Light stimulates the rapid formation of inositol trisphosphate in squid retinas

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To test the hypothesis that inositol trisphosphate (InsP_3) mediates adaptation and excitation in invertebrate photoreceptors, we measured its formation on a rapid time scale in squid retinas. For squid, excitation and adaption occurs within 0.1 and 1–2 s respectively. We could detect an elevation in InsP_3 within 200 ms of a bright flash. This increase is about 240% over dark basal levels and is maintained for at least 2 min after a flash. The increase probably occurs in the photoreceptors, which are the only neurons in squid retinas. Analysis by h.p.l.c. indicates that the light-regulated isomer is $\text{Ins}(1,4,5)\text{P}_3$, which is formed by the hydrolysis of phosphatidylinositol bisphosphate (PtdInsP_2).

INTRODUCTION

myo-Inositol trisphosphate (InsP_3) has been proposed to be an intracellular messenger for invertebrate photoreceptors (Brown *et al.*, 1984; Fein *et al.*, 1984). Injection of InsP_3 into dark-adapted ventral photoreceptors of *Limulus* (horseshoe crab) leads to rapid depolarization of the cell membrane (excitation) and to a decrease in the receptor's sensitivity to light (adaptation). The effects of InsP_3 can be clearly attributed to the release of Ca^{2+} from intracellular stores (Rubin & Brown, 1985; Payne *et al.*, 1986a,b). The physiological importance of Ca^{2+} in the adaptation of these cells was previously established by Lisman & Brown (1972, 1975). Recent work indicates that Ca^{2+} may also serve in a separate capacity as a messenger for excitation (Payne *et al.*, 1984, 1986a). Thus an intracellular messenger is clearly needed for initiating Ca^{2+} release within *Limulus* receptors, and this function may be served by InsP_3 .

If InsP_3 performs such a role, its intracellular concentration should increase during the time course of excitation and adaptation. We set out to measure light-stimulated formation of InsP_3 in invertebrate receptors, using the eyes of squid, which contain about five orders of magnitude more receptors than the ventral eyes of *Limulus*. The squid eye superficially resembles its vertebrate counterpart in its anatomy. The major difference is that the squid retina contains only one kind of neuron, the photoreceptors (Zonana, 1961; Cohen, 1973). The distal segments of the receptors are the first structures to encounter light that is incident on the retina and they contain the microvilli, where both excitation and adaptation are localized (Hagins *et al.*, 1962). Due to their size, the distal segments occupy more than half of the intracellular volume of the retina. The choice of squid was also motivated by the preliminary observations of Vandenberg & Montal (1984), who found some evidence for light-stimulated turnover of PtdInsP_2 .

EXPERIMENTAL

Materials

myo-[2- ^3H] Ins (14 Ci/mmol) and *myo*-[2- ^3H (n)] Ins -(1,4,5) P_3 (1–5 Ci/mmol) were purchased from American Radiolabeled Chemicals and New England Nuclear respectively.

Tissue incubations

Squids (*Loligo pealei*) were supplied by the Department of Marine Resources at the Marine Biological Laboratory. One dark-adapted animal was used per experiment. The eyes were excised under dim red light and the two eyecups were cut to yield four half-eyecups of nearly equal size. The eyecup consists of the retina and its tightly attached sclera. The four half-eyecups were placed into 1 ml of artificial seawater containing 100 μCi of [^3H] Ins and incubated for 3 h with gentle agitation. The composition of the seawater was 393 mM-NaCl, 12 mM-KCl, 20 mM-MgCl₂, 26 mM-MgSO₄, 10 mM-CaCl₂, 9 mM-glucose and 10 mM-Hepes, pH 7.4 (Shoukimas *et al.*, 1977). Because squid photoreceptors rapidly deteriorate under low O_2 partial pressures and at room temperature, the incubations were routinely performed under O_2 at 10 °C. The temperature of the solution was maintained with a water-cooled thermoelectric assembly and was continuously monitored with a thermocouple. Under these conditions, retinas remained physiologically active, as judged by their ERGs.

To facilitate turnover of inositol phosphates in the photoreceptors, the eyecups were incubated under dim red light for 1 h and then kept in darkness for 2 h. Subsequent steps up to and including extraction with trichloroacetic acid were performed under i.r. illumination with the aid of image converters. After incubation, retinas were mounted on a 'freezing apparatus' (Bridgman & Reese, 1984) with the receptors facing a strobe

Abbreviations used: InsP_1 , InsP_2 , InsP_3 , InsP_4 , InsP_5 and InsP_6 , inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates respectively; GroPIns , glycerophosphoinositol; PtdInsP_2 , phosphatidylinositol bisphosphate; ERG, electroretinogram.

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unit. Two of the half-eyecups were flash-illuminated with the strobe before their reactions were quenched by plunging them into liquid Freon 22 kept below -100°C . Stimulus was a flash of white light (1 ms in duration), which passed through a heat-absorbing filter and which photoactivated over 10% of the rhodopsin. The two test eyecups were usually frozen at different predetermined times after the flash. The other two half-eyecups were unstimulated controls and were processed the same way as the test eyecups, except for omission of the flash. The frozen tissues were transferred to 2 ml of ice-cold 15% (w/v) trichloroacetic acid for extraction for 1 h.

The freezing rates of the tissues were measured with fine thermocouples (copper/constantan, 0.076 mm diam.), whose response time was about 15 ms. The time interval for freezing the distal segments was calculated to be 66 ± 32 ms (mean $\pm$ s.d.). When this is added to the minimum delay time between stimulus and tissue immersion (125 ms), the minimum quenching time in our experiments was about 200 ms after a flash.

ERG recording

To ensure that retinas remained physiologically active under our experimental protocol, the ERG was recorded in several experiments. The light flash described above elicited an ERG response whose peak amplitude was about 10 mv. The photoresponse peaked at about 100 ms after the flash and subsequently decayed back to zero over tens of minutes. The ERG correlates with the receptor's membrane potential during the rising phase of the response (Pynsent & Duncan, 1977; Duncan & Pynsent, 1979). Light-adaptation has a slower time course. In *Limulus* it follows the decline of the response from peak to plateau (Lisman & Brown, 1975) and appears to occur within a few seconds in squid (Pinto & Brown, 1977). Although the intensity of our stimulus was several orders of magnitude greater than necessary for saturating the receptor response, the flash did not damage the cells. If the receptors were allowed to dark-adapt for 1 h between flashes, several responses could be elicited from the same tissue. We chose the stimulus intensity to optimize the detection of InsP_3 formation.

Measurement of inositol phosphates

The trichloroacetic acid extracts were prepared and their inositol phosphates separated on anion-exchange columns as described by Downes & Michell (1981). For analysis, the amount of $[\text{^3H}]\text{InsP}_3$ for each half-eyecup was expressed as a percentage of total radioactivity for all inositol phosphates, which include GroPIns , InsP_1 , InsP_2 and InsP_3 . The use of this percentage value was necessary to correct for any variability among the eyecups regarding $[\text{^3H}]\text{Ins}$ uptake, Ins metabolism and extraction efficiency. On average, about 2000 c.p.m. was found in the InsP_3 pool per half-eyecup, corresponding to a percentage value of 5–30% respectively.

To determine light's effect on InsP_3 levels, light/dark ratios were calculated. The average percentage value for InsP_3 was first derived for the two control half-eyecups (herein given as $\bar{D}$) and then the percentage value of InsP_3 for the illuminated retinas (L) was divided by $\bar{D}$. Because in most experiments the two stimulated retinas were quenched at different time intervals, they were not averaged. The derived ratio of $L/\bar{D}$ can be converted to a fractional increase by the relationship $(L/\bar{D}) - 1$. For

Table 1. Effect of light on InsP_3 content

Interval between flash and time of immersion*	Variability between control eyecups ($D/\bar{D}$)†	Light effect on test eyecup ($L/\bar{D}$)‡
–1.0 s‡	1.03 ± 0.27 (6)	1.20 ± 0.48 (6)
0.125 s	0.98 ± 0.02 (4)	3.60 ± 1.23 (4)
0.3 s	0.88 ± 0.07 (3)	1.92 ± 0.69 (3)
3 s	0.94 ± 0.15 (27)	3.41 ± 1.61 (28)
10 s	1.01 ± 0.23 (4)	5.34 ± 1.58 (4)
2 min	0.92 ± 0.10 (5)	2.26 ± 1.09 (5)

* Measurements of tissue freezing indicate that the enzymic activity within the microvilli is quenched within 66 ± 32 ms of immersion.

† These ratios were calculated for each experiment as described in the Experimental section. D is the percentage value for an unstimulated half-eyecup, $\bar{D}$ is the average percentage value for unstimulated half-eyecups, and L is the percentage value for a stimulated half-eyecup. The distribution of these ratios is given here mean $\pm$ s.d. (n), where n is the number of experiments.

‡ To test the effectiveness of freezing in quenching the light-stimulated reactions, freezing preceded illumination for these eyecups.

calculating $L/\bar{D}$ for InsP_2 , InsP_3 was excluded in the denominator for the derivation of the percentage value of InsP_2 , because increases in InsP_3 would have artificially decreased the percentage value of InsP_2 .

H.p.l.c. and high-voltage electrophoresis on paper were performed as described by others (Agranoff *et al.*, 1983; Heslop *et al.*, 1985; Rittenhouse & Sasson, 1985).

RESULTS AND DISCUSSION

Table 1 indicates that the InsP_3 content of control retinas were nearly identical within an experiment and varied only about 15%. By contrast, light caused elevation of InsP_3 levels, which at 3 s were on the average 3.4 times greater than in the control. This is equivalent to a 240% increase. If InsP_3 is involved in phototransduction, light should regulate its levels over the time course of excitation and adaptation. To measure the kinetics of its formation, we varied the time interval between flash and quenching for one test retina in each experiment. Light was found to increase InsP_3 levels significantly when the tissue was immersed into liquid Freon as soon as 125 ms after a flash. If the delay of 66 ± 32 ms for freezing the receptors is taken into account, our earliest measurement occurs at about 200 ms after the flash. Due to the magnitude of the light effect, appreciable amounts of InsP_3 must have formed during the time interval when excitation occurred. However, our time resolution is insufficient to determine whether InsP_3 forms before the light response. In our experiments we did not find a significant decrease in InsP_3 for up to 2 min after the flash. During this time interval the amplitude of the ERG gradually decreased to 40% of the peak response. Variability between animals and half-eyecups prevents precise measurement of the time course of InsP_3 with these experiments.

Because the protocol we employed for ion-exchange chromatography does not resolve the various isomers of

Fig. 1. Separation of inositol phosphates by h.p.l.c.

Elution profile of water extract from an unstimulated half-eyecup (●) and from its light-stimulated pair (○) that was quenched 3 s after the flash; ○, overlapping data points. The left ordinate refers to the radioactivity in eluted fractions whose values were corrected for variations in counting efficiency. The right ordinate refers to solvent strength shown by the thick broken line. Sample volumes were 0.75 ml (23% of total extract) and were 'spiked' with ATP. The flow rate through the column was 2 ml/min. Eluted peaks were identified by comparison with standards. Arrows indicate times for elution of ATP and InsP_4 – InsP_6 standards.

InsP_3 and InsP_4 , we performed additional analysis by h.p.l.c. and high-voltage electrophoresis. Fig. 1 illustrates that the only isomer of InsP_3 that could be detected by h.p.l.c. in our samples was $\text{Ins}(1,4,5)\text{P}_3$. Presumably, this is the only isomer involved in the light reaction. In addition, radioactively labelled InsP_4 , InsP_5 and InsP_6 were undetectable in the extracts of either dark-adapted or light-stimulated retinas. This observation was confirmed by both h.p.l.c. and high-voltage electrophoresis. As shown in Fig. 1, the radioactivity associated with InsP_3 was more than three orders of magnitude greater than for any of the pools of InsP_4 – InsP_6 . These polyphosphorylated inositols may be better labelled with longer incubations, such as those employed by Heslop *et al.* (1985). However, under our conditions very little, if any, radioactive InsP_4 was formed for up to 2 min after stimulation. This allowed us to employ the standard protocol for ion-exchange chromatography without the need to resolve the co-eluted species. A second consequence of the absence of radioactive InsP_4 was that it simplified the interpretation of the light-induced reaction. The absence of preformed radioactive InsP_4 in dark retinas eliminates InsP_4 as the source of InsP_3 formed by light.

In the absence of InsP_4 , two enzymic pathways remain for the formation of InsP_3 : hydrolysis of PtdInsP_2 and phosphorylation of InsP_2 . For the latter process to occur, light should lead to a decrease in the levels of InsP_2 . However, such an effect was not observed. For example, the $L/\bar{D}$ ratio for InsP_2 was found to be 1.03 ± 0.22 ($n = 21$) at 3 s after the flash. This result suggests that formation of InsP_3 proceeds by the hydrolysis of PtdInsP_2 as in many other systems. We also did not detect any correlation with light for any of the other inositol phosphates.

Several reports have already appeared on light-stimulated formation of InsP_3 or hydrolysis of PtdInsP_2 in photoreceptors (Brown *et al.*, 1984; Ghalayini & Anderson, 1984; Hayashi & Amakawa, 1985). However, all these measurements were made at long time intervals after stimulation and so could not test the role of InsP_3 in phototransduction. Our results are the first to indicate that the levels of InsP_3 increase sufficiently fast for the molecule to participate in the adaptation of the photoreceptor and possibly also in its excitation.

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