Activation of TRP Channels by Protons and Phosphoinositide Depletion in Drosophila Photoreceptors

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Summary

Background: Phototransduction in microvillar photoreceptors is mediated via G protein-coupled phospholipase C (PLC), but how PLC activation leads to the opening of the light-sensitive TRPC channels (TRP and TRPL) remains unresolved. In Drosophila, InsP₃ appears not to be involved, and recent studies have implicated lipid products of PLC activity, e.g., diacylglycerol, its metabolites, or the reduction in PIP₂. The fact that hydrolysis of the phosphodiester bond in PIP₂ by PLC also releases a proton is seldom recognized and has neither been measured in vivo nor implicated previously in a signaling context.

Results: Following depletion of PIP₂ and other phosphoinositides by a variety of experimental manipulations, the light-sensitive channels in Drosophila photoreceptors become remarkably sensitive to rapid and reversible activation by the lipophilic protonophore 2,4-dinitrophenol (DNP) in a pH-dependent manner. We further show that light induces a rapid (<10 ms) acidification originating in the microvilli, which is eliminated in mutants of PLC, and that heterologously expressed TRPL channels are activated by acidification of the cytosolic surface of inside-out patches.

Conclusions: Our results indicate that a combination of phosphoinositide depletion and acidification of the membrane/boundary layer is sufficient to activate the light-sensitive channels. Together with the demonstration of light-induced, PLC-dependent acidification, this suggests that excitation in Drosophila photoreceptors may be mediated by PLC's dual action of phosphoinositide depletion and proton release.

Introduction

The light-sensitive channels in microvillar photoreceptors are activated via a G protein-coupled phospholipase C (PLC) cascade, but how PLC activity is linked to channel activation is unresolved. In Drosophila, the most intensively studied model of microvillar phototransduction (reviewed in [1–3]), rhodopsin- and Gq-mediated activation of PLC leads to the gating of two light-sensitive “transient receptor potential” channels, TRP and TRPL, localized in ~30,000 microvilli, which form the transduction compartment (rhabdomere) of the cell. TRP and TRPL are the founding members of the TRP ion channel family [4–6], with 28 mammalian members distributed among six subfamilies (reviewed in [7, 8]). Of these, TRP and TRPL define the canonical TRP (TRPC) subfamily.

PLC hydrolyzes phosphatidyl-inositol (4,5) bisphosphate (PIP₂) to generate soluble inositol (1,4,5) trisphosphate (InsP₃) and diacylglycerol (DAG), which remains in the membrane. InsP₃ seems not to be involved in Drosophila phototransduction [9, 10], focusing attention on lipid products of PLC. Although genetic evidence has implicated DAG in excitation [11, 12], exogenous DAG has little effect on TRP and TRPL channels [13] (but see [14]). By contrast, polyunsaturated fatty acids (PUFAs), such as arachidonic acid, are potent activators [15]. Although PUFAs might in principle be released from DAG via a DAG lipase, this has yet to be demonstrated in the photoreceptors. More generally, DAG is widely recognized as an activator of a subset of mammalian TRPCs, but whether its action is direct remains controversial. In addition, recent evidence implicates PIP₂ as a regulator of TRPC channels (including TRP and TRPL), with both excitatory and inhibitory actions reported [13, 16–18].

It is seldom recognized that hydrolysis of the phosphodiester bond in PIP₂ by PLC also releases a proton (see Figure S1 available online; [19]). To our knowledge, the potential resultant acidification has never been measured in vivo or implicated as a signal in any system. Here we report a rapid light-induced and PLC-dependent acidification in Drosophila photoreceptors. Strikingly, we show that following depletion of PIP₂ and other phosphoinositides, lipophilic protonophores rapidly and reversibly activate TRP and TRPL channels in situ, and that recombinant TRPL channels are activated by acidification of excised inside-out patches. Although not specifically excluding roles for DAG or PUFAs, our evidence indicates that phosphoinositide depletion in combination with acidification of the membrane/boundary layer is sufficient to activate the light-sensitive channels, suggesting an unexpected novel mechanism for microvillar phototransduction.

Results

Actions of 2,4-Dinitrophenol
It is well known that the light-sensitive TRP and TRPL channels in Drosophila become activated following metabolic inhibition [20, 21], for example by bath application of mitochondrial uncouplers. Uncouplers, such as 2,4-dinitrophenol (DNP), are weak-acid lipophilic protonophores, which not only dissipate the proton gradient across the mitochondrial inner membrane but also facilitate proton transport across the plasma membrane. To test whether cytosolic pH changes were induced by DNP under our conditions, we measured whole-cell fluorescence of the pH indicator 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS) [22, 23], loaded into dissociated Drosophila photoreceptors via the patch pipette. To avoid activation by the excitation light or DNP, we recorded from norpA;trp double mutants lacking PLC and the TRP channel. DNP (100 µM) applied by puffer pipette induced a rapid, reversible acidification, detectable within ~200 ms. The pH shift (0.28 pH units) induced by DNP buffered at
induced first a small noise-free inward current of \(w\) cell voltage-clamped wild-type, \(trp\) Similarly, TRPL channels (in a cell) (Ac).

Evoked substantial channel activity beyond an increase in dark noise (all from the same cell) (Ac).

Subsequent application of oligomycin (5 \(\mu\)M, 30 s) to the same cell had little direct effect (Ab). However, following oligomycin exposure, DNP no longer evoked substantial channel activity beyond an increase in dark noise (all from the same cell) (Ac).

(Ba and Bb) Similarly, TRPL channels (in a trp mutant) were activated \(\sim 10\) s after perfusion with DNP (Ba), but following 30 s exposure to oligomycin (data not shown), DNP no longer activated channels beyond an increase in dark noise (same cell) (Bb). Prolonged (\(\geq 40\) s) exposure to DNP failed to activate channels following 30 s oligomycin exposure in all cells tested (\(n = 4\) trpl and \(n = 6\) trp cells). Without oligomycin exposure, repeated doses of DNP were always effective in repeatedly activating channels (data not shown). The downward deflection at the start of each trace is the response to a test flash containing \(\sim 50\) effective photons. Data were recorded in photoreceptors clamped at resting potential (\(-70\) mV) with nucleotide additives (including 4 mM ATP) in the electrode solution.

Because the electrode solution contained ATP, this interpretation presumes that the mitochondrial uncoupling induced by DNP results in the \(F_1\)-\(F_0\) ATP synthetase operating in reverse, converting ATP to ADP. If this is the case, the irreversible ATP synthetase inhibitor oligomycin should prevent activation by DNP. In confirmation, following saturating exposure to oligomycin (5 \(\mu\)M for 30 s), DNP (pH 6.45) no longer activated substantial channel activity in either trp or trpl mutants under control conditions (Figures 1Ac and 1Bb).

Channel Activation by DNP Is Profoundly Enhanced after Phosphoinositide Depletion

In *Drosophila* photoreceptors, Ca\(^{2+}\) influx via TRP channels mediates inhibition of PLC [18]. When this inhibition is prevented by removing extracellular Ca\(^{2+}\) or in trp mutants lacking the more Ca\(^{2+}\)-permeable TRP channel, moderate illumination, corresponding to \(\sim 1\) effectively absorbed photon per microvillus, results in near-total depletion of phosphoinositides (PIP\(_2\), as well as the PI and PIP reserve) in the rhabdomere. Depletion of PLC’s substrate leads to decay of the light response (transient receptor potential phenotype) and complete refractoriness to subsequent light flashes. Sensitivity then returns as phosphoinositides are resynthesized with a half-time of \(\sim 1\) min in the dark [18].

In wild-type or trp photoreceptors recorded under control conditions (1.5 mM Ca\(^{2+}\), ATP in electrode), brief pulses (<5 s) of DNP failed to activate channel activity either before or 20 s after bright illumination (e.g., see Figure S5B, right), which would have depleted phosphoinositides in a trp mutant. Remarkably however, following phosphoinositide depletion by such illumination in trp mutants, sensitivity to DNP was profoundly enhanced, and although responses to light were abolished, large currents were now very rapidly and reversibly activated by only brief (1–2 s) “puffs” of DNP (Figure 2A). Activation was strongly pH dependent, with \(\sim 10\times\) greater currents elicited when DNP was buffered at pH 6.45 compared to pH 7.15 (Figure 2C). However, acid-buffered bath solution (pH 6.0) without DNP and DNP buffered at pH 8.5 failed to activate currents. When buffered at pH 6.45, brief puffs of DNP were effective at concentrations as low as 20 \(\mu\)M with an EC\(_{50}\) of \(\sim 30\) \(\mu\)M (Figure S2D). The currents activated by DNP were clearly identifiable as TRPL channels from their biophysical properties (e.g., rectification; Figure S3) and their absence in trpl/trp double mutants (data not shown). In addition, TRPL channels are strongly inhibited by Ca\(^{2+}\) influx [24, 25]; correspondingly, in Ca\(^{2+}\)-free bath, currents elicited by DNP following phosphoinositide depletion reached even larger values (3930 \(\pm\) 490 pA, \(n = 4\); Figure S4A).

Significantly, whereas prior exposure to oligomycin always prevented the slow activation of channels by DNP under control conditions (Figure 1), the profoundly sensitized activation following phosphoinositide depletion appeared unaffected by oligomycin (\(n = 5\); Figure 2A). This implies that activation under these conditions was no longer caused by ATP depletion, suggesting instead that, following phosphoinositide depletion, the channels might be gated by cytosolic acidification.

Similar results were obtained for TRP channels recorded in trpl mutants. Here we achieved phosphoinositide depletion (which requires prevention of Ca\(^{2+}\) influx) either by first illuminating the cells in Ca\(^{2+}\)-free bath or by exploiting a point mutation in the pore of the TRP channel (TRP\(_{D621G}\)), which eliminates Ca\(^{2+}\) permeability [26]. In both cases, large TRP-mediated currents were rapidly activated by brief pulses of
DNP in a pH-dependent manner after phosphoinositide depletion, but not before (Figure 2B; Figures S3, S4D, and S4E).

Sensitivity to DNP is inversely correlated with phosphoinositide levels

The currents that could be activated by DNP in trp photoreceptors were maximal up to ~30 s following phosphoinositide-depleting stimuli but then declined to control level over 2–3 min. This closely mirrored the time course for recovery of sensitivity to light (Figure 3), which has previously been shown to track the time course of PIP2 resynthesis [18, 27]. Following recovery, repeated phosphoinositide-depleting stimuli always resensitized the channels to rapid activation by DNP for the lifetime of the recording (n > 20, data not shown).

These results suggest that sensitivity to DNP is inversely correlated with the level of phosphoinositides in the microvillar membrane; however, the light stimulation used to deplete phosphoinositides has other effects, such as generation of DAG and InsP3. We therefore used a variety of other manipulations to deplete phosphoinositides. First, we made recordings without ATP in the patch electrode. Under these conditions, kinases required for PIP2 synthesis (see Figure 3C) fail, and PIP2 is more gradually, but now irreversibly, depleted in the dark by basal PLC activity [27]. Under these ATP-deprived conditions, all light sensitivity in trp mutants was progressively...
activated by DNP as PIP2 was depleted. Inset (right) shows examples of DNP-a small ionophore current; however, TRPL channels were increasingly acti-

taneously depleted by basal phospholipase C (PLC) activity in the dark over

Figure 4. Sensitivity to DNP in ATP-Depleted Cells
(A) Recording from trp photoreceptor recorded without nucleotide additives
(–ATP) in the pipette. Under these conditions, phosphoinositides are sponta-
eously depleted by basal phospholipase C (PLC) activity in the dark over a few minutes, reflected in gradual loss of response to dim test flashes
(small arrows). Before depletion, DNP (2 s, 100 µM, pH 6.45) induced only a small ionophore current; however, TRPL channels were increasingly acti-
vated by DNP as PIP2 was depleted. Inset (right) shows examples of DNP-
evoked currents before and after depletion on expanded scale.

(B) Same recording on a slower timescale: sensitivity to DNP was main-
tained for >10 min.
(C) Currents evoked by DNP near the start and end of the recording (starred
in B) were similar.
(D) Responses to 2 s DNP in another ATP-depleted cell before (control) and
after exposure to oligomycin (30 s, 5 µM).
(E) Mean ± SEM from 5 trp photoreceptors exposed to 1 s, 50 µM DNP (pH
6.45) induced only 6.45) puffs at 30 s intervals is shown, averaged after aligning at time taken to
reach 50% of maximum response to DNP. For comparison, the dotted
curve shows time course of decay of PIP2 monitored by PIP2-sensitive ion
channels (Kir2.1R228Q) genetically targeted to the photoreceptors (data from [30]). Sensitivity to DNP developed in parallel with the decline in sensi-
tivity to light and was maintained for the lifetime of the recordings.

lost in parallel with the loss of PIP2 (as monitored by genetically targeted PIP2-sensitive Kir2.1 channels [27], and the channels
(TRPL) remained closed [20]. Strikingly however, in parallel
with the loss of sensitivity to light, TRPL channels became
very sensitive to rapid, reversible activation by DNP and
remained so indefinitely (Figure 4). Activation by DNP under
these conditions was again unaffected by oligomycin
(Figure 4D).

Next, we investigated mutants of CDP-DAG synthase (cds),
an enzyme required for PI and PIP2 resynthesis [28] (Figure 3C).
Metabolism of DAG and InsP3 should be largely unaffected in
cds mutants; however, phosphoinositide depletion in cds was
irreversible on the timescale of our experiments [11, 28], and
we therefore predicted that DNP would be effective indefinitely
following depleting stimuli, despite ATP in the electrode. In
confirmation, following phosphoinositide depletion induced
by light in cds photoreceptors, DNP again induced rapid and
reversible channel activation and now continued to do so for
the lifetime of the recording (Figure S5A).

We also recorded from mutants of protein kinase C (PKC)
encoded by the inaC gene. INAC is required for the Ca2+-
dependent inhibition of PLC, and consequently phosphoino-
isitides are depleted in inaC mutants by similar light regimes to
those that are effective in trp mutants [29]. As usual, brief
pulses of DNP were ineffective in dark-adapted inaC mutants,
but, following a depleting stimulus, large rapidly reversible
currents were elicited that now decreased with time in the
dark as PIP2 was resynthesized (Figure S5B). These results
also show that INAC-dependent phosphorylation/dephos-
phorylation cycles (e.g., of the TRP channel) do not underlie
the reversible activation of channels by DNP.

In summary, a range of conditions that deplete phosphoino-
isitides all profoundly sensitized the photoreceptors to rapid
activation by DNP, showing an inverse correlation with phos-
phoinositide levels during both depletion and resynthesis
phases. Other products or consequences of PLC hydrolysis
of PIP2 (including InsP3, Ca2+, DAG and its metabolites, and
phosphorylation state of elements of the cascade) would
have varied greatly under the various conditions used. This
suggests that phosphoinositide depletion directly or indirectly
sensitizes channels to activation by protonophores.

Light-Induced pH Changes
Although the PLC reaction is expected to release a proton
(Figure S1), to our knowledge this has never been demon-
strated in vivo in any system. To measure possible pH changes
induced by light, we used the fluorescent pH indicator HPTS,
the excitation light (470 nm) simultaneously serving as a satu-
rating light stimulus (Figure 5). Under control conditions,
a rapid light-induced decrease in whole-cell HPTS fluores-
cence was invariably recorded in both wild-type and trp
photoreceptors, equivalent to an acidification of ~0.1 pH units.
This developed with a short latency (<20 ms) similar to that of the
light response and was largely complete within 100–
200 ms (Figures 5A and 5C). Following red illumination (to
photoreisomerise metarhodopsin back to the rhodopsin state)
and a brief (10–20 s) dark adaptation period, repeated 470 nm
stimuli continued to give similar pH shifts through several
cycles. The acidification seemed to originate in the microvilli
because pH measured from the rhabdomere decreased
much more rapidly than measurements from the cell body
(Figure 5B).

To control for possible artifacts due to the large light-
induced ion fluxes associated with these measurements,
we first loaded cells with a pH-insensitive fluorescent dye
(1 mM lucifer yellow) but detected no significant light-induced
change in fluorescence (n = 6, data not shown). Next we
recorded HPTS fluorescence from wild-type photoreis,
perfused with Ca2+-free solution and also from trpl photore-
ceptors in the presence of La3+ (50 µM), which blocks TRP
channels, thereby abolishing the light-induced current. In
both cases, pH dropped with essentially identical kinetics
over the first ~50 ms but then continued to decrease so that
the final pH shift was nearly doubled (Figures 5A and 5C).
Because Ca2+ normally inhibits PLC [18], the enhanced pH
signal without Ca2+ influx is consistent with the acidification
activation could not be reliably detected. This suggests either that a factor required for pH regulation may wash out following excision or that the repeated acidification of excised patches was often main- 
maximum

being mediated by PLC. To test this further, we recorded from mutants lacking PLC (norpA22) and found that the pH shift was abolished (Figure 5C). We also recorded pH signals in inaC mutants in which PLC fails to be rapidly inactivated by Ca2+ [29]. Again, we saw an enhanced pH decrease similar to that recorded in the absence of Ca2+ influx (Figure 5D). Finally, the pH shift was eliminated when phosphoinositides were first depleted by prior illumination in the absence of Ca2+ influx (in trpl recorded in presence of La3+, n = 4, data not shown). These results indicate that the light-dependent acidification is a consequence of PIP2 hydrolysis by PLC.

Interestingly, although the light-induced TRP current was abolished in trpl in the presence of La3+ or in trpl;trp double mutants, the bright stimuli used to excite HPTS fluorescence induced a small (5–10 pA) transient outward current with similar kinetics to the light response itself (Figure 5C, inset). Like the pH signal, this transient outward current was absent in norpA mutants and also if PIP2 was first depleted (data not shown). We suggest that it represents the electrical signature of the charge displacement on the inner leaflet of the microvillar membrane as PIP2 is cleaved, releasing negatively charged InsP3 into the cytosol.

**pH-Dependent Activation of Heterologously Expressed TRPL Channels in S2 Cells**

The rapid, pH-dependent but oligomycin-independent activation of TRP and TRPL channels by DNP following phosphoinositide depletion suggested that the channels are activated by the associated pH change rather than metabolic inhibition. We sought further evidence for regulation by pH in TRPL channels heterologously expressed in Drosophila S2 cells, which allow routine excised patch recordings that are not practicable in photoreceptors.

Heterologously expressed TRPL channels show some spontaneous activity even when unstimulated. This activity was rapidly and reversibly increased up to ~5-fold in a pH-dependent fashion by DNP in whole-cell recordings (Figures 6A, 6B, and 6F). As in photoreceptors, the response to DNP was unaffected by prior application of oligomycin (n = 5 cells, data not shown) and was always evoked with or without ATP in the electrode. Significantly, DNP also rapidly increased channel activity to a similar extent in both outside-out and inside-out patches, excluding any mitochondrial involvement (Figures 6C and 6E). In inside-out patches, it was possible to show that activation by DNP depended upon an appropriate proton gradient, because the identical solution (100 μM DNP, pH 6.45) applied to the cytosolic surface was only effective when pHc (i.e., pH of the patch electrode solution) was more acidic (Figure 6E). This represents compelling evidence that activation by DNP is via membrane protonophore action and excludes the possibility that DNP acts as a direct channel-activating ligand. In all cases, analysis of channel openings showed that upregulation was due to an increase in channel open probability (NPo) rather than single channel conductance. In fact, single channel conductance decreased at acid pH (103 ± 6 pS at pH 7.15; 88 ± 10 pS at pH 6.45; n = 5), indicative of a modest pore block by protons.

We next asked whether TRPL channels in inside-out patches could be directly activated by protons. Indeed, in ~75% of patches NPo was rapidly and reversibly increased simply by cytosolic acidification. At the lowest pH tested (pH 5.0), NPo was increased on average by ~10-fold (maximum ~60-fold), but the channels then usually became rapidly but reversibly desensitized (Figures 6D and 6F). Sensitivity to repeated acidification of excised patches was often maintained for many minutes, but not always indefinitely, and in a minority (15 of 65) of patches, clear activation could not be reliably detected. This suggests either that a factor required for pH regulation may wash out following excision or that the
channels may enter a state that can no longer be regulated by pH. The increase in \( N_{Po} \) was again mainly due to an increase in channel opening frequency, with only a modest increase in mean channel open time (e.g., from 0.64 ± 0.07 ms at pH 7.15 to 1.05 ± 0.12 ms at pH 6.45 at +60 mV, \( n = 5 \)).

Finally, we explored the phosphoinositide dependence of TRPL channel activity in inside-out patches. Our initial expectation was that PIP\(_2\) would inhibit channel activity in patches, as reported in the only previous study of the effects of PIP\(_2\) on heterologously expressed TRPL channels [13]. However, under the conditions of our experiments, we found that diC\(_8\)PIP\(_2\) rapidly increased \( N_{Po} \) by up to 10-fold (EC\(_{50}\) ~ 1.5 \( \mu \)M) even when tested at acid pH (Figures 7A and 7B). Interestingly however, diC\(_8\) phosphatidyl inositol (PI) and diC\(_8\)PI(4)P both reversibly inhibited TRPL channel activity in inside-out patches. PI was the more potent, with an IC\(_{50}\) of ~25 \( \mu \)M and ~8-fold reduction in \( N_{Po} \) measured at 100 \( \mu \)M (Figures 7C and 7D).

Discussion

To our knowledge, our measurements of rapid, light-induced and PLC-dependent acidification represent the first evidence for in vivo, PLC-induced proton release in any system. Although we do not exclude additional sources from reactions operating downstream of PLC, calculation suggests that the protons released by hydrolysis of PIP\(_2\) are sufficient to account for the magnitude of the observed pH shift (Figure S1). We also found that the lipophilic protonophore DNP rapidly and reversibly activated both TRP and TRPL channels, following phosphoinositide depletion in situ, whereas heterologously expressed TRPL channels could be activated not only by DNP but also by direct acidification of excised inside-out patches. Together, these results suggest an unexpected novel mechanism for microvillar phototransduction, namely that the channels might be gated by a combination of phosphoinositide depletion and protons.

As well as DNP, we tested a variety of “conventional” weak acids, which also cause cytosolic acidification. Propionic (20 mM) and benzoic (10 mM) acid failed to activate channels; however, octanoic acid (5 mM), which was the most lipophilic weak acid tested, induced modest TRPL channel activation following phosphoinositide depletion in \( trp \) mutants (\( n = 6 \); Figure S6). Significantly, we were able to show activation of recombinant TRPL channels by protons without recourse to
pharmacological agents in inside-out patches from S2 cells. However, the pH required to achieve robust activation (Figures 6D and 6F) was more acidic than the bulk pH changes induced by DNP or light in the photoreceptors. To reconcile these observations, we suggest that a putative pH-sensitive site on the channels or associated proteins is close to the site of PIP2 hydrolysis (and proton release) within the membrane or boundary layer and is relatively protected from the bulk pH of the aqueous phase. We suggest that the particular efficacy of DNP relates to its lipophilic nature and to the fact that even the anionic (deprotonated) form is lipid soluble and adsorbs to the bilayer [30]. This means that protonated DNP can ionize and donate protons (possibly by direct charge transfer) in the membrane or the boundary layer, whereas conventional weak acids only ionize and release protons in the aqueous phase. Consistent with this interpretation, carbonyl cyanide m-chlorophenylhydrazone, which is another lipophilic weak acid protonophore with broadly similar mode of action to DNP [31], also rapidly and potently activated TRPL channels following phosphoinositide depletion (Figure S6C). We also note that although the bulk light-induced pH shift was only 0.1–0.2 pH units, our results clearly show a pH gradient from the microvilli to cytosol (Figure 5B). It seems likely that the immediate pH change in the membrane or boundary layer where PIP2 is hydrolyzed may be yet greater. A further consequence of PIP2 hydrolysis suggested by our results is a transient local increase in positive charge density due to negatively charged InsP3 being cleaved from the membrane (Figure 5C). We do not exclude the possibility that this might also contribute to excitation.

We found that a range of manipulations that deplete PIP2 and other phosphoinositides resulted in profound sensitization of the channels to activation by DNP (Figures 2 and 3; Figure S5). However, the mechanism by which phosphoinositide depletion results in sensitization to protonophores requires further study. Although TRPL channels have been reported to be inhibited by exogenous PIP2 [13], in our hands diC8PIP2 robustly activated heterologously expressed TRPL channels in excised patches. At first sight, this seems difficult to reconcile with our in vivo data; however, the EC50 (1–2 μM PIP2) probably corresponds to only ~5%–10% of endogenous resting PIP2 levels and might reflect a constitutive requirement for PIP2 unrelated to any role in activation. We also note that depletion of PIP2 inevitably depletes the upstream phosphoinositide reserve pool (PI and PI(4)P) and that diC8 analogs of these were effective inhibitors of heterologously expressed TRPL channels in inside-out patches (Figure 7). This raises the possibility that the knockon depletion of PI and PI(4)P by PI and PIP kinases, rather than PIP2, may be a key event in channel activation. Another possibility is that the effects of PIP2 and/or other lipids on channel activity might be mediated, not by ligand binding but by altering the properties of the lipid bilayer [14, 21, 32]. In this respect, there are major differences between the lipid environment of TRP channels in situ (in signaling complexes within microvilli) and heterologously expressed channels in cell lines. For example, the depletion and resynthesis of phosphoinositides in the inner leaflet of the highly curved microvillar membrane may affect bilayer properties such as membrane stress curvature in a manner that cannot be readily mimicked in an expression system or by exogenous application of lipids.

A role for PIP2 depletion in invertebrate phototransduction has been discussed previously [13, 18, 33, 34], but the dominant hypothesis is that activation is mediated by DAG or downstream metabolites (PUFAs), such as arachidonic acid (AA), which are potent activators of TRP and TRPL channels [15]. Recently, mutants in a DAG lipase (inaE) were found to have reduced sensitivity to light [35]. However, INAE is an sn-1 lipase, which generates 2-arachidonoyl glycerol rather than AA, and it remains unclear whether it is directly involved in phototransduction. An excitatory role for DAG has been strongly suggested by the phenotypes of mutants in DAG kinase (rdgA), which include constitutive channel activity [10] and the rescue of light responses in hypomorphic mutants of

Figure 7. Effects of Phosphoinositides on Heterologously Expressed TRPL Channels

Excised multichannel patches held at +60 mV were exposed to different concentrations of diC8PIP2 applied by puffer pipette at times indicated by bars. (A) Significant (~2–3-fold) reversible upregulation was observed at 1 μM; 10 μM applied to the same patch was ~3x more effective (NPo plot from same data).

(B) Dose response function measured with diC8PIP2 buffered either at bath pH (7.15) or at pH 6.25 (mean ± SEM, n = 2–5 patches per data point). Data are expressed as relative increase in NPo compared to spontaneous activity in bath. Perfusing the same patches with pH 6.25 (without PIP2) caused a 2.3-fold increase in NPo (data not shown), which can account for the enhanced activation seen when PIP2 was applied buffered at pH 6.25.

(C) diC8 phosphatidyl inositol (PI; 40 μM) applied to cytosolic surface of excised patch reversibly inhibited TRPL activity.

(D) Dose response function for inhibition by diC8PI; data fitted with inverse Hill function, IC50 25 μM, n = 2.3 (mean ± SEM, 4–6 patches per data point); also shown is inhibition by 40 μM diC8PI(4)P (n = 9). All solutions were buffered with 200 μM free 1,2-bis(2-aminophenoxy)ethane N,N,N,N-tetraacetic acid to give free [Ca2+]o of ~60 nM.
PLC or Gq [11], both of which could be interpreted in terms of increased production of DAG. Exogenous DAG has also recently been reported to activate channels, albeit slowly, in patches excised from rhodobinocytes [14]. However, in other studies DAG had little, if any, effect either in situ or on heterologously expressed TRPL channels (unpublished data; [13]). We also note that the product of DAG kinase, namely phosphatidic acid, is both a precursor for phosphoinositol resynthesis (Figure 3C) and an allosteric activator of PI(4)P-5-kinase [36], i.e., rdgA mutants may also be expected to have defects in the (rapid) resynthesis ofPIP$_2$ [37]. Nevertheless, given the emerging consensus that TRP channels are polymodally regulated (e.g., [38]), the possibility should also be considered that protons, phosphoinositide depletion, DAG, and/or PUFAs may all contribute to excitation.

Conclusions
Our results suggest an unexpected new paradigm for phototransduction in Drosophila, namely that the channels may be activated combinatorially by the simultaneous reduction in PIP$_2$ and/or other phosphoinositides, combined with localized PLC-dependent proton release in the membrane boundary layer. Drosophila TRP and TRPL channels are the prototypical members of the TRPC family, widely expressed throughout the body [7]. As is the case in Drosophila, TRPCs are gated downstream of PLC, but by unknown mechanisms. Although DAG can activate a subset of mammalian TRPCs [39], whether this action is direct is unclear, and even less is understood about the mechanism of activation of the DAG-insensitive TRPC6 and TRPC4 [16], TRPC4 [40], and TRPC5 [17] have all recently been reported to be inhibited by PIP$_2$. To our knowledge, the effects of cytosolic pH on any TRPC channel have yet to be investigated—though TRPC4 and TRPC5 were reported to be potentiated by extracellular acidification [41]. Extracellular pH has also been reported to upregulate several members of the broader TRP channel family, including TRPV1, TRPV4, TRPP2, and TRPA1 (reviewed by [42]), but the only TRP channels previously reported to be regulated by cytosolic pH are TRPM7 [43] and TRPM2 [44], which were both inhibited by protons. It will be interesting to see whether any mammalian TRPC channels are coregulated by phosphoinositides and pH in a manner similar to that suggested here.

Experimental Procedures

Files
The wild-type strain was Oregon w$^{1118}$. Mutants (also white-eyed) included trp$^{343}$ and trp$^{507}$, null mutants for TRP and TRPL channels [24]; TRP$^{RD21G}$, a point mutation that eliminates Ca$^{2+}$ permeability [28]; inac$^{209}$. a null mutant for PKC [45]; csh$^{-1}$, lacking CDP-DAG synthase [28]; and norpA$^{251}$, a protein null mutant of PLC [46].

Solutions
For photocyte recordings, the bath contained (in mM): 120 NaCl, 5 KCl, 10 N-Tris(hydroxymethyl)-methyl-2-amino-ethanesulfonic acid (TES), 4 MgCl$_2$, 1.5 CaCl$_2$, 25 proline, and 5 alanine, pH 7.15. For Ca$^{2+}$-free bath, CaCl$_2$ was omitted and 1.6 mM Na$_4$EGTA added. The intracellular electrode solution was (in mM): 140 K gluconate, 10 TES, 4 MgATP, 2 MgCl$_2$, 1 NAD, and 0.4 NaGTP, pH 7.15. For ATP deprivation, all nucleotide additives (ATP, NAD, and GTP) were omitted.

For S2 cell whole-cell recordings, the bath contained (in mM): 125 Na gluconate, 5 K gluconate, 2 MgCl$_2$, 15 mannitol, and 10 TES, pH 7.15. For excised patch recordings, cells were bathed in 140 K gluconate, 2 MgCl$_2$, 13 mannitol, 10 TES, 2 PIPES, 260 mM BAPTA, and 60 mM CaCl$_2$, pH adjusted as required with gluconic acid. Free Ca$^{2+}$ was ~60 nM.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cub.2009.12.019.

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References


Supplemental Information

Activation of TRP Channels by Protons and Phosphoinositide Depletion in *Drosophila* Photoreceptors

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Supplemental Experimental Procedures

**Flies**

Flies, *Drosophila melanogaster* were raised in the dark at 25°C. The wild-type strain was Oregon *w*1118. Mutants (also on white-eyed backgrounds) included *trp*343 and *trpl*302 – null mutants for TRP and TRPL channels respectively [ref 24 main text]; *TRP*<sub>D621G</sub> – a TRP transgene with a point mutation in the pore that eliminates Ca<sup>2+</sup> permeability, expressed under control of the *Rh1* promoter on a *trpl;trp* double mutant background [ref 26 main text]; *inaC*<sup>209</sup>, a null mutant for eye-specific PKC[ref 43 main text]; *cds1*<sup>1</sup>, defective in CDP-DAG synthase [ref 28 main text] and *norpA*<sup>P24</sup>, a near null mutant of PLC [ref 44 main text].

**Whole-Cell Photoreceptor Recordings**

Dissociated ommatidia were prepared as previously described from newly eclosed flies [ref 18 main text] and transferred to a recording chamber on an inverted microscope. Rapid perfusion was achieved using puffer pipettes positioned close (< 50 μm) to the cells. The intracellular pipette solution was (in mM): 140 K gluconate, 10 TES, 4 MgATP, 2 MgCl<sub>2</sub>, 1 NAD and 0.4 NaGTP, pH 7.15. In some experiments nucleotide additives (NAD, GTP and ATP) were omitted and osmolarity compensated using sucrose. Working solutions of DNP were diluted from a 50 mM ethanolic stock. All chemicals were obtained from Sigma-Aldrich. Whole-cell voltage clamp recordings were made at 20° ± 1°C at -70 mV (including correction for -10 mV junction potential) using electrodes of resistance ~10-15 MΩ. Series resistance values were generally below 30 MΩ and routinely compensated to >80%. Data were collected and analyzed using Axopatch 200 or 2D amplifiers and pCLAMP software (Molecular Devices, Union City CA).

**S2 Cell Electrophysiology**

Recordings were made from a clonal *Drosophila* S2 cell line expressing TRPL channels and the Dm1 muscarinic receptor under control of a metallothionein promoter as previously described.
Expression was induced 1-10 days before recording by addition of 0.6 mM CuSO₄. For whole-cell recordings, the bath solution contained (in mM) 125 Na gluconate, 5 K gluconate, 2 MgCl₂, 15 mannitol, 10 TES pH 7.15. The pipette solution was the same used for photoreceptor recordings. Electrodes were pulled from thin-walled 1 mm O.D. glass and fire-polished to resistances of ~5 MΩ. For excised patch recordings cells were bathed in 140 K gluconate, 2 MgCl₂, 13 mannitol, 10 TES, 2 PIPES, 260 μM BAPTA and 60 μM CaCl₂ pH 7.15 (free Ca²⁺ ca. 60 nM). Identical solutions were used for perfusing inside-out patches at different pH except that the BAPTA:Ca²⁺ ratio was adjusted to maintain free Ca²⁺ and free BAPTA concentrations using WinMAXC (http://www.stanford.edu/~cpatton/), pH was adjusted using gluconic acid. The patch electrode contained a similar solution but without BAPTA or added Ca²⁺. DiC₈ phosphoinositide solutions (Echelon or Cayman) were made fresh daily from 1 mM aqueous stocks kept at –20°C. Excised inside-out patches were passed briefly through the air/water interface before recording. For single channel analysis, data were sampled at 5-10KHz, filtered at 1-2 kHz; channel openings were idealised and analysed using Clampfit v.10.

**Fluorescent pH Measurements**

The fluorescent pH indicator 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS) was loaded into photoreceptors at a concentration of 500 μM via the patch electrode, allowing ~2-3 minutes for equilibration before making measurements. Excitation light (470nm) was delivered from a 75W Xe arc lamp coupled to a monochromator and fluorescence from the whole-cell measured via a photon counting photomultiplier (Photon Technology International N.J. U.S.A.) at wavelengths above 520 nm using a Nikon DM510 dichroic mirror. Background auto-fluorescence (typically <5% of signal) was measured from unloaded cells and subtracted before further analysis. Although HPTS is a ratiometric dye, single wavelength fluorimetry was preferred because of the superior signal-to-noise ratio, which was essential for time resolving responses to light. Absolute pH shifts ($ΔpH$) can be estimated using single wavelength fluorimetry if the starting pH value ($pH_{start}$) is known [ref 23 main text]

$$ΔpH = \log(F/F₀)-\log(1-(F/F₀-1) × 10^{pH_{start}-pKa}) \quad \text{Eq.1}$$

where $F$ is the instantaneous fluorescence and $F₀$ fluorescence at the start of the recording. The pKa value for HPTS was taken as 7.18 [ref 23 main text]. We assumed starting pH was 7.15 (pH of the bath and patch pipette solutions). An error, e.g. of ± 0.2 pH units in the estimated pH$_{start}$ would have resulted in ca 30% errors in the estimated pH shift – which would not substantially affect any of our arguments or conclusions.
Figure S1.

The Phospholipase C Reaction Releases a Proton
Hydrolysis of the phosphodiester bond in PIP$_2$ generates diacylglycerol (DAG) and inositol (1,4,5) trisphosphate (InsP$_3$). From measured pKa values of InsP$_3$ [1] and PIP$_2$ [2] there is predicted to be a net release of 0.8 proton at physiological pH (7.2). Proton release by PLC has occasionally been used as an in vitro assay (ref. 19 main text), but to our knowledge has not previously been measured in vivo.

Can Proton Release from PIP$_2$ Account for the Measured pH Shift?
The rhabdomere contains ca. 30-50000 microvilli, on average ca. 1.2 $\mu$m long and 60 nm in diameter – a membrane area of ~0.2 $\mu$m$^2$. On the assumption that PIP$_2$ and PI, PI(4)P reserve account for 3% of membrane phospholipids, and assigning an area of 70 Å$^2$ per phospholipid there are estimated to be ca 10$^4$ phosphoinositides per microvillus, or 4x10$^8$ in the rhabdomere as a whole. Under Ca$^{2+}$ free conditions essentially all of these will be hydrolysed by PLC in response to the saturating 470 nm stimulus used to measured HPTS fluorescence (ref 27 main text). Within the volume of the cell (5 x 10$^{-13}$ litre, assuming 50% aqueous fractional volume), and unbuffered this would represent a proton concentration of 1.1 mM. The measured bulk pH shift under Ca$^{2+}$ free conditions was 0.18 pH units, which would imply a buffering power of ~6 mM / pH unit, which is slightly lower than typical estimates of cellular buffering capacity. Given uncertainties in the quantitative assumptions, this seems a reasonable correspondence, but may also imply there are additional proton(s) released on this timescale during phototransduction (eg conversion of DAG to phosphatidic acid by DG kinase).
Figure S2. Rapid Acidification of Photoreceptors by 2,4-Dinitrophenol (DNP) and Dose Response Function for Channel Activation by DNP

(A) rapid and reversible acidification of photoreceptor induced by puffer application of DNP (100 μM) buffered at bath pH (7.15); the pH shift was 2-3 fold larger when buffered at pH 6.45 (same cell).

(B) Comparison of pH shift induced by 100 μM DNP (onset at $t = 0$) buffered at pH 6.45 and acid buffered bath (pH 6.45) alone on faster timebase.

© Summary of pH shifts measured after 10 sec perfusion with 100 μM DNP buffered at pH 6.45 ($n = 12$), pH 7.15 ($n = 7$) and pH 8.5 ($n = 3$) as well as pH 6.45 alone (no DNP, $n = 3$). Mean ± SEM; pH calculated from HPTS fluorescence (Eq. 1) recorded from norpA;trp photoreceptors loaded with 500 μM HPTS via patch electrodes.

(D) Dose response function for TRPL channel activation in phosphoinositide depleted trp photoreceptors; constructed by comparing responses to alternating 2s pulses of 100 μM DNP (buffered at pH 6.45) and a test concentration (20 μM in example shown in inset). Responses expressed as % response to 100 μM DNP (mean ± SEM $n = 3$ to 5 cells for each data point). Average response to 100 μM was 1041 ± 265 pA ($n = 9$). Curve fitted with a Hill plot with $EC_{50}$ 28 μM and $n = 4.4$. This should be considered as an “operational” dose response function (that underestimates sensitivity to DNP), because steady-state conditions were not reached during the short exposures (used to exclude any longer term effects of DNP). Data obtained from recordings without nucleotide additives in the electrode >10 minutes after establishing whole-cell configuration. Although a full dose response was not performed, responses to brief (1s) pulses of DNP were still reliably elicited in phosphoinositide depleted cells at concentrations as low as 25 μM when buffered at bath neutral pH 7.15.
Figure S3. Current Voltage (I-V) Relationships of Light-Induced Currents and Currents Induced by DNP following Phosphoinositide Depletion

(A) TRPL channels (isolated in trp mutant) and (B) TRP channels exploiting the Ca\(^{2+}\) impermeable TRP\(^{D621G}\) mutant (TRP\(^{D621G}\) transgene expressed in trp;trpl double mutant background) to allow convenient phosphoinositide depletion. The rectification characteristics of the light and DNP induced currents were similar in each case. Note the particularly unusual and diagnostic I-V characteristic of the TRP\(^{D621G}\) mutant (ref 26 main text).

Data obtained using voltage ramps from –100 to + 100 mV (duration 1s) recorded in normal bath with pipette solution containing 120 mM Cs Gluconate, 15 mM TEACl, 2 MgCl\(_2\), 4 MgATP 0.4 NaGTP and 1 mM NAD. A template ramp recorded in the dark and prior to DNP application was subtracted from each trace, but data have not otherwise been scaled. Representative of 3 cells each.
Figure S4. Activation of TRPL (A-C) and TRP (Channels D,E) by DNP after Phosphoinositide Depletion in Absence of Ca\textsuperscript{2+} Influx

(A) 2s pulse of 100 μM DNP (pH 6.45, arrowheads) applied before depletion in Ca\textsuperscript{2+} free bath (left) failed to activate channels in a trp photoreceptor recorded with nucleotide additives (+ATP). 5s illumination (~\text{10}^5 effective photons, bar) generated a saturating response that rapidly decayed as phosphoinositides were depleted. A second 2s pulse of DNP now rapidly activated a large current. 1 ms test flashes (arrows) monitor sensitivity. Mean maximum current activated by DNP under these conditions was 3.9 ± 0.49 nA (mean ± SEM, \( n = 5 \) cells).

(B) Recording from trp photoreceptor in Ca\textsuperscript{2+} free bath without nucleotide additives in the electrode (-ATP). 2 s pulses of 100 μM DNP (pH 6.45, arrows) initially activated no channels, but then currents up to ~14 nA were repeatedly evoked for the lifetime of the recording.

(C) Peak light-induced currents (normalised to \( I_{\text{max}} \)) and DNP evoked currents as function of time after establishing whole-cell (averaged data from B plus two other cells recorded in Ca\textsuperscript{2+} free bath without ATP). Sensitivity to DNP developed as sensitivity to light (which tracks PIP\textsubscript{2} levels) declined. The currents activated (>10 nA) under these conditions were at least as large as the maximum light-induced currents in trp mutants.

(D) Recording (with nucleotide additives) from trpl mutant (to isolate TRP channels) in Ca\textsuperscript{2+} free bath: 2s application of DNP (100 μM, pH 6.45,arrows) induced only a small current (left), but
following phosphoinositide depletion by repeated 1 ms flashes (each containing ~10^4 effective photons), the same dose evoked a large rapid current (mean 1.08 ± 0.36 nA n = 3 cells).

(E) Recording from photoreceptor expressing the Ca^{2+} impermeable TRP_{D621G} channels recorded in normal bath (1.5 mM Ca^{2+}). Phosphoinositide depletion was achieved by two 5s light flashes (bars, each containing ~10^5 effective photons). DNP (2s, 100 μM, pH 6.45;arrowheads) applied prior to depletion evoked only a 20 pA ionophore current, but evoked large currents during and immediately after the depleting stimuli. Currents returned to control level over a period of 1-2 minutes as PIP_2 was resynthesised. Representative of results in 4 cells.

Figure S5. Sensitivity to DNP in cds and inaC Mutants

(A) In a cds mutant three bright flashes (each containing ~ 5x10^5 effective photons) (delivered during bar) depleted phosphoinositides irreversibly. Thereafter, the cell became insensitive to further illumination, but highly sensitive to DNP (2s 100 μM pulses, pH 6.45) and remained so for the rest of the recording (~10 min) despite presence of ATP in the electrode. Inset (right) shows responses to DNP shortly after depletion (dotted) and ~9 minutes later on expanded timescale. The response to DNP before depletion (ionophore current only) is also shown. Representative of n = 6 cells.

(B) Left: in the PKC mutant (inaC), sensitivity to DNP (2s pulses at arrows) developed after phosphoinositide depletion induced by a single 5s stimulus (5x 10^5 effective photons). The sensitivity to DNP then declined over 2-3 minutes as sensitivity to light (reflecting PIP_2 resynthesis) recovered (small transient downward deflections). Right: in a wild-type (wt) photoreceptor the same 5s light stimulus no longer resulted in increased sensitivity to DNP (2s pulses at arrows). The response to brief flashes had also fully recovered within ~20s. Representative of n = 4 cells each.
Figure S6 Effects of Weak Acids on TRPL Channels in trp Mutants

(A) Octanoic acid (4s, 5mM buffered at pH 6.45) induced only a small inward current in a dark adapted trp cell ca. 2 minutes after establishing whole-cell configuration with an electrode lacking ATP or other nucleotide additives (left). But 10 minutes later, following phosphoinositide depletion (right) rapidly reversible activation of TRPL channels was routinely observed; albeit much less than could be activated by DNP (representative of n = 6 cells, recorded without ATP or other nucleotide additives). Test flashes (arrows) delivered prior to the acid pulse indicate sensitivity to light was abolished and that phosphoinositides were effectively depleted at this stage.

(B) in another trp cell ca 20 minutes after onset of recording without nucleotide additives, octanoic acid again induced a similar current; the less lipophilic benzoic acid (10 mM pH 6.45) induced a slight increase in noise (n = 3); whilst propionic acid (n = 4) failed to activate any channels under these conditions.

(C) CCCP (1s, 2 μM, pH 6.45, n = 4), which like DNP is a mitochondrial uncoupler with a membrane permeable anionic (charged) state, generated a large (>500 pA) current following phosphoinositide depletion (right) but only an ionophore current before (left). As predicted from its relative potency as a protonophore (ref 31 main text) it was ca. 2 orders of magnitude more potent than DNP. However, because its effects were less rapidly reversible, we preferred DNP for most experiments.
Supplemental References

