A Common Mechanism Underlies Vertebrate Calcium Signaling and *Drosophila* Phototransduction

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Drosophila phototransduction is an important model system for studies of inositol lipid signaling. Light excitation in *Drosophila* photoreceptors depends on phospholipase C, because null mutants of this enzyme do not respond to light. Surprisingly, genetic elimination of the apparently single inositol trisphosphate receptor ($InsP_3R$) of *Drosophila* has no effect on phototransduction. This led to the proposal that *Drosophila* photoreceptors do not use the $InsP_3$ branch of phospholipase C (PLC)-mediated signaling for phototransduction, unlike most other inositol lipid-signaling systems. To examine this hypothesis we applied the membrane-permeant $InsP_3R$ antagonist 2-aminoethoxydiphenyl borate (2-APB), which has proved to be an important probe for assessing $InsP_3R$ involvement in various signaling systems. We first examined the effects of 2-APB on *Xenopus* oocytes. We found that 2-APB is efficient at reversibly blocking the robust $InsP_3$-mediated Ca^{2+} release and store-operated Ca^{2+} entry in *Xenopus* oocytes at a stage operating after production of $InsP_3$ but before the opening of the surface membrane Cl^- channels by Ca^{2+}. We next demonstrated that 2-APB is effective at reversibly blocking the response to light of *Drosophila* photoreceptors in a light-dependent manner at a concentration range similar to that effective in *Xenopus* oocytes and other cells. We show furthermore that 2-APB does not directly block the light-sensitive channels, indicating that it operates upstream in the activation of these channels. The results indicate an important link in the coupling mechanism of vertebrate store-operated channels and *Drosophila* TRP channels, which involves the $InsP_3$ branch of the inositol lipid-signaling pathway.

Key words: inositol lipid signaling; $InsP_3$ receptor; 2-APB; TRP; Drosophila *phototransduction;* Xenopus *oocytes*

Drosophila phototransduction has been an important model system for studies of the ubiquitous inositol lipid-signaling pathway. In this system hydrolysis of the phospholipid PIP_2 by phospholipase C (PLC) produces two second messengers: 1,4,5-inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG), each eliciting a unique signaling pathway (Berridge and Irvine, 1984). Genetic (Bloomquist et al., 1988), biochemical, and electrophysiological studies (Devary et al., 1987) have shown that a G-protein-activated PLC is essential for generation of the response to light. However, the involvement of downstream stages of the signaling pathway leading to opening of surface membrane channels remains elusive in *Drosophila* as it does in the coupling of entry channels in vertebrate PLC-coupled receptor responses.

It has been recently suggested that in contrast to other inositol lipid-signaling cascades, *Drosophila* phototransduction does not use $InsP_3$ for excitation because genetic elimination of the apparently single $InsP_3$ receptor of *Drosophila* has no effect on the response to light (Acharya et al., 1997; Raghu et al., 2000). Studies aimed at investigating the role of the $InsP_3$ branch in *Drosophila* phototransduction (Devary et al., 1987) have been difficult because of the complex, highly compartmentalized morphology of the *Drosophila* microvillar region containing the phototransduction signaling molecules and the inability to pharmacologically probe this region (our unpublished observations). This situation has changed with discovery of the membrane-permeant $InsP_3R$ antagonist 2-aminoethoxydiphenyl borate (2-APB), which has proven remarkably effective as a probe for assessing the involvement of the $InsP_3R$ in intact cells. 2-APB at 75 μM blocked receptor-mediated Ca^{2+} store emptying in intact human embryonic kidney (HEK) 293 cells and several other cells tested (Ma et al., 2000). In broken cells, 2-APB directly blocks $InsP_3R$-mediated Ca^{2+} release from endoplasmic reticulum (ER), although at high concentrations (>50 μM) it seems also to release Ca^{2+} from internal stores. 2-APB has no effect on $InsP_3$ binding, does not alter $InsP_3$ production through agonist-sensitive PLC, and does not modify the function of ryanodine receptors or voltage-gated Ca^{2+} channels (Maruyama et al., 1997; Ma et al., 2000). All the above features of 2-APB, together with very fast penetration into the signaling region inside the cell, make 2-APB an ideal reagent for studies of the involvement of the $InsP_3R$ in *Drosophila* phototransduction.

In the present study we reveal first that 2-APB reversibly and efficiently blocks the robust $InsP_3$-mediated signaling pathway of *Xenopus* oocytes at a stage operating after production of $InsP_3$ but before its action in mediating the rise in cellular Ca^{2+}. We demonstrate next that 2-APB is highly effective at reversibly blocking the response to light of *Drosophila* flies in a light-dependent manner at a concentration range that coincides with its effectiveness in oocytes. We show furthermore that 2-APB does

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not block the light-sensitive channels themselves indicating that it operates upstream of the channels. We propose that *Drosophila* photoreceptors use the $InsP_3$ branch of the inositol lipid-signaling pathway for light excitation either via a hitherto unknown $InsP_3R$ subtype or a protein intimately involved in mediating the action of $InsP_3$ on entry channels.

MATERIALS AND METHODS

Electrophysiological measurements using voltage-clamped Xenopus *oocytes.* The method used in the present study has been previously described (Gillo et al., 1987). Briefly, oocytes were impaled with two glass microelectrodes, which were filled with 3 M KCl with a resistance of 0.5–2.0 MΩ. The cells were voltage-clamped using standard technique. For pressure injection of solutions, a third and sometimes a fourth micropipette with tip diameter broken to ~3–4 μm diameter, were introduced into the oocytes. 1,4,5-Inositol trisphosphate, 3-deoxy-3-fluoro ($InsP_3$-F; 100 μM in the pipette, 6.5 nM, final concentration in the oocyte) or solution containing Ca^{2+} (10 mM in the pipette, 0.65 μM, final concentration in the oocyte) were pressure-injected by a pulse of pressure adjusted to release 65 pl of solution. When both $InsP_3$-F and Ca^{2+} were injected, two separate micropipettes were used. Drugs were added externally to the perfusate. For Ca^{2+} store depletion, previous injection of $InsP_3$-F into oocytes, bathed in Ca-free medium was performed at least 15 min before the electrophysiological recordings. $InsP_3$-F (10 μM, final concentration in the oocytes) was injected into the oocyte with a Drummond 10 μl microdispenser. Alternatively, thapsigargin (1 μM) or ionomycin (2 μM) were applied to the Ca^{2+}-free medium for 1 hr or 15 min respectively, before the electrophysiological recordings. When the oocytes were bathed in Ca^{2+}-free medium, the ND96 medium was used containing (in mM): 96 NaCl, 2 KCl, 5 HEPES, 10 $MgCl_2$, and 0.2 EGTA. When Ca^{2+}-containing solution was used, EGTA was replaced with 5 mM $CaCl_2$, and $MgCl_2$ was reduced to 5 mM. All chemicals were obtained from Sigma (St. Louis, MO) except for thapsigargin, which was obtained from Alomone Labs (Jerusalem, Israel).

Whole-cell recordings in Drosophila. Dissociated ommatidia were prepared from newly eclosed white-eyed adult flies (<1 hr after eclosion) (Hardie, 1991). Whole-cell patch-clamp recordings were performed as previously described (Hardie and Minke, 1992; Peretz et al., 1994a). Signals were amplified with an Axopatch-1D or 200B (Axon Instruments, Foster City, CA) patch-clamp amplifier, sampled at 900 Hz, and filtered at <5 kHz. The bath solution contained (in mM): 120 NaCl, 5 KCl, 10 *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES; pH 7.15), 4 $MgSO_4$ and 1.5 $CaCl_2$ (except when Ca^{2+} was removed from the medium). In part of the experiments (Fig. 6) the pipette solution included ions needed to block K^+ channel activity and contained (in mM): 100 CsCl, 15 tetraethyl ammonium (TEA) chloride, 2 $MgSO_4$, 10 TES, pH 7.15, 4 MgATP, 0.4 Na_2GTP, and 1 NAD. In other experiments the pipette solution contained (in mM): 120 K gluconate, 2 $MgSO_4$, 10 TES, pH 7.15, 4 MgATP, 0.4 Na_2GTP, and 1 NAD. In some experiments the ATP and NAD were removed from the pipette.

Electroretinogram and light stimulation. Electroretinogram (ERG) recordings were applied to intact flies as described previously (Peretz et al., 1994b). Orange light (OG 590 Schott edge filter) from a Xenon high-pressure lamp (PTI, LPS 220; operating at 50 W) was delivered to the compound eye by a fiber optic. The maximal luminous intensity at the eye surface was ~2.5 logarithmic intensity units above the intensity for a half-maximal response of the major photoreceptors (R1–6). For whole cell recordings a similar light source was used, and the orange stimulating light of similar intensity was applied via the objective lens (40× Olympus) and attenuated by Schott neutral density filters.

RESULTS

2-APB blocks the inositol-lipid signaling of *Xenopus* oocytes

The permeant $InsP_3$ receptor antagonist 2-APB has been studied in several different cells and tissues, however, its action on an intact native system such as the *Xenopus* oocytes, which contain a robust $InsP_3$-mediated signaling pathway (Gillo et al., 1987) has not been investigated. Thus, *Xenopus* oocytes constitute a powerful model system to study the effects of 2-APB and to accurately localize its site of action. Such information is considered essential for interpreting the effects of 2-APB on *Drosophila* photoreceptors described later.

Pressure injection into *Xenopus* oocytes of hydrolysis-resistant $InsP_3$ analog $InsP_3$-F activated the native Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) in a typical manner (Gillo et al., 1987). The final concentration of $InsP_3$-F in the oocytes (6.5 nM) was 1500-fold lower than that used to deplete the Ca^{2+} stores (see below). The $I_{Cl,Ca}$ induced by $InsP_3$ was typically composed of two phases: an initial relatively fast phase that rapidly declined toward baseline, followed by a slower and prolonged phase which was composed of current oscillations (Fig. 1*A*) (Dascal et al., 1984, 1985; Gillo et al., 1987). Both the initial transient and the oscillations reflect release of Ca^{2+} from internal stores, because both of them

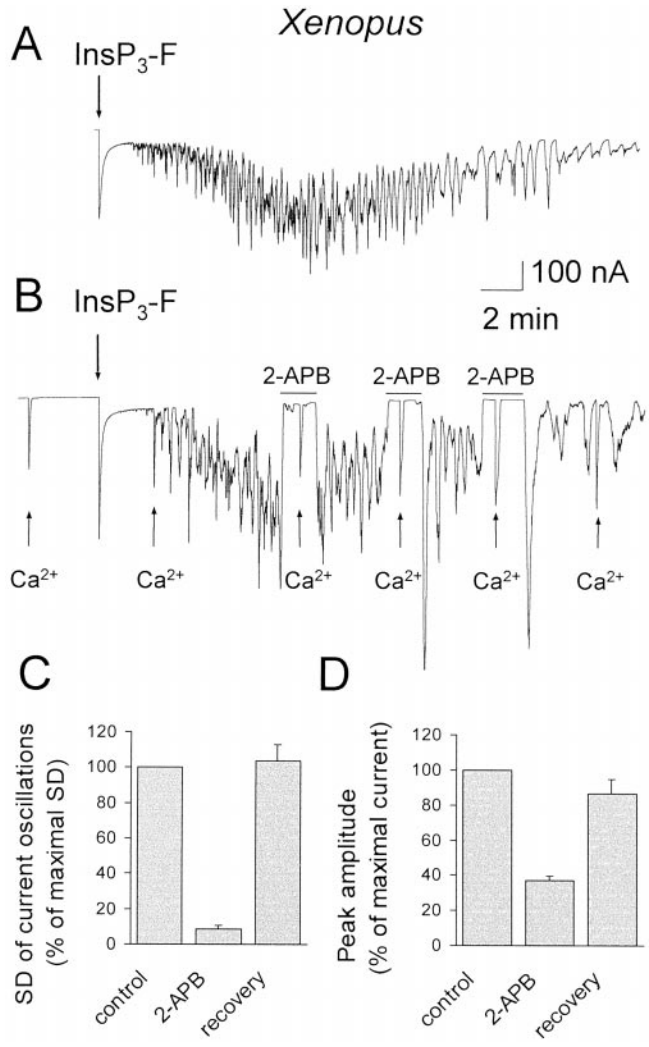

Figure 1. 2-APB blocks the inositol lipid signaling of *Xenopus* oocytes at the level of the $InsP_3$-sensitive Ca^{2+} stores. *A*, *B,* Measurements of $InsP_3$-F and Ca^{2+}-induced activation of $I_{Cl,\ Ca}$ currents in voltage-clamped oocytes at −70 mV membrane potential. *A* is a control trace. *B* shows the effect of 2-APB (50 μM in Figs. 1, 2) applied during current oscillations (*horizontal bars*). Pressure injection of Ca^{2+} during application of 2-APB induced $I_{Cl,\ Ca}$ that was similar to the control (*B, left*). *C,* Histogram showing the normalized SD of the current oscillations in different oocytes measured for 1 min before application of 2-APB (control), 1 min during (2-APB), and 1 min after removal of 2-APB (recovery). *D,* Histogram showing the normalized peak amplitude of the initial current phase induced by repeated injections of $InsP_3$-F, before (control), during application (2-APB), and 19 min after removal of 2-APB (recovery). The error bars are SEM in all figures.

remain unchanged at zero external Ca^{2+} (Gillo et al., 1987). However, whereas the initial transient reflects only Ca^{2+} release, the oscillations reflect release and reuptake of Ca^{2+} into the $InsP_3$-sensitive stores (Lechleiter et al., 1991; Jafri et al., 1992). Also, the initial transient and oscillations have different properties, probably reflecting Ca^{2+} release from different pools (Gillo et al., 1987) or different gating mechanism of $I_{Cl,Ca}$ (Boton et al., 1990). When experiments similar to those of Figure 1*A* were undertaken without external Ca^{2+} the prolonged current and oscillations were very similar to those of Figure 1*A* ($n = 16$). Figure 1*B* (left) shows that $I_{Cl,Ca}$ can also be induced by pressure injection of Ca^{2+} into the oocyte by a short pulse of pressure. This current was short and smooth without oscillations because Ca^{2+} injection is known to bypass the stages of the cascade which involve Ca^{2+} release from the ER stores by $InsP_3$ and to directly activate the surface membrane Cl^- channels (Dascal et al., 1985). Subsequent injection of $InsP_3$ induced again the typical responses with two phases. Strikingly, application of 2-APB (50 μM) to the bath almost completely suppressed the current oscillation during application (Fig. 1*B*). Because in several oocytes the current oscillations appeared at zero mean current, measuring the SD of the oscillations turned out to be an accurate measure of this signal. Figure 1*C* summarizes the effect of 2-APB in various cells by calculating the SD of the oscillations before application of 2-APB (control), during application of 50 μM 2-APB (2-APB), and after removal of 2-APB (recovery). The histogram shows a very pronounced block of the $InsP_3$-induced current oscillations. The initial transient response to $InsP_3$ was also inhibited by 2-APB, but the effect was less pronounced (Fig. 1*D*). Removal of 2-APB from the bath resulted in immediate recovery of the oscillations close to the control level (Fig. 1*C*, recovery). Repeated injections of $InsP_3$ during application of 2-APB (data not shown) revealed suppression of the initial peak transient phase of $I_{Cl,Ca}$ (Fig. 1*D,* 2-APB) ($n = 4$), which recovered much more slowly after removal of 2-APB (e.g., 19 min) than the recovery of current oscillations (Fig. 1*D*, recovery) ($n = 4$). Interestingly, pressure injection of Ca^{2+} during application of 2-APB when the oscillations were completely suppressed (Fig. 1*B*) showed that 2-APB had no significant effect on the surface membrane Cl^- channels. This is revealed by the waveform and amplitude of $I_{Cl,Ca}$ that remained either similar or was insignificantly depressed during 2-APB application when evoked by Ca^{2+} injection ($n = 7$), relative to injections before the induction of the oscillations (Fig. 1*B*, *left*) ($n = 4$).

Figure 1, thus, shows that 2-APB efficiently, rapidly, and reversibly blocks the inositol lipid signaling of the oocytes at a stage that involves activation of the $InsP_3$-sensitive stores after the action of $InsP_3$ but before the effects of the released Ca^{2+}. The most likely site of action of 2-APB is therefore the $InsP_3R$, as previously suggested for other cellular systems (Maruyama et al., 1997; Ma et al., 2000).

In mammalian cells, the modification of $InsP_3$ receptors by 2-APB has provided important evidence that the activation of store-operated Ca^{2+} channels (SOCs) (Putney, 1990) in response to store emptying is mediated through the $InsP_3R$ (Ma et al., 2000; van Rossum et al., 2000), supporting the conclusions of other recent reports (Kiselyov et al., 1998, 1999). In *Xenopus* oocytes, a robust activity of endogenous SOC channels has been demonstrated by monitoring $I_{Cl,Ca}$ (Petersen and Berridge, 1994) after store depletion by the Ca^{2+} pump inhibitor thapsigargin (Jackson et al., 1988). It was therefore important to assess any effects of 2-APB on the activation of SOCs in *Xenopus* oocytes. Figure 2*A* shows the typical pattern of activation of SOC channels in *Xenopus* oocytes. Oocytes were preincubated for 1 hr in the presence of thapsigargin (1 μM) in Ca^{2+}-free medium. After store depletion, application of a Ca^{2+} pulse to the external medium of the treated cells resulted in Ca^{2+} influx as manifested by a large $I_{Cl,Ca}$ that was rapidly inactivated during the Ca^{2+} pulse because of inactivation of the Cl^- channels (Petersen and Berridge, 1994), and this procedure could be repeated many times (Fig. 2*A*). Without store depletion at zero external Ca^{2+}, application of Ca^{2+} pulse did not induce any inward Cl^- current ($n = 17$) (Gillo et al., 1996a,b). Application of 2-APB to the external medium strongly suppressed $I_{Cl,Ca}$ (85% ± 6.9% suppression; $n = 5$) in a partially reversible manner (52 ± 6.8% recovery within a period of 30 min; $n = 5$) (Fig. 2*B*). Similar results were obtained when store depletion was obtained by previous application of the Ca^{2+} ionophore ionomycin (2 μM for 15 min), which has been widely used for activation of SOC channels (Ma et al., 2000) or by injection of $InsP_3$-F (10 μM) in

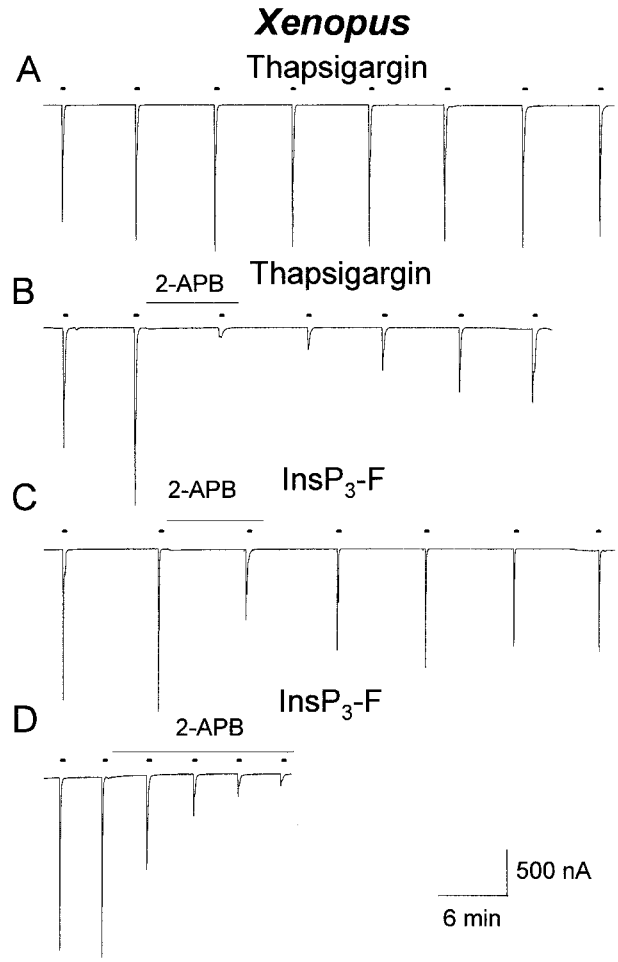

Figure 2. 2-APB reversibly blocks store-operated Ca^{2+} entry in *Xenopus* oocytes. *A,* Measurements of $I_{Cl,\ Ca}$ in thapsigargin-treated oocytes bathed in Ca^{2+}-free medium (ND 96) at −70 mV membrane potential. Application of Ca^{2+}-containing solution (5 mM Ca^{2+}, *short horizontal bars*) elicited $I_{Cl,\ Ca}$ of similar amplitudes. *B,* Similar paradigm as in *A* except that 50 μM 2-APB was added to the medium during a period indicated by *horizontal bar*. *C,* Similar paradigm as in *B* except that store depletion was obtained by previous (15 min) injection of $InsP_3$-F (10 μM). *D,* When Ca^{2+} pulses were applied to the external medium more frequently (every 3 min) the suppression of $I_{Cl,\ Ca}$ was much more pronounced, but the recovery was not evident ($n = 5$).

Ca^{2+}-free medium 15 min before the experiment. Although ionomycin renders the plasma membrane somewhat leaky to Ca^{2+}, in *Xenopus* oocytes this effect is very small, and the resulting Ca^{2+} influx is very slow and cannot be confused with the robust effect of store depletion caused by ionomycin or other Ca^{2+} ionophores (Boton et al., 1990). Suppression in $InsP_3$-F-treated cells reached 77 ± 4.8% (n = 14), and the recovery reached 50% after 28 min (n = 8). When Ca^{2+} pulses were applied to the external medium more frequently (every 3 min), the suppression of $I_{Cl,Ca}$ in oocytes treated with $InsP_3$ was much more pronounced, but the recovery was very slow (Fig. 2, compare *C*, *D*).

We took advantage of the relatively short time required to deplete the Ca^{2+} stores by ionomycin, to measure a dose–response for the effect of 2-APB. The dose–response data are presented in Figure 5 in comparison with similar data obtained for *Drosophila* (see below). The results of Figure 2 are consistent with those of Figure 1, and both show that 2-APB is a powerful antagonist of the inositol lipid signaling of *Xenopus* oocytes operating at the level of the $InsP_3$-sensitive Ca^{2+} stores, and likely on the $InsP_3R$ itself.

2-APB reversibly blocks the response to light of *Drosophila* photoreceptors

To examine whether 2-APB has an effect on *Drosophila* phototransduction, we took advantage of the ability to examine its effect on the intact animal using the ERG. The ERG is the sum of the electrophysiological response to light of the entire retina *in vivo*. Application of 2-APB to the intact eye by two pulses of pressure injections below the cornea (10 mM in the pipette, ~200 μM in the eye) (Fig. 3*A*, *arrows*) almost abolished the response to light ~10 min after application. The inhibitory effect was partially reversible after ~15 min and almost completely recovered after an additional 45 min (Fig. 3*A*).

To investigate whether inhibition of the ERG originated from blocking the light response of the photoreceptor cells, we investigated the effect of 2-APB using whole-cell patch clamp recordings from single photoreceptor cells. Figure 3*B* shows a train of light-induced currents (LICs) in response to orange light pulses of constant intensity. The amplitudes of the LICs were similar in all responses. Figure 3*C* shows the effect of 100 μM 2-APB applied to the internal solution of the recording pipette during whole-cell recordings. The initial three responses to light were only little affected. A small but significant slow inward current (*arrow*) was observed in the dark in most cells, after application of 2-APB at concentration >50 μM. Additional light pulses applied during the slow inward current resulted in a drastic reduction in response amplitude, which eventually led to total abolition of the response to light even when very intense white light was applied (data not shown). The desensitization produced by 2-APB cannot be a secondary consequence of Ca^{2+} influx, which may accompanied the slow and small inward current induced in the dark by 2-APB (Fig. 3*C, arrow*) because 2-APB inhibits the LIC also at concentrations <50 μM, which did not induce any detectable inward current. In some experiments we applied 2-APB at zero external Ca^{2+} and found that application of 2-APB combined with intense light (−logI = 1.0) at zero external Ca^{2+} caused rapid deterioration of the response to light and spontaneous openings of the light-sensitive channels (Hardie and Minke, 1994a). To prevent these effects and still examine the effect of 2-APB at zero external Ca^{2+}, we applied 200 μM of 2-APB and tested its effects using dimmer light of −logI = 2.0. Under these conditions, which kept the cells in good shape, we still observes a large suppression of the LIC (91.4 ±1.64% suppression; n = 5) ~13 min after application of 2-APB, thus indicating that Ca^{2+} influx cannot explain the suppression of the LIC. Figure 3*D* shows partial recovery of the response to light when 2-APB (75 μM) was applied to the bath for ~6 min, and constant orange light pulses (−logI = 1.0) were used to test its effect. Typically, the light-induced current was slower than normal when 2-APB caused a significant reduction in response amplitude, as manifested by a slow rise time and a slow response termination (Fig. 3*C,D*).

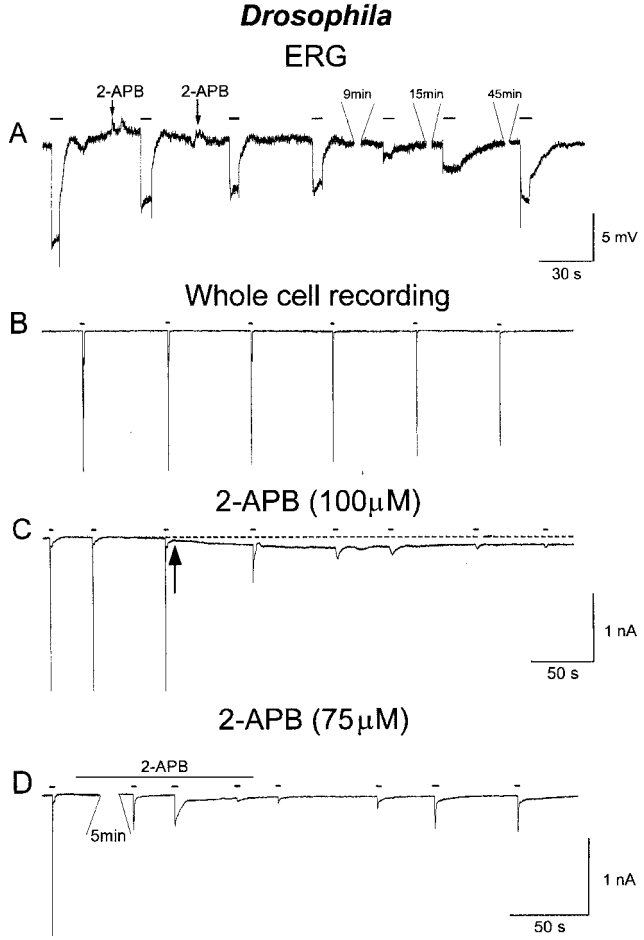

Figure 3. 2-APB reversibly blocks the response to light of *Drosophila* photoreceptors. *A,* ERGs were recorded in response to orange lights (−logI = 1.0) before (*left trace*) and after application of 2-APB into the eye by two short pressure injections as indicated. Partial recovery was observed after 15 min, and almost complete recovery was observed after additional 45 min. *B,* Whole-cell patch-clamp recordings (established ~30 sec before the recorded traces in *B* and *C*) in response to orange light pulses (−logI = 1.0). *C,* Similar paradigm as in *B* except that 100 μM 2-APB was included in the recording pipette. The *dotted line* indicates the control baseline level, and the *arrow* marks the induction of small inward current by 2-APB. *D,* Similar paradigm as in *B* except that 2-APB was applied to the bath for ~6 min as indicated (*horizontal bar*).

The effect of 2-APB is light-dependent, and it operates in the micromolar range

A pronounced suppression of the response to light by 2-APB could be observed within 3 min, provided that intense light was used to test its effect. This raised the possibility that its effect is light-dependent. To test this possibility we compared the amplitudes of the LIC to dim (−logI = 3) and to more intense orange

light pulses (−logI = 1) as a function of time, during application of 50 or 100 μM 2-APB to the pipette. Figure 4 presents the averaged amplitudes of the LIC in response to the dim and more intense light pulses (as indicated), as a function of time from application of 100 μM 2-APB. At both test lights the amplitude of the LIC declined with time, but the decline was much faster when stronger test light was used, indicating that the effect of 2-APB is light-dependent, suggesting that inhibition by 2-ABP requires that the $InsP_3R$ will be in its activated form.

We also found that when a relatively large (>50 μM) concentration of 2-APB was used, in addition to the slow inward current mentioned above (Fig. 3*C, arrow*), facilitation of the response to light was observed before the blocking action was evident. This phenomenon is manifested in Figure 4 by the large SEM and slight increase in averaged amplitude of the responses to light 90 sec after application of 2-APB. The large SEM reflects the large variability in amplitudes of the responses to light at this time, because a significant fraction of the responses to light were ~30% larger than control. This transient facilitation was not observed at low concentration of 2-APB (<50 μM) or when dim lights were used.

To compare the concentration dependence of the blocking effect of 2-APB in *Drosophila* to that of *Xenopus* oocytes and various vertebrate cells we measured curves similar to that of Figure 4 in response to the more intense light (−logI = 1.0) using various concentrations of 2-APB. To reduce the effect of facilitation in *Drosophila* we used the averaged amplitude of the LIC, 3 min after application of 2-APB as a measure for its effect. The dose–response curve was not sensitive to the time (>3 min) of measurements, and a similar curve was obtained when the averaged amplitude was measured at 5 min after application of 2-APB (data snot shown). Figure 5 plots the relative peak amplitude of the LIC (expressed as percentage of maximal current for each cell) in response to the more intense light (−logI = 1.0) as a function of concentration of 2-APB. Figure 5 also plots the dose–response curve for 2-APB measured from *Xenopus* oocytes after store depletion by ionomycin measured 6 min after application of 2-APB. The dose–response relationship was similar for the two species, and this similarity also fits the dose–response relationship found in other species (Maruyama et al., 1997). As yet there is very little data on 2-APB (Ma et al., 2000), and the results of the present study support the notion of previous studies that its effects are similar and quite specific to $InsP_3R$, SOC, and activation of TRP channels in all the tested species.

2-APB operates upstream to the light-sensitive channels TRP and TRPL

If 2-APB is a specific inhibitor of the $InsP_3R$, we expect that its application will not affect the light-sensitive channels. To test this notion it is required to activate the light-sensitive channels directly and not via the phototransduction cascade. Recently, it has been found that *Drosophila* TRP and TRPL channels can be activated in the dark by inducing metabolic stress after elimination of NAD from the pipette solution combined with depletion of ATP caused by illumination. The mitochondrial uncoupler

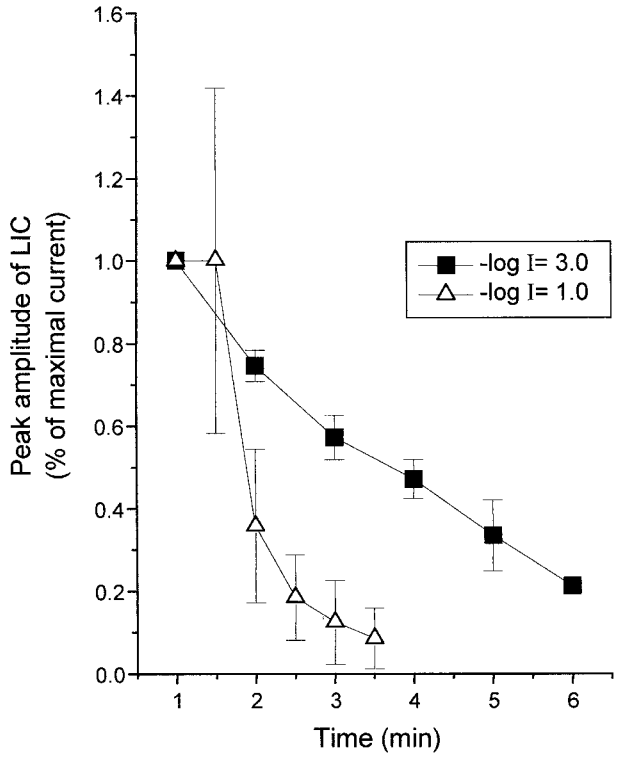

Figure 4. The effect of 2-APB is light-dependent. The relative peak amplitude of the LIC recorded from different cells is presented as a function of time from application of 100 μM 2-APB. Two intensities of orange test lights were used as indicated. The error bars were calculated from sample of four to seven cells for each point. The relatively large error bar of the second point (Δ) arises from a transient facilitation of the LIC in part of the cells.

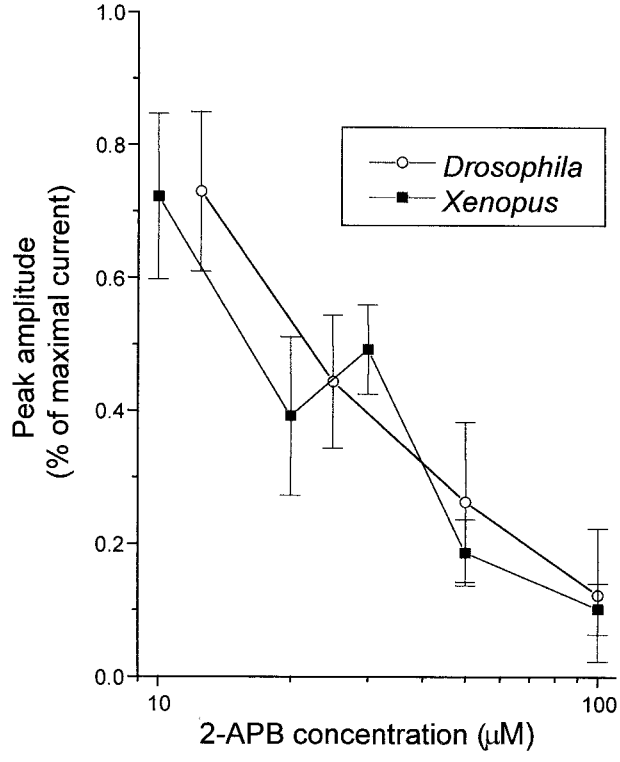

Figure 5. Dose–response curves for the inhibitory effect of 2-APB in both *Drosophila* and *Xenopus* oocytes. The relative peak amplitude of the LIC recorded from different cells of *Drosophila* in response to orange light pulses (−logI = 1.0), 3 min after application of 2-APB, is presented as a function of 2-APB concentration. The normalized peak current of *Xenopus* oocytes in response to pulses of solution containing 5 mM external Ca^{2+} was recorded 6 min after application of 2-APB at various concentrations, in oocytes treated with ionomycin to deplete the Ca^{2+} stores. The error bars for the various points were calculated from 3–11 oocytes.

dinitrophenol (DNP) is also a very potent reagent for direct activation of the TRP and TRPL channels (Agam et al., 2000).

Figure 6 demonstrates activation of the light-sensitive channels in the dark by metabolic stress obtained by application of DNP (Fig. 6*A*). Without metabolic stress, stepping the holding voltage from −100 to 80 mV in steps of 20 mV (Fig. 6*B*) during whole-cell recordings in the dark revealed only small leak current (Fig. 6*A*, control). However, after metabolic stress in the same cells, stepping the holding voltage at normal Ringer's solution (1.5 mM Ca^{2+}) elicited large outwardly rectifying currents when the holding voltages were stepped to the positive range, indicating that the channels are constitutively open (Fig. 6*A*, DNP). These currents are the typical manifestation of active TRP and TRPL channels (Hardie and Minke, 1992, 1994b; Reuss et al., 1997) that were readily blocked by 10 μM La^{3+} (Fig. 6*A*, DNP + 2-APB+ La^{3+}). Application of 2-APB up to a concentration of 100 μM had no significant effect on these currents (Fig. 6*A*, DNP + 2-APB). Data similar to those of Figure 6, which were obtained from different cells, are summarized as follows. The amplitude of the outward current at + 80 mV during metabolic stress in the presence of 2-APB was divided by the corresponding current measured from the same cell under metabolic stress before application of 2-APB. The use of the above ratios reduced the variability in the outward currents measured from different cells. The geometric average (and not the arithmetic average) is the correct way to calculate average of ratios. The geometric average of the ratios was 1.02 ± 0.26 ($n = 7$), indicating that 2-APB had no significant blocking effect on the opening of the light-sensitive channels. Metabolic stress was obtained by DNP or by elimination of NAD and ATP from the cells. The effect of 2-APB was usually measured 2 min after continuous application at 50 μM. In two cells, after 2 min of application, the concentration of 2-APB was increased to 75 μM and in two additional cells, to 100 μM for 5 min. In all these cases no significant effect of 2-APB on the constitutive current was observed.

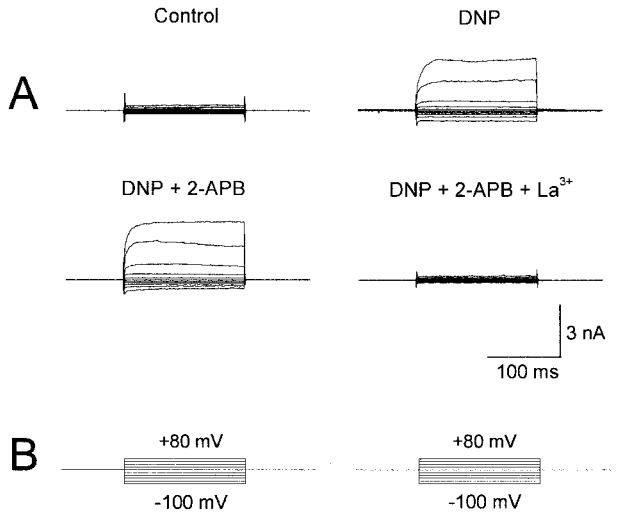

Figure 6. 2-APB operates upstream to the light sensitive channels TRP and TRPL. *A*, *B,* Series of nine voltage steps were applied from a holding potential of −20 mV in 20 mV steps, as indicated (*B*). The *left traces* (*A*, *Control*) show the current traces without metabolic stress. Metabolic stress induced by DNP (0.1 mM) resulted in constitutive activation of the light-sensitive channels TRP and TRPL (*A*, *DNP*). Application of 100 μM 2-APB had no inhibitory effect (*A*, *DNP + 2-APB*); the slight enhancement was not statistically significant (see Results). The *right traces* (*A, DNP + 2-APB+ La*$^{3+}$) show complete suppression of the currents by 10 μM La^{3+}.

DISCUSSION

In the present study we show that 2-APB is an efficient inhibitor of *Drosophila* phototransduction, operating both in intact cells and in isolated ommatidia, and that this inhibition partially reverses when the inhibitor is removed. The great interest in 2-APB arises from its reported function as a powerful probe for assessing involvement of $InsP_3$ receptors in cell signaling (Maruyama et al., 1997; Ma et al., 2000). Indeed, the reversible inhibition of $InsP_3$-induced current oscillations in *Xenopus* oocytes strongly supports previous studies showing that 2-APB blocks Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores (Maruyama et al., 1997). Furthermore, the failure of 2-APB to block the Ca^{2+}-activated surface membrane Cl^- channels while it suppresses the $InsP_3$-induced activity indicates that the action of 2-APB is confined to the signaling stages downstream of $InsP_3$ production, but upstream of the Ca^{2+} release-activated processes.

The inhibitory effect of 2-APB on $I_{Cl,Ca}$-induced by Ca^{2+} store depletion (Fig. 2) indicates that activation of native SOC channels of the oocyte is inhibited. This action is highly analogous to the inhibition of SOC channel activation by 2-APB reported in HEK293 cells after store depletion, which provided evidence for an interaction between the 2-APB-inhibited $InsP_3R$ and SOC channels (Ma et al., 2000). The relatively slow recovery of SOC activity after 2-APB inhibition that was found in oocytes in the present study (Fig. 2) and the experiments on HEK293 cells (Ma et al., 2000) also supports a common mechanism of 2-APB inhibition in the two systems.

Importantly, the concentration range of 2-APB action was similar for *Drosophila* and *Xenopus* oocytes as for other reported cells. Furthermore, in *Drosophila*, 2-APB did not seem to directly inhibit the surface membrane, light-sensitive channels. The resistance of the light-sensitive channels to 2-APB is reminiscent of the resistance of the mammalian TRP3 channel to direct blockade by 2-APB when this channel is activated directly by the OAG analog of DAG (Ma et al., 2000). Furthermore, the lag of a few minutes in the blocking effect of 2-APB in *Drosophila* (Fig. 3) does not support a direct effect on the TRP channels. This evidence also strongly suggests that the mode of action of 2-APB is similar in all the cells studied and that in each system its action may be a target closely associated with the function of $InsP_3$ receptors (Maruyama et al., 1997; Ma et al., 2000).

The mode of action and the identity of the specific ER protein with which 2-APB interacts are not clear. Previous studies suggest that the action of 2-APB is on the $InsP_3$ branch and not the DAG branch of inositol lipid signaling (Ma et al., 2000), however, it has not been possible to eliminate the possibility that 2-APB targets channels other than the $InsP_3$ receptor. For *Drosophila* phototransduction a major question has been whether the $InsP_3$ branch of the inositol lipid signaling is necessary for excitation. The present results and previous studies on the characteristics of 2-APB inhibition provide evidence for the hypothesis that *Drosophila* photoreceptors use the $InsP_3$ branch of the inositol lipid-signaling pathway for excitation in consistence with previous studies on the *Limulus* (Payne et al., 1986; Payne and Fein, 1987) and bee (Walz et al., 1994) photoreceptors. In addition, the observation that a high concentration of 2-APB can release Ca^{2+} from $InsP_3$-sensitive stores (Maruyama et al., 1997) provides further evidence that Ca^{2+} release can mediate light excitation in *Drosophila*. A possible explanation for the release of Ca^{2+} by

2-APB is that it binds to the open state of the $InsP_3$ receptor and locks it in the open state. So far, demonstration of a significant light-induced release of Ca^{2+} from ER stores (Cook and Minke, 1999), and its participation in excitation was hampered as a result of the small size of the putative $InsP_3$-sensitive Ca^{2+} stores of *Drosophila* and the difficulty of introducing exogenous chemicals to the highly compartmentalized region of these stores. Importantly, the small inward current induced in the dark by 2-APB (Fig. 3*C*) and the transient facilitation of the LIC (Fig. 4) provide significant support for the hypothesis that Ca^{2+} release can induce excitation. Recent evidence indicates that 2-APB can indeed act as a partial activator of the $InsP_3$ receptor inducing some release of Ca^{2+} (D. L. Gill, unpublished observations).

An interesting finding is that the blockade of phototransduction by 2-APB was facilitated by light, suggesting that 2-APB inhibits the $InsP_3R$ by blocking the pore region in the open state.

The conclusion that *Drosophila* phototransduction uses the $InsP_3$ branch of the inositol-lipid-signaling pathway for light excitation is not consistent with two recent reports. The *Drosophila* genomic sequence identifies only one $InsP_3$ receptor gene in the *Drosophila* genome (Adams et al., 2000), and mutations in this gene are lethal (Acharya et al., 1997; Venkatesh and Hasan, 1997; Raghu et al., 2000). However, it is possible to generate mutant photoreceptors in mosaic patches by inducing mitotic recombination in heterozygotes. Intracellular recordings from photoreceptors in such mosaic patches revealed no differences in light response from wild-type leading the authors to conclude that the $InsP_3$ receptor played no role in phototransduction (Acharya et al., 1997). A more detailed study using mosaic eyes homozygous for a deficiency of the $InsP_3$ receptor of *Drosophila* confirmed by RT-PCR, Western blot analysis, and immunocytochemistry, showed that the $InsP_3$ receptor was indeed eliminated without any effect on the response to light as tested by several functional tests using patch-clamp whole cell recordings (Raghu et al., 2000). In experiments on vertebrate DT40 cells, knock-out of all three known $InsP_3$ receptors did not prevent what appears to be normal functioning of store-operated channels (Sugawara et al., 1997). However, it has been suggested that these cells could be expressing an N-terminal portion of the $InsP_3$ receptor perhaps involved in coupling to plasma membrane entry channels but not functional as a Ca^{2+} store release channel (Kiselyov et al., 1998). The reconciliation of these apparently conflicting data are likely to shed important new light on the mechanism of activation of light-sensitive channels. One possibility is that a second, still undiscovered, novel $InsP_3$ receptor exists because sequencing of the *Drosophila* genome has not been completed, the heterochromatin (about a third of the genome) has not been sequenced yet because of technical difficulties (Adams et al., 2000). Another possibility is that 2-APB interacts with a protein that can associate with the $InsP_3$ receptor but is not the $InsP_3$ receptor itself. Such a target may play an obligatory role in mediating the coupling process that results in activation of light-sensitive channels. It is also possible that other as yet unidentified $InsP_3$-responsive proteins exist that may be targets for 2-APB. The important principle finding is that 2-APB blocks activation of mammalian, *Xenopus*, and likely all vertebrate SOCs, and in addition it blocks activation of mammalian TRP channels as well as the TRP channels mediating the light induced current in *Drosophila*. However, in each case, 2-APB does not appear to directly modify channel activity. These observations allow us to conclude that there is a fundamentally conserved step in the activation process for each of these channels. In vertebrate cells, the activation appears to use input from the $InsP_3$ receptor, whereas in *Drosophila* phototransduction, the input from known $InsP_3$ receptors is not a requirement for channel activation. Whether a different $InsP_3$ binding protein mediates the inositol lipid-signaling branch in *Drosophila* phototransduction remains a further important question to address.

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