Inositol Incorporation into Phosphoinositides in Retinal Horizontal Cells of *Xenopus laevis*: Enhancement by Acetylcholine, Inhibition by Glycine

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ABSTRACT The absorption of light by photoreceptor cells leads to an increased incorporation of [2-^{3}H]inositol into phosphoinositides of horizontal cells in the retina of *Xenopus laevis* in vitro. We have identified several retinal neurotransmitters that are involved in regulating this response. Incubation with glycine, the neurotransmitter of an interplexiform cell that has direct synaptic input onto horizontal cells, abolishes the light effect. This inhibition is reversed by preincubation with strychnine. Acetylcholine added to the culture medium enhances the incorporation of [2-^{3}H]inositol into phosphoinositides in horizontal cells when retinas are incubated in the dark. This effect is inhibited by preincubation with atropine. However, atropine alone does not inhibit the light-enhanced incorporation of [2-^{3}H]inositol into phosphoinositides in the retina. γ-Aminobutyric acid, the neurotransmitter of retinal horizontal cells in *X. laevis*, as well as dopamine and norepinephrine, have no effect on the incorporation of [2-^{3}H]inositol into phosphoinositides. These studies demonstrate that the light-enhanced incorporation of [2-^{3}H]inositol into phosphoinositides of retinal horizontal cells is regulated by specific neurotransmitters, and that there are probably several synaptic inputs into horizontal cells which control this process.

In some cells, the interaction of extracellular stimuli with specific receptors on the plasma membrane leads to the hydrolysis of phosphoinositides to 1,2-diglycerides and inositol phosphate(s). The physiological function of this hydrolysis is still unknown, although it is most often found in cells involved in stimulus-coupled secretion events in which Ca^{++} may participate as an internal messenger (1, 17). In neural tissues, this response was first observed as a stimulation by acetylcholine of the incorporation of $^{32}PO_4$ into phosphatidic acid and phosphoinositides in brain slices (13). However, other studies have shown that phosphoinositide hydrolysis is the initial event in both neural (7) and non-neural (2, 21) tissues, and the apparent increase in label incorporation is a recovery reaction. Since it is difficult to demonstrate phosphoinositide hydrolysis in complex tissues such as the pineal (30), brain (1, 17), and retina (3, 5, 27), the recovery reaction offers a convenient way to study stimulus-mediated changes in phosphoinositide metabolism, especially when autoradiography is used to identify specific neurons involved in the response (3, 5).

In 1981, we reported that light is a stimulus for this "phosphoinositide effect" in the retina of the clawed toad *Xenopus laevis* (3). The cell type involved in the response was identified by light and electron microscope autoradiography to be the horizontal cell (3, 5). Because the stimulus (light) is absorbed by visual pigments in the outer segments of the photoreceptor cell, and the response occurs in a second-order neuron, information transfer between these two cells must be involved in initiating the phosphoinositide effect. To identify the neurotransmitters that mediate this information exchange, we evaluated the effect of several putative retinal neurotransmitters on this response.

MATERIALS AND METHODS

Animals: *X. laevis* juvenile toads (snout-vent length 5–6 cm) were maintained in a constant temperature incubator on a 12-h light:12-h dark cycle at 21°C for at least 1 wk before any experiment. Animals were killed in the dark just before the normal onset of light, and retinas were dissected under dim red light and pooled. Dissections usually took 30–45 min, and the retinas were maintained in total darkness in cold incubation media during this period. These retinas were randomly divided into groups of at least six retinas each and transferred to the appropriate incubation media in a large bore capillary pipet.

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Care was taken that the retinas were never subjected to the shear forces of an air–aqueous interface.

Incubation Conditions: Retinas were incubated at 21°C for 2 h in a medium containing three parts of a modified Ringer's–bicarbonate–pyruvate and one part Wolf–Quemby amphibian tissue culture medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). Ringer's–bicarbonate–pyruvate media contained 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM pyruvate. This combined media was found to give better preservation of tissue for morphological studies than Ringer's–bicarbonate–pyruvate alone. The incubation medium was gassed with 95% O_2:5% CO_2 in the cold for 10–15 min before use. Incubations were carried out in translucent plastic tubes or in tubes that had been double wrapped with black electrical tape. Each tube was continuously gassed during the period of incubation (1 ft^3/h per 20 cm^2 of surface area). The usual incubation contained 1 ml of media, *myo*-[2-^{3}H]inositol (Amersham Corp., Arlington Heights, IL, 16.4 Ci/mmol, 1.2 μM, 20 μCi/ml), and the appropriate agonist/antagonist. The light stimulus was an incandescent bulb which yielded 20 footcandles illumination at the surface of the incubation bath. Further explanation of the incubation conditions is given in the figure legends.

Lipid Procedures: Incubations were terminated by the addition of ice-cold trichloroacetic acid to give a final concentration of 10%. Individual retinas were removed and homogenized with chloroform/methanol/concentrated HCl (100:100:6, by volume), followed by chloroform:methanol (2:1, vol/vol). The combined extracts were shaken with 0.2 vol of 1 N HCl. The lower lipid phase was washed three times with synthetic upper phase (chloroform/methanol/0.2 N HCl; 3:47:50, by volume), evaporated, and made up to a known volume with chloroform/methanol/water (9:7:2, by volume). Aliquots were removed for phosphorus assay (24) and for the determination of radioactivity. Radioactivity is expressed as disintegrations per minute (DPM)[1] per microgram of total lipid phosphorus. In some experiments, lipids were separated by thin-layer chromatography and identified and counted as previously described (5).

Light Microscope Autoradiography: In some experiments, retinas incubated for 2 h were fixed in 2% formaldehyde–2% glutaraldehyde in cold 0.1 M cacodylate buffer containing 0.025% calcium chloride. Tissue was processed for autoradiography, using a procedure described previously (5), which had been modified from the one of Gould and Dawson (10). We have determined that <5% of lipids labeled with [^{3}H]inositol are extracted during fixation and imbedding.

Quantitative grain count analysis of specific retinal lamina was made with a Zeiss Videoplan image analysis system (Carl Zeiss, Inc., New York). At least 20 different regions per retina from three to five retinas were counted for each experimental variable. The data are expressed as grain counts per unit area of retina.

Statistical Analysis: Biochemical experiments contained at least six individual retinas per treatment group. Mean values and standard deviations were calculated from the specific activity of single retinas from several experiments, and comparisons were made with other treatment groups by a two-tailed Student's *t* test. The data are expressed in the histograms as mean DPM per microgram of lipid phosphorus ± standard error of the mean.

RESULTS

Phosphoinositide Labeling in Response to Light

Light enhanced the incorporation of [2-^{3}H]inositol into phosphoinositides in the retina of *X. laevis*. The light microscope autoradiograms in Figs. 1 and 2 show the incorporation of label into lipid. Silver grains indicative of radioactive phosphoinositides are present in all layers of the retina but are more concentrated in the horizontal cells (arrows) in retinas incubated in the light (Fig. 1) compared with those incubated in the dark (Fig. 2). Previous quantitative studies have shown that the horizontal cells are the only retinal cells that show a light-dark difference in lipid labeling from [2-^{3}H]inositol (3, 5). We have also determined previously that none of the radioactive inositol is incorporated into protein or nonphosphoinositide lipids in these in vitro experiments, so the silver grains are representative of labeling in phosphoinositides (5).

The autoradiography data are supported by biochemical studies showing an increase in incorporation of [2-^{3}H]inositol into retinal phosphoinositides in the light compared to darkness (Fig. 3). The light values averaged 38% higher than the dark values, although there was no effect of light on the uptake of inositol into the whole retina. Thin-layer chromatography of lipid extracts from each of eight retinas incubated for 2 h in light or darkness showed that the label was present in phosphatidylinositol (85%), phosphatidylinositol 4-phosphate (3%), and phosphatidylinositol 4,5-bisphosphate (12%). Light stimulated the labeling of all three phosphoinositides.

Labeling of Phosphoinositides in Response to Glycine

Addition of glycine (final concentration 100 μM) to the incubation media resulted in an inhibition of the light-enhanced incorporation of [2-^{3}H]inositol into retinal phosphoinositides. As shown in Fig. 3, the radioactivity of phospholipids of retinas incubated in light in the presence of glycine was the same as that of retinas incubated in the dark with or without glycine. The addition of strychnine (final concentration 20 μM), a specific antagonist of glycine, prevented the glycine inhibition of the light effect (Fig. 4).

Labeling of Phosphoinositides in Response to Acetylcholine

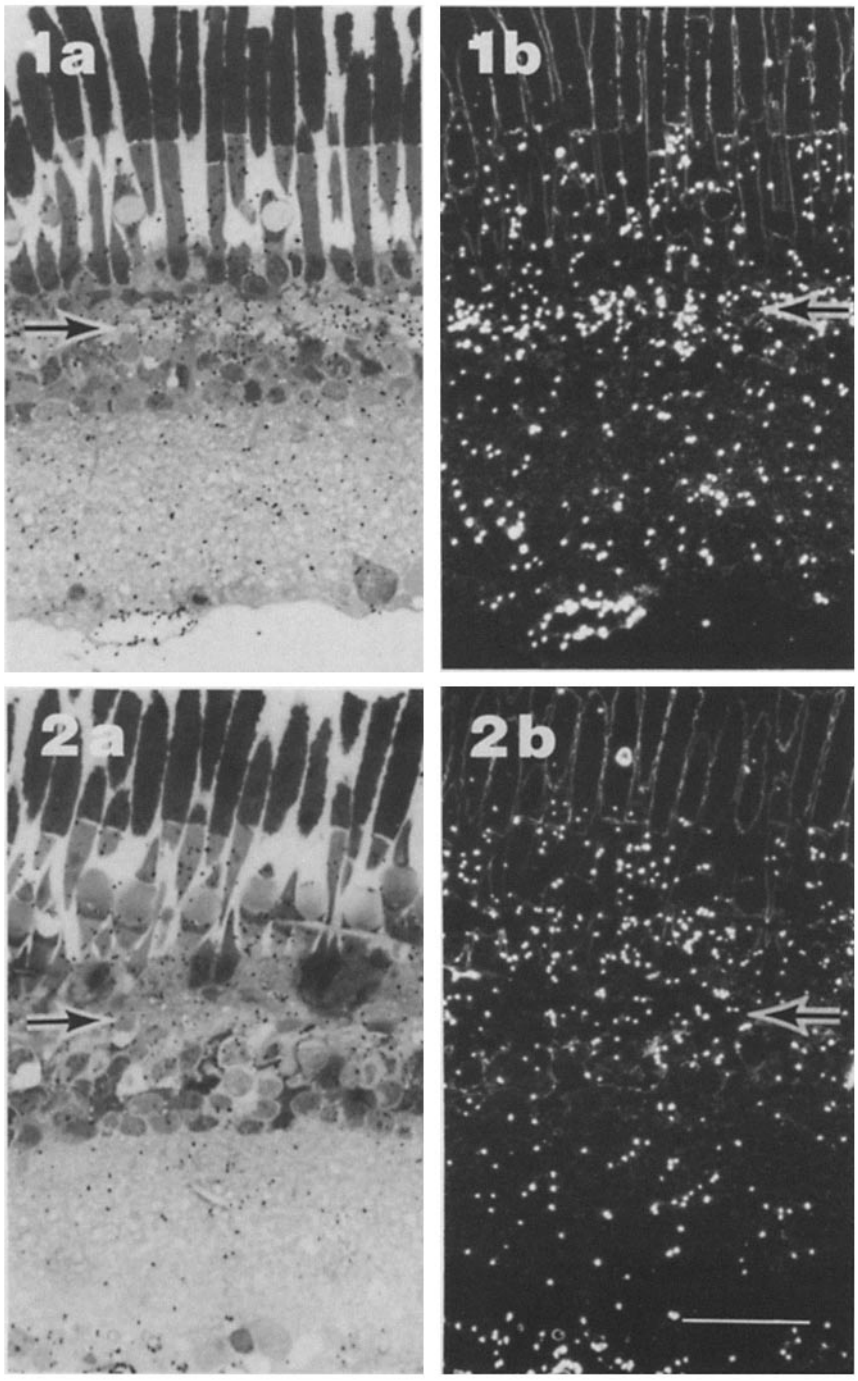
Retinas incubated in the dark with 50 μM acetylcholine incorporated significantly more [2-^{3}H]inositol into phosphoinositides than retinas incubated in the dark (Fig. 5). In the presence of 20 μM atropine, a specific antagonist of acetylcholine at muscarinic receptors, the incorporation of label into lipid returned to dark values. The grain count distribution of [2-^{3}H]inositol in lipids after 2 h of incubation in the dark in standard media or in media containing 50 μM acetylcholine is shown in Fig. 6. Significant labeling of phosphoinositides occurred throughout the retina. However, there is an increase in silver grains over the horizontal cells in the retinas incubated with acetylcholine. This labeling pattern is identical to that seen with light stimulation (Figs. 1 and 2; references 3 and 5).

Atropine alone does not inhibit the light effect. In the presence of 20 μM atropine, there was a significant increase in incorporation of [2-^{3}H]inositol into phosphoinositides in the light (2,694 ± 529, $n = 22$) compared with retinas incubated in the dark (1,787 ± 433, $n = 35$) (DPM ± SD/μg of lipid phosphorus, $P < 0.001$).

Labeling of Phosphoinositides in Response to γ-Aminobutyric Acid (GABA), Dopamine, and Norepinephrine

GABA, the neurotransmitter of the horizontal cell in *X. laevis* (14), has no effect on the incorporation of [2-^{3}H]inositol in phosphoinositides. As seen in Fig. 7, there is no significant difference in the incorporation of label into lipids of retinas incubated in the light with or without 100 μM GABA. The incubation of retinas for 2 h in light or dark with 100 μM dopamine or norepinephrine showed no effects of these compounds on the incorporation of [2-^{3}H]inositol into retinal phosphoinositides.

[1] *Abbreviations used in this paper:* DPM, disintegrations per minute; GABA, γ-aminobutyric acid.



FIGURES 1 and 2 Light microscope autoradiograms of retinas of *Xenopus laevis* incubated for 2 h with [2-^{3}H]inositol in the light (Fig. 1) or in the dark (Fig. 2). The arrows point to the nuclei of horizontal cells. Dark-field autoradiograms of these same retinas are presented in Figs. 1*b* and 2*b*, respectively. Increased labeling of the lipids in horizontal cells in retinas incubated in the light is evident in these micrographs. Bar, 30 μm. × 570.

DISCUSSION

This and previous reports (3, 5) from our laboratory have described the effects of light on the biosynthesis of phosphoinositides in the retina of *X. laevis*. Light stimulates the incorporation of [2-^{3}H]inositol into all classes of phosphoinositides. After 2 h of incubation, the specific activity in phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate, determined on the basis of total lipid phosphorus, was significantly higher in retinas incubated in the light compared with dark-incubated controls. Most of the label was present in phosphatidylinositol, which is quantitatively the most abundant phosphoinositide. Incubation with $^{32}PO_4$ resulted in significant labeling in phosphatidic acid, phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate (5). These four lipid classes accounted for >90% of the radioactive lipids isolated from the retina. There was a significant increase in the radioactivity in each of these phospholipid classes, determined as DPM per microgram of total lipid phosphorus, isolated from retinas incubated in light compared to darkness. As was expected, the distribution of the label was somewhat

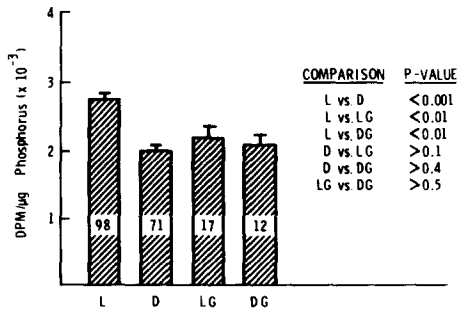


FIGURE 3 Histogram of DPM of label incorporated per microgram of total lipid phosphorus from retinas of *X. laevis* that had been incubated for 2 h with [2-^{3}H]inositol in light (*L*) or dark (*D*). In some experiments, 100 µM glycine was added just before light onset, and retinas were incubated for 2 h in light (*LG*) or dark (*DG*).

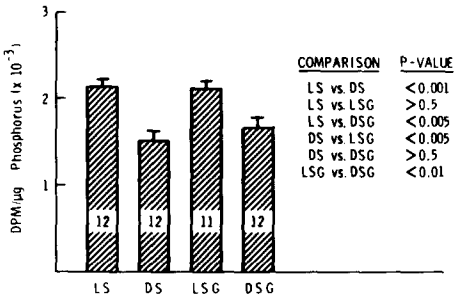


FIGURE 4 DPM of label incorporated per microgram of total lipid phosphorus of retinas preincubated for 15 min in the dark with 20 µM strychnine, and then incubated for 2 h with [2-^{3}H]inositol in light (*LS*) or dark (*DS*) in the presence (*LSG* and *DSG*) or absence of 100 µM glycine.

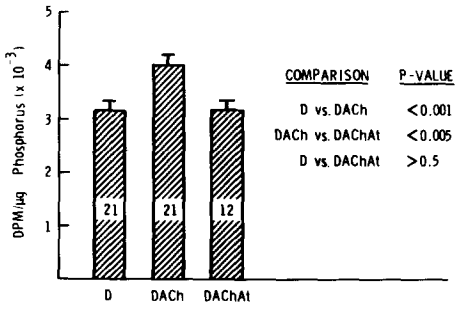


FIGURE 5 DPM of label incorporated per microgram of total lipid phosphorus of retinas incubated for 2 h with [2-^{3}H]inositol in the dark (*D*) with 50 µM acetylcholine (*DACh*) or with 50 µM ACh plus 20 µM atropine (*DAChAt*). Retinas were preincubated 15 min with atropine before the addition of ACh and inositol. All incubation tubes contained 50 µM eserine sulfate.

different when $^{32}PO_4$ was used; phosphatidylinositol 4,5-bisphosphate contained ~60% of the label, followed by phosphatidylinositol (17–18%), phosphatidylinositol 4-phosphate (13–15%), and phosphatidic acid (6–8%). Light had no effect on the incorporation of radioactive glycerol into any of the retinal glycerolipids (5). Neither was there an effect on the incorporation of ethanolamine, serine, or choline into their respective phospholipid classes (5). Thus, in the *Xenopus* retina, light does not stimulate *de novo* lipid biosynthesis. Rather, the effect of light is restricted to phosphoinositides and their precursor, phosphatidic acid. This pattern of phosphoinositide biosynthesis is similar to that observed in nu-

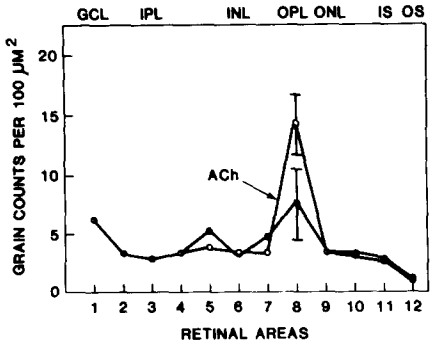


FIGURE 6 Grain count distributions in retinas incubated in the dark for 2 h with [2-^{3}H]inositol with and without 50 µM acetylcholine. *GCL*, ganglion cell layer; *IPL*, inner plexiform layer; *INL*, inner nuclear layer; *OPL*, outer plexiform layer; *ONL*, outer nuclear layer; *IS*, inner segment; and *OS*, outer segment.

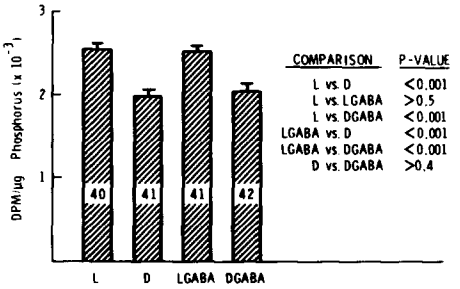


FIGURE 7 DPM of label incorporated per microgram of total lipid phosphorus of retinas incubated for 2 h with [2-^{3}H]inositol in the light (*L*) or dark (*D*) in the presence (LGABA, DGABA) or absence (*L* and *D*) of 100 µM GABA.

merous other tissues which respond to extracellular stimuli (15).

Although Hokin (12) demonstrated by autoradiography the acetylcholine-stimulated labeling of phosphoinositides with [2-^{3}H]inositol in cells of the superior cervical ganglion of the rat, the identity of specific cell types involved in stimulus-mediated phosphoinositide metabolism in neural tissues is not known. This is due in part to the heterogeneity of neuroneal cell types, the lack of information on synaptic circuitry, and difficulties in utilizing autoradiographic techniques which preserve lipid. We applied the autoradiographic technique of Gould and Dawson (10), which was designed for studies on lipid metabolism in myelin, for the study of phosphoinositide metabolism in the retina at both the light and the electron microscope autoradiographic level. After 2 h of incubation with [2-^{3}H]inositol in the light or the dark, there was a significant increase in silver grains in the light group over the outer plexiform layer. Although the outer plexiform layer is classically defined as comprising the outer neuropile of the retina consisting of the lateral extensions of horizontal cells, the bipolar dendrites, and the terminals of the photoreceptor cell in the *Xenopus* retina, the horizontal cell bodies are frequently entrapped within this neurite meshwork and are clearly outside of the area delineated as the inner nuclear layer. Horizontal cell bodies in this location are present in the photomicrographs presented in Figs. 1 and 2. Electron microscope autoradiography revealed unequivocally that the increased labeling was confined to the horizontal cells (5), thus establishing that the horizontal cell of the *Xenopus* retina responds to photon capture in the photoreceptor cell by in-

creasing the incorporation of radioactive inositol into phosphoinositides.

Since the stimulus (light) is absorbed by photopigments in the photoreceptors and the response is in a second-order neuron, intercellular communication between these neurons must have occurred, presumably by chemical neurotransmission. The effect of light on the photoreceptor cell is to hyperpolarize the cell (11, 20) and decrease the release of neurotransmitter at the photoreceptor synapse (23, 26, 31). In an earlier study (5), we observed that incubation of retinas in the dark with divalent cations known to decrease the photoreceptor release of neurotransmitter (Mn^{++}, Mg^{++}, and Co^{++}) resulted in an increased incorporation of [2-^{3}H]inositol into horizontal cell phosphoinositides. Ba^{++} and Ca^{++}, divalent cations that allow neurotransmitter release by exocitosis (18), had no effect on phospholipid labeling (5). The simple interpretation of these experiments is that a reduction in neurotransmitter release from photoreceptor terminals is the signal for stimulation of phosphoinositide metabolism in horizontal cells. This interpretation was supported by experiments which showed that Mg^{++} increased incorporation of [2-^{3}H]inositol and $^{32}PO_4$, but not of 2-^{3}H-glycerol (unpublished) or 3-^{3}H-serine into retinal phospholipids (5). Furthermore, light and electron microscope autoradiography showed the Mg^{++}-stimulated labeling with [^{3}H]inositol was primarily in retinal horizontal cells (5), although some increase in labeling was also observed in photoreceptor cells. Nevertheless, it was not possible in this study to distinguish between a direct effect of divalent cations on phospholipid biosynthesis and a specific effect mediated via inhibition of neurotransmitter release.

In the present study, we have identified two retinal neurotransmitters that affect phosphoinositide metabolism in the horizontal cell and several neurotransmitters that have no effect. GABA, the neurotransmitter of retinal horizontal cells in *Xenopus* (14), has no effect on phosphoinositide metabolism in this cell. Dopamine, which has been shown to stimulate adenylate cyclase in carp retinal horizontal cells (32, 34), has no effect on phosphoinositide metabolism. Likewise, norepinephrine, which is known to elicit a phosphoinositide response in other neural tissues (17, 30), was not effective in producing a phosphoinositide response in the retina.

The light-stimulated increase in phosphoinositide metabolism was inhibited when glycine was present in the incubation media. The light response could be elicited in the presence of glycine only if the retinas were preincubated with strychnine, a specific antagonist of glycine. Rayborn et al. (22) have previously shown that glycinergic interplexiform cells in *Xenopus* retinas have direct synaptic input onto horizontal cells. These cells release glycine when depolarized by K^+, and the release is blocked by Co^{++}. Thus, glycinergic interplexiform cells may act directly on the horizontal cells to control the phosphoinositide response. It is not known if glycine is released from these interplexiform cells in the light or in darkness. If glycine is released in the dark, the effect of light would be to remove the inhibition by decreasing glycine release. However, if this is true, we would expect that incubation of retinas in the dark in the presence of strychnine would result in an increase in [2-^{3}H]inositol incorporation into phospholipids similar to that seen in the light. This is not the case, so it seems most likely that glycine is released in response to light, and its physiological effect on the phosphoinositide response is to dampen the magnitude of the response, rather than to completely abolish it as was observed in these pharmacological studies on isolated retinas.

Acetylcholine, perhaps the most widely studied of all effectors of the phosphoinositide response in neural tissues, mimicked the light response when added to retinas incubated for 2 h in the dark with [2-^{3}H]inositol. This stimulation was inhibited by atropine, indicating that muscarinic receptors are involved. Acetylcholine-containing amacrine cells have been identified in rabbit retinas by Masland and Mills (16). Gerschenfeld and Piccolino (9) observed that 5 mM atropine hyperpolarized the L-type horizontal cell and diminished the light response in the turtle retina. Nicotinic antagonists were not effective, leading them to suggest that the response was due to muscarinic cholinergic receptors on the horizontal cells. Acetylcholine synthesis has been shown in *Xenopus* retinas (25), and we have preliminary data demonstrating acetylcholine receptors in homogenates of *Xenopus* retinas. However, we have no indication as to which cells in the *Xenopus* retina are cholinergic or whether they make direct synaptic contact with horizontal cells. Although acetylcholine stimulated the labeling of phosphoinositides in horizontal cells in the dark, incubation of retinas in concentrations of atropine which inhibited this response did not prevent the light stimulation of phosphoinositide labeling. Thus, there must be a pathway of communication between photoreceptor and horizontal cells that does not involve a cholinergic mechanism.

Glutamic and aspartic acids have been suggested as neurotransmitter candidates for cone photoreceptor cells in several vertebrate retinas (19, 28, 29). We tested both acidic amino acids in the experimental protocol used for glycine. If either were the photoreceptor transmitter in *Xenopus*, we would expect an inhibition of the light effect. However, the result of several incubations did not show any consistent effect on the light response. In view of our previous studies showing that both aspartate and glutamate block protein (4) and lipid (unpublished) synthesis in *Xenopus* neural retina, the results of our inositol labeling experiments are difficult to interpret. Resolution of the question of a role for these acidic amino acids in light-mediated inositol incorporation into horizontal cell phosphoinositides can best be made using isolated horizontal cells.

In neural tissues where phosphoinoside turnover is elicited by neurotransmitters, it is interesting to speculate that the lipid turnover is involved in neurotransmitter release. However, there are little data to support such a role, and it is now clear that the PI response is postsynaptic (8, 30). Thus, neurotransmitter-stimulated phosphoinositide turnover is most likely associated with cellular activities other than transmitter release. In horizontal cells from carp retina, the regulation of adenylate cyclase activity has been demonstrated to be mediated by input from dopaminergic interplexiform cells (6, 32, 34) as well as by vasoactive intestinal peptide (VIP) (15, 33). Since the effects of these two compounds on accumulation of cyclic AMP in isolated horizontal cells is not additive (12), it is likely that receptors for both are on the same cell. Furthermore, dopamine antagonists were ineffective in blocking vasoactive intestine peptide-stimulated accumulation of cyclic AMP in carp retina (33) and isolated horizontal cells (15), suggesting that dopamine and vasoactive intestine peptide receptors are separate and distinct. Thus, it is now clear that neurotransmitter input into horizontal cells regulates at least two identified metabolic activities. Dopamine and vasoactive intestinal peptide stimulate adenylate cyclase activity in the carp retina, while glycine, acetylcholine, and light

control phosphoinositide metabolism in *Xenopus* retina. The functional consequences of neurotransmitter control of these specific metabolic activities remain to be elucidated.

These data suggest that at least three inputs into *Xenopus* retinal horizontal cells control phosphoinositide metabolism: (*a*) direct inhibitory control via a glycinergic mechanism, (*b*) direct or indirect stimulatory control through some noncholinergic mechanism, and (*c*) direct or indirect stimulatory control through a cholinergic mechanism. Multiple inputs into a single cell controlling phosphoinositide metabolism is unprecedented. However, to our knowledge, these are the first studies that have attemped to evaluate a variety of presynaptic inputs which affect the phosphoinositide response in an identified neuron.

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