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A Direct Signaling Role for Phosphatidylinositol 4,5-Bisphosphate (PIP_2) in the Visual Excitation Process of Microvillar Receptors*

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In microvillar photoreceptors the pivotal role of phospholipase C in light transduction is undisputed, but previous attempts to account for the photoresponse solely in terms of downstream products of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis have proved wanting. In other systems PIP_2 has been shown to possess signaling functions of its own, rather than simply serving as a precursor molecule. Because illumination of microvillar photoreceptors cells leads to PIP_2 breakdown, a potential role for this phospholipid in phototransduction would be to help maintain some element(s) of the transduction cascade in the inactive state. We tested the effect of intracellular dialysis of PIP_2 on voltage-clamped molluscan photoreceptors and found a marked reduction in the amplitude of the photocurrent; by contrast, depolarization-activated calcium and potassium currents were unaffected, thus supporting the notion of a specific effect on light signaling. In the dark, PIP_2 caused a gradual outward shift of the holding current; this change was due to a decrease in membrane conductance and may reflect the suppression of basal openings of the light-sensitive conductance. The consequences of depleting PIP_2 were examined in patches of light-sensitive microvillar membrane screened for the exclusive presence of light-activated ion channels. After excision, superfusion with anti-PIP_2 antibodies induced the appearance of single-channel currents. Replenishment of PIP_2 by exogenous application reverted the effect. These data support the notion that PIP_2, in addition to being the source of inositol trisphosphate and diacylglycerol, two messengers of visual excitation, may also participate in a direct fashion in the control of the light-sensitive conductance.

In microvillar invertebrate photoreceptors the key enzymatic step for light transduction is a light-regulated phospholipase C (PLC)[1] that hydrolyzes phosphatidylinositol bisphosphate (PIP_2). A pivotal clue was the isolation of a blind *Drosophila* mutant, *norpA* (1), and the demonstration that this gene encodes a PLC-β expressed in the retina (2). PLC activity and light responses are rescued in *norpA* mutants induced to express the NORPA protein (3), and light-induced hydrolysis of PIP_2 has been shown in *Drosophila* (4) and squid (5). Although a great deal of research efforts have focused on the role of inositol trisphosphate (IP_3) (6–8) and internally released calcium (9–12) as messengers in photoexcitation, a number of shortcomings have become evident. (i) Buffering intracellular calcium attenuates and slows down the photocurrent but does not abolish it (9, 13). (ii) Low molecular weight heparin, an inhibitor of the IP_3 receptor, does not depress the plateau of the photoresponse (14, 15). (iii) In some species, such as *Balanus* and *Drosophila*, removal of extracellular calcium virtually abolishes the light-evoked $\Delta[Ca^{2+}]_i$ (increase in intracellular calcium) (16, 17), suggesting it is solely the consequence of the opening of light-activated channels, which are calcium-permeable (Refs. 18 and 19; see also Ref. 20). (iv) A null mutation of the IP_3 receptor does not adversely affect the light response (21, 22). These findings argue against IP_3 and calcium release being indispensable for visual excitation and prompted a search for alternative signaling molecules. The potential role of diacylglycerol (DAG), the other messenger generated by PIP_2 hydrolysis, has recently emerged; in *Lima* microvillar photoreceptors DAG analogs elicit a robust inward current, the properties of which resemble those of the photocurrent or a component thereof (23). DAG can in turn be metabolized by DAG lipase, releasing fatty acids and glycerol, and in *Drosophila* photoreceptors polyunsaturated fatty acids can activate a current that shares significant features with the photocurrent (24); heterologously expressed TRPL channels (believed to underlie a component of the light-dependent conductance) were also responsive to the same agents. However, the generality of the involvement of polyunsaturated fatty acids is unclear, as the results could not be confirmed in *Limulus* (25).

Despite the tantalizing evidence for DAG and/or its downstream products in visual transduction and the synergistic role of calcium, in no instance has application of such chemical stimuli fully reproduced the remarkable size and speed of the photocurrent. This may imply that yet another signal may be missing from the proposed schemes. In other systems PIP_2 has been shown to possess signaling functions of its own, independent from those of its hydrolysis products. The seminal observation concerns PIP_2 requirements of kinase-mediated desensitization of G protein-coupled receptors (26, 27). Subsequently, a role for PIP_2 becomes apparent also in ion channel gating. For a group of channels PIP_2 acts as a co-agonist; these include members of the inward rectifier family, such as ATP-inhibited potassium channels (28–30) and G protein-gated potassium channels (31, 32), and the same behavior is exhibited by sodium-activated cationic channels in lobster olfactory neurons (33) and by retinal cGMP-gated channels (34). Other channels, by contrast, are inhibited by PIP_2; interestingly, these are all related to the PLC cascade, such as neuronal IP_3 receptors (35), calcium release-activated channels (which mediate calcium in-

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[1] The abbreviations used are: PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; TRPL, transient receptor potential-like.

flux following depletion of the endoplasmic reticulum) (36), and heterologously expressed TRPL channels of *Drosophila* photoreceptors (37). These observations prompted the conjecture that in microvillar photoreceptors PIP_2 may help keep the channels closed and its hydrolysis could promote their opening. In the present report, we examined the consequences of manipulating PIP_2 on membrane currents and light responsiveness in isolated photoreceptors from *Pecten* and *Lima*. Our results are consistent with the participation of PIP_2 as a negative messenger for visual excitation, targeting at least in part the light-dependent conductance. Initial aspects of this study were presented in preliminary form (38).

EXPERIMENTAL PROCEDURES

Cell Dissociation Procedures—Complete eyecups of *Lima scabra* (Carolina Biological, Burlington, NC) were dissected under dim red light ($\lambda > 650$ nm), incubated in 0.6% collagenase (Worthington type II) and 0.4% trypsin (Sigma type III) for 40 min at 24 °C, washed in 3% fetal calf serum, and gently triturated with a fire-polished Pasteur pipette, as described previously (39). Retinas of *Pecten irradians*, obtained from the Aquatic Resources Center at the Marine Biological Laboratory (Woods Hole, MA), were dissociated after incubation in Pronase (Roche Applied Science, 850 p.u.k./ml) for 40–50 min at 22 °C (40). After plating, the recording flow chamber was continuously superfused with artificial seawater containing 480 mM NaCl, 10 mM KCl, 49 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose, pH 7.8 (NaOH).

Electrophysiology—Whole-cell patch pipettes were fabricated from thin wall borosilicate glass (Garner Glass 7052, outer diameter 1.5 mm, inner diameter 1.1 mm), fire-polished, and filled with an intracellular solution containing 100 mM KCl, 200 mM potassium glutamate, 5 mM $MgCl_2$, 5 mM Na_2ATP, 12 mM NaCl, 1 mM EGTA, 300 mM Sucrose, 10 mM HEPES, 0.2 mM GTP, pH 7.3 (KOH). To block potassium channels, cesium replaced potassium on an equimolar basis. Electrode resistance measured in artificial seawater was 2–4 megohms; series resistance was routinely compensated (maximum residual error <2 mV). For single channel recording thick wall glass capillaries (outer diameter 1.5 mm, inner diameter 0.75 mm) were pulled to a resistance of 10–15 MΩ (measured in symmetrical artificial seawater). All of the recordings were made at room temperature (20–22 °C).

Calcium Measurements—Changes in cytosolic Ca^{2+} were monitored with the fluorescent indicator Oregon Green 2 (Molecular Probes, Eugene, OR). The octapotassium salt of the probe was dissolved in the intracellular solution filling the patch electrode, at a concentration of 65–100 μM. Excitation light was provided by a 75-watt xenon arc lamp (PTI, South Brunswick, NJ) filtered by a dichroic reflector to reject wavelengths longer than 670 nm (Omega Optical, Brattleboro, VT) and by an interference filter (480 nm, 40 nm bandwidth; Chroma Technology, Brattleboro, VT). The beam was brought to the epi-illumination port of the microscope via a liquid light-guide (Oriel Corp., Stratford, CT); the dichroic reflector in the microscope turret had a cutoff wavelength of 505 nm. Emission light collected by a ×100, 1.3 numerical aperture oil immersion objective (Nikon) was filtered sequentially by an additional dichroic to reject $\lambda > 610$ nm and by a 535-nm interference filter, 50-nm bandwidth (all supplied by Chroma). An adjustable rectangular mask (Nikon) located at a conjugated image plane was positioned under infrared visualization to restrict the collection of fluorescence emission to the region of interest. The fluorescence signal was detected by a photomultiplier tube (model R4220 PHA, Hammamatsu, Bridgewater, NJ) operated at 800 V in photon-counting mode, using a window discriminator and a rate meter (models F-100T and PRM-100, Advanced Research Instruments, Boulder, CO). An analog voltage proportional to the counts accumulated in bins of programmable duration (typically, 10^{-5} s) was fed to the analog to digital interface of the computer.

Chemical Stimulation—For local superfusion, a dual puffer pipette (tip diameter ≈2–3 μm) was lowered automatically to a pre-set position near the target cell (≈30–50 μm) by a programmable positioner (Patchman, Eppendorf, Hamburg, Germany); test solutions were ejected by applying pressurized nitrogen under the control of a solenoid-activated valve. For intracellular dialysis, substances were added to the electrode-filling solution from freshly made stocks and perfused via patch pipette, sealed onto the somatic lobe of the cell in the whole-cell configuration. With a variety of small molecules, ranging from inorganic ions to fluorescent dyes, we have previously shown that the cytosol of the light-sensitive microvillar lobe fully equilibrates with the internal solution within 2–3 min (23, 41). Brain PIP_2 (99.5% purity by high pressure liquid chromatography) was obtained from Avanti Polar Lipids (Alabaster, AL), dissolved in chloroform, aliquoted, and stored at −70 °C. For each experiment, an aliquot was extensively dried under nitrogen and the lipid sonicated in buffer solution. The final "concentration" used was in the range of 10–50 μM, which compares favorably with the effective concentration range reported for a variety of PIP_2-sensitive G protein-coupled receptor kinases (26–27) and ion channels (28–36). Anti-PIP_2 antibodies produced by PerSeptive Biosystems (Framingham, MA) and distributed through Assay Designs (Ann Arbor, MI) were used at a dilution of 1:200 in intracellular solution.

Light Stimulation—The optical stimulator consisted of a 100-watt tungsten-halogen light source (Oriel, Stratford, CT), the beam of which was passed through a heat-absorbing filter (>95% rejection for $\lambda > 800$ nm). A solenoid-driven shutter (Vincent Associates, Rochester, NY) and calibrated neutral density filters (Melles Griot, Irvine, CA) were used to control the duration and intensity of stimulation. A pinhole and a field lens restricted the illuminated region to a focused spot on the chamber (≈150 μm). A beam splitter above the microscope condenser combined this beam with that of the microscope illuminator. The intensity of stimulating light was measured with a radiometer (UDT, Hawthorne, CA) and is expressed either as $\log_{10}$ attenuation or as the equivalent flux of effective photons at 500 nm. During experimental manipulations the cells were illuminated with near infrared light using a long pass filter ($\lambda > 780$ nm; Andover Corp., Salem, NH) and viewed with the aid of a TV camera (Panasonic, Secaucus, NJ). The infrared illuminator was turned off for 3–4 min before testing light responses.

RESULTS

We first determined whether PIP_2 altered the basal membrane conductance in voltage-clamped *Lima* microvillar photoreceptors. For this purpose a freshly sonicated stock of PIP_2 was added to the standard intracellular solution used to fill the patch electrode. The pipette tip was front-filled with regular internal solution to forestall problems with seal formation. As shown in Fig. 1*A*, 10–15 s after gaining access to the cell interior the holding current gradually shifted in the outward direction; control cells displayed a stable holding current. The effect was concentration-dependent as shown in the bar graph in Fig. 1*B*; with 10 μM PIP_2 in the pipette the current stabilized at a mean value (±S.E.) of 180 ± 112 pA ($n = 3$). Increasing PIP_2 to 50 μM augmented the average change in holding current to 329 ± 117 pA ($n = 6$). In control cells the current drifted by an average of 13 ± 7 pA ($n = 7$). To monitor the changes in membrane resistance that accompanied such a shift in holding current, a repetitive rectangular command step (4–10 mV in amplitude, 10 Hz) was superimposed on the −50 mV holding potential. As shown in Fig. 1*C*, the current steps induced by the perturbations in the command voltage progressively decreased in amplitude, indicating that the slow shift in membrane current was due to a decrease in membrane conductance. No changes in input resistance were detectable in control cells ($n = 3$). Fig. 1*D* shows that, on average, g_m (membrane conductance) was reduced ≈3.6-fold, from 8.4 nanosiemens to 1.7 nanosiemens with 50 μM PIP_2 ($n = 4$), whereas the effect was more modest with 10 μM (≈1.9-fold, $n = 2$).

Because light-dependent activation of a PLC is recognized as a necessary step in visual excitation of microvillar photoreceptors, it follows that if PIP_2 does function as a signal for the photoresponse, it has to be a negative one, *i.e.* it needs to antagonize the excitatory process. We examined the effect of dialyzing PIP_2 into *Lima* photoreceptors on the light response. The *top trace* of Fig. 2*A* is the current evoked by a half-saturating light stimulus applied to a cell internally perfused with 50 μM PIP_2; light responsiveness was substantially reduced, compared with a control cell (*bottom trace*). The bar graph in Fig. 2*B* compares the average saturating amplitude of the photocurrent measured in the two conditions, pooling different cells ($n = 5$ and 11, respectively). The reduction in the size of the light response by PIP_2 was >80%.

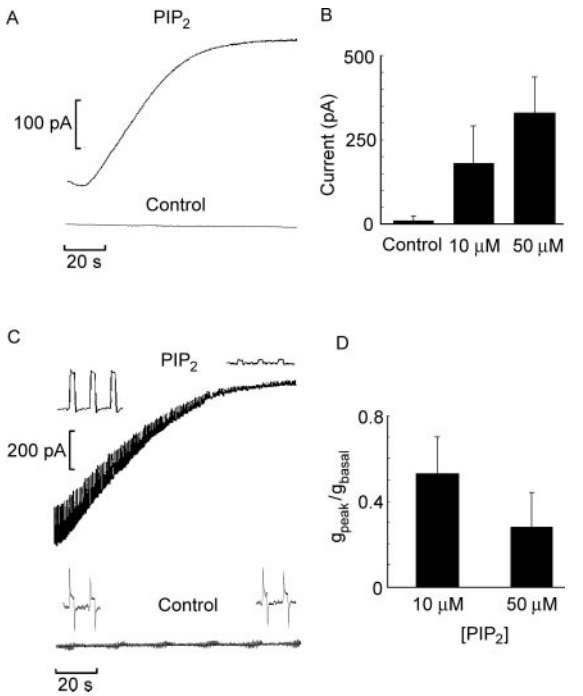

FIG. 1. **PIP$_2$-induced changes in basal membrane conductance.** Freshly sonicated PIP$_2$ was diluted in standard intracellular solution to a final concentration of 10–50 μM and dialyzed intracellularly via the patch pipette. *A*, 15 s after rupturing the membrane patch to attain the whole-cell configuration (just before the start of the trace), the holding current gradually drifted in the outward direction (*top trace*). In control cells, the holding current remained stable throughout the dialysis period (*bottom trace*). *B*, mean amplitude of the change in holding current obtained from photoreceptor cells internally perfused with control solution or with 10 or 50 μM PIP$_2$. *Error bars* represent S.E. *C*, input resistance was monitored during intracellular perfusion with PIP$_2$ (*top trace*) by superimposing a repetitive (10 Hz) rectangular voltage perturbation (10 mV) on the steady holding (−50 mV). The progressive reduction in the amplitude of the response to the repetitive command pulses (*insets*) indicated that the gradual change in membrane current reflected a decrease in membrane conductance. A similar procedure applied to a control cell (4-mV steps) revealed no change in membrane conductance (*bottom trace*). *D*, the effects of PIP$_2$ on membrane conductance were concentration-dependent. The conductance measured at the peak (g_{peak}) of the PIP$_2$-induced current change was normalized by the base line value (g_{basal}) at the start of the recording and averaged across cells treated with the two concentrations of PIP$_2$. *Error bars* represent the S.E.

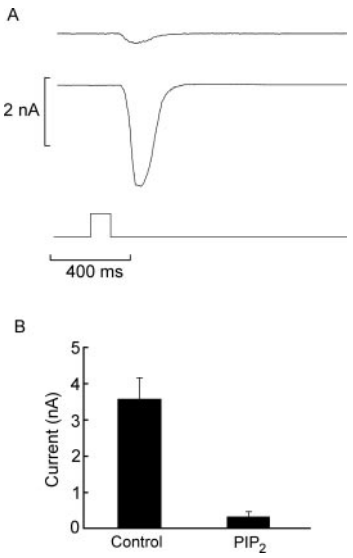

FIG. 2. **The light-dependent current is reduced by PIP$_2$.** *A*, membrane current evoked by a 100-ms flash (1.5×10^{11} photons $\times$ s^{-1} $\times$ cm^{-2}) in a voltage-clamped *Lima* microvillar photoreceptor internally perfused with 50 μM PIP$_2$ (*top trace*) and in an untreated cell (*bottom trace*). Holding potential, −50 mV. *B*, average amplitude of the saturating photocurrent pooled for PIP$_2$-treated and control cells (n = 4 and 11, respectively). *Error bars* indicate S.E.

The observed decrease in membrane conductance in the dark and the reduction in the amplitude of the photocurrent could conceivably occur if dialysis with the PIP$_2$-containing solution caused membrane re-sealing, thus compromising access to the cell interior. However, monitoring the access resistance via the capacitance and series resistance circuitry of the amplifier did not reveal that access to the cell interior suffered any degradation. Alternatively, the observed outcome could reflect a generalized impairment of membrane conductance. To rule out nonspecific effects on ion channels, we examined the consequence of PIP$_2$ application on non-light-dependent currents. *Lima* microvillar photoreceptors express depolarization-activated calcium and potassium channels (42). Fig. 3*A* illustrates a measurement in a cell that was internally perfused with Cesium to block potassium currents and with the fluorescent indicator Oregon Green 2 to monitor calcium changes. The *top traces* in Fig. 3*A* are the nearly identical calcium inward currents elicited by applying the same voltage step twice, from −60 to 0 mV. The *bottom traces* in Fig. 3*A* are the corresponding fluorescence measurements restricted to a small optical window (3 $\times$ 3 μm) that was positioned either on the light-sensitive microvillar lobe or on the soma. The voltage-dependent calcium channels are primarily found in the microvillar region, as indicated by the larger amplitude and the more rapid rise of the fluorescence change. Pooled data are shown in the bar graph in Fig. 3*B* (n = 5). Potassium channels, by contrast, seem more widely distributed throughout the cell, as gauged by recordings of single channel currents from either cellular region (data not shown). Fig. 3*C* shows voltage-dependent calcium and potassium currents measured in the dark either in control conditions (namely, with standard intracellular solution) or with PIP$_2$. Administration of depolarizing steps of increasing amplitude from a holding potential of −60 mV (after the effects of PIP$_2$ dialysis stabilized) elicited a calcium inward current that was not different from control cells either in mean amplitude (peak 237 ± 95 pA, n = 4 *versus* 180 ± 28 pA, n = 11) or in time course. Likewise, the outward potassium current retained its normal amplitude (990 ± 87 pA at +20 mV, n = 4) compared with untreated cells (1155 ± 218 pA, n = 11). These data are summarized in the bar graph in Fig. 3*D*. Because no effects were observed either on calcium channels, which co-localize with the light-sensitive conductance, or on potassium channels, which also express in the soma (and hence are even less liable to accessibility problems), it appears that PIP$_2$ selectively targets the light transduction pathway.

The observations reported above are consistent with the notion that PIP$_2$ antagonizes the light transduction cascade. A critical test of this proposition entails assessing the consequence of decreasing functional PIP$_2$ in the membrane; the working hypothesis predicts that such manipulation should either directly activate or synergistically facilitate the light-dependent conductance. In principle, two alternative strategies are available for this purpose. The obvious one, which parallels the physiological situation, would be to hydrolyze PIP$_2$ by exogenous application of a PLC. Unfortunately, this approach suffers from two shortcomings. First, phospholipases that are commercially available are either nonselective and target a variety of phospholipids or, like the phosphoinositide-specific bacterial PLC, are not effective on multiply phosphorylated forms of phosphatidylinositol. Second, even if a suitable (and constitutively active) PLC-β were readily available, a more insidious issue is that such stimulation would inevitably be

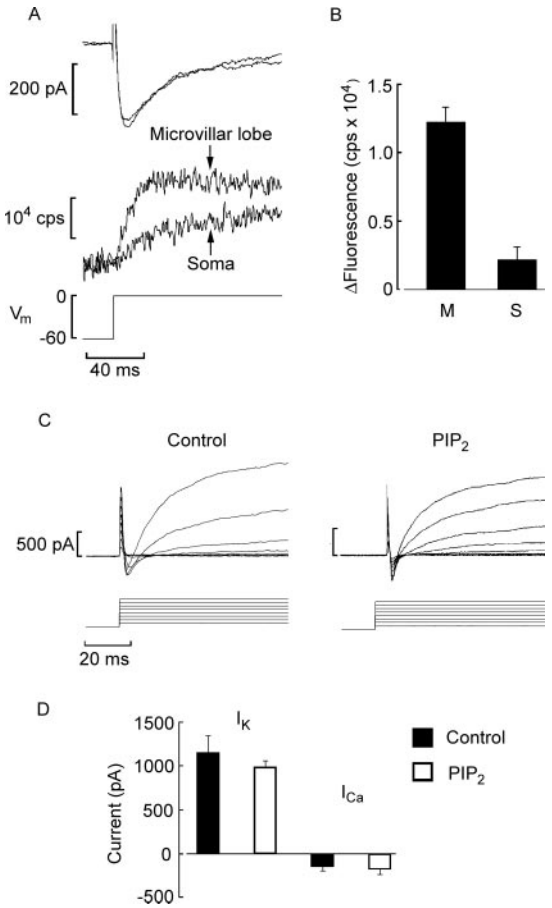

FIG. 3. **Voltage-dependent currents are not affected by PIP_2.** *A* and *B*, demonstration that depolarization-activated calcium channels are confined to the light-sensitive microvillar lobe (*M*). A photoreceptor was internally perfused with 300 mM Cesium and 83 μM Oregon Green 2, a fluorescent calcium indicator. Repetitive membrane depolarization from a holding potential of −60 to 0 mV reproducibly elicited an isolated calcium current (*top traces*); simultaneously, a calcium fluorescence signal was monitored either from the microvillar lobe or from the cell body by displacing an optical mask with a 3 × 3-μm window. The average amplitude of the calcium transient measured in the two regions is shown in *B* ($n = 5$). V_m, membrane voltage; *cps*, counts per second; *S*, soma. *C*, families of membrane currents evoked by depolarizing steps of increasing amplitude (10-mV increments from a holding potential of −60 mV) administered in the dark to photoreceptors perfused either with control intracellular solution (*left*) or with 50 μM PIP_2 (*right*). *D*, mean peak amplitude of the outward (*left columns*) and inward currents (*right columns*) elicited by a voltage step to +20 mV in control cells and cells internally dialyzed with PIP_2.

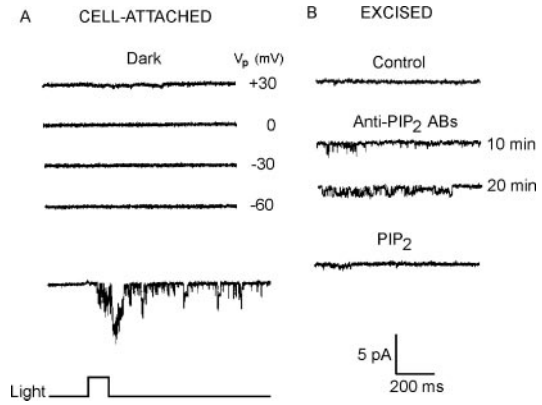

FIG. 4. **Activity of light-dependent channels is stimulated by PIP_2 depletion and suppressed by PIP_2 replenishment.** *A*, a patch pipette was sealed onto the light-sensitive membrane of the microvillar lobe, and different voltages were applied to screen for the presence of voltage-dependent channels. No activity was observed in the dark, within a voltage range spanning 90 mV. Application of a 100-ms light flash (2.1×10^{12} photons $\times$ s^{-1} $\times$ cm^{-2}) elicited a distinct burst of single-channel currents (*bottom trace*). V_p, pipette potential. *B*, after superfusing the chamber with an intracellular solution, the patch was excised and approached with a double-barrel pipette. One barrel contained anti-PIP_2 antibodies (1:200, diluted in intracellular solution); the other was filled with PIP_2 (50 μM). Sustained pressure-ejection of the antibody (*AB*) solution gradually gave rise to the appearance of single channel currents. At that point, the ejection solution was switched to PIP_2, resulting in the silencing of all channel activity.

accompanied by the production of DAG and IP_3, thus confounding the effects of PIP_2 reduction with those due to the generation of its downstream messengers.

An alternative strategy is to use antibodies raised against PIP_2, which have been found to be functional in a number of studies cited previously (31, 34–36). To evaluate the effects of functional depletion of PIP_2, we chose a technique that would circumvent the need of intracellular dialysis, which would be expected to be unacceptably sluggish because of the large molecular mass of the antibodies. Experiments were therefore conducted on patches of microvillar membrane from the photosensitive lobe of *Pecten* visual receptors, a preparation in which we had previously succeeded in resolving single channel currents activated by light (40–41). Fig. 4 illustrates the experiment. A patch pipette was sealed onto the microvillar membrane, and the current was monitored as the voltage across the patch was held at different values in a range spanning 90 mV (hyperpolarizing it by up to 30 mV and depolarizing it by up to 60 mV) in the dark. The *top traces* in Fig. 4*A* demonstrate that voltage stimulation alone was ineffective in eliciting any channel activity, thus ruling out the presence of voltage-gated channels in the patch. Next, a light stimulus was applied. The *bottom trace* in Fig. 4*A* shows that photostimulation evoked a distinct burst of single channel currents (at least three channels were present in the patch), which could not be due to changes in the cell membrane potential during the light response. The properties of these channels were previously shown to correspond to those of the light-dependent macroscopic current (40). At that point, the recording flow chamber was superfused with an intracellular solution, and the membrane patch was excised from the cell, in the inside-out configuration; all of the currents subsided (Fig. 4*B*, *top trace*). A dual puffer pipette was then lowered near the excised patch, and an intracellular solution containing the anti-PIP_2 antibodies was steadily pressure-ejected. Gradually, single channel currents reappeared. Their amplitude was smaller than that of the light-activated unitary currents recorded in the cell-attached configuration; this can be expected from the reduced driving force, because upon excision the contribution of the membrane potential of the cell to the total *trans*-patch potential, ≈50 mV (39), is lost. Subsequently, pressure ejection from the second barrel of the puffer pipette assembly containing 50 μM PIP_2 was initiated and caused the patch to become quiet, suggesting that the channel activity was indeed related to the depletion of PIP_2. A second patch in which the full experiment could be completed yielded nearly identical results. Three more patches confirmed to contain only light-dependent channels also displayed distinct channel activity when stimulated with anti-PIP_2 antibodies after excision, but they did not survive long enough to test the subsequent application of PIP_2.

DISCUSSION

Elucidation of the transduction cascade of microvillar photoreceptors has proved a most challenging enterprise, and progress has been sluggish in comparison with the swift breakthroughs that have provided, in a short time span, a clear picture of visual excitation in rods and cones (43). The

unexpected hurdles stem largely from the essential nonlinearity of the process and the multitude of regulatory loops involved; these complexities reflect the extraordinary performance required from these cells in terms of the speed of the response, the huge amplification, and the enormous dynamic range (44). Early attempts to account for the visual transduction process in terms of a simple linear cascade stemming from PLC activation and a single downstream messenger proved wanting, prompting the realization that a multiplicity of signaling molecules is likely to be implicated. Recent research points to the DAG branch of PIP_2 hydrolysis as the most promising primary pathway for activation of the light-sensitive conductance (23, 24), with calcium elevation (either from IP_3-mediated internal release or from influx through light-dependent channels) serving as a key synergistic modulator (23, 45, 46). Notwithstanding, persistent shortcomings suggest the involvement of additional players. PIP_2, long regarded simply as the substrate of PLC-β and the precursor of the key signaling molecules DAG and IP_3, may be a missing link and could function as a negative messenger contributing to the sequence of events by which light stimulation ultimately leads to a large increase in membrane conductance. Our observations in molluscan photoreceptors support such contention. We found that the application of PIP_2 reduced the photocurrent, whereas voltage-dependent calcium and potassium currents remained unaffected. A modest reduction in basal membrane conductance was also found and could reflect suppression of the background pool of open light-sensitive channels. Because the activity of PLC in the photoreceptor cell is low in the dark (47), significant degradation of the applied PIP_2 is unlikely. Under the assumption that the PIP_2 is incorporated into the plasma membrane, these effects are consistent with a role of this lipid as a negative regulator of light transduction.

Further support for the proposed scheme was provided by the test with antibodies designed to deplete PIP_2 without the concomitant generation of its downstream messengers. These experiments benefited from the unique opportunity that *Pecten* photoreceptors offer for recording light-dependent single channel currents in patches of microvillar membrane. Application of anti-PIP_2 antibodies induced channel openings; the time course of this effect was slow (5 min); in a variety of PIP_2-sensitive potassium channels, the effects of the same antibodies have been reported to develop within intervals ranging from 30 s to as much as 4 min (31, 33–36). The present observations are decidedly at the upper limit of this range; this could be attributable to the complex geometry of the microvilli in the membrane patch, which would be expected to impose a formidable barrier to the diffusion of a large molecule. The excitatory effects of the antibodies were subsequently reverted by PIP_2 replenishment. Because patch excision causes the washout of soluble regulatory elements of the phototransduction cascade and decouples the channels from rhodopsin stimulation (as evidenced by the loss of the light response), these observations suggest that the target site of PIP_2 is located downstream in the cascade. Therefore, early elements such as G$\beta\gamma$, which in other systems is susceptible to PIP_2 effects (but whose role in microvillar receptors is poorly understood), would not be prime candidates. An obvious potential target is the final link of the pathway, namely, the light-sensitive conductance. Two lines of evidence support this contention: (i) the lack of inhibitory effects of this lipid on non-light-dependent currents in the case of *Lima* intact photoreceptors; and (ii) the screening to ascertain the presence of light-dependent but not of voltage-dependent ion channels in the patches from *Pecten* in which excitatory effects were subsequently induced by anti-PIP_2 antibodies. This conclusion also agrees with the finding that in PIP_2-treated intact cells the maximum amplitude of the current that can be elicited by light is reduced. Moreover, it dovetails with the reported effects of PIP_2 on heterologously expressed TRPL (37), which are putative light-dependent channels in *Drosophila*. One interpretation would envision a direct interaction between PIP_2 and the channel protein, mirroring that found in a growing number of ion channels that are capable of binding PIP_2 via a pleckstrin homology domain and alter their gating. However, until more detailed molecular information becomes available, one cannot dismiss the possibility of indirect effects; for example, the depletion of PIP_2 could lead to the release of other PIP_2-binding proteins, which in turn could be responsible for the functional effects. It is also conceivable that aspects other than gating may be implicated, *e.g.* it has been demonstrated recently that TRPL channels are subject to a novel form of regulation via translocation to and from the plasma membrane (48). Because PIP_2 has been implicated in trafficking (49), an effect on channel delivery could be proposed; however, the time scale of the reported channel mobilization (>15–30 min) seems too slow to explain our observations.

Although a plasma membrane-delimited mechanism is suggested, there is no reason to exclude additional sites of action of PIP_2 in light signaling. A second potential target is the light-induced calcium release process, as suggested by inhibitory interactions between PIP_2 and IP_3 receptors in other systems (35, 36). In view of the divergent mechanisms utilized by microvillar photoreceptors in different species, some of which seem not to rely on calcium release from intracellular stores (16, 17), the generality of such an additional role may be limited. It could, however, be relevant to molluscan visual cells, in which calcium release is likely to be the sole source of light-induced calcium elevation (23, 41, 50). Finally, a rhodopsin kinase, GPRK1, has been recently identified in *Drosophila* (51); it contains a pleckstrin homology domain capable of binding inositol phospholipids and resembles β-adrenergic receptor kinase, which is stimulated by PIP_2 (26). Because GPRK1 phosphotransferase activity can down-regulate the light response (51), such a mechanism could have contributed to the observed PIP_2-induced desensitization of the photocurrent (Fig. 2).

An issue to be considered is the viability and efficiency of a scheme that includes lipid hydrolysis as a component of visual excitation. PIP_2 content in microvillar membrane has been estimated at ≈3% of total phospholipids, about 30 nmol/g retinal tissue (52); to put this figure into perspective, in the microvillar membrane it represents a 3–4-fold molar excess over rhodopsin. Not surprisingly, photostimulation leading to a "massive" accumulation of IP_3 in physiological terms (*i.e.* micromolar) is not accompanied by a detectable decrease in PIP_2 (52). Indeed, lowering the bulk concentration of this lipid would seem hopefully slow and wasteful. However, the emerging picture of the spatial organization of the phototransduction machinery suggests a solution to this impasse. It has been found in *Drosophila* that many of the proteins involved in transduction are clustered into a large macromolecular complex via a scaffolding protein, InaD, which binds, among others, the light-dependent TRP and TRPL channels (53–55), as well as PLC (56). This arrangement has multiple implications. (i) It provides for rapid and efficient interactions, overcoming diffusional delays. (ii) It preserves the functional specificity of otherwise ubiquitous signaling molecules, avoiding cross-talk between pathways that share similar enzymatic machinery (56). Of special relevance to the proposed role of PIP_2 in phototransduction is the fact that, if PLC is tethered to the same complex that contains the putative light-sensitive channels, PIP_2-mediated signaling could function economically and effi-

ciently by virtue of a highly localized enzymatic action. So, the question is whether physiologically meaningful light-induced changes in PIP_2 levels actually occur. Fusion constructs of green fluorescent protein with pleckstrin homology domains have been used in other cells for this purpose (57), but specificity issues have been raised because of the propensity of pleckstrin homology domains to bind IP_3 (58), and the approach may lack the sensitivity to respond to highly localized changes. It has recently become possible to monitor PIP_2 levels by targeted expression of a PIP_2-sensitive potassium channel, Kir 2.1, in *Drosophila* photoreceptors under the control of the rhodopsin promoter (59). The results indicate that although PIP_2 levels are quickly replenished after illumination, interfering with the recycling pathway can reveal a marked drop in concentration, which is sensed by the gating machinery of the reporter channels.

Finally, although the proposed involvement of PIP_2 in visual transduction is in line with the growing recognition of the importance of lipid signaling in a variety of systems, we emphasize that PIP_2 by itself is unlikely to be the primary controller of gating of the light-sensitive conductance. Even though we clearly observed channel openings induced by PIP_2 depletion under conditions that are not expected to lead to any DAG production (see Fig. 4), the activity was considerably less pronounced than that evoked by light when the patch was in the cell-attached configuration (40). The removal of the inhibitory action of PIP_2, by itself, seems to modestly shift the P_o and induce some channel openings while still remaining in a gating regime far below that of light-induced excitation. Conversely, we had shown that DAG analogs can open the light-sensitive conductance presumably in the absence of any induced PLC activity (23), but the amplitude of the resulting current, although sizable, fell short of that evoked by light stimulation. It would appear that the synergistic interaction between the several controlling factors is required to attain the full opening probability of the channels when light is presented.

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