Inositol 1,4,5-trisphosphate–induced Calcium Release Is Necessary for Generating the Entire Light Response of *Limulus* Ventral Photoreceptors

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ABSTRACT The experiments reported here were designed to answer the question of whether inositol 1,4,5-trisphosphate (IP_3)-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors. For this purpose the membrane-permeable IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2APB) (Maruyama, T., T. Kanaji, S. Nakade, T. Kanno, and K. Mikoshiba. 1997. *J. Biochem. (Tokyo).* 122:498–505) was used. Previously, 2APB was found to inhibit the light activated current of *Limulus* ventral photoreceptors and reversibly inhibit both light and IP_3 induced calcium release as well as the current activated by pressure injection of calcium into the light sensitive lobe of the photoreceptor (Wang, Y., M. Deshpande, and R. Payne. 2002. *Cell Calcium.* 32:209). In this study 2APB was found to inhibit the response to a flash of light at all light intensities and to inhibit the entire light response to a step of light, that is, both the initial transient and the steady-state components of the response to a step of light were inhibited. The light response in cells injected with the calcium buffer 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) was reversibly inhibited by 2APB, indicating that these light responses result from IP_3-mediated calcium release giving rise to an increase in Ca_i. The light response obtained from cells after treatment with 100 μM cyclopiazonic acid (CPA), which acts to empty intracellular calcium stores, was reversibly inhibited by 2APB, indicating that the light response after CPA treatment results from IP_3-mediated calcium release and a consequent rise in Ca_i. Together these findings imply that IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors.

KEY WORDS: phototransduction • microvillar photoreceptors • inositol 1,4,5-trisphosphate receptor • 2-aminoethoxydiphenyl borate • calcium buffers

INTRODUCTION

The microvillar photoreceptors of invertebrates respond to light stimulation with a graded depolarization, called the receptor potential. Microvillar photoreceptors utilize the phosphoinositide cascade to couple photon absorption by rhodopsin to the activation of the ion channels in the plasma membrane that give rise to the receptor potential (for reviews see Fein and Cavar, 2000; Hardie and Raghu, 2001). The phosphoinositide cascade utilizes phospholipase C to generate two intracellular messengers via the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2),* soluble inositol 1,4,5-trisphosphate (IP_3), and membrane-bound diacylglycerol (DAG). For *Limulus* ventral photoreceptors the DAG branch of the phosphoinositide cascade does not appear to be involved in the activation of the ion channels that give rise to the receptor potential (Dabdoub and Payne, 1999; Fein and Cavar, 2000). Rather, the evidence indicates that calcium release from intracellular stores, mediated by IP_3, plays an important role in the generation of the receptor potential. Intracellular pressure injection of IP_3 (Brown et al., 1984; Fein et al., 1984) or calcium (Payne et al., 1986a) into the R-lobe of *Limulus* ventral photoreceptors activates an ionic conductance with a reversal potential similar to that of the light-induced conductance. Moreover, injection of IP_3 into the R-lobe releases calcium from intracellular stores (Brown and Rubin, 1984; Payne and Fein, 1987) and previous injection of calcium buffers effectively block excitation produced by a subsequent injection of IP_3 (Payne et al., 1986b).

If IP_3-mediated calcium release is solely required for generating the entire light response of *Limulus* ventral photoreceptors, it must then be true that IP_3-mediated calcium release is both necessary and sufficient for generating the light response. Previous studies aimed at testing whether IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors have yielded conflicting results that make one suspicious of the conclusions based on these studies (Frank and Fein, 1991; Faddis and Brown, 1993). These studies used the aminoglycoside antibiotic neomycin, which is thought to act by binding to

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**Abbreviations used in this paper:* A-lobe, light-insensitive arhabdomeral lobe; ASW, artificial sea water; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CPA, cyclopiazonic acid; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3-R, IP_3-receptor; PIP_2, phosphatidylinositol-4,5-bisphosphate; R-lobe, light-sensitive rhabdomeral lobe; 2APB, 2-aminoethoxydiphenyl borate.

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PIP_2, thereby preventing the production of IP_3 and also heparin an inhibitor of IP_3-induced calcium release, which appears to function by binding to the IP_3-R (Frank and Fein, 1991; Faddis and Brown, 1993). The findings in these studies led to a similar suggestion, that IP_3-induced calcium release only mediates a portion of the light response in *Limulus* ventral photoreceptors or, stated differently, that visual excitation can occur in the absence of IP_3-induced calcium release (Frank and Fein, 1991; Faddis and Brown, 1993). Although these studies used the same agents, a number of the experimental findings were significantly different and the reasons for these differences have never been determined; consequently, the conclusions based on these findings are suspect. The purpose of the present study was to reexamine the question of whether IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors using an inhibitor that was unavailable at the time when these earlier studies (Frank and Fein, 1991; Faddis and Brown, 1993) were undertaken. The experimental evidence as to whether IP_3-induced calcium release is sufficient for generating the light response is considered in DISCUSSION.

The membrane-permeable IP_3-R antagonist 2APB (Maruyama et al., 1997) has proven to be an effective inhibitor of IP_3-mediated calcium release in intact cells of vertebrates and invertebrates (Maruyama et al., 1997; Ma et al., 2000; Koganezawa and Shimada, 2002). 2APB was not found to alter either agonist-mediated IP_3 production or IP_3 binding to its receptor (Maruyama et al., 1997; Ma et al., 2000). Moreover, 2APB was found to rapidly penetrate *Xenopus* oocytes to inhibit IP_3-mediated calcium mobilization and recovery was rapid and essentially complete when 2APB was washed out (Chorna-Ornan et al., 2001). When *Limulus* ventral photoreceptors were exposed to 2APB (Wang et al., 2002) it was found that the light-activated current was inhibited in a concentration-dependent manner. Moreover, 100 μM 2APB reversibly inhibited both light- and IP_3-induced calcium release as well as the current activated by pressure injection of calcium into the R-lobe. Thus, 2APB has the advantage, in *Limulus* ventral photoreceptors, of inhibiting both IP_3-induced calcium release and the current activated by the released calcium. For these reasons 2APB was chosen to reexamine the question of whether IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors.

MATERIALS AND METHODS

Methods for removing and preparing the ventral nerves containing the photoreceptors of *Limulus* have been described previously (Fein and DeVoe, 1973). Briefly, ventral nerves were dissected out, desheathed, treated with 1% pronase, and pinned to Sylgard in the bottom of a plexiglass chamber containing artificial sea water (ASW) (in mM, 435 NaCl, 10 KCl, 10 $CaCl_2$, 25 $MgSO_4$, 20 $MgCl_2$, 10 HEPES, pH 7.0). The chamber had a volume of 1.0 ml and ASW superfused through the chamber at a rate of 3 ml/min. Calcium-free ASW, that is EGTA-ASW, was made by replacing the $CaCl_2$ with 1mM EGTA (Sigma-Aldrich). An Axoclamp-2A current and voltage clamp amplifier (Axon Instruments, Inc.) was used for intracellular recording of membrane potential or for recording membrane current when performing a two electrode voltage clamp.

Cells were stimulated with white light from a 45-W tungsten-halogen bulb focused on the preparation (Fein and Charlton, 1975). The intensity of the unattenuated beam was 3.5 mW/cm^2, and stimulus intensities are given as $\log_{10} I/I_0$, where I_0 is the unattenuated light intensity. Cells were illuminated throughout the experiments with infrared light from a substage illuminator, allowing for visualization of the photoreceptors (and substances injected into them) with an infrared-sensitive video camera (Corson and Fein, 1983). Cells were impaled with either one or two single barreled microelectrodes, which were formed on a P-97, Flaming/Brown micropipette puller (Sutter Instrument Co.). Pressure pulses of 20–40 psi having duration of 100–200 ms were typically used to inject substances into cells from such electrodes.

Previous work has shown that ventral photoreceptors are segmented into two lobes: an A-lobe and an R-lobe (Calman and Chamberlain, 1982; Stern and Lisman, 1982). The concentration of substances injected into cells is given as the concentration in the injection electrode. Because the cells are compartmentalized and because there is no information about how rapidly substances are metabolized by the cell actual intracellular concentrations of injected substances are not given (Frank and Fein, 1991).

PClamp 8 was used to record experimental data by computer (Axon Instruments, Inc.). Data from pClamp was output to Origin 7.0 (Microcal Software) and Coreldraw 11 (Corel Corp.) for final preparation of figures.

2APB (CALBIOCHEM) was stored as a 20 mg/ml stock solution in DMSO, which was diluted in either ASW or calcium-free ASW immediately before use. Cells were exposed to varying concentrations of 2APB for 10 min, the exposure time used in a previous study that examined the effects of 2APB on *Limulus* ventral photoreceptors (Wang et al., 2002). This ensured that the results reported here could be directly compared with these previous findings. Cyclopiazonic acid (CALBIOCHEM) was stored as a 10 mM stock solution, which was diluted in calcium-free ASW immediately before use.

RESULTS

The effects of 100 μM 2APB on the initial transient and the steady-state components of the response to a 6-s step of light are shown in Fig. 1. If IP_3-mediated calcium release is necessary for generating the entire light response then one would expect 2APB to block both the transient and steady-state components of the response at all light intensities. The light responses, measured under voltage clamp, at two different light intensities differing by a thousand fold in light intensity ($\log_{10} I/I_0 = -5.0$ and $\log_{10} I/I_0 = -2.0$) are shown in Fig. 1. Fig. 1, A and C, shows the initial components of the response on an expanded time scale and low gain because the initial light-induced currents turn on and off rapidly and are one to two orders of magnitude

greater than the steady-state currents at these light intensities. Fig. 1, B and D, shows the steady-state components of the light responses at these intensities on a much slower time scale and much higher gain. It took ~40 min for the cell in Fig. 1 to recover from exposure to 100 μM 2APB. It is quite clear in Fig. 1 that 100 μM 2APB reversibly inhibits the entire light response at these two light intensities. It can also be seen that 2APB slows the rising phase of the light response.

For this particular cell the light responses after washout of 2APB exceeded those before exposure to 2APB; this was not a consistent finding. Typically, when recording from cells the light sensitivity will often decrease as time progresses; however, sometimes, as in this cell, the sensitivity to light will improve a small amount over time. Also note that after the initial peak current there is a small overshoot before the current reaches a plateau (Fig. 1 D) and this overshoot is greatly reduced in the presence of 2APB. This was a consistent finding in an additional five cells studied. These overshoots are only seen with bright lights, compare Fig. 1 B with Fig. 1 D, for which there is a 1,000-fold increase in light intensity. This observation was not studied further.

The data in Fig. 1 are from an experiment that examined the effects of 2APB on the light response over a much wider range of light intensities, the results of which are summarized in the graph of Fig. 2. The experiment was performed on a total of 14 cells, 8 of which did not exhibit any recovery of sensitivity after exposure to 2APB. Moreover, these eight cells exhibited the greatest degree of desensitization in the presence of 2APB. Experience over many years has shown that cells impaled with two microelectrodes and stimulated with the high intensity lights used in these experiments don't survive very well for the times (40–60 min) required for these experiments. Therefore these eight cells were classified as damaged and the data obtained from them was excluded from further analysis. The remaining six cells all exhibited approximately the same degree of desensitization in the presence of 2APB (see Fig. 2) and all exhibited significant recovery of sensitivity after exposure to 2APB, with two of the six exhibiting complete recovery of sensitivity. The absolute values of both the peak inward current and steady-state inward current, for these six cells, are plotted as a function of the intensity of a 6-s step of light in Fig. 2. The intensity response curves of Fig. 2 were obtained by stimulating the photoreceptor once a minute with steps of light of increasing intensity. The graphs in Fig. 2 demonstrate that 100 μM 2APB reversibly inhibits both the initial transient and steady-state components of the light response at all intensities, except perhaps at the highest intensities (see also Fig. 1 A in Wang et al., 2002).

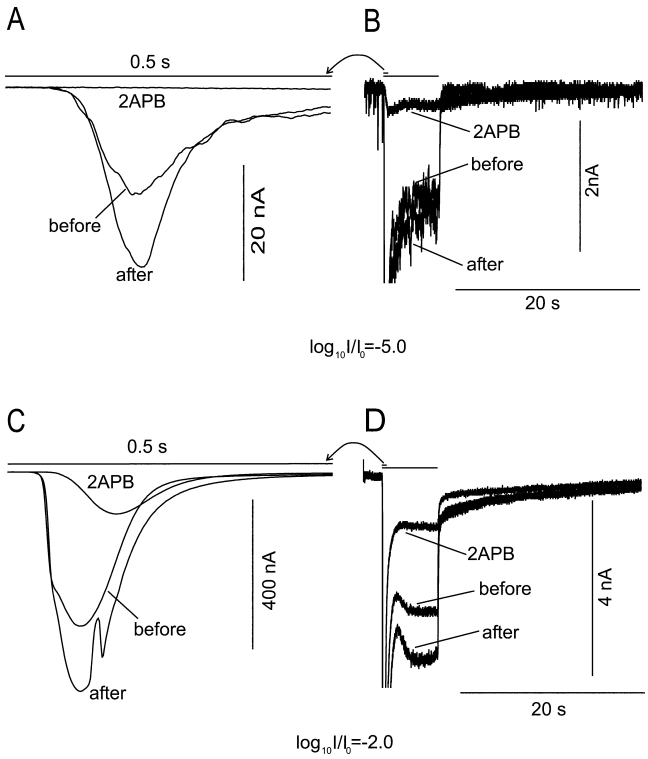


FIGURE 1. Light-induced currents measured under voltage clamp before, in the presence of 100 μM 2APB, and ~40 min after recovery from exposure to 2APB. In B and D the initial component of the responses go off scale except for the response at $\log_{10}I/I_0 = -5.0$ in the presence of 2APB. The responses are presented in this way in B and D so that the effect of 2APB on the steady-state components of the responses can be visualized. The bar above the records in B and D shows the time of occurrence of the 6-s light stimulus. The first 0.5 s of the responses during the light stimulus in B and D are shown on an expanded time scale and at much lower gain in A and C, respectively. The responses are shown this way in A and C so that the effect of 2APB on the initial transient components of the responses can be visualized.

To determine whether 2APB inhibited the light response for the highest intensities it was necessary to examine the effects of concentrations higher than 100 μM. To go to higher 2APB concentrations and still have reversibility, the experiment was performed under current clamp. Cells, impaled with only one microelectrode used for current clamp, typically survive much better than cells impaled with two microelectrodes. Measurements made in current clamp underestimate the desensitization caused by 2APB as a result of inhibition of the voltage gated outward current, thereby increasing the input resistance of the cell for depolarizing current responses (Wang et al., 2002). 2APB inhibits the light-activated current but since it increases the input resistance of the cell the inhibition of the receptor potential will be less than that of the light-activated current, hence the desensitization will be underestimated. Cells were exposed to varying concentrations of

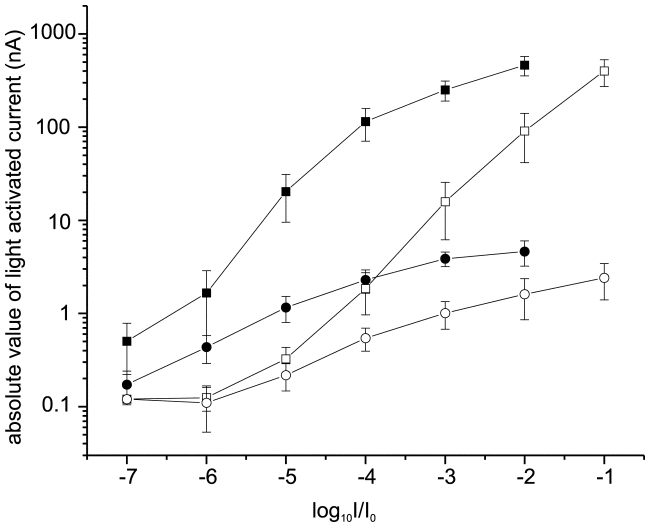
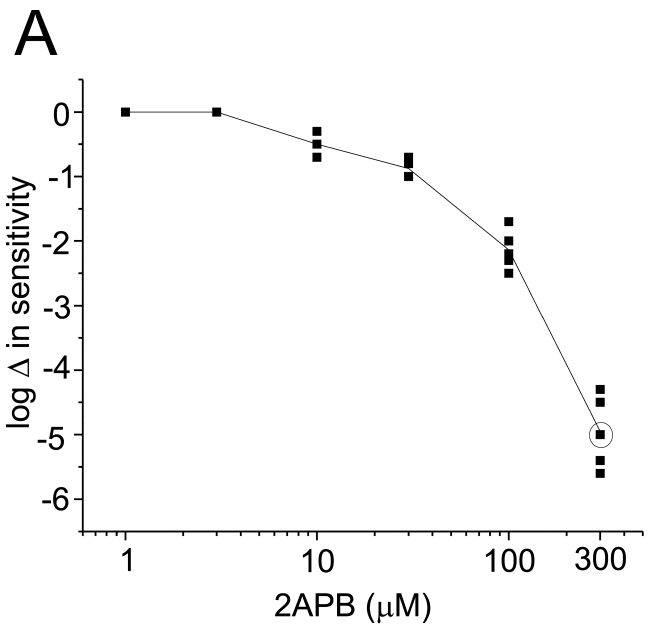


FIGURE 2. Intensity response function for the absolute value of the initial transient (■, control; □, 100 μM 2APB) and steady-state (●, control; ○, 100 μM 2APB) components of the light response measured under voltage clamp. The data were obtained before and in the presence of 100 μM 2APB for each of six cells. Data obtained 40 min after returning to ASW after exposure to 2APB is also included for the two cells that completely recovered from exposure to 2APB. Each intensity response function was obtained by stimulating the photoreceptor once each minute with a 6-s step of light of increasing intensity. The intensity response function in the presence of 2APB was obtained starting 10 min after first exposing the cell to 2APB. The absolute values of the peak of the initial transient and of the steady-state before turning off the light stimulus was measured at each light intensity. The values for $\log_{10}I/I_0 = -5.0$ and -2.0 include the data in Fig. 1. The data are plotted as the mean ± SD.



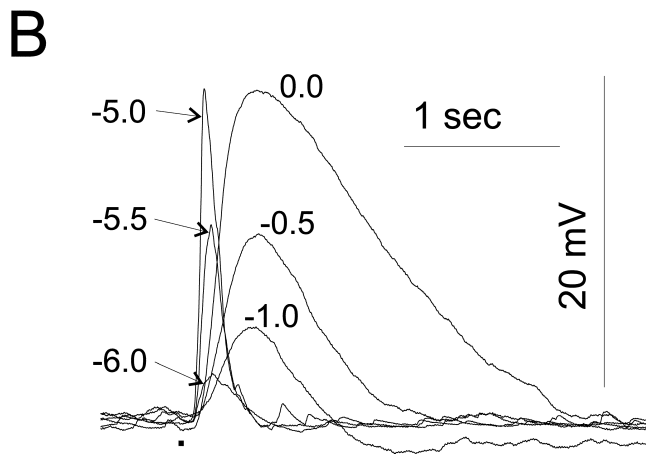


FIGURE 3. (A) Changes in sensitivity to a criterion response induced by 2APB. The change (Δ) in sensitivity was measured as the $-\log_{10}$ of the change in light intensity needed to evoke a 10-mV criterion response to a 40-ms flash of light at different concentrations of 2APB. The change in sensitivity was measured after 10 min of exposure to 2APB. The recovery time from exposure to 2APB varied from ~15 min at 10 μM 2APB to ~1 h and 45 min at 300 μM 2APB. (B) Dark-adapted light responses at and above threshold in the presence and absence of 300 μM 2APB. These light responses correspond to the circled data point in A.

2APB for 10 min and the effect on the sensitivity to a 40 ms flash of light was determined (Fig. 3 A). At 1 and 3 μM 2APB was without any appreciable effect on the sensitivity to light. At 10 μM 2APB the photoreceptors were desensitized about threefold and at 100 μM the desensitization was ~100-fold. This degree of desensitization is significantly less than what was measured under voltage clamp at these same concentrations (Wang et al., 2002), which is consistent with current clamp measurements giving an under estimate of the desensitization caused by 2APB. At 300 μM 2APB the sensitivity was decreased around 100,000-fold. Fig. 3 B juxtaposes dark-adapted light responses at and above threshold in the presence and absence of 300 μM 2APB. The responses in the absence of 2APB were obtained before exposure to 2APB. It took this cell ~1 h and 45 min to fully recover from exposure to 300 μM 2APB. The light response for a 40-ms flash of intensity $\log_{10}I/I_0 = 0.0$, the maximum intensity available from the light source, is reduced to only ~20 mV (Fig. 3 B), indicating that 2APB inhibits the light response at all light intensities. The slowing of the light response in the presence of 2APB (Fig. 3 B) was a consistent finding at all concentrations of 2APB that desensitized the photoreceptor. In contrast to this slowing of the light response by 2APB, desensitization of the photoreceptor by light or calcium injection is associated with a speeding up of the entire light response (Fein and Charlton, 1977).

The role of a light-induced Ca_i increase in visual excitation of *Limulus* ventral photoreceptors has been intensely investigated independent of whether or not the increase results from IP_3-induced calcium release. Phar-

macological means have been used to either empty the intracellular calcium store or to block the rise in Ca_i. Intracellular injection of calcium buffers, to inhibit the light-induced rise in Ca_i, greatly inhibit the light response for dim light flashes and slows the time course of the response at all light intensities (Lisman and Brown, 1975; Payne and Fein, 1986; Frank and Fein, 1991; Shin et al., 1993). However, no matter how much calcium buffer is injected into the photoreceptor, the light response for intense flashes persists, although the time course of the response is greatly slowed. CPA, which acts to empty intracellular calcium stores (Mason et al., 1991), also inhibits the response to a flash at dim intensities and slows the response to intense flashes (Ukhanov and Payne, 1995; Dorlochter et al., 1999; Payne and Demas, 2000). If intracellular-injected calcium buffers and exposure to CPA are only partially effective at blocking the light-induced rise in Ca_i near the plasma membrane then the light response remaining in their presence might result from a residual Ca_i increase that is greatly slowed by these agents. Assuming that the light response remaining in the presence of these agents is in fact the result of a residual IP_3-induced Ca_i increase, then the residual response should be blocked by 2APB.

The light-induced currents in Fig. 4, A and B, were obtained from two ventral photoreceptors that were injected with the calcium buffer BAPTA. The amount of injected BAPTA was greater for the cell in Fig. 4 B. The light responses labeled "before" were obtained immediately after BAPTA injection and are typical of the light responses observed in cells loaded with calcium buffers (Lisman and Brown, 1975; Payne and Fein, 1986; Frank and Fein, 1991; Shin et al., 1993). The rising and falling phases of the responses are greatly slowed (compare with the time course of the responses in Fig. 1) and the initial transient is decreased in amplitude, whereas the amplitude of the steady-state component of the response is greatly increased in amplitude (compare with the data in Fig. 2 for the same light intensity). The cell in Fig. 4 B was injected with BAPTA until one could not distinguish the initial transient from the steady-state of the response. During exposure to 100 μM 2APB the light-induced currents were greatly reduced in amplitude in their entirety, and upon return to ASW the responses partially recovered. Similar results were seen in 4 other cells, and in all cases there was only partial recovery of the light response upon returning to ASW after exposure to 2APB. This finding is consistent with the idea that the light responses in cells injected with calcium buffers are the result of IP_3-induced calcium release giving rise to a Ca_i increase.

The same protocol as used previously (Ukhanov and Payne, 1995; Dorlochter et al., 1999; Payne and Demas, 2000) to try and empty the intracellular calcium stores

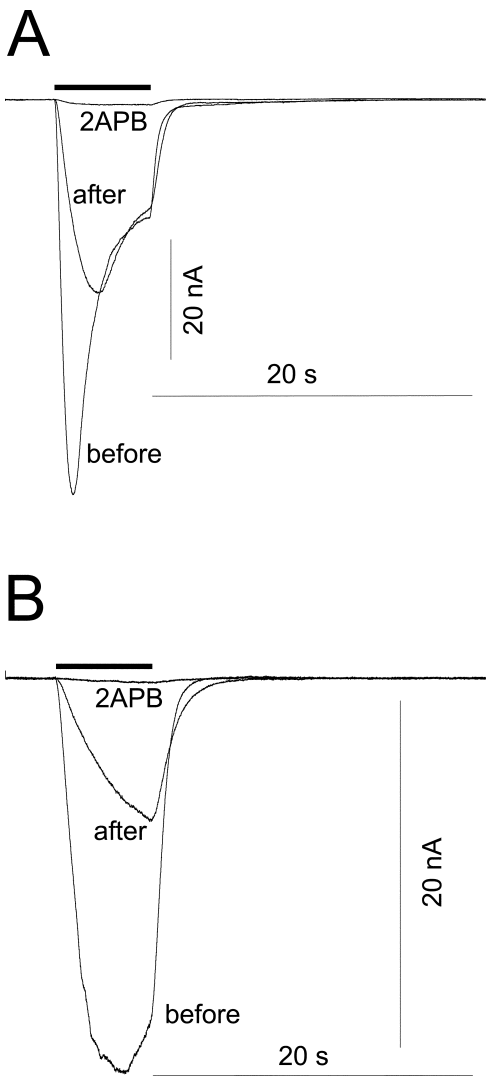


FIGURE 4. 2APB inhibits the response to a step of light in photoreceptors loaded with BAPTA. The cells in A and B were impaled with two microelectrodes, a current passing electrode containing 2 M KCl, and an injection electrode containing the injection solution (see MATERIALS AND METHODS) to which 100 mM BAPTA was added. The injection electrode was also used for recording membrane potential during voltage clamp. The cells were repeatedly stimulated once a minute by a 6-s step of light of intensity $\log_{10}I/I_0 = -4.0$, the occurrence of which is indicated by the bar above the superimposed responses in A and B. The cells were injected with BAPTA until the responses labeled before were obtained. These light responses are typical of those obtained from cells injected with BAPTA or other calcium buffers. The responses labeled 100 μM 2APB were obtained 10 min after switching to ASW + 100 μM 2APB and the responses labeled recovery were obtained ~20 min after returning to ASW. In these two cells and in another four cells studied similarly there was only partial recovery from the desensitization caused by exposure to 100 μM 2APB.

was used in the experiment illustrated in Fig. 5. After obtaining a stable recording, the cell was repeatedly stimulated once a minute with an intense ($\log_{10}I/I_0 = -2.0$) 40-ms flash and switched to a bathing solution of

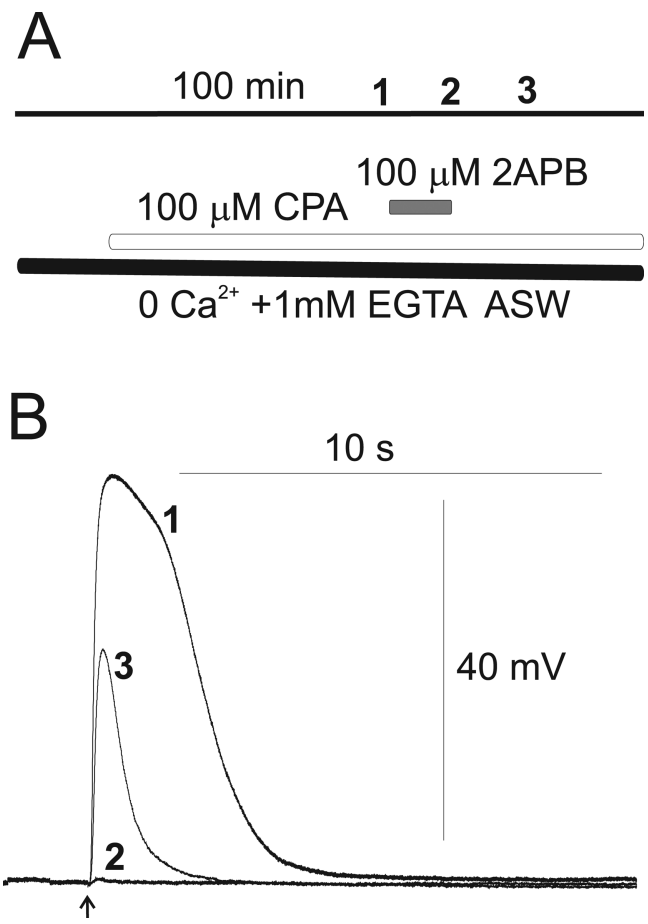


FIGURE 5. 2APB inhibits the response to a light flash in a photoreceptor treated with CPA to empty the Ca^{2+} stores. The cell was impaled with a 2M KCl containing microelectrode that was used to record the light response to a 40-ms flash (indicated by the black arrow below the superimposed traces) of intensity $\log_{10}I/I_0 = -2.0$, repeated once a minute. The cell was then exposed to the indicated bathing solutions for the times indicated in A. The times at which each of the superimposed light responses labeled 1, 2, and 3 in B were recorded are indicated in A.

0 Ca^{2+} + 1 mM EGTA (see MATERIALS AND METHODS). After 15 min in this solution the cell was bathed for an additional 45 min in the same 0 Ca^{2+} + 1 mM EGTA solution to which 100 μM CPA was added to try and empty the Ca^{2+} stores. After 45 min in CPA, as expected, a large slow light response was obtained (response 1 in Fig. 5 B). The cell was then exposed to a bathing solution of 0 Ca^{2+} + 1 mM EGTA + 100 μM CPA to which 100 μM 2APB was added. After 10 min in this solution the light response was dramatically suppressed (response 2 in Fig. 5 B). 15 min after returning the cell to a bathing solution of 0 Ca^{2+} + 1 mM EGTA + 100 μM CPA the light response recovered substantially, albeit not completely (response 3 in Fig. 5 B). Results similar to those in Fig. 5 were seen in four other cells, and in all cases there was only partial recovery of the light response. This experiment was done in current clamp because cells impaled with only one electrode exhibited significantly better recovery after these exposures than cells impaled with two electrodes. This finding is consistent with the idea that the light response in cells treated with CPA, to empty the Ca^{2+} stores, is actually the result of residual IP_3-induced calcium release giving rise to an increase in Ca_i.

DISCUSSION

The purpose of the experiments described in this study was to determine whether IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors. For this purpose the membrane-permeable IP_3-R antagonist 2APB was employed. 2APB has been shown to inhibit calcium release by the IP_3-R in *Limulus* ventral photoreceptors and also to inhibit the current activated by pressure injection of calcium into the light sensitive lobe of the photoreceptor (Wang et al., 2002). Thus, it is an excellent tool for investigating the role of IP_3-mediated calcium release in visual excitation of the photoreceptor. In accord with the hypothesis being tested, 2APB was found to inhibit the response to a flash of light at all light intensities (see Fig. 3) and to inhibit the entire light response to a step of light, that is both the initial transient and the steady-state components of the response, at all intensities tested (see Figs. 1 and 2).

2APB has been found to inhibit IP_3-mediated calcium release in the concentration range from 10–300 μM in intact cells and broken cells of both vertebrates and invertebrates (Maruyama et al., 1997; Ma et al., 2000; Chorna-Ornan et al., 2001). This is precisely the concentration range over which 2APB inhibits the light response in *Limulus* ventral photoreceptors (Wang et al., 2002) and also see Fig. 3. This finding suggests that the effects of 2APB in ventral photoreceptors result in part from its ability to block IP_3-mediated calcium release.

As mentioned in RESULTS, the intracellular injection of calcium buffers has been used to examine the role of a rise in Ca_i in generating the light response of *Limulus* ventral photoreceptors. In a number of cases the inability of calcium buffers to block the response to bright lights has been interpreted to indicate that a rise in Ca_i is not necessary for generating the light response of *Limulus* ventral photoreceptors (Lisman and Brown, 1975; Payne and Fein, 1986; Frank and Fein, 1991; Faddis and Brown, 1993). This interpretation was reconsidered under the assumption that calcium release during steady light in *Limulus* ventral photoreceptors is sufficiently great to saturate the calcium buffers even when the buffers are injected to concentrations greater than 20 mM (Shin et al., 1993). With mathematical modeling of the effects of calcium buffers on the rise in Ca_i in a well-mixed compartment, together with the assump-

tion that calcium influx into the compartment is sufficient to saturate the calcium buffer, these authors proposed that a rise in Ca_i is necessary for generating the light response of *Limulus* ventral photoreceptors. Consistent with this proposal are the findings in Fig. 4, which show that light responses obtained in the presence of the calcium buffer BAPTA are reversibly inhibited by 2APB, indicating that these light responses result from IP_3-mediated calcium release giving rise to an increase in Ca_i.

The inability of treatment of *Limulus* ventral photoreceptors with CPA, an inhibitor of endoplasmic reticulum calcium pumps, to block the light response for bright flashes has been interpreted to indicate that a rise in Ca_i is not necessary for generating the light response of *Limulus* ventral photoreceptors (Ukhanov and Payne, 1995; Dorlochter et al., 1999; Payne and Demas, 2000). This interpretation was dependent on the finding that treatment with CPA blocked the flash-induced rise in Ca_i, detected with calcium-sensitive dyes, yet failed to block the membrane depolarization resulting from the flash (Ukhanov and Payne, 1995; Payne and Demas, 2000). Just because a rise in Ca_i is not detected does not necessarily imply that a rise in Ca_i is not occurring, it might be that the method used is not sensitive enough to detect the reduced rise in Ca_i after treatment with CPA. Consistent with this possibility are the findings in Fig. 5 that show that the light response obtained after CPA treatment is reversibly inhibited by 2APB, indicating that the light response results from IP_3-mediated calcium release and a consequent rise in Ca_i.

How can it be that the rise in Ca_i that activates the ion channels in the plasma membrane still occurs in the presence of calcium buffers injected to concentrations >20 mM and after treatment with CPA, which makes the Ca_i rise itself undetectable? The answer most likely lies in the structure of the light-sensitive R-lobe of *Limulus* ventral photoreceptors (Calman and Chamberlain, 1982). The R-lobe is specialized for light sensitivity and is covered by visual pigment containing microvilli over its external surface. For this discussion the other important structure of the R-lobe is the palisade of closely apposed endoplasmic reticulum that lies just below and within a distance of ~0.1 μm of the microvillar membrane. Narrow cytoplasmic bridges connect the narrow compartment between microvilli and the endoplasmic reticulum to the rest of the cytoplasm. In response to light, IP_3 is produced in the microvilli from which it diffuses to the endoplasmic reticulum causing calcium to be released into the narrow space between the endoplasmic reticulum and the microvilli. Under normal circumstances the released calcium diffuses from this space throughout the cytoplasm of the R-lobe (Payne et al., 1988). However, it is only the Ca_i rise at the plasma membrane in this narrowly confined space that is the actual signal responsible for the opening of the light-activated ion channels in the plasma membrane. Calcium released from the endoplasmic reticulum only needs to saturate the calcium buffer present in this narrowly confined space in order to open the ion channels in the plasma membrane. Furthermore, the laser beam, which fails to detect the Ca_i rise after exposure to CPA, is measuring the average Ca_i in a much greater volume of the cytoplasm than this narrowly confined space.

Previously, the only really significant evidence against the suggestion that IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors was the evidence with CPA and calcium buffers discussed above. If the results of this study are accepted then the evidence reviewed below, together with these results, imply that IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors. It should be kept in mind that the experimental findings in this study are totally dependent on the specificity of 2APB.

First, calcium injection appears to activate the light-sensitive conductance, and the light-induced rise in Ca_i has the necessary properties for it to play the role of an intracellular messenger of visual excitation.

(a) The light-induced rise in Ca_i has been detected with a variety of experimental methods (Brown and Blinks, 1974; Brown et al., 1977; Levy and Fein, 1985; Ukhanov et al., 1995), and this rise in Ca_i is largely confined the R-lobe (Payne and Fein, 1987) and is dominated by calcium release from intracellular stores (Brown and Blinks, 1974).

(b) The rise in Ca_i precedes the electrical response to light over a wide range of light intensities (Ukhanov and Payne, 1995; Payne and Demas, 2000).

(c) The light-induced rise in Ca_i during steady illumination is graded with the intensity of light over a wide range of light intensities (Levy and Fein, 1985; Ukhanov et al., 1995).

(d) Reduction of the amount of calcium in the intracellular stores reduces the sensitivity to light (Bolsover and Brown, 1985; Dorlochter et al., 1999).

(e) Intracellular pressure injection of calcium activates a conductance with reversal potential and sodium permeability similar to that of the light sensitive conductance (Payne et al., 1986a).

(f) Intracellular pressure injection of calcium buffers with free calcium of 5 or 45 μM irreversibly activate sustained inward currents with reversal potentials similar to that of the light-sensitive conductance (Shin et al., 1993).

(g) Rapid release of calcium by flash photolysis of caged calcium activates an inward current within a few milliseconds (Ukhanov and Payne, 1997).

Second, the light-induced release of calcium from intracellular stores in *Limulus* ventral photoreceptors ap-

pears to result from IP_3-induced calcium release. Moreover, IP_3-induced calcium release appears to activate the light-sensitive conductance via a rise in Ca_i.

(a) There is a light-induced rise in IP_3 (Brown et al., 1984).

(b) Intracellular pressure injection of IP_3 causes a transient rise in Ca_i (Brown and Rubin, 1984; Payne et al., 1986b).

(c) 2APB reversibly inhibits both light- and IP_3-induced calcium release (Wang et al., 2002).

(d) Intracellular pressure injection of IP_3 transiently activates a conductance with reversal potential and sodium permeability similar to that of the light-sensitive conductance (Brown et al., 1984; Fein et al., 1984; Payne et al., 1986b), and prior injection of calcium buffer inhibits the activation of the conductance by IP_3 (Payne et al., 1986b).

Although the evidence strongly supports the conclusion that IP_3-induced calcium release is necessary for generating the entire light response of *Limulus* ventral photoreceptors, these findings should not be interpreted to indicate that IP_3-induced calcium release is also sufficient for generating the entire light response.

Stimulation of *Limulus* ventral photoreceptors with a step of light results in a steady elevation of Ca_i and a maintained depolarization (Levy and Fein, 1985). When measured under voltage clamp a steady inward current is found to be responsible for the maintained depolarization (see Fig. 1). If IP_3-mediated calcium release is sufficient for generating the entire light response then one might predict that it should be possible to mimic the maintained depolarization and maintained rise in Ca_i by intracellular pressure injection of IP_3 into the photoreceptor. In contrast to this prediction, intracellular injection of IP_3 in *Limulus* ventral photoreceptors often induces discrete bursts of depolarization (Brown et al., 1984; Fein et al., 1984) that are accompanied by discrete bursts of increases in Ca_i (Corson and Fein, 1987). Likewise, injection of hydrolysis-resistant analogs of IP_3 result in the production of similar bursts of depolarization that are also accompanied by bursts of increases in Ca_i (Payne and Potter, 1991; Vallet and Fein, 1997). In all of these studies neither a maintained steady depolarization nor a steady elevation of Ca_i was ever observed. Until the reason for this discrepancy is determined it cannot be concluded that IP_3-induced calcium release is sufficient for generating the entire light response of *Limulus* ventral photoreceptors. It is possible that the discrepancy arises somehow from differences in the way that light and intracellular pressure injection elevate the concentration of IP_3 in the cytoplasm. For light, one would expect the IP_3 concentration to be raised near the plasma membrane in the vicinity of each rhodopsin that effectively absorbs a photon. Whereas pressure injection of IP_3 delivers a large bolus of IP_3 to one location in the cytoplasm.

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REFERENCES

Bolsover, S.R., and J.E. Brown. 1985. Calcium ion, an intracellular messenger of light adaptation, also participates in excitation of *Limulus* photoreceptors. *J. Physiol.* 364:381–393.

Brown, J.E., and J.R. Blinks. 1974. Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. Detection with aequorin. *J. Gen. Physiol.* 64:643–665.

Brown, J.E., P.K. Brown, and L.H. Pinto. 1977. Detection of light-induced changes of intracellular ionized calcium concentration in *Limulus* ventral photoreceptors using arsenazo III. *J. Physiol.* 267:299–320.

Brown, J.E., and L.J. Rubin. 1984. A direct demonstration that inositol-trisphosphate induces an increase in intracellular calcium in *Limulus* photoreceptors. *Biochem. Biophys. Res. Commun.* 125: 1137–1142.

Brown, J.E., L.J. Rubin, A.J. Ghalayini, A.P. Tarver, R.F. Irvine, M.J. Berridge, and R.E. Anderson. 1984. myo-Inositol polyphosphate may be a messenger for visual excitation in *Limulus* photoreceptors. *Nature.* 311:160–163.

Calman, B.G., and S.C. Chamberlain. 1982. Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. *J. Gen. Physiol.* 80:839–862.

Chorna-Ornan, I., T. Joel-Almagor, H.C. Ben-Ami, S. Frechter, B. Gillo, Z. Selinger, D.L. Gill, and B. Minke. 2001. A common mechanism underlies vertebrate calcium signaling and *Drosophila* phototransduction. *J. Neurosci.* 21:2622–2629.

Corson, D.W., and A. Fein. 1983. Quantitative pressure injection of picoliter volumes into *Limulus* ventral photoreceptors. *Biophys. J.* 44:299–304.

Corson, D.W., and A. Fein. 1987. Inositol 1,4,5-trisphosphate induces bursts of calcium release inside *Limulus* ventral photoreceptors. *Brain Res.* 423:343–346.

Dabdoub, A., and R. Payne. 1999. Protein kinase C activators inhibit the visual cascade in *Limulus* ventral photoreceptors at an early stage. *J. Neurosci.* 19:10262–10269.

Dorlochter, M., W. Yuan, and H. Stieve. 1999. Effects of calcium and cyclopiazonic acid on the photoresponse in the *Limulus* ventral photoreceptor. *Verlag der Zeitschrift fur Naturforschung.* 54c: 446–455.

Faddis, M.N., and J.E. Brown. 1993. Intracellular injection of heparin and polyamines. Effects on phototransduction in limulus ventral photoreceptors. *J. Gen. Physiol.* 101:909–931.

Fein, A., and S. Cavar. 2000. Divergent mechanisms for phototransduction of invertebrate microvillar photoreceptors. *Vis. Neurosci.* 17:911–917.

Fein, A., and J.S. Charlton. 1975. Local adaptation in the ventral photoreceptors of *Limulus*. *J. Gen. Physiol.* 66:823–836.

Fein, A., and J.S. Charlton. 1977. A quantitative comparison of the effects of intracellular calcium injection and light adaptation on the photoresponse of *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 70:591–600.

Fein, A., and R.D. DeVoe. 1973. Adaptation in the ventral eye of *Limulus* is functionally independent of the photochemical cycle, membrane potential, and membrane resistance. *J. Gen. Physiol.* 61:273–289.

Fein, A., R. Payne, D.W. Corson, M.J. Berridge, and R.F. Irvine.

1984. Photoreceptor excitation and adaptation by inositol 1,4,5-trisphosphate. *Nature.* 311:157–160.

Frank, T.M., and A. Fein. 1991. The role of the inositol phosphate cascade in visual excitation of invertebrate microvillar photoreceptors. *J. Gen. Physiol.* 97:697–723.

Hardie, R.C., and P. Raghu. 2001. Visual transduction in *Drosophila*. *Nature.* 413:186–193.

Koganezawa, M., and I. Shimada. 2002. Inositol 1,4,5-trisphosphate transduction cascade in taste reception of the fleshfly, *Boettcherisca peregrina*. *J. Neurobiol.* 51:66–83.

Levy, S., and A. Fein. 1985. Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors. A quantitative study using Ca-selective microelectrodes. *J. Gen. Physiol.* 85:805–841.

Lisman, J.E., and J.E. Brown. 1975. Effects of intracellular injection of calcium buffers on light adaptation in *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 66:489–506.

Ma, H.T., R.L. Patterson, D.B. van Rossum, L. Birnbaumer, K. Mikoshiba, and D.L. Gill. 2000. Requirement of the inositol trisphosphate receptor for activation of store-operated Ca2+ channels. *Science.* 287:1647–1651.

Maruyama, T., T. Kanaji, S. Nakade, T. Kanno, and K. Mikoshiba. 1997. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. *J. Biochem. (Tokyo).* 122:498–505.

Mason, M.J., C. Garcia-Rodriguez, and S. Grinstein. 1991. Coupling between intracellular Ca2+ stores and the Ca2+ permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* 266:20856–20862.

Payne, R., D.W. Corson, and A. Fein. 1986a. Pressure injection of calcium both excites and adapts *Limulus* ventral photoreceptors. *J. Gen. Physiol.* 88:107–126.

Payne, R., D.W. Corson, A. Fein, and M.J. Berridge. 1986b. Excitation and adaptation of *Limulus* ventral photoreceptors by inositol 1,4,5 triphosphate result from a rise in intracellular calcium. *J. Gen. Physiol.* 88:127–142.

Payne, R., and J. Demas. 2000. Timing of Ca(2+) release from intracellular stores and the electrical response of *Limulus* ventral photoreceptors to dim flashes. *J. Gen. Physiol.* 115:735–748.

Payne, R., and A. Fein. 1986. The initial response of *Limulus* ventral photoreceptors to bright flashes. Released calcium as a synergist to excitation. *J. Gen. Physiol.* 87:243–269.

Payne, R., and A. Fein. 1987. Inositol 1,4,5 trisphosphate releases calcium from specialized sites within *Limulus* photoreceptors. *J. Cell Biol.* 104:933–937.

Payne, R., and B.V. Potter. 1991. Injection of inositol trisphosphorothioate into *Limulus* ventral photoreceptors causes oscillations of free cytosolic calcium. *J. Gen. Physiol.* 97:1165–1186.

Payne, R., B. Walz, S. Levy, and A. Fein. 1988. The localization of calcium release by inositol trisphosphate in *Limulus* photoreceptors and its control by negative feedback. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 320:359–379.

Shin, J., E.A. Richard, and J.E. Lisman. 1993. Ca2+ is an obligatory intermediate in the excitation cascade of limulus photoreceptors. *Neuron.* 11:845–855.

Stern, J.H., and J.E. Lisman. 1982. Internal dialysis of *Limulus* ventral photoreceptors. *Proc. Natl. Acad. Sci. USA.* 79:7580–7584.

Ukhanov, K., and R. Payne. 1995. Light activated calcium release in *Limulus* ventral photoreceptors as revealed by laser confocal microscopy. *Cell Calcium.* 18:301–313.

Ukhanov, K., and R. Payne. 1997. Rapid coupling of calcium release to depolarization in *Limulus* polyphemus ventral photoreceptors as revealed by microphotolysis and confocal microscopy. *J. Neurosci.* 17:1701–1709.

Ukhanov, K.Y., T.M. Flores, H.S. Hsiao, P. Mohapatra, C.H. Pitts, and R. Payne. 1995. Measurement of cytosolic Ca2+ concentration in *Limulus* ventral photoreceptors using fluorescent dyes. *J. Gen. Physiol.* 105:95–116.

Vallet, A.M., and A. Fein. 1997. A role for hydrolysis of inositol 1,4,5-trisphosphate in terminating the response to inositol 1,4,5-trisphosphate and to a flash of light in *Limulus* ventral photoreceptors. *Brain Res.* 768:91–101.

Wang, Y., M. Deshpande, and R. Payne. 2002. 2-Aminoethoxydiphenyl borate inhibits phototransduction and blocks voltage-gated potassium channels in *Limulus* ventral photoreceptors. *Cell Calcium.* 32:209.