INHIBITORS OF cGMP-PHOSPHODIESTERASE AND GUANYLATE CYCLASE ALTER *DROSOPHILA* LIGHT RESPONSES

by

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A large body of evidence shows that *Drosophila* visual transduction occurs through a G-protein linked pathway involving phosphoinositidyl chemistry. However, recent studies indicate that the small second messenger molecule cGMP may also play a role in *Drosophila* vision. To further test this hypothesis, we used cGMP metabolic inhibitors to examine whether endogenous cGMP levels contribute to excitation or regulation of the light response. We used whole-cell patch-clamp technology to record electrical responses to light. LY83583, an inhibitor of cGMP synthesis, increased light response amplitudes in a Ca^{2+} and dosage dependent manner. Zaprinast, an inhibitor of cGMP degradation, increased light response amplitudes during short light flashes, but decreased light response amplitudes during long light flashes. Additionally, in some cells, Zaprinast caused an inward shift in the baseline current. These results support the notion that cGMP has an important role in *Drosophila* vision.

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Introduction

Communication is important on many different levels--society to society, person to person, brain to body, cell to cell, and molecule to molecule. Our bodies gain information about their internal and external world using sensory cells that detect signals (stimuli) such as chemicals, heat, light, or pressure. The flow and regulation of information through the cell gives rise to a physiologic response; however, the pathway by which the information flows and is regulated is not obvious from a macroscopic point of view. Signals cross the cell membrane and often travel to important sub-cellular regions. Additionally, optimizing the accuracy of acquiring information depends on the regulation of the physiologic response; for instance, our eyes must adapt to bright light before we can clearly detect objects in brightly lit environments. The cellular mechanism for detecting and responding to signals is called "transduction"--a complex set of biochemical reactions that allow for the regulation and route of information flow inside a cell.

A general type of cell-signalling used by the body for many essential functions, including vision, metabolism, and cell proliferation, involves G-protein linked pathways (Figure 1A) (Alberts et al., 1994). First, a stimulus activates a receptor protein in the cell membrane enabling the receptor to bind to and activate one or more G-protein molecules. Each G-protein activates an effector enzyme. Effector enzymes are responsible for the synthesis, degradation, or release of second messengers, such as Ca^{2+}, cAMP, or cGMP. Second messengers activate the target protein in the pathway that gives rise to the observed physiologic response. Figure 1B provides a more

specific illustration of what occurs in the cell. Second messengers also stimulate regulatory proteins that can influence the activity of the receptor, effector, target, or other regulatory proteins. In addition, key proteins in the pathway may bind together and regulate each other's activity. Finally, a stimulus, for example a single photon of light, leads to the activation of thousands of target proteins through an amplification process (Figure 1C). The intrinsic characteristics of each receptor, effector, and target protein and the unique orchestration of these molecules account for the diversity among G-protein linked pathways and their functions.

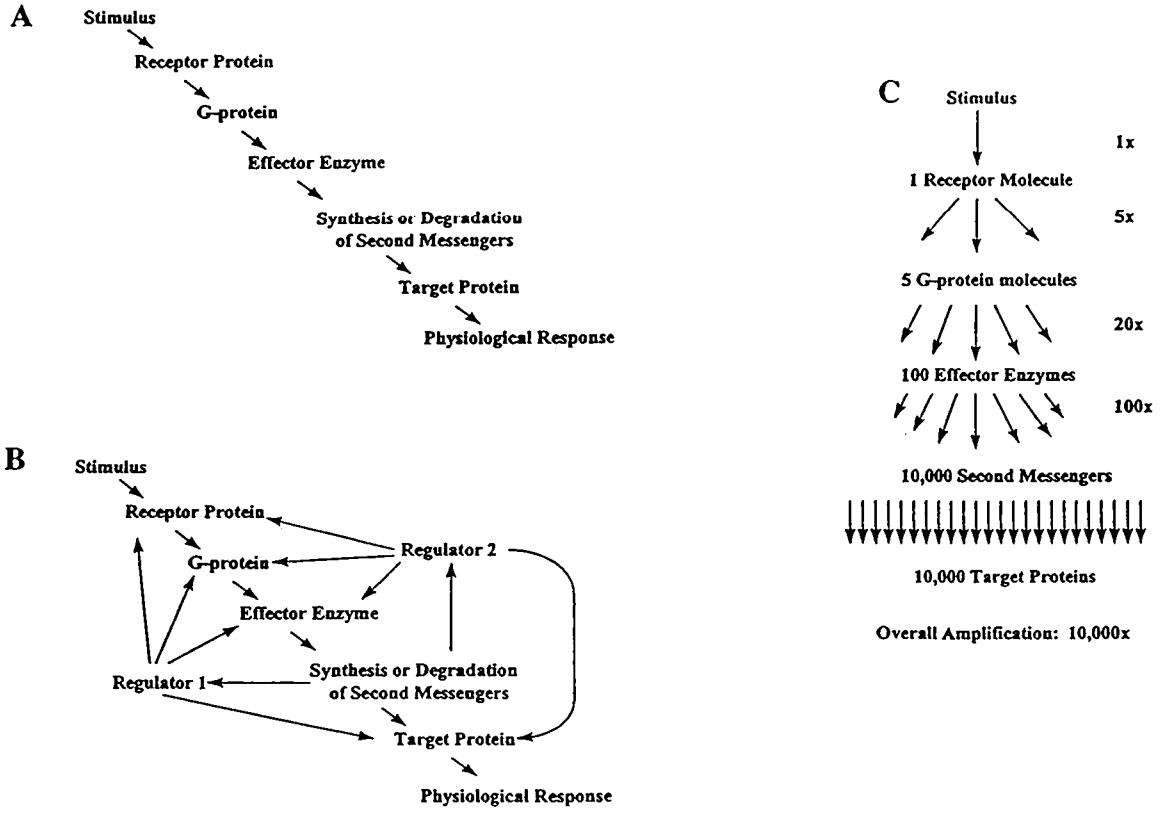

Figure 1. G-protein linked pathways. A, G-protein linked pathways primarily consist of a receptor, G-protein, effector, and target protein and second messengers. B, Regulatory proteins regulate the flow of information by influencing the activities of the primary proteins in the pathway. C, G-protein signalling pathways amplify the stimulus through a series of smaller amplifications.

One widely studied class of G-protein linked pathways is necessary for vision. In acquiring and processing visual stimuli, photoreceptor cells, located in the retina of the eye, absorb light and transfer it into a nerve signal. This transformation process, called visual transduction, occurs through a G-protein linked pathway that converts electromagnetic energy (light) into electrochemical energy (nerve signal). The nerve impulse then travels to higher orders in the brain for further processing. Although this general sequence of events occurs in invertebrate and vertebrate vision, there are differences among the visual transduction pathways of various organisms.

The fruit fly, *Drosophila* melanogaster, is often used to study phototransduction because many visual mutants exist, a short life-cycle allows for rapid genetic manipulation, and their photoreceptors are relatively easy to isolate and prepare for various experimental assays. Several excellent reviews discuss the investigations and details surrounding the known pathway in *Drosophila* visual transduction (Hardie and Minke, 1995; Minke and Selinger, 1996; O'Day et al., 1997; Zuker, 1996).

A large body of evidence suggests that *Drosophila* vision is mediated by the phosphoinositidyl (PI) pathway (Figure 2). Rhodopsin (RH), a receptor protein in the plasma membrane, absorbs a photon of light (hv) and undergoes a conformational change leading to the activation of the trimeric G-protein, DGQ. DGQ activates the effector enzyme, phospholipase C (PLC), which cleaves the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into a lipid soluble component, diacylglycerol (DAG), and a water soluble component, inositol(1,4,5)-trisphosphate (IP_3). IP_3 releases internal Ca^{2+} stores by binding to the IP_3-Receptor (IP_3R), and DAG activates protein kinase C (PKC). The target protein in this pathway is an ion channel that, when activated, allows ions to flow across the cell membrane, thus generating the

physiologic response (a nerve signal) to light. The steps subsequent to Ca^{2+} release and PKC activation that ultimately give rise to the opening of channels and the identity of the light-activated channel(s) remain a mystery.

Several recent findings suggest that the small second messenger molecule cyclic guanosine 3':5'-monophosphate (cGMP) is involved in *Drosophila* vision. First, a gene encoding the β-subunit of a soluble guanylate cyclase (a cGMP-producing protein) is expressed in *Drosophila* retina (Yoshikawa et al., 1993). Second, a cGMP-gated channel, normally expressed in *Drosophila* retina, was expressed in frog oocytes and found to have electrical properties that resemble the light response (Baumann et al., 1994). Finally, exogenously applied membrane permeable cGMP facilitated excitation (Bacigalupo et al., 1995).

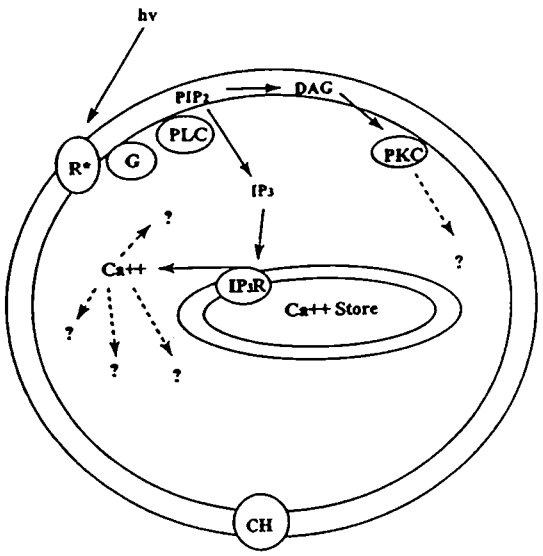

Figure 2. The known *Drosophila* visual transduction pathway depicted inside of a photoreceptor. **hv** light stimulus; **R*** receptor protein, rhodopsin; **G** trimeric G-protein, DGQ; **PLC** effector enzyme, phospholipase C; **PIP_2** membrane phospholipid, phosphatidylinositol 4,5-bisphosphate; **IP_3** water soluble second messenger, inositol-(1,4,5)trisphosphate; **IP_3R** a Ca^{2+} channel imbedded in the membrane of Ca^{2+} stores, IP_3-receptor; **Ca^{++}** water soluble second messenger, calcium ion; **DAG** lipid soluble second messenger, diacylglycerol; **PKC** protein kinase C; **CH** target protein, ion channel(s).

Our study further investigates the potential role(s) of cGMP in *Drosophila* vision using inhibitors of cGMP metabolism to examine whether endogenous cGMP

levels contribute to excitation or regulation. The proteins guanylate cyclase (GC) and cGMP-phosphodiesterase (PDE) control the intracellular concentration of cGMP through synthesis and degradation, respectively. LY83583 (LY) and Zaprinast are drugs that inhibit GC and PDE activity, respectively (Figure 3). If cGMP is involved in *Drosophila* phototransduction, then these inhibitors should affect the wild-type (normal) light response and therefore provide clues about the biochemistry underlying vision. Our preliminary results are consistent with the hypothesis that cGMP has an important role in *Drosophila* visual transduction. This work was done in collaboration with graduate student Joan Haab and Professor Peter O'Day, some of which has appeared previously in abstract form (Haab et al., 1996).

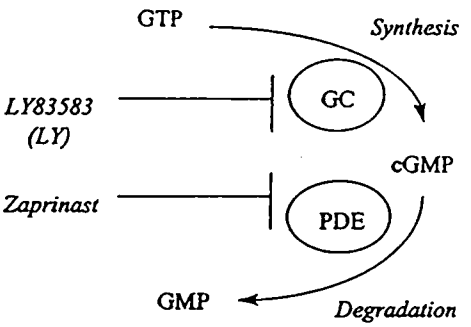

Figure 3. Drug action on cGMP metabolism. LY83583 inhibits the cGMP synthesis protein, guanylate cyclase (**GC**), and Zaprinast inhibits the cGMP degradation protein, cGMP-phosphodiesterase (**PDE**).

Materials and Methods

Flies

Wild type (normal) strain Oregon red or white eyed flies were raised at ~30°C on a standard cornmeal and molasses diet. Late stage pupae were used for all experiments. The eyes were extracted and scraped gently with a dissecting probe to

isolate ommatidia--eight cell photoreceptor clusters. The cells were contained in a bubble of standard bath solution in a dish designed for electrical recordings (Bacigalupo et al., 1995).

Solutions

Bath solution (pH=7.15) contained 120mM NaCl, 5mM KCl, 10mM $MgSO_4$, 10mM TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid), 5mM L-proline, 25mM sucrose, and $CaCl_2$ concentrations as detailed in the figure legend of each experiment. Recording electrodes were filled with either "internal w/TEA" (120mM $CsCl_2$, 15mM TEA-Cl, 2mM $MgSO_4$, 10mM TES, 0.1mM EGTA, 0.5mM GTP, 2mM ATP, pH=7.15) or "internal w/EGTA and Ca^{2+}" (120mM $CsCl_2$, 2mM $MgSO_4$, 10mM TES, 0.06 $CaCl_2$, 0.1mM EGTA, 0.5 GTP, 2mM ATP, pH=7.15). Zaprinast was dissolved in 0.27M sodium hydroxide to prepare a 50mM stock solution and LY was dissolved in a 1:1 water/ethanol solution to prepare a 25mM stock solution. The drugs were further diluted to various concentrations using bath solution.

Solution Control Jet (spritzer)

The spritzer consists of a four-barrel glass pipette and an adjacent single barrel glass pipette that provide an "in flow" and an "out flow" of solution, respectively (Figure 4). One barrel was loaded with control (bath) solution while the other three barrels were loaded with drug solutions at various dosages or calcium concentrations. Two peristaltic pumps regulated the movement of solution. The application of either a control or drug solution was under electronic control.

Whole-cell Recordings of Electrical Responses

We used whole-cell patch clamp (voltage clamp) technology to measure the electrical responses to light stimuli. Voltage clamp allowed us to control the transmembrane voltage while quantitatively measuring the amount of current flowing across the cell membrane as a function of time (Nicholls et al., 1992). Patch pipette resistances varied from ~7-12 MΩ and seal resistances were typically 5GΩ or higher. Current was measured and amplified by patch clamp, AXOPATCH 200 (Axon Instruments). Data acquisition and analysis were performed using pClamp Version 6.0 software (Axon Instruments). The light source--white light--was logarithmically filtered using neutral density (ND) filters ranging from 0-4 NDs (0 corresponding to no attenuation of light and 4 corresponding to a 10,000 fold attenuation of light), as described previously (Bacigalupo et al., 1995).

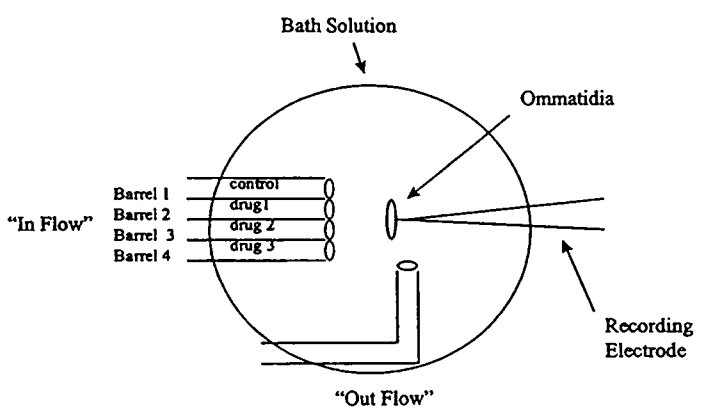

Figure 4. A schematic of the experimental setup. An ommatidia rests in the center of a bubble in a dish designed for electrical recordings. The spritzer's four barrel glass pipette and adjacent single barrel glass pipet provide an inflow and outflow of control or drug solution, respectively. A recording electrode measures current flowing across the cell membrane and sends this information to an amplifier and then a computer.

Results

To investigate the effects of cGMP metabolic inhibitors, control (normal) light

responses must be characterized. Typical light responses from wild-type (WT) photoreceptors are shown in Figure 5. The waveform is characterized by the amplitude (size) and shape of the light response. WT responses vary with internal and external Ca^{2+} concentrations and the duration and intensity of the light stimulus. Additionally, there is some variability in responses from cell to cell. Therefore, we used one of several WT responses immediately preceding drug application as a standard in analyzing the effects of a given drug spritz.

Figure 5. Wild-type responses to a short and long duration light stimulus. A, A light response to a short (400ms) light flash. B, A response to a long (3s) light flash.

Exogenous application of the GC inhibitor, LY, enhanced light response amplitudes. We exposed WT photoreceptors to constant intervals of short (400ms) or long light (3s) flashes in the presence of bath until we observed consistent light response waveforms. Next, we spritzed (sprayed) the cell with drug solution until we observed a change in the light response and a stabilization of the drug effect. We then re-spritzed the cell with bath solution. The external Ca^{2+} and drug concentrations and

the light-intensity were kept constant. In the presence of LY, light response amplitudes increased during both short (Figure 6A) and long (Figure 6B) test flashes. These results were surprising because earlier studies indicate that exogenously added cGMP increases the light response amplitude (Bacigalupo et al., 1995), thus leading us to predict that a decrease in endogenous cGMP concentrations, due to LY, would cause a decrease in light response amplitudes.

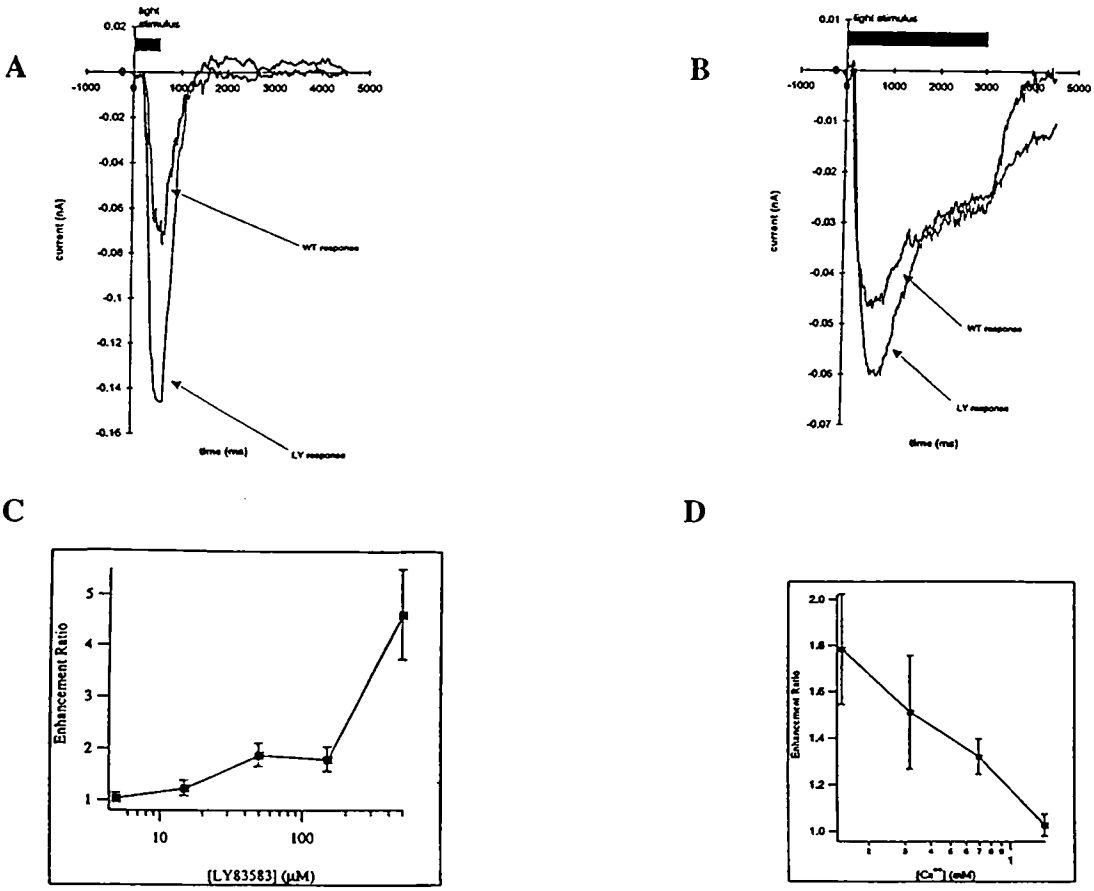

Figure 6. LY effects. A, LY application to photoreceptors during short light flashes resulted in an increase in the light response amplitudes. The WT response was taken prior to the drug spritz as a standard for comparison. B, During long light flashes, photoreceptors, in the presence of LY, again showed larger light response amplitudes. C, LY effects were dosage dependent. The ratio of the amplitude of the LY response to a WT response (enhancement ratio) was plotted versus LY concentrations of 15μM, 50μM, 150μM, and 500μM. 500μM LY yielded the greatest effects. D, LY effects were also Ca^{2+}-dependent. The enhancement ratio was plotted versus spritzer Ca^{2+} concentrations of 0.15mM, 0.32mM, 0.70mM, and 1.5mM. No LY effects were seen at 1.5mM Ca^{2+}, whereas the greatest LY effects were observed at 0.15mM Ca^{2+}.

The LY effect was dosage-dependent. For this experiment, we exposed photoreceptors to successively higher concentrations of LY following a bath control spritz. Spritzer Ca^{2+} concentrations did not vary. Figure 6C plots the enhancement ratio (the relative increase in light response amplitude for a given photoreceptor) as a function of the spritzer concentration of LY. The general trend indicates that higher LY concentrations result in greater light response enhancement.

The LY effect was also dependent on the external Ca^{2+} concentration. We loaded each of the three available spritzer barrels with the same concentration of LY, but with three different Ca^{2+} concentrations. After a control spritz, we applied LY continuously while successively increasing the Ca^{2+} concentration. Figure 6d illustrates that the enhancement ratio varied with the concentration of Ca^{2+} in the bath solution. At high Ca^{2+} concentrations (1.5mM) there was no obvious effect of LY whereas at low Ca^{2+} concentrations (0.15mM) LY had the greatest effect.

The LY effect showed no apparent dependence on the intensity of the light stimulus. To test the light intensity dependence of the drug effect, we compared LY data conducted with various neutral density filters (see MATERIALS AND METHODS). The extent of LY effects on light responses was the same for all NDs (data not shown).

To further investigate the effects of inhibiting cGMP metabolism, we examined the effects of introducing a specific inhibitor of PDE, Zaprinast. Zaprinast reversibly altered the baseline current and inconsistently affected the light response amplitude. The protocol used here is identical to the one used to detect effects of LY at constant light-intensity and Ca^{2+} and drug concentrations. Figure 7A shows a large inward shift in baseline current as a result of a Zaprinast spritz. Shortly after the termination of

the spritz, the baseline returned to the original position. Figure 7B shows that Zaprinast increased the light-response amplitude during short light flashes. Figure 7C illustrates that Zaprinast decreased the light response amplitude and caused a dramatic change in the shape of the light response during long light flashes.

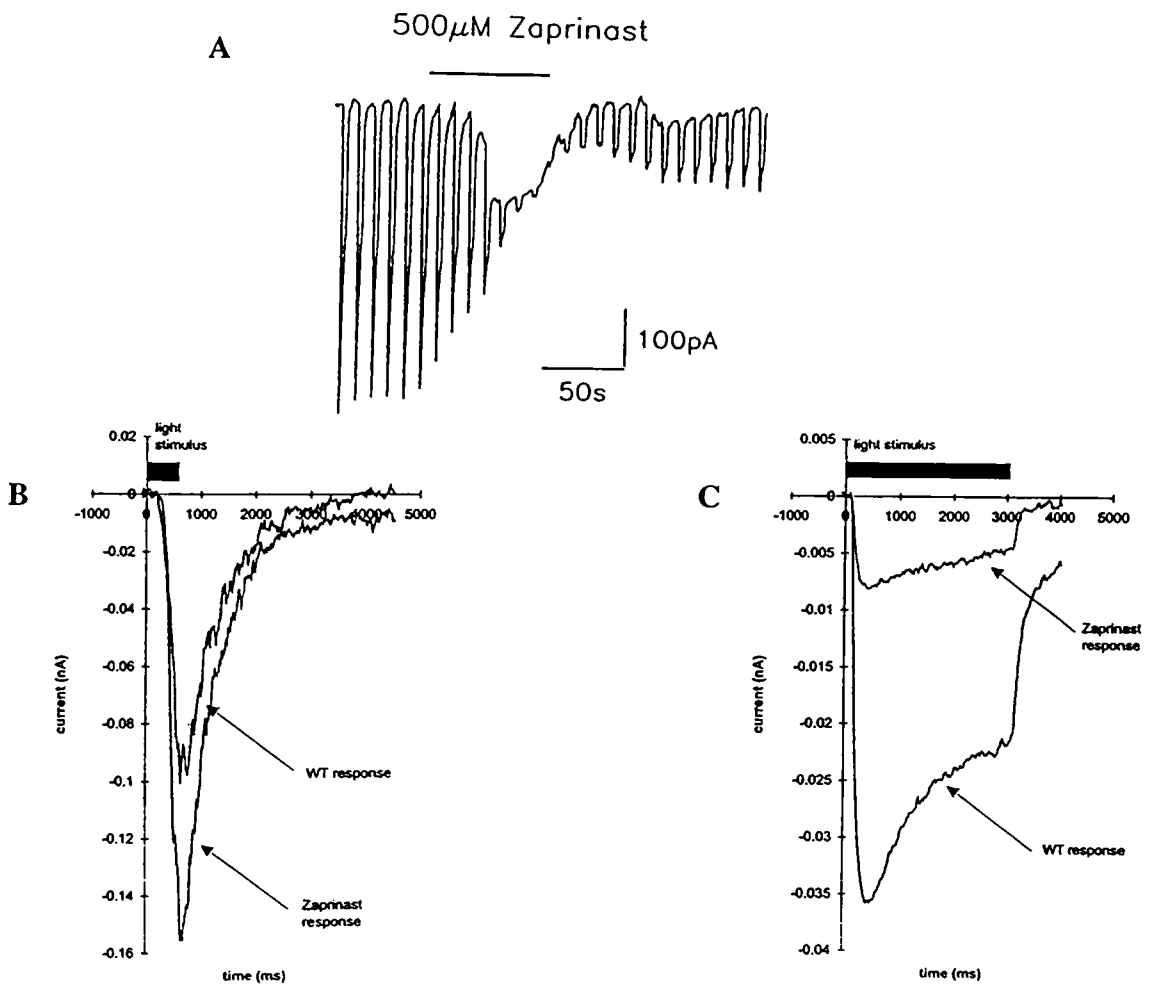

Figure 7. Zaprinast effects. A, Zaprinast induced an inward current. While successively flashing a photoreceptor with long light flashes, a Zaprinast application (the black bar) caused the baseline to shift inward (downward). Upon termination of the spritz, the baseline returned to its original level. B, Zaprinast increased light response amplitudes during short light flashes. A WT response taken prior to the drug spritz is superimposed for comparison. C, During long light flashes, Zaprinast decreased light response amplitudes and dramatically changed the shape of the light response.

Again, based on earlier studies with exogenously added cGMP (Bacigalupo et al., 1995), we expected that a rise in endogenous cGMP, due to Zaprinast, would

increase the light response amplitude. Although Zaprinast increased light response amplitudes during short light flashes, it did not during long light flashes. Later, we discovered that we had inadvertently used two different internal solutions, one with Ca^{2+} and the other without Ca^{2+}, in the experiments depicted by Figure 7B and 7C, although all other conditions were identical. This difference in solutions most likely accounts for the apparent discrepancy of the results. Nevertheless, these results are important to consider because they reveal the sensitivity of the drug-induced effect to the internal Ca^{2+} concentration (see Discussion).

The drug-dosage, light-intensity, and Ca^{2+}-dependencies of the Zaprinast effect have not yet been determined due to insufficient data. However, early results indicate that a higher drug dosage corresponds to a more dramatic effect. In addition, Zaprinast is Ca^{2+}-dependent in studies done on the marine invertebrate *Limulus* (Johnson and O'Day, 1995); thus, we expect that Zaprinast is also Ca^{2+}-dependent in *Drosophila* because light response phenomenonology is conserved among invertebrates, specifically among species having photoreceptor structures called rhabdomeres--the sub-cellular region where transduction takes place (Bacigalupo and O'Day, 1996). Finally, in some cases, Zaprinast had no observable effect on light-induced excitation.

Discussion

These preliminary results suggest that cGMP is involved in *Drosophila* visual transduction and that cGMP-related chemistry may interact with another important transduction component, Ca^{2+}. Although the results presented here and by others do not provide a clear model of cGMP involvement, they contribute to a growing pool of

evidence suggesting that cGMP plays a significant role in excitation, regulation, or both.

One possibility is that cGMP functions as a channel gating ligand--a small molecule that binds to channels allowing them to open. If this is true, we would expect that an increase in intracellular cGMP concentration would induce an inward current and increase the magnitude of the light response. It is likely that Zaprinast application effectively raises cGMP levels because it inhibits cGMP degradation. Therefore, Zaprinast should induce an inward current and increase the light response amplitude. Photoreceptors exposed to short light flashes in the presence of Zaprinast indeed showed increased light-response amplitudes and in some experiments Zaprinast induced inward currents. However, Zaprinast decreased the light response amplitude during long light flashes. The use of two different internal solutions may account for this apparent discrepancy because one solution contained Ca^{2+} and the other did not. Intracellular Ca^{2+} concentrations influence the flow of ions through an ion channel, especially if the channel primarily conducts Ca^{2+} ions. Additionally, Ca^{2+} is known to regulate enzyme and ion channel activity through several mechanisms, including binding to cofactor regulatory sites or Ca^{2+} binding proteins that modulate the activity of other proteins (Berridge, 1993). Therefore, it is plausible that changes in intracellular Ca^{2+} concentrations influence PDE activity, and thus alter Zaprinast effects. LY effectively lowers cGMP concentrations by blocking cGMP synthesis and should thus lead to a decrease in the magnitude of the light response, according to the ligand theory. Yet, the results showed increased light response amplitudes. The LY and Zaprinast results do not provide strong support for the notion that cGMP-gated channels are responsible for the light response, although the data do not necessarily

refute the idea.

Alternatively, cGMP may be involved in regulating the light response. Since the wild-type light response and the LY effects are Ca^{2+}-dependent and the apparent inconsistency in some of the Zaprinast data may be due to differences in intracellular Ca^{2+} concentrations, cGMP may regulate Ca^{2+} levels--an important part of excitation and adaptation. For example, cGMP may act via a kinase--a general class of enzymes that modulate other proteins' activities via phosphorylation (the addition of a phosphate chemical group to a protein). Protein Kinase G (PKG) is a sub-class of kinases specifically activated by cGMP (Lohmann et al., 1997). Based on studies done in other systems, PKG is known to act on classes of proteins that are involved *Drosophila* phototransduction (Figure 8), including the IP_3R, sodium/calcium exchanger, and Ca^{2+}-ATPase (O'Day et al., 1997). These proteins are responsible for emptying and replenishing internal Ca^{2+} stores. By activating PKG, cGMP may regulate the movement of Ca^{2+} into and out of the internal Ca^{2+} stores and the extracellular space, and therefore modulate vision.

Another possibility is that *Drosophila* vision involves two simultaneously functioning pathways (O'Day et al., 1991), the PI and a cGMP pathways (Johnson et al., 1997; Johnson and O'Day. 1995). Pharmacological studies on *Limulus* and physiological investigations of *Drosophila* (Bacigalupo et al., 1995) suggest that invertebrate vision is PI and cGMP-dependent. Two or more channels may be involved in the light response, one of which is gated by cGMP. Additionally, both Ca^{2+} and cGMP may be required to activate channels. Recent controversial studies, however, suggest that the PI pathway may not directly give rise to the light response. One investigation shows that IP_3, and thus at least half of the PI pathway, is not

essential for *Drosophila* vision (Acharya et al., 1997). Additionally, in whole-cell recordings, IP_3, Ca^{2+}, or DAG do not mimic excitation in *Drosophila* (Hardie and Minke., 1995; Bacigalupo and O'Day, unpublished observations). It is possible that the PI pathway is necessary for cell-membrane maintenance and integrity (O'Tousa, 1990; Ranganathan et al., 1995), without which the generation of a light responses would not occur (O'Day et al., 1997). If further studies support the notion that the PI pathway does not directly give rise to *Drosophila* light responses, then a cGMP-dependent pathway will be the only known pathway directly relevant to the generation and/or regulation of the light response.

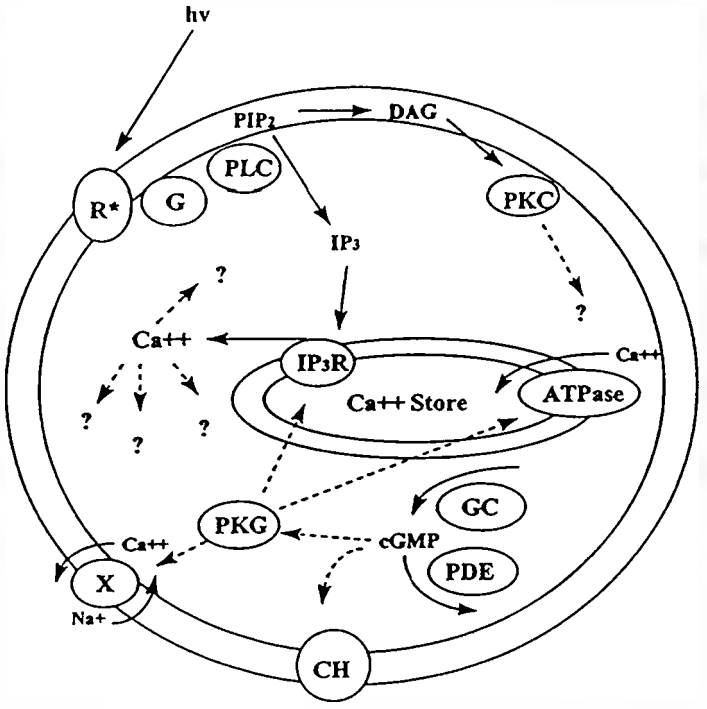

Figure 8. Potential cGMP targets. cGMP may activate protein kinase G (**PKG**) which is known to phosphorylate a Na^+/Ca^{2+} exchanger (**X**), **IP_3R**, and Ca^{2+} ATPase (**ATPase**) in other systems. PKG modulation of the these proteins may regulate the light response by altering Ca^{2+} movement into and out of the internal Ca^{2+} stores and extracellular space.

The general significance of researching fruit fly vision is quite broad, relating back to the idea that clear and efficient communication is central to our well-being.

Recent studies suggest that defective PI and cGMP pathways are causative agents of dysfunctional cell-signalling diseases such as cancer, heart disease, Alzheimer's disease, and retinal degeneration (Powis, 1991; Ghoshal, 1995; Cohen and Downey, 1996; Wong, 1994; Wellman et al., 1996; Barger and Mattson, 1995; Barger et al., 1995; Pacheco, 1996). An understanding of the biochemical and molecular basis of *Drosophila* vision may provide further insights into the pathology of such diseases and development of therapeutic solutions. In addition to aiding biomedical science, *Drosophila* vision research has expanded our general knowledge of biology and will most likely continue to do so in the future. *Drosophila* visual mutants *trp* and *trp-l* have led to the discovery of new ion channels types in the human nervous system (Zitt et al., 1996). Additionally, since Ca^{2+} regulation is a ubiquitous part of nearly all known cell-signalling pathways, a model of Ca^{2+} regulation in *Drosophila* vision will most likely have widespread implications. It is clear, by the complexity of the present study's and others' results, that future investigations will need to continue to draw upon many disciplines, including biochemistry, genetics, pharmacology, and electrophysiology, to help solve the mysteries of cell-signalling and aid biomedicine in finding cures to debilitating and deadly diseases.

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