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THE MECHANISMS OF THE TRANSFORMATION OF PHOTOSIGNALS IN VISUAL PHOTORECEPTOR CELLS

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A review of the data on the light control of phosphoinositide metabolism in disc membrane was presented. It was shown that light stimulates both synthesis and hydrolysis of membranous PtdIns, PtdInsP and PtdaInsP$_2$ was found to be most sensitive to the light regulatory action. Light regulates these metabolic reactions indirectly via rhodopsin as a photoreceptor and βγ-transducum complex as a mediator. It was suggested that phosphoinositide metabolism is in crosstalk with cGMP phototransduction cascade with help of transducin, two components of which, Tα and Tβγ control appropriate enzyme systems in ROS.

INTRODUCTION

A trigger event of visual excitations in vertebrates is *cis-trans* isomerization of retinal, a chromophore of visual pigment rhodopsin. A photochemical reaction induces a chain of the reactions in a visual cell called phototransduction which is completed by the rise of the receptor potential. Phototransduction occurs with the participation of a messenger which is cGMP as it was shown in many works in last decade (Stryer, 1991). Ca^{2+} fulfil an auxiliary role: these ions modulate the phototransduction controlling its distinct stages and the light sensitivity of visual cells as a whole (reviewed by Volotovski & Konev, 1986; Volotovski, Khovratovich & Baranova, 1992). In relation to the role of Ca^{2+} in visual phototransduction the attention of the researches was attracted to the phosphoinosites, the metabolic products of which were found to control the cellular Ca^{2+} homeostasis. Inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG) are the second messengers linking a flow of information from many extracellular signalling molecules (hormones, growth factors and neurotransmitters) with the targets within the cell (Berridge, 1993). $InsP_3$ and DAG were also shown to be involved into the functioning of the photoreceptor cells of invertebrates (reviewed by Yoshioka, Inoue, Das & Schichi, 1989). The metabolism of the phosphoinositides in vertebrate photoreceptor cells turned out to be less studied though the disc membranes were shown to contain their rapidly exchangeable pool (Ghalayni & Anderson, 1984; Hayashi & Amakawa, 1985). Furthermore, there were indications that IP_3 is likely to mobilize Ca^{2+} from retinal rod discs and other non-mitochondrial rod Ca^{2+} stores (Volotovski & Khovratovich, 1993) and DAG-activated protein kinase C phosphorylated rhodopsin and retinal outer segment (ROS) cGMP-phosphodiesterase (Udovichenko, Cunnik, Gonzales & Takemoto, 1994). This means that light sensitivity of $InsP_3$ and DAG formation in retinal rods could be an indication of the involvement of phosphoinositide turnover into light-triggered processes closely associated with phototransduction. This review summarizes the experimental data concerning the turnover of phosphoinositides in the disc membranes of bovine retinal rods.

cGMP CASCADE IN VERTEBRATE RETINAL RODS

The structure of vertebrate rod photoreceptor cells indicates that photo-receptor visual pigments and ion channels are not located in close proximity in the same cell membrane. Light is absorbed by rhodopsin associated with the disc membrane and the sodium channels, which are primarily responsible for light-elicited membrane hyperpolarization, are localised to the plasma membrane. Because of this spatial separation, an intracellular messenger is needed to convey the light-induced signal from the disc to the plasma membrane. Understanding of vertebrate visual transduction has been greatly advanced by the discovery by Fesenko, Kolesnikov and Lubarski (1985) that cGMP maintains the

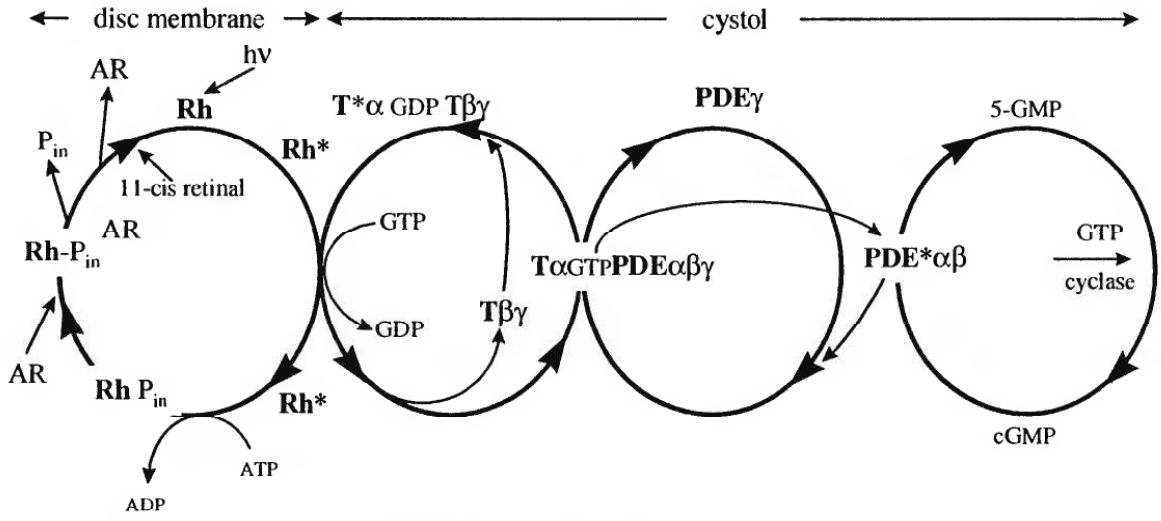

Fig. 1 cGMP cascade of phototransduction in visual rod cell of vertebrates (according to Stryer, 1991). Rh — rhodopsin, T — transducin, AR — arrestin, PDE — cGMP phosphodiesterase. In the upper part of the scheme the localization of partial reactions of the pathway in ROS is shown. The low sentence indicates the amplification taken place on different levels of the cascade.The asterisk means the active state of a molecule.

cation channels in plasma membrane of vertebrate retinal rod outer segment (ROS) in the open state. Levels of cGMP in ROS are regulated by a light-driven cascade involving bleached rhodopsin, G protein (transducin) and a cGMP phosphodiesterase (Fig. 1). According to this scheme, photoexcitation of rhodopsin is coupled to the activation of cGMP phosphodiesterase through transducin, leading to the hydrolysis of cGMP to modulate the activity of cGMP-gated cation channels (reviewed by Stryer, 1991). Transducin is a member of heterotrimeric G-proteins which are regarded as the coupling proteins for cellular effector systems. It contains three(α, β γ) subunits in one complex (Tαβγ) that can easily dissociate into two components, Tα and Tβγ. More often transducin controls the activity of effector systems by α-subunits (Simon, Strathman & Gautam, 1991). There are several ways to stop the functioning of the cGMP cascade. For that the regulatory proteins like rhodopsin kinase, arrestin etc. are used. The activity of transducin depends on whether its α–subunits is associated with GTP or not. The exchange of GDP for GTP on Tα is triggered by bleached rhodopsin provided Tα is bound to Tβγ. Tα·GDP is an activator of cGMP phosphodiestarese. The activation of this enzyme switches on the GTPase activity of Tα that leads to the transformation of GTP into GDP on Tα. In turn Tα·GDP has affinity for Tβγ high enough to stay associated with bleached rhodopsin. It should be stressed that on the level of this cascade the extremely high amplification of the primary photochemical signal occurs. It is as high as 10^5-10^6.

PHOSPHOINOSITIDE CASCADE AND ITS PRODUCTS

The phosphoinositide involvement into transduction of receptor signals within the cells was suggested by Hokin and Hokin (1953) but only in 20 years the products of the enzyme transformation of phosphoinositides began to be considered as intracellular messengers (Michell, 1975). Biological membranes contain three main classes of phosphoinositides, phosphatidylinositol (PtdIns), phosphatidylinositol 5-monophosphate (PtdInstdP), and phosphatidyl 4,5-bisphos-phate ($PtdInsP_2$) differing in their levels of phosphorylation. The crucial role is played by $PtdInsP_2$. In many cell types, $PtdInsP_2$-specific phospholipase C brings about the formation of $InsP_3$ and DAG. In some cells, phospholipase C is regulated by G proteins (by their α-subunits with bound GTP) which mediate effects of extracellular agonists. Therefore, both GTP and G-proteins mimic to some extent the action of the ligands. $InsP_3$ was found to stimulate the release of calcium ions from intracellular stores (reviewed by Berridge, 1993). DAG is an activator of protein kinase C responsible for the phosphorylation of a number of intracellular proteins that is followed by modulation of their activities (reviewed by Nishizuka, 1988). While Ca^{2+} activate protein kinase C, $InsP_3$ controls indirectly the activity of this enzyme. The scheme presented on Fig.2 illustrates the main stages of the turnover of phosphoinositides in biological membranes.

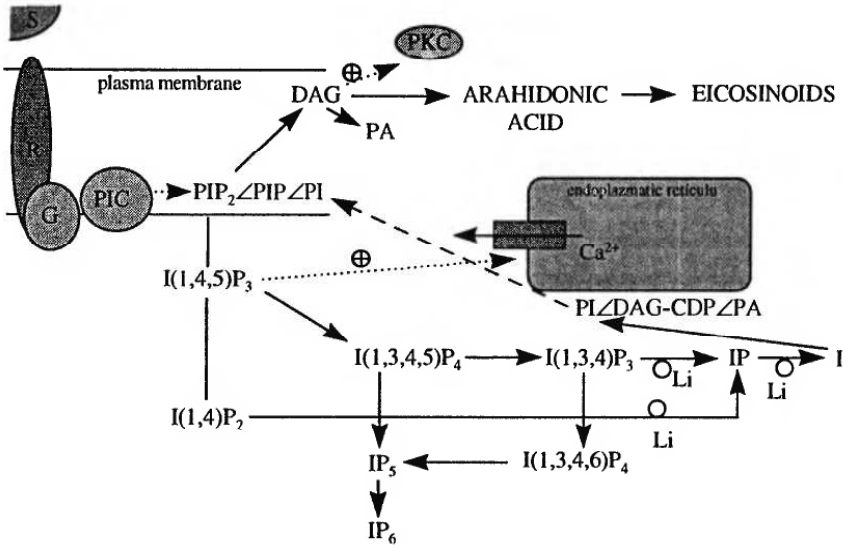

Fig. 2. Polyphosphoinositide turnover (according to Berridge, 1987). R — receptor, G - G-protein, PIC — phosphoinositidase (phospholipase C), PKC-protein kinase C, DAG-CDP — diacylglycerol-cytidine diphosphate, $I(1,4,5)P_3$, $I(1,3,4,5)_4$, $I(1,3,4)P_3$, $I(1,3)P_2$, $I(1,4)P_2$, IP_5, $I(1,3,4,6)P_4$, IP_6 — phosphorylated inositols.

PHOSPHOINOSITIDE TURNOVER IN PHOTORECEPTOR CELLS OF VERTEBRATES AND PHYSIOLOGICAL EFFECTS OF ITS PRODUCTS

The first indications on the possible involvement of phosphoinositides into molecular mechanisms of vision in vertebrates appeared at the beginning of last decade. Below the main data obtained before the investigations of our institute are presented:

1. Illumination of the retina prelabelled with [^{3}H]-inositol or [^{32}P]H_3PO_4 showed that $PtdInsP_2$ hydrolysis occurred in ROS of frog, bovine, rat and chick in response to light (Ghalayni & Anderson, 1984; Hayashi & Amakawa, 1985; Millar, Fisher, Muir, Edwards & Hawthorne, 1988)
2. Investigations with [^{3}H]inositol indicated that light-stimulated phospho-inositide turnover occured in rat photoreceptors (mainly in the inner segment). Dibutyryl cGMP and IBMX (cGMP phosphodiesterase inhibitor) suppressed light-stimulated turnover (Schmidt, 1983).
3. Immunostaining of the retina with anti-$PtdInsP_2$ antibodies indicated that the staining intensity of the outer segment layer is markedly reduced by 1 ms flash illumination and was restored within several minutes of dark adaptation of rats, suggesting possibly rapid turnover of $PtdInsP_2$ (Das, Yoshioka, Samuelson, Cohen & Schichi, 1987).
4. Pressure-injection of $ItdPns_3$ into the dark-adapted rod of salamander induced a transient hyperpolarization of the membranes in the dark (Waloga & Anderson, 1985).
5. Enzymes involved in polyphosphoinositide turnover are present in ROS (Hayashi & Amakawa, 1985; Seyfred, Kohnken, Collins & McConnel, 1984; Guisto & Ilinchela de Boshero, 1986; Gehm and Mc Connel, 1990).

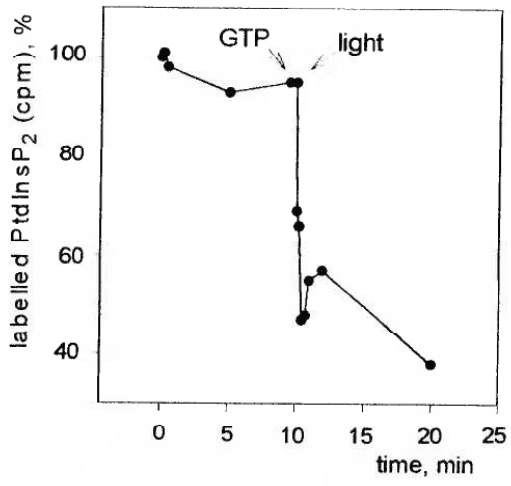

Fig. 3.The time course of light-induced breakdown of $PtdInsP_2$. The concentration of the GTP added was as low as 100 μM. ROS was bleached using a 100 W tungsten lamp (Grigorjev et al., 1995).

The data above were used to suggest the schemes for the phosphoinositide participation in the functioning of visual cells. At the same time particularities of the metabolic transformation of the phosphoinositides in retinal rod photoreceptor membranes and especially the regulation of its light-dependent stages remained to be elucidated.

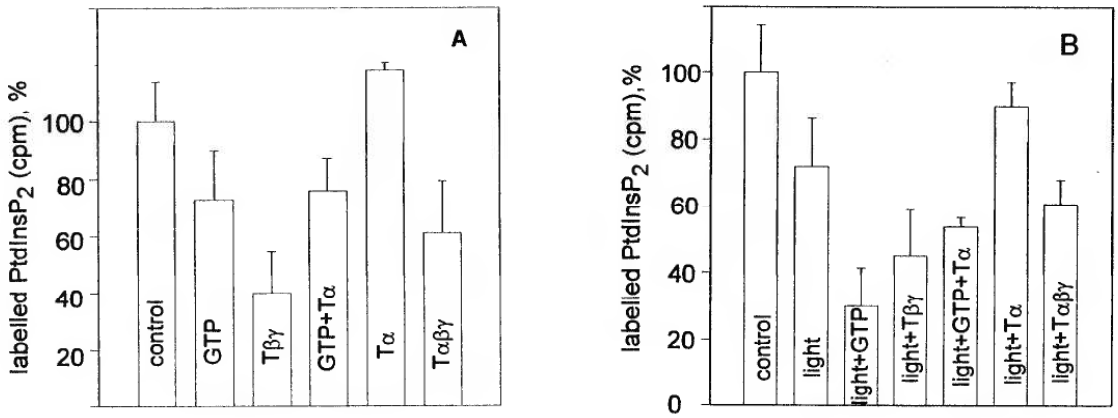

Fig. 4.Effect of light, GTP (100 μM), transducin (0.02 mg/ml) and its subunits (0.01 mg/ml) on the $PtdInsP_2$ hydrolysis in dark-adapted (A) and bleached (B) ROS as measured using TLC separation and scintillation counting (Grigorjev *et al.*, 1995).

PHOTOCONTROL OF PHOSPHOINOSITIDE TURNOVER IN PHOTORECEPTOR MEMBRANES

In the presence of [^{32}P]-γ-ATP or [^{32}P] H_3PO_4 rapid exchange of phosphate in phosphoinositides of photoreceptor membranes occurs which is the reflection of the processes of their synthesis and breakdown. A half-time of this exchange is as short as several minutes, compared with much longer half-times of that typical for other phospholipids (12-36 hours). The most rapid [^{32}P] incorporation is observed for PtdInsP as compared with those for $PtdInsP_2$ and phosphatidic acid (PA).

The first aim of our studies was to evaluate the influence of light on the turnover of phosphoinositides in photoreceptor membranes. For that purpose separate registration of light-induced changes in the amounts of the metabolic products formed during their synthesis and hydrolysis was carried out (Khovratovich, Grigoriev, Korotkina, Shejko & Volotovski, 1990). When light was switched on and ^{32}P-labelled ATP was simultaneously added, the increase in radioactivity of individual phospholipids reflected the influence of light on their synthesis. To show the effect of light on phosphoinositide hydrolysis, ^{32}P-labelled ATP was added to the ROS suspension in the dark to saturate the lipids with ^{32}P. After 60 min incubation, light was switched on and 10 min later the decrease in ^{32}P radioactivity of a lipid was measured.

The experiments have shown that light stimulated both synthesis and hydrolysis of phosphoinositides. Fig. 3 demonstrates the kinetics of light-induced $PtdInsP_2$ hydrolysis in ROS. The radioactivity of ^{32}P once incorporated into $PtdInsP_2$ and kept in the dark was maintained at a constant level. However, after light switching on in the presence of GTP, a rapid drop (by 60%) in the radioactivity was registered within not longer than 1 min. Subsequently, a slow decrease in the radioactivity was observed during next 10 minutes. It means that light induced a breakdown of $PtdInsP_2$, presumably activating phospholipase C as it was shown previously for other systems. The sensitivities of the product turnover to light are given by the following series:

For synthesis - $PtdInsP_2$ > PtdInsP > PtdIns > PA
and
For hydrolysis - $PtdInsP_2$ > PtdInsP > PtdIns > PA

It should be also noted that light exerts its influence more effectively in ROS membranes than in the discs. This implies that photoreceptor contains the enzymes responsible for these reactions (phosphokinases and phospholipases C) and that there is a specific factor(s) weakly associated with photoreceptor memb-ranes which is required to provide the activities of these enzymes. Actually, a wash of ROS membranes by hypotonic buffer which removes the all peripheral proteins from the disc membrane diminished the efficacy of light for the stimulation both of hydrolysis and synthesis of $PtdInsP_2$ just as it was found for light stimulation of phosphodiesterase activity (Stryer, 1991). The

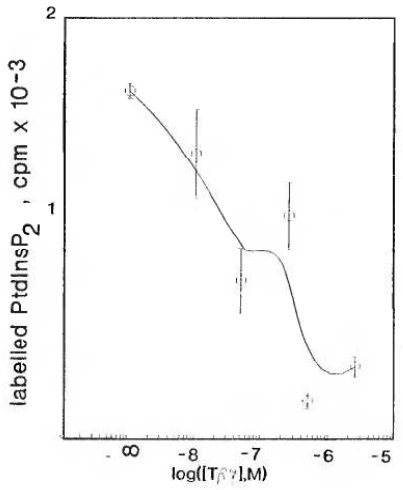

Fig..5. The dependence of the $PtdInsP_2$ hydrolysis in dark-adapted ROS on Tβγ concentration (Grigorjev *et al.*, 1995).

more washes the lower was the efficacy of light. Moreover, the effect of light on phosphoinositide turnover was also closely related to transducin. So, the addition of transducin and GTP to the membrane suspension resulted in the strengthening of the stimulation that indicated strongly the involvement of this G-protein in the stimulation of phospholipase C. Furthermore, cholera and pertussis toxins modulated the stimulation effect of transducin in accordance with their inhibitory influence on G-proteins .

The action of light on phosphoinositide synthesis appears to be due to stimulation of the enzymes responsible for the consecutive formation of PtdInsP and $PtdInsP_2$ from PtdIns. It implies that several phosphokinases are strongly associated with photoreceptor membrane since the synthesis reaction is still occurring after repetitive hypotonic washes of the membranes. Unfortunately, these data are in contradiction with those obtained by Gehm and McConnel (1990) and Panfoli, Morelli and Pepe (1990) that can be explained by partial bleaching of the photoreceptor membranes in the experiments of these authors. Moreover, Gehm and McConnel (1990) used exogenous [^{3}H] $PtdInsP_2$ in liposomes as a substrate for phospholipase C. Taking into account the existence of at least two kinds of phospholipases C (membrane-bound and soluble) in ROS, $PtdInsP_2$ could be hydrolyzed by an enzyme of the second type which is presumably non-sensitive to light action because it has no connection with rhodopsin.

Thus, it can be concluded that the disk membranes contain the autonomous system of light-sensitive phosphoinositide turnover which produces the biologically active products, $InsP_3$ and DAG.

βγ-TRANSDUCIN MEDIATES THE LIGHT REGULATION OF PHOSPHOINOSITIDE TURNOVER

The following experiments were done to study the role of transducin subunits in light control of phosphoinositide turnover. Fig.4ab demonstrates the influence of light, Tα and Tβγ on the $PtdInsP_2$ hydrolysis in dark-adapted and bleached ROS. The amount of $PtdInsP_2$ in dark-adapted ROS is decreased after adding GTP and Tβγ. On the contrary, Tαβγ when added to ROS in the dark with-

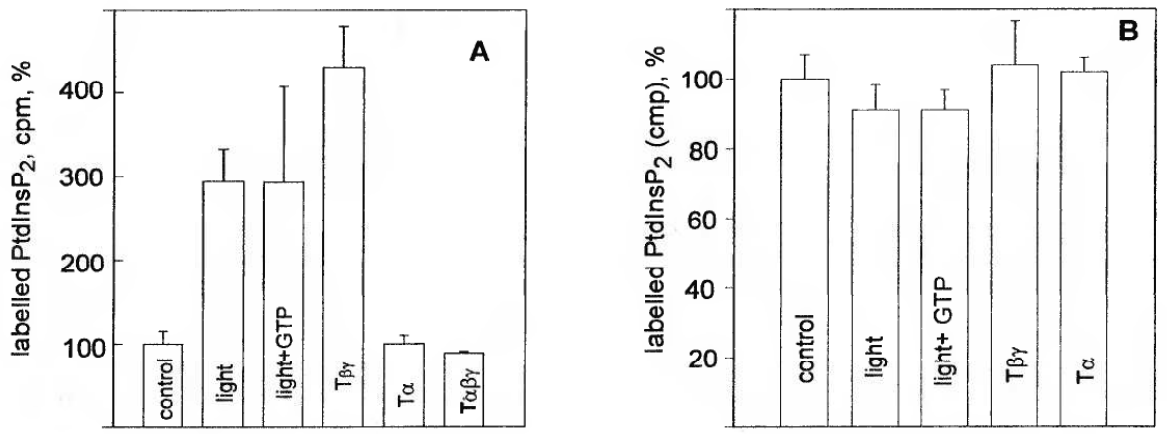

Fig. 6.Effect of light, GTP (100 μM), transducin (0.02 mg/ml) and its subunits (0.01 mg/ml) on $PtdInsP_2$ synthesis in ROS (A) and washed ROS (B) as measured using TLC separation and scintillation counting. (Grigorjev *et al.*, 1995).

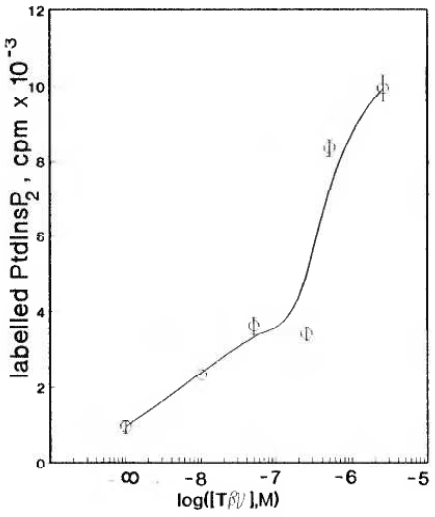

Fig. 7. The dependence of the ^{32}P incorporation into PIP_2 in dark-adapted ROS on Tβγ concentration (Grigorjev *et al.*, 1995).

out any GTP did influence the ^{32}P incorporation to a smaller extent than Tβγ. Tα GDP slightly inhibited the stimulation caused by GTP and did not exert any stimulating effect on the $PtdInsP_2$ hydrolysis when it was added to ROS in the dark. These facts mean that the only stimulator of $PtdInsP_2$ hydrolysis is Tβγ (Fig.4a). Therefore, the $PtdInsP_2$ hydrolysis was enhanced by Tβγ even in the dark, and light-induced stimulation was reduced in the presence of GDP-liganded Tα. In contrast, Tαβγ did not reduced the stimulating effect of light as compared with the effect of Tα. In fact, both illumination of ROS suspension, and light with GTP mimicked the action of Tβγ stimulation of the $PtdInsP_2$ hydrolysis (Fig. 4b). Moreover, the addition of Tβγ, but not of Tα, to bleached ROS did not lead to a decrease in the hydrolysis. The light stimulation of $PtdInsP_2$ hydrolysis can be explained by dissociation of Tαβγ into two components, Tα and Tβγ, under the action of bleached rhodopsin and GTP. In the presence of GTP an additional increase in the light stimulation of the $PtdInsP_2$ hydrolysis was detected (Fig.4b) since exogenous GTP facilitated the complete breakdown of Tαβγ and Tβγ formation which occurred after the ROS illumination and exchange of GDP for GTP in Tα·GDP complex (Stryer, 1991). On the contrary, Tα·GDP -inhibited the activating effect because it bound Tβγ to reassociate Tαβγ. The addition of Tβγ to the suspension of bleached ROS resulted in an additional stimulation of the hydrolysis as compared to the light effect (Fig.4b). Unfortunately, it remains unclear why the efficacy of light+GTP in hydrolysis stimulation was higher than that of light+Tβγ (Fig. 4b, columns 3 and 4). Perhaps, the amount of endogenous Tβγ was sufficient to attain a saturating effect. Actually, the rhodopsin concentration in the samples was about 100 μM. and that of endogenous transducin was as low as 10 μM.

The action of Tβγ on $PtdInsP_2$ hydrolysis in ROS is consistent with the data by Camps *et al.* (1992), Katz, Wu and Simon (1992) and Carozzi, Camps, Gierchik and Parker (1992), Camps, Carozzi, Schnabel, Scheer, Parker & Gierschik (1992) have shown that both Gβγ and Tβγ stimulated phosphoinositide phospholipase C activity (presumably phospholipase $C\beta_2$) derived from HL60 cells. Similar effect was observed with the solubilized enzyme (Camps *et al.*, 1992). Tβγ was also reported to stimulate phospholipase A_2 in ROS (Jelsema & Axelrod, 1978).

Fig. 5 presents the influence of Tβγ concentration on the $PtdInsP_2$ hydrolysis in dark-adapted ROS. All conditions for this experiment were as described in Fig.4 legends. Using the curve obtained, the EC_{50} for the $PtdInsP_2$ hydrolysis was estimated to be $1.25 \cdot 10^{-7}$ M which is consistent with the EC_{50} determined for phospholipase $C\beta_2$ stimulation as opposed to the stimulation of phospholipase $C\beta_1$ by nM concentration of $G\alpha_q$ (Sternwise & Smrcka, 1992). Moreover, this EC_{50} was similar to the concentration of Tβγ required to control bleached rhodopsin-catalyzed binding of GTP to Tα (Fawzi, Fay, Murphy, Tamir, Erdos & Northup, 1991). These data strengthen the above conclusion that $PtdInsP_2$ hydrolysis in ROS is catalysed by phospholipase C belonging to β_2 family since the ^{32}P incorporation is decreased by Tβγ within the range of concentrations required for the interaction of Tβγ with GDP-liganded Tα (Fawzi *et al.*, 1991). Thus, both Tα and Tβγ appear to be involved in light regulation of the activity of the appropriate enzymes, phosphodiesterase and phosphoinositidase (phospholipase C). To study the influence of light, GTP, Tα and Tβγ on the $PtdInsP_2$ synthesis, both ROS and hypotonically washed ROS were used. As it was already stated the washed ROS avoided any kind of cytoplasmic (phospholipase C) and disc peripheral membrane proteins. ADP-ribosylation of washed ROS by pertussis toxin did not also show the presence of membrane-bound Tα.

The illumination, GTP and Tβγ, but again not Tα·GDP or Tαβγ, stimulated $PtdInsP_2$ synthesis in intact ROS as compared with dark control (Fig.6a). Absolutely different results were obtained with washed ROS (Fig.6b). The effect of light stimulation disappeared and neither GTP nor Tβγ did stimulate ^{32}P incorporation into $PtdInsP_2$. The comparative analysis of data presented in Figs. 4 and 6 shows a difference in the degrees of Tβγ modulation of $PtdInsP_2$ hydrolysis and synthesis (65 and 400%, respectively). It means that Tβγ appears to stimulate two different target enzymes responsible for both hydrolysis and synthesis of $PtdInsP_2$, PtdInsP phospholipase C and phosphatidylinositol 4-monophosphate 5-Chinese. It also seems likely that Tβγ could stimulate a previous (before PtdInsP) step of $PtdInsP_2$ formation since the ^{32}P incorporation into PtdInsP was also under control of light. But this stimulation was negligible. Another important conclusion which comes from the data on the ^{32}P incorporation into $PtdInsP_2$ in washed ROS is that the enzyme catalysing this reaction is strongly membrane-bound. Actually, ^{32}P incorporation into $PtdInsP_2$ was observed after the wash of all peripheral proteins out of the disc membrane. However, the stimulation of this enzyme by Tβγ appears to be related to a additional factor(s) removed during the hypotonic wash of ROS, after which neither light nor Tβγ reveal any stimulating action on the PtdInsP synthesis (Fig.6b).

Fig. 7 shows the dependence of the ^{32}P incorporation into $PtdInsP_2$ on the Tβγ concentration. As in the case for the hydrolysis the EC_{50} for stimulation of ^{32}P incorporation into $PtdInsP_2$ was about $2.5 \cdot 10^{-7}$ M which is also consistent with the sensitivity of the effector phospholipase C to the stimulation action of Tβγ (Sternweis & Smrcka, 1992). What must be emphasised, however, is that Tβγ stimulation of $PtdInsP_2$ hydrolysis and synthesis did not occur when the ROS suspension was saturated by Tα·GDP and, thus, was not a factor in these situa-tions.

According to generally agreed view βγ dimer of G-proteins regulates the different effectors. For example, it was shown that Gβγ controls the activities of K^+ channels (Lee *et al.*, 1992), phospholipase A_2 (Jelsema & Axelrod, 1987), pheromone responce (Whiteway, Hougan & Dignard, 1989), phospholipase $C\beta_{1-3}$ (Camps *et al.*, 1992), kinases of muscarinic (Haga & Haga, 1992) and β-adrenergic receptors (Pitcher, Inglese & Higgins, 1992). These findings testified that activated G proteins seem to produce a bifurcating signal allowing for both subtle regulation and complex feedback control of the effectors and receptors. Actually, some effectors are regulated by Gα only, Gβγ only , by Gα and Gβγ separately or by Gα and Gβγ together that can produce divergent signals.

Unfortunately, besides our work there are no more data concerning transducin participation in mediating of the light action on ROS phospholipase C. Nevertheless, it is likely that situation described above also exists in visual cells. Two effector systems, cGMP phosphodiesterase and phospholipase C of ROS are sensitive to light action by virtue of Tα(Gα) and Tβγ(Gβγ) mediating its regulatory effect.

The findings of this article elucidated the relationship between phototransduction and phosphoinositide pathways and possible interaction between them on the way (Fig. 8). Thus, light through dissociation of Tαβγ into Tα and Tβγ, is capable of influencing the cGMP and phosphatidylinositide pathways, respectively. The hydrolysis of $PtdInsP_2$ seems to generate two second messengers, $InsP_3$ and DAG to mobilize Ca^{2+} within the visual cell

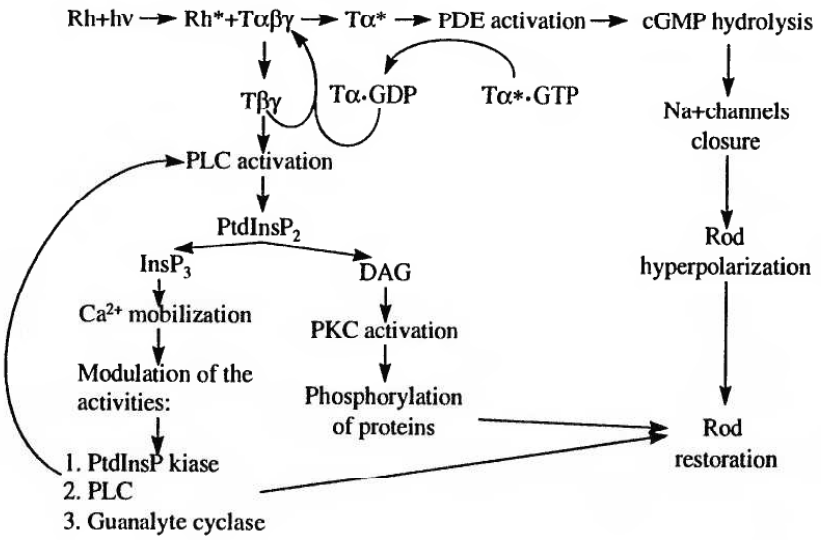

Fig. 8 The possible cross-talk between cGMP and phosphoinositide cascades.

and to activate protein kinase C. The Tβγ involvement into the $PtdInsP_2$ breakdown also explains why the hydrolysis reachs only a certain level. The reason is that Tα·GDP was formed after GTP hydrolysis on Tα. It is tempting to speculate that the affinity of Tβγ for Tα·GDP is much higher than that for $PtdInsP_2$ phospholipase C. After the phosphodiesterase activation, the Tα·GDP formed appears to fulfil the function of terminating the $PtdInsP_2$ hydrolysis. If it is the case, Tαβγ can be considered as the bifurcation point at which a primary signal from rhodopsin is split into two to take part in the light activation of two enzyme cascades with a possible cross-talk between them. This is very likely, and can be inferred from the phosphorylation of rhodopsin and ROS phosphodiesterase by protein kinase C as it was indicated by Udovichenko *et al.* (1994). Furthermore, Tβγ appears to induce through $InsP_3$ a release of Ca^{2+} from the internal stores to control indirectly guanylate cyclase activity and, consequently, the light adaptation of the visual cell. Perhaps, the retinal discs may play a role of the Ca^{2+} stores since the calcium concentration within the discs is thought to be high (Szuts & Cone, 1977; Schroeder & Fain, 1984; Somlyo & Walz, 1985). Ratto, Payne, Owen and Tsien (1988) found that the discs contain up to 13% of the total dye in Fura2-loaded rod outer segments. Moreover, the disc membrane is likely to contain Ca^{2+}-dependent ATPase which facilitates the Ca^{2+} accumulation in the retinal discs (Baranova, Shejko, Volotovski, Konev, Pokudin & Orlov, 1984; Panfoli *et al.*, 1994).

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