Nitric Oxide and the Control of Firefly Flashing

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Bioluminescent flashing is essential for firefly reproduction, yet the specific molecular mechanisms that control light production are not well understood. We report that light production by fireflies can be stimulated by nitric oxide (NO) gas in the presence of oxygen and that NO scavengers block bioluminescence induced by the neurotransmitter octopamine. NO synthase is robustly expressed in the firefly lantern in cells interposed between nerve endings and the light-producing photocytes. These results suggest that NO synthesis is a key determinant of flash control in fireflies.

Firefly courtship depends on a remarkable flash communication system involving precisely timed, rapid bursts of bioluminescence. The duration of a single flash is typically a few hundred milliseconds, and flash patterns vary among firefly species (*1*, *2*). Bursts of neural activity stimulate release of the primary neurotransmitter octopamine, which triggers the firefly light-producing organ (the lantern) in the abdomen to emit light through the luciferin-luciferase reaction (*3*–*5*). However, the pathway between neurotransmitter release and light production remains unknown.

The ability to modulate flash duration is correlated with distinctive anatomical features of the firefly abdominal lantern (*6*). Light production involves an adenosine triphosphate– and O_2-dependent luciferin-luciferase reaction occurring within photocytes in the firefly lantern (*3*). Theories of flash control have focused on the regulation of O_2 supply to luciferin-containing organelles (peroxisomes) within photocytes (*6*, *7*). The peripheral cytoplasm of photocytes is densely packed with mitochondria, which have been proposed to act as gatekeepers that control O_2 access to the light-producing reactions in the more centrally located peroxisomes (*6*, *7*). Neurons that innervate the lantern do not terminate directly on the photocytes themselves but synapse on tracheolar cells surrounding terminal branch points of the tracheal air supply (*8*). The activation of photocytes therefore requires a signal to pass from the tracheolar cells to the peroxisomes, a distance of about 17 μm (*9*). One potential transmitter that can penetrate cell membranes and quickly cross such distances is the free radical gas nitric oxide (NO).

The effect of NO gas on the flashing behavior of intact adult *Photuris* sp. fireflies (*10*) was examined by placing individual fireflies into an observation chamber in the dark with a steady flow of N_2/O_2 (80%/20%) for 5 min. When NO gas was introduced at 70 parts per million (ppm) (a dose commonly used for NO inhalation in human patients), flashing began almost immediately (Fig. 1). Most fireflies (10 of 13 tested) exhibited a continuous lantern glow, accompanied by an increase in firefly motor activity, when exposed to NO. Flashing was more sustained and rapid (up to two per second) than normal flash patterns. The effect of NO on firefly light production depended on the simultaneous presence of O_2 (*11*).

Because NO is an important signal in the peripheral and central nervous systems (CNS) of many insects (*12*–*14*), increased flash rate and locomotor behavior in fireflies exposed to NO gas might represent CNS effects rather than direct effects on the lantern. Therefore, we explored whether isolated lanterns maintained in insect saline also responded to NO or nitric oxide synthase (NOS) inhibitors. However, lanterns separated from their tracheal air supply and hemolymph glowed continuously (*4*), and it was difficult to evoke consistent flashes in response to the primary lantern neurotransmitter, octopamine. Despite disrupted O_2 levels in photocytes resulting from cut tracheae and exposure to insect saline, isolated lanterns exhibited brighter glow in the presence of the NO donors diethylamine NONOate, NOC-12, and NOC-7 (*15*, *16*). The effect of NO appears to be independent of guanylyl cyclase because assays of cyclic guanosine monophosphate (cGMP) levels in NO-treated lanterns failed to detect increases over the very low basal levels (*15*, *16*).

Because fully dissected firefly lanterns yielded inconsistent bioluminescent responses, we developed a partially dissected preparation in which descending neural inputs were removed but the tracheal air supply to the lantern remained largely intact. When placed in the observation chamber, these exposed lanterns showed no spontaneous flash activity, but when NO was introduced, they glowed brightly. Application of octopamine (10 to 100 μM) evoked transient light production (Fig. 2), which was abrogated by treatment with the NO scavenger carboxy-PTIO (CPTIO; 10 to 100 μM). Prolonged washing of firefly lanterns that had been treated with CPTIO restored the bioluminescent response to octopamine (Fig. 2).

The firefly lantern contains thousands of cylindrical units, each consisting of several photocytes arranged radially around a central air-conducting trachea (Fig. 3). These trache-

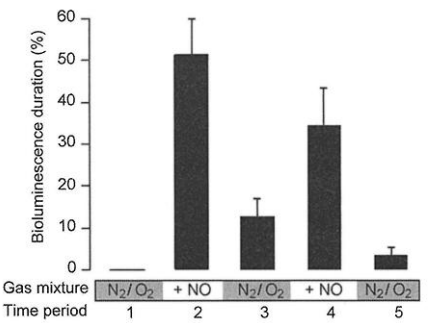

Fig. 1. Bioluminescence response of adult fireflies to NO gas. Lantern bioluminescence (mean percentage + 1 SE of total period duration) for *Photuris* sp. fireflies (n = 13) exposed to gas mixtures for ~4-min periods. During period 1, fireflies showed no bioluminescence when exposed to 80% O_2, 20% nitrogen, but shortly after NO gas (70 ppm) was introduced to the chamber at the start of period 2, lantern bioluminescence (flash and/or glow) increased markedly in all fireflies tested. Bioluminescence diminished when NO was turned off in period 3, returned in period 4 (reexposed to NO), and diminished again without NO in period 5. Differences in bioluminescence response between period 1 (preexposure), periods 2 and 4 (exposed to NO), and periods 3 and 5 (without NO) were highly significant [repeated-measures analysis of variance $F(2,24)$ = 30.8, $P < 0.0001$]. Fireflies were tested individually in a 14-ml plexiglass chamber connected to a multiple flowmeter that allowed precise control of gas mixture; gas flow rate (110 to 120 cm^3/min) through the test chamber and O_2 concentration were kept constant. NO and O_2 concentrations were calibrated in test chamber outflow with a clinical nitroximeter and oximeter. Lantern bioluminescence duration was quantified by recording the number of flashes and glow duration with event recording software, and total bioluminescence duration (seconds) for each period was calculated as glow duration + (number of flashes × 170 ms mean flash duration). A video showing firefly courtship behavior in the field and documenting experimental bioluminescent response to NO is available on *Science* Online (*30*).

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ae originate dorsally and divide repeatedly as they permeate the lantern tissue, ultimately becoming fine tracheoles that radiate out between the photocytes. The distal end of each tracheal branch is ringed by tracheal end cells, each of which encloses an innervated tracheolar cell surrounding a tracheolar branch point. Light production takes place in centrally located photocyte peroxisomes. Photocyte mitochondria are clustered in the peripheral cytoplasm, especially concentrated at locations proximal to the central trachea and along the radiating tracheoles. We used an NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) diaphorase assay as an activity stain for NOS (*17–19*) in firefly lantern preparations. Positive NADPH diaphorase staining (dark precipitate) was observed in tracheal end cells, in tracheolar cells, in the mitochondrial zone of photocytes adjacent to the main trachea, and in a stellate pattern along photocyte edges that may represent either the lateral mitochondrial zone of the photocyctes or tracheolar cells projecting between photocytes (arrowheads in Fig. 3C). No NADPH diaphorase staining was observed when the flavoprotein inhibitor diphenylene iodonium chloride (DPI) was included to inhibit NOS oxidation of NADPH (Fig. 3, B and D) or when NADPH was omitted. Immunocytochemical analyses with an antibody directed against a peptide sequence common to NOS isoforms in multiple species (*18*, *20*) showed a similar pattern (Fig. 3, F and G). Controls (Fig. 3E, no primary antibody) showed no immunofluorescence, except for the autofluorescence characteristic of tracheal walls. This NADPH diaphorase and NOS antibody staining in the firefly lantern suggests that NO is produced in cells interposed between synapses on tracheolar cells and the photocytes.

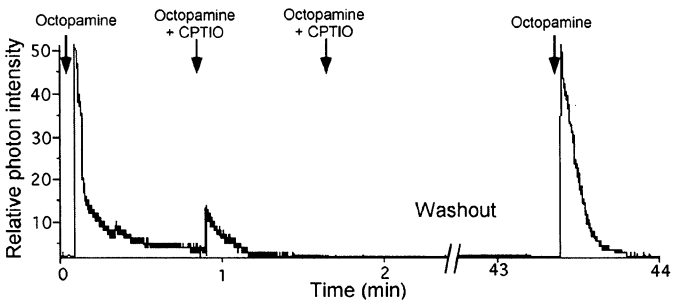

Fig. 2. Response of the firefly lantern to the primary lantern neurotransmitter, octopamine, and the NO scavenger, carboxy-PTIO (CPTIO). Partially dissected lanterns were prepared by decapitating fireflies, removing wings and legs, and removing the cuticle from the dorsal abdominal surface. These in situ lanterns emitted light in response to the application of octopamine (100 μM final concentration, dissolved in Grace's Insect Media), and this response was reproducibly blocked by concurrent application of CPTIO (100 μM). After firefly lanterns were treated with CPTIO, prolonged washout restored the response to octopamine. Light intensity was quantified with a PMT-300 photomultiplier tube (IonOptix) mounted on a Nikon Diaphot microscope. Data shown are representative of four experiments.

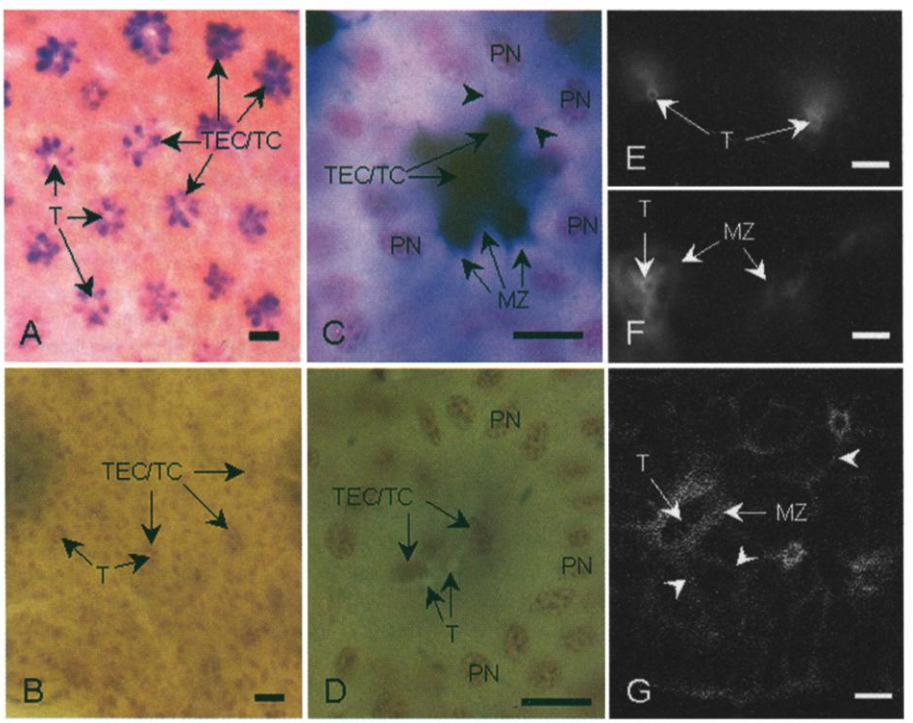

Fig. 3. Localization of NO synthase in the firefly lantern. (**A** to **D**) NADPH diaphorase activity in *Photuris* sp. (A) and (B) show multiple cylinders with tracheal end cells (TEC) and tracheolar cells (TC) surrounding a central main trachae (T). (C) and (D) show higher magnification of single cylinders (scale bars, 20 μm). (A) and (C) show NADPH diaphorase activity localized in the TECs and TCs, in the photocyte mitochondrial zones [MZ in (C); this cytoplasmic region, here termed the mitochondrial zone, has also been called the differentiated zone], and in regions projecting between photocytes [arrowheads in (C)]. (B) and (D) show controls in which NADPH diaphorase activity was inhibited by DPI (1 μM). (B), (C), and (D) were also stained with safranin O to reveal nuclei [photocyte nuclei labeled PN in (C) and (D)]. (**E**), (**F**), and (**G**) show immunolabeling with antibody to uNOS in the firefly lantern viewed by conventional microscopy in *Photinus ignitus* (E and F) and by confocal microscopy in *Photuris* sp. (G). Tracheae (T) of each cylinder autofluoresced in both the controls [(E), no primary antibody] and in antibody-labeled preparations (F and G). Antibody-specific fluorescence appears as a bright halo in the photocyte mitochondrial zone [MZ in (F) and (G)] adjacent to the main trachea. The confocal view (G) shows well-resolved fluorescence along the borders between photocytes [arrowheads in (G)]. In (E) to (G), the focal plane is such that the tracheolar cells and tracheal end cells cannot be clearly seen; in other planes (*15*), fluorescence was also evident in the tracheolar and tracheal end cells. NADPH diaphorase staining, n = 21 preparations; immunolabeling, n = 9 preparations.

The results reported here document an important role for NO in firefly flash control. It is well established that O_2 availability is the immediate biochemical trigger for light production, and we propose that the role of NO is to transiently inhibit mitochondrial respiration in photocytes and thereby increase O_2 levels in the peroxisomes. This is consistent with the distinctive spatial arrangement of NOS-containing cells, the known NO-mediated inhibition of cytochrome c oxidase (*21–23*), and the fact that firefly luminescence can be induced by cytochrome c oxidase inhibitors, such as cyanide and carbon monoxide (*6*, *7*). The kinetically complex termination of a flash (*24*) will involve the relief of respiratory inhibition by NO degradation (*25*), perhaps chemically accelerated by the O_2 increase (*23*). Because NO inhibition of mammalian cytochrome c oxidase is readily reversed by white light (*23*, *26*), it is possible that in the firefly lantern, the flash itself may contribute to the off signal. It remains to be determined how neurally released octopamine activates NOS and what the role might be of octopamine-induced cyclic adenosine monophosphate in the lantern (*27*, *28*). This flash control model is consistent with the dimensions of the lantern functional units (*9*), speed of NO diffusion (*25*, *29*), and time course of the light flash (*6*, *24*). It is fitting

that the remarkable chemical and physical properties of NO, which have only recently been linked to signaling between cells, appear to have long been exploited by fireflies to control signaling between individuals.

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10. *Photuris* fireflies used in this study have been identified by J. E. Lloyd (University of Florida, Gainesville) as belonging to a species group that cannot be distinguished with current taxonomic criteria: We refer to it here as *Photuris* sp.
11. *Photuris* sp. fireflies held for ~2.5 min in a mixture of N_2 and NO did not glow or flash until O_2 was reintroduced. This was not a "pseudoflash" response to O_2 deprivation as previously described by Buck (6), as O_2 reintroduction to insects maintained in pure N_2 for as long as 2 min did not evoke any bioluminescent response.
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16. Isolated, perfused lanterns were exposed to 0.5 mM of each NO donor diluted in saline from a stock solution of 100 mM in 1 mM NaOH. Light emission increased progressively throughout a 5-min application. Control application of saline containing only added NaOH had no effect on light production. cGMP levels were measured in lantern extracts with an enzyme immunoassay with samples from the nerve cord of the insect *Manduca sexta* used as a positive control. The lanterns contained about 10 fM cGMP per microgram of protein, and this was unaffected by prior treatment with the NO donor SNAP (1 mM).
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31. We thank J. Buck and J. Lloyd for inspiration, A. Gustafson and R. Willson for assistance with histochemistry and confocal microscopy, B. Michel and Z. Michel for help in firefly collecting, and J. Buck and H. Ghiradella for discussion and comments on the manuscript. This work was supported in part by grants from the NIH/National Institute of Neurological Disorders and Stroke and NSF to B.A.T., grants to T.M. from the NIH and the Burroughs Wellcome Fund, an NSF grant to S.M.L., and an APA Minority Fellowship in Neuroscience to R.M.Z.

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Femtomolar Sensitivity of Metalloregulatory Proteins Controlling Zinc Homeostasis

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Intracellular zinc is thought to be available in a cytosolic pool of free or loosely bound Zn(II) ions in the micromolar to picomolar range. To test this, we determined the mechanism of zinc sensors that control metal uptake or export in *Escherichia coli* and calibrated their response against the thermodynamically defined free zinc concentration. Whereas the cellular zinc quota is millimolar, free Zn(II) concentrations that trigger transcription of zinc uptake or efflux machinery are femtomolar, or six orders of magnitude less than one atom per cell. This is not consistent with a cytosolic pool of free Zn(II) and suggests an extraordinary intracellular zinc-binding capacity. Thus, cells exert tight control over cytosolic metal concentrations, even for relatively low-toxicity metals such as zinc.

Zinc is an essential element for living organisms (*1*) and is the second most abundant transition metal in seawater and in humans. It is considerably less toxic than redox-active metals such as copper and is more soluble in oxygenated buffers than iron. Zinc serves as a cofactor in all six classes of enzymes as well as several classes of regulatory proteins (*2*, *3*). Several families of integral membrane proteins transport Zn(II), moving it across membranes into and out of cells (*4*, *5*). Less is known about the intracellular chemistry and mechanisms by which Zn(II) is sensed, stored, or incorporated as a cofactor. A common assumption is that Zn(II)-requiring enzymes and transcription factors passively acquire this essential cofactor from a cytosolic pool thought to be 10^{-5} to 10^{-12} M in free Zn(II) (*4*, *6–9*). Direct measurements of cytosolic zinc pools have proved difficult because fractionation can lead to cross contamination between intracellular sites.

Several Zn(II)-responsive transcription factors are known to mediate zinc homeostasis in vivo (*10–15*) and are thought to do so by monitoring changes in this hypothetical pool of free zinc. The mammalian MTF1 sensor is estimated to have a dissociation constant K_d below 90 μM (*4*). Estimates of the zinc sensitivity of the *Synechococcus* PCC7942 SmtB protein vary from <0.01 nM to 3.5 μM (*7*, *8*). Expression of *E. coli* zinc uptake and export genes is regulated by Zur and ZntR metalloregulatory proteins, respectively (*16–20*). We report here the mechanism of Zur and the calibration of both of these zinc-sensing metalloregulatory proteins to directly establish their functional "set point" relative to $[Zn(II)]_{free}$. The femtomolar sensitivity of the pair indicates that intracellular fluxes of zinc between metalloenzymes and metal sensor, storage, and transport proteins are likely to involve direct transfer of the ion between proteins in kinetically controlled substitution reactions.

The total zinc content of *E. coli*, also known as the zinc quota, was established by inductively coupled plasma mass spectrometry (ICP-MS) analysis of whole cell lysate. Cells accumulate each transition element to a different extent, but zinc is concentrated by the greatest factor (Fig. 1). Growth in a metal-depleted medium establishes the minimal quota for this element, or 2×10^5 atoms of zinc per cell [determined here as a colony-forming unit (CFU)] (*21*, *22*) (Fig. 1A). These quotas can be interpreted as total cellular concentrations by dividing the moles per cell by a maximum volume for a cell grown in this medium (Fig. 1, B and C). In the case of minimal medium, the minimal $[Zn(II)]_{total}$ corresponds to 0.2 mM. This value is ~2000 times the ambient total zinc concentration in this depleted medium (Fig. 1B). The ability of microbes to accumulate metals such as iron to such high concentrations under starvation conditions is well established, but not documented for zinc. Cells grown in a medium replete with metals accumulate twice as much zinc per cell; however, the cell volume also doubles (*23*, *24*), leaving the total zinc concentration unchanged (Fig. 1C). Thus, the total concentration of Zn(II) is

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