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Light-induced changes in the content of inositol phosphates in squid (*Loligo pealei*) retina

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Illumination induced an increase in inositol polyphosphates, inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate, in the photoreceptors of the squid, *Loligo pealei*. There was a concomitant decrease in phosphatidylinositol 4,5-bisphosphate, but no light-induced change in any other phosphoinositide. None of these stimulus-induced changes were altered by treatment *in vivo* with pertussis toxin, which ADP-ribosylated a M_r-39000-peptide. These findings support the hypothesis that inositol 1,4,5-trisphosphate participates as either a messenger or a modulator in transduction in invertebrate photoreceptors.

INTRODUCTION

Inositol polyphosphates have been proposed to participate in phototransduction in invertebrate photoreceptors. Brown *et al.* (1984) reported that illumination caused a significant rise in the content of inositol trisphosphate (InsP_3) and a concomitant decrease in the content of phosphatidylinositol bisphosphate (PtdInsP_2) in *Limulus* (horseshoe crab) ventral eyes. Moreover, intracellular injection of Ins(1,4,5)P_3 induced electrophysiological responses that mimicked several aspects of excitation and adaptation in *Limulus* ventral photoreceptors (Brown *et al.*, 1984; Fein *et al.*, 1984; Payne *et al.*, 1986). For example, intracellular pressure injection of Ins(1,4,5)P_3 into intact *Limulus* photoreceptors induced an increase in intracellular Ca^{2+} resembling that rise induced by light (Brown & Rubin, 1984; Payne *et al.*, 1986).

Previous studies have reported light-induced changes in phosphoinositides in several broken-cell preparations of invertebrate photoreceptors. Yoshioka *et al.* (1981, 1983) found that illumination induced a decrease in ^{32}P labelling of polyphosphoinositides in photoreceptor membranes prepared from the squid (*Watasenia scintillans*). Also, in photoreceptor membranes isolated from squid (*Loligo opalescens*), light has been shown to influence the radiolabelling of phosphoinositides by [γ-^{32}P]ATP added after illumination (Vandenberg & Montal, 1984). More recently, Wood *et al.* (1985) and Szuts *et al.* (1986) reported that light induces an increase in the content of InsP_3 in intact isolated retinas of the squid (*Loligo pealei*).

GTP-binding regulatory proteins (G-proteins) appear to couple some cell-surface receptors to membrane-bound phospholipase C (for reviews, see Litosch & Fain, 1986; Cockcroft, 1987). G-proteins have been suggested to participate in phototransduction in invertebrates (Calhoon *et al.*, 1980; Bolsover & Brown, 1982; Saibil & Michel-Villaz, 1984; Bentrop & Paulsen, 1986; Fein, 1986; Tsuda *et al.*, 1986). Some G-proteins can be identified by the use of bacterial toxins; for example, pertussis toxin catalyses the transfer of an ADP-ribose moiety from NAD^+ to the α-subunit of some G-proteins (Katada & Ui, 1982; Malbon *et al.*, 1984; Tsuda *et al.*, 1986). In the present paper, we confirm that light induces changes in the content of InsP_3 in squid retinas; we also find other inositol phosphates whose concentrations are influenced by light, and we examine the effect of treatment *in vivo* with pertussis toxin on the production of the inositol polyphosphates.

MATERIALS AND METHODS

Squids (*Loligo pealei*; mantle length 12–18 cm) were held in aquaria containing 2.2 litres ($\frac{1}{2}$ gallon) of fresh sea water at 18 °C continuously bubbled with 100% O_2. Each aquarium held a single squid and was covered by an opaque black shroud. Two procedures were used to incubate the retina with [^{3}H]inositol. For the procedure *in vivo*, 0.05 ml of artificial sea water (Pinto & Brown, 1977) was injected into each squid eye; the injection solution contained [^{3}H]inositol (0.125 mCi per eye). Most of the squid retina consists of photoreceptor cells (Cajal, 1917; Cohen, 1973). Therefore our incubation procedure *in vivo* ought to optimize the labelling of the photoreceptor cells with [^{3}H]inositol. For half the animals, the injection solution also contained pertussis holotoxin (List Biological Laboratory, Campbell, CA, U.S.A.; 1 μg per eye). After the intraocular injections, the animals were kept in the dark for 2 or 4 h. At the end of the incubation *in vivo*, an animal was decapitated in dim red light and the eyes were rapidly dissected into oxygenated artificial sea water. All subsequent manipulations were done under i.r. illumination (> 850 nm) by using an i.r.-sensitive television system (RCA Ultricon). The front half of each eyeball was cut away, the lens was removed and the remaining eye cup was bisected. Half of each eye cup was placed receptor-side down in a glass scintillation vial. Then 1 ml of chloroform/methanol/conc. HCl (80:40:1, by vol.) at solid-CO_2 temperature was injected into the vial to freeze

Abbreviations used: PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; PtdIns$P$, phosphatidylinositol 1-phosphate; PtdIns, phosphatidylinositol; Ins(1,4)P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4)P_3, inositol 1,3,4-trisphosphate; Gro$P$Ins(4,5)P_2, glycerophosphoinositol 4,5-bisphosphate; G_t, guanine-nucleotide-binding (M_r 39000) regulatory protein of vertebrate retinal rod outer segments.

and fix the tissue. The other half of each eye cup was illuminated for 5 s (2.4 mW/cm^2; 360–750 nm); at the end of the illumination the tissue was frozen and fixed.

For the procedure *in vitro*, pieces of eye cups from dark-adapted animals were removed and incubated in artificial sea water containing [^{3}H]inositol (0.5 mCi/ml). Each quarter or half of an eye cup was placed in a glass tube containing 0.2 ml of incubation medium, and a stream of 100% O_2 saturated with water vapour was continuously passed into the tube. Tissue was incubated for 2–4 h at 18 °C in darkness, illuminated or not, fixed and processed as described for the procedure *in vivo*.

Fixed tissue was stored on solid CO_2 until all eyes had been processed, then stored for 1 h to overnight at −20 °C. Samples were then warmed to room temperature, and 0.16 ml of 1 M-HCl was added to separate the aqueous and chloroform phases. The upper, aqueous, phase was aspirated and pooled with two 0.4 ml washes of 'Folch upper phase' solution. The pooled samples were evaporated to dryness (Savant Speed Vac apparatus), dissolved in 0.1 M-HCl and clarified by centrifugation in a Beckman Airfuge operated at 100 kPa (15 lb/in^2). Samples were analysed by h.p.l.c. on Partisil 10 SAX by using an ammonium formate/H_3PO_4 gradient (Irvine *et al.*, 1985*b*). ^{32}P-labelled Ins(1,4)P_2 or Ins(1,4,5)P_3 prepared from human red-cell ghosts (Downes *et al.*, 1982) was included in each sample to determine the efficiency of recovery of inositol polyphosphates. The lower, chloroform, phase was collected and evaporated to dryness under a stream of dry N_2 at room temperature. Each sample was dissolved in 1 ml of chloroform/methanol/1 M-HCl (20:9:1, by vol.) and a 0.1 ml sample was counted for radioactivity by liquid-scintillation spectrometry to determine the total ^{3}H incorporated into lipids. ^{3}H-labelled lipids were separated by t.l.c. on oxalate-treated silica-gel plates (Gonzalez-Sastre & Folch-Pi, 1968), with chloroform/methanol/conc. NH_3/water (360:280:5:76, by vol.) as the mobile phase. After chromatographic separation, plates were dried, exposed to iodine vapour to locate non-radioactive standards of PtdIns, PtdIns*P* and PtdInsP_2 (Sigma), sprayed with En^{3}Hance (DuPont), and autoradiograms were made on to Kodak X-Omat AR film at −70 °C. The bands identified on the autoradiograms were scraped from the t.l.c. plates and counted for radioactivity by liquid-scintillation spectrometry.

Pieces of the eye cups from several animals were frozen on solid CO_2 for analysis of the efficacy of pertussis intoxication. The pieces of retina were rapidly thawed in 0.15 ml of a buffer composed of 250 mM-KH_2PO_4, pH 7.8, leupeptin (5 μg/ml), aprotinin (5 μg/ml) and 0.1 mM-phenylmethanesulphonyl fluoride. This mixture was maintained in an ice bath and homogenized in a Radnotti glass mortar fitted with a ground-glass pestle. Portions (80–140 μg of protein) of homogenate were incubated in a reaction mixture containing 125 mM-KH_2PO_4, pH 8.0, 20 mM-arginine, 20 mM-thymidine, 2.5 mM-$MgCl_2$, 20 mM-ATP, 0.05 mM-GTP, 5 μM-[^{32}P]NAD$^+$ (10 μCi), and vehicle (20 mM-dithiothreitol) alone or in combination with pertussis toxin (1 μg/ml). The pertussis toxin was activated by prior incubation in 20 mM-dithiothreitol for 10 min. The labelling reaction was conducted at 30 °C for 60 min. The reaction was terminated and the samples were subjected to SDS/polyacrylamide-gel electrophoresis, as described previously (Rapiejko *et al.*, 1986). Bands were identified by autoradiography of the gels. The identified bands were excised and the amount of radioactivity in each band was quantified by liquid-scintillation spectrometry.

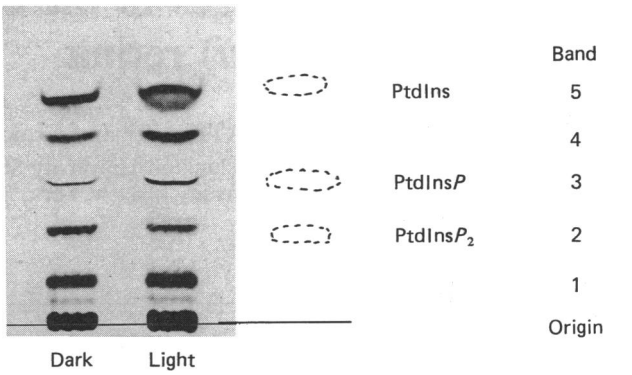

Fig. 1. Autoradiogram of t.l.c. separation of [^{3}H]inositol-labelled lipids from a squid retina

The lane labelled 'Dark' contains material extracted from the un-illuminated half of the eye cup; that labelled 'Light' contains extracts from the illuminated half. The dotted outlines show the positions of the iodine-stained standards of PtdIns, PtdIns*P* and PtdInsP_2.

RESULTS

Phosphoinositides

The autoradiogram of ^{3}H-labelled lipids extracted from a squid eye cup is shown in Fig. 1. In addition to lipids that co-chromatographed with PtdIns, PtdIns*P* and PtdInsP_2 standards, there are several radiolabelled lipids whose identities have not been established. The ^{3}H radioactivity (c.p.m.) in each band was normalized by the total ^{3}H radioactivity in the lane on the t.l.c. plate (minus the ^{3}H left at the origin) to account for differences in the amount of material between samples. For illuminated and un-illuminated samples from each eye, the light/dark ratio of normalized ^{3}H radioactivity was calculated for each band. The only phosphoinositide whose content was changed by illumination was PtdInsP_2 (Table 1). Although illumination induced a change in content of PtdInsP_2, the PtdInsP_2 was a very small fraction of the total [^{3}H]lipids ('dark' samples, 0.009 ± 0.003, $n = 19$; 'light' samples, 0.007 ± 0.003, $n = 19$). That is, the light-induced changes in PtdInsP_2 were about as small a fraction of total [^{3}H]lipid as was the error in scintillation counting of the total [^{3}H]lipid. The light/dark ratios for total [^{3}H]lipid, total [^{3}H]lipids separated by t.l.c. minus the component left at the origin, or any normalized [^{3}H]lipid component other than PtdInsP_2 did not differ significantly from unity. Therefore the total [^{3}H]lipid values provide a measure of total labelled retinal material that is not biased significantly by light-induced changes.

Inositol polyphosphates

The water-soluble compounds that were extracted from pieces of squid eye cups and that contained [^{3}H]-inositol were separated by ion-exchange h.p.l.c. A typical chromatogram of [^{3}H]inositol-labelled compounds extracted from both dark-adapted and light-stimulated pieces of the same eye cup is shown in Fig. 2.

Table 1. Light/dark ratios of [^{3}H]inositol-labelled lipids from squid retinas

The bands are labelled as in Fig. 1. The numbers were normalized by the total ^{3}H-labelled material in each lane on the t.l.c. plate (minus the ^{3}H radioactivity that remained at the origin). The light/dark ratio for total [^{3}H]lipids (before chromatography) was 1.08 ± 0.42 ($n = 20$), and for total ^{3}H-labelled material in each lane (minus the ^{3}H radioactivity that remained at the origin) was 1.13 ± 0.49 ($n = 18$). Results are given as means ± S.D., for the numbers of samples in parentheses. Only the PtdInsP_2 value differs significantly ($P \leqslant 0.05$) from unity.

[^{3}H]Lipid component		
Band labelled on Fig. 1	Co-migrates with standard of:	Light/dark ratio
1	–	0.85 ± 0.42 (17)
2	PtdInsP_2	0.63 ± 0.16 (19)
3	PtdIns$P$	1.17 ± 0.25 (18)
4	–	1.11 ± 0.27 (19)
5	PtdIns	1.11 ± 0.17 (16)

There are six main peaks in the chromatograms. Peak 4 co-chromatographed with authentic Ins(1,4)P_2 prepared from human red-cell ghosts (Downes *et al.*, 1982). Peak 6 co-chromatographed with authentic Ins(1,4,5)P_3. The material in each of these peaks increased in the light-stimulated samples. For these two peaks, the average ratios of radioactivity above background for the light-stimulated sample to that for the dark-adapted sample from each eye cup are shown in Table 2. The data were normalized for both recovery of ^{32}P-labelled inositol polyphosphate [either Ins(1,4)P_2 or Ins(1,4,5)P_3] and for the total amount of ^{3}H incorporated into lipid. This latter factor corrects for differences in the mass of retinal tissue in each sample and for any differences in viability of the retinas during incubation.

The chemical identity of the materials in peaks 1 and 5 has not been established. However, both peaks decreased in size when the samples were illuminated (Fig. 2 and Table 2). The material in peak 5 does not co-migrate with Gro*P*Ins(4,5)P_2 on high-voltage electrophoresis (R. F. Irvine, personal communication), nor does it have the chromatographic mobility expected of Ins(1,3,4)P_3 (Irvine *et al.*, 1985*b*). The material in peak 1 comprised a substantial fraction of the total ^{3}H-containing water-soluble materials. In control samples, the fraction was 0.19 ± 0.11 ($n = 36$). Rather than normalizing by the total ^{3}H-containing material in the aqueous phase, as has occasionally been done by others (Szuts *et al.*, 1986), we chose to normalize the ^{3}H-containing materials in each peak by the total [^{3}H]lipid material extracted into the chloroform phase. Table 2 shows that the two normalization procedures give somewhat different values.

Pertussis-toxin effects

Incubating homogenates of squid retina with activated pertussis toxin and [^{32}P]NAD$^+$ resulted in the ADP-ribosylation of a peptide with the same electrophoretic mobility (M_r 39000) as the α-subunit of the major G-protein of vertebrate retinal rod outer segments, α-G_t,

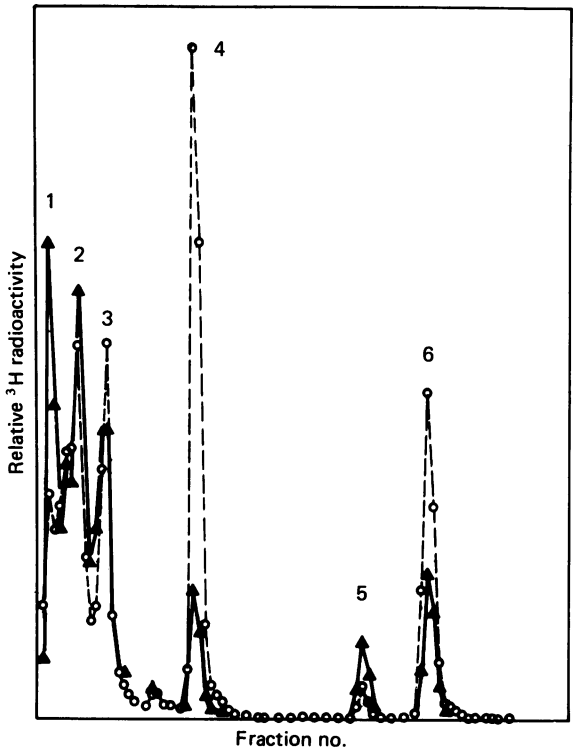

Fig. 2. H.p.l.c. separation of water-soluble compounds that contain [^{3}H]inositol from extracts of pieces of a squid eye cup

The relative ^{3}H radioactivity is plotted for each 0.25 min fraction (flow rate 2 ml/min). The data for each sample were normalized to total [^{3}H]lipid and also normalized to the recovery of ^{32}P-labelled Ins(1,4)P_2. This latter normalization corrects each sample for recovery of a known addition of inositol polyphosphate through the chromatographic procedure. The normalization for total [^{3}H]lipid corrects for the difference in amount of radiolabelled tissue in each sample. For these samples, peak 4 co-chromatographed with authentic Ins(1,4)P_2. For other samples, peak 6 co-chromatographed with authentic Ins(1,4,5)P_3. ○, Chromatogram of the extract of the illuminated (light) piece of the eye cup; ▲, chromatogram of the extract of the un-illuminated (dark) piece of the eye cup. Note that peaks 4 and 6 are increased for the illuminated sample, whereas peaks 1 and 5 are decreased.

that had been treated in the same fashion (results not shown). However, intraocular injection of 1 μg of pertussis holotoxin and treatment for periods of up to 4 h failed to alter significantly the changes in inositol polyphosphates induced by illumination (Table 2).

It is difficult to quantify ADP-ribosylation that occurs *in vivo* by endogenous NAD$^+$ and that is catalysed by pertussis toxin. Living cells that have been intoxicated by pertussis holotoxin can be secondarily labelled by breaking the cells and incubating with [^{32}P]NAD$^+$ and activated pertussis toxin; this secondary labelling assays for remaining unlabelled G-protein. The amount of remaining G-protein reflects the remaining ability of cells to respond to stimulation of a G-protein-dependent effector (for example, the inhibition by adenosine of adenylate cyclase in fat-cells from hypothyroid rats; Malbon *et al.*, 1985). When incubated with 5 μM-NAD$^+$ and activated pertussis toxin, homogenates of retinas

Table 2. Light/dark ratios of inositol phosphates in squid retinas

The peaks are labelled as in Fig. 2. All values were normalized for recovery of ^{32}P-labelled Ins(1,4)P_2 or Ins(1,4,5)P_3, after the chromatographic procedures. Data are presented as means ± S.D., for the numbers of experiments in parentheses.

		Light/dark ratios			
		Per total [^{3}H]lipid		Per total ^{3}H-labelled water-soluble material	
Inositol polyphosphate	Incubation condition	Control	Pertussis-toxin-treated	Control	Pertussis-toxin-treated
[^{3}H]Ins$P$ (peak 6)	In eye	2.06±0.72 (17)	1.93±0.76 (10)	1.34±0.52 (18)	2.10±0.90 (9)
[^{3}H]Ins$P$ (peak 4)	In eye	2.68±1.07 (13)	2.01±1.10 (10)	1.87±0.64 (15)	2.05±1.21 (9)
^{3}H (peak 5)	In eye	0.56±0.46 (17)	0.46±0.32 (8)	0.37±0.22 (16)	0.67±0.50 (9)
^{3}H (peak 1)	In eye	0.79±0.31 (13)	0.65±0.43 (9)	0.69±0.25 (14)	0.64±0.33 (8)
[^{3}H]Ins$P$ (peak 6)	*In vitro*	1.73±0.94 (12)		2.01±1.00 (14)	
[^{3}H]Ins$P$ (peak 4)	*In vitro*	1.60±0.74 (14)		1.51±0.64 (14)	
^{3}H (peak 5)	*In vitro*	0.91±0.60 (14)		0.80±0.36 (14)	
^{3}H (peak 1)	*In vitro*	0.89±0.22 (12)		0.91±0.14 (13)	

from eyes that had been injected with vehicle only ([^{3}H]inositol) contained 82±7 fmol of ADP-ribose/mg of protein (mean±S.E.M., $n = 3$) incorporated into the M_r-39000 peptide. Homogenates of retinas from eyes into which pertussis toxin previously had been injected ([^{3}H]inositol + 1 μg of pertussis holotoxin) contained 21±10 fmol of ADP-ribose/mg of retinal protein incorporated into the M_r-39000 peptide, a 75% decrease from control values. Thus, pertussis toxin injected intraocularly catalyses *in vivo* the ADP-ribosylation of the M_r-39000 peptide by endogenous NAD$^+$. Despite demonstrable intoxication of the squid retinal tissue, there was no significant change in light-induced changes of inositol polyphosphates (Table 2).

DISCUSSION

Metabolic labelling *in vivo* of squid eyes by intraocular injection of [^{3}H]inositol was explored as a system for studying light-induced changes in the content of inositol phosphates in squid retina. The viability of excised squid retinal tissue is difficult to maintain (Pinto & Brown, 1977). Our technique for labelling *in vivo* eliminates many of the difficulties encountered in a system *in vitro*. The experiments of Szuts *et al.* (1986) and Wood *et al.* (1985), as well as our own, in which excised retinas were incubated in oxygenated artificial sea water containing [^{3}H]inositol, found large variability in the light/dark ratios of InsP_3 between samples. However, our data suggest that this variability is not simply due to tissue viability *in vitro*, because the variability was not substantially decreased in experiments in which labelling was performed *in vivo*.

PtdInsP_2 was the only ^{3}H-inositol-labelled lipid in squid retina that was dramatically changed with illumination; it comprises only about 1% of the total [^{3}H]lipids. Neither the light/dark ratios for total [^{3}H]lipids nor any normalized [^{3}H]lipid component other than PtdInsP_2 differed significantly from unity. These findings establish a reliable and valid means of evaluating changes that were observed in water-soluble inositol-containing compounds after illumination. Light-induced changes in inositol-containing lipids of squid retina have not been evaluated in previous studies. The finding that illumination does not change the PtdIns or PtdIns$P$ content of squid retinas indicates that the stimulated phospholipase activity principally produces InsP_3, unlike in some other systems (Majerus *et al.*, 1986), and that the increase in InsP_2 content that we detect probably arises from degradation of InsP_3.

Our h.p.l.c. separation of inositol polyphosphates extracted from squid retinas revealed that the predominant isomers whose concentrations were increased by illumination are Ins(1,4,5)P_3 and Ins(1,4)P_2. Szuts *et al.* (1986) did not find a light-stimulated increase in InsP_2. However, our experiments differ from theirs in incubation, stimulus and fixation protocols. Unlike the studies of Szuts *et al.* (1986), we did not attempt to facilitate turnover of phosphoinositides by preincubating the photoreceptors under dim red light for 1 h before dark-adaptation. Also, the stimulus protocols were very different in the different studies. Wood *et al.* (1985) and Szuts *et al.* (1986) delivered a very bright 1 ms strobe flash and froze the tissue not less than, but often much more than, 200 ms later; they fixed and extracted with 'ice-cold' 15% trichloroacetic acid. On the other hand, we stimulated with a 5 s light and froze the tissue after a brief, undetermined, delay by addition of chloroform/methanol/HCl at −70 °C; the tissue was extracted at −20 °C. Szuts *et al.* (1986) report there are only small changes in inositol polyphosphates during progressively increased intervals in darkness after a light flash, so it is unlikely that our delay before freezing accounts for the differences. How the various differences between protocols may have contributed to differences in the data in the two reports is unknown.

Linkage to effector systems via G-proteins appears to be a characteristic common to a number of cell-surface receptors (Gilman, 1984; Stryer & Bourne, 1986; Litosch & Fain, 1986; Cockcroft, 1987). G-proteins appear to mediate visual excitation in vertebrate rods (Kuhn, 1986; Stryer & Bourne, 1986). Phototransduction in invertebrates also may involve a G-protein (Bolsover & Brown, 1982; Fein, 1986). In several systems, stimulation of

PtdIns metabolism has been shown to be attenuated by treatment with pertussis toxin, whereas in other systems it is not (Litosch & Fain, 1986; Cockcroft, 1987).

In squid retinas, pertussis toxin catalysed the ADP-ribosylation of a M_r-39000 peptide that co-migrated on SDS/polyacrylamide-gel electrophoresis with α-G_t purified from bovine rod outer segments. A large fraction of squid retinal G-protein was effectively labelled by pertussis holotoxin *in vivo*. However, pertussis-toxin treatment did not alter the light-induced changes in InsP_3; this finding suggests that the M_r-39000 peptide that is ADP-ribosylated may not mediate light-induced changes in PtdIns metabolism in squid retina. Tsuda *et al.* (1986) identified a M_r-41000 substrate for pertussis-toxin-catalysed labelling in octopus photoreceptors; this M_r-41000 substrate was not recognized by rabbit polyclonal antisera raised against the M_r-39000 α-subunit of G_t. The effects of pertussis toxin on a light-induced electrophysiological response or phosphoinositide metabolism in the octopus system have not been reported. Identification of a G-protein that mediates visual excitation in invertebrates remains an important goal.

Although an increase in the content of Ins(1,4,5)P_3 has been observed in squid retina (Wood *et al.*, 1985; Szuts *et al.*, 1986) and confirmed in the present report, there is no evidence for Ins(1,3,4)P_3, as is found in *Limulus* photoreceptors (Irvine *et al.*, 1985*a*). Most interesting is the discovery of another compound (peak 5 in Fig. 2) whose concentration is decreased by illumination. Analyses of InsP_3 that rely on separation on Dowex-1 resin will be confounded by this compound. The role of inositol polyphosphates in the mechanism of transduction in invertebrate photoreceptors remains to be elucidated.

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