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17. Identification of any commercial product does not imply recommendation or endorsement by either the National Cancer Institute, National Institutes of Health, or by the National Bureau of Standards. We thank C. C. Brown, Biometry Branch, Field Studies and Statistics Program, Division of Cancer Cause and Prevention, National Cancer Institute, for suggestions regarding the statistical analysis of the results, M. J. Wilson and J. Brown for help in growing the hepatocyte lines, and F. M. Williams and D. R. Bender for help in preparing the manuscript.

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Excitation of *Limulus* Photoreceptors by Vanadate and by a Hydrolysis-Resistant Analog of Guanosine Triphosphate

Abstract. *Discrete voltage fluctuations that occur spontaneously or in response to dim lights can be recorded from the ventral photoreceptors of* Limulus. *The injection of vanadate or the hydrolysis-resistant analog of guanosine triphosphate, GTP-γ-S, into ventral photoreceptors induces the production of discrete waves in the dark. The chemically induced discrete waves are similar to those induced by light. Ventral photoreceptors may contain a guanyl nucleotide binding protein whose activation by vanadate or GTP-γ-S induces the discrete waves.*

Discrete fluctuations of transmembrane potential that occur spontaneously or in response to dim lights have been recorded from various invertebrate photoreceptors (*1*). The discrete voltage fluctuations induced by light appear to result from the absorption of single photons (*2*) and hence are often referred to as quantum bumps or more simply bumps. Shot noise analysis of the response to steady light of the ventral photoreceptors of *Limulus* suggests that the light response results from a summation of these bumps (*3*). Thus, the cellular process responsible for the production of bumps appears to be fundamental to the phototransduction process.

A series of chemical reactions may intervene between absorption of light and the subsequent response (bumps). We sought to manipulate these chemical reactions by exposing the ventral photoreceptors of *Limulus* to pharmacologic agents. When we discovered that exposure of ventral photoreceptors to fluoride ions (F^-) induced bumps in the absence of light (*4*), we suspected that fluoride activated the biochemical pathway that was normally affected by light. However, fluoride affects many biochemical reactions, and we could not define the particular pathway being activated in the photoreceptor. To identify the biochemical steps involved in phototransduction, we looked for other specific agents that might produce bumps. We now report that vanadate (VO_3^-) and guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S), a hydrolysis-resistant analog of guanosine-5′-triphosphate (GTP), induce bumps in the absence of light. All experiments were carried out on the ventral photoreceptors of *Limulus* (*5*).

Figure 1 shows that injection of GTP-γ-S (*5*) induces bumps within the photoreceptor in the absence of light. The recording in Fig. 1A was taken before injection and that in Fig. 1C after the injection; both were taken in the dark. For comparison, the response of the cell (before injection) to a steady dim light is shown in Fig. 1B. The bumps induced by the injection of GTP-γ-S have a similar time course and, on the average, a smaller amplitude than those induced by light. Exposure of cells to fluoride (*4*) also induced bumps of similar time course and smaller amplitude than those induced by light. The effects of GTP-γ-S last for a long time. For the cell in Fig. 1, no sign of recovery was seen for more than 3.5 hours after injection.

Vanadate induces bumps either when added to the artificial seawater (ASW) that bathed the preparation or when injected intracellularly by iontophoresis (*5*). The bumps in Fig. 2 were induced by VO_3^- injection. Injection of vanadate (Fig. 2B) caused a dramatic rise in the rate of occurrence of bumps. The cell recovered only partially from the effects of the VO_3^- injection (Fig. 2, C and D). We found it experimentally easier to control the effects of VO_3^- by adding it to the ASW that bathed the receptor. When added to the ASW at concentrations as

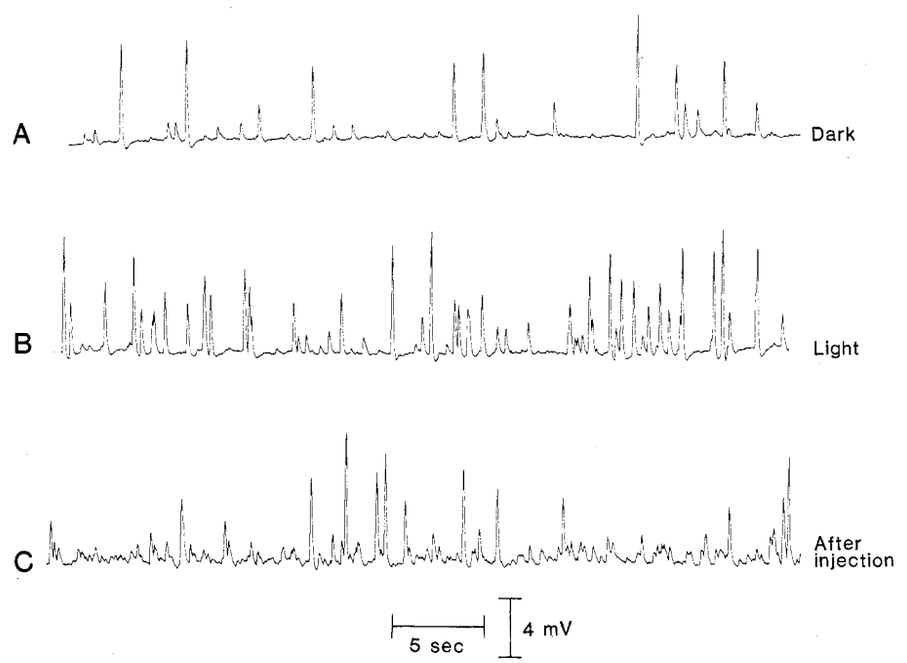

Fig. 1. Discrete waves (bumps) induced by GTP-γ-S. Intracellular recordings from a photoreceptor (A and B) before and (C) after injection of GTP-γ-S into the photoreceptor. The GTP-γ-S was injected into the cell iontophoretically by a 1-nA, 5-second hyperpolarizing current pulse; the pulse was repeated once every 10 seconds for 15 minutes. For purposes of comparison, (B) shows bumps elicited by a steady white light whose intensity was attenuated 8 log units below the maximum intensity available from the light source.

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low as 200 μM, VO_3^- typically induced the production of bumps within 10 to 20 minutes. After the receptor was washed with ASW, the effects of VO_3^- were usually reversed completely in about 1 hour.

We tested for the specificity of GTP-γ-S by injecting other cells with GTP, adenosine triphosphate (ATP), guanylyl imidodiphosphate (GMP-PNP), or adenosine-5′-*O*-(3-thiotriphosphate) (ATP-γ-S) (*5*). For similar injections, none of these produced effects similar to those of GTP-γ-S. The finding that ATP-γ-S was ineffective indicates that the system being affected by GTP-γ-S is selective for guanyl nucleotides. The ineffectiveness of GTP suggests the GTP-γ-S is effective because it resists hydrolysis. We will discuss the finding with GMP-PNP later.

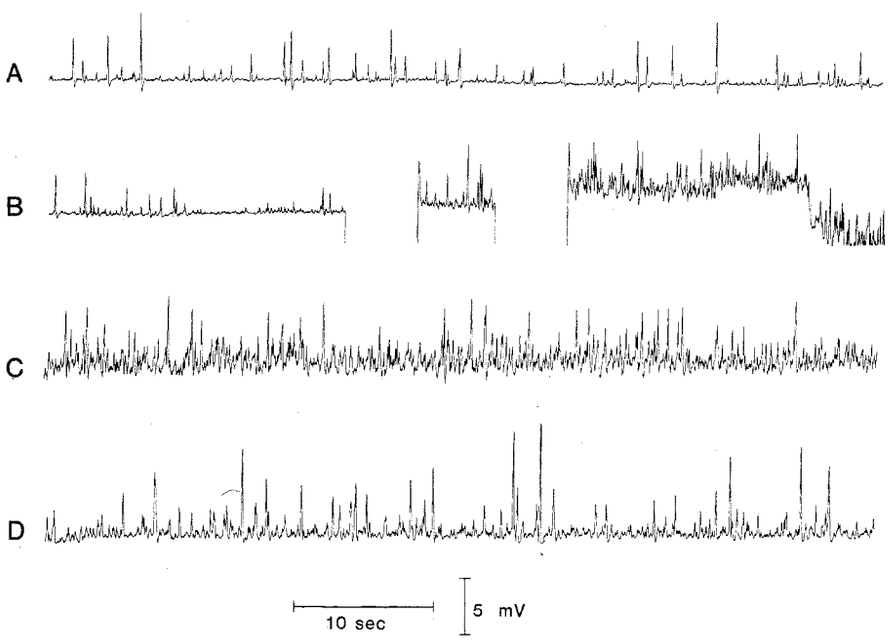

Fig. 2. Bumps induced by vanadate injection. All the recordings shown in this figure were obtained by use of an intracellular KCl-filled microelectrode (A) before and (B) after a second microelectrode containing vanadate was inserted into the cell in the dark. Vanadate was iontophoretically injected into the photoreceptor by 1-nA, 5-second hyperpolarizing current pulses. Injections are indicated in (B) by breaks in the records; these breaks occur because the injection current hyperpolarized the photoreceptor membrane beyond the range of the chart recorder. After each vanadate injection, the rate of occurrence of bumps increases. After the two vanadate injections, the vanadate electrode was removed from the cell in the dark. The membrane hyperpolarization at the end of (B) occurred as the vanadate electrode was being removed, and the recording bottomed out of the range of the chart recorder at the end of (B). Record (C) was taken 30 seconds after removal of the vanadate electrode, and (D) was taken 14 hours later. There appears to be only partial recovery from the vanadate injection.

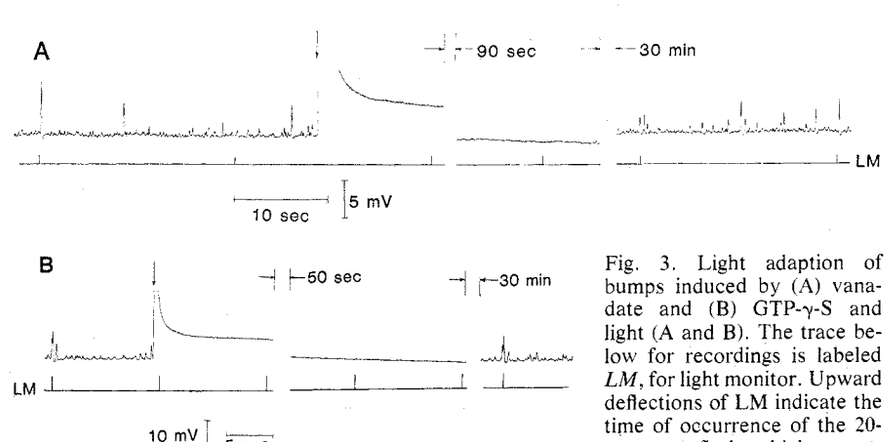

Fig. 3. Light adaption of bumps induced by (A) vanadate and (B) GTP-γ-S and light (A and B). The trace below for recordings is labeled *LM*, for light monitor. Upward deflections of LM indicate the time of occurrence of the 20-msec test flash, which was attenuated 6 log units below the maximum intensity available from the white light source. The vertical arrow above the recordings shows the time of the 20-msec adapting flash, which was attenuated 2 log units below the maximum intensity available from the white light source. In both (A) and (B), the response of the cell to the adapting flash went off scale. The data are excerpts from a continuous recording from the receptor, and the time breaks shown during recovery are solely for the purpose of illustration. The data in (A) and (B) are from different cells.

If the bumps induced by VO_3^- and GTP-γ-S are indeed similar to those induced by light, then it should be possible to light-adapt them. Both the chemically induced bumps and the light-induced bumps become greatly attenuated after exposure to a bright adapting flash, and both take many minutes to recover (Fig. 3).

What conclusions can we draw from the observation that three chemicals as diverse as F^-, VO_3^-, and GTP-γ-S have similar effects on the photoreceptor? To our knowledge the only known common denominator of these three agents is their positive action on hormone-regulated adenylate cyclase. Having said this, we must be careful to stress that we do not mean to suggest that our findings imply that cyclic nucleotides are involved in the effects of these three agents. The reason for this disclaimer will become clear as we proceed with the interpretation of our findings.

To avoid any confusion surrounding the nature of our conclusions, we first briefly review the current view of the way in which hormonal signals are transduced into adenosine 3′,5′-monophosphate (cyclic AMP) synthesis. At least three classes of membrane protein are involved: hormone receptors (R), catalytic adenylate cyclase (C), and a third component (N) required for coupling R and C (*6*). After the hormone (H) is bound to R (H-R), activation of C is controlled by a regulatory cycle consisting of two reactions occurring at N: an H-R–induced formation of an N-GTP complex that activates C, and a subsequent turn-off reaction in which hydrolysis of the bound GTP returns the system to the inactive state (*7*). A specific guanyl nucleotide binding component (N) is the site where GTP is bound and subsequently hydrolyzed. As discussed below, the available evidence indicates that F^-, VO_3^-, and GTP-γ-S activate adenylate cyclase by interacting with N. By analogy, we suggest that these three agents activate ventral photoreceptors (induce bumps) by interacting with an N-like protein.

Adenylate cyclase is activated by GTP-γ-S in a quasi-irreversible manner that results from its resistance to hydrolysis and its attachment to the binding site of N (*7*). Fluoride activates adenylate cyclase by interacting with N (*8*). Vana-

date activates adenylate cyclase (*9*) and, in other systems, inhibits a number of phosphohydrolases (*10*); therefore, its activation of adenylate cyclase probably involves binding to N. We suggest that the ventral photoreceptors of *Limulus* contain a protein similar or perhaps identical to the guanyl nucleotide binding component, N, of hormone-regulated adenylate cyclase. Furthermore, we suggest that GTP-γ-S, fluoride, and vanadate induce bumps by interacting with the proposed guanyl nucleotide binding component. Obviously, such an interpretation is highly speculative.

If the proposed GTP-binding protein participates in the production of light-induced bumps, then F^-, VO_3^-, and GTP-γ-S might modify the cell's light response. We reported earlier that F^- prolongs the response to a dim flash (*4*), and in preliminary experiments, we determined that in some cells VO_3^- and GTP-γ-S also prolong the response to a dim flash. Thus it appears that the proposed GTP-binding protein is normally involved in visual excitation.

According to the above interpretation, GMP-PNP might be expected to have effects similar to those of GTP-γ-S, since GMP-PNP is an analog of GTP known to resist enzymatic hydrolysis. However, GMP-PNP dissociates from the guanyl nucleotide binding site some 50 times faster than GTP-γ-S does (*11*). Thus, similar effects on ventral photoreceptors might require 50 times as much GMP-PNP as GTP-γ-S. This probably explains why, with equal injections, we found GTP-γ-S to be effective and GMP-PNP ineffective. For ventral photoreceptors injected with either GMP-PNP or GTP-γ-S and exposed to bright light, there is an increase in bump frequency in the dark (*12*). For some cells a second bright illumination is necessary before the increase in bump frequency is observed in the dark (*12*). We interpret these findings to imply that light enhances the binding of GMP-PNP and GTP-γ-S to our proposed GTP-binding protein and thereby induces bumps.

The reason for our cautious disclaimer about cyclic nucleotides should now be clear. The effects of F^-, VO_3^-, and GTP-γ-S are only suggestive of the presence of N in the photoreceptor. These effects say nothing about the catalytic moiety C that is being regulated by N. Cyclic nucleotides can be shown to be involved in excitation only by demonstrating a specific effect of introducing the nucleotide to the interior of the photoreceptor. To our knowledge the results of such experiments have not as yet been reported for ventral photoreceptors. Another reason for caution is the finding that in other systems N may regulate effector molecules other than catalytic adenylate cyclase (*6*).

If an N-like protein is present in invertebrate photoreceptors, there must be biochemical evidence for its existence, in addition to the pharmacologic evidence presented above. In the hormonal system described above, N exhibits hormone-activated guanosine triphosphatase activity. By analogy, photoreceptors would be expected to exhibit a light-activated guanosine triphosphatase activity, and such activity has been reported in the photoreceptors of the octopus (*13*), an invertebrate.

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5. Standard recording and stimulation techniques that have been described in detail were used [A. Fein and J. S. Charlton, *J. Gen. Physiol.* **66**, 823 (1975); *ibid.* **69**, 553 (1977)]. GTP-γ-S, ATP-γ-S, and GMP-PNP were obtained from Boehringer Mannheim (Indianapolis, Ind.), and ATP (adenosine 5′-triphosphate) and GTP were obtained from Sigma (St. Louis, Mo.). Microelectrodes for intracellular injection and recording were filled with 100 m*M* solutions of the above compounds. All solutions were titrated to within 0.5 *p*H unit of *p*H 7.0 with sodium hydroxide or hydrochloric acid. All injections were done iontophoretically. Sodium vanadate was obtained from Fisher Scientific (Fairlawn, N.J.); VO_3^- was injected intracellularly by use of electrodes filled with either 100 m*M* $NaVO_3$ or 10 m*M* $NaVO_3$ mixed with either 100 m*M* KCl or 100 m*M* KAc. Similar results were obtained with all three solutions. Cells were also exposed to VO_3^- by adding $NaVO_3$ to the ASW (*4*) that bathed the preparation. All solutions of the above chemicals were freshly prepared before each experiment.
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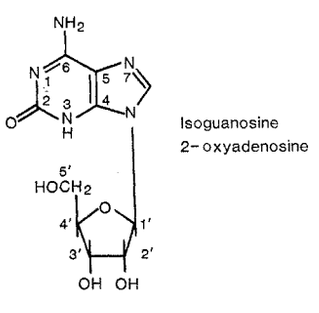
Isoguanosine: Isolation from an Animal

Abstract. *Isoguanosine (oxyadenosine or crotonoside), previously known to occur in nature only in the croton bean, was isolated from an animal, the marine nudibranch mollusk* Diaulula sandiegensis.

When Emil Fischer synthesized isoguanine (oxyadenine) in 1897, he predicted that it would soon be found in nature (*1*). In fact, isoguanine has been isolated only from butterfly wings (*2*). A report that it occurs in pig blood (*3*) could not be confirmed (*4*). The riboside of isoguanine, isoguanosine (crotonoside), has hitherto been known in nature only as a constituent of the croton bean, *Croton tiglium* L. (*5*). We now report isolation of isoguanosine (**1**) from an animal, the marine nudibranch mollusk *Diaulula sandiegensis*. The biological role of isoguanosine has been neglected, probably because this purine riboside has never before been known to occur in an animal. Nevertheless, isoguanosine has most of the attributes of other purine ribosides. In mammals it produces hypotension, bradycardia, and relaxation of smooth muscle (*6*), but it is more potent and much longer-acting than adenosine. Like adenosine and certain other of its analogs, it stimulates accumulation of adenosine 3′,5′-monophosphate (cyclic AMP) in brain tissue (*7*). It is reported to have negligible antitumor activity (*8*).



Isoguanosine
2-oxyadenosine

1

Approximately 100 specimens of *Diaulula sandiegensis* Cooper, a rather common dorid nudibranch from the Pa-

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