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RAPID COMMUNICATION

Protein kinase C and IP_3 in Photoresponses of Functionally Intact Rod Outer Segments: Constraints about Their Role

H. JINDROVÁ, P.B. DETWILER

Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195

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Summary

Protein kinase C and polyphosphoinositide metabolism are reported to affect light-activated processes in cell free systems. To investigate their role in phototransduction under more physiological conditions the effects of nonhydrolyzable inositol trisphosphate (IP_3) analogs as well as of protein kinase C and phospholipase C inhibitors on the characteristics of the electrical light response were studied. Rod outer segments were dialyzed in whole-cell voltage clamp and photoresponses in the presence and absence of the tested compounds were compared. None of the compounds influenced the light responses suggesting that neither IP_3 nor protein kinase C participate in the phototransduction cascade. A number of different proposals about the participation of protein kinase C and inositol trisphosphate (IP_3) in the phototransduction process based on a wide variety of *in vitro* experiments should therefore be reevaluated.

Key words

Vertebrate phototransduction – Photoresponse – Protein kinase C – Inositol trisphosphate – Rhodopsin

Abbreviations

ATP	adenosine 5'-triphosphate
cGMP	guanosine 3',5'-cyclic monophosphate
GTP	guanosine 5'-triphosphate
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine, protein kinase inhibitor
H-8	N-[2-(methylamino)ethyl)]-5-isoquinolinesulfonamide, protein kinase C inhibitor
HEPES	N-(2-hydroxyethyl)piperazine-N'-(5-ethanesulfonic acid)
IP_3	D-*myo*-inositol 1,4,5-trisphosphate
PDE	cGMP specific phosphodiesterase
PMA	phorbol-12-myristate-13-acetate, protein kinase C activator
R*	metarhodopsin II, an active photolyzed form of rhodopsin
ROS	rod outer segment

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Fax +420 2 293 894
E-mail physres@sun1.biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

There are numerous reports about the participation of the polyphosphoinositide and protein kinase C pathways in the phototransduction cascade but their light-dependent regulation is a source of controversy. The literature contains evidence for and against their role in the phototransduction process which led us to examine the effects of protein kinase C and phospholipase C inhibitors and inositol trisphosphate (IP_3) analogs on the electrical light response of functionally intact rod outer segments (ROS).

Protein kinase C is a ubiquitous serine/threonine kinase that is significantly less specific than protein kinase A (reviewed by Hug and Sarre 1993, Newton 1995, 1997). It has been purified from bovine ROS (Kelleher and Johnson 1985) and phosphorylation of ROS proteins by endogenous protein kinase C has been shown in *in vitro* experiments (Kapoor and Chader 1984). Protein kinase C is less abundant in ROS than rhodopsin kinase, with molar ratios of 1 protein kinase C : 2000 rhodopsins versus 1 rhodopsin kinase : 100 rhodopsins (Palczewski *et al.* 1992, Kelleher and Johnson 1985). Guanylyl cyclase and an inhibitory PDEγ subunit of cGMP specific phosphodiesterase (PDE) have been reported to be activated by protein kinase C mediated phosphorylation (Wolbring and Schnetkamp 1995, Udovichenko *et al.* 1996). Arrestin and transducin were also found to be phosphorylated by protein kinase C (Zick *et al.* 1986, Sagi-Eisenberg *et al.* 1989, Weyland and Kuhn 1990).

It has been proposed that protein kinase C is involved in photoresponse recovery and in light adaptation (Newton and Williams 1993a, Hurley 1994). Although many proteins of the phototransduction cascade have been shown to be phosphorylated by protein kinase C *in vitro*, the main target for protein kinase C is thought to be rhodopsin. Rhodopsin phosphorylation by protein kinase C has been postulated to play an important role in the shut-off light-activated rhodopsin (R*) (Newton and Williams 1993a, 1993b). Protein kinase C has been shown to phosphorylate both bleached and nonbleached rhodopsin *in vitro* (Kelleher and Johnson 1986, Newton and Williams 1991, Greene *et al.* 1995). It has been suggested that protein kinase C mediated phosphorylation of rhodopsin is important at low light levels when the number of R* is low, and that rhodopsin kinase catalyzed phosphorylation of R* dominates at high levels of illumination (Newton and Williams 1991). There is a question whether the protein kinase C phosphorylation occurs under physiological conditions *in vivo* and if so, whether this phosphorylation participates in rhodopsin shut-off and contributes to the recovery of the photoresponse.

Hydrolysis of phosphatidylinositol bisphosphate by phospholipase C generates two products important for protein kinase C regulation, diacylglycerol and IP_3. Diacylglycerol stimulates protein kinase C directly and IP_3 releases calcium from intracellular stores. The resulting rise in cytosolic calcium plays a key role in the activation of protein kinase C (Hug and Sarre 1993, Nishizuka 1995, Newton 1997). There are many studies about the involvement of polyphosphoinositide metabolism in phototransduction in cell free systems but very few were done on functionally intact rods. It has been reported that light increases phosphoinositide specific phospholipase C activity in vertebrate ROS (Ghalayini and Anderson 1984, Hayashi and Amakawa 1985, Millar and Hawthorne 1985, Millar *et al.* 1988). The activities of enzymes involved in polyphosphoinositide metabolism, diacylglycerol kinase, phosphatidylinositol kinase and phosphatidylinositol-4-phosphate kinase, are present in frog ROS (Choe *et al.* 1990). However, Ferreira and Pak (1994) found phosphoinositide specific phospholipase C in bovine cones but not in rods, while Peng *et al.* (1997) detected phospholipase C β4 immunoreactivity in bovine as well as rat, mouse and rabbit ROS. It has been shown that the photoresponses recorded from rods of mice lacking phospholipase Cβ4 did not differ from those of control mice but the affected mice expressed impaired vision presumably caused by more central deficits (Jiang *et al.* 1996). Waloga and Anderson (1985) reported that intracellular injections of IP_3 in salamander rods trigger a transient increase in the membrane potential by an unknown mechanism. It is clear from the preceding survey that much of the work that has been focused on photoreceptor polyphosphoinositide metabolism is contradictory and contains no direct evidence supporting its participation in the events that underlie the generation and recovery of the light response in the intact photoreceptor.

To test the possible roles of protein kinase C and polyphosphoinositide metabolism in phototransduction the effects of protein kinase C and phospholipase C inhibitors as well as nonhydrolyzable IP_3 analogs on the properties of the electrical light response were studied in functionally intact ROS. If protein kinase C played a major role in the R* shut-off, then a protein kinase C inhibitor should extend R* lifetime prolonging the rising phase of the photoresponse to increase its peak amplitude and duration. If polyphosphoinositide metabolism is involved in either stimulation of protein kinase C in R* shut-off or in some other recovery process, IP_3 analogs would be expected to facilitate the R* shut-off and therefore to have opposite effects on the photoresponse as those described above for the inhibition of protein kinase C.

The ROS were isolated from dark-adapted nocturnal lizards (*Gekko gekko*) and all procedures were performed in the dark under infrared illumination using night-vision goggles and image converters. The detached ROS, mechanically dissociated from the inner

segments, were dialyzed under whole-cell voltage clamp as described previously (Rispoli *et al.* 1993, Gray-Keller and Detwiler 1994, Jindrová and Detwiler 1998). Briefly, after a gigaseal formation the whole-cell recording was established using a brief pulse of suction to break the cell membrane at the pipette tip. Typical value of seal resistance was 5–35 GΩ and access resistance was 25–50 MΩ. A holding potential was −29 mV after correction for liquid junction potential (Rispoli *et al.* 1993). A stable dark current developed after breakthrough and test flash protocol was started after 10 min of the whole-cell dialysis. All experiments were performed at 16–18 °C.

The output signal of either a L/M-EPC 7 amplifier (Adams & List Associates, Westbury, NY) or an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) was low-pass filtered at 30 Hz. Indec Laboratory Data Acquisition System with Fastlab software (Indec Systems, Sunnyvale, CA) controlled the amplifier output, the light stimuli and the data acquisition.

Gecko saline solution contained 160 mM NaCl, 3.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM $CaCl_2$, 2.8 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH (Rispoli *et al.* 1993). The control internal dialysis solution contained 120 mM KAsp, 7 mM KCl, 6.05 mM $MgCl_2$, 5 mM HEPES, 5 mM ATP, 1 mM GTP, pH adjusted to 7.4 by KOH (Rispoli *et al.* 1993).

Visual observation was provided by an infrared-sensitive video camera (RCA, Lancaster, PA; Unicon model TC2055UC) using long wavelength illumination >850 nm. The ROS were uniformly illuminated using 20 ms flashes of 514 nm nonpolarized light. The unattenuated intensity of the stimulus light source was 7.57×10^5 photons/μm^2.s.

Stimulus intensities are reported as the number of photolyzed rhodopsin (R*) per flash using the measured light intensity and the effective collecting area for gecko ROS of 22.8 μm^2 (Sather 1988).

The data were analyzed using program SigmaPlot (Jandel Scientific) and are presented as means ± S.E.M. (n equals to the number of samples). Flash responses are expressed as fractional suppression of the maximum light sensitive current. In some cases, the dim flash responses are normalized as fractional suppression per R*.

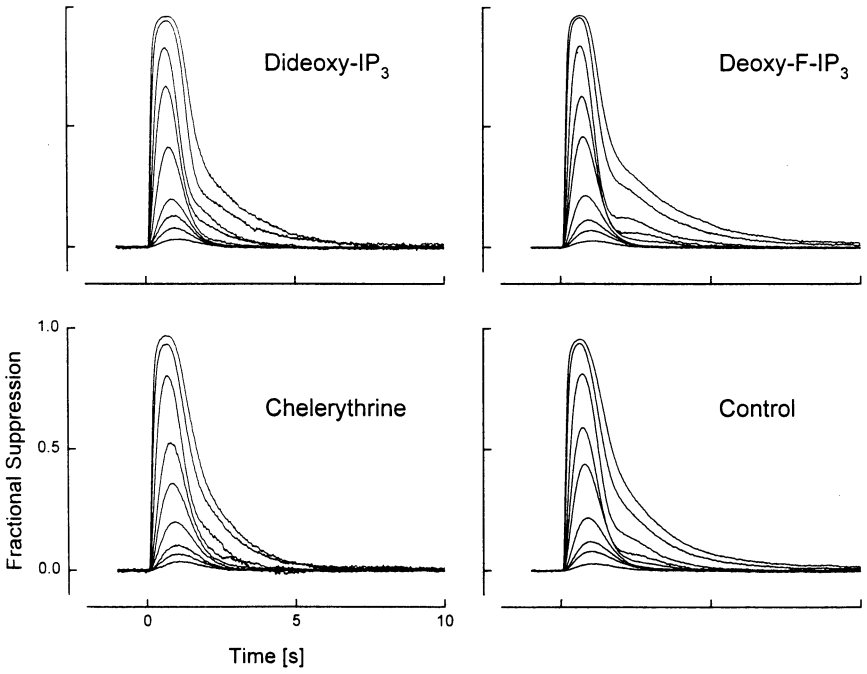

Fig. 1. *Comparison of flash response families in the presence and absence of nonhydrolyzable IP_3 analogs and protein kinase C inhibitor. The flash intensities are 6, 22, 40, 91, 261, 471, 1001, 2834 and 5093 R*/flash.ROS and the responses are expressed as fractional suppression of the resting dark current. The cells were dialyzed with standard internal solution containing in the tested cells 20 μM Dideoxy-IP_3, 20 μM Deoxy-F-IP_3 and 10 μM chelerythrine. The control traces represent the averages of 7 separate experiments. The Dideoxy-IP_3, Deoxy-F-IP_3 and chelerythrine traces are averages of 3, 4 and 2 experiments, respectively.*

Light-evoked electrical responses were recorded from functionally intact rod outer segments (ROS). Detached ROS, dialyzed in whole-cell voltage clamp with a standard intracellular solution containing 5 mM ATP and 1 mM GTP, synthesize cGMP and maintain a circulating inward dark current that is

suppressed by light and gives rise to electrical responses that have the same activation kinetics, sensitivity, recovery time and adaptational behavior as intact rod (Rispoli *et al.* 1993). Flash families were recorded from functionally intact ROS after 10 min of whole-cell dialysis. Control cells were dialyzed with the standard internal solution, while the test cells were dialyzed with the same internal solution supplemented with either 20 μM 2,3-dideoxy-D-myo-inositol-1,4,5 trisphosphate (Dideoxy-IP_3), 20 μM 3-deoxy-3-fluoro-D-myo-inositol 1,4,5-trisphosphate (Deoxy-F-IP_3), 10 μM chelerythrine or 50 μM U-73122. Light responses in the presence and absence of the compounds were compared.

Dideoxy-IP_3 and Deoxy-F-IP_3 are nonhydrolyzable IP_3 analogs that serve as potent agonists in releasing calcium from the intracellular stores. Deoxy-F-IP_3 has a 3-position blocked by fluorine and cannot be converted to IP_4 by a 3-kinase. Chelerythrine is a highly effective and selective protein kinase C inhibitor (Jarvis *et al.* 1994). U-73122 is a phospholipase C inhibitor and inhibits production of IP_3 (Tatrai *et al.* 1994).

Figure 1 shows flash families recorded from cells dialyzed with standard internal solution compared with cells dialyzed with Dideoxy-IP_3, Deoxy-F-IP_3 and chelerythrine. Flashes were applied from the dimmest to the brightest intensity. There was no difference in the amplitude or kinetics of the flash responses recorded in the absence and presence of either IP_3 analogs or the protein kinase C inhibitor. This can be seen in Figure 2 which compares linear range dim responses recorded from control cells with cells dialyzed with Dideoxy-IP_3, Deoxy-F-IP_3 and chelerythrine. The photoresponses were normalized as fractional suppression of dark current per R*.

The tested compounds had no significant effect (at the 0.05 level as determined by t-Test) on the amplitude of the resting dark current which was 110.8±4.2 pA (n=11) for control cells, 98.6±7.3 pA (n=6) for Dideoxy-IP_3 dialyzed cells, 120.4±7.9 pA (n=4) for Deoxy-F-IP_3 dialyzed cells and 116.8±1.6 pA (n=2) for chelerythrine dialyzed cells.

Experiments with phospholipase C inhibitor U-73122 turned out to be difficult and the cells dialyzed with the inhibitor gave variable results for unknown reasons, therefore we did not continue with the experiments with U-73122 and its inactive analog U-73343.

The results of experiments with a protein kinase C inhibitor and the IP_3 analogs indicate that the polyphosphoinositide signaling pathway and protein kinase C do not participate in phototransduction in functionally intact ROS. The results with the protein kinase C inhibitor agree with preliminary results from this laboratory (Rispoli and Detwiler, unpublished observation) which showed that neither phorbol ester (PMA), a protein kinase C activator, nor H-7, H-8 and staurosporine, protein kinase C inhibitors, had any effect on the ROS light response. Our results are also consistent with those of Binder *et al.* (1989), who found that R* was not phosphorylated by activated protein kinase C. Ohguro *et al.* (1996) came to the same conclusion based on the finding that neither protein kinase C activation by phorbol ester nor inhibition by H-7 influenced rhodopsin phosphorylation in bovine ROS. Our results are also in agreement with the findings of Nakatani *et al.* (1997) who reported that several phorbol esters had no effect on flash responses recorded from salamander rods using suction electrode recordings.

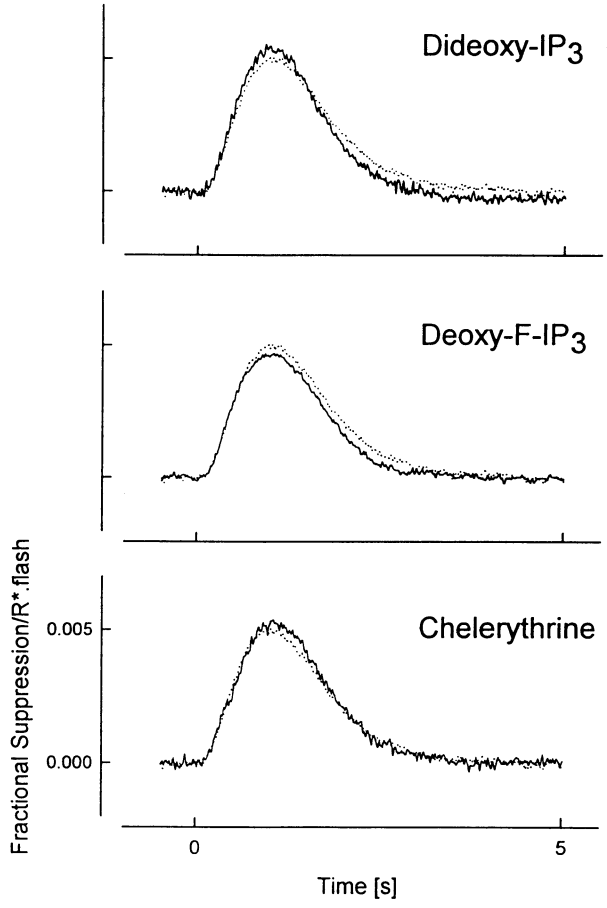

Fig. 2. *Comparison of dim flash responses in the presence and absence of nonhydrolyzable IP_3 analogs and a protein kinase C inhibitor. The two dimmest flash responses (6 and 22 R*/flash.ROS) in the intensity series from Figure 1 were normalized as fractional suppression of the dark current per R* and averaged together. The traces compare control flash responses (dotted lline) with flash responses of cells dialyzed with either 20 μM Dideoxy-IP_3, 20 μM Deoxy-F-IP_3 or 10 μM chelerythrine (full line).*

The results reported here do not support the hypothesis that protein kinase C is involved in R* shut-

off and light adaptation (Newton and Williams 1993a). This proposal is based mostly on work by Newton and Williams (1991, 1993b) and Greene *et al.* (1995). Newton and Williams (1991) claimed that the activation of protein kinase C by phorbol esters in the intact retina alters the phosphorylation state of rhodopsin in a light-dependent manner. Newton and Williams (1991, 1993b) and Greene *et al.* (1995) found that protein kinase C has the same affinity for bleached and nonbleached rhodopsin *in vitro* and assert that the greatest effects of protein kinase C are at low light levels where most of the rhodopsin is in its nonbleached form. This conclusion is clearly not consistent with the results in Figure 2. The protein kinase C proposal also has difficulty accounting for the fact that the fall in calcium during a photoresponse would be expected to inhibit protein kinase C rather than to accelerate it. The results of our experiments suggest that protein kinase C does not participate in phototransduction in the gecko ROS.

Our results also contradict the proposed role of the inositol phosphate branch of polyphosphoinositide metabolism in phototransduction (Waloga and Anderson 1985, Ghalayini and Anderson 1984, Hayashi and Amakawa 1985, Millar and Hawthorne 1985, Millar *et al.* 1988). Our conclusion that the polyphosphoinositide pathway does not participate in phototransduction is supported by Van Rooijen and Bazan (1986) who were unable to find any light dependent changes in polyphosphoinositide metabolism in bovine ROS. There is also no evidence for the presence of the IP_3 receptors in the rod outer segment (Peng *et al.* 1991, Day *et al.* 1993).

The results of *in vitro* assays published by several groups using purified proteins or ROS membranes, which support a role of protein kinase C and polyphosphoinositides in the phototransduction process are difficult to completely discount. They may be an artifact of the cell free systems used for the studies or they may reflect reactions that are important in light-dependent processes other than those directly involved in the electrical light response. For example, protein kinase C and polyphosphoinositides might participate in the control of cytoskeleton rearrangements that are involved in the diurnal regulation of disk formation and shedding.

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Reprint requests

H. Jindrová, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic.