATP}$ ,  $\text{Na}_2\text{GTP}$ ,  $\text{MgCl}_2$ , and Na-aspartate, as specified in Table I. The ATP was the ultrapure sodium salt from Boehringer-Mannheim Biochemicals, Indianapolis, IN. In an attempt to avoid systematic errors from contaminants, we also used ATP and GTP (both from Sigma Chemical Co., St. Louis, MO) prepared by different techniques, but we detected no obvious differences.

For conventional recording from undialyzed cells, 10-M $\Omega$  microelectrodes filled with 3 M KCl were used. For pressure injection, microelectrodes were filled with the solutions described in Table I. Solutions were microinjected into a cell by delivering 100-ms, 5–20-psi pressure pulses to the back of the microelectrode. Injections were monitored under infrared illumination (Corson and Fein, 1983c). Extracellular application of solutions was via a constant-flow superfusion system.

ATPase and GTPase activity of alkaline phosphatase (P4502; Sigma Chemical Co.) or protein kinase inhibitor (P5015; Sigma Chemical Co.) (both at 10 U/ml) was measured as inorganic phosphate liberation after incubation with ATP or GTP under ionic conditions similar to those encountered during an internal dialysis experiment. The resulting solution was acid-quenched. Phosphate was then determined colorimetrically by the method of

TABLE I  
*Composition of Solutions (Concentrations in mM)*

ASW*	NaCl	KCl	CaCl <sub>2</sub>	MgCl <sub>2</sub>	MgSO <sub>4</sub>	Tris	pH	
	425	10	10	22	26	15	7.8	
Internal solution <sup>‡</sup>	K-aspar- tate	Sucrose	Gluta- thione	MgCl <sub>2</sub>	K <sub>2</sub> PO <sub>4</sub>	Na <sub>2</sub> ATP	Na <sub>2</sub> GTP	pH
	300	200	15	9	5	5	1	7.3
Injection solutions	K-aspar- tate	Sucrose	Gluta- thione	MgCl <sub>2</sub>	Na <sub>2</sub> ATP	HEPES	pH	
ATP injection	300	0	0	25	25	2.5	7.3	
Enzyme injection	300	200	10	2	0	5	7.3	

\* The pH was 7.0 during the metabolic poisoning experiments using 2-DG.

<sup>‡</sup> When nucleotide concentrations were varied, free [Mg] was kept constant by adjusting the amount of MgCl<sub>2</sub> added such that the total Mg added equaled the concentration of nucleotides (to which Mg binds) plus the desired free Mg concentration. Total [Na] was adjusted by substituting 2 mol of Na for each mole of nucleotide so that each solution in a single experiment had [Na] equal to the solution containing the most nucleotide.

Fiske and SubbaRow (1925) as modified by Stanton (1968) for the determination of inorganic phosphate in the presence of ATP. Ammonium molybdate/sulfuric acid and 1,2,4-aminonaphtholsulfonic acid solutions were obtained from Sigma Chemical Co.

In internal dialysis experiments, only cells that had spontaneous quantum bump rates of <0.8/s within the first 4 min of dialysis were selected for further experimentation. In all experiments, we used only cells that generated large quantum bumps (maximum amplitudes >5 mV or >0.5 nA). Quantum bumps were counted manually. Deflections having an amplitude of at least twice the peak-to-peak baseline noise were counted as bumps. Rates were usually measured over sample periods of 1 min. All experiments were conducted at room temperature (20–23°C).

## RESULTS

Cells were prepared for internal dialysis as described in Methods. After obtaining a seal between the pipette and the cell, the patch of membrane within the pipette was disrupted and the cell was voltage-clamped to resting potential. Spontaneous quantum bumps were readily observed in the current trace under these conditions. These spontaneous events had variable sizes, as previously described for intact photoreceptors (Adolph, 1964). Fig. 1 shows that the rate of spontaneous

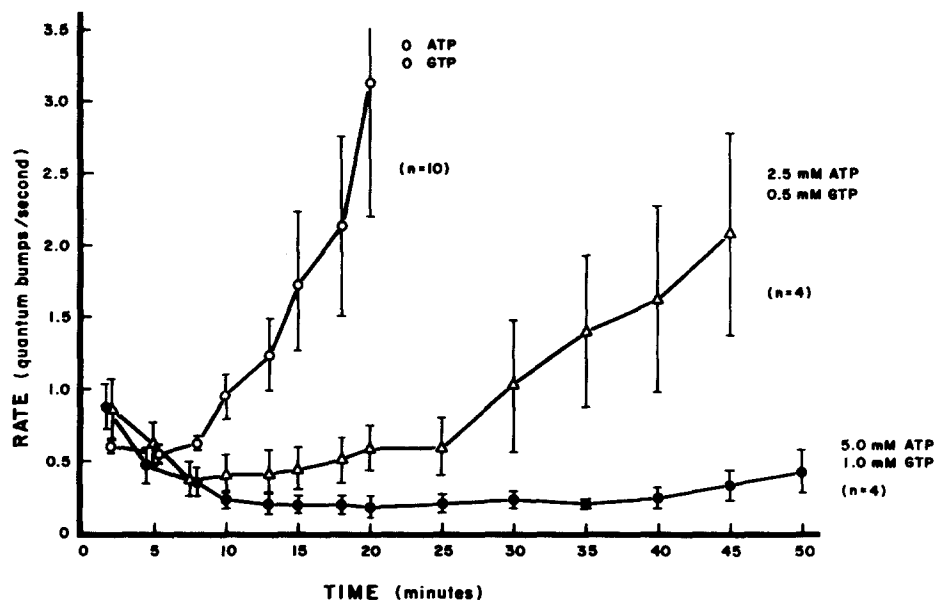


FIGURE 1. The spontaneous quantum bump rate is plotted against time of dialysis. Each line represents a set of experiments using different concentrations of ATP and GTP as indicated. Each data point is the mean rate for  $n$  cells and the error bars show the SEM.

quantum bumps depended in a complex way on the duration of dialysis and on the concentration of high-energy nucleotides in the dialysis solution. A few minutes after the start of experiments on healthy cells, the spontaneous rate was 0.2–0.8 bumps/s, irrespective of whether nucleotides were included in the dialysis solution. This rate is comparable to that recorded using conventional intracellular recording (Table II). If cells were dialyzed with an internal solution containing

TABLE II  
*Intracellular Injection of ATP Reduces the Spontaneous Quantum Bump Rate (Bumps/s) of Undialyzed, Unpoisoned Photoreceptors (at 20–23°C)*

Cell*	Initial rate	Rate after ATP injection
A1	0.11	0.03
A2	0.28	0.05
A3	0.48	0.13
A4	1.92	0.06
A5	1.25	0.06
A6	1.80	0.16
A7	4.50	0.12
B	0.80	0.08
C	0.52	0.10
D	0.38	0.08

\*Cells A1–A7 were recorded from sequentially on the same nerve over a 5-h period.

5 mM ATP and 1 mM GTP, the rate stayed at this low level for ~40 min; only after 40–60 min was there a tendency of the rate to rise. In contrast, the rate of spontaneous quantum bumps rose dramatically after only 10–20 min of dialysis with nucleotide-free internal solution (Fig. 1). Internal solutions containing 2.5 mM ATP and 0.5 mM GTP gave results intermediate between the nucleotide-free solution and the 5 mM ATP, 1 mM GTP solution.

The effect of removing nucleotides on the spontaneous bump rate could be reversed by addition of nucleotide. Fig. 2 is a plot of the bump rate vs. time in an individual cell as the nucleotide concentration was switched back and forth

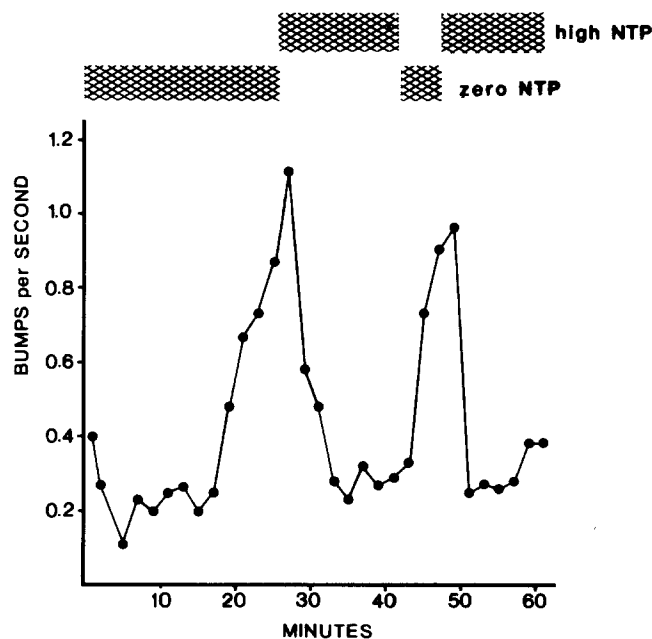


FIGURE 2. Quantum bump rate is plotted vs. time in an individual cell as NTP is changed. The high-NTP solution contained 10 mM ATP and 2 mM GTP, and zero-NTP solutions lacked ATP and GTP.

between a high-nucleotide solution (10 mM ATP, 2 mM GTP) and a nucleotide-free solution. During the initial 26 min of the experiment, the cell was dialyzed with nucleotide-free solution. The bump rate was initially low, but after 18 min the rate rose rapidly, as in Fig. 1. When the internal solution was then switched to the high-nucleotide solution, the bump rate fell rapidly from 1.1 to 0.3 bumps/s. The cell was then again dialyzed with nucleotide-free solution and the bump rate rose within minutes to a high level, until the bump rate was again reduced by reintroduction of nucleotides. Reversible effects of nucleotide were observed in 19 of 25 experiments. In four of these, it was possible to go through several cycles of nucleotide changes, as in Fig. 2. In 6 of the 25 experiments, introduction of nucleotides produced no apparent reduction in bump rate. Traces illustrating the spontaneous bumps in the low- and high-nucleotide solution are shown in

Fig. 3. The bump shapes and amplitude under the two conditions were roughly similar, but a quantitative comparison was not made.

In dialysis experiments similar to those of Fig. 1, the effect of low-energy nucleotides was examined. Dialysis with solutions containing 10 mM AMP and 2 mM GMP kept the spontaneous rates low for 30–40 min, a period during which a high rate would have developed in the absence of any nucleotide. Therefore, the monophosphates were also effective at suppressing spontaneous bumps. From

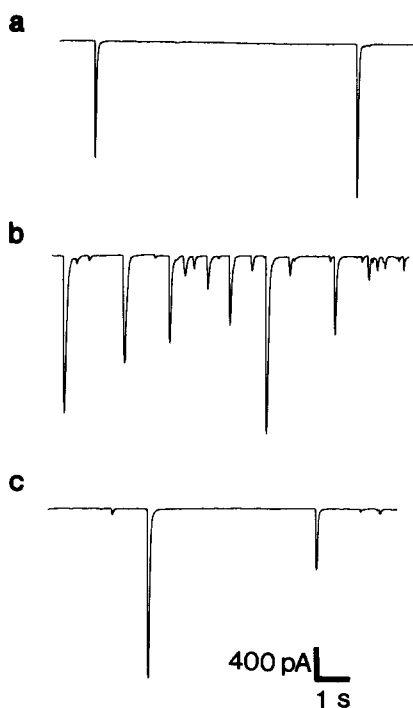


FIGURE 3. Spontaneous quantum bumps measured under voltage clamp. (a) "Control" trace 10 min after the start of the experiment in zero-NTP solution. (b) Trace after 20 min in zero-NTP solution showing the elevated rate of bumps. (c) The spontaneous rate 5 min after changing to an internal solution containing 10 mM ATP and 2 mM GTP. Data are from the same cell as Fig. 2.

the dialysis experiments alone, it is unclear whether both low- and high-energy nucleotides actually suppress bumps or whether the low-energy nucleotides are converted to high-energy nucleotides in the cell and thus only indirectly suppress bumps.

#### *Effect of Metabolic Inhibitors on Quantum Bump Rate in Intact Cells*

It was of interest to determine how metabolic inhibitors affect the rate of spontaneous bumps in intact cells. Previous work on *Limulus* photoreceptors showed that inhibition of metabolism desensitized cells and eventually led to a complete abolition of the receptor potential (Borsellino and Fuortes, 1968; Wong

et al., 1979). Since desensitization occurs by reduction of quantum bump size (Dodge et al., 1968), neither spontaneous nor light-induced quantum bumps can be observed during metabolic inhibition, and it is therefore not possible to measure their rate. It nevertheless seemed possible that as cells recovered from metabolic inhibition, there might be a period during which the cell had recovered from the desensitizing effect of metabolic inhibition, but during which other effects were still present.

Experiments were conducted using the metabolic inhibitor 2-deoxyglucose (2-DG). This inhibitor is taken up by cells and phosphorylated. It inhibits metabolism by competitively inhibiting a key enzymatic step in glycolysis and by becoming phosphorylated to such an extent that it depletes the cell of ATP and phosphate. The inhibition of glycolysis by 2-DG can be partially reversed by addition of glucose (for a review of 2-DG metabolic effects, see Webb, 1966). The following protocol was used to study the effects of 2-DG. Cells were bathed in ASW containing 5 mM 2-DG for 20–30 min. Toward the end of this period, cells lost their responsiveness to light and quantum bumps were abolished. The superfusate was then changed back to ASW for 20 min. At this point, 2.5 mM glucose was added to the ASW. Typically, within 30–60 min after the addition of glucose, the sensitivity to light recovered and spontaneous quantum bumps were observed. Fig. 4 shows that under these conditions the rate of spontaneous quantum bumps was 2–3/s, considerably higher than before application of 2-DG. Similar results were obtained in five cells. Control experiments showed that the elevation of bump rate was not due to glucose alone.

If the increase in spontaneous rate induced by 2-DG was due to a reduction in high-energy nucleotides, it should be possible to reverse the effect by injection of such nucleotides. Fig. 5 shows the results of such an experiment. After application of 2-DG, the bump rate was considerably elevated. The cell was then impaled with an electrode containing ATP (25 mM). This solution was injected using 100-ms pressure pulses (at arrows). Injections were confirmed by the optical method of Corson and Fein (1983c) and were always <0.1 cell volume. In the experiment of Fig. 5, the injection of ATP dramatically lowered the bump rate. Similar results were obtained in four other experiments. If ATP was not injected, the bump rate stayed high for at least 30 min. There was considerable variation in the onset of the suppression of bump rate by ATP injection. In some cells, the bump rate fell within a few minutes after the injection, as in Fig. 5; in others, the decrease was not apparent for 5–10 min. The decrease in bump rate was sometimes accompanied by an increase in baseline noise (see Fig. 5). The ability of ATP to reduce the quantum bump rate strongly suggests that 2-DG lowered the concentration of high-energy nucleotides and raised the level of low-energy nucleotides, as demonstrated directly in other systems (Letnansky, 1964; Gibbins, 1982). It would therefore follow that the suppressive effect of nucleotides is due to the high-energy form of these compounds.

#### *Effect of Nucleotide Injection*

Experiments in the section above suggest that lowering the level of high-energy nucleotides leads to an increase in the rate of spontaneous quantum bumps. This raises the question of whether the converse experiment, raising the level of high-

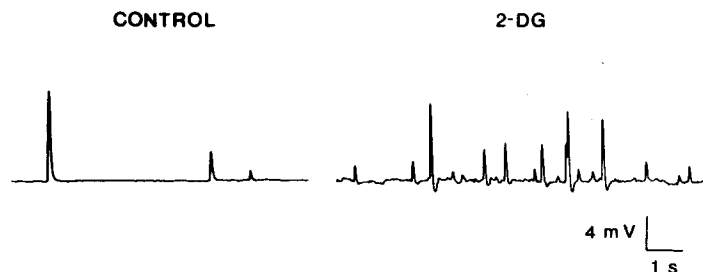


FIGURE 4. The rate of spontaneous quantum bumps is increased by 2-DG. Voltage was monitored using conventional intracellular recording techniques.

energy nucleotides in intact cells, leads to a lowering of the bump rate. To answer this question, ATP was pressure-injected into unpoisoned, intact, dark-adapted cells. As summarized in Table II, the rate of spontaneous bumps fell in all cells studied. The rate fell within <10 min after the injection but did not increase again for the remainder (30 min) of the experiments. Similar injections of a control solution without nucleotides had no effect. In a few experiments, we injected the low-energy nucleotide AMP into intact ventral photoreceptors and found effects similar to those produced by ATP injection. Because the pressure injections were not calibrated, it was not possible to compare the effectiveness of AMP and ATP. Our experiments with ATP confirm a brief report by Fein and Corson (1982), which showed that injection of high-energy nucleotides can reduce the rate of spontaneous bumps.

It is of interest to note in passing that during experiments on cells from an individual nerve (cells A1–A7 in Table II), there was a progressive increase in the resting rate of spontaneous quantum bumps. Similar increases in the rate have also been seen during long-term recordings from single ventral photoreceptors (M. Goldring, personal communication).

*Effect of Alkaline Phosphatase and Protein Kinase Inhibitor on Spontaneous Rate*

Involvement in protein phosphorylation reactions is one possible mechanism of ATP's effect on spontaneous quantum bump rate. As a preliminary examination

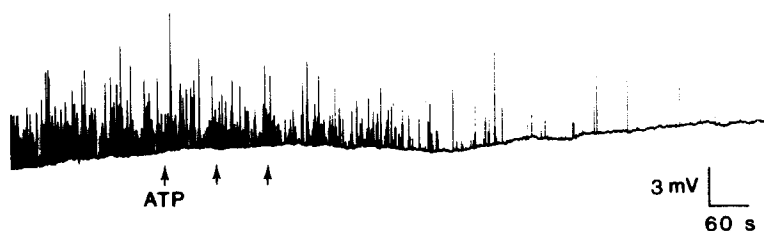


FIGURE 5. The elevated rate of spontaneous quantum bumps caused by exposure to 2-DG was reduced by intracellular injection of ATP. An elevated bump rate was induced by 2-DG as described in text. ATP was pressure-injected (for 100 ms at each arrow) into the photoreceptor.



of this possibility, we dialyzed ventral photoreceptors with alkaline phosphatase (68,000 mol wt), which dephosphorylates most phosphoproteins, and protein kinase inhibitor (26,000 mol wt), which inhibits cyclic AMP-dependent protein kinase. A greater than fivefold increase in the rate of spontaneous bumps was consistently observed within 15 min of dialysis with solutions containing both 2 U/ml alkaline phosphatase and 10 U/ml protein kinase inhibitor. Much higher activities were required in order to obtain the same effect with solutions containing only one of the enzymes, at least 10 U/ml phosphatase or 50 U/ml kinase inhibitor (six experiments).

An important criticism of the experiments involving alkaline phosphatase, which we became aware of only after the experiments were completed and presented in abstract form (Stern and Lisman, 1982a), is that the alkaline phosphatase we used has ATPase activity. In an internal solution containing 1 mM ATP or 1 mM GTP, alkaline phosphatase (10 U/ml) liberated 1 mol of phosphate per mole of nucleotide within 10 min and two to three phosphates in <1 h (see Methods). Since this contaminant NTPase hydrolyzed the nucleotides in the dialysis solutions, it is possible that the effect of these solutions was due to the lowered nucleotide levels rather than to the enzymes themselves. On the other hand, since dialysis with mononucleotides is able to suppress bumps effectively, it is possible that the action of phosphatase was direct and was not due to nucleotide hydrolysis.

#### DISCUSSION

Experiments using the internal dialysis method show that the spontaneous quantum bump rate was greatly elevated when nucleotides were omitted from the dialysis solution (Fig. 1) and that restoration of nucleotides suppressed the rate (Fig. 2). These experiments provide a direct demonstration of the importance of nucleotides in the suppression of the spontaneous quantum bump rate. Further support for this connection comes from several independent lines of less direct experiments: after intact cells were treated with the metabolic inhibitor 2-DG, there was an increase in the rate of spontaneous bumps. This increase could be reversed by ATP, which is consistent with the notion that 2-DG raised the bump rate by lowering the level of high-energy nucleotides (Fig. 4). Conversely, increasing the level of nucleotides in unpoisoned cells lowered the bump rate (Table II).

These results clearly demonstrate the importance of nucleotides in the suppression of spontaneous events, but questions regarding the mechanism of suppression and the identity of the nucleotide responsible remain to be answered. The difficulty stems from the ability of cells to regulate their own nucleotide levels and the existence of pathways that couple various components of nucleotide metabolism. For instance, Fig. 2 shows that after 20 min of dialysis, the bump rate was normal only if nucleotides were present. In early parts of the experiment, however, the rate was normal even in the absence of exogenous nucleotide. This may indicate that the cell can initially maintain intracellular nucleotide levels using its endogenous metabolic machinery. For energy, the cell could use endogenous glycogen stores and the reduced glutathione in the dialysis solutions.

Related considerations regarding endogenous metabolism bear on the question of whether low- or high-energy nucleotides are responsible for the suppression of spontaneous bumps. For many types of energy-rich cells, the factor that limits the production of high-energy nucleotides is the availability of low-energy nucleotides (Whittam and Wiley, 1968; Lund et al., 1975). By analogy, the suppressive effect of AMP and GMP on spontaneous bumps in *Limulus* may be due to a secondary elevation of high-energy nucleotides. A more direct argument that the high-energy nucleotides rather than the low-energy nucleotides suppress bumps comes from the 2-DG experiments. If low-energy nucleotides directly suppressed bumps, inhibition of metabolism would reduce the frequency of spontaneous bumps, but just the opposite occurs (Fig. 5). Thus, it seems likely that the rate of spontaneous bumps is suppressed by some form of high-energy nucleotide.

The dependence of the spontaneous bump rate on nucleotides would seem to be inconsistent with the view that spontaneous bumps originate exclusively from the spontaneous isomerization of rhodopsin (Srebro and Behbehani, 1972). Rather, it seems more reasonable to imagine that spontaneous bumps originate from some early step in transduction that is nucleotide dependent. A model concerning the mechanism of the nucleotide effect is presented in the companion paper (Lisman, 1985) and is summarized briefly here. Suppose that light converts rhodopsin to an active metarhodopsin ( $M^*$ ) capable of activating the enzyme cascade that produces a quantum bump, and that  $M^*$  is inactivated by multiple phosphorylation reactions. Because the ventral photoreceptors used in our experiments were dissected under white light and because *Limulus* metarhodopsin is thermally stable, all cells contained  $\sim 10^9$  inactivated metarhodopsin molecules (Lisman and Sheline, 1976; Lisman and Bering, 1977). According to the model, a spontaneous quantum bump occurs whenever any of these  $10^9$  metarhodopsin molecules becomes spontaneously dephosphorylated. Because ATP provides the free energy that keeps metarhodopsin in the inactive, multiply phosphorylated state, lowering the ATP concentration would increase the probability of metarhodopsin becoming completely dephosphorylated and would thereby elevate the spontaneous rate.

Previous work has shown that a wide variety of pharmacological agents can affect the rate of spontaneous bumps. These include fluoride, vanadate, and molybdate (Corson and Fein, 1983*b*), diamide (Hanani and Fein, 1981), pH (Corson and Fein, 1980), and the poorly hydrolyzable nucleotide analogues GTP- $\gamma$ -S (Fein and Corson, 1981) guanylyl imidodiphosphate, and ATP- $\gamma$ -S (Bolsover and Brown, 1982). Since many of these drugs affect GTP-binding regulatory proteins of the kind identified in both vertebrate (Fung and Stryer, 1980) and invertebrate photoreceptors (Vandenberg and Montal, 1982), it has been suggested that these drugs induce bumps by affecting a G-protein (Corson and Fein, 1983*a*). However, given that some, and possibly all, of these drugs can inhibit metabolism (DeMaster and Mitchell, 1973; Shearer, 1974), it is important to consider the possibility that some or all these drugs act by reducing the concentration of high-energy nucleotides. Several very recent experiments have addressed this question. Corson and Fein (1983*b*) showed that the increase in frequency of quantum bumps produced by vanadate could be reversed by

injection of ATP. However, Rubin and Brown (1983) showed that the increase in spontaneous events induced by fluoride was not accompanied by a decrease in ATP levels. Thus, further work will be required to clarify this issue.

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