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 triphosphate (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 ∆[Ca$^{2+}$] (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 Limna 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-
flux following depletion of the endoplasmic reticulum) (36), and heterologously expressed TRPL channels of *Drosophila* photoceptors (37). These observations prompted the conjecture that in microvillar photoceptors 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 photoceptors 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 (λ > 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 irroratus*, 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 Na$_2$ATP, 10 mM CaCl$_2$, 5 mM MgCl$_2$, 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$_2$ATP, 12 mM NaCl, 1 mM EGTA, 300 mM sucrose, 10 mM HEPES, 120 mM Na$_2$HPO$_4$, 5 mM MgCl$_2$, 5 mM HEPES, and 5 mM glucose, pH 7.8 (NaOH).

**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 shorter 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 epifluorescence port of the microscope via a liquid light-guide (Oriel Corp., Stratford, CT) the beam of which was turned off for 3–4 min before testing light responses. 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 λ > 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 fractional changes in effective photons. During the experiments at photoreceptor axon terminals the cells were illuminated with near infrared light using a long pass filter (λ > 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 photoceptors. 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 artificial seawater to forestall problems with seal formation. As shown in Fig. 1A, 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. 1B; 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 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 mean (±S.E.) holding current (Fig. 1C). The current steps induced by the perturbations in the membrane voltage progressively decreased in amplitude, indicating that the slow shift in membrane conductance was due to a decrease in membrane conductance. No changes in input resistance were detectable in control cells (n = 3). Fig. 1D shows that, on average, g_m (membrane conductance) was reduced ~3.6-fold, from 8.4 nS to 1.7 nS 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 photoceptors, it follows that if PIP$_2$ does function as a signal for the photoreceptor, 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* photoceptors on the light response. The top trace of Fig. 2A 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. 2B 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%.
The conductance measured at 16786 patch pipette.

Whole-cell configuration (just before the start of the trace), the holding current gradually drifted in the outward direction. (A) A nearly identical 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. 3D. Because no effects were observed either on calcium channels, which colocalize 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 PIP2 selectively targets the light transduction pathway.

The observations reported above are consistent with the notion that PIP2 antagonizes the light transduction cascade. A critical test of this proposition entails assessing the consequence of decreasing functional PIP2 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 PIP2 by exogenous application of a PLC. Unfortunately, this approach suffers from two shortcomings. First, phospholipases that are commercially available are either nonspecific or 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...
accompanies the production of DAG and IP₃, thus confounding the effects of PIP₂ reduction with those due to the generation of its downstream messengers.

An alternative strategy is to use antibodies raised against PIP₂, 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 PIP₂, we chose a technique that would circumvent the need for 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. 4A 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. 4A 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. 4B, top trace). A dual puffer pipette was then lowered near the excised patch, and an intracellular solution containing the anti-PIP₂ 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 PIP₂ was initiated and caused the patch to become quiet, suggesting that the channel activity was indeed related to the depletion of PIP₂. 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 burst channel activity when stimulated with anti-PIP₂ antibodies after excision, but they did not survive long enough to test the subsequent application of PIP₂.

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 PIP₂ hydrolysis as the most promising primary pathway for activation of the light-sensitive conductance (23, 24), with calcium elevation (either from IP₃-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. PIP₂, long regarded simply as the substrate of PLC-β and the precursor of the key signaling molecules DAG and IP₃, 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 PIP₂ 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 PIP₂ is unlikely. Under the assumption that the PIP₂ 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 PIP₂ 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-PIP₂ antibodies induced channel openings; the time course of this effect was slow (5 min); in a variety of PIP₂-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 PIP₂ 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 PIP₂ is located downstream in the cascade. Therefore, early elements such as Gβγ, which in other systems is susceptible to PIP₂ 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 Limna 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-PIP₂ antibodies. This conclusion also agrees with the finding that in PIP₂-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 PIP₂ on heterologously expressed TRPL (37), which are putative light-dependent channels in Drosophila. One interpretation would envision a direct interaction between PIP₂ and the channel protein, mirroring that found in a growing number of ion channels that are capable of binding PIP₂ 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 PIP₂ could lead to the release of other PIP₂-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 PIP₂ 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 PIP₂ in light signaling. A second potential target is the light-induced calcium release process, as suggested by inhibitory interactions between PIP₂ and IP₃ 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 PIP₂ (26). Because GPRK1 phosphotransferase activity can down-regulate the light response (51), such a mechanism could have contributed to the observed PIP₂-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. PIP₂ 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 IP₃ in physiological terms (i.e. micromolar) is not accompanied by a detectable decrease in PIP₂ (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 PIP₂ in phototransduction is the fact that, if PLC is tethered to the same complex that contains the putative light-sensitive channels, PIP₂-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 PIP2 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 IP3 (58), and the approach may lack the sensitivity to respond to highly localized changes. It has recently become possible to monitor PIP2 levels by targeted expression of a PIP2-sensitive potassium channel, Kir 2.1, in Drosophila photoreceptors under the control of the rhodopsin promoter (59). The results indicate that although PIP2 concentration, which is sensed by the gating machinery of the reporter channels, it has recently become possible to monitor PIP2 levels by targeted expression of a PIP2-sensitive potassium channel, Kir 2.1, in Drosophila photoreceptors under the control of the rhodopsin promoter (59). The results indicate that although PIP2 concentration, which is sensed by the gating machinery of the reporter channels, is sensed by the gating machinery of the reporter channels.

Finally, although the proposed involvement of PIP2 in visual transduction is in line with the growing recognition of the importance of lipid signaling in a variety of systems, we emphasize that PIP2 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 PIP2 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 PIP2, by itself, seems to modestly shift the P0 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 present.

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