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) 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 O₂-dependent luciferin-luciferase reaction occurring within photocytes in the firefly lantern (3). Theories of flash control have focused on the regulation of O₂ 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 O₂ 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 tracheal cells surrounding terminal branch points of the tracheal air supply (8). The activation of photocytes therefore requires a signal to pass from the tracheal 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 Photurus sp. fireflies (10) was examined by placing individual fireflies into an observation chamber in the dark with a steady flow of N₂/O₂ (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 O₂ (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 flowed continuously (4), and it was difficult to evoke consistent flashes in response to the primary lantern neurotransmitter, octopamine. Despite disrupted O₂ 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 carboxypatitol (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-
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 photocytes or tracheolar cells projecting between photocytes (arrowheads in Fig. 3C).

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 trachea (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 ([F], 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 O2 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 O2 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 O2 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

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Reports
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.

References and Notes
11. Isolated, perfused lanterns were exposed to 0.5 mM N2 and NO did not glow or flash until O2 was reintroduced. This was not a “pseudoflash” response to O2 deprivation as previously described by Buck (6), as O2 reintroduction to insects maintained in pure N2 for as long as 2 min did not evoke any bioluminescent response.
16. 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 Kd below 90 μM (4). Estimates of the zinc sensitivity of the Synecococcus 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 ZnrR metalloregulatory proteins, respectively (16–20).
17. The femtomolar sensitivity of the pair indicates that intracellular fluxes of zinc between metallothioneins and metal sensor, storage, and transport proteins are likely to involve direct transfer of the ion between proteins in kinetically controlled substitution reactions.
18. 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 × 105 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)]min 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...