

Protein Kinase C Activators Inhibit the Visual Cascade in *Limulus* Ventral Photoreceptors at an Early Stage

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The phosphoinositide cascade mediates visual transduction in invertebrate photoreceptors. Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol biphosphate, producing inositol trisphosphate (InsP₃) and diacylglycerol (DAG). Protein kinase C (PKC) is a major target of DAG in many cell types. We have used PKC activators to investigate the function of the kinase in the phototransduction cascade in *Limulus polyphemus* ventral photoreceptors. Extracellular application of (–)-indolactam V (0.03–30 μM) or phorbol-12,13-dibutyrate (10 μM) reversibly reduced the sensitivity of the electrical response of the photoreceptors to light by up to 1000-fold. The inert stereoisomer (+)-indolactam V and 4α-phorbol had no effect. The effect of (–)-indolactam V was antagonized by the PKC inhibitors bisindolylmaleimide I and Gö 6976. Coapplication of bisindolylmaleimide V, used as a negative control compound for PKC inhibition, did not reduce the effectiveness of (–)-

indolactam V. These findings are consistent with (–)-indolactam V activating PKC and desensitizing the light response. Furthermore, our pharmacological results indicate that PKC activation does not appear to play a role in light adaptation. We localized the position of the target of PKC in the visual cascade. We chemically excited the cascade at various stages to determine the kinase's target. PKC activation by (–)-indolactam V decreased the light-induced elevation of intracellular calcium but had no effect on the photoreceptor's excitatory response to intracellular injection of InsP₃. However, the PKC activator greatly reduced the excitation caused by GTP-γ-S injection. We propose that PKC inhibits the visual transduction cascade at the G-protein and/or PLC stage.

Key words: *Limulus polyphemus*; photoreceptor; PKC; phototransduction; phosphoinositide pathway; visual inhibition; desensitization; adaptation

In invertebrate microvillar photoreceptors, absorption of light by rhodopsin activates phospholipase C (PLC) via a GTP-binding protein (Bloomquist et al., 1988; Ranganathan et al., 1995). PLC catalyzes the hydrolysis of phosphatidylinositol biphosphate producing inositol trisphosphate (InsP₃) and diacylglycerol (DAG) (Berridge, 1993). The role of the DAG branch of the cascade is not clear. In many cell types DAG is an activator of the enzyme protein kinase C (PKC) (Nishizuka, 1988), which in turn phosphorylates a variety of targets. PKCs make up a family of serine/threonine kinases that are involved in the regulation of many cellular processes.

Light initiates three general processes in invertebrate microvillar photoreceptors: the activation of cation channels in the microvillar membrane (excitation), desensitization of the visual cascade (adaptation), and long-term changes in the structure of the microvillar membrane, such as the shedding and renewal of rhabdom at dawn. PKC has been proposed to play a role in all of these processes. Application of PKC activators to photoreceptors of the clam *Lima* evokes an inward current that shares some of the characteristics of the light-induced current (Gomez and Nasi, 1998). Studies of the *Drosophila* mutant inactivation-no-afterpotential C (*inaC*), which lacks an eye-specific PKC (Smith et al., 1991), have indicated a role for PKC in adaptation

and response deactivation (Smith et al., 1991; Hardie et al., 1993). In the horseshoe crab *Limulus polyphemus*, PKC activators induce rhabdom shedding when injected into the compound lateral eye before dawn (Jinks et al., 1996). PKC activators have also been reported to reduce potassium channel activity and promote changes in photoreceptor morphology in the marine mollusc *Hermisenda* (Farley and Auerbach, 1986; Lederhendler et al., 1990; Etcheberrigaray et al., 1992).

Recent biochemical experiments have demonstrated putative PKC activity in *Limulus* photoreceptors. The addition of calcium and phospholipid to homogenates of *Limulus* ventral and lateral eyes resulted in the phosphorylation of several proteins, and this phosphorylation was inhibited by the PKC inhibitor peptide PKC 19–36 (Calman et al., 1996). The giant ventral nerve photoreceptors of *Limulus* have several advantages for a pharmacological study of the action of PKC. The physiological and anatomical consequences of illumination have been well defined. In *Limulus* ventral photoreceptors, light-induced production of InsP₃ causes the release of calcium from intracellular stores (Brown and Rubin, 1984; Payne et al., 1986b), leading to the opening of nonspecific cation channels in the plasma membrane and a resulting depolarization of the photoreceptor's membrane potential. Calcium release subsequently reduces the photoreceptors' sensitivity, initiating light adaptation (Brown and Lisman, 1975). Prolonged bright illumination also leads to profound, reversible changes in rhabdom structure (Herman, 1991). The stability of electrophysiological recordings from isolated ventral photoreceptors permits investigation of the reversibility of the effects of PKC activators, whereas the response to pulsed intracellular injection of intermediates, such as InsP₃, allows the identification of the level of the visual cascade at which phosphorylation by PKC might act.

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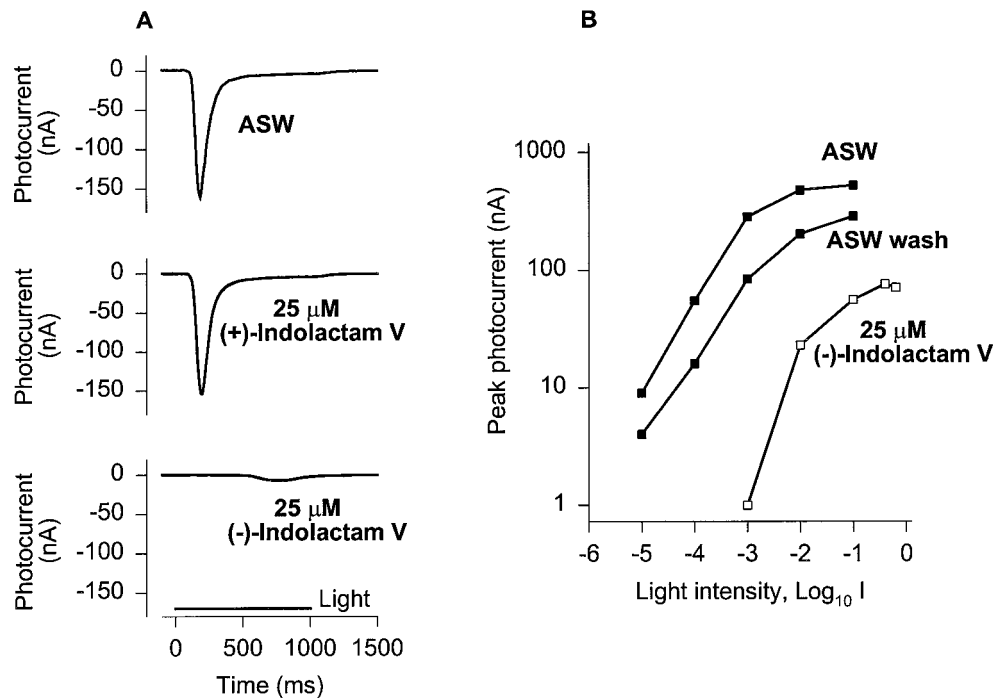


Figure 1. (–)-Indolactam V reversibly and stereospecifically inhibits the light response causing a shift in the intensity–response curve. *A*, The photoreceptor's response to a 1 sec light stimulus ($\text{log}_{10} -3$) under voltage-clamp conditions in ASW (*top*) is unaffected by 25 μ M (+)-indolactam V, the inactive stereoisomer (*middle*). The response to that same stimulus is strongly reduced (-7 nA) in the presence of 25 μ M (–)-indolactam V (*bottom*). *B*, (–)-Indolactam V at 25 μ M causes a shift in the intensity–response curve to the *right* by $\sim 2.5 \text{ log}_{10}$ units and a reduction in the saturated light-induced current of $\sim 0.8 \text{ log}_{10}$ units. The effects of (–)-indolactam V were partially reversible after 2 hr of washing in ASW. The light stimulus was 1 sec long, delivered at 1 min intervals.

Parts of this paper have been published previously (Dabdoub and Payne, 1998).

MATERIALS AND METHODS

Preparation of the ventral nerve photoreceptors was performed as described by Millecchia and Mauro (1969a,b). Nerves were placed in artificial seawater (ASW) that contained (in mM): 435 NaCl, 10 KCl, 20 MgCl₂, 25 MgSO₄, 10 CaCl₂, and 10 HEPES at pH 7.3. Phorbol-12,13-dibutyrate (PDBu), 4 α -phorbol, bisindolylmaleimide I and V, and Gö 6976 were obtained from Calbiochem (San Diego, CA). (–)-Indolactam V was obtained from both Calbiochem and Sigma (St. Louis, MO), and (+)-indolactam V was obtained from Sigma. All these substances were dissolved in DMSO as a 5–10 mM stock solution and kept at -20°C . Test substances were applied extracellularly by superfusion (1 ml/min; 5–10 times bath vol) followed by a 10 min wait before continuing with the experiment.

White light from a 100 W quartz–halogen source (model 6333; Oriol Corporation, Stratford, CT) illuminated the photoreceptors. The light passed through a heat filter (Schott KG3; Ealing Optics, South Natick, MA), neutral density (ND) filters, and a shutter before being focused onto the specimen plane. The intensity of the light at the specimen, with no intervening ND filters, was 80 mW/cm². Light intensities are quoted in this paper as log_{10} units of attenuation relative to this intensity. To view the preparation with an infrared-sensitive video camera, we also continuously illuminated cells by an infrared beam, created by passing a second beam of light from the quartz–halogen lamp through an infrared filter (Schott RG1000) before focusing it onto the specimen. Attenuation of the light by a $\text{log}_{10} -8.5$ ND filter resulted in a rate of one single photon event per second.

For intracellular recording, the micropipette contained 3 M KCl. For voltage-clamp experiments, cells were impaled with a second microelectrode that also contained 3 M KCl (resistance $< 10 \text{ M}\Omega$) and were voltage clamped at the dark resting membrane potential using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). The voltage-clamped current or the membrane potential was filtered at 300 Hz and sampled at 1 kHz using a Digidata 1200 (Axon Instruments) analog-to-digital board installed in a personal computer. The pCLAMP (Axon Instruments) software programs Clampex and Fetchex (version 6.0.3) were used for data acquisition.

All chemicals injected into the cell were dissolved in a carrier solution that contained 100 mM potassium aspartate and 10 mM HEPES, at pH 7.0. Injections were monitored using the infrared video camera (Corson and Fein, 1983a). InsP₃ was obtained from Research Biochemicals (Natick, MA), dissolved into a 10 mM stock solution, and kept at -20°C .

Poorly hydrolyzable L-Ins(1,3,4)P₃ was the gift of Dr. Barry Potter (University of Bath, Bath, United Kingdom). GTP- γ -S was obtained from Sigma, dissolved into a 1 mM stock solution, and kept at -70°C . For excitation by InsP₃, 10 or 100 μ M InsP₃ in carrier solution was pulse pressure injected into the light-sensitive rhabdomeral lobe of the photoreceptor as described by Fein et al. (1984). For activation of G-proteins, 100 μ M GTP- γ -S in carrier solution was pressure injected into the cell. After multiple injections, nucleotide exchange was activated by light flashes after which the photoreceptor was dark adapted (Bolsover and Brown, 1982).

Experiments using confocal imaging and a calcium fluorescent indicator were performed as described previously (Ukhanov and Payne, 1995). Briefly, cells were injected with 500 μ M Oregon green-5N (Molecular Probes, Eugene, OR), a fluorescent calcium indicator dye, in carrier solution. The dissociation constant K_d of Oregon green-5N was determined to be 18 μ M in a solution containing 400 mM KCl and 10 mM HEPES at pH 7.0. The photoreceptors were viewed with a Zeiss LSM 410 laser-scanning confocal microscope equipped with a 488 nm argon laser (Uniphase) focused through a Zeiss Neofluor 10 \times , 0.3 numerical aperture objective lens using the stationary spot recording mode of the microscope.

RESULTS

Activators of PKC inhibit the electrical response to light

(–)-Indolactam V is a potent and selective activator of PKC that serves as a mimic of the endogenous activator DAG. (–)-Indolactam V activates PKC by binding to the same site on the enzyme as phorbol esters (Heikkilä and Åkerman, 1989). The specificity of (–)-indolactam V can be verified by performing control experiments using the stereoisomer (+)-indolactam V. Figure 1 illustrates the effects of addition of both isomers. A ventral nerve photoreceptor was dark adapted and voltage clamped to its resting membrane potential. A 1 sec light flash (intensity, $\text{log}_{10} -3$) excited an inward current, which peaked at an amplitude of -160 nA and then declined as adaptation proceeded (Fig. 1*A*, *top*). Superfusing the cell with the inactive stereoisomer (+)-indolactam V (25 μ M) had no effect on the photoreponse (Fig. 1*A*, *middle*). Superfusing the cell with the same concentration of the active stereoisomer (–)-indolactam V

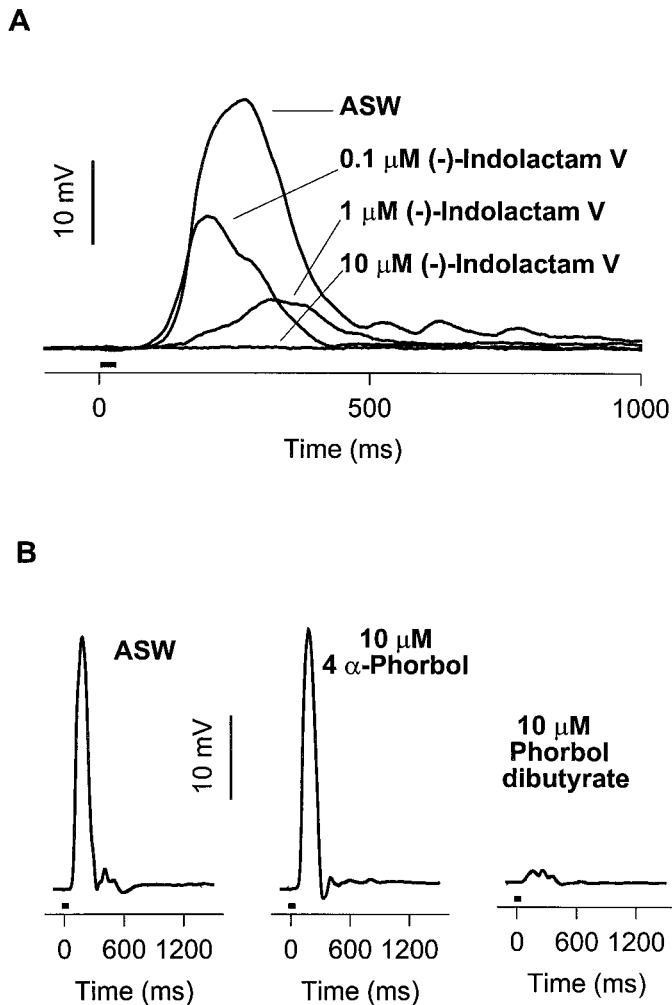


Figure 2. PKC activators decrease the photoreceptor's sensitivity to light. *A*, The receptor potential, under current-clamp recording, decreases with increasing (–)-indolactam V concentrations. *B*, PDBu also decreases the receptor potential, whereas the inactive 4 α -phorbol has no effect. The horizontal bar below the trace represents the duration of the light flash (20 msec; $\log_{10} -5.4$). Compounds were dissolved in DMSO and applied extracellularly to the bath. The percent DMSO was 0.18% for the different concentrations of (–)-indolactam V, 0.2% for PDBu, and 0.1% for 4 α -phorbol.

reduced the peak response amplitude to -7 nA and increased its latency (Fig. 1*A*, bottom). (–)-Indolactam V affected all photoreceptors tested in a similar manner. The reduction of sensitivity induced by $25 \mu\text{M}$ (–)-indolactam V was analyzed in another cell by plotting the relationship between the peak response and light intensity. The effect of $25 \mu\text{M}$ (–)-indolactam V could be described approximately as a shift of the intensity–peak response curve to the right by $2.5 \log_{10}$ units coupled with a reduction of the saturated light-induced current by $0.8 \log_{10}$ units (Fig. 1*B*, $n = 4$). The effects of (–)-indolactam V were partially reversible after a 2 hr wash.

In current-clamp recordings of membrane potential, (–)-indolactam V decreased the photoresponse in a dose-dependent manner. Receptor potentials elicited by a 20 msec light flash ($\log_{10} -5.4$) decreased with increasing (–)-indolactam V concentrations (Fig. 2*A*). Desensitization of cells after application of (–)-indolactam V was not accompanied by significant (>4 mV) changes in resting membrane potential or a reduction in electrical

input resistance, measured with brief -0.3 – 3 nA current pulses. To check whether inhibition of the photoresponse is a common characteristic of PKC activators, we used the phorbol ester PDBu, a chemically distinct molecule that is also a specific PKC activator. Extracellular application of $10 \mu\text{M}$ PDBu inhibited the photoresponse in a manner similar to that of (–)-indolactam V (Fig. 2*B*, $n = 4$). The effects of PDBu persisted for hours and were partially reversible after an 8 hr wash (data not shown). The inert 4 α -phorbol had no effect on the photoresponse ($n = 3$), further confirming the specificity of the effects of PKC activators.

We examined whether the inhibition caused by DAG surrogates was indeed the result of PKC activation. We pretreated the photoreceptors with PKC inhibitors and tested the cell's response to (–)-indolactam V. We used bisindolylmaleimide I, a selective PKC inhibitor that acts as a competitive inhibitor to the ATP-binding site of PKC (Toullec et al., 1991). As a control, we used bisindolylmaleimide V (Davis et al., 1992). We also used Gö 6976, an indolocarbazole, another highly potent and selective inhibitor of PKC (Martiny-Baron et al., 1993).

For these experiments, we decided to monitor the sensitivity of the cell under current clamp, rather than two-electrode voltage clamp. Impaling the cell with a single micropipette minimized damage to the cell, resulting in less variation in the dark-adapted sensitivity to light and a greater chance that sensitivity would be stable for long time periods. However, the use of current-clamp recording complicates the analysis of the responses. For dark-adapted *Limulus* ventral photoreceptors, the relationship between receptor potential and flash intensity is highly nonlinear. Voltage-sensitive conductances are active even during single photon signals of >10 mV in amplitude. Above this depolarization, rectification and the approach to the reversal potential compress the intensity–response curve (Stieve and Pflaum, 1978). This means that a small change in receptor potential amplitude might reflect a large change in the sensitivity of the cell. Rather than measure receptor potential amplitude in response to flashes of fixed intensity, we therefore quantified the effects of the inhibitors by monitoring the sensitivity of the cell as the inverse of the intensity of a 20 msec flash required to elicit a 30 mV criterion response. The effects of voltage-dependent conductances are then the same for all the measurements, and changes in the sensitivity of the criterion response should reflect changes in the sensitivity of the underlying photocurrent, which displays a much larger linear dynamic range. This seems to hold for our data. The $3.3 \log_{10}$ unit decrease in sensitivity to a 30 mV criterion response induced by $30 \mu\text{M}$ (–)-indolactam V (Fig. 3*B*) can be compared with the $2.5 \log_{10}$ unit reduction in the voltage-clamp current after a $-3 \log_{10}$ unit flash induced by $25 \mu\text{M}$ (–)-indolactam V (Fig. 1*B*).

Without addition of inhibitor, sensitivity decreased progressively as (–)-indolactam V concentration increased from 0.03 to $30 \mu\text{M}$ (Fig. 3*A*, top; $n = 4$ – 6 cells for each concentration). The inactive stereoisomer (+)-indolactam V had no effect (Fig. 3*B*, $n = 3$). The dependence of sensitivity to light on (–)-indolactam V concentration was then reexamined in the presence of the two PKC inhibitors (Fig. 3*A*, C). The PKC inhibitors alone induced a small increase in the photoreceptors' sensitivity ($0.5 \pm 0.4 \log_{10}$ units; $n = 9$, pooled data using either inhibitor). The inhibitors greatly reduced the effectiveness of (–)-indolactam V (Fig. 3*A*, C; $n = 4$ – 5 for each group). In comparison with cells that were not bathed in inhibitors (Fig. 3*B*), the relative sensitivity was increased by $1.3 \log_{10}$ units in the presence of $1 \mu\text{M}$ (–)-indolactam V and by $2.2 \log_{10}$ units in the presence of $10 \mu\text{M}$ (–)-indolactam V. We obtained similar results using chelerythrine and calphostin

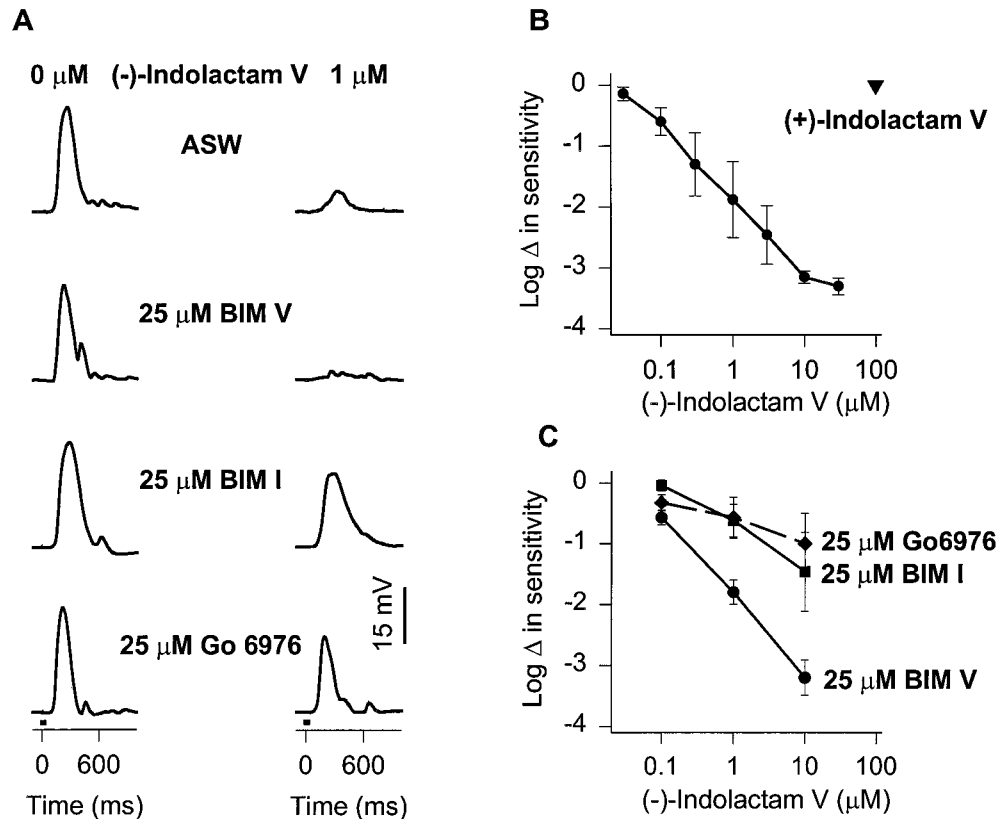


Figure 3. The effectiveness of (-)-indolactam V is decreased by selective PKC inhibitors. *A*, The PKC inhibitors bisindolylmaleimide (*BIM*) I and Gö 6976 significantly reduced the effectiveness of (-)-indolactam V when either was coapplied. *BIM* V, a negative control compound for PKC inhibition, did not alter the effectiveness of (-)-indolactam V. The horizontal bar below the bottom receptor potential traces represents the duration of the light flash (20 msec); the intensity of the flashes was the same before and after application of 1 μ M (-)-indolactam V. *B*, Changes in sensitivity to a criterion response induced by (-)-indolactam V (●) were measured. A 30 mV criterion response to a 20 msec light flash was chosen, and the change in light intensity required to evoke the criterion response was measured (0.5% DMSO at all concentrations). At a much higher concentration, the inactive stereoisomer (+)-indolactam V (▼) had no effect on the photoreceptor's sensitivity to light (1.8% DMSO). *C*, The change in the photoreceptor's sensitivity was measured for the same criterion response in the presence of specific PKC inhibitors as well as different concentrations of (-)-indolactam V. Sensitivity changes are shown relative to that measured after application of the inhibitor but before application of (-)-indolactam V and therefore do not reflect a small sensitization induced by the inhibitor alone.

C, a PKC inhibitor that interacts with the kinase's regulatory domain by competing at the binding site of DAG and phorbol esters (data not shown). Bisindolylmaleimide V had no significant effect on the desensitization caused by (-)-indolactam V (compare Fig. 3*B,C*). Taken together, these results are consistent with (-)-indolactam V activating PKC and desensitizing the photoresponse.

PKC inhibitors do not antagonize light adaptation

We investigated the effects of the PKC inhibitor Gö 6976 on the kinetics of the electrical response to sustained bright light and on light adaptation. Light adaptation was investigated by delivering paired 20 msec light flashes to voltage-clamped, dark-adapted photoreceptors. The response to the second flash in the pair was greatly reduced by light adaptation (ASW, first flash, -202 ± 5 nA; second flash, -11.3 ± 0.6 nA; $n = 3$; Fig. 4*A*). Superfusion with ASW containing 25 μ M Gö 6976 resulted in a relatively small but significant (t test, $p < 0.05$) increase in the peak response to the first flash, consistent with the increase in the sensitivity of dark-adapted photoreceptors described above. However, the PKC inhibitor had no significant effect (t test, $p > 0.05$) on the amplitude of the light-adapted response to the second flash (25 μ M Gö 6976, first flash, -247 ± 19 nA; second flash, -8.7 ± 1.9 nA; $n = 3$; Fig. 4*B*). In other experiments, bisindolylmaleimide I had

effects on dark-adapted sensitivity similar to those shown in Figure 4, whereas bisindolylmaleimide V had no significant effect (data not shown).

We were concerned that in the experiment of Figure 4, *A* and *B*, the slightly larger amplitude of the first, dark-adapted response in the presence of the PKC inhibitor might also result in a larger adapting effect, thus masking any relief of adaptation induced by the PKC inhibitor. Therefore in experiments on three cells, we compensated for the increase in light-induced current after application of 25 μ M Gö 6976 by slightly reducing the intensity of the light to maintain a -50 nA response to a test flash. We also correspondingly reduced the intensity of a bright, adapting flash. We found that the adapting flash reduced the response to the test flashes from -50 to -5 ± 0.25 nA in ASW and to -7 ± 3.5 nA in the presence of the PKC inhibitor. Thus this modified protocol also failed to demonstrate any significant relief of light adaptation by Gö 6976. We also used adaptation protocols in which the sensitivity of the photocurrent was examined in the presence of steady background illumination. For background intensities of $\log_{10} -3$ and $\log_{10} -2$, which desensitized voltage-clamped photoreceptors by 2–3 \log_{10} units, application of 25 μ M Gö 6976 had no significant effect on the peak amplitude of the light-adapted responses to test flashes (data not shown).

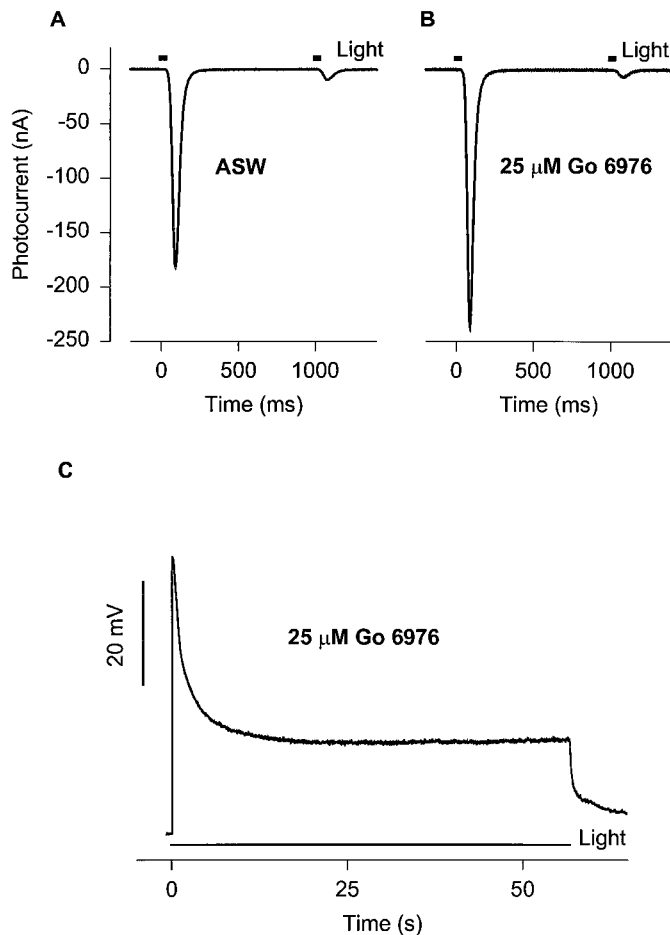


Figure 4. PKC inhibitors sensitize the photoreceptor and do not appear to affect light adaptation. *A*, Photocurrents elicited by two 20 msec light flashes ($\log_{10} -3.5$; 1 sec apart) under voltage-clamp conditions in ASW are shown. *B*, The response to the first flash is increased in the presence of 25 μM Gö 6976, whereas the second flash is unchanged. *C*, Receptor potential elicited by a prolonged intense light ($\log_{10} -1$), after overnight incubation with 25 μM Gö 6976.

The rapid transition during sustained bright illumination from the initial peak transient phase of the response to the lower steady-state phase is another consequence of light adaptation. In the *Drosophila inaC* mutant the latter phase is missing; instead the response to sustained bright light decays slowly to baseline (Hardie et al., 1993). Ventral photoreceptors that were incubated overnight in 25 μM Gö 6976 displayed receptor potentials in response to prolonged bright light ($\log_{10} -1$) that still showed a rapid transition from a transient phase to a sustained steady-state phase (Fig. 4C, $n = 4$). These responses are indistinguishable from control responses, as described by others (Millecchia and Mauro, 1969a).

(–)-Indolactam V inhibits the phototransduction cascade at the G-protein and/or PLC stage

Because phosphorylation of ion channels by protein kinase C is a well known mechanism for regulating channel activity, we examined whether the PKC acted only at a late stage in the cascade, after InsP_3 -induced calcium release. In this case, the activators should not influence the amount of calcium released as a result of light stimulation. To measure light-induced calcium release, we pressure injected ventral photoreceptor cells with the calcium-

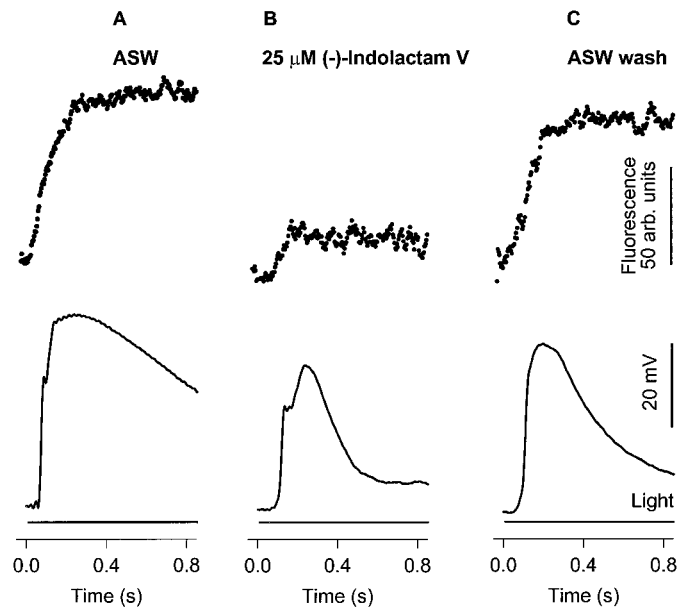


Figure 5. (–)-Indolactam V reduces light-induced calcium release. Simultaneous recordings were made of membrane potential (*bottom*) and the fluorescence of the calcium-indicator dye Oregon green-5N (*top*). The cell was pressure injected with the calcium indicator, and confocal spot measurements of Oregon green-5N fluorescence were made in the light-sensitive rhabdomeral lobe of the ventral photoreceptor. Membrane potential was sampled every 5 msec, and fluorescence was sampled every 4.5 msec. *A*, Photoreceptor bathed in ASW. *B*, After treatment with (–)-indolactam V. *C*, After a 2 hr wash with ASW. *arb.*, Arbitrary.

sensitive fluorescent dye Oregon green-5N. Confocal spot measurements of the fluorescence of Oregon green-5N were made in the light-sensitive rhabdomeral lobe. The delayed increase in the fluorescence of calcium-sensitive dyes observed during the first few hundred milliseconds of confocal laser illumination has been shown previously to indicate light-induced release of intracellular calcium ions from intracellular stores (Ukhanov and Payne, 1995; Ukhanov et al., 1995). Figure 5 shows the simultaneous recording of the membrane potential (*bottom*) and Oregon green-5N signal (*top*). Measurements were first taken before superfusion with (–)-indolactam V (Fig. 5A). Both the electrical response and the calcium signal decreased in amplitude in the presence of 25 μM (–)-indolactam V (Fig. 5B, $n = 4$). The effects were partially reversible after a 2 hr wash (Fig. 5C). We conclude that (–)-indolactam V inhibits the visual cascade at a stage that is upstream from calcium release.

We next determined whether PKC activation blocked InsP_3 -induced calcium release. If this was the case, then PKC activation should strongly inhibit the excitation caused by intracellular injection of InsP_3 , just as it inhibits the light response. Excitation by InsP_3 injection was insensitive to 25 μM (–)-indolactam V (Fig. 6, $n = 5$). In addition, 25 μM (–)-indolactam V also had no effect on repetitive bursts of depolarization caused by injecting 100 μM of the poorly hydrolyzable InsP_3 analog L- $\text{Ins}(1,3,4)\text{P}_3$ (data not shown) (Riley et al., 1994). These results indicate that the primary target of PKC is upstream from InsP_3 -induced calcium release.

Finally, we examined the effect of PKC activation on excitation caused by the G-protein activator GTP- γ -S (Pfeuffer and Helmreich, 1975). Injecting the photoreceptor with 100 μM GTP- γ -S followed by exposure to light increases the frequency of discrete

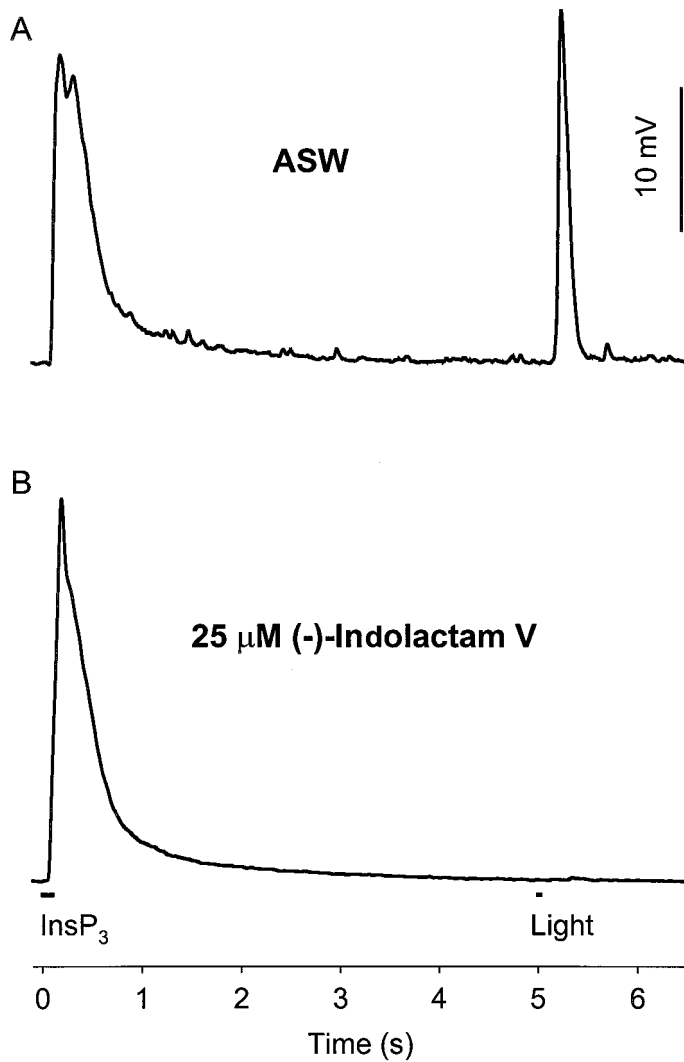


Figure 6. The desensitization of the photoreceptor by (-)-indolactam V is upstream from the InsP₃ receptor in the cascade. Membrane potential was recorded during the delivery of an intracellular pressure injection (100 msec) of 100 μM InsP₃ followed by a 20 msec light flash ($\log_{10} -5$). *A*, Photoreceptor bathed in ASW. *B*, After treatment with 25 μM (-)-indolactam V.

events in the dark (Fig. 7*A,C*) (Corson and Fein, 1983b). These events are smaller on average than spontaneous or light-induced events (Fig. 7*A,B*), probably because of the reduced amplification associated with the activation of the G-protein rather than rhodopsin (Kirkwood and Lisman, 1994). The induction of these events by GTP- γ -S injection is irreversible, and for large injections, the events fuse to form a noisy sustained depolarization (Corson and Fein, 1983b). The amplitude of GTP- γ -S-generated events was reduced by superfusion with the PKC activator (-)-indolactam V (Fig. 7*D*, $n = 4$), reducing the level of noise in darkness. After a 2 hr wash with ASW, the noise recorded in darkness increased, suggesting recovery of the amplitude or frequency of GTP- γ -S-generated events, but individual events could not be distinguished (Fig. 7*E*). The variance of this noise was transiently reduced from 0.16 to 0.06 mV² after 20 msec flashes of light ($\log_{10} -4.4$) consistent with the adaptation of GTP- γ -S-induced events (Fein and Corson, 1981). We therefore conclude that desensitization by (-)-indolactam V is downstream from rhodopsin.

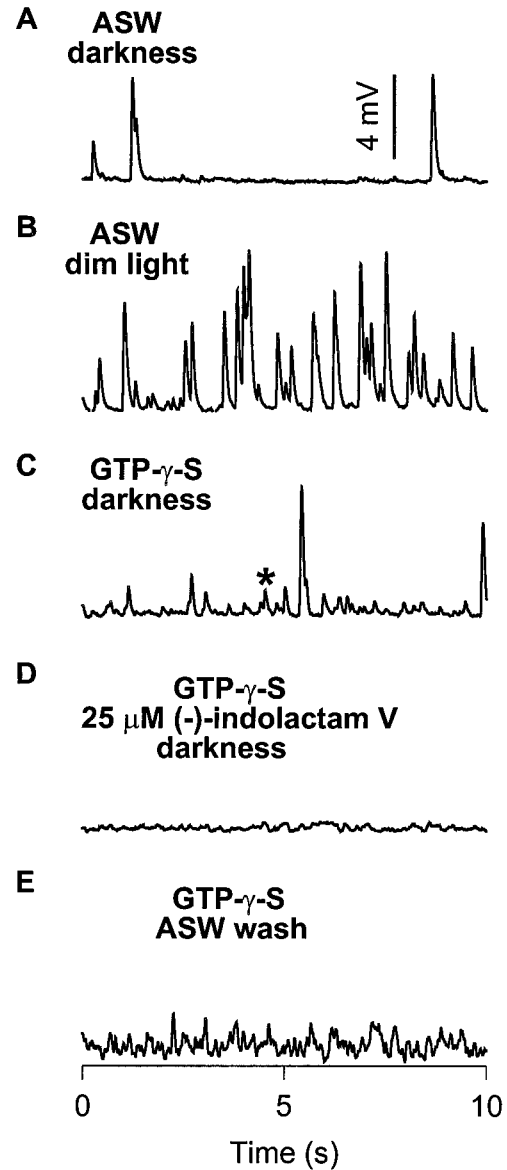


Figure 7. Desensitization by (-)-indolactam V is downstream from rhodopsin. *A*, Recording of membrane potential shows spontaneous depolarizing discrete events recorded in darkness. *B*, Light-induced discrete events ($\log_{10} -8.4$) are shown. *C*, The cell was injected with GTP- γ -S, and nucleotide exchange was activated by a series of light flashes, after which the cell was dark adapted. This resulted in a sustained increase in the frequency of small discrete events (*). *D*, Extracellular application of (-)-indolactam V reduced the amplitude of the GTP- γ -S-generated events. *E*, After 2 hr of wash, the noise level of the trace increased, consistent with the recovery of GTP- γ -S-induced events.

DISCUSSION

PKC modulates photoreceptor sensitivity to light

We have demonstrated that the light response of *Limulus* ventral nerve photoreceptor cells can be reversibly desensitized by several orders of magnitude by DAG surrogates as was reported in molluscan photoreceptors (Gomez and Nasi, 1998). We found this by using two distinct and potent activators of PKC, (-)-indolactam V, an indole alkaloid, and PDBu, a phorbol ester. PDBu and (-)-indolactam V did not excite ventral photoreceptors or induce a membrane current, indicating that PKC does not mediate excitation in these photoreceptors. This is in contrast to

Limulus photoreceptors in which it was proposed recently that PKC mediates, at least in part, photoexcitation (Gomez and Nasi, 1998). The lack of an effect of the inert stereoisomer (+)-indolactam V and 4 α -phorbol on photoreceptor sensitivity establishes the specificity of the result. In addition, PKC inhibitors antagonized the desensitization caused by (–)-indolactam V, confirming that the desensitization was indeed the result of PKC activation.

The PKC isozyme activated in this study is not an atypical PKC. There are 11 isozymes in the PKC family, all requiring phosphatidylserine (PS), a lipid found on the cytoplasmic side of membranes, for activation. These isozymes are divided into three subclasses based on their biochemical properties as follows: conventional, isozymes requiring both DAG and calcium for activation in addition to PS; novel, isozymes requiring DAG for activation in addition to PS; and atypical (aPKC), isozymes requiring only PS for activation (for review, see Newton, 1997). It is therefore possible to distinguish between the subclasses based on their cofactor requirement. The PKC activation that we described here was induced by DAG surrogates, suggesting that the PKC investigated does not belong to the aPKC subclass. Whether the kinase belongs to the conventional or novel subclass remains to be determined.

PKC activators do not mimic light adaptation, nor do PKC inhibitors prevent it

Exposure to bright light initiates a number of processes that modulate the sensitivity of a photoreceptor. The term “light adaptation” generally refers to the rapid decline in sensitivity that follows bright illumination. Light adaptation is usually accompanied by a reduced response latency and duration (Fuortes and Hodgkin, 1964). In *Drosophila* photoreceptors, the presence of an eye-specific PKC appears to be necessary for light adaptation (Hardie et al., 1993). Our pharmacological results do not support the concept that PKC mediates light adaptation in *Limulus* ventral photoreceptors. First, although (–)-indolactam V decreases the cell’s sensitivity to light, it does not reduce response latency or duration (Fig. 2A; A. Dabdoub and R. Payne, unpublished observations). Second, light adaptation desensitizes the cell’s response to InsP₃ (Fein et al., 1984), whereas (–)-indolactam V does not (Fig. 6B). Third, although PKC inhibitors slightly sensitize the photoresponse and antagonize the effect of PKC activators, they do not reverse the decrease in sensitivity associated with light adaptation for the adaptation protocols that we describe. Finally, injection of calcium ions alone into the photoreceptor can fully mimic light adaptation (Brown and Lisman, 1975; Fein and Charlton, 1977) and suppress the response to InsP₃ (Payne et al., 1986a) without any requirement for the addition of DAG or a surrogate. However, each of these results is subject to caveats, and it is not possible to conclude from these results that PKC plays no role in adaptation. In particular, desensitization mediated by PKC could be a minor component of light adaptation for the adaptation protocols that we chose. It may require more prolonged or intense illumination, or it may be redundant to calcium-mediated desensitization. It is also possible that the PKC activated by light is not as susceptible to attack by PKC inhibitors as that activated by (–)-indolactam V. Calman et al. (1996) found that the PKC inhibitors hypericin, chelerythrine, and calphostin C did not inhibit PKC phosphorylation of endogenous substrates in *Limulus* photoreceptor extracts, whereas the PKC peptide inhibitor PKC 19–36 blocked phosphorylation. In addition, we found incomplete reversal of PKC activation by the noncompet-

itive inhibitors bisindolylmaleimide I and Gö 6976 (Fig. 3C), indicating that there might be a component of the putative *Limulus* PKC activity that is resistant to the inhibitors used.

Site of action of PKC and its physiological role

We have physiologically localized the site of desensitization of light-induced calcium release and depolarization by PKC activators to an intermediate that is downstream from rhodopsin but upstream from InsP₃-induced calcium release. Therefore, we propose that PKC activation results in the inhibition of the phototransduction cascade at the level of the G-protein and PLC. One possibility is that PKC might be directly phosphorylating the G-protein and/or PLC in the cascade. PKC-mediated phosphorylation of PLC β isoforms has been proposed to decrease the activation of PLC β by the respective G-proteins (Ryu et al., 1990). Alternatively, PKC-mediated phosphorylation of the G-protein’s α subunit may decrease its activity (Aragay and Quick, 1999).

Injection of PKC activators into the lateral eyes of *Limulus* induces structural changes leading to the internalization of microvillar membrane (Jinks et al., 1996), mimicking the natural shedding of microvillar membrane observed at dawn. Light-induced internalization of the microvillar membrane has also been observed in isolated *Limulus* ventral nerve photoreceptors (Herman, 1991). The desensitization that we observe may therefore be a correlate or prelude to these anatomical changes. Another possibility is that PKC activation may induce the translocation from the rhabdom of one of the intermediates in the phosphoinositide cascade (Terakita et al., 1996), resulting in a slow, long-term uncoupling of the phosphoinositide cascade from rhodopsin that is distinct from the rapid calcium-mediated reduction in sensitivity that is normally associated with light adaptation.

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