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INDUCTION OF PHOTORESPONSE BY THE HYDROLYSIS OF POLYPHOSPHOINOSITIDES IN THE *HERMISSENDA* TYPE B PHOTORECEPTOR

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Direct evidence that the photoresponse of the *Hermissenda* type B photoreceptor cell is triggered directly by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) was obtained. Neomycin and spermine, which inhibit PIP_2 breakdown, suppressed light response, while injection of inositol 1,4,5-trisphosphate (IP_3), guanosine 5'-(3-O-thio)triphosphate (GTPγS), guanosine 5'-(2-O-thio)diphosphate (GDPβS), cAMP, cGMP did not alter the light-induced Na^+ influx underlying the photoresponse. Suppression of the photoresponse was also observed with decrease of total amount of membraneous PIP_2 induced by injection of the phosphoinositides (PI) turnover inhibitors, isobutylmethylxanthine (IBMX), LiCl and R 59022.

Despite a considerable number of studies, understanding of phototransduction in the invertebrate photoreceptor has remained elusive. The major reason for this is the difficulty of identifying the second messengers involved (1-3). We propose a hypothesis in which the invertebrate photoresponse is triggered by a perturbation of the membrane field caused by the hydrolysis of PIP_2. This can account for a variety

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Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; GTPγS, guanosine 5'-(3-O-thio)triphosphate; GDPβS, guanosine 5'-(2-O-thio)diphosphate; PI, phosphoinositides; IBMX, isobutylmethylxanthine; DG, diacylglycerol; PLC, phospholipase C; CNS, circumesophageal nervous system.

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of properties of the photoresponse and stems from two previous apparently contradictory results; (i) PIP_2-specific phospholipase C (PLC) activity is necessary (4,5), (ii) but IP_3, the hydrolyzed product of PIP_2, is not necessary for the generation of photoreceptor potential (6, 7). Although the cellular PIP_2 content is very low (less than 1% of total phospholipids), IP_3 release from the inner surface of the plasma membrane might be expected to have quite a large effect on the membrane field around the Na^+-selective channel underlying the photoresponse, since PIP_2 has 5 negative charges in the physiological pH range (8).

We hypothesize that the photoresponse is triggered by activation of Na^+-selective channels, induced by local IP_3 release. If this were the case, inhibition of PIP_2 breakdown by screening of the negative charges of PIP_2 by positively charged compounds or reduction of PIP_2 production rate by various PI turnover inhibitors should suppress the photoresponse. In order to test this idea we injected various kinds of negative-charge chelators of PIP_2 and inhibitors of PI turnover into the *Hermissenda* type B photoreceptor cell, and observed the effects on the photoresponse.

Materials and Methods

Materials PIP_2, and spermine were purchased from Sigma. Neomycin was from Wako Pure Chemical Industries Ltd. (Japan). R 59022 was from Research Biochemicals Inc. IP_3 was from Amersham. GTPγS and GDPβS were from Boehringer Mannheim. [^{3}H]PIP_2 was from NEN. Other chemicals were all reagent grade.

Electrophysiology Photoreceptor somata were isolated from all synaptic interactions by axotomy as previously described (9). After enzymatic digestion of the enveloping connective tissue sheath, a microelectrode was inserted, also as in preceding studies (9). IBMX and R 59022 were dissolved in dimethyl sulfoxide (DMSO), and diluted with the intracellular solution (67 mM Na-acetate, 400 mM K-gluconate, 10 mM $MgCl_2$, 10 mM HEPES, 1.5 mM $CaCl_2$, 1.5 mM EGTA, pH 7.4) to 1 mM. Other drugs were dissolved in the intracellular solution. Drugs were put into the electrodes and iontophoretically injected with DC currents of 2.5 nA for 3 min. The intracellular solution alone or DMSO did not modulate the light response at all.

Assay of PLC activity Ten circumesophageal nervous systems were homogenized with solution A, containing 0.25 M sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM EGTA and 25 mM Tris-HCl, pH 7.5 and centrifuged at 100,000 x *g* for 60 min. The precipitate was suspended with solution A. PIP_2-PLC activity was measured as described previously (10) with a slight modification. The reaction mixture contained 100 mM KCl, 1 μM $CaCl_2$, 20 μM [^{3}H]PIP_2, 50 mM Tris-HCl, pH 7.5 and the enzyme in a final volume of 50 μl. The incubation was carried out for 30 min at room temperature. The reaction was stopped and then the water-soluble product was extracted as described previously (10).

Results

Inhibition of photoresponse by negative-charge chelators of PIP_2

In the *Hermissenda* eye, there are two type A photoreceptors and three type B photoreceptors (Fig. 1). The type B cell is more light-sensitive than type A (11). The normal light response of the type B photoreceptor is shown in Fig. 1. A previous voltage clamp study of the type B cell (12) showed that the initial phase is due to a light-induced inward Na^+ current, while the delayed phase is due to a light induced reduction of an outward K^+ current. Thus light elicits an initial increase of a Na^+ conductance and a delayed, prolonged decrease of a Ca^{2+}-dependent K^+ conductance (12).

Initially we examined the effect of cationic aminoglycoside antibiotics on the photoresponse. When neomycin, which has 6 positive charges at pH 7 (13), was injected into the type B cell, the response to light was almost completely suppressed, although the resting potential was unchanged (Fig. 2). The amplitude of the initial response recovered gradually, but not completely, while the delayed response did not recover within 10 min after injection. This might be due to the direct interaction of the injected positively charged neomycin with the K^+ channel (13). The rapid initial

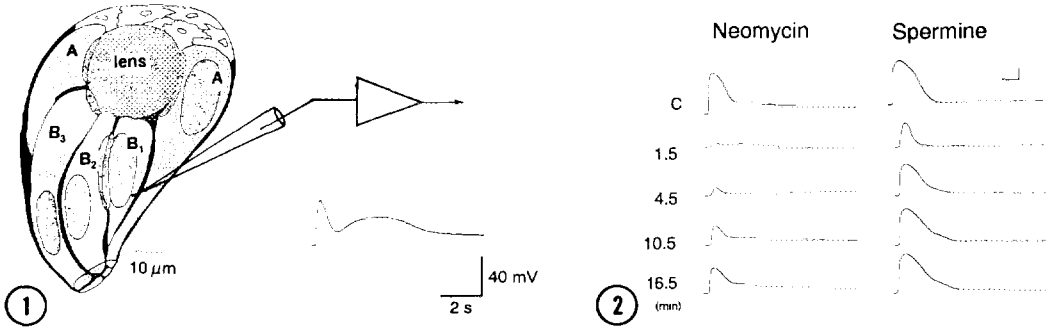


Fig. 1. Schematic representation of the eye of *Hermissenda crassicornis* illustrating the arrangement of the two type A and the three type B photoreceptors (28). The position of the microelectrode and the control response to 100 ms duration of 580 $\mu W/cm^2$ at 510 nm of flash light are also shown. Heavily stippled regions of the photoreceptors adjacent to the lens and between B1 and B2 represent rhabdomeres.

Fig. 2. Photoresponses from the type B photoreceptor of *Hermissenda* were suppressed by the injection of neomycin (1 mM) and spermine (1 mM). The top panel shows the control response. The number on the left represents time in minutes after injection. The calibration bar corresponds to 1 s and 10 mV.

recovery may be explained by the fast turnover rate of PIP_2 (within 4 min) (14). More concentrated neomycin injection gave a slower recovery. Gentamicin, a stronger chelator for negative charge, also produced a more prolonged inhibitory effect on the photoresponse (data not shown).

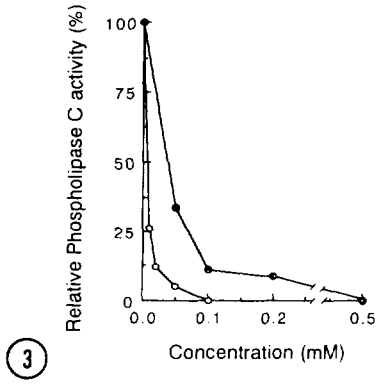
In order to confirm the hypothesis that negative charge screening of PIP_2 suppresses the photoresponse, we examined the effect of polyamines on the photoresponse. These are chemically very different from the aminoglycoside antibiotics. Injection of spermine, which has 4 positive charges at neutral pH (13), produced a partial suppression of the photoresponse without changing the resting potential (Fig. 2). The amplitude of the initial response was suppressed to 50%, but recovered to 90% of the control response within 10 min after the injection (Fig. 2). Injection of spermidine, a weaker chelator for negative charge, gave a smaller (less than 40%) inhibition of the photoresponse (data not shown).

Inhibition of PLC activity by negative charge chelators for PIP_2

To assure that the inhibitory effect of neomycin and spermine on the photoresponse is due to the inhibition of PIP_2 breakdown by PLC, and not to direct inactivation of the IP_3 as a second messenger, we examined the biochemical effects of these drugs on *Hermissenda* PLC. These drugs are already known to inhibit PLC-mediated PIP_2 breakdown by binding to PIP_2 (15-17). Since the *Hermissenda* eye is too small for assay of enzyme activity, we used the *Hermissenda* circumesophageal nervous system (CNS), which contains ganglia, eyes and statocysts. Since about 60% of the total PLC activity in the CNS was recovered in the particulate fraction, the precipitate obtained after centrifugation at 100,000 x *g* for 60 min was used for the assay of membrane-associated PLC activity. Neomycin inhibited PLC activity completely at concentrations higher than 0.1 mM (Fig 3). Spermine was a weaker inhibitor than neomycin, and complete inhibition was obtained at a concentration of 0.5 mM.

Changes of photoresponse by PI turnover inhibitor

In the next step, the hypothesis was examined by a different approach, i. e. through reduction of PIP_2 production rate with inhibitors of PI turnover. When we



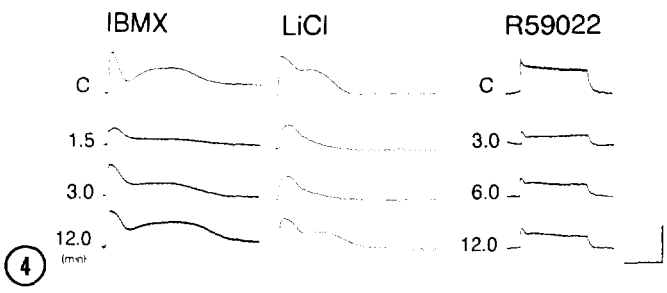


Fig. 3. The effects of neomycin and spermine on membrane-associated PIP_2-specific PLC activity in the *Hermissenda* CNS. Here, 100% corresponds to 0.39 nmol/min/mg protein. The results shown are representatives of three independent experiments. ○, neomycin; ●, spermine

Fig. 4. Dynamics of the light response were modulated by the iontophoretic injection of PI turnover inhibitors, IBMX, LiCl and R 59022 of 1 mM in an electrode. LiCl and R 59022 were injected under a repetitive light stimulus, every 1 s in 0.5 s duration for 1 min, in order to promote PI turnover. Top panel shows the light response before the injection of drugs, taken as a control. The number on the left of each trace represents the time in minutes after injection. The calibration bar corresponds to 2 s and 40 mV for IBMX and LiCl, and 30 s and 40 mV for R59022.

injected 1 mM of IBMX into the photoreceptor, the photoresponse was suppressed (Fig. 4), as we have observed previously in octopus photoreceptors (18). Both initial and delayed responses recovered quickly. In this experiment, IBMX acted as a suppressor of PI turnover, as previously shown (18), not as an inhibitor for cyclic nucleotide phosphodiesterase. This is supported by the fact that injected cAMP or cGMP did not affect the *Hermissenda* photoresponse (Fig. 5). In the presence of IBMX, ^{32}P incorporation into PIP_2 and phosphatidylinositol 4-monophosphate in *Hermissenda* CNS was found to be reduced to 60% of control value (data not shown).

We also examined the effect of LiCl (an inhibitor of inositol phosphate phosphatase) and R 59022 (a DG kinase inhibitor) on the photoresponse. Injection of R 59022 (1 mM) into the photoreceptor showed a largely prolonged and reduced photoresponse with a poor recovery, while LiCl (1 mM) injection showed an effect intermediate between that of IBMX and R 59022 (Fig. 4).

Effects of some chemicals to photoresponse

Since a number of experiments have indicated that a light-activated G-protein modulates PLC activity (19), we injected GTPγS intracellularly, but without effect (Fig

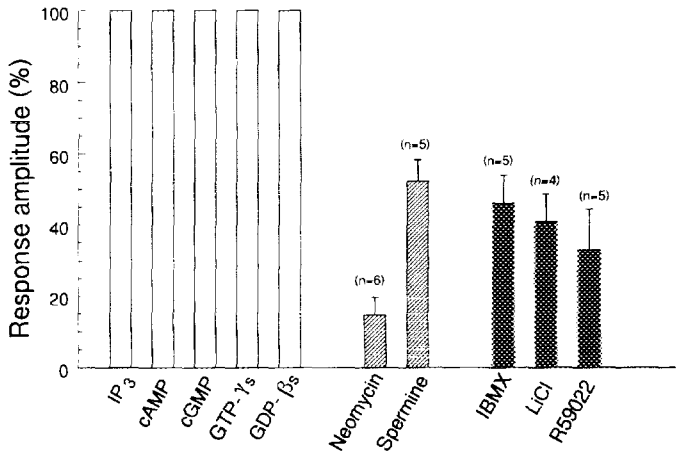


Fig. 5. The effect of various chemical compounds injected into the type B photoreceptor cell on the initial light-response amplitude. Data represent means of the most suppressed light response amplitude with bars showing standard errors.

5). Injection of GDPβS also showed no effect (Fig. 5). Furthermore, the effect of IP_3 injection was examined in order to exclude the possibility that IP_3 might be a second messenger in invertebrate phototransduction. Injection of IP_3 showed no effect (Fig. 5), as was expected.

Discussion

Suppression of photoresponses induced by drugs used in this experiment was listed in Fig. 5. These results directly support the idea that the photoresponse is triggered by the breakdown of PIP_2 by PLC and suggest that a direct interaction between PIP_2 and a Na^+-selective channel is an essential step in the generation of the *Hermissenda* photoresponse. The Na^+-selective channel could be greatly activated when the most closely associated polar group (e.g. IP_3) was removed from membrane, as is shown in the fixed-charge model of potential profile near Na^+-selective channels (20). On the basis of these discussions, a following working hypothesis for invertebrate phototransduction is proposed (Fig. 6). Rhodopsin (R) is activated by light. Active rhodopsin (R*) may directly activate PLC. PIP_2, which has 5 negative charges (8), is hydrolyzed to IP_3, which has 5 negative charges, and DG, which has no negative charge. The membrane field perturbation induced by negative charge release will activate a Na^+-selective channel (g1). IP_3-induced

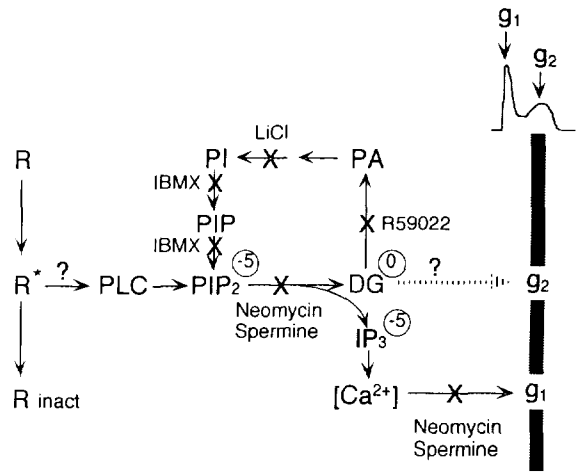


Fig. 6. A novel model of *Hermissenda* phototransduction.

Ca^{2+} release from intracellular stores increases intracellular Ca^{2+}, which may turn on Ca^{2+}-activated K^+ channels, thus causing the delayed response (g2). Here we assumed that PIP_2 (or IP_3) has 5 negative charges at neutral pH, although Toner et al. (21) proposed that PIP_2 has 3 negative charges in the presence of K^+ and Mg^{2+} at neutral pH using NMR and surface potential measurement. The fact that no change in the resting potential follows the injection of positively charged materials supports the idea that the fixed charge model holds in this case.

IP_3 was initially thought to be a second messenger in invertebrate phototransduction. Evidence for this comes from injection of IP_3 into *Limulus* ventral eye photoreceptor cell (22, 23), from using the *Drosophila* visual transduction mutant (4), and from chemical analysis of the IP_3 content on a rapid time scale in squid retina (24). IP_3 was found to cause an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in *Limulus* ventral eye (25, 26). However, this idea was problematic, because EGTA blocks excitation by IP_3 (6), but EGTA does not block the light response (26). Many attempts to resolve this contradiction have failed (1-3). Our working hypothesis, shown in Fig. 6, is that the photoresponse is triggered by an activation of the Na^+-selective channel induced by breakdown of PIP_2 on the membrane. This hypothesis is consistent with all of these data. Recently Faddis and Brown (27) injected heparin and polyamine into *Limulus* ventral photoreceptors and

concluded that excitation can proceed in the absence of IP_3-induced $[Ca^{2+}]_i$ increase. These findings also fully support our hypothesis. Further study is needed to get direct evidence how IP_3 released from the membrane changes the membrane field around Na^+-selective channels.

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