Calcium-dependent Inactivation of Light-sensitive Channels in *Drosophila* Photoreceptors

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ABSTRACT Whole-cell voltage clamp recordings were made from photoreceptors of dissociated *Drosophila* ommatidia under conditions when the light-sensitive channels activate spontaneously, generating a "rundown current" (RDC). The Ca^{2+} and voltage dependence of the RDC was investigated by applying voltage steps (+80 to −100 mV) at a variety of extracellular Ca^{2+} concentrations (0–10 mM). In Ca^{2+}-free Ringer large currents are maintained tonically throughout 50-ms-long voltage steps. In the presence of external Ca^{2+}, hyperpolarizing steps elicit transient currents which inactivate increasingly rapidly as Ca^{2+} is raised. On depolarization inactivation is removed with a time constant of ~10 ms at +80 mV. The Ca^{2+}-dependent inactivation is suppressed by 10 mM internal BAPTA, suggesting it requires Ca^{2+} influx. The inactivation is absent in the *trp* mutant, which lacks one class of Ca^{2+}-selective, light-sensitive channel, but appears unaffected by the *inaC* mutant which lacks an eye-specific protein kinase C. Hyperpolarizing voltage steps applied during light responses in wild-type (WT) flies before rundown induce a rapid transient facilitation followed by slower inhibition. Both processes accelerate as Ca^{2+} is raised, but the time constant of inhibition (12 ms with 1.5 mM external Ca^{2+} at −60 mV) is ~10 times slower than that of the RDC inactivation. The Ca^{2+}-mediated inhibition of the light response recovers in ~50–100 ms on depolarization, recovery being accelerated with higher external Ca^{2+}. The Ca^{2+} and voltage dependence of the light-induced current is virtually eliminated in the *trp* mutant. In *inaC,* hyperpolarizing voltage steps induced transient currents which appeared similar to those in WT during early phases of the light response. However, 200 ms after the onset of light, the currents induced by voltage steps inactivated more rapidly with time constants similar to those of the RDC. It is suggested that the Ca^{2+}-dependent inactivation of the light-sensitive channels first occurs at some concentration of Ca^{2+} not normally reached during the moderate illumination regimes used, but that the defect in *inaC* allows this level to be reached.

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INTRODUCTION

Invertebrate photoreceptors respond to light via activation of a phosphoinositide signaling cascade (Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Fein, Payne, Corson, Berridge, and Irvine, 1984; Devary, Heichal, Blumenfeld, Cassel, Suss, Barash, Rubinstein, Minke, and Selinger, 1987; reviewed in Payne, Walz, Levy, and Fein, 1988; Minke and Selinger, 1991), which results in the inositol 1,4,5 trisphosphate ($InsP_3$)-induced release of Ca^{2+} from localized intracellular stores and activation of cation permeable channels in the plasma membrane (Millecchia and Mauro, 1969; reviewed in Nagy, 1991). In microvillar photoreceptors, the Ca^{2+} stores are represented by specialized endoplasmic reticular structures abutting the base of the microvilli, called submicrovillar cisternae or SMC (Payne et al., 1988; Baumann and Walz, 1989). Ca^{2+} plays a vital role in transduction: it appears to be directly involved in excitation, possibly gating light-dependent channels in the plasma membrane (Payne, Corson, and Fein, 1986; Nagy, 1991; Hardie and Minke, 1993). Ca^{2+} has also been proposed to play a positive feedback role, accelerating the rising phase of the response in both *Limulus* (Payne and Fein, 1986) and *Drosophila* (Hardie, 1991). In addition, it has long been recognized that Ca^{2+} is a major mediator of light adaptation, acting via negative feedback to decrease the gain of phototransduction while simultaneously shortening latency and accelerating the light response (Lisman and Brown, 1972; Wong and Knight, 1980).

In *Limulus* the light-dependent channels have little, if any, permeability to Ca^{2+} (Brown and Blinks, 1974; Deckert and Stieve, 1991) and the SMC are presumably the major source of Ca^{2+} for phototransduction. In all other invertebrates investigated, however, there is a significant light-induced influx of Ca^{2+} into the photoreceptors (barnacle, Brown and Blinks, 1974; Brown and Rydqvist, 1990; Werner, Suss-Toby, Rom, and Minke, 1992; bee, Minke and Tsacopoulos, 1986; Ziegler and Walz, 1989; fly, Sandler and Kirschfeld, 1988; Hardie, 1991). In *Drosophila*, at least, this influx apparently occurs via the light-dependent channels themselves, which are highly permeable to Ca^{2+} (Hardie, 1991; Ranganathan, Harris, Stevens, and Zuker, 1991). The permeability to Ca^{2+}, however, is drastically reduced in the *trp* mutant (Hardie and Minke, 1992), leading to the suggestion that, as in other invertebrate photoreceptors (Nasi, 1991; Nasi and Gomez, 1991; Deckert, Nagy, Helrich, and Stieve, 1992), there are in fact two (or more) classes of light-dependent channels in *Drosophila*, and that one is highly selective for Ca^{2+} and encoded by the *trp* gene (Hardie and Minke, 1992; Montell and Rubin, 1989; Phillips, Bull, and Kelly, 1992). Evidence from *Drosophila* indicates that Ca^{2+} entering the cell via the Ca^{2+} selective *trp*-dependent channels during the light response is largely responsible for the feedback roles of Ca^{2+} in phototransduction. One argument is that all the classical Ca^{2+}-mediated manifestations of light adaptation are blocked in the *trp* mutant (Minke, 1982; reviewed in Minke and Selinger, 1991). Furthermore, in wild-type (WT) photoreceptors the kinetics of voltage-clamped flash responses shows a strong dependence upon both voltage and external Ca^{2+} concentration. Both the onset and the decay of flash responses are greatly accelerated as Ca^{2+} is raised or as the cell is hyperpolarized (Hardie, 1991; Ranganathan et al., 1991). The effect of voltage is probably secondary since it is absent in Ca^{2+}-free Ringer and presumably reflects the

increased driving force for Ca^{2+} entry as the cell is hyperpolarized (Hardie, 1991; Ranganathan et al., 1991). This voltage dependence is largely abolished in the *trp* mutant indicating that influx via the *trp*-dependent channels is responsible for the feedback (Hardie and Minke, 1992).

Although the importance of Ca^{2+} for the feedback mechanisms is well established, the sites at which it acts are not. Payne, Flores, and Fein (1990) showed that Ca^{2+} inhibits $InsP_3$-induced-Ca^{2+} release in *Limulus* ventral photoreceptors. This is also a potential target for positive feedback since the $InsP_3$ receptor has been reported to have a bell-shaped dependency on Ca^{2+}, being facilitated up to ~300 nM and inhibited by higher concentrations (Baumann and Walz, 1989; Bezprozvanny, Watras, and Ehrlich, 1991; Finch, Turner, and Goldin, 1991). A second potential target is protein kinase C (PKC), which is known to be activated by a combination of Ca^{2+} and diacylglycerol (DAG), itself generated by phospholipase C (reviewed in Berridge, 1987; Nishizuka, 1988). It was recently shown that, *inaC,* a mutant defective in eye-specific PKC (Smith, Ranganathan, Hardy, Marx, Tsuchida, and Zuker, 1991), has an abnormally slow response termination (Ranganathan et al., 1991). Despite initial reports (Smith et al., 1991), it was further shown that light adaptation was severely reduced in *inaC* and the defect localized to the failure of single quantum bumps to terminate, such that each quantum bump has a residual tail consisting of apparently random channel openings (Hardie, Peretz, Suss-Toby, Rom-Glas, Bishop, Selinger, and Minke, 1993*b*). To explain these and other findings we suggested that quantum bumps corresponded to quantal release of Ca^{2+} from the SMC and that PKC was required to effectively terminate this process (Hardie et al., 1993*b*).

A third potential site of feedback is at the level of the light-sensitive channels themselves, as has recently been suggested from experiments on excised patches from *Limulus* ventral photoreceptors (Johnson and Bacigalupo, 1992). Previously the properties of the light-sensitive channels had not been investigated in *Drosophila* inasmuch as they have proved inaccessible to the patch pipette, whereas the properties of the light-induced current recorded in whole-cell recordings are dominated by the kinetic and noise properties of the quantum bumps. However, in the preceding article we showed that the light-dependent channels activate spontaneously during prolonged recording, thereby becoming uncoupled from the transduction machinery (Hardie and Minke, 1994). In this article we exploit this finding to explore the voltage and Ca^{2+} dependency of these channels in more detail, and compare them to the properties of the light-induced current (LIC) in WT, *trp,* and *inaC* photoreceptors. We argue that the *trp*-dependent channels in particular are subject to a rapid and powerful Ca^{2+}-dependent inactivation that may be expected to play a role in light adaptation.

MATERIALS AND METHODS

With minor differences detailed below, the methods used are essentially the same as in the previous paper (Hardie and Minke, 1994).

Flies

The WT strain was Oregon R, white-eyed (*w*). The mutants used included trp^{CM} (Cosens and Manning, 1969), lacking a light-dependent Ca^{2+} channel (Montell and Rubin, 1989; Hardie

and Minke, 1992) and *inaC*^{P209}, which is a null mutant of an eye-specific PKC (Pak, 1979; Smith et al., 1991). Recently eclosed adult flies (<4 h posteclosion) or late-stage pupae (<10 h preeclosion) were used. Beyond quantitative differences in current amplitudes, no significant relevant differences were noted between adults and p15 pupae.

Electrophysiology

Whole-cell recordings were made at 19–21°C as described previously (Hardie, 1991; Hardie and Minke, 1994). Solutions are described in the accompanying article (Hardie and Minke, 1994). Most experiments were made using Cs/TEA Cl–based intracellular solutions to block K channel activity. For most recordings these solutions included 100 μM EGTA. For the experiment of Fig. 3, the pipette solution included (in mM): 10 BAPTA, 40 CsOH, 80 CsCl, 15 tetraethylammonium (TEA) Cl, 2 $MgSO_4$, 10 TES, pH 7.15. Series resistances were typically 7–15 MΩ. For some of the largest currents evoked in this study (particularly for positive voltage steps applied after the development of rundown; e.g., see Fig. 1), only recordings with series resistance <10 MΩ and series compensation >75% were accepted (resulting in errors of <12.5 mV for 5-nA currents). Even so some distortion of the largest currents can be expected, both because of series resistance errors, and also because of deteriorating space clamp (calculated length constant ~1.5–2 times length of cell during 5-nA currents). This probably accounts for some of the variability observed in their time course. For some experiments involving instantaneous voltage steps during light responses, leak currents were subtracted using templates generated by applying identical voltage protocols in the darkness. Otherwise capacitive transients were carefully compensated, and, unless otherwise stated (see figure legends), a linear leak subtracted digitally off-line. Time constants were determined by fitting single exponential functions to data traces using proprietary software (Clampfit, Axon Instruments, Inc., Burlingame, CA).

Illumination was via a 50-W halogen lamp filtered by Wratten ND filters (Eastman Kodak Co., Rochester, NY), and an OG 530 yellow filter, and was delivered to the preparation by a light guide positioned 2 cm over the bath. Relative intensities were calibrated using a photodiode with a strictly linear output. Absolute calibration in terms of effective photons per second delivered to the preparation was achieved by counting quantum bumps when the light was heavily attenuated. Log −6.0 attenuation corresponds to 5–10 effectively absorbed photons per second in adult WT photoreceptors, p15 pupae may be up to 10× less sensitive (Hardie, Peretz, Pollock, and Minke, 1993*a*).

RESULTS

Ca^{2+} and Voltage Dependence of the RDC

In order to explore the Ca^{2+} and voltage dependence of the light-dependent channels, we exploited the finding of the preceding article: namely that after some minutes of whole-cell recording the light-dependent channels activate spontaneously, generating what we have called a "rundown current" or RDC. The noise properties of the RDC are consistent with the random openings of channels with a mean open time of ~1–2 ms suggesting that channel activity is effectively uncoupled from the transduction cascade (Hardie and Minke, 1994).

WT. Fig. 1 shows the currents elicited by 50-ms voltage pulses in the range +80 to −100 mV, applied from a holding potential of −20 mV after the development of the rundown current. As already seen (Hardie and Minke, 1994), such voltage steps induce characteristic transient currents; here we show that they are also strongly

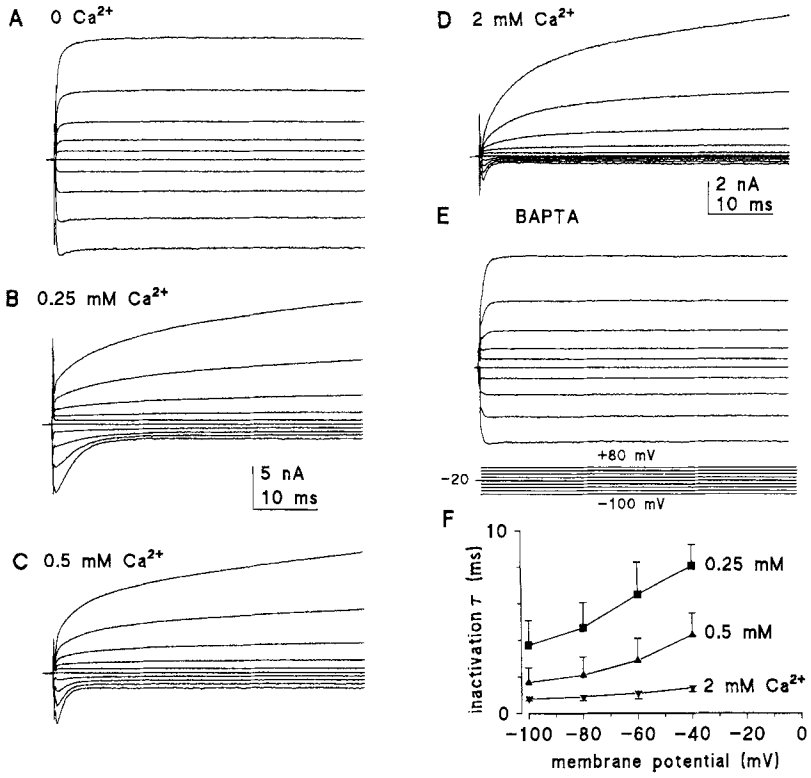

FIGURE 1. Ca^{2+} and voltage dependence of rundown currents in adult WT photoreceptors. Currents elicited by 50-ms voltage steps (+80 to −100 mV) applied after rundown, from a holding potential of −20 mV. (*A*) Virtually no dynamic behaviour is seen in 0 Ca^{2+} Ringer (100 μM EGTA, no added Ca^{2+}), although the currents reveal a characteristic dual (inward and outward) rectification. As bath Ca^{2+} is raised (*B–D*) inward currents inactivate increasingly rapidly, whilst more slowly activating outward currents are observed as the cells are depolarized. Traces *A* and *B* are from the same cell: rundown was recorded initially in 0.25 mM Ca^{2+} Ringer which was then substituted for 0 mM Ca^{2+} Ringer and shows that the absolute size of the RDC is increased when external Ca^{2+} is removed. Remaining traces from different cells. (*E*) Traces recorded in 1.5 mM external Ca^{2+}, but with internal Ca^{2+} buffered by 10 mM BAPTA (all other traces recorded with pipette solution containing Cs/TEA Cl and 100 μM EGTA). (*F*) Inactivation time constants derived from single exponential fits to traces as in *B–D* at three different Ca^{2+} concentrations, plotted against membrane potential. Note the marked Ca^{2+} and voltage dependence. Curves are based on the mean (±SD) of between 4 and 14 cells. To facilitate averaging, these data have not been corrected for series resistance errors (maximally ~+10 mV at −100 mV). The junction potential (−3 mV) has also been ignored. Recordings were filtered at 5 kHz and sampled at 20 kHz. Traces have not been leak subtracted (leak currents prior to rundown are typically <5% of the rundown currents and largely linear [Hardie and Minke, 1994]).

dependent upon Ca^{2+}. In the presence of external Ca^{2+}, hyperpolarizing voltage steps induce rapidly inactivating transient currents. In the absence of external Ca^{2+} there is little or no inactivation (Fig. 1 *A*); whilst the rate and degree of inactivation increase as Ca^{2+} is raised. With high external Ca^{2+} (10 mM), inactivation already appears saturated at −20 mV, as no transient inward currents can be elicited by hyperpolarizing steps (not shown). In most cases the inactivation time courses are well approximated by a single exponential decay. The time constants of such fits are plotted in Fig. 1 *F* and show a marked dependence on both Ca^{2+} and voltage. The voltage dependence is particularly marked with low (0.25 mM) extracellular Ca^{2+}. With physiological concentrations of Ca^{2+} (2 mM) and physiological voltages (−40 mV), the time constant of inactivation is ca. 1.5 ms.

The marked Ca^{2+} dependency of the inactivation suggests that it is mediated by Ca^{2+} entering the channels and that the effect of hyperpolarization is simply to increase the driving force for Ca^{2+} entry. This interpretation is further supported by the observation that after rundown at a given Ca^{2+} concentration and voltage, the steady-state RDC is increased by reducing extracellular Ca^{2+} (and vice versa). For example, the series of traces in Fig. 1, *A* and *B*, were obtained from the same photoreceptor: the cell initially ran down in 0.25 mM Ca^{2+} and the i-V series of Fig. 1 *B* was recorded; subsequently the Ringer was replaced with Ca^{2+}-free Ringer (100 μM EGTA, no added Ca^{2+}) and the traces in Fig. 1 *A* were recorded. At negative holding potentials the steady-state RDC in 0 Ca^{2+} is about fourfold larger, but almost identical at +80 mV. A similar behavior was observed in all four photoreceptors tested in this way. Note, however, that a characteristic dual (inward and outward) rectification is still observed in the absence of external Ca^{2+} (Fig. 1 *A*), suggesting that this feature represents an intrinsic voltage-dependent property of the underlying conductance (see also Hardie and Minke, 1994).

Depolarizing steps, which presumably relieve the Ca^{2+}-dependent inactivation, produce the opposite behavior to hyperpolarizing steps. Currents increase in size during depolarizing pulses, following a relatively slow time course (single exponential time constant typically ≈10 ms at +80 mV), but now with little obvious dependence on external [Ca^{2+}] between 0.25 mM (=10.8 ± 3.3 ms [n = 14]) and 10 mM (=11.7 ± 1.6 ms [n = 5]). The precise time course is somewhat variable between cells and not always adequately fitted by a single exponential, probably reflecting poor voltage control with the very large conductances activated under these circumstances (see Materials and Methods).

Ca^{2+}-dependent inactivation of the RDC could be mediated by at least two mechanisms: a voltage-dependent channel block of the pore by external Ca^{2+}, or at the cytoplasmic face by Ca^{2+} having permeated the channels. To distinguish between these possibilities we investigated the effect of buffering internal Ca^{2+} with BAPTA. This should have no effect on a Ca^{2+} binding site in the pore but should alleviate inactivation mediated by Ca^{2+} entering the cell. Fig. 1 *E* shows the voltage dependence of the RDC with 1.5 mM external Ca^{2+} and 10 mM internal BAPTA. The inactivation is clearly almost abolished, although once again the currents shows a characteristic dual inward and outward rectification. In fact, the currents now very closely resemble those elicited with normal pipette solution and Ca^{2+}-free external solution (c.f. Fig. 1 *A*). A similar behavior was seen in two further cells tested in this

way, whereas another two cells showed some residual inactivation, which was, however, slower and much less severe than in controls. These results suggest that the inactivation is mediated on the cytoplasmic side by Ca^{2+} entering the cell.

trp mutants. After rundown in *trp* the currents induced by hyperpolarizing steps also show a transient behavior; however, it is less pronounced than in WT (Fig. 2 *A*), and in many cases could hardly be distinguished from the residual capacitive

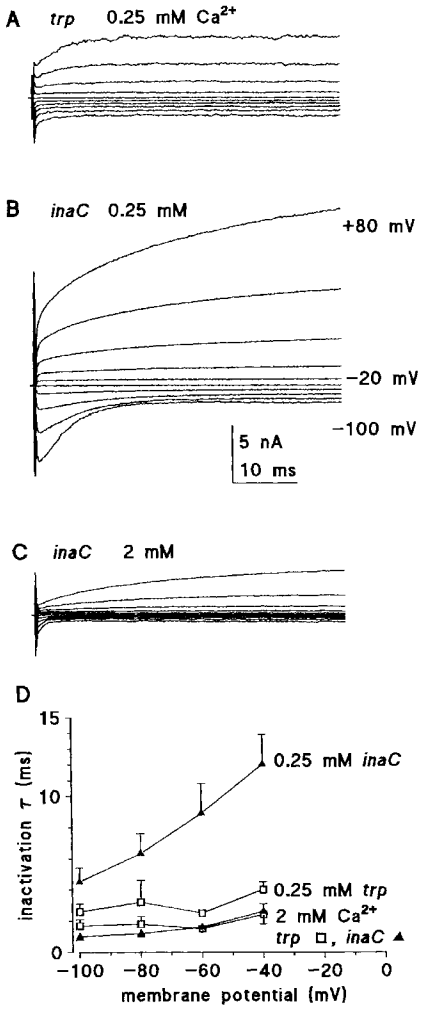

FIGURE 2. Voltage dependence of rundown currents in *trp* and *inaC*. Currents elicited following rundown: (*A*) *trp* in 0.25 mM external Ca^{2+}; (*B*) *inaC* in 0.25 mM Ca^{2+} Ringer; and (*C*) *inaC* in 2 mM Ca^{2+} Ringer. The behaviour in *inaC* is similar to that in WT (c.f. Fig. 1). In *trp* only very small inward transient currents are elicited (see also preceding article, Hardie and Minke, 1993), whereas the outward rectification develops more rapidly and is characterized by conspicuous noisy fluctuations. (*D*) Inactivation time constants derived from traces as in *A–C* at 0.25 and 2 mM Ca^{2+} Ringer. Each curve based on mean ± SD from three to five cells. Other details as in Fig. 1.

transients. Where relaxation time constants could be reliably measured, they were in the range 1–2 ms with no longer any marked dependence on external Ca^{2+} and a much smaller dependence on voltage (Fig. 2 *D*). As described in the accompanying article (Hardie and Minke, 1994), depolarizing steps reveal an outward rectification in *trp*, however, this develops with a faster time course (τ = ~4 ms).

inaC mutants. The Ca^{2+}-mediated deactivation of the light response is defective in mutants of the *inaC* gene (Ranganathan et al., 1991; Hardie et al., 1993*b*), which encodes an eye-specific PKC (Smith et al., 1991). We wondered whether PKC might also be required for the Ca^{2+}-dependent inactivation of the rundown current; however, this appears not to be the case. RDCs in the *inaC* mutant appeared very similar to those in WT, and after rundown, voltage steps elicited similar, Ca^{2+}-dependent transient currents (Fig. 2, *B–D*). At most, there appeared to be slight quantitative differences in the time constants of inactivation (c.f. Figs. 1 and 2), however, further studies would be required to substantiate this point.

Ca^{2+} and Voltage Dependence of the LIC

Previous studies have shown that the kinetics of responses to flashes of light are accelerated as external Ca^{2+} is raised, or as the cell is hyperpolarized (Hardie, 1991). This was interpreted as a sequential positive and negative feedback of the transduction cascade mediated by Ca^{2+} influx via the *trp*-dependent light sensitive channels (Hardie, 1991; Hardie and Minke, 1992). Hardie (1991) also showed that it was possible to probe the kinetics of these Ca^{2+}-mediated feedback processes by making hyperpolarizing voltage steps during the light response in order to instantaneously increase the Ca^{2+} influx. The finding that the channels themselves appear to be subject to Ca^{2+}-dependent inactivation prompted us to reexamine the Ca^{2+}-mediated feedback of the LIC in more detail for comparison. Figs. 3 and 4 show the results of systematic measurements of this sort at a variety of external Ca^{2+} concentrations.

WT. The time course of the positive and negative feedback was explored as described previously (Hardie, 1991), by delivering a step of light at a relatively depolarized potential (usually −20 mV) and then hyperpolarizing the cell (usually to −60 mV) during the response. A control response to an identical light step was also recorded with the cell clamped at −60 mV throughout. The leak currents due to the voltage protocol alone were then recorded in the dark and subtracted from the traces. As seen in Fig. 3, when the voltage is stepped to −60 mV during the response, there is a rapid and pronounced facilitation (~300%), which then decays more slowly to overlap the control response elicited at −60 mV throughout.

The time course of both processes clearly accelerates markedly as external Ca^{2+} is raised from 0.25 to 2 mM. For example, at 0.25 and 0.5 mM Ca^{2+}, the time course of the positive feedback can be clearly resolved, whereas at higher Ca^{2+} concentrations, it could often no longer be distinguished from the residual capacitive artifact. The positive feedback was quantified simply by determining the time-to-peak after the voltage step, with the realization that at higher external Ca^{2+} concentrations this may be limited by the clamp time constant. A time constant for the slower decaying phase was derived by subtracting the control response at −60 mV (Fig. 3 *B*) and fitting a single exponential to the decay. The values of both the time-to-peak of the transient currents and the time constant of their inhibition are plotted against external Ca^{2+} in Fig. 3 *C*. Although the Ca^{2+} and voltage dependent inhibition of the LIC is qualitatively similar to the Ca^{2+}-dependent inactivation of the RDC, the time constants are an order of magnitude longer (Fig. 3 *C*), and there was no indication of

a rapid component to the decay which might be equated with the rapidly decaying currents observed in the RDC.

Depolarizing the cell during the light response alleviates the inhibition experienced at the prevailing voltage. Fig. 4 shows the voltage and time dependence of this recovery process. The time course of recovery was probed by depolarizing (to 0 mV) for varying durations during the steady-state response to a moderate light intensity, initiated at −60 mV. The degree of recovery was assessed by the size of the transient

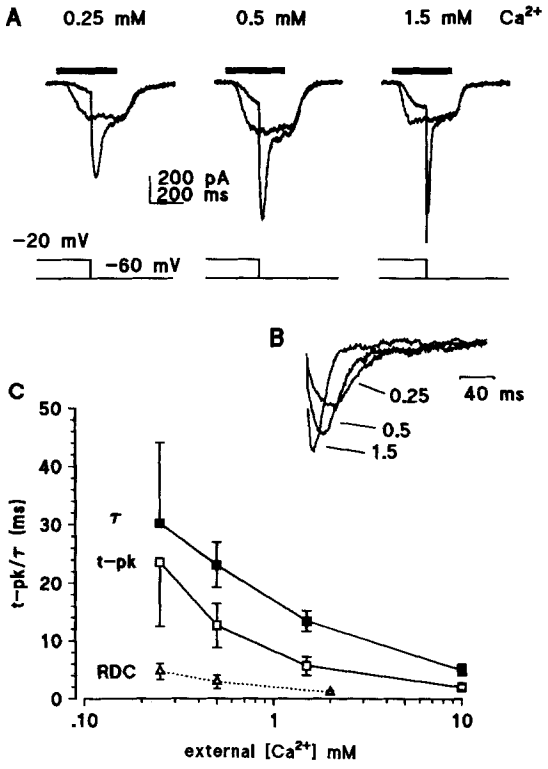

FIGURE 3. Ca^{2+} and voltage dependence of the light-induced current. (*A*) Recordings from three different adult WT photoreceptors to identical light stimuli (log −2.5, duration indicated by black bars). The first flash is initiated with the cell clamped at −20 mV and the holding potential stepped to −60 mV during the response plateau. A control response is then recorded at −60 mV throughout. Leak currents have been subtracted using a template recorded using the same voltage protocols applied in the dark. (*B*) The transient, hyperpolarization-induced current for all three Ca^{2+} concentrations are superimposed and shown on a faster time scale. In each case the control response at −60 mV has been subtracted. (*C*) Averaged data from traces, as in *B* plotted against external Ca^{2+} concentration (log scale). Time-to-peak of the transient currents induced by the voltage steps (□) were estimated by eye; a single exponential was fitted to the decaying phase to generate the time constant of inhibition (■). For comparison, the dotted curve shows the time constant of inactivation of the RDC at −60 mV (△, data from Fig. 1).

currents observed on repolarizing to −60 mV (Fig. 4 *A*). As seen in Fig. 4 *C* the time course of recovery is strongly dependent upon external Ca^{2+} being complete in <100 ms with physiological external Ca^{2+} (2 mM), but taking several hundred milliseconds with 0.25 mM Ca^{2+}. The voltage dependence of the recovery process was probed similarly by applying long (250 ms) depolarizing steps to different voltages during the steady-state response initiated at −80 mV (Fig. 4, *B* and *D*). The degree of recovery was again assessed on repolarizing to −80 mV. Fig. 4 *D* plots the

potential of the depolarizing pulse against the normalized peak of the transient current on repolarizing to −80 mV and shows that inhibition is removed over a narrow range of voltages which shifts depending on the prevailing extracellular Ca^{2+} concentration.

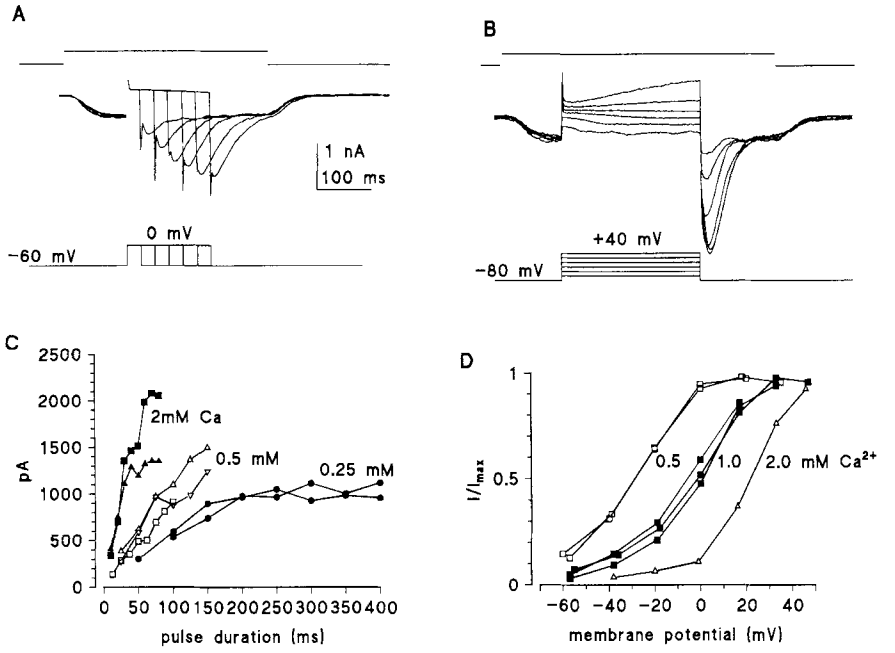

FIGURE 4. Voltage and time dependence of recovery from Ca^{2+}-dependent inhibition of the light response (*A* and *B*) WT adult photoreceptors recorded in 0.5 mM Ca^{2+} Ringer. In each case the cell is clamped at a negative holding potential (−60 or −80 mV) and stimulated with a moderate intensity light step (log −3.0). In *A* the holding potential is stepped to 0 mV for 12.5 ms and then repolarized to −60 mV. This protocol cycle is then repeated, each time incrementing the duration of the step to 0 mV by 25 ms. In *B* the cell is depolarised by increasing amounts (20 mV steps between −60 and +40 mV) for 250 ms with each successive flash. The transient current elicited on repolarizing (to −60/−80 mV) increases with longer or greater depolarizations. The traces have not been leak subtracted. In *C* and *D* this behavior is quantified at different Ca^{2+} concentrations for several different cells. The rate of recovery accelerates with higher external Ca^{2+}, whereas the range of voltages over which inhibition is removed shifts systematically as Ca^{2+} is raised.

trp mutants. Similar voltage jumps during the light response in *trp* failed to reveal any significant time- and voltage-dependent processes (Fig. 5). In some cases a small rapidly decaying transient was observed after hyperpolarizing steps; however, this was seldom reliably distinguished from the capacitive artefact.

inaC mutants. As previously described (Ranganathan et al., 1991; Hardie et al., 1993*b*), the response in *inaC* photoreceptors is complex. The onset kinetics of flash

responses are completely normal but instead of the usual rapid decay the response abruptly changes trajectory leaving a long residual tail lasting up to ~1 s. Responses to longer steps of dim light are characterized by abnormally slow rising and decaying phases. This phenomenology is explained by an underlying defect in the shape of individual quantum bumps which themselves fail to terminate, and have the same basic form as the flash response (Hardie et al., 1993*b*).

The Ca^{2+}-mediated inhibition of the light response is greatly altered in *inaC* and in addition depends critically upon the phase of the response. In order to demonstrate this, repeated flashes were given and hyperpolarizing voltage steps (to −60 or −80 mV) were made at different times during the response. In *inaC,* such hyperpolarizing commands presented during the initial phase of the response reveal transient currents which decay relatively slowly. In some cells at least, the time constant of the decay of these "early transients" closely approximates that observed under the same conditions in WT (Fig. 6, *E* and *F*). As the voltage steps are made progressively later during the response, the currents decay more rapidly. The latest steps, now made during the slowly decaying tail of the light response, elicit transient currents which

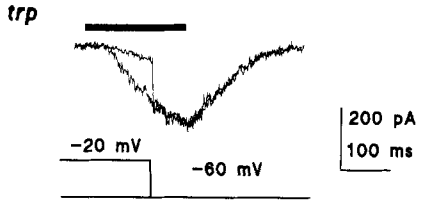

FIGURE 5. Lack of voltage dependence in the light response of *trp*. A voltage step from −20 to −60 mV (c.f. Fig. 6) applied during the response to a step of light (*solid bar*, log −2.0) in an adult *trp* photoreceptor elicits no transient facilitation or inhibition. Bath Ringer 0.25 mM Ca^{2+}. Traces have been leak subtracted using a template recorded in the dark.

decay with a time constant of only 2–3 ms (at 0.5 mM Ca^{2+}), which is very similar to the time constant determined during the rundown current at similar Ca^{2+} concentrations and voltages (e.g., Fig. 1 *F*). The same protocols performed in WT photoreceptors (Fig. 6, *C* and *D*) generate transient currents which decay with a relatively long time constant (~20 ms with steps to −60 mV) that changes little throughout the response (see also Fig. 4). The behavior in WT and *inaC* is compared quantitatively in Fig. 6, *E* and *F*, where the time constants of the decaying phases are plotted as a function of time of the voltage step after onset of the light flash for a number of different cells with voltage steps to both −60 and −80 mV.

Voltage steps made during the steady-state of responses to longer steps of illumination gave variable results in *inaC* (Fig. 7). Very occasionally (two cells), at the outset of recording, a behavior similar to that in WT was observed with relatively slow time constants. However, after several minutes recording and in most cells (eight tested in this way) from the very outset of recording, only rapidly decaying transients were observed. When probed with the voltage protocol used to characterize the RDC, the time constants of the LIC in *inaC* now come to very closely approximate those of the RDC in WT, suggesting that properties of the channels themselves, uncoupled

from the transduction cascade, are now being observed. Which behavior prevailed appeared to correlate with the quality of the recording. Thus a typical feature of whole-cell recordings from *inaC* is that initially very clear quantum bumps are observed; these are of similar amplitude (10 pA) and initial rising phase to WT, but characterized by a long residual tail of apparently random channel activity (Hardie et

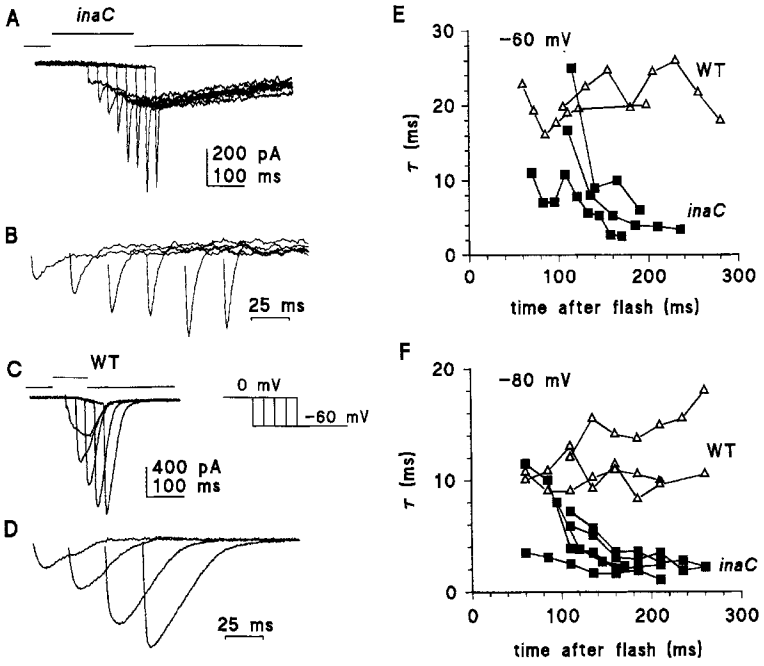

FIGURE 6. Transient currents measured in response to voltage steps at different times of the light response in *inaC* (*A* and *B*) and WT p15 pupal photoreceptors (*C* and *D*). In each case the response was initiated at 0 mV and the cell stepped to −60 mV at different times in subsequent repeated flashes (log −2.5). In *B* and *D* the transient currents (after subtraction of control response at −60 mV throughout) are plotted on an expanded time scale. Both cells recorded in 0.5 mM Ca^{2+} Ringer. In *E* and *F* the time constants of the decaying phases of these transient currents (determined by single exponential fits) are plotted as a function of time of the voltage step after onset of the flash (two to five different cells in each case from both adult and pupal ommatidia); (*E*) for voltage jumps from 0 to −60 mV recorded with Cs/TEA Cl electrodes; (*F*) for voltage jumps from −40 to −80 mV recorded using K-gluconate electrodes. In WT (△), transient currents similar to those described in Fig. 6 are seen which have a similar form at all times during the response decaying with a time constant of ~20 ms (−60 mV) or 12 ms (−80 mV). In *inaC* (■), initially responses have a relatively slow decay time constant, in some cells indistinguishable from WT, but at later stages of the response the decay is much more rapid.

al., 1993*b*). However, after some minutes the bumps deteriorate and the response appears to consist entirely of channel noise. When tested, cells in the latter state always showed the rapidly decaying transient currents in response to voltage steps applied during steady-state light responses.

DISCUSSION

Ca²⁺-dependent Inactivation of the trp-dependent Channels

As discussed in the preceding article (Hardie and Minke, 1994), at least a subpopulation of the light-dependent channels in *Drosophila* photoreceptors become active spontaneously during prolonged whole-cell recording, generating a so-called "run-down current." A striking feature of the RDC in WT photoreceptors is the dynamic

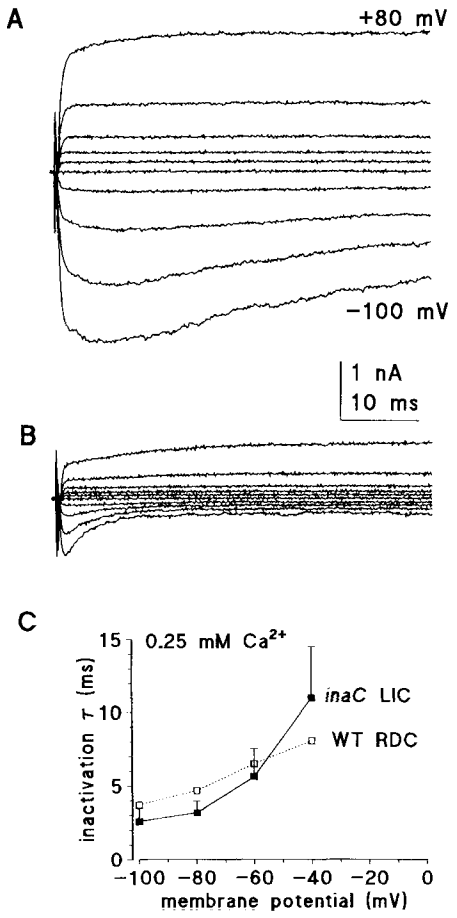

FIGURE 7. Voltage dependence of the steady-state LIC in *inaC*. (*A* and *B*) currents elicited by voltage steps applied during the steady-state of a response to a continuous dim background (log −3.0) in two *inaC* photoreceptors, bathed in 0.5 mM Ca^{2+} Ringer. The voltage protocol is the same as that used for characterization of the RDC (c.f. Fig. 1). Most cells showed the behavior in *B* with rapidly inactivating currents, however, occasionally cells showed slower transient currents similar to those recorded in WT under similar conditions (not shown). (*C*) Time constants of inactivation of the light response determined from *inaC* photoreceptors cells showing the more typical phenomenology of *B*, plotted as a function of voltage. *Dotted line:* data from WT RDC (Fig. 1) for comparison.

rectification observed with instantaneous voltage steps (Fig. 1). Such behavior can in principle be expected for currents mediated by channels with a voltage-dependent open time, in which case the currents should relax with a time constant equal to the open time at the new voltage (Colquhoun and Hawkes, 1977). However, unlike values of the open time derived by noise analysis (Hardie and Minke, 1994), the relaxation time constants are strongly dependent upon Ca^{2+}. Since inactivation is also absolutely dependent upon extracellular Ca^{2+}, we suggest instead that the rapid inactiva-

tion observed with hyperpolarizing voltage steps reflects a process of Ca^{2+}-mediated inactivation. Similarly, we suggest that the slower activation observed with depolarizing steps reflects removal of this inactivation. Since the inactivation is reduced or eliminated by internal Ca^{2+} buffering with BAPTA (Fig. 1 *E*), the inactivation appears to mediated on the cytoplasmic side of the membrane, i.e., it is presumably mediated by Ca^{2+} influx through the light sensitive channels. A similar conclusion has been reached in excised patches from *Limulus* ventral photoreceptors, in which the light-sensitive channels have entered an irreversible spontaneously active state. The open probability of channels in such patches is reduced by application of Ca^{2+} to the cytoplasmic face of the patch (Johnson and Bacigalupo, 1992).

Voltage steps applied after the development of rundown currents in *trp* photoreceptors reveal only very small transient currents. The relaxation time constants of these currents varied only slightly between ~4 ms at +80 mV and 1–2 ms at −100 mV. They showed little dependence upon Ca^{2+} and were similar to estimates of open time achieved by fitting Lorentzian functions to the power spectra of noise spectra (Hardie and Minke, 1994). These results can be interpreted as the reflection of a direct voltage dependence of channel open time.

Although the absence of Ca^{2+}-dependent inactivation in *trp* indicates that Ca^{2+} influx via the *trp*-dependent channels is required for inactivation, it does not necessarily indicate which channels are being inactivated in WT. Although the RDC in WT is almost completely eliminated by La^{3+} (Hardie and Minke, 1994), which specifically blocks the *trp*-dependent channels (Hardie and Minke, 1992), it is still possible that non-*trp*-dependent channels are also active during rundown. Thus, as discussed in the preceding article (Hardie and Minke, 1994) we suspect that the RDC in WT includes a component mediated by non-*trp*-dependent channels activated by Ca^{2+} influx via the *trp*-dependent channels. This component is unlikely to be large however, and on the assumption that the noise in the RDC derives from non-*trp*-dependent channels can be estimated as < 10% (Hardie and Minke, 1994). Examination of Fig. 1, however, shows that as much as 90% of the RDC may be inactivated during hyperpolarizing voltage commands. This strongly suggests that the Ca^{2+}-dependent inactivation is a specific property of the *trp*-dependent channels.

Ca^{2+}-dependent or facilitated inactivation is a phenomenon described in a variety of Ca^{2+} permeable channels, including voltage activated Ca^{2+} channels (Gutnick, Lux, Swandulla, and Zucker, 1989; Imredy and Yue, 1992; reviewed in Eckert and Chad, 1984), cyclic nucleotide-activated cation channels in olfactory receptors (Zufall, Shepherd, and Firestein, 1991) and also a phosphoinositide-mediated Ca^{2+} entry conductance recently described in mast cells (Hoth and Penner, 1992). Where studied, the inactivation represents a decrease in the open probability of the underlying channels rather than their conductance or mean open time (Eckert and Chad, 1984; Zufall et al., 1991). Such a mechanism would suggest that the mean open time of the *trp*-dependent channels should be shorter than the inactivation time constant (i.e., < 2 ms at physiological voltages and external Ca^{2+} concentrations). A Ca^{2+}-dependent block of a rather different nature occurs in the cyclic-GMP–gated channels of vertebrate photoreceptors. Here the effective channel conductance is greatly reduced by a voltage dependent block of the pore mediated by external divalent cations (reviewed in Yau and Baylor, 1989).

Ca^{2+}-dependent Feedback Control of the LIC

The discovery of Ca^{2+}-dependent inactivation of the light-dependent channels prompted us to reexamine the Ca^{2+} feedback control of the transduction cascade. As previously described, Ca^{2+} influx via the *trp*-dependent channels during the light response mediates an early facilitation (positive feedback) followed by a somewhat slower inhibition of the light-induced current (Hardie, 1991; Hardie and Minke, 1992). Although the Ca^{2+} dependency of this latter, negative feedback process is qualitatively similar to the Ca^{2+} dependent inactivation of the RDC, the time constants are an order of magnitude longer (Fig. 3) and presumably, therefore this behavior reflects feedback at an earlier point in the transduction cascade. We suggest the $InsP_3$ receptor as a possible candidate since Ca^{2+} mediated inhibition of $InsP_3$-induced Ca^{2+} release has been demonstrated in several systems including bee and *Limulus* photoreceptors (Baumann and Walz, 1989; Payne et al., 1990; see also Bezprozvanny et al., 1991), and is likely to represent one mechanism of light adaptation. Although we have emphasized that this inhibition is slow compared to inactivation of the channels, in fact the time constants are still remarkably fast ($\sim$12 ms at -60 mV with physiological Ca^{2+}) and must contribute to the exceptionally rapid response kinetics of insect photoreceptors (Hardie, 1991). Recovery from this sort of inhibition probably represents one component of dark adaptation, and appears to be complete within 50–100 ms at physiological Ca^{2+} concentrations (Fig. 4). Possibly this time course reflects some native Ca^{2+} buffering mechanism (sequestration, extrusion or binding). The strong Ca^{2+} dependence of the recovery time course (Fig. 4 *C*), whereby rate of recovery increases as external Ca^{2+} is raised, suggests that the underlying mechanism itself is subject to Ca^{2+}-mediated regulation.

Multiple Feedback Roles of Ca^{2+} in Light Adaptation

If, as our evidence strongly suggests (Hardie and Minke, 1994), the RDC and the LIC are mediated by the same channels, why is the rapid inactivation seen in the RDC not observed when hyperpolarizing commands are applied during responses to light? One possible explanation is that at the moderate intensities used for the experiments on the LIC, Ca^{2+} in the subplasmalemmal space does not reach values which induce significant inactivation—due, for example, to the local density of active channels, or the operation of more effective buffering mechanisms, such as Ca^{2+} sequestration or extrusion, in the physiologically intact preparation. Support for this suggestion is found in the unusual behavior of the LIC in the *inaC* mutant (Fig. 6), which lacks an eye-specific PKC (Smith et al., 1991). On completely independent evidence we have recently suggested that the defect in *inaC* may represent failure to terminate the light-induced rise in cytosolic Ca^{2+}, possibly owing to a defect in Ca^{2+} sequestration or failure to terminate $InsP_3$-induced Ca^{2+} release (Hardie et al., 1993*b*). In *inaC*, hyperpolarizing voltage commands made during the initial phases of light-induced responses induce transient currents which decay with a relatively slow time constant. At later stages in the response, however, the currents inactivate very rapidly with time constants similar to those of the Ca^{2+}-dependent inactivation of the RDC (Fig. 6, *E* and *F*) suggesting that properties of the channels themselves, uncoupled from the transduction cascade, are now being observed. Which behavior prevails during

steady-state responses is characteristically labile. Our previous interpretation of the defect in *inaC* now suggests an explanation for this complex phenomenology, provided that, as suggested above, direct Ca^{2+}-mediated inactivation of the light-sensitive channels only comes into play at some higher level of subplasmalemmal Ca^{2+}. In other words, we suggest that in *inaC* the light-induced rise in cytosolic Ca^{2+} is not effectively curtailed (Hardie et al., 1993*b*) and, a finite time after the onset of the light response, subplasmalemmal [Ca^{2+}] reaches values that excede the threshold for Ca^{2+}-mediated inactivation of the channels. The lability of this behaviour could reflect the efficiency of the native Ca^{2+} buffering capability of individual cells, itself likely to be dependent on the metabolic integrity of the cell. An alternative hypothesis, which cannot be excluded, is that the channels enter a different functional state during rundown, and that this altered state is also adopted in the absence of PKC.

We conclude by emphasizing the importance of Ca^{2+} influx via the *trp*-dependent channels. Since virtually all manifestations of light adaptation are absent in the *trp* mutant (reviewed in Minke and Selinger, 1991), this source of Ca^{2+} appears to play an essential role in light adaptation. The sites of feedback, however, are likely to be several. Previously we have shown that PKC, itself presumably activated via the light-induced rise in cytosolic Ca^{2+} (Ranganathan et al., 1991), is required for adaptation as the classical manifestations of light adaptation are severely reduced in the *inaC* mutant (Hardie et al., 1993*b*). In this paper we have identified two further Ca^{2+}-mediated processes, namely Ca^{2+}-dependent inactivation of the channels themselves, and the slower inhibition of the light-induced current, tentatively attributed to inhibition of the $InsP_3$ receptor. As Ca^{2+}-mediated negative feedback processes, these both appear well-suited to play roles in adaptation. Although PKC does not appear to be directly required for either mechanism, in the present study we show that the interplay and balance between these two negative feedback processes collapse in the *inaC* mutant. Together with our earlier findings (Hardie et al., 1993*b*), this suggests that PKC is required to coordinate the activation and physiological operation of the two feedback mechanisms by controlling the Ca^{2+} level in photoreceptor, thereby shaping the waveform of the bump.

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