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Light-stimulated inositolphospholipid turnover in *Samanea saman* leaf pulvini

(phototransduction/inositol metabolism/phosphatidylinositol/phytochrome)

M. J. MORSE*, RICHARD C. CRAIN, AND RUTH L. SATTER

Department of Molecular and Cell Biology, U-42, University of Connecticut, Storrs, CT 06268

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ABSTRACT **Leaflets of *Samanea saman* open and close rhythmically, driven by an endogenous circadian clock. Light has a rapid, direct effect on the movements and also rephases the rhythm. We investigated whether light signals might be mediated by increased inositolphospholipid turnover, a mechanism for signal transduction that is widely utilized in animal systems. *Samanea* motor organs (pulvini) labeled with [^{3}H]inositol were irradiated briefly (5–30 sec) with white light, and membrane-localized phosphatidylinositol phosphates and their aqueous breakdown products, the inositol phosphates, were examined. After a 15-sec or longer light pulse, labeled phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate decreased and their labeled metabolic products inositol 1,4-bisphosphate and inositol 1,4,5-trisphosphate increased, changes characteristic of inositolphospholipid turnover. We conclude that inositolphospholipid turnover may act as a phototransduction mechanism in *Samanea* pulvini in a manner that is similar to that reported in animal systems.**

Inositolphospholipid turnover has proven to be a central biochemical mechanism for the transduction of neurotransmitter, hormone, and light signals in animal tissues (1). When an appropriate agonist interacts with its receptor, breakdown of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) into two active moieties, water-soluble inositol 1,4,5-trisphosphate (InsP_3) and membrane-bound diacylglycerol, is stimulated. The increase in InsP_3 occurs within seconds; steady-state levels are generally reestablished within a few minutes (2). During the period that levels of InsP_3 and diacylglycerol are elevated, each of these metabolites initiates a cascade of biochemical responses: InsP_3 mobilizes intracellular Ca^{2+}, and diacylglycerol activates protein kinase C. Both have regulatory functions. Increased intracellular free Ca^{2+} affects a large number of cell functions (3), while protein kinase C phosphorylates proteins and this, in turn, activates or inactivates additional enzymes (4, 5).

Recently, several investigators have suggested that inositolphospholipid turnover may also act as a signal-transduction mechanism in plants. All of the requisite metabolic intermediates [phosphatidylinositol phosphates (6–8), inositol phosphates (6–9), and protein kinase C (10)] have been isolated from plants. In addition, isolated plant membranes and protoplasts exhibit appropriate responses to exogenously supplied InsP_3—that is, increased Ca^{2+} efflux from carrot protoplasts (11) and from zucchini (12) and corn (13) microsomes. Alterations in the levels of inositol phosphates resulting from prolonged exposure to cytokinin have also been described (9). However, these changes may represent constitutive differences rather than the transient changes associated with signal transduction (2). Direct evidence for rapid inositolphospholipid turnover in plants in response to a brief stimulus has been lacking. We now present evidence that a short light treatment elicits a rapid change in inositolphospholipid-cycle components in the legume *Samanea saman*.

Samanea has paired leaflets that usually open in the light and close in the dark, although the movements persist with a circadian periodicity in the absence of environmental cues (14). Phytochrome is one of the pigments whose absorption of light affects leaflet movements. A brief pulse of red light that converts the Pr (red-absorbing) form of phytochrome to Pfr (the far-red-absorbing form) rephases the rhythm (15) and also promotes rapid leaflet closure at certain times in the circadian cycle (16). These phytochrome effects may be associated with an increase in intracellular free Ca^{2+}, as reported for phytochrome-mediated events in other plants (17, 18). Such an increase in free Ca^{2+} could result from accelerated inositolphospholipid turnover (1).

Leaflet movements in *Samanea* are dependent upon changes in the volume of cells on opposite sides of the pulvinus, in turn reflecting changes in the distribution of a number of ions, principally K^+ and Cl^- (14, 19). An excised pulvinus moves (bends and straightens) rhythmically and responds to light (19). Each pulvinus contains a circadian clock, the photoreceptors that interface with the clock, and the ions and metabolic machinery for effecting cell volume changes (14–16). The pulvinus also contains all of the components of the inositolphospholipid cycle (6).

MATERIALS AND METHODS

Plant Material. *S. saman* plants were grown in a control chamber with 16 hr light/8 hr dark cycles (cool white fluorescent lamps, photon flux density of 200 μmol·m^{-2}·sec^{-1}) at 26° ± 1.5°C. A single terminal secondary pulvinus (14) was used for each experimental treatment. Pulvini from the second to the fifth leaf, counting from the uppermost mature leaf, were used for all experiments. Manipulations conducted during the dark period were performed using dim green "safelight" [two 15-W cool white fluorescent tubes covered with two layers of green Roscolene celluloid, no. 874 (Roscoe Laboratories, Port Chester, NY)].

Experimental Protocol. The pulvinus and an attached 2.5-cm section of rachilla were excised at hour 4.5 of the dark period, and the cut end of the rachilla was incubated in distilled water (6, 15). Seventeen and one-half hours later (at hour 14 of the next light period), the water was replaced with a solution containing *myo*-[2(n)-^{3}H]inositol (60 μCi/ml; 1 μCi = 37 kBq), and the pulvinus was kept in this solution until the experiment ended 8 hr later. In most cases, the sample cups in which the pulvini were incubated were placed in a

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Abbreviations: PtdIns, phosphatidylinositol; PtdIns*P*, phosphatidylinositol 4-phosphate; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; InsP_2, inositol 1,4-bisphosphate; InsP_3, inositol 1,4,5-trisphosphate.

*To whom reprint requests should be addressed.

desiccator containing Drierite to maintain constant low humidity, which increased fluid uptake.

At hour 6 of the dark period, the experimental pulvini were exposed to white light [Reichert fiber-optics light source (EJA bulb, 150 W, 21 V), photon flux density of 300 μmol·m^{-2}·sec^{-1}] for 5–30 sec, while the control pulvini were kept in the dark. Each pulvinus with its attached rachilla was then rapidly frozen in liquid nitrogen, and the rachilla was broken off and discarded. The pulvinus was pulverized over dry ice with a mortar and pestle and then extracted with chloroform/methanol/3.4 M HCl (6:6:1, vol/vol) and separated into organic and aqueous phases, as described (6).

Separation and Identification of Inositolphospholipids and Inositol Phosphates. The organic phase was dried under nitrogen, dissolved in chloroform/methanol (1:1, vol/vol), and applied to a silica gel 60 (Merck) thin-layer plate and developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, vol/vol). Inositolphospholipid bands were identified by comparison with authentic phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns*P*), and phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) and then scraped into scintillation vials. Radioactivity was quantitated in 10 ml of Scintiverse E scintillation fluid (Fisher) after sonication of the silica gel powder in 1 ml of water.

The aqueous phase was either dried under nitrogen or lyophilized before reconstitution in water. The inositol phosphates were separated by Dowex anion-exchange column chromatography (Fig. 1) (6), or by HPLC using a Partisil SAX anion-exchange column (Figs. 2–4) (20, 21). Fractions were collected and radioactivity was quantitated by liquid scintillation counting.

Although light-induced changes in the labeled inositol profiles could be detected with either Dowex chromatography or HPLC, HPLC was preferable in that it gave baseline separation of the inositol phosphates from each other and from other inositol metabolites. Peaks separated by Dowex chromatography using a conventional four-step gradient represented incompletely resolved inositol metabolites. Because the InsP_3 peak obtained with HPLC was small, especially in extracts from dark controls, and because InsP_3 may be rapidly hydrolyzed to inositol bisphosphate (InsP_2) (2, 22), data in Figs. 3 and 4 are presented as the sum of InsP_2 plus InsP_3.

Presentation of Data. To normalize for variations in the tissue mass and in the uptake and incorporation of [^{3}H]-inositol in individual pulvini, we calculated the ratio of radioactivity (dpm) in PtdIns*P*, PtdInsP_2, or InsP_2 plus InsP_3 to the sum of the dpm in all of the inositolphospholipids (PtdIns plus PtdIns*P* plus PtdInsP_2). In Figs. 3 and 4, the dark controls were averaged, and their average value was designated as 100%. Values for light-stimulated pulvini are expressed as a percent of the dark control.

RESULTS

In our first experiments, the inositol phosphates were separated by Dowex anion-exchange chromatography, which is routinely used for separation and analysis of inositol phosphates (22). Inositol phosphates from pulvini that were irradiated with white light for 30 sec are compared with dark controls in Fig. 1. The dark controls exhibited minor variability. A 30-sec light pulse caused a substantial increase in the level of inositol phosphates that were coeluted with erythrocyte standards for InsP_2 and InsP_3 (6). However, the Dowex column did not give adequate baseline separation of the various metabolites, whereas HPLC did (21). Therefore, we separated the inositol phosphates on HPLC in our remaining experiments.

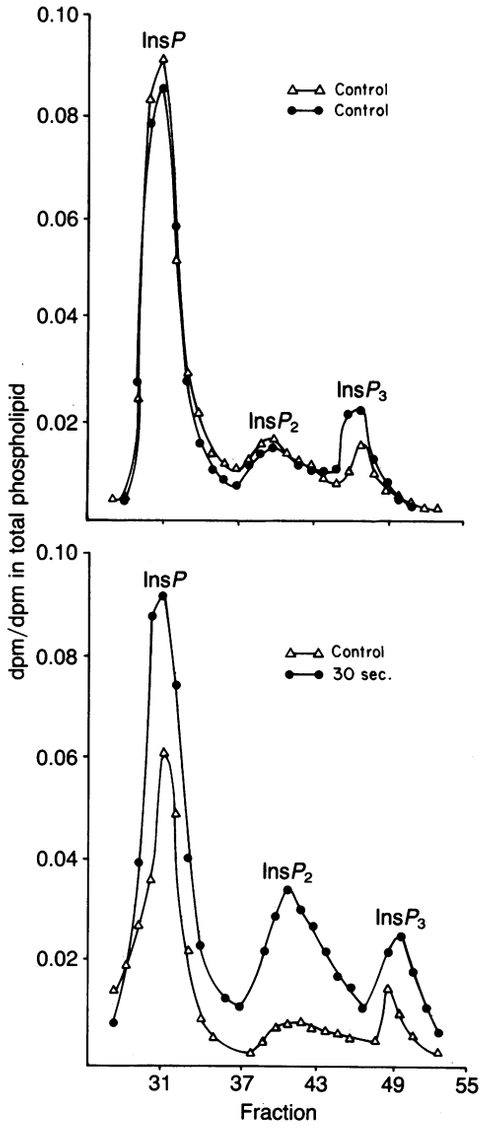

FIG. 1. Dowex anion-exchange chromatography of [^{3}H]inositol phosphates extracted from pulvini not exposed to light (control) or exposed to white light for 30 sec. Data are presented as dpm in each fraction normalized to dpm in the total phospholipid extract. Each experiment was repeated three or more times; typical results are shown. Ins*P* represents inositol monophosphate and other inositol metabolites. (*Upper*) Data from two dark controls, to test variability. (*Lower*) Data from a pulvinus exposed to 30 sec of light and from its dark control.

Fig. 2 shows representative InsP_2 and InsP_3 peaks from pulvini maintained in darkness for 6 hr with or without a subsequent 30-sec white light pulse. The light stimulus increased the levels of both InsP_2 and InsP_3. Fig. 2 *Inset* illustrates the entire HPLC profile from the light-stimulated pulvinus. The metabolites are well separated. The inositol phosphate peaks comigrate with authentic standards (21).

Light-stimulated changes in InsP_2 plus InsP_3 increased as the duration of the irradiation increased from 5 to 30 sec (Fig. 3). After a 30-sec exposure to light, the radioactivity in InsP_2 plus InsP_3 had increased by 30% relative to dark controls (statistically significant at $P < 0.01$).

Fig. 4 displays the amount of label in PtdIns*P* and PtdInsP_2 in light-stimulated pulvini as compared to dark controls. After 5–15 sec of light, PtdIns*P* was not affected significantly, whereas PtdInsP_2 had decreased to 76% of the control level. After 30 sec of light, PtdIns*P* had dropped to 72% of the

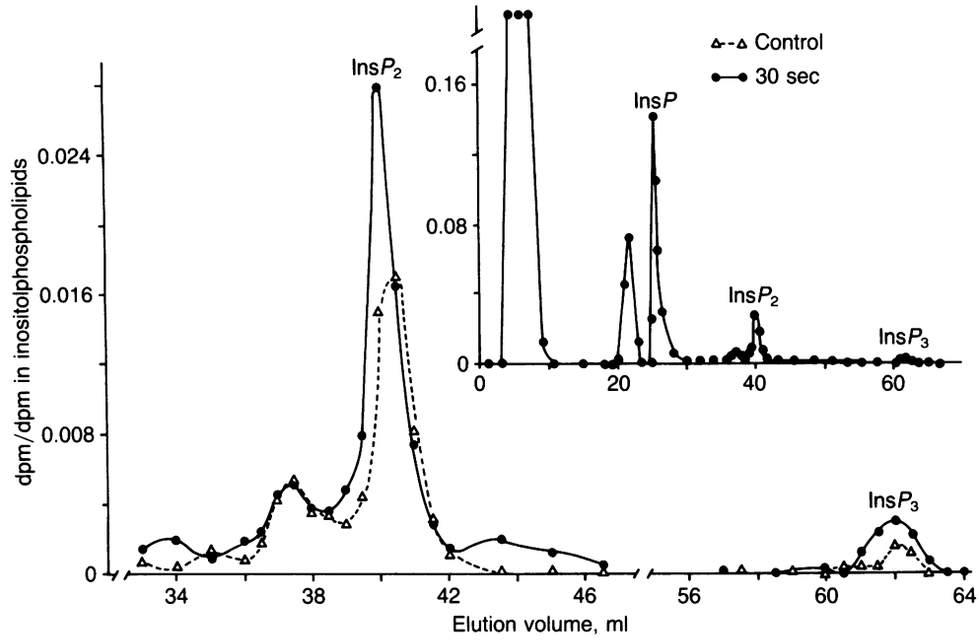

FIG. 2. Comparison of HPLC profiles of the labeled inositol phosphates InsP_2 and InsP_3 from a pulvinus exposed to a 30-sec pulse of white light and from its dark control. (*Inset*) Chromatographic separation of all ^{3}H-labeled compounds from the light-stimulated pulvinus. Ins*P*, inositol monophosphate. Data are normalized to dpm in total inositolphospholipids (PtdIns + PtdIns*P* + PtdInsP_2).

control level, while PtdInsP_2 had decreased still further, to 40% of the control level.

DISCUSSION

We have demonstrated that a brief (15- to 30-sec) white light pulse promotes inositolphospholipid turnover—i.e., decreases in membrane-localized phosphatidylinositol phosphates accompanied by increases in aqueous inositol phosphates. The 30% increase in InsP_2 plus InsP_3 that we detected is smaller than the increases reported for most animal systems (1, 2, 20). However, the pulvinus is a heterogeneous tissue composed of vascular, collenchyma, cortical, and epidermal tissue; furthermore, there are two populations of cortical motor cells (extensor and flexor) that exhibit different responses to light (19). It may be that the light-stimulated increase in InsP_2 plus InsP_3 occurs only in a select population of pulvinar cells and is much larger than 30% in these cells. We did not attempt to differentiate cell types before analyzing inositol phosphates but instead extracted the entire pulvinus, because injury might, of itself, act as a stimulus for inositolphospholipid turnover.

Our data support the possibility that inositolphospholipid turnover may be an early step in the catena of events that couples absorption of light to ion transport and other bio-

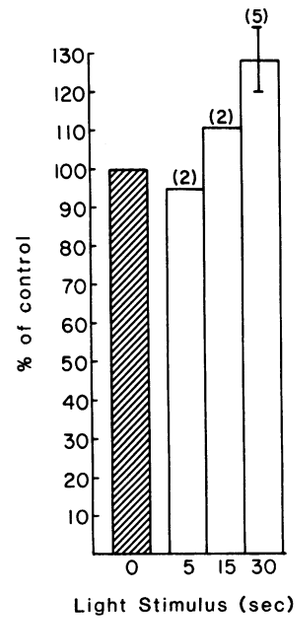

FIG. 3. Levels of ^{3}H-labeled InsP_2 plus InsP_3 in pulvini exposed to light for 5, 15, or 30 sec. Inositol phosphates were analyzed by HPLC. Radioactivity in InsP_2 plus InsP_3 were normalized to radioactivity in PtdIns plus PtdIns*P* plus PtdInsP_2 and are presented as a percent of the averaged control (0 sec) values, as described in *Materials and Methods*. Numbers in parentheses indicate number of samples (pulvini) analyzed.

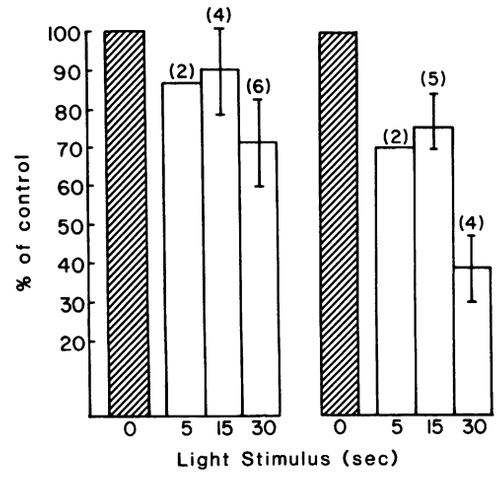

FIG. 4. Levels of ^{3}H-labeled PtdIns*P* (*Left*) and PtdInsP_2 (*Right*) in pulvini exposed to light for 5, 15, or 30 sec. Data were normalized to radioactivity in PtdIns plus PtdIns*P* plus PtdInsP_2 and are presented as a percent of the averaged control values, as described in *Materials and Methods*. Average control values for PtdIns, PtdIns*P*, and PtdInsP_2 were 26,750, 5520, and 690 dpm, respectively, representing 90% recovery of radiolabel applied to the TLC plate.

chemical responses that affect leaflet movement in *Samanea*. That a brief signal triggers inositolphospholipid turnover is reassuringly similar to the signal-transduction mechanism described in animal systems (1, 2). The inositolphospholipid cycle as a pathway for signal transduction appears to be very highly conserved. The details of the turnover cycle and metabolic constituents are the same in organisms ranging from invertebrates to mammalian vertebrates (1). It is tantalizing to speculate that this mechanism may be utilized as widely in plants, despite the fact that inositol metabolism is quite different in plants and animals. In plants, inositol functions not only in the inositolphospholipid cycle but also as an intermediate in both phytate and cell wall syntheses. Its multiple use does not preclude its function in signal transduction, although it adds a number of complexities to its metabolic regulation.

To understand the details and consequences of inositolphospholipid-turnover activation in *Samanea* pulvini, it will be necessary to identify the pigment whose absorption of light promotes this turnover. Phytochrome may be involved, since it is a photoreceptor for rhythmic phase shifting, and a brief red irradiation at hour 6 of the dark period (the time the light stimulus was presented) promotes a substantial phase shift (15). While red-light-promoted phase shifts may be potentiated by a transient signal-transduction mechanism, there also may be rhythmic changes in either the intermediates or the responsiveness of the inositolphospholipid cycle. The turnover response we observed occurs even more rapidly than the most rapid phytochrome-mediated event reported thus far for *Samanea* pulvini, the red-light-induced hyperpolarization of the membrane potential (23). Alternatively, a blue-absorbing pigment might be the photoreceptor; blue irradiation at hour 6 of the dark period promotes leaflet opening (24) and membrane depolarization. It is also possible that chlorophyll is the photoreceptor, although absorption of light by chlorophyll *decreases* cytoplasmic free Ca^{2+} in *Nitellopsis* (25), presumably by promoting active Ca^{2+} uptake into the chloroplast. In other systems, exogenously supplied InsP_3 stimulates Ca^{2+} flow down its electrochemical gradient (26). Clearly, it will be necessary to repeat our experiments with light of defined wavelengths to determine which pigment is involved.

It also will be important to ascertain whether the light-stimulated increases in InsP_2 plus InsP_3 result in increased intracellular free Ca^{2+}, which, acting alone or in concert with calmodulin, regulates a myriad of cellular activities (17, 18). In *Samanea*, the plasma membrane contains K^+-specific channels (27), which may respond to intracellular Ca^{2+}. Increases in cytosolic Ca^{2+} have been associated with changes in membrane conductance in other systems. Injection of InsP_3 into the *Limulus* ventral photoreceptor increases intracellular free Ca^{2+} and the conductance of ionic channels (28). This mimics the effect of light absorbed by rhodopsin, and inositolphospholipid turnover has been implicated as the phototransduction mechanism (29).

The discovery of agonist-stimulated inositolphospholipid turnover was an important breakthrough in understanding signal transduction and regulation in animals, and if it proves to be as ubiquitous in plants, it will represent a much more fundamental regulatory mechanism than previously appreciated.

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