Light- and GTP-activated Hydrolysis of Phosphatidylinositol Bisphosphate in Squid Photoreceptor Membranes*

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Light stimulates the hydrolysis of exogenous, [3H] inositol-labeled phosphatidylinositol bisphosphate (PtdInsP2) added to squid photoreceptor membranes, releasing inositol trisphosphate (InsP3). At free calcium levels of 0.05 μM or greater, hydrolysis of the labeled lipid is stimulated up to 4-fold by GTP and light, but not separately. This activity is the biochemical counterpart of observations on intact retina showing that a rhodopsin-activated GTP-binding protein is involved in visual transduction in invertebrates, and that InsP3 release is correlated with visual excitation and adaptation. Using an in vitro assay, we investigated the calcium and GTP dependence of the phospholipase activity. At calcium concentrations between 0.1 and 0.5 μM, some hydrolysis occurs independently of GTP and light, with a light- and GTP-activated component superimposed. At 1 μM calcium there is no background activity, and hydrolysis absolutely requires both GTP and light. Ion exchange chromatography on Dowex 1 (formate form) of the water-soluble products released at 1 μM calcium reveals that the product is almost entirely InsP3. Invertebrate rhodopsin is homologous in sequence and function to vertebrate visual pigments, which modulates the concentration of cyclic GMP through the mediation of the GTP-binding protein transducin. While there is some evidence that light also modulates PtdInsP2 content in vertebrate photoreceptors, the case for its involvement in phototransduction is stronger for the invertebrate systems. The results reported here support the scheme of rhodopsin → GTP-binding protein → phospholipase C activation in invertebrate photoreceptors.

Photoexcitation of the visual pigment, rhodopsin, triggers a cascade of enzyme reactions which both mediate phototransduction and play a universal role of transmembrane signalling in animal cells (Stryer and Bourne, 1986; Stieve, 1986). Rhodopsin is a member of a family of receptor proteins which includes the visual pigments (Findlay and Pappin, 1986), β-adrenergic receptor (Dixon et al., 1986), and muscarinic acetylcholine receptor (Kubo et al., 1986). These transmembrane receptors are coupled, through the mediation of a family of GTP-binding proteins (G-proteins) on the cytoplasmic surface of the membranes, to effector enzymes which modulate cyclic nucleotides, phosphoinositides and ion channels.

In the vertebrate retina, photoexcited rhodopsin catalyzes GDP-GTP exchange on transducin, the rod G-protein. The GTP binding α-subunit activates a cyclic GMP phosphodiesterase, resulting in the closure of cyclic GMP-controlled sodium channels and the hyperpolarizing visual signal in vertebrate rods (Stryer, 1986).

In the invertebrate retina the sequence of events is somewhat different. Rhodopsin and G-protein are present (Tsuda et al., 1986; Vandenberg and Montal, 1984a: Saibil and Michel-Villaz, 1984) within microvillar structures which, in the squid, support a highly ordered membrane-cytoskeleton network (Saibil, 1982; Saibil and Hewat, 1987). Activation of G-protein is involved in the generation of the invertebrate photoreceptor potential (Blumenfeld et al., 1985), but it has not been established which effector enzyme(s) are activated by this G-protein and whether more than one variant of G-protein plays a role in invertebrate phototransduction. Both cholera toxin (Vandenberg and Montal, 1984a) and pertussis toxin substrates (Tsuda et al., 1986) have been separately described in cephalopod photoreceptors. In contrast to the dual toxin sensitivity of transducin, these toxins cause labeling of two distinct substrates in the invertebrate photoreceptor membrane preparation.

Considering the possible effector enzymes activated, Vandenberg and Montal (1984b) observed a light-induced loss of PtdInsP2 in squid photoreceptors, and both a light-stimulated increase of cyclic GMP (Saibil, 1984; Johnson et al., 1986) and of InsP3 (Szuts et al., 1986) have been reported in the squid retina. Both of these cytoplasmic messenger candidates have been associated with the depolarizing light response in Limulus ventral photoreceptor (Brown et al., 1984; Payne et al., 1986; Johnson et al., 1986), although the effects of InsP3 are more pronounced and more consistent.

Exogenous, labeled PtdInsP2 has been used as a substrate for the in vitro investigation of phospholipase C in blowfly salivary membranes, where release of inositol phosphates was observed (Litosch and Fain, 1985). In order to study the rhodopsin-activated phospholipase in purified membranes highly specialized for visual transduction, we have measured light-stimulated hydrolysis of exogenous PtdInsP2 incubated with squid photoreceptor membranes, a preparation in which rhodopsin is the major protein.

MATERIALS AND METHODS

Membrane Preparation—Living, dark-adapted squid (Alloteuthis sublata, about 10-cm body length) were obtained at the Laboratory of the Marine Biological Association, Plymouth, and were dark-adapted for at least 4 h before decapitation. Under dim red light (Kodak filter 1A), the eyes were rapidly dissected and frozen intact.

1. The abbreviations used are: G-protein, GTP-binding protein; PtdInsP2, phosphatidylinositol bisphosphate; PtdInsP3, phosphatidylinositol monophosphate; PA, phosphatidic acid; InsP3, inositol trisphosphate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MOPS, 3-N-morpholino)propanesulfonic acid.

2. J.M. Fyles and H. R. Saibil, unpublished observations.

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including optic lobes, in liquid nitrogen-cooled hexane, and stored in liquid nitrogen until use. For isolation of retinal photoreceptor membranes, 2-6 eyes were fractured while still frozen, to expose the retinas, which were immediately placed in EGTG-containing buffer (0.4 M NaCl, 10 mM EGTA, 50 mM MOPS, 2.5 mM MgCl2, 1 mM dithiothreitol, pH 6.9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this preparation is shown in Fig. 9 of Saibil and Hewat (1987). For 1 h prior to the assays, the membranes were incubated in the dark at 4° C in assay buffer containing 1 mM ATP. The rhodopsin concentration was measured by the absorbance change at 494 nm on illumination at pH 10. All manipulations of the membranes were done in very deep red light (Kodak filter 2, with a 25-watt bulb; blacked out on bulb side) or in dim light, except bleaching flashes, until completion of the assay.

**Phospholipase Assay and Ion Exchange Chromatography**—[3H]Inositol and [3H]inositol-PtdInsP2 by squid photoreceptor membranes—The lipids were separated by two-dimensional thin layer chromatography, by the method of Mitchell et al. (1983) and the rhodopsin/rhodopsin complex, which was consistent with the calculated values in the micromolar range. Arsenazo III (17 μM, Sigma) was used to check the effect of deoxycholate, which was found to be negligible in the 10 μM range.

**RESULTS AND DISCUSSION**

**Endogenous Polyphosphoinositide Content of Squid Photoreceptor Membranes—**After [γ-32P]ATP incubation of the membranes in our assay buffer, PtdInsP was readily detected and rapidly phosphorylated, with a smaller amount of label incorporated into PA. Using the values for lipids and protein composition of Paulsen et al. (1983) and the rhodopsin/total microsomal protein from Saibil and Hewat (1987), we estimate that a membrane suspension containing 0.3 mg/ml rhodopsin contains about 1 μmol PtdInsP. However PtdInsP was not detectable in these samples, for which our sensitivity is about 1 pmol of labeled PtdInsP. We conclude that there is at least a 10-50-fold excess of exogenous PtdInsP, so that the specific activity of PtdInsP in our assay is close to that of the exogenous substrate. Using higher Mg2+ concentrations, label can be detected in PtdInsP, in agreement with the observations of Yandenberg and Montal (1984b).

**Calcium- and GTP-dependent Release of [3H]Inositol from PtdInsP in Squid Photoreceptor Membranes in the Light—**Reactions were initiated by mixing illuminated squid photoreceptor membranes with PtdInsP, which had been sonicated in the presence of deoxycholate, which appears necessary to allow the phospholipase access to exogenous lipid (Hofmann and Majerus, 1982). We were unable to keep the exogenous PtdInsP dispersed in our assay buffer without deoxycholate present at levels close to its critical micelle concentration. However, the concentration used was too low to solubilize the membranes. Upon phase separation of the assay samples, a progressive loss of radioactivity from the lipid phase and an increase in the water-soluble phase was observed. Under favorable conditions, 20% of the radioactivity was released from the lipid phase in 20 min. In Fig. 1 the rates of inositol release, obtained from a set of time series as described under “Materials and Methods”, are plotted as a function of the free calcium concentration. Very little release was observed below 0.1 μM free Ca2+. Between 0.1 and 1.0 μM, substantial release occurred in 1 mM GTP, and at 0.1-0.5 μM a somewhat lower release was observed in the absence of GTP. At 1.0 μM the effect of GTP was most dramatic, with an activity ratio +GTP/−GTP = 2.85 ± 0.78 (S.D., n = 9). In some experiments GTP-stimulated activity remained at the maximal level up to 0.1 mM free Ca2+ (data not shown).

**Light-stimulated Inositol Release—**The time series for [3H]inositol release by dark and light-activated photoreceptor membrane...
branes at 1 μM free Ca\(^{2+}\) with 0 or 1 mM GTP are shown in Fig. 2. Light had no effect unless GTP was present, in which case it stimulated inositol release up to 4-fold. The light/dark activity ratio was 2.9 ± 0.8 (S.D., n = 6), similar to the result obtained by Szuts et al. (1986) for InsP\(_3\) content of rapidly frozen retinas. Addition of GTP in the dark had little effect (note that these experiments were not performed in total darkness). At 0.1–0.5 μM Ca\(^{2+}\), light activation was also observed, superimposed on the GTP- and light-independent background (data not shown). Thus we have clear evidence for a light-stimulated PtdInsP\(_3\) phospholipase C with an absolute requirement for GTP. Although the InsP\(_3\) increase in the living squid retina occurs within 200 ms of a light flash (Szuts et al., 1986) and is expected to be transient, the persistent activation of inositol release observed in our membrane preparations is not unexpected. Activation of cyclic GMP phosphodiesterase by transducin in suspensions of rod outer segment disc membranes persists for hours if adequate GTP is present (Sitaramayya and Liebman, 1983). This can probably be explained by the absence of soluble, deactivating enzymes such as rhodopsin kinase and arrestin (Stryer, 1986).

**Anion Exchange Chromatography of the Deacylated Lipids and Released Inositol Phosphates**—Reactions were set up at 1 μM Ca\(^{2+}\), 1 mM GTP in light and dark, and at 0.1 μM Ca\(^{2+}\) without GTP. After 30 min the products were extracted, the lipids were deacylated, and aliquots of water-soluble and deacylated lipid products were run on Dowex columns with the same elution protocol, in which glycerol-heid groups elute in advance of inositol phosphates. The elution profiles in Fig. 3 show that (a) the deacylated lipid runs mainly as glycerol-InsP\(_3\), with a small glycerol-InsP\(_2\) peak; (b) the receptor-stimulated release yields practically a single broad peak migrating as InsP\(_3\), with a small InsP\(_2\) peak; (c) in the dark only a small InsP\(_3\) peak is detected; and (d) the GTP-independent activity produced several unidentified products eluting in positions similar to InsP\(_3\), glycerol-InsP\(_3\), and InsP\(_3\). These results suggest that there may be two distinct phospholipase activities in this membrane preparation. The rhodopsin/GTP-stimulated phospholipase C is active at 0.1 μM free Ca\(^{2+}\) and above, similar to that observed in other systems (e.g., Uehling et al., 1986), while the unidentified, GTP- and light-independent phospholipase C is only observed between 0.1 and 0.5 μM free Ca\(^{2+}\) (see Fig. 1).

In summary, we have observed a very distinct light- and GTP-stimulated phospholipase C in isolated squid photoreceptor membranes which appears to generate almost pure InsP\(_3\) from exogenous PtdInsP\(_3\). In addition, our data provide preliminary evidence for a second, less well characterized activity with unusual calcium dependence, which produces unidentified products. It has recently been reported that transducin mediates light activation of phospholipase A\(_2\) in vertebrate rods (Jelsema, 1987). Thus the current data suggest...
that rhodopsin regulates both cyclic nucleotide and phospholipid metabolism in vertebrate and invertebrate photoreceptors. There is now strong evidence for the rhodopsin-phospholipase C pathway in the invertebrate photoreceptors, and the squid retina provides an excellent model system for studying the relations between G-proteins and the phosphoinositide and cyclic GMP pathways in cell signalling.

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