

# Light-Dependent Modulation of Shab Channels via Phosphoinositide Depletion in *Drosophila* Photoreceptors

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## SUMMARY

The *Drosophila* phototransduction cascade transforms light into depolarizations that are further shaped by activation of voltage-dependent K<sup>+</sup> (Kv) channels. In whole-cell recordings of isolated photoreceptors, we show that light selectively modulated the delayed rectifier (Shab) current. Shab currents were increased by light with similar kinetics to the light-induced current itself (latency ~20 ms), recovering to control values with a  $t_{1/2}$  of ~60 s in darkness. Genetic disruption of PLC $\beta$ 4, responsible for light-induced PIP<sub>2</sub> hydrolysis, abolished this light-dependent modulation. In mutants of CDP-diacylglycerol synthase (*cds*<sup>1</sup>), required for PIP<sub>2</sub> resynthesis, the modulation became irreversible, but exogenously applied PIP<sub>2</sub> restored reversibility. The modulation was accurately and reversibly mimicked by application of PIP<sub>2</sub> to heterologously expressed Shab channels in excised inside-out patches. The results indicate a functionally implemented mechanism of Kv channel modulation by PIP<sub>2</sub> in photoreceptors, which enables light-dependent regulation of signal processing by direct coupling to the phototransduction cascade.

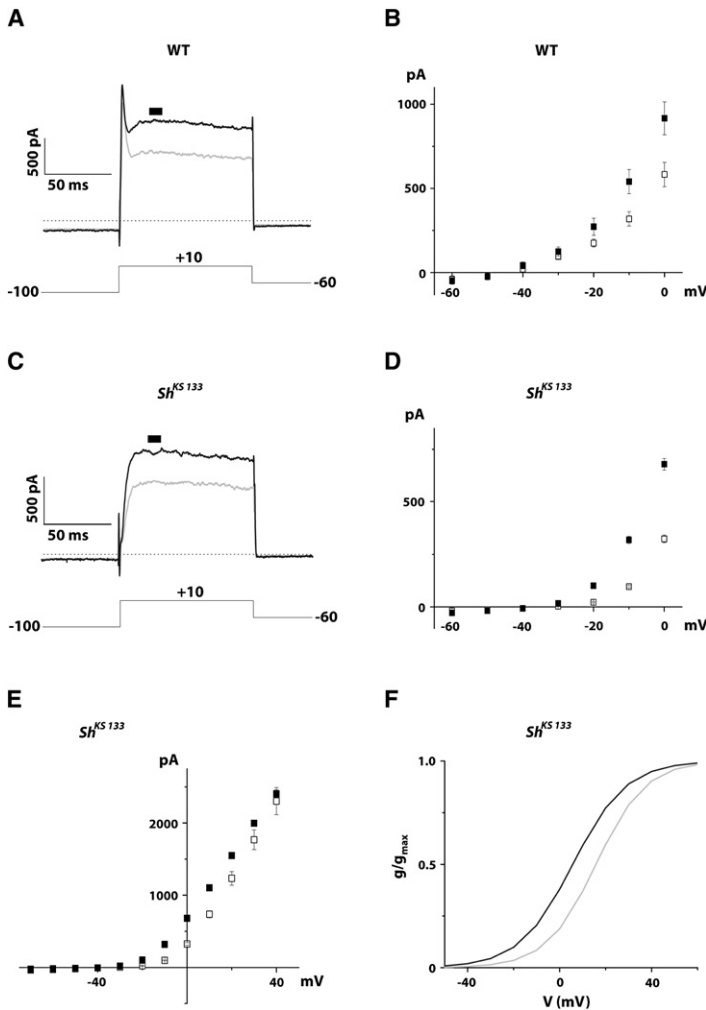
## INTRODUCTION

In *Drosophila* photoreceptors, light-dependent activation of TRP and TRPL channels produces an inward current, carried mainly by calcium and sodium ions, creating a light-dependent depolarization. This voltage-change is opposed by opening of at least four types of voltage-activated K<sup>+</sup> channels (Kv channels): a rapidly inactivating A current mediated by Shaker channels (Hardie et al., 1991), a slow delayed rectifier (Shab), and two others responsible for a fast delayed rectifier and a slow, noninactivating current (Hardie, 1991; Vähäsöyrinki et al., 2006). Shaker and Shab are the prototypical members of the Kv1 and Kv2 voltage-activated K<sup>+</sup> channel subfamilies, respectively (Wei et al.,

1990). The functional significance of these K<sup>+</sup> channels in the photoreceptors has turned out to be rather complicated. Currents mediated by Shaker and Shab have been proposed to play various roles in adaptation, regulation of gain and frequency response, and in optimizing the response voltage range (Weckström et al., 1991; Laughlin and Weckström, 1993; Weckström and Laughlin, 1995; Niven et al., 2003; Vähäsöyrinki et al., 2006).

Many K<sup>+</sup> channels, including those in *Drosophila* neurons (Yao and Wu, 2001; Park et al., 2006; Jonas and Kaczmarek, 1996), have been reported to be targets of modulation by intracellular signaling molecules. The transduction cascade, with its various enzymatic and nonenzymatic components, could be a potential modulator of the K<sup>+</sup> channels. This has not been investigated systematically in *Drosophila* photoreceptors, although one report suggested that the delayed rectifier was modulated by CaMKII (Peretz et al., 1998), while the fast Shaker current can be modulated by serotonin (Hevers and Hardie, 1995). Phototransduction in *Drosophila* photoreceptors is mediated by a canonical phospholipase C (PLC) signaling cascade located in the microvillar part of the photoreceptors (rhabdomeres), where temporal fluctuations in the number of absorbed photons are transformed into modulation of membrane voltage (Hardie and Raghu, 2001; Niven et al., 2003). In the cascade, light triggers (via rhodopsin isomerization and G proteins) the activation of the phospholipase C isoform  $\beta$ 4 (PLC $\beta$ 4, encoded by the *norpA* gene). This catalyzes hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) into soluble inositol-1,4,5,-triphosphate (InsP<sub>3</sub>) and membrane-bound diacylglycerol (DAG). Of these, DAG or one of its metabolites leads eventually to opening of the Ca<sup>2+</sup>- and Na<sup>+</sup>-permeable TRP and TRPL channels (encoded by the *trp* and *trpl* genes), while most available evidence suggests that InsP<sub>3</sub> plays no role in the photoreceptor's light response (for reviews see Hardie and Raghu, 2001; Hardie, 2007; Wang and Montell, 2007). The continuous functionality of the photoreceptors is maintained by regeneration of PIP<sub>2</sub> in a cyclic, enzymatic pathway (the PI cycle, see Figure 9). This means that illumination of the photoreceptor activates turnover of its membrane lipids and dynamically modulates their composition (Wu et al., 1995; Hardie et al., 2001, 2004).

In the present work, we show that the delayed rectifier K<sup>+</sup> channel (encoded by *shab*) is selectively, rapidly, and reversibly upregulated by light stimulation. Our analysis indicates that this



**Figure 1. Light Stimulation Increases the Outward Current of *Drosophila* Photoreceptors**

(A) Voltage-clamp current traces of wild-type photoreceptors in response to voltage pulses from  $-100$  to  $+10$  mV before (gray) and after 10-fold standard light stimulation (black). The dotted line represents the zero current level. The black bar indicates the time, at which the Shab peak current is depicted.

(B) The current-voltage dependency of the Shab peak current in wild-type photoreceptors before (open symbol) and after 10-fold standard light stimulation (filled symbol) in a physiological voltage range ( $n = 4 \pm$  SEM).

(C) Voltage-clamp current traces of  $sh^{KS133}$  photoreceptors in response to voltage pulses from  $-100$  to  $+10$  mV before (gray) and after 10-fold standard light stimulation (black). The dotted line represents the zero current level. The black bar indicates the time, at which the Shab peak current is depicted.

(D) The current-voltage dependency of the Shab peak current in  $sh^{KS133}$  photoreceptors before (open symbol) and after 10-fold standard light stimulation (filled symbol), in a physiological voltage range ( $n = 4 \pm$  SEM).

(E) Plot of (D) over the complete measured voltage range.

(F) The averaged fitted conductance voltage dependency of the peak outward current in  $sh^{KS133}$  before (gray) and after illumination (black). Conductance has been calculated according to Equation 1, fitted according to Boltzmann (Equation 2), normalized to maximal conductance, and then averaged (no error bars shown).

### Light Stimulation Boosts the Voltage-Dependent Potassium Current in *Drosophila* Photoreceptors

In order to test whether illumination of photoreceptors could modulate Kv channels, we recorded Kv currents before and after stimulation by light. Standard light stimulation (consisting of ten repeated 100 ms light flashes, defined in [Experimental Procedures](#)) of wild-type (WT) photoreceptors increased the Kv currents activated by  $+10$  mV command voltage pulses by up to 30%. This effect was most obvious for the late component of the Kv

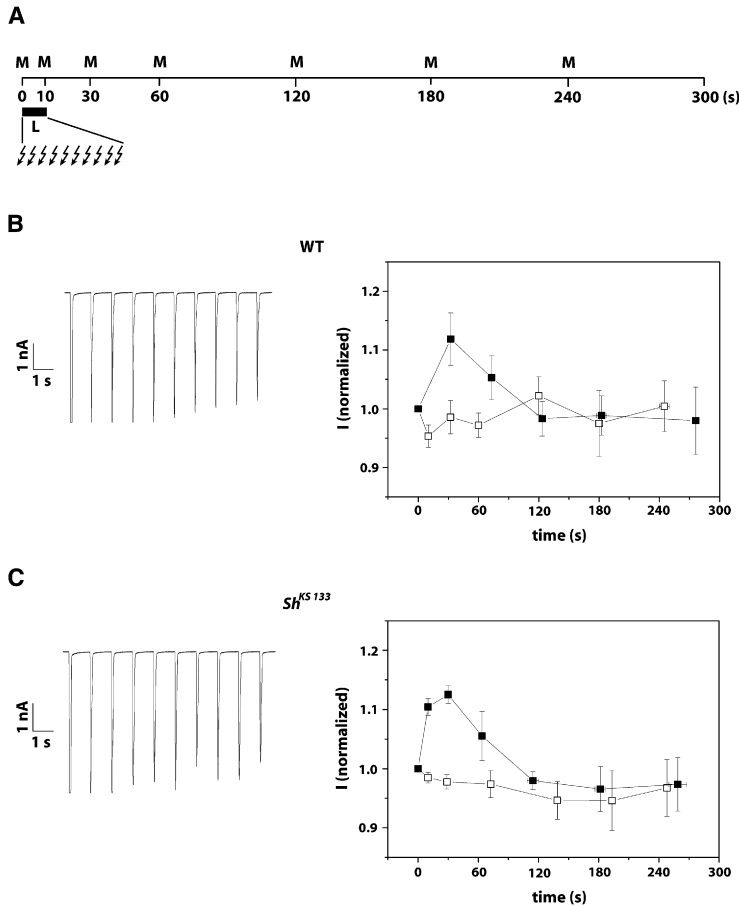
current response, which is dominated by Shab (Figure 1A). After light stimulation, the I/V curve of the late, presumptive Shab peak current clearly shifted to hyperpolarized potentials (leftward shift) (Figure 1B). Particularly at low but physiologically relevant potentials ( $-40$  to  $-20$  mV), this parallel shift resulted in a large ( $\sim 3$ -fold) relative increase of conductance. The increase in the Kv current usually developed fully by the time of the first measurement after standard illumination, namely within 10 s of stimulation (see further below). Repeated measurements in the dark showed that the current typically recovered to baseline levels within  $\sim 2$  min ( $\tau_{1/2} \sim 60$  s) (Figure 2) and when tested could be induced at least a second time in the same photoreceptor (data not shown). The described effects did not depend on the eye color (data not shown).

## RESULTS

In whole-cell patch-clamp recordings of *Drosophila* photoreceptors, positive command voltages evoke large voltage-dependent potassium (Kv) currents. These are conducted by potassium channels encoded by *shaker* and *shab* genes and by one or more unidentified channels that make only minor contributions to the whole-cell currents (Hardie, 1991; Vähäsöyrinki et al., 2006). It has been shown that Shaker is the dominant component of the A-type current (Hardie, 1991; Hardie et al., 1991) and that Shab channels conduct the slow delayed rectifier current (Vähäsöyrinki et al., 2006) (Figure 1). Activation and inactivation kinetics of Shaker channels are unusually rapid (a few milliseconds) compared to most other  $K^+$  channels, whereas, depending on the potential, Shab requires  $\sim 10$ – $30$  ms to fully activate and several seconds to inactivate.

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In principle, the apparent increase of the presumptive Shab current could also be caused by reduction of the residual inactivation of the Shaker channels. The inactivation time course of the Shaker current consists of a fast N-type inactivation and a slow C-type inactivation, which is superimposed on the time course of the slow delayed rectifier current (Roepker et al., 1997). In order to test whether the apparent light-dependent modulation of the slow delayed rectifier current is caused



**Figure 2. Light-Dependent Modulation Is Reversible**

(A) Design of the experiment: M = measurement to elicit Shab peak currents, +10 mV pulses with –100 mV prepulses were applied at the indicated points of time; L = standard light stimulation.

(B) CS WT. (Left) A typical current response upon standard light stimulation of a single photoreceptor. The first response is cut off due to amplifier saturation. (Right) Increase and recovery of Shab peak currents. Filled symbols represent experiments with standard light stimulation ( $n = 4-11$ , and 1 refers to  $1213 \pm 50$  pA SEM). Open symbols represent control experiment without light stimulus ( $n = 4-7$ , and 1 refers to  $1024 \pm 31$  pA SEM).

(C) *sh<sup>KS133</sup>*. (Left) A typical current response upon standard illumination of a single photoreceptor. (Right) Increase and recovery of Shab peak currents. Filled symbols represent experiments with standard light stimulation ( $n = 4-17$ , and 1 refers to  $957 \pm 39$  pA SEM). Open symbols represent control experiment without light stimulus ( $n = 5-11$ , and 1 refers to  $986 \pm 47$  pA SEM).

by a modulation of Shaker C-type inactivation, we repeated the experiment using the *sh<sup>KS133</sup>* mutants. In *sh<sup>KS133</sup>*, the channel protein carries a V429D point mutation, which results in expression of nonfunctional Shaker channels (Lichtinghagen et al., 1990). Nevertheless, standard light stimulation induced a similar leftward shift of the I/V curve to that measured in WT (Figures 1C and 1D), indicating that the modulation was not mediated by Shaker channels. When the data were plotted in terms of conductance ( $g$ ) to generate the voltage dependence of activation (Figures 1E and 1F), it was apparent that absolute  $g_{max}$  values were not altered by illumination (before light,  $20.3 \pm 1.7$  nS SEM; after light,  $20.1 \pm 0.4$  nS SEM;  $n = 4$ ) but that the modulation could be described by a parallel leftward shift of  $\sim 10$  mV and that an increase of open probability  $P_o$  of the channels rather than a change in single-channel conductance is responsible for the enhanced currents.

These findings showed that illumination of photoreceptors in WT and *sh<sup>KS133</sup>* mutant flies resulted in a leftward shift of the activation curve, leading to an increase in the delayed rectifier current at a given potential. Below, this phenomenon will be called light-dependent modulation (LDM) of the delayed rectifier current.

### LDM Is Specific to Shab Channels

To further test whether the LDM was specific to Shab channels, we recorded Kv currents in *shab<sup>2</sup>* and *shab<sup>1</sup>* mutants. In *shab<sup>2</sup>*,

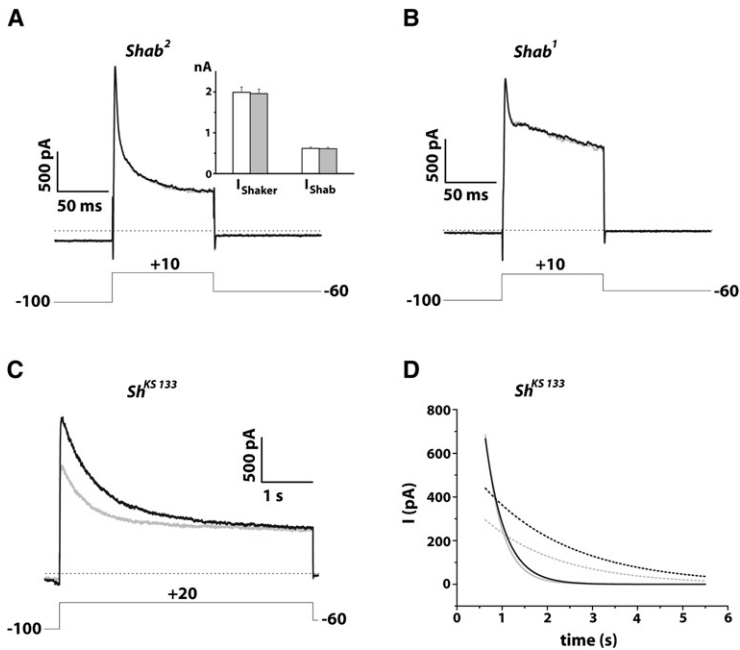
the channel protein carries a V608D point mutation in the entryway to the pore (Hegde et al., 1999). In photoreceptors of *shab<sup>2</sup>* mutants, no Shab current could be detected. The residual current, which is probably conducted by the other Kv channels, was 54% of the amplitude of Shab peak current in WT with voltage commands to +10 mV (*shab<sup>2</sup>*:  $617 \pm 36$  pA SEM,  $n = 7$ ; WT:  $1140 \pm 39$  pA SEM,  $n = 18$ ,  $p < 0.01$ ). In marked contrast to the results obtained in WT or *sh<sup>KS133</sup>* mutants, standard light stimulation of *shab<sup>2</sup>* photoreceptors now no longer influenced the remaining Kv current, with both the Shaker peak current and the following residual currents remaining unaltered (Figure 3A, cf. Figures 2B and 2C). Thus, LDM of the slow delayed rectifier is specific to the Shab channels.

In the *shab<sup>1</sup>* mutant, the channel protein carries an R435Q point mutation in the last amino acid N-terminal to the first transmembrane segment. In *shab<sup>1</sup>* photoreceptors, the mutant channel protein still generated substantial currents, which were only reduced by 13% (currents at +10 mV *shab<sup>1</sup>*:  $982 \pm 37$  pA SEM,  $n = 17$ ; WT:  $1140 \pm 39$  pA SEM,  $n = 18$ ,  $p < 0.01$ ). However, standard light stimulation no longer influenced the Shab current in *shab<sup>1</sup>* mutant photoreceptors, indicating that Arg<sup>435</sup> is required for induction of LDM (Figure 3B).

**Dependence of LDM on Channel Kinetics**  
To investigate the modulation of kinetic parameters of the Shab current, long activating voltage pulses (5 s) were applied to photoreceptors of *sh<sup>KS133</sup>* mutant flies. This allowed monitoring of activation and complete inactivation of the Shab current (Figure 3C). The kinetics of activation, in terms of time to peak, did not change upon standard illumination.

### Dependence of LDM on Channel Kinetics

Inactivating currents of a set of nine photoreceptors were fitted with a two-component exponential function (Equation 3) (Hardie, 1991; Immke et al., 1999). The averaged parameters of the fitted function are presented in Table 1. After standard light stimulation, only the amplitude of the slow inactivating



**Figure 3. LDM Is Specific to Shab Channels**

(A) Typical current traces of a single *shab*<sup>2</sup> photoreceptor in response to voltage pulses from  $-100$  to  $+10$  mV before (gray) and after standard light stimulation (black) show perfect overlay. The dotted line represents the zero current level. The inset shows that neither Shaker peak current  $I_{\text{Shaker}}$  ( $n = 6 \pm \text{SEM}$ ) nor residual Shab current  $I_{\text{Shab}}$  ( $n = 7 \pm \text{SEM}$ ) is regulated by light stimulation.  $I_{\text{Shab}}$  is depicted as described in [Experimental Procedures](#).

(B) Typical current traces of a single *shab*<sup>1</sup> mutant photoreceptor in response to voltage pulses from  $-100$  to  $+10$  mV before (gray) and after standard light stimulation (black). The dotted line represents the zero current level. Note that in *shab*<sup>1</sup> the residual Shab current is large but shows no modulation.

(C) Representative current traces of a single *sh*<sup>KS133</sup> mutant photoreceptor in response to 5 s voltage pulses from  $-100$  to  $+20$  mV before (gray) and after standard light stimulation (500 Hz sampling rate, 200 Hz filter). The dotted line represents the zero current level.

(D) The fast (solid line) and slow decaying (dashed line) components of Shab current before (gray) and after standard light stimulation (black), calculated from parameters given in [Table 1](#).

component of Shab ( $A_{\text{slow}}$ ) was significantly modulated. A graphical representation of the parameters of [Table 1](#) is shown in [Figure 3D](#). It has been shown previously that, depending on the occupational state of the pore, Shab-type channels can exist in two conformations, one of which inactivates much more slowly than the other ([Immke et al., 1999](#)). Selective upregulation of the amplitude of  $A_{\text{slow}}$  indicates that only those channel proteins residing in the slow inactivating conformation were affected by LDM.

As previously described, a fast delayed rectifier, which inactivates over a time course of tens of milliseconds and which is probably mediated by Shal channels, is also found in  $\sim 50\%$  of the photoreceptors ([Hardie, 1991](#); [Vähäsöyrinki et al., 2006](#)). We recorded such a current in four of the nine measured photoreceptors, but since the inactivation time constant of this current is much faster than of Shab ([Table 1](#)), the random appearance of the putative Shal current does not affect the conclusion drawn above.

In summary, experiments using *sh*<sup>KS133</sup>, *shab*<sup>1</sup>, and *shab*<sup>2</sup> mutant flies showed that LDM in photoreceptors is specific to the Shab current and that a point mutation (R435Q) in the N terminus of the Shab protein abolishes the regulation. Kinetic analysis implies that LDM relies on a selective upregulation of the slow inactivating conformation of the Shab channel.

### LDM of Shab Channels Is Initiated Downstream of PLC $\beta$ 4

What is the mechanism whereby light stimulation causes a leftward shift of the activation curve of Shab and the concomitant increase in  $K^+$  current? The light response in *Drosophila* photoreceptors results in massive  $Ca^{2+}$  influx via TRP channels, which is believed to raise the intracellular  $Ca^{2+}$  concentration throughout the cell by at least  $10 \mu\text{M}$  and in the microvilli by as much as  $1 \text{mM}$  ([Hardie, 1996](#); [Oberwinkler and Stavenga, 2000](#); [Postma et al.,](#)

[1999](#)). In addition, many  $Ca^{2+}$ -sensitive regulatory pathways and  $Ca^{2+}$ -sensitive enzymes, including CaMKII and PKC, are present in *Drosophila* photoreceptors ([Matsumoto et al., 1994](#); [Hardie et al., 2001](#)). Therefore, we first asked whether TRP-mediated  $Ca^{2+}$  influx might induce the LDM of Shab channels as proposed by [Peretz et al. \(1998\)](#). However, recording the Shab current in  $Ca^{2+}$ -free external solution did not support this hypothesis ([Figure 4A](#)), since robust and reversible LDM was still observed under these conditions. Even though the decay of the LDM seemed to be impeded, it was apparent that at the same time the control Kv current (with no light stimulation) gradually increased under these conditions. After taking this into account, the LDM also showed typical decay to baseline under  $Ca^{2+}$ -free (extracellular) conditions. The light-induced current response plotted in [Figure 4A](#) (left) is typical for recordings in  $Ca^{2+}$ -free solution and is explained by the absence of  $Ca^{2+}$ -dependent negative feedback (e.g., [Hardie et al., 2001](#); [Gu et al., 2005](#)).

To complement the results obtained with  $Ca^{2+}$ -free external solution, we measured Kv currents in *trpl;trp* double mutants, lacking both classes of light-sensitive channel ([Niemeyer et al., 1996](#); [Reuss et al., 1997](#); [Leung et al., 2000](#)). Illumination of *trpl;trp* photoreceptors leads to activation of the complete phototransduction cascade, but does not result in any light-induced current ([Figure 4B](#), left) or  $Ca^{2+}$  influx. However, also here Shab channels showed a clear LDM. Thus, the initiator of LDM is located upstream of TRP/TRPL in the phototransduction cascade and does not require  $Ca^{2+}$  influx.

Interestingly, in both  $Ca^{2+}$ -free solution and the *trpl;trp* double mutant, the LDM of Shab channels was more pronounced than in WT photoreceptors in physiological,  $Ca^{2+}$ -containing solutions (e.g., compare [Figure 2B](#) and [Figure 4B](#)). This would be consistent with the LDM being mediated by PLC, since net  $\text{PIP}_2$  hydrolysis by PLC is greatly increased in the absence of  $Ca^{2+}$  influx,

**Table 1. Kinetic Inactivation Parameters**

	$A_{\text{fast}}$	$\tau_{\text{fast}}$	$A_{\text{slow}}$	$\tau_{\text{slow}}$	$I_{\text{SS}}$
Before light	686 ± 46	0.379 ± 0.014	296 ± 33	1.654 ± 0.212	504 ± 26
After light	666 ± 75	0.43 ± 0.021	440 ± 48	1.946 ± 0.426	500 ± 33
Significant difference ( $p \leq 0.05$ )	–	–	+	–	–

Parameters are derived from fitting the decay of Shab outward currents with Equation 3.  $A_{\text{fast}}$  and  $\tau_{\text{fast}}$  are amplitude and time constant of the fast inactivating component,  $A_{\text{slow}}$  and  $\tau_{\text{slow}}$  of the slow inactivating component, respectively.  $I_{\text{SS}}$  is the steady-state current (mean ± SEM,  $n = 9$ ).

which normally strongly inhibits PLC (Hardie et al., 2001, 2004). The fact that the modulation measured in the *trpl;trp* mutant was even greater than in  $\text{Ca}^{2+}$ -free solutions may reflect the fact that in the latter situation the light response overlaps slightly with the first recording after standard illumination (10 s). This leads to reduced early Kv current measurements (10 s) and becomes apparent in a flattened and slowed time course of LDM. In contrast, in *trpl;trp* photoreceptors in the absence of any light response, the early (10 s) measurement after standard illumination represents more accurately the instantaneous Shab channel regulation.

To test more directly the role of PLC in light-dependent Shab channel regulation, we performed experiments in *norpA<sup>P24</sup>*, which is a null or near null mutant of PLC $\beta$ 4 (Pearn et al., 1996; Leung et al., 2000). We actually used a *norpA;trp* double mutant, since there is a low level of spontaneous TRP channel activity in *norpA* (Hardie et al., 2003). Upon standard light stimulus, neither a light response nor any indication of LDM could be observed (Figure 4C). This confirms the requirement for PLC and suggests that its substrate,  $\text{PIP}_2$ , or its products, DAG or  $\text{InsP}_3$ , are involved in the initiation of LDM.

Taken together, these experiments indicate that the LDM is initiated by either reduction of  $\text{PIP}_2$  or increase of DAG or  $\text{InsP}_3$ . Any requirement for light-induced and TRP-mediated  $\text{Ca}^{2+}$  influx can be excluded.

### LDM Is Not Mediated by $\text{Ca}^{2+}$ Release from $\text{InsP}_3$ -Sensitive Stores

In order to test the possible role of  $\text{InsP}_3$  as initiator of LDM of Shab channel, we employed a mosaic null mutant of the only known *Drosophila*  $\text{InsP}_3$  receptor, *itpr<sup>90B.0</sup>* (Raghu et al., 2000). After standard light stimulation, photoreceptors of *itpr<sup>90B.0</sup>* showed LDM of Shab peak current indistinguishable from that observed in WT (Figure 5A). This excludes a possible role of the  $\text{InsP}_3$  receptor in LDM. As the most familiar role of  $\text{InsP}_3$  is to mediate release of  $\text{Ca}^{2+}$  from intracellular stores, we also measured LDM of Shab channels using pipettes containing BAPTA (2 mM free BAPTA, 110 nM free  $\text{Ca}^{2+}$ ) to buffer intracellular  $\text{Ca}^{2+}$ . These measurements were made in *trpl;trp* double mutants to prevent  $\text{Ca}^{2+}$  influx from the extracellular space. Following standard light stimulation, the Shab current recorded with BAPTA in the pipette showed similar regulation to control experiments performed without BAPTA (Figure 5B). Similarly, inclusion of EGTA (8 mM) in the patch pipette, calculated to buffer the intracellular free- $\text{Ca}^{2+}$  concentration to 55 nM, did not impede LDM (data not shown). We also tested the effect of the Ca-ATPase inhibitor thapsigargin (2  $\mu\text{M}$ ), which has been shown to deplete intracellular  $\text{Ca}^{2+}$  stores and raise internal  $\text{Ca}^{2+}$  by

~200 nM (Hardie, 1996). However, the typical light-dependent Shab channel regulation was again unaffected (data not shown). Thus, neither the  $\text{InsP}_3$  receptor nor any other  $\text{Ca}^{2+}$  release mechanism seems to be involved in the LDM of Shab current.

### LDM of Shab Channels Is Correlated with $\text{PIP}_2$ Depletion

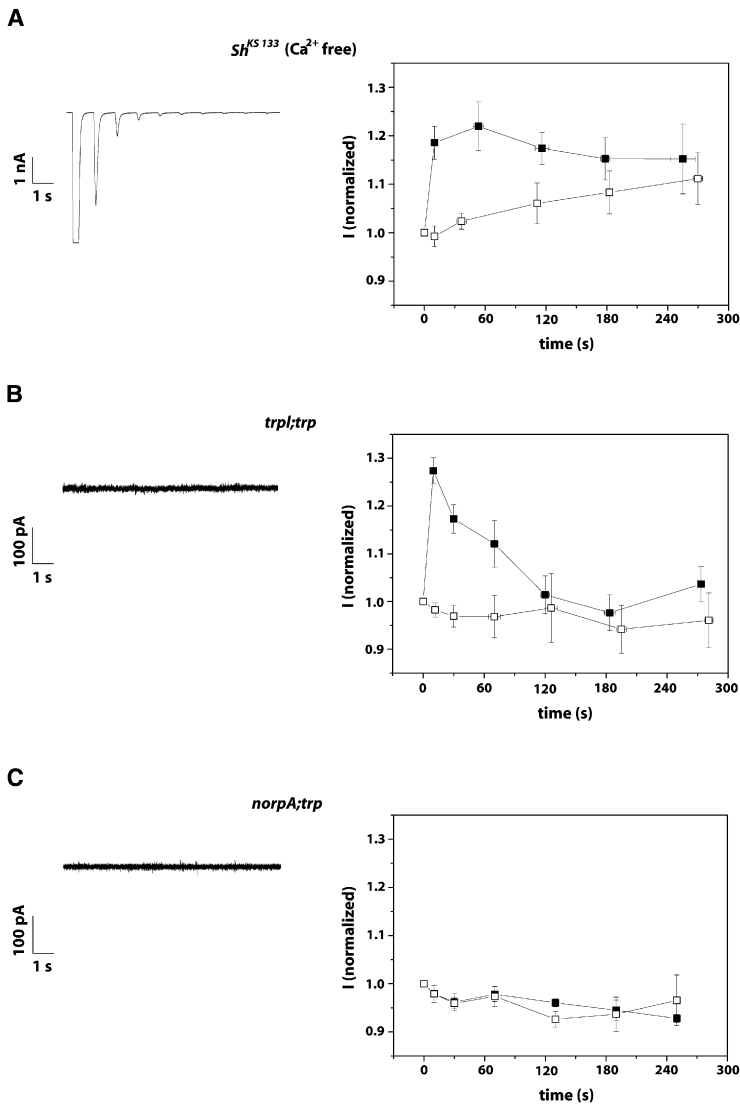
The apparent lack of involvement of  $\text{InsP}_3$  suggests that one or other of the membrane-delimited consequences of PLC activity, namely DAG generation or  $\text{PIP}_2$  reduction, may mediate the LDM. To distinguish these possibilities, we performed experiments in the *cds<sup>1</sup>* mutant, a null mutant of CDP-diacylglycerol synthase (CDS), catalyzing an essential step in the  $\text{PIP}_2$  synthesis cycle in *Drosophila* photoreceptors (Figure 9) (Wu et al., 1995). Dark-adapted *cds* mutants have near-normal levels of microvillar  $\text{PIP}_2$ , probably due to an alternate CDS isoform not specialized for phototransduction; however, following illumination,  $\text{PIP}_2$  becomes depleted in *cds<sup>1</sup>* mutants and does not recover on the timescale of the physiological experiments (Wu et al., 1995; Hardie et al., 2002). While DAG is initially generated during illumination, the exhaustion of substrate ( $\text{PIP}_2$ ) means that DAG generation cannot be sustained. The *cds<sup>1</sup>* mutants were reared in constant darkness to prevent light-induced  $\text{PIP}_2$  depletion, thus allowing light responses initially similar to those in WT. After standard light stimulation, no LDM of Shab could be observed. However, increasing the light level by a factor of ten caused an irreversible upregulation by up to 35% (Figure 6), suggesting depletion of  $\text{PIP}_2$  as responsible mediator of LDM of Shab. The same experiment (10-fold light intensity) in *trpl;trp* double mutants resulted in an upregulation of about 30% and recovery to baseline within 3 min (Figure 7D). Under these light stimulation conditions, the LDM in both WT and Oregon Red photoreceptors also recovered to baseline within 3 min (data not shown). Hence, an unspecific irreversible regulation by excessive illumination can be excluded.

To further test the role of  $\text{PIP}_2$ , we repeated the experiment with 26  $\mu\text{M}$  di- $\text{C}_8$   $\text{PIP}_2$  in the intracellular solution in an attempt to replace the membrane  $\text{PIP}_2$  in *cds<sup>1</sup>* mutant photoreceptors after light-induced  $\text{PIP}_2$  depletion. Under these conditions, standard light stimulation caused upregulation by only about 15%, which decayed nearly completely to baseline in 4 min (Figure 6).

Together, irreversibility of LDM of Shab in *cds<sup>1</sup>* mutants and its recovery upon application of exogenous di- $\text{C}_8$   $\text{PIP}_2$  strongly suggest that LDM was initiated by depletion of membrane  $\text{PIP}_2$ .

### Intensity Dependence and Onset of LDM

The intensity dependence of  $\text{PIP}_2$  depletion in *Drosophila* has previously been measured by using the  $\text{PIP}_2$ -sensitive ion channel Kir2.1 as a genetically targeted biosensor (Hardie et al., 2001,



**Figure 4. LDM of Shab Is Initiated by Activation of PLC $\beta$ 4**

(A) *sh<sup>KS133</sup>* with nominal Ca<sup>2+</sup>-free extracellular solution. (Left) A typical current response upon standard light stimulation of a single photoreceptor. (Right) Increase and recovery of Shab peak currents. Filled symbols represent experiments with standard light stimulation ( $n = 3-7$ , and 1 refers to  $1457 \pm 51$  pA SEM). Open symbols represent control experiment without light stimulus ( $n = 6-7$ , and 1 refers to  $1450 \pm 69$  pA SEM). Note the rise of Shab peak current in the control experiment.

(B) *trpl;trp*. (Left) A typical current response upon standard light stimulation of a single photoreceptor. Due to genetic disruption of TRP and TRPL, no light response, and thereby no Ca<sup>2+</sup> influx, occurs. (Right) Increase and recovery of Shab peak currents. Filled symbols represent experiments with standard light stimulation ( $n = 3-8$ , and 1 refers to  $1131 \pm 93$  pA SEM). Open symbols represent control experiment without light stimulus ( $n = 4-6$ , and 1 refers to  $1137 \pm 102$  pA SEM).

(C) *norpA;trp*. (Left) A typical current response upon standard light stimulation of a single photoreceptor. Due to genetic disruption of PLC $\beta$ 4, no hydrolysis of PIP<sub>2</sub> takes place and therefore no light response occurs. (Right) Shab peak currents in response to light. Filled symbols represent experiments with standard light stimulation ( $n = 5-6$ , and 1 refers to  $1336 \pm 34$  pA SEM). Open symbols represent control experiment without light stimulus ( $n = 5$ , and 1 refers to  $1320 \pm 92$  pA SEM). Note the absence of LDM of Shab.

2004). If, as our results suggest, the Shab conductance is also regulated by PIP<sub>2</sub> depletion, we would predict that the LDM should show similar intensity dependence. We made these measurements in *trpl;trp* double mutants (Figures 7A–7D), which allow complete, but reversible, depletion of microvillar PIP<sub>2</sub>, because of the lack of Ca<sup>2+</sup>-dependent inhibition of PLC (Hardie et al., 2004). The amplitude at 10 s, i.e., maximal activation, is shown in Figure 7E, plotted against light intensity. A curve fit (Hill plot) gives a light intensity for half-maximal Shab current increase of about 10,000 photons absorbed per photoreceptor. Strikingly, this coincides almost exactly with measurements previously made of PIP<sub>2</sub> levels in *trpl;trp* mutants using Kir2.1 biosensors (50% depleted by  $\sim 9000$  effectively absorbed photons) (Hardie et al., 2004).

If Shab channels are modulated by PIP<sub>2</sub>, it would also be predicted that the recovery (LDM decay) after illumination should reflect the time course of PIP<sub>2</sub> resynthesis. Following modulation induced by intermediate intensities (standard light stimulation), the LDM typically decayed with a half time of  $\sim 60$  s, which is

again very similar to the estimates of PIP<sub>2</sub> resynthesis obtained using Kir2.1 channels ( $t_{1/2} \sim 50$  s; Hardie et al., 2004). With increasing light intensities, and also under Ca<sup>2+</sup>-free conditions, the LDM decay increased further in duration. This would again be consistent with PIP<sub>2</sub> resynthesis, as the ability to resynthesize PIP<sub>2</sub> (as monitored by Kir2.1 channels) following repeated bright illumination and under Ca<sup>2+</sup>-free conditions typically deteriorates in the whole-cell recording situation (Hardie et al., 2004; R.C.H., unpublished data).

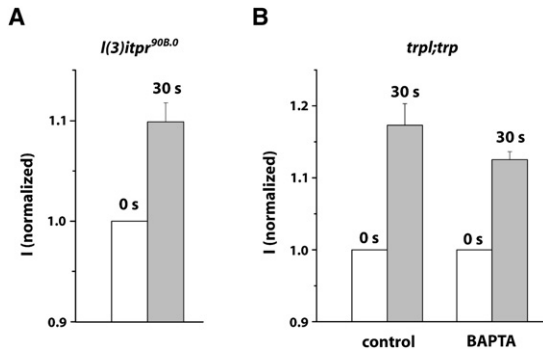
In all the experiments above, the light stimuli were delivered over a period of 10 s, and the increase of Shab current fully developed within that time. To determine the onset of LDM more accurately, we again em-

ployed *trpl;trp* double mutants and applied a single intense 1 ms light flash emitting  $\sim 160,000$  effective photons to induce LDM. The onset of LDM was remarkably rapid with a latency of  $\sim 20$  ms and reached maximum amplitude within  $\sim 200$  ms. Although surprisingly fast, this time course in fact almost perfectly overlapped with the onset of the light-induced current recorded under similar Ca<sup>2+</sup>-free conditions (gray trace in Figure 7F) and which can be taken as a good indicator for the onset of PIP<sub>2</sub> hydrolysis and depletion in the microvilli.

Taken together, the intensity dependence and onset of LDM all support the hypothesis that Shab channels could be modulated by PIP<sub>2</sub>. The very fast activation, matching the development of the light-induced current, suggests that the Shab channels are likely to be located within or in very close proximity to the photo-transduction compartment.

#### Modulation of Shab Channels by PIP<sub>2</sub> in Excised Patches

Our results thus far suggest that the Shab channels are negatively regulated by PIP<sub>2</sub>. To address this hypothesis more



**Figure 5. LDM of Shab Is Not Related to  $\text{InsP}_3$**

(A) *Itp-r83A<sup>90B.0</sup>*. Averaged Shab peak current in response to voltage pulses from  $-100$  to  $+10$  mV before (open,  $n = 6$ , and 1 refers to  $1153 \pm 62$  pA SEM) and after standard light stimulation (filled). Note the Shab modulation is similar to CS WT.

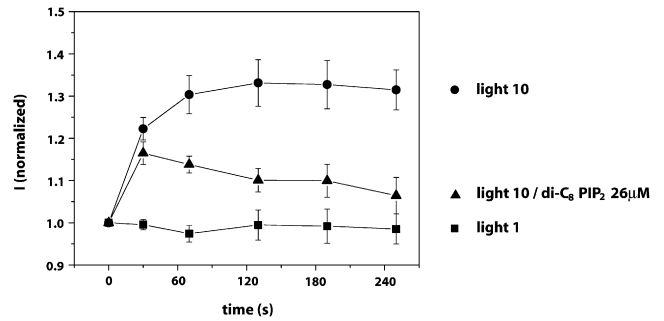
(B) *trpl;trp*. Averaged Shab peak current in response to voltage pulses from  $-100$  to  $+10$  mV before (open,  $n = 6$ , and 1 refers to  $1299 \pm 66$  pA SEM) and after standard light stimulation (filled) in the presence of 2 mM free BAPTA. Control is taken from Figure 4B.

directly, we tested whether  $\text{PIP}_2$  applied to the cytosolic surface of excised inside-out patches could modulate Shab channels and mimic the LDM. Because Shab channels are virtually never encountered in patches from the photoreceptors (Hardie, 1991), we expressed recombinant Shab channels in *Drosophila* S2 cells to enable stable inside-out patches to be routinely obtained. Strikingly, the results of these excised patch experiments (Figure 8) accurately recapitulated the properties of the LDM measured in situ.

Previous studies using known  $\text{PIP}_2$ -sensitive ion channels (Kir2.1) indicated that, following patch excision, endogenous  $\text{PIP}_2$  is rapidly lost in inside-out patches from S2 cells, presumably due to endogenous PLC and/or lipid phosphatase activity (Hardie et al., 2004). When measured within 30 s of patch excision, Shab channels in inside-out patches from S2 cells typically showed a threshold for activation between  $-30$  mV and  $-40$  mV; however, within a few minutes, this had shifted to more negative potentials ( $\sim -50$  mV) (Figure 8E). In the majority of patches (10/14), perfusion of the cytosolic surface with diC8- $\text{PIP}_2$  ( $40 \mu\text{M}$ ) then resulted in a rapid and reversible  $+10$  mV shift in the voltage dependency that closely recapitulated the in vivo modulation by light (Figure 8B; cf. Figure 1). An even more pronounced shift was observed using  $100 \mu\text{M}$  diC8- $\text{PIP}_2$ , restoring the I/V relationship close to that measured immediately after patch excision (Figure 8E). As in the photoreceptors, the modulation of the current amplitude at relatively depolarized potentials was small, but at physiologically relevant voltages near threshold (e.g.,  $-40$  mV), activity was reversibly modulated in a dose-dependent fashion by up to 800% (Figures 8D and 8F). By contrast a diacylglycerol analog (OAG  $40 \mu\text{M}$ ) failed to significantly alter channel activity under the same conditions ( $n = 3$ , data not shown).

## DISCUSSION

In this study, we have discovered a regulatory mechanism of a delayed rectifier type Kv channel in *Drosophila* photoreceptors



**Figure 6. Shab-Channel-Specific LDM Is Caused by Depletion of  $\text{PIP}_2$**

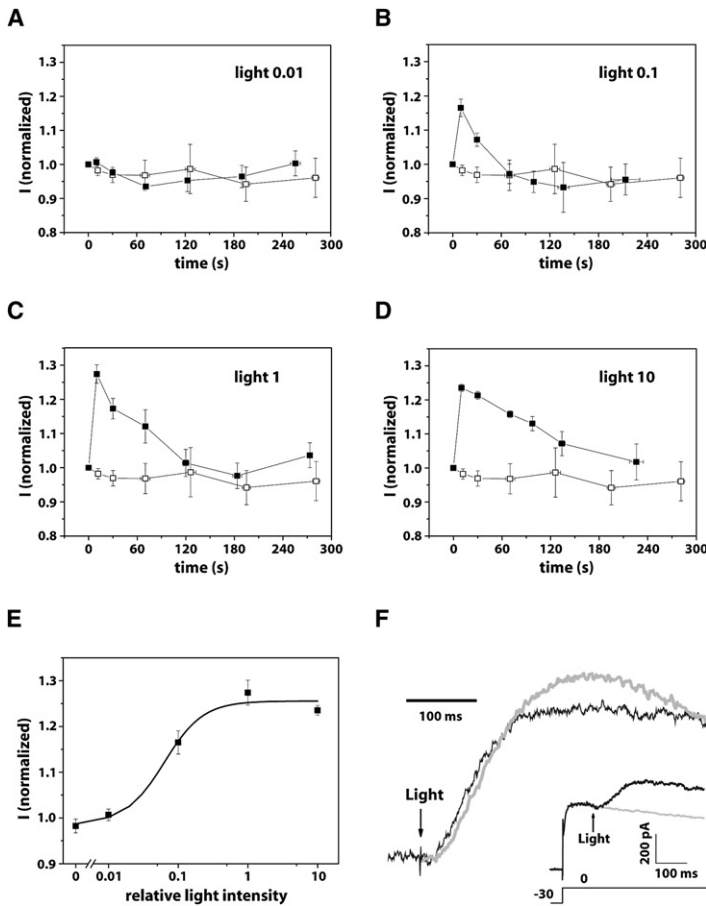
*cds<sup>1</sup>*. Filled squares represent experiments with standard light stimulation ( $n = 4-6$ , and 1 refers to  $1520 \pm 148$  pA SEM). Filled circles represent experiments with 10-fold standard light stimulation ( $n = 10-14$ , and 1 refers to  $1411 \pm 90$  pA SEM). Filled triangles represent experiments with 10-fold standard light stimulation and  $26 \mu\text{M}$  di-C<sub>8</sub>  $\text{PIP}_2$  in the patch pipette ( $n = 6-10$ , and 1 refers to  $1557 \pm 80$  pA SEM). To elicit Shab peak currents,  $+10$  mV pulses with  $-100$  mV prepulses were applied at the indicated points of time.

and used genetic and electrophysiological approaches to reveal its molecular mechanism. We showed that, upon illumination, the delayed rectifier current mediated by Shab channels (Vähäsöyri et al., 2006) was specifically increased. Genetic and pharmacological dissection of the underlying pathway indicated that the light-dependent modulation (LDM) of Shab current was mediated by PLC and the resulting reduction in  $\text{PIP}_2$ . Finally, direct application of  $\text{PIP}_2$  to inside-out patches containing heterologously expressed Shab channels precisely mimicked the modulation, suggesting that  $\text{PIP}_2$  may interact directly with the channels. The LDM can be argued to be an economical adaptation mechanism, because it is tightly linked to the phototransduction cascade that uses the same cycle. In the following, we will discuss these aspects of our findings.

## Molecular Mechanism of Regulation

Disruption of PLC $\beta$ 4 function completely blocked LDM, but neither  $\text{Ca}^{2+}$  influx nor  $\text{Ca}^{2+}$  release was required for LDM induction, while a null mutation in the  $\text{InsP}_3$  receptor had no effect. Although this does not completely exclude the possibility of other  $\text{InsP}_3$ -mediated processes, at this stage we could conclude that the substrate ( $\text{PIP}_2$ ) or one of the lipid products of PLC $\beta$ 4 activity is the relevant mediator (Figure 9).

Strikingly, LDM of Shab became irreversible in mutants of the *cds* gene encoding CDP-DAG synthase, a vital enzyme of the PI cycle required for  $\text{PIP}_2$  synthesis (Figure 9). This suggests that LDM is initiated by a reduction in  $\text{PIP}_2$ , since  $\text{PIP}_2$  has been shown to be irreversibly depleted by light in *cds<sup>1</sup>* mutants (Wu et al., 1995; Hardie et al., 2002). This was strongly supported by finding that exogenous di-C<sub>8</sub>  $\text{PIP}_2$  in the intracellular medium could restore the recovery from LDM in *cds<sup>1</sup>* mutants. The proposal that LDM is initiated by  $\text{PIP}_2$  depletion is also fully consistent with its intensity dependence, which closely matched the intensity dependence of  $\text{PIP}_2$  depletion, while the time course of recovery of LDM also closely matched the time course of  $\text{PIP}_2$  re-synthesis previously monitored using  $\text{PIP}_2$  biosensors (Hardie et al., 2004).



**Figure 7. Light Dependency and Onset of LDM of Shab in *trp1;trp* Photoreceptors**

(A–E) Filled symbols represent experiments with light stimulation of different intensities; open symbols are control experiment without light stimulus ( $n = 4-6$  and 1 refers to  $1137 \pm 102$  pA SEM).

(A) Relative light intensity 0.01 ( $n = 4-12$ , and 1 refers to  $1080 \pm 55$  pA SEM).

(B) Relative light intensity 0.1 ( $n = 3-6$ , and 1 refers to  $993 \pm 48$  pA SEM).

(C) Relative light intensity 1 = standard light stimulation ( $n = 3-8$ , and 1 refers to  $1131 \pm 93$  pA SEM).

(D) Relative light intensity 10 ( $n = 3-6$ , and 1 refers to  $1139 \pm 51$  pA SEM).

(E) Light dependency of the maximal amplitude LDM of Shab (10 s). The solid line represents a curve fit according to the Hill equation.

(F) Onset of LDM of Shab compared to light-induced current. Shab currents in *trp1;trp* mutant were elicited by a voltage step to 0 mV following a  $-30$  mV prepulse to inactivate the Shaker channels (see inset). Maximal LDM was then induced by a brief (1 ms) flash containing 160,000 effective photons (arrow). The black trace shows the Shab current after subtraction of control currents elicited by voltage steps without light stimulation (average of eight flash responses from three cells). The gray trace shows the waveform of the light-induced current (mediated by TRP channels) in response to a 1 ms flash containing  $\sim 50$  effective photons recorded under  $\text{Ca}^{2+}$ -free conditions in a *trp1* mutant (average of ten flashes). The response has been inverted and scaled by eye to align the rising phases (the vertical axis is hence arbitrary). The inset shows sample traces of Shab current with (black) and without light stimulation (gray) showing the LDM.

Strikingly, we found that we could accurately recapitulate the LDM by applying exogenous  $\text{PIP}_2$  to the cytosolic surface of excised patches containing recombinant Shab channels expressed in *Drosophila* S2 cells. While we cannot exclude the possibility of additional factors in excised patches, the robust, rapid, and reversible effects using ATP-free solutions suggest that the effects of  $\text{PIP}_2$  may be mediated by direct interactions with the Shab channels themselves. The effective  $\text{PIP}_2$  dose-response function showed significant inhibition only with quite high concentrations (see Figure 8)—i.e., Shab would appear to have rather low affinity for  $\text{PIP}_2$ —but this is exactly what is required to generate a sensitive and rapid response to  $\text{PIP}_2$  depletion.

In summary, five independent lines of positive evidence (mutant analysis, intensity dependence, recovery time course, application of exogenous di- $\text{C}_8$   $\text{PIP}_2$  to photoreceptors, modulation of the activation curve of Shab channels in patches), along with evidence against alternative possibilities, lead us to conclude that LDM of Shab is mediated by a reduction of membrane  $\text{PIP}_2$ , quite possibly by direct interaction with the channel itself.

### The Biophysical Basis of Light-Dependent Modulation of Shab Channel

Figures 1–3 show that the light-dependent increase of the Shab current is caused by selective increase in amplitude of its slowly inactivating component. This, in turn, originates from a change of voltage dependence of its open probability rather than a change

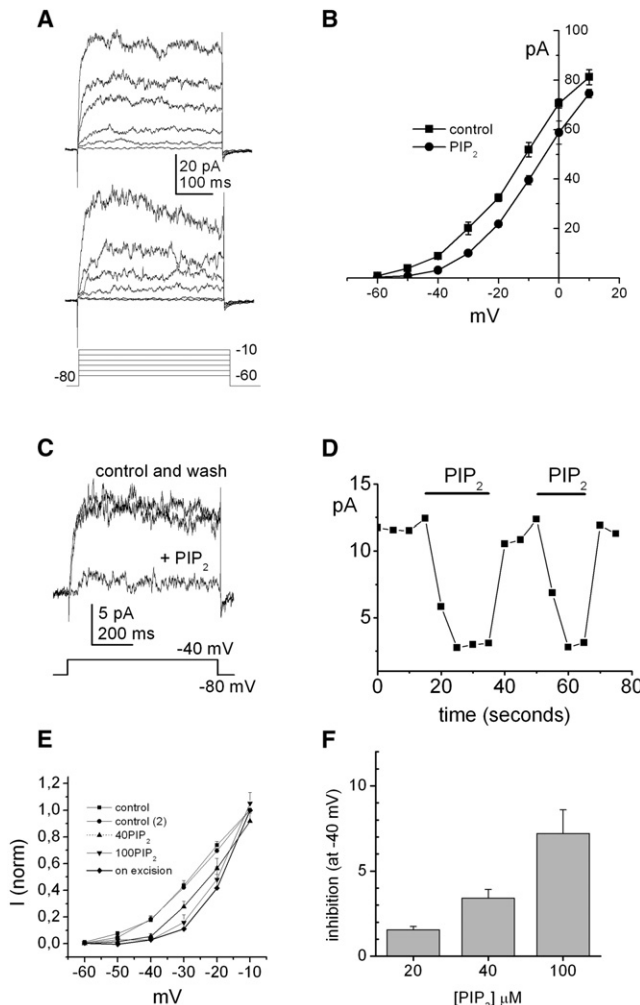
of the open channel conductance, and manifests in a leftward shift of the voltage dependence of activation.

It has been shown previously that currents mediated by Shab and its mammalian homolog, Kv2.1, decay with two time constants (Hardie, 1991; Immke et al., 1999). Immke

et al. (1999) attributed these two different kinetic components to two separately functioning conformations. Our results now suggest that one of these components can be specifically and independently regulated. Thus, in *Drosophila* photoreceptors, *shab* codes for one channel protein, which presumably depending on its pore occupancy, operates in two different kinetic modes.

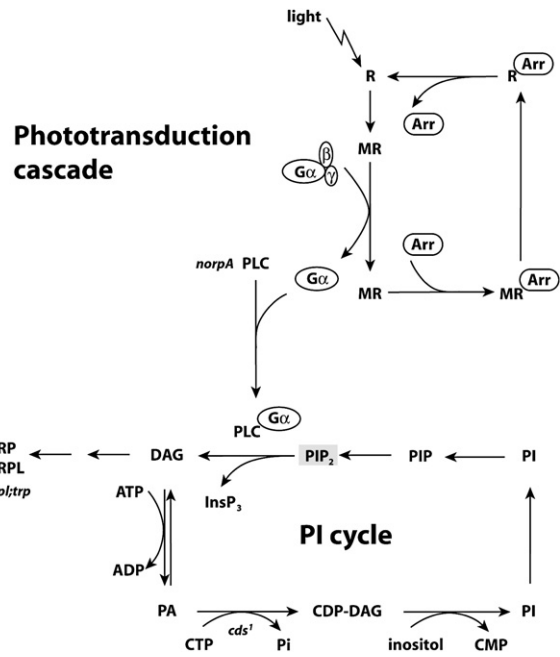
The increase in Shab conductance we observed upon  $\text{PIP}_2$  depletion is in direct contrast to the only previous reports of modulation of Kv channels by  $\text{PIP}_2$ , namely members of the rapidly inactivating mammalian Kv1 and Kv3 families, which were reported to increase their open probability upon application of  $\text{PIP}_2$  (Oliver et al., 2004). The mechanism proposed by Oliver et al. was a specific interaction of the negatively charged head group of  $\text{PIP}_2$  with the positively charged N terminus, the so-called ball-and-chain motif. Since the slow inactivating Shab channel lacks this motif, it is not surprising that we did not observe such positive modulation by  $\text{PIP}_2$ . The upregulation of Shab observed upon  $\text{PIP}_2$  depletion indicates that members of the Kv2 channel subfamily may instead be inhibited by  $\text{PIP}_2$ .

Interestingly, the LDM was eliminated in *shab1* mutants, although these mutants still generate a considerable Shab current. The R435Q point mutation in *shab1* is at the border of the  $\text{NH}_2$  terminus and the first membrane segment, suggesting that  $\text{PIP}_2$  evokes LDM via the  $\text{NH}_2$  terminus. This region of the N terminus is generally believed to be involved in heteromultimerization within subfamilies in 6TM  $\text{K}^+$  channels (Choe, 2002), which



**Figure 8. Modulation of Shab Channels by PIP<sub>2</sub> in Excised Patches**  
 (A) Currents evoked by voltage steps (−60 to −10 mV) from a holding potential of −80 mV in an inside-out patch containing multiple channels from an S2 cell expressing Shab cDNA: above in control bath solution and below during perfusion with 40 μM di-C8 PIP<sub>2</sub> dissolved in bath solution. Bath solution based on 140 K gluconate (Ca<sup>2+</sup> and ATP free), pipette, 120 Na gluconate, 5 K gluconate (see Experimental Procedures).  
 (B) Current voltage relationship from the same patch: mean ± SE, n = 3 determinations alternating between control bath solution and PIP<sub>2</sub> perfusion. The ~10 mV right shift induced by PIP<sub>2</sub> recapitulates the behavior in the photoreceptors (cf. Figure 1).  
 (C) Currents evoked by voltage step from −80 to −40 mV before and after (two larger traces) and during perfusion of 40 μM PIP<sub>2</sub> (smaller, dotted trace).  
 (D) Mean current induced by −80 to −40 mV steps during repeated application of 40 μM PIP<sub>2</sub>.  
 (E) Normalized and averaged I/V curves show data from patches on excision, controls, and then after addition of 40 (n = 6) or 100 μM PIP<sub>2</sub> (n = 3)—the latter almost completely returns the curve to the situation on excision. The two controls are independent and averages from patches tested with 40 or 100 μM PIP<sub>2</sub>, respectively.  
 (F) The dose-response data in terms of the degree of block for a step to −40 mV. OAG (40 μM) was ineffective in three patches.

might suggest that subunit interactions are involved in LDM. In addition, the point mutation of the *shab*<sup>1</sup> mutant is located within a region rich in basic residues. Similar arginine- and lysine-rich



**Figure 9. The Phototransduction Cascade and PI Cycle**

R, MR, and Arr stand for rhodopsin, metarhodopsin, and arrestin, respectively. Gαβγ represents the G protein with its subunits. Other abbreviations have their usual biochemical meaning. The PI cycle is spatially separated into cell membrane and submicrovillar cisternae. DAG, PIP<sub>2</sub>, PIP, and PI reside in the outer cell membrane.

motifs are known in several inward rectifier channels to mediate interaction with PIP<sub>2</sub> (Baukowitz et al., 1998; Huang et al., 1998; Shyng and Nichols, 1998; Zhang et al., 1999), and neutralization of just one basic residue can disrupt PIP<sub>2</sub> binding. It will be interesting to see whether PIP<sub>2</sub> interacts directly with this region in Shab and, if so, whether neutralization of Arg<sup>435</sup> disrupts the interaction. This region is highly conserved with mammalian Kv2.1 channels; it will also be interesting to see whether this proposed mode of Kv channel modulation by PIP<sub>2</sub> is more general.

**Location of Shab Channels in *Drosophila* Photoreceptors**

The onset of LDM is remarkably fast, taking place within a few tens of milliseconds after the light stimulus, and is at least as rapid as the phototransduction current itself when recorded under Ca<sup>2+</sup>-free conditions (Figure 7). This fast onset would be consistent with a direct effect of PIP<sub>2</sub> on the channel and in addition argues for a close spatial proximity to PLCβ4, which itself is localized to the microvilli (Schneuwly et al., 1991). Given the relatively slow diffusion coefficient of PIP<sub>2</sub> (Golebiewska et al., 2008) and the fact that phototransduction in flies is already generally recognized as the fastest known G protein-coupled signaling cascade, this speed of response would be difficult to understand unless the Shab channels were localized to the microvillar rhabdomere where the phototransduction machinery is located (for review see Hardie and Raghu, 2001). Such a localization would also explain an earlier puzzling finding, namely that, in marked contrast to Shaker channels, Shab channels were

almost never found in excised patches from the exposed, basal membrane of the photoreceptor (Hardie, 1991). Location of the Shab channels in the rhabdomeral microvilli or base rather than in the basal plasma membrane (where Shaker channels are densely expressed) would also support the idea that LDM plays a relevant role in vivo.

### Functional Implications of LDM of Shab in *Drosophila* Photoreceptors

The modulation of Shab current (LDM) is tightly linked to the phototransduction cascade that uses the same cycle. Thus, whenever the phototransduction cascade is activated by light, Shab channels are upregulated without requirement of additional signal transduction machinery. At the same time, it automatically confers a dependence on light intensity, thus matching the upregulation of the delayed rectifier conductance to the level of activation of phototransduction cascade.

In intracellular recordings of the intact retina, sustained stimuli of ~30,000 effective photons per second are sufficient to depolarize the photoreceptor membrane to a maintained plateau potential of about 30 mV above resting potential (Juusola and Hardie, 2001). In the experiments reported here, similar light intensities resulted in an ~3-fold increase in the Shab conductance at these potentials. This may, however, overestimate the degree of modulation in vivo, since it is likely that the isolated photoreceptors in whole-cell patch-clamp are more sensitive to PIP<sub>2</sub> depletion than cells in the intact retina. With this caveat, we can expect that under daylight conditions in vivo, the available Shab conductance that can be activated by voltage excursions from the plateau potential should be considerably larger than in the dark and would be expected to decrease in a graded fashion as the ambient intensity decreases. Therefore, adaptive mechanisms previously attributed to delayed rectifier in photoreceptors (Weckström et al., 1991; Weckström and Laughlin, 1995; Vähäsöyrinki et al., 2006) should be increased during progressive light adaptation. These include, for example, the speeding up of responses due to the widened bandwidth of the filter properties of the membrane and the less depolarized membrane voltage upon illumination. In this context, the LDM can be viewed as a feed-forward mechanism promoting adaptation (i.e., reduction of depolarization caused by light), because it tends to increase the activation of Shab channels that are already being activated by depolarization.

### Conclusion

This study shows that in *Drosophila* photoreceptors the slow inactivating component of Shab channel, responsible for the main delayed rectifier current, is increased selectively and independently of other Kv channels upon illumination. The modulation is dependent on light intensity, and it can be evoked even by relatively dim light. The regulation of Shab is initiated by the same PLCβ4 responsible for phototransduction itself. Our evidence suggests that a reduction in the membrane lipid PIP<sub>2</sub> is the key regulating factor and is consistent with a direct action of PIP<sub>2</sub> on Shab channels. We further propose that light-dependent Shab regulation enhances mid- to long-term light adaptation in fly vision.

### EXPERIMENTAL PROCEDURES

#### Flies

All experiments were performed with newly eclosed (<2 hr) *Drosophila* adults. If not stated otherwise, flies were raised on standard medium at 19°C with a 12 hr dark-light rhythm. Canton S was used as wild-type strain; in some occasion, as indicated, Oregon Red and white Oregon Red were used as control flies as well. To disrupt voltage-dependent potassium channel (Kv) function, we used *sh<sup>KS133</sup>*, *shab<sup>1</sup>*, and *shab<sup>2</sup>* mutants (Hegde et al., 1999). To interrupt the phototransduction cascade, we used *trp<sup>302</sup>;trp<sup>343</sup>* and *norpA;trp* double mutants. Due to the *cn bw* mutations on chromosome 2, *trp<sup>302</sup>;trp<sup>343</sup>* is a white eye mutant. To disrupt the only known InsP<sub>3</sub> receptor, we used *l(3)itpr<sup>90B.0</sup>* mutants. The InsP<sub>3</sub> receptor mutant is a whole eye mosaic mutant that is generated by crossing the two constructs *yw;;itp-r83A90B.0/TM6Tb* and *yw;EGUF;5253/Tb*. Photoreceptors of F1 adult flies are homozygous for *l(3)itpr<sup>90B.0</sup>*. They were selected for experiment (for detailed description see Raghu et al., 2000). To interrupt the PI cycle, we used *cds<sup>1</sup>* mutant (Wu et al., 1995).

#### Isolation and Electrophysiology

Dissociated ommatidia were prepared as described previously (Hardie, 1991). Briefly, retinæ were dissected with a flattened insect pin under red light conditions. After an incubation time of 20 min in extracellular solution complemented with BSA 10% and 10 mg/ml sucrose, retinæ were gently triturated until ommatidia fell off. Separate ommatidia were allowed to settle in the recording chamber on the stage of an inverted microscope (Axiovert 35 M, Zeiss, Germany). Recording electrodes had a resistance of 12–15 MΩ and allowed recordings in whole-cell configuration with an access resistance of less than 25 MΩ. Access resistance was monitored throughout the experiments. Seal resistance was typically greater than 10 GΩ, membrane resistance in darkness was about 1 GΩ. Signals were amplified with an Axopatch 1-D amplifier (Axon Instruments, USA), digitized and recorded on a personal computer using clampex9 software (Axon Instruments, USA). If not stated otherwise, 100 ms prepulse to –100 mV was used to deactivate Kv channels, 100 ms voltage pulses were used to activate Kv channels, data were low-pass filtered at 1 kHz and sampled at 10 kHz. The 100 ms prepulse to –100 mV is sufficient to fully deactivate Shab channels (data not shown). For all measurements, a series resistance compensation of 80% was applied. No liquid junction potential (LJP) was corrected. To receive absolute values of voltage-dependent processes, an LJP correction of –12 mV has to be implemented. Photoreceptors were clamped to a holding potential of –60 mV.

Excised patch recordings from S2 cells were made using Sylgarded thin-walled glass pipettes, fire-polished to resistances of 5–10 MΩ.

#### Light Stimulus

Cells were stimulated with a green light-emitting diode (LED) via the fluorescence port of the microscope. Effective light intensities of the LED were calibrated by counting single quantum bumps upon dim light and with a linear extrapolation to experimental light conditions. Ten 100 ms light flashes applied within 10 s emitted 160,000 effective photons per photoreceptor (n = 7) (relative intensity 1). Throughout this study, we refer to this procedure as “standard light stimulation.”

#### Solutions

*Drosophila* Ringer solution contained (in mM) 120 NaCl, 5 KCl, 4 MgCl<sub>2</sub>, 1.5 CaCl<sub>2</sub>, 10 N-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid (TES), 25 proline, and 5 alanine, pH was adjusted to 7.15 (NaOH). Ca<sup>2+</sup>-free solution contained (in mM) 120 NaCl, 5 KCl, 4 MgCl<sub>2</sub>, 10 TES, 25 proline, and 5 alanine, pH 7.15 (NaOH). Intracellular solution contained (in mM) 140 K-glucuronate, 10 TES, 2 MgCl<sub>2</sub>, 4 Mg-ATP, 0.4 Na-GTP, and 1 NAD, pH was adjusted to 7.15 (KOH). EGTA-buffered intracellular solution contained 10.7 mM EGTA and 2.675 mM Ca<sup>2+</sup>, which resulted in free concentrations of 8 mM EGTA and 55 nM Ca<sup>2+</sup>. BAPTA-buffered intracellular solution contained 3 mM BAPTA and 1 mM Ca<sup>2+</sup>, which resulted in free concentrations of 2 mM BAPTA and 110 nM Ca<sup>2+</sup>. The WEBMAXC STANDARD program (<http://www.stanford.edu/~cpatton/maxc.html>) was used to calculate the free divalent ion concentration, and pH of all solutions was adjusted to 7.15. When working with intracellular EGTA, we complemented the extracellular solution with an appropriate

amount of alanine to ensure iso-osmotic conditions. Exogenously applied membrane lipid was di-C<sub>8</sub> phosphatidylinositol (4,5)-biphosphate (di-C<sub>8</sub> PIP<sub>2</sub>). All chemicals were obtained from Sigma Co. (USA). Solution change was performed with RSC 200 (BioLogic, France).

For recording excised patches from S2 cells, the bath contained 140 K-gluconate, 10 TES, 2 MgCl<sub>2</sub>, 20 mM sucrose. The patch electrode contained 120 Na-gluconate, 5 K-gluconate, 1.5 mM CaCl<sub>2</sub>, and 4 mM MgCl<sub>2</sub>. Exogenously applied membrane lipid was di-C<sub>8</sub> phosphatidylinositol (4,5)-biphosphate (di-C<sub>8</sub> PIP<sub>2</sub>) obtained from Sigma Co. (USA) or Echelon Biosciences (USA). All other chemicals were obtained from Sigma Co. (USA). Solution change was performed with RSC 200 (BioLogic, France), or for the case of excised patches using a puffer pipette (~5 μm tip diameter) placed within 10 μm of the excised patch.

### Cell Lines

*Drosophila* S2 cells (Schneider, 1972) were maintained at 25°C in Shield & Sang M3 growth medium supplemented with 12.5% fetal-calf serum as previously described (Hardie and Raghu, 1998). EGFP (Clontech) and Shab cDNA (Islas and Sigworth, 1999) were subcloned into the *Drosophila* metallothionein expression vector PMT/V5-HisA (Invitrogen). Wild-type S2 cells were transiently transfected in 6-well plates using FuGene 6 transfection reagent (Roche) with the 0.5 μg (microgram) of both the PMT/V5-Shab construct and also PMT/V5-eGFP. Two to five days before recording, expression was induced by addition of 0.6 mM CuSO<sub>4</sub> to the growth medium. Patch-clamp recordings were subsequently made from GFP-positive cells using an inverted fluorescence microscope (Nikon TM300).

### Data Analysis

Shab peak current was defined as an average of 100 data points from 23.6 to 33.6 ms after the onset of the activating voltage pulse (Figures 1A and 1C). Activation curves were calculated according to

$$g = \frac{I}{E - E_{Nernst}} \quad (1)$$

with conductance  $g$ , recorded current  $I$ , command potential  $E$ , and calculated Nernst potential  $E_{Nernst}$ .

Activation curves were fitted with Boltzmann equation

$$g = \frac{-g_{max}}{1 + e^{(E - E_{1/2})zF/RT}} + g_{max} \quad (2)$$

with conductance  $g$ , maximal conductance  $g_{max}$ , command potential  $E$ , half activation potential  $E_{1/2}$ , and valence  $z$ .  $F/RT$  has its usual thermodynamical meaning. Subsequently, activation curves were normalized with the fitted  $g_{max}$  value.

Decay of Shab inactivation was fitted according to

$$I = I_{ss} + A_{fast}e^{-(x/\tau_{fast})} + A_{slow}e^{-(x/\tau_{slow})} \quad (3)$$

with current  $I$ , steady-state current after complete inactivation  $I_{ss}$ , amplitude and time constant of the fast inactivating component  $A_{fast}$  and  $\tau_{fast}$ , respectively, amplitude and time constant of the slow inactivating component  $A_{slow}$  and  $\tau_{slow}$ , respectively, and time  $x$ .

Data analysis was performed with Origin 7.0 (OriginLab Corp., Northampton, MA).

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