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 bisphosphate, producing inositol trisphosphate (InsP$_3$) 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 effect of (-)-indolactam V was antagonized by the PKC inhibitors bisindolylmaleimide I and Go6976. 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 InsP$_3$. 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 bisphosphate producing inositol trisphosphate (InsP$_3$) 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 InsP$_3$ 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 Lismian, 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 InsP$_3$, allows the identification of the level of the visual cascade at which phosphorylation by PKC might act.
that contained 100 mM potassium aspartate and 10 mM HEPES, at pH data acquisition.

Software programs Clampex and Fetchex (version 6.0.3) were installed in a personal computer. The pCLAMP (Axon Instruments) 1 kHz using a Digidata 1200 (Axon Instruments) analog-to-digital board current or the membrane potential was filtered at 300 Hz and sampled at clamped at the dark resting membrane potential using an Axoclamp-2A electrode that also contained 3M KCl (resistance

Voltage-clamp experiments, cells were impaled with a second microelec-

trode that also contained 3M KCl (resistance

100 mM GTP-γ-S was obtained from Sigma, dissolved into a 1 mM stock solution, and kept at 20°C. Injections were monitored using the infrared video camera (Corson and Fein, 1983a). InsP3 was obtained from Research Biochemicals (Eugene, OR), a fluorescent calcium indicator dye, in carrier solution. The photoreceptor as described by Fein et al. (1984). For activation of G-proteins, 100 mM GTP-γ-S in carrier solution was pressure injected into the light-sensitive rhabdomeric lobe of the photoreceptor as described by Fein et al. (1984). For activation of G-proteins, 100 mM 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 indica-

tor were performed as described previously (Ukhanov and Payne, 1995). Briefly, cells were injected with 500 mM Oregon green-5N (Molecular Probes, Eugene, OR), a fluorescent calcium indicator dye, in carrier solution. The dissociation constant $K_o$ of Oregon green-5N was determined to be 18 μM in a solution containing 400 mM KC1 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× objective lens using the stationary spot recording mode of the microscope.

RESULTS

Activators of PKC inhibit the electrical response to light

(−)-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 ($log_{10} -3$) under voltage-clamp conditions in ASW (top) is unaffected by 25 μM (−)-indolactam V, the inactive stereoisomer (middle). The response to that same stimulus is strongly reduced under 25 μM (−)-indolactam V at 25 μs causes a shift in the intensity–response curve to the right by $-2.5 log_{10}$ units and a reduction in the saturated light-induced current of $-0.8 log_{10}$ units. The effects of (−)-indolactam V were partially reversible after 2 hr of wash-

ing in ASW. The light stimulus was 1 sec long, delivered at 1 min intervals.
PKC activators inhibit phototransduction.

Reduced peak response amplitude to -7 nA and increased its latency (Fig. 1A, bottom). (-)-Indolactam V affected all photoreceptors tested in a similar manner. The reduction of sensitivity induced by 25 μM (-)-indolactam V was analyzed in another cell by plotting the relationship between the peak response and light intensity. The effect of 25 μM (-)-indolactam V could be described approximately as a shift of the intensity–peak response curve to the right by 2.5 log₁₀ units coupled with a reduction of the saturated light-induced current by 0.8 log₁₀ units (Fig. 1B, 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 photosresponse in a dose-dependent manner. Receptor potentials elicited by a 20 msec light flash (log₂₁₀ = -5.4) decreased with increasing (-)-indolactam V concentrations (Fig. 2A). 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 photosresponse 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 μM PDBu inhibited the photosresponse in a manner similar to that of (-)-indolactam V (Fig. 2B, n = 4). The effects of PDBu persisted for hours and were partially reversible after an 8 hr wash (data not shown). The inert 4α-phorbol had no effect on the photosresponse (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 II (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 a long period. 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 log₁₀ unit decrease in sensitivity to a 30 mV criterion response induced by 30 μM (-)-indolactam V (Fig. 3B) can be compared with the 2.5 log₁₀ unit reduction in the voltage-clamp current after a -3 log₁₀ unit flash induced by 25 μM (-)-indolactam V (Fig. 1B).

Without addition of inhibitor, sensitivity decreased progressively as (-)-indolactam V concentration increased from 0.03 to 30 μM (Fig. 3A, top; B, n = 4–6 cells for each concentration). The inactive stereoisomer (+)-indolactam V had no effect (Fig. 3B, n = 3). The dependence of sensitivity to light on (-)-indolactam V concentration was then reexamined in the presence of the two PKC inhibitors (Fig. 3A,C). The PKC inhibitors alone induced a small increase in the photoreceptors’ sensitivity (0.5 ± 0.4 log₁₀ units; n = 9, pooled data using either inhibitor). The inhibitors greatly reduced the effectiveness of (-)-indolactam V (Fig. 3A, C; n = 4–5 for each group). In comparison with cells that were not bathed in inhibitors (Fig. 3B), the relative sensitivity was increased by 1.3 log₁₀ units in the presence of 1 μM (-)-indolactam V and by 2.2 log₁₀ units in the presence of 10 μM (-)-indolactam V. We obtained similar results using chelerythrine and calphostin.
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 \( (\pm) \)-indolactam V (compare Fig. 3B,C). Taken together, these results are consistent with \((\pm)\)-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, \(-202 \pm 5\) nA; second flash, \(-11.3 \pm 0.6\) nA; \(n = 3\); Fig. 4A). Superfusion with ASW containing 25 \(\mu\)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 \(\mu\)M Gö 6976, first flash, \(-247 \pm 19\) nA; second flash, \(-8.7 \pm 1.9\) nA; \(n = 3\); Fig. 4B). 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 \(\mu\)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 \(-5 \pm 0.25\) nA in ASW and to \(-7 \pm 3.5\) 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 \(\log_{10} 2^3\) and \(\log_{10} 2^2\), which desensitized voltage-clamped photoreceptors by \(2 \pm 3\ log_{10}\) units, application of 25 \(\mu\)M Gö 6976 had no significant effect on the peak amplitude of the light-adapted responses to test flashes (data not shown).
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 G6976 displayed receptor potentials in response to prolonged bright light (log10 21) 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 InsP3-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-sensitive fluorescent dye Oregon green-5N. Confocal spot measurements of the fluorescence of Oregon green-5N 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.

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.

We next determined whether PKC activation blocked InsP3-induced calcium release. If this was the case, then PKC activation should strongly inhibit the excitation caused by intracellular injection of InsP3, just as it inhibits the light response. Excitation by InsP3 injection was insensitive to 25 μM (-)-indolactam V (Fig. 6, n = 5). Additionally, 25 μM (-)-indolactam V also had no effect on repetitive bursts of depolarization caused by injecting 100 μM of the poorly hydrolyzable InsP3 analog 1-Ins(1,3,4)P3 (data not shown) (Riley et al., 1994). These results indicate that the primary target of PKC is upstream from InsP3-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
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 rho-dopsin (Kirkwood and Lisman, 1994). The induction of these events by GTP-\(\gamma\)-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-\(\gamma\)-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-\(\gamma\)-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 mV\(^2\) after 20 msec flashes of light (log\(\_10\) -4.4) consistent with the adaptation of GTP-\(\gamma\)-S-induced events (Fein and Corson, 1981). We therefore conclude that desensitization by (\(-\))-indolactam V is downstream from rhodopsin.

**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
Lima 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 4α-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 photoreponse 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 noncompetitive 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 PLCβ isoforms has been proposed to decrease the activation of PLCβ 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.

**REFERENCES**


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