In Vivo Light-induced and Basal Phospholipase C Activity in Drosophila Photoreceptors Measured with Genetically Targeted Phosphatidylinositol 4,5-Bisphosphate-sensitive Ion Channels (Kir2.1)*

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The phosphatidylinositol 4,5-bisphosphate (PIP2)-sensitive inward rectifier channel Kir2.1 was expressed in Drosophila photoreceptors and used to monitor in vivo PIP2 levels. Since the wild-type (WT) Kir2.1 channel appeared to be saturated by the prevailing PIP2 concentration, we made a single amino acid substitution (R228Q), which reduced the effective affinity for PIP2 and yielded channels generating currents proportional to the PIP2 levels relevant for phototransduction. To isolate Kir2.1 currents, recordings were made from mutants lacking both classes of light-sensitive transient receptor potential channels (TRP and TRPL). Light resulted in the effective depletion of PIP2 by phospholipase C (PLC) in approximately three or four microvilli per absorbed photon at rates exceeding 150% of total microvillar phosphoinositides per second. PIP2 was resynthesized with a half-time of ~50 s. When PIP2 resynthesis was prevented by depriving the cell of ATP, the Kir current spontaneously decayed at maximal rates representing a loss of ~40% loss of total PIP2 per minute. This loss was attributed primarily to basal PLC activity, because it was greatly decreased in norpA mutants lacking PLC. We tried to confirm this by using the PLC inhibitor U73122; however, this was found to act as a novel inhibitor of the Kir2.1 channel. PIP2 levels were reduced ~5-fold in the diacylglycerol kinase mutant (rdgA), but basal PLC activity was still pronounced, consistent with the suggestion that raised diacylglycerol levels are responsible for the constitutive TRP channel activity characteristic of this mutant.

Phototransduction in Drosophila is mediated by a G-protein-coupled phospholipase C (PLC) cascade, resulting in activation of two classes of light-sensitive channels TRP and TRPL. These are the prototypical members of the large and diverse family of “transient receptor potential” (TRP) channels many of which, including all of the most closely related “canonical” TRPC subfamily, are also regulated by PLC (reviewed in Refs. 1–3). Although in most cases the exact mechanism of TRP channel gating remains unresolved, mounting evidence, both in Drosophila and in at least a subset of mammalian TRP homologues, now suggests that diacylglycerol (DAG) rather than inositol 1,4,5-trisphosphate (InsP3) is the critical product of phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis (4, 5), whereas some evidence suggests that the reduction in PIP2 itself may also be important (3, 6, 7). Because of its central role in phototransduction and PLC signaling generally, it is important to understand the dynamics of PIP2 turnover. In recent years a number of attempts have been made to monitor PIP2 levels in vivo, many making use of the GFP-tagged PIP2 binding PH domain from PLCβ (8). While this has provided valuable insight into dynamic and spatial aspects of PIP2 mobilization, the PLCβ PH domain also binds InsP3 with high affinity, and it is not always clear whether PIP2 or InsP3 levels are being monitored (Ref. 9, but see also Ref. 10). Fluorescence-based technologies are also of restricted value in photoreceptors, because the excitation light represents a saturating and usually damaging stimulus for the cell. Therefore we adopted an alternative approach by using an electrophysiological biosensor in the guise of the well characterized PIP2-sensitive ion channel, Kir2.1 (7). Like all members of the inward rectifying Kir family, these channels require phosphoinositide binding for their activity, but Kir2.1 has the highest specificity for PIP2 showing, for example, no detectable activation by PI or phosphatidylinositol 3,4-bisphosphate and <5% activation by phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol 3,4,5-trisphosphate (11, 12). In an initial study we expressed eGFP-tagged Kir2.1 channels in Drosophila photoreceptors under the control of the rhodopsin promoter and found them to be specifically targeted to the light-transducing microvillar membrane. In whole cell recordings from dissociated cells, the channels generated large constitutive currents, which could be rapidly and reversibly suppressed by light, representing PIP2 hydrolysis by PLC and its subsequent resynthesis (7).

In the present study we have developed this technology further by expressing a mutant version of the Kir2.1 channel with 1,4,5-trisphosphate; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; GFP, green fluorescent protein; eGFP, enhanced GFP; WT, wild-type; TES, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)methylamino)ethanesulfonic acid; LIC, light-induced current; PI, phosphatidylinositol.
In Vivo PLC Activity in Drosophila Photoreceptors

reduced affinity for PIP2 with a dynamic range that more effectively covers the range of physiologically relevant PIP2 levels. We expressed the channels in several mutant backgrounds, including flies lacking the light-sensitive TRP and TRPL channels so that Kir2.1 currents could be recorded in isolation. Our results indicate that activated PLC can deplete PIP2 at rates well in excess of 100% s⁻¹. In addition our data provide in vivo measurements of basal rates of PLC activity that, although they are orders of magnitude less than that of activated PLC, can still deplete all detectable PIP2 within minutes if PIP2 resynthesis is blocked. As well as providing unique quantitative information on the in vivo activity of PLC, the results also shed light on some recent studies concerning the mechanism of phototransduction.

EXPERIMENTAL PROCEDURES

Flies—Drosophila melanogaster were raised in the dark at 25 °C. The eGFP-Kir2.1R228Q construct was generated from the wild-type (WT) eGFP-Kir2.1 construct previously described (7, 13) using the QuikChange mutagenesis system (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Following mutagenesis, the presence of the R228Q mutation and no other was confirmed by sequencing of the full gene. The eGFP-Kir2.1R228Q gene was then subcloned into pUAST and injected into yw embryos to obtain transformants as previously described (7, 13). Expression was driven by the Gal4-UAS system (14) under control of the rhodopsin promoter (p[Rh1-Gal4]) flies provided by J. Treisman and F. Pichaud; pUAS Kir2.1, p[Rh1-Gal4] recombinants for both WT and R228Q Kir2.1 channels were generated on both second and third chromosomes to facilitate crossing into a variety of mutant backgrounds, including trpnull, a null mutant of the dominant light-sensitive and Ca²⁺ permeable channel (15); trpnull, null mutant of the second class of light-sensitive channel (15); rdgA², the most severe allele of the photoreceptor DAG kinase (16); norpA², a null or near-null allele of the photoreceptor phospholipase C (17), and Gaq², a second allele of the photoreceptor G protein with <1% protein (18).

Whole Cell Recordings—Dissociated ommatidia were prepared as previously described from recently eclosed adult flies (7, 19) and transferred to the bottom of a recording chamber on an inverted Nikon Diaphot microscope. Unless otherwise stated, the bath was composed of (in mM): 110 NaCl, 10 KCl, 4 CaCl₂, 10 TES, 4 MgCl₂, 1.5 CaCl₂, 25 proline, and 5 alanine. The standard intracellular solution was (in mM): 140 potassium gluconate, 10 TES, 2 MgCl₂, 1 NaCl, and 0.4 Na-GTP. In some experiments nucleotide additives (ATP, GTP, and NAD) were omitted and replaced with sucrose to maintain osmolarity. The pH of all solutions was 7.15. Whole cell clamp recordings were made using electrodes of ~10–15 MΩ resistance, and series resistance was generally below 25 MΩ and were routinely compensated to >80%. Data were collected and analyzed using Axopatch 1D or 200 amplifiers and pCLAMP 8 or 9 software (Axon Instruments, Foster City, CA). Cells were stimulated via one of two green light-emitting diodes, with maximum effective intensity of ~2 × 10³ and ~8 × 10³ photons s⁻¹ per photoreceptor, respectively. Relative intensities were calibrated using a linear photodiode and converted to absolute intensities in terms of effectively absorbed photons by counting quantum bumps at low intensities in WT flies (e.g. Ref. 20).

With normal bath solutions, the constitutive currents generated by Kir2.1 channels were often very large (>5 nA), potentially leading to the background-subtracted GFP fluorescence measured from at least 10 randomly selected ommatidia from the same preparations using a microfluorimetry system (Photon Technology International) incorporating a photomultiplier, which measured the fluorescence above 510 nm induced by 485-nm excitation from a 75-watt xenon arc lamp.

RESULTS

The Dynamic Range of Kir2.1R228Q Matches Endogenous PIP2 Levels—To track PIP2 levels in vivo we previously expressed the wild-type eGFP-tagged Kir2.1 channel (Kir2.1WT) in Drosophila photoreceptors under control of the rhodopsin (Rh1) promoter. The channels localize almost exclusively to the light-transducing microvilli, where they generate large constitutive inwardly rectifying currents, which can be suppressed by light as PIP2 is hydrolyzed by PLC, and recover in the dark as PIP2 is resynthesized. Most notably, when Ca²⁺ influx via TRP channels was prevented by blocking them with La³⁺, modest stimulation by light resulted in near complete suppression of Kir currents, indicating essentially total depletion of PIP2, as well as PI and PIP. This suggested that Ca²⁺ influx via TRP channels is normally required to inhibit PLC and/or facilitate PIP2 resynthesis (7). We concluded that the resulting light-induced depletion of PIP2 in the trp mutant was the underlying cause of the long-debated trp (“transient receptor potential”) phenotype (21), whereby the response to maintained light in trp mutants decays to baseline, and thereafter the photorecep-
The Kir 2.1 WT channel the response to test flashes (LIC: open symbols) suppression, whereas with Kir2.1R228Q there is only a relative shift of PIP2, we generated a point mutation (R228Q) in Kir2.1, firm this and in an attempt to generate a more accurate probe for PIP2, we generated a point mutation (R228Q) in Kir2.1, which had previously been reported to substantially reduce the activity of Kir2.1R228Q channel activity in the photoreceptors could be further increased at least 2-fold by application of exogenous PIP2 while Kir2.1 WT channels seemed to be saturated (i.e. additional PIP2 did not significantly increase currents). Furthermore, like the WT channel (23), Kir2.1R228Q channels have a PIP2 dose response function with a Hill coefficient close to 1.0 (Supplemental Fig. S1); together with the low (<50%) level of activation, this means that the current should be approximately directly proportional to physiological PIP2 levels.

We quantitatively compared the ability of calibrated light stimuli to suppress Kir currents on the one hand and to inactivate the LIC on the other. With Kir2.1 WT channels, increasing intensities of light, delivered to cells in the presence of La3+ to block TRP channels, progressively suppressed both Kir current and the LIC; however, ~9× higher intensities were required to suppress the Kir current (Fig. 2). By contrast, in flies expressing Kir2.1R228Q, suppression of sensitivity and Kir current were much more closely matched with only a 2.5-fold shift. A very similar behavior (2-fold shift) was also seen in trp mutants expressing Kir2.1R228Q and recorded in the absence of La3+.

We also compared the time courses of the recovery of Kir current (representing PIP2 resynthesis) and the LIC following stimuli inducing substantial PIP2 depletion (Fig. 3). Currents mediated by Kir2.1 WT channels recovered relatively rapidly (t1/2 ~ 30 s), but significant sensitivity to light only began to be

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2 Y. Gu, M. Postma, G. Thomas, P. Raghu, and R. C. Hardie, manuscript in preparation.

Fig. 2. Comparison of Kir2.1 WT and Kir 2.1R228Q channels. In the presence of La3+, a 5-s light stimulus (bar, ~60,000 photons) resulted in a partial “trp decay” and an associated ~70% suppression of the responses to dim test flashes (arrows). In a cell expressing Kir2.1 WT (A) the Kir current was only suppressed by ~10%; however, in a cell expressing Kir2.1R228Q (B), the Kir current was reduced by ~50%. The upper dotted lines (zero current) represent the resting current after total suppression. The graphs (C and D) plot the intensity of the “depleting” stimulus versus normalized suppression of both Kir current (open symbols) and the response to test flashes (LIC; closed symbols) (mean ± S.E. n = 6–7 cells). For the Kir 2.1 WT channel the fitted curves (Equation 1) indicate an 8.6-fold mismatch between sensitivity of the LIC and the Kir current to suppression, whereas with Kir2.1R228Q there is only a relative shift of ~2.5-fold. Similar experiments using the trp mutant expressing Kir2.1R228Q in the absence of La3+ revealed a similar behavior (shift of 1.98; n = 5; data not shown).
restored after the Kir current had almost fully returned to pre-stimulation levels. By contrast, using Kir2.1R228Q the LIC and Kir current recovered over a similar time course (t½ = 30 s) whereas Kir2.1WT recovery (B) was slower. The simultaneously recorded responses to brief test flashes (vertical deflections) recovered with similar time course in cells expressing Kir2.1WT, but the Kir2.1WT current almost completely recovered before significant recovery of the light response (note the initial phase of resynthesis in B is masked by the slow component of the Kir2.1 response to PIP2 depletion). C, the increase of sensitivity to light flashes and Kir2.1R228Q current recorded during “run up” shortly after establishing the whole cell recording configuration (time after w-c) were closely matched. Dotted lines represent zero current (i.e. after complete suppression of Kir current). Normalized time courses of Kir current recovery (solid symbols) and light response (open symbols) are shown on the accompanying graphs. D, half times (t½) for 50% recovery of the light response (LIC), Kir2.1WT and Kir2.1R228Q currents following 50–75% suppression by PIP2 “depleting” stimuli in the presence of 20–40 μM La3+ and (for Kir2.1R228Q only) also following a maximum intensity stimulus under “physiological” conditions in the absence of La3+ (see also Fig. 4) (mean ± S.E., n = 7–11 cells).
In summary, the activity of Kir2.1 R228Q channels shows a close quantitative correlation with the sensitivity to light over a range of PIP2 levels, and the channels’ operating range thus appears to effectively cover the PIP2 levels relevant to phototransduction. Although Kir2.1 WT channels are more sensitive to low levels of PIP2, e.g. during the initial period of PIP2 resynthesis following depletion, they seem to be largely saturated by the dark-adapted resting level of PIP2. We also note that the large differences in sensitivity to suppression by light and the time course of the subsequent recovery, following a point mutation in Kir2.1 known to reduce its effective affinity for PIP2, represent strong additional confirmation that Kir2.1 channels are directly monitoring PIP2 levels under these conditions.

Light-induced PLC Activity—We previously found that, under control “physiological” conditions with both TRP and TRPL function intact, even very bright stimuli only suppressed the Kir2.1WT current by ~20%. It could also not be excluded that even this modest suppression might have represented modulation by the large light-induced ion fluxes (which include Na+, K+, Ca2+, and Mg2+) rather than PIP2 depletion. We therefore repeated these experiments using flies expressing Kir2.1 R228Q, reasoning that if PIP2 levels were in fact reduced under these conditions, there should now be substantially more suppression of the Kir current. Indeed, the Kir2.1 R228Q channels were significantly more sensitive to suppression by light; nevertheless, even the brightest stimuli tested (~10^6 photons, roughly equivalent to full daylight intensities) failed to suppress more than ~40% of the current (Fig. 4). The currents then recovered with the typical time course seen in the presence of La3+ (t₁/₂ ~ 50 s: Figs. 3D and 4B). These results indicate that there is indeed a significant, though not debilitating loss of PIP2 during light adaptation under physiological conditions.

In experiments described thus far, the Kir current was simultaneously recorded with the LIC. Although this approximates physiological conditions, it compromises accurate measurement of the Kir current, because it cannot be cleanly separated from the LIC and because the Kir current may be indirectly influenced by ion fluxes associated with the LIC. To isolate the Kir current we expressed and recorded from Kir2.1 channels in trp;trpl double mutants or in trpl mutants in the presence of La3+. In either case this results in total elimination of all native light-sensitive currents (15, 26), and the electrophysiological response to light now consisted solely of a suppression of the constitutive Kir current uncontaminated by any other conductance. Suppression and recovery of the Kir current should now provide basic quantitative information on rates of PIP2 hydrolysis and synthesis in the absence of feedback by Ca2+. Both the overall suppression and the rate of suppression increased with brief flashes of increasing intensity (Fig. 5). In terms of the overall suppression reached, the data could be reasonably well fitted by a simple equation that as-
assumes that each absorbed photon effectively depletes a number \( n \) of microvilli of their PIP2; namely in Equation 1 (see Ref. 7),

\[
I_{n,\text{max}} = e^{-n p/(m+1)}
\]

(Eq. 1)

where \( p \) is the number of effectively absorbed photons, \( m \) is the total number of microvilli (estimated at 45,000), and \( I_{n,\text{max}} \) is the normalized residual current after suppression. Using Kir2.1R228Q channels, recorded under control conditions, the data were fitted assuming \( -3 - 4 \) microvilli were depleted of PIP2 (and PI and PIP) per effectively absorbed photon (Fig. 5D). Using the higher affinity Kir2.1WT channel, a value of only \(-0.6\) microvilli per photon was estimated.

The suppression of both Kir2.1WT and Kir2.1R228Q currents in response to brief flashes showed complex kinetics. At relatively low intensities, suppression was rather slow (several seconds) and approximately monoexponential, but with brighter flashes a biphasic time course became increasingly apparent with an initial rapid suppression of maximally \(-70\%\) of total current, lasting only \(-500\) ms at the brightest intensities, and a slower suppression that continued for \(-20\) s (Fig. 5C). The maximum slope of the overall response reached rates well in excess of 100% total current \( s^{-1} \) (150 ± 7% \( s^{-1} \), \( n = 9 \)) before saturating at intensities roughly equivalent to one absorbed photon per microvillus (Fig. 5E). These kinetics are probably limited by the Kir2.1 channel response to PIP2 and its gating kinetics, which are relatively slow (e.g. mean open time \(-200-300\) ms (27, 28)). Consequently, even the rapid phase can only be taken as a minimum estimate of the rate of PLC activity. In particular, it should be emphasized that it is very unlikely that the slow response with dimmer flashes, or the slow component of fast flashes, are reflected in similarly slow kinetics of PLC activity and PIP2 depletion, which probably

occur on a sub-second time scale. A direct indication of this can be seen in traces such as Fig. 3B where the LIC typically starts to recover (presumably representing PIP2 resynthesis) while the Kir current is still decreasing.

**Basal PLC Activity—In vitro** Basal activity of many PLC isoforms, including the Drosophila NORPA PLC/\( \alpha \) (29), has often been reported in biochemical experiments; however, to our knowledge basal PLC activity has not previously been monitored in real-time in living cells. We reasoned that by tracking PIP2 levels in the dark under conditions where PIP2 synthesis was blocked, it would be possible to detect and measure the unstimulated basal rate of PLC activity in vivo. We first established that PIP2 levels, as monitored by Kir currents, remained stable under control conditions in the dark. Indeed, currents mediated by both Kir2.1WT and Kir2.1R228Q channels remained stable for considerable periods in photoreceptors recorded with the standard nucleotide enriched pipette solution. Kir2.1WT currents remained stable indefinitely (>15 min), while at most a very gradual loss of current amounting to less than 1% maximum current/min. After the initial run-up (see above), currents mediated by Kir2.1R228Q channels did eventually decay, but only very slowly (maximum rate of \(-5\%\)/min; Fig. 6). This apparent slow loss of PIP2 is presumably non-physiological and may reflect “wash-out” of some factor required for resynthesis. This is suggested by the observation that PIP2 resynthesis rates following PIP2 depleting flashes almost invariably slowed down with repeated flashes or prolonged recording times (e.g. Fig. 5A).

Under control conditions, therefore, it appears that any loss of PIP2 by basal PLC activity must be largely replaced by resynthesis of PIP2 and to detect basal PLC activity, it is first necessary to prevent this resynthesis. We found that this could
be simply achieved without significantly affecting PLC activity by omitting ATP and other nucleotide additives from the electrode. Because the TRP channels activate spontaneously after several minutes of whole cell recordings under these conditions, generating a so-called rundown current (30, 31), measurements were made in trp mutants or in the presence of La3+/H11001 to block TRP channels. We also made measurements in trpl mutants in the presence of La3+/H11001 where no light-sensitive channel activity is possible at all. Under these conditions, the Kir current still initially increased for 2–3 min after turning off the red light, which we interpret as PIP2 resynthesis mediated by the cells endogenous ATP reserves. However, soon afterward, the current decayed spontaneously, usually reaching baseline after a further 3–4 min (Fig. 6). As independent confirmation that this decay represented loss of PIP2, in several cases in WT photoreceptors exposed to La3+/H11001 (n/6) we simultaneously monitored the response to brief test flashes and found that the decay was invariably paralleled by a similar loss in sensitivity.
In Vivo PLC Activity in Drosophila Photoreceptors

Kir2.1WT and Kir2.1R228Q emphasizes that even this residual activity. The significant difference in behavior between delivered a stimulus sufficient to deplete substantial PIP2. Also tried to prevent PIP2 resynthesis using wortmannin, reduced. By contrast the light-induced activity of PLC in cells recorded without ATP (as measured by the rate and extent of Kir current suppression by light) was similar to controls with ATP in the electrode. We also attempted to accelerate the loss of ATP by applying the mitochondrial inhibitor dinitrophenol, which should rapidly reduce any residual ATP levels; however, this appeared to make negligible difference to the maximum rates indicating that omitting ATP from the electrode alone was sufficient to effectively deplete the cell of ATP. Initially we also tried to prevent PIP2 resynthesis using wortmannin, reported to block PI 4-kinase at concentrations above 10 μM (32, 33). However, PIP2 resynthesis following depleting stimuli appeared unaffected even by 50 μM wortmannin applied over several minutes (recovery t50 = 51 ± 5 s, n = 6) suggesting that the PIP2 levels in the microvilli are insensitive to wortmannin.

Spontaneous Rundown Is PLC-dependent—In principle the spontaneous loss of PIP2 could be mediated by basal PLC activity or, e.g., basal activity of lipid phosphatases. To distinguish these we expressed Kir2.1 channels in norpAP24, a severe PLC mutant with no detectable biochemical activity (17). In norpA flies expressing Kir2.1WT, and recorded without ATP in the electrode, Kir currents were stable for at least 15 min of recording (n = 6); in norpA photoreceptors expressing Kir2.1R228Q a small residual decay (9.5 ± 2.2% min−1 n = 6) could be detected after a delay of ~5 min (Fig. 6D). This 4-fold reduction compared with controls suggests that most of the PIP2 loss in WT or trpl backgrounds can be attributed to PLC activity. The significant difference in behavior between Kir2.1WT and Kir2.1R228Q emphasizes that even this residual Kir current rundown represents PIP2 depletion, in this case probably mediated by lipid phosphatases and/or alternative PLC isoforms.

We also tried to confirm that the spontaneous PIP2 loss was due to PLC by applying the PLC inhibitor U73122. Surprisingly, however, rather than preventing the spontaneous decay, U73122 (6 μM), but not the control compound U73443, caused a rapid suppression of Kir currents even in the presence of nucleotide additives. This action was independent of PLC, because it was equally pronounced in norpA mutants and appears to represent a direct and novel action of U73122 as a Kir2.1 inhibitor (Supplemental Fig. 2). From dose response data based on the rate of suppression the IC50 was estimated at ~1.5 μM for Kir2.1R228Q and ~10–15 μM for Kir2.1WT. The increased sensitivity in Kir2.1R228Q may indicate that U73122 acts by inhibiting the PIP2 channel interaction as suggested for a similar inhibitory action of U73122 on another member of the Kir family (the acetylcholine-activated K channel or GIRK) (34, 35).

Basal activity of PLC could be due to spontaneous activity of PLC itself or spontaneous activation of G protein. However, we found that the rate of spontaneous PIP2 loss in a mutant of the Gq protein a subunit, Gaq′, was indistinguishable from that measured in WT or trpl backgrounds (Fig. 6D) suggesting that the basal PLC activity is a property of the PLC molecule itself. Not surprisingly, the ability of light to suppress Kir currents was essentially eliminated (tested with flashes containing up to ~2 × 105 effective photons) in both Gaq and norpA mutants, representing a genetic demonstration that suppression of Kir2.1 currents by light is G protein- and PLC-dependent.

Basal PLC Activity in rdgA Mutants—The primary genetic evidence for a role of DAG as a messenger in Drosophila photoreceptors comes from studies of mutants of the retinal degeneration A (rdgA) gene, which encodes DAG kinase. TRP channels are constitutively active in rdgA mutants, which in principle would be consistent with the suggestion that DAG is an excitatory messenger, because DAG might be expected to accumulate in the absence of metabolism by DAG kinase (36). This presupposes, however, that there is sufficient PIP2 to act as substrate for PLC and that there is still significant PLC with basal activity. Neither can be taken for granted, because conversion of DAG to phosphatidic acid by DAG kinase is also the first step in PIP2 resynthesis, whereas the severe degeneration seen in rdgA results in virtual elimination of the transduction compartment, i.e., the microvilli. We therefore expressed both WT and R228Q Kir2.1 channels in the rdgA mutant. Despite the severe retinal degeneration, the channels were still successfully expressed and targeted to the residual rhabdomeres as judged by GFP fluorescence. After blocking the constitutive TRP channel activity with La WT, substantial Kir currents remained indicating that PIP2 was still present, but absolute PIP2 levels appeared to be reduced as GFP-normalized Kir currents in rdgA cells expressing Kir2.1R228Q were ~5–10 × lower than in WT controls (7.8 ± 2.4-fold, n = 7 cells from three flies). Importantly, in cells recorded without nucleotide additives, the Kir currents, whether Kir2.1WT or Kir2.1R228Q, decayed spontaneously with a time course at least as fast as that seen in WT flies (Fig. 6D). To confirm that this decay was PLC-dependent, we also expressed Kir2.1WT channels in a norpA, rdgA double mutant and observed no decay of the Kir current even after >10 min recording time without nucleotide additives (n = 4, data not shown).

DISCUSSION

The present results build on a previous study, which introduced PIP2-sensitive Kir2.1 channels as electrophysiological biosensors for PIP2 (7). We first demonstrated that a point mutation previously shown to reduce the effective affinity of Kir2.1 for PIP2 generates channels with a dynamic range matched to the PIP2 levels relevant for phototransduction and then used these to derive in vivo estimates of both light induced and basal PLC activity as well as PIP2 resynthesis. In the following we discuss the suitability of Kir2.1WT and Kir2.1R228Q as PIP2 biosensors and explore the significance of the results for our current understanding of phototransduction.

Kir2.1R228Q Is a More Appropriate PIP2 Sensor—The Kir2.1WT channel proved very informative as a PIP2 biosensor (7); however, several lines of evidence indicated that the Kir2.1 channel is more or less saturated by the prevailing dark-adapted PIP2 levels in Drosophila photoreceptors, prompting...
us to investigate a mutant Kir2.1 channel with reduced affinity for PIP2. Extensive studies from several laboratories have described a number of point mutations that reduce the effective affinity of Kir2.1 for PIP2. From these we chose the R228Q mutation (22) and confirmed that the effective affinity was reduced ~4-fold (Supplemental Fig. S1). Because the Kir2.1R228Q channel was less than 50% saturated by the prevailing dark-adapted PIP2 levels and was found to have a Hill coefficient of close to 1.0 (Supplemental Fig. S1), this means that in the steady state, the Kir2.1R228Q current should be approximately proportional to PIP2 over the entire dynamic range and in principle can be further corrected by transforming the data via the Hill equation. Importantly, the close quantitative correlation between sensitivity to light and currents mediated by Kir2.1R228Q (e.g. Figs. 3 and 6) also implies that, under certain conditions, such as in the trp mutant or in recordings made in Ca2+-free solutions, the sensitivity to light itself can also be interpreted as an approximately linear indicator of PIP2 levels.

Rate of PIP2 Hydrolysis by Light-activated PLC—In the present study, not only did we use a probe with an approximately linear response to physiological levels of PIP2, but we also expressed the channels on genetic/pharmacological backgrounds that eliminated TRP and TRPL channel activity allowing us to record the Kir current in isolation. In principle this should provide a measure of the dynamic changes in PIP2 during illumination; however, the kinetics of rapid changes in PIP2 following stimulation by light are almost certainly compromised by the kinetics of the Kir2.1 channel. This has a mean open time reported to be between 200 and 300 ms (27) and multiple closed states, with a mean of ~10 s (28). In addition, although the kinetics of Kir2.1-PIP2 interactions are far from fully understood, the response time of Kir2.1 to PIP2 sequestration by PIP2 antibodies takes tens of seconds (22, 37).

For relatively slow changes in PIP2, such as resynthesis and basal depletion, these kinetics appeared to have little impact, as witnessed by the close correlation with measurements based on the sensitivity to light (e.g. Figs. 3 and 6); however, the kinetics of the light-induced PLC activity, particularly at low stimulus strength, are likely to be significantly underestimated. Even the maximum slope of the suppression of Kir current (Fig. 5E), which reached saturating values of ~150% maximum current s−1, should be considered only as a minimum estimate for the maximal rate of PIP2 depletion. Thus, these rates saturated with stimuli representing only ~1 absorbed photon per microvillus but would be expected to increase further with multiple hits per microvillus, which should recruit additional PLC molecules. The slow channel kinetics probably also result in a slight underestimation of the total light-induced suppression, because there may be significant resynthesis before the channels have completely adjusted to the new level (e.g. Fig. 3B). This seems likely to account for the slight (~2-fold) residual mismatch between sensitivity to suppression of Kir2.1R228Q and the response to light (Fig. 2D).

As discussed previously (7) the prolonged recovery time (t1/2 ~50 s) following depletion, and the lack of any substantial recovery in rdgB mutant defective in PI transfer protein, strongly suggest that the estimated rates of PLC activity reflect not only the immediately available PIP2 but also all PI and PIP that we propose is rapidly converted to PIP2 “on demand” on a sub-second timescale. As will be discussed in more detail elsewhere, together with quantitative estimates of PI and PIP2 levels in the microvilli, the present results allow a conservative estimate of the absolute rate of PLC hydrolysis in Drosophila phototransduction of PIP2 as greater than 104 molecules s−1 photon−1. These rates of PLC were measured in the absence of Ca2+ influx by blocking or genetically eliminating both TRP and TRPL channels; as previously reported the Ca2+ influx associated with the LIC rapidly inhibits PLC and under physiological conditions prevents this precipitous depletion of PIP2 (7). Presumably, however, these rates of hydrolysis are also approached during the 20- to 100-ms latent period of the response, resulting in large transient and localized increases in DAG and InsP3.

Basal PLC Activity—Previous measurements of basal, unstimulated PLC activity have relied on biochemical experiments (e.g. Ref. 33). In the present study we were able to estimate the rate of spontaneous PIP2 depletion in vivo in real-time by depriving the cells of ATP required for PIP2 resynthesis. The rundown of Kir2.1 and other Kir channels in the absence of ATP has been widely reported, particularly in excised patches (37), but has generally been attributed to depletion of PIP2 by lipid phosphatase activity. Because the spontaneous decay of Kir2.1 channels was either blocked or greatly slowed in the near null PLC mutant, norpA224, it seems likely that the spontaneous PIP2 loss in the photoreceptors is largely due to basal PLC activity. Formally, however, we cannot exclude the possibility that PLC activity under these conditions also indirectly promotes some other mode of PIP2 degradation (e.g. DAG might stimulate a lipid phosphatase). The maximum rates of PIP2 loss amounted to ~40% of the total PIP2 per minute with loss of all detectable PIP2 even by the higher affinity Kir2.1WT occurring typically within 10 min of break-in.

Although this apparent high rate of basal turnover may at first seem surprising, it is in keeping with the few available measurements in other cells. For example, a recent comprehensive biochemical study of PI turnover (33) estimated that the total cellular pool of PIP2 in human neuroblastoma cells was turned over every 5 min under basal conditions. In fact, compared with the maximum light-activated rate (150% s−1, Fig. 5), basal turnover in Drosophila photoreceptors appears exceptionally low. Thus, although the light-activated rate is believed to represent not only PIP2 but also the microvillar PI and PIP pool, the estimate of basal activity presumably represents just PIP2. If, as in most cells, PIP2 represented only 5–10% of the total PI pool, this would imply that the basal rate is ~2000–4000 times less than the maximal activated rate. This compares, for example with a basal rate in neuroblastoma cells that is only ~20-fold less than the maximum activated rate (33). The fact that the low basal rate in Drosophila has the potential to deplete all detectable PIP2 within 10 min is presumably a reflection of the extremely high density of PLC in the microvillar membrane (~100 copies per microvillus (38)).

Under normal conditions, the basal activity of PLC may be of little consequence for the cell, because the PIP2 is continually replaced. However, the demonstration of such a significant overall rate of basal activity is important for the interpretation of some recent studies on the mechanism of phototransduction. First, it contributes to the debate over the long-standing finding that the light-sensitive TRP channels activate spontaneously in the absence of ATP or when cells are challenged with metabolic inhibitors (30, 31). On the one hand, it has been proposed that this is due to protein dephosphorylation, possibly of the light-sensitive channels themselves (31, 39). However, in a recent study we found that activation of TRP channels by metabolic inhibition was dependent upon PLC, and we proposed that the spontaneous activation under these conditions was most likely due to failure of DAG kinase to metabolize DAG (40). Because substantial other genetic evidence is consistent with an excitatory role for DAG (reviewed in Refs. 3, 36, 41), this seems a plausible explanation, but it assumes the existence of a substantial basal PLC activity.
for which there was no evidence. This conclusion has since been challenged (39). The present study strongly indicates that there is indeed a high level of basal PLC activity, consistent with this interpretation. Second, the finding that the TRP channels in the rdgA mutant were constitutively active represented the first genetic evidence for the excitatory role of DAG in Drosophila phototransduction (36). It presupposed, however, both that there were significant amounts of PIP2 remaining in rdgA mutants and that there was basal PLC activity, which could convert this to DAG. Again our results provide direct evidence supporting both these assumptions, thus consolidating our previous conclusions and adding strength to the proposal that DAG is an excitatory messenger in Drosophila phototransduction.

**Concluding Remarks**—The present study has demonstrated the potential of the Kir2.1 PIP2-sensitive ion channel, and in particular the mutant Kir2.1R218Q as a biosensor for tracking PIP2 in vivo providing unique quantitative insight into the dynamics of PIP2 turnover. Their usefulness in other systems remains essentially unexploited; however, Kobrinsky et al. (35) demonstrated that a similarly engineered Kir2.1 channel (R218Q) also responded to agonist stimulation of PLC when expressed in Xenopus oocytes. Drosophila phototransduction has long been an influential model for PLC signaling. The ability to directly monitor both the activity of PLC and the PIP2 resynthesis machinery in vivo, coupled with the rich repertoire of transduction mutants should provide the opportunity to dissect many features of the regulation that have previously been intractable to analysis. We also note that the present study has established conditions whereby PIP2 levels can be rapidly and quantitatively manipulated in vivo. PIP2 is increasingly implicated in the regulation of an ever-growing list of membrane proteins, including a variety of ion channels and transporters. Targeted expression of proteins such as Kir2.1 in Drosophila photoreceptors may provide a useful approach for exploring their PIP2 dependence under physiological conditions.

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