FEBS Letters 356 (1994) 39–42

FEBS 14826

5-Hydroxytryptamine affects turnover of polyphosphoinositides in maize and stimulates nitrate reductase in the absence of light

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Received 23 October 1994

Abstract Incubation of etiolated maize leaves for 5 min in 5-hydroxytryptamine increased phosphatidylinositol-4,5-bisphosphate levels but on longer incubation its level decreased and a corresponding increase in inositol-trisphosphate was observed. The increase in phosphatidylinositol-4,5-bisphosphate by 5-hydroxytryptamine was similar to that obtained after short irradiation of leaves with red light. Nitrate-inducible and phytochrome-stimulated enzyme nitrate reductase could be stimulated in darkness if the leaves were incubated in the presence of nitrate and 5-hydroxytryptamine. These results indicate that one of the initial events in phytochrome-mediated enzyme stimulation could be through the generation of 'signals' from the turnover of the phosphoinositide cycle.

Key words: Nitrate reductase; Phytochrome; Polyphosphoinositide cycle; *Zea mays*

1. Introduction

Phytochrome regulates a large number of enzymes in plants [1,2] by mediating its effect at the level of transcription [3]. However, the mechanism of action of the active form of phytochrome (Pfr), especially the nature of the transmitter and signal transduction chain induced, is still largely unknown. In animal systems, turnover of polyphosphoinositides leads to the generation of second messengers [4]. It has been suggested that inositol phospholipid turnover may be involved in the signal transduction chain in plants [5–7], and the turnover of phoshatidyl inositol has been reported to be stimulated by exogenous factors [8–11]. It is thought that phosphoinositides may be important in signal transduction in plant cells by altering the cytoplasmic Ca^{2+} level, and the importance of involvement of Ca^{2+} in light-mediated processes has been reported [11,12].

It was shown earlier that Pfr affects Ca^{2+} uptake [13] and the polyphosphoinositide (PPI) cycle in maize [14]. In animal systems, the PPI cycle is affected by 5-hydroxytryptamine (5-HT) [15] and we reported that it also affects Ca^{2+} uptake in maize protoplasts [13]. In the present work the effect of 5-HT on the PPI cycle was studied and experiments were performed to check if 5-HT could replace the light requirement for the stimulation of an enzyme, nitrate reductase (NR).

2. Materials and methods

2.1. Materials

[^{32}P]Phosphate was purchased from Bhabha Atomic Research Centre, India. Both *myo*-[^{3}H]inositol and *myo*-[^{3}H]inositol-1,4,5-triphosphate (IP_3) assay systems were purchased from Amersham, UK. Chemicals and lipid standards were purchased from Sigma Chemical Co., USA. Maize seeds Ganga-5 (*Zea mays*) were purchased from National Seed Corporation, New Delhi, India.

2.2. Plant material and incubations with 5-HT

Seeds were grown in complete darkness and 8- to 9-day-old etiolated primary leaves were taken as starting material. Red light was obtained from 100 W tungsten lamps filtered through CBS 650 (Carolina Biological Supply, Co., USA) filter (λ_{max} 650 nm) as described earlier [16]. The intensity of red light was 1.47 $\mu W \cdot cm^{-2}$. All manipulations were done under green safe light (0.01 $W \cdot m^{-2}$) which was obtained by filtering the light from a cool fluorescent tube with several layers of green cellophane paper (λ_{max} 500 nm).

For treatments with 5-HT or irradiation with red light or incubation with [^{32}P]phosphate, respectively, primary opened etiolated leaves (8–9 days old) were used. The leaves were cut from the base (length of cut leaves was 4–5 cm) before giving any treatment. For labelling with *myo*-[^{3}H]inositol, leaves were cut into 0.5–1 cm pieces for better uptake. Incubations with 5-HT were done in HEPES buffer (10 mM, pH 7.5) at 25°C in darkness.

2.3. Extraction of lipids

Labelling, extraction and separation of lipids were done as described earlier [14]. For ^{32}P labelling experiments, the tissue was incubated for 90 min in 10 mM HEPES (pH 7.5) containing 500 μCi of the label (total volume 2 ml). For *myo*-[^{3}H]inositol experiments, the leaf pieces (0.5–1 cm) were incubated in 20 ml of 10 mM HEPES (pH 7.5) containing 200 μCi of the radiolabel. After labelling, the tissue was thoroughly washed with distilled water and used for further treatments. All manipulations were done under green safe light at 25°C.

2.4. Analysis of phosphoinositides

Following the different treatments, the reaction was terminated by rapidly freezing the tissue in liquid nitrogen. Lipids were extracted in a solution of chloroform:methanol:conc. HCl (33:66:1) in a 1:2 ratio (w/v). The extract was centrifuged at 5000 rpm for 30 min. KCl (2 M) was added to the supernatant and the upper white layer was removed. This process was repeated till turbidity disappeared. The chloroform layer was dried under nitrogen. The lipids were subsequently dissolved in a minimum amount of chloroform and the samples were applied to silica gel H plate impregnated with potassium oxalate. Loading was done both on an equal cpm and equal lipid basis. Chloroform:acetic acid:methanol:acetone:water (160:60:52:48:32) was used as the solvent to resolve the lipids [17]. Lipid standards of phosphatidylinositol, phosphatidylinositol-bisphosphate, phosphatidylinositol-trisphosphate, phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid, were run along with the samples to identify the spots. After chromatography, the plates were subjected to autoradiography using Indu X-ray films and high intensity screens. The identification of the phosphoinositide species was done by using unlabelled lipid standards. After labelling with *myo*-[^{3}H]inositol, silica gel was scraped from TLC plates in portions of 1 cm and counted in a LKB liquid scintillation counter.

2.5. Measurements of IP_3

IP_3 levels were measured after 5-HT treatment. The treated leaves were frozen in liquid nitrogen and the inositol phosphates were extracted by 15% ice-cold TCA (w/v). The proteins were sedimented by

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SSDI 0014-5793(94)01213-X

centrifugation at 2000 rpm for 15 min at 4°C. Supernatants were extracted three times with 10 vols. of water-saturated diethyl ether (cold) and titrated to pH 7.5 by the addition of 1 N NaOH and after the excess ether was boiled off. IP_3 measurements were done according to the *myo*-[^{3}H]inositol-1,4,5-triphosphate (IP_3) assay system as per the instructions provided by the supplier (Amersham, UK).

2.6. Extraction and assay of nitrate reductase (NR)

Induction, extraction and assay of NR was done as described previously [18]. Etiolated leaves of 8- to 9-day-old seedlings were excised and floated on nitrate solution (60 mM) for induction of NR. Excised etiolated leaves were subjected to red light irradiation and 5-HT treatment. After extraction of NR it was assayed in vitro as described earlier [18]. The assay mixture contained 1.5 ml of 0.1 M phosphate buffer (pH 7.5), 0.2 ml of 0.1 M KNO_3, and 0.2 ml of NADH (1 mg/ml). Nitrate reductase activity (NRA) is expressed as nmol nitrite produced $\cdot h^{-1} \cdot mg^{-1}$ protein. Protein was estimated according to Bradford's method [19]. Nitrate content was estimated by the zinc dust method [20].

2.7. Analysis of data presented

Experiments were done 2–3 times, in duplicate, and S.D. was calculated from three different independent experiments done in duplicate. Quantitation of radioactivity was performed by scanning the X-ray films using a Hirchmann Scanner (Germany) at 523 nm wavelength.

3. Results and discussion

3.1. Effect of 5-HT on phosphoinositides and IP_3

The tissue was prelabelled with either [^{32}P]phosphate or *myo*-[^{3}H]inositol and then incubated in 5-HT (30 mM) for specific time periods followed by lipid extraction. Fig. 1 shows the separation pattern of three phosphatidylinositol lipids along with others on TLC. Leaves that were treated with 5-HT for 5 min showed a significant increase in the incorporation of [^{32}P]phosphate in phosphatidylinositol-4,5-bisphosphate (PIP_2) compared to other inositol lipids. The level of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidic acid (PA) also increased. However, if the leaves were incubated in 5-HT for longer time periods (1 and 2 h), the level of PIP_2

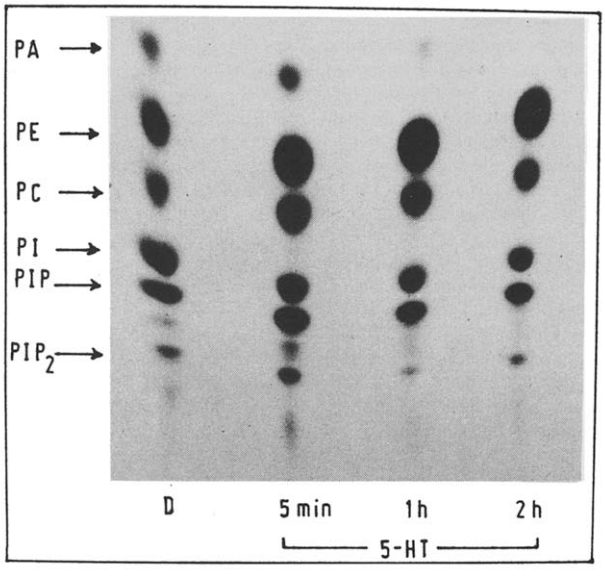

Fig. 1. An autoradiograph showing the separation of phosphoinositides by TLC. Cut leaves were incubated with 5-HT (30 mM) for 5 min, 1 h and 2 h in darkness and in the presence of [^{32}P]phosphate before being frozen. After separation, the TLC plates were exposed to X-ray films. D, dark without 5-HT.

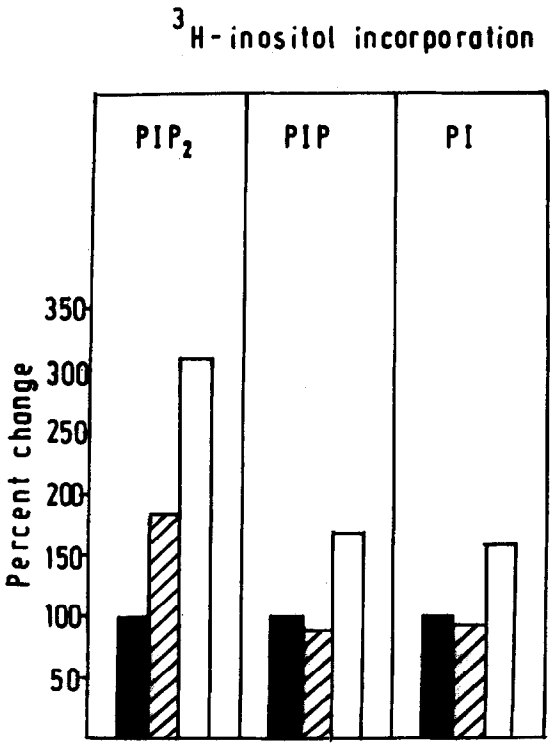

Fig. 2. Effect of short treatments with 5-HT (30 mM) on phosphoinositide levels. Etiolated cut leaves of 8- to 9-day-old plants were preincubated with *myo*-[^{3}H]inositol (10 μCi/ml) for 24 h. Lipids were extracted as described in section 2. Black bars, control (without 5-HT); hatched bars, 30 s (with 5-HT); open bars, 5 min (with 5-HT).

and also that of PA decreased. There was no significant changes in PE or PC levels. Clearly, short treatments with 5-HT increased the level of PIP_2. The level of PI increased only 18% but PIP and PIP_2 levels increased by 46% and 119%, respectively, as compared to untreated controls. To confirm this observation inositol labelling experiments were performed and 5-HT treatment was given for short time periods. Even a 30 s treatment resulted in an increase in PIP_2 levels as compared to the control (Fig. 2), and the increase in PIP_2 level after 5 min of 5-HT treatment was similar to the ^{32}P labelling experiment. Interestingly, the increase in the PIP_2 level after 5 min incubation of leaves with 5-HT was similar to that found in leaves irradiated with red light [14].

When leaves were treated with 5-HT for a shorter time period, the level of IP_3 increased by only 15%. However, longer treatments with 5-HT led to a decrease in the level of PIP_2 and an increase in that of IP_3 (Fig. 3). With treatments for time periods longer than 1 h, there was no further decrease in PIP_2 but a sharp decrease in the level of IP_3. This could be due to a faster turnover of IP_3 compared to PIP_2. The results show that 5-HT treatment initially leads to an increase in the level of PIP_2 which later decreases and results in the production of IP_3.

3.2. Stimulation of nitrate reductase activity (NRA) by 5-HT in the absence of light

Since treatment of leaves with 5-HT for short time periods resulted in an initial change in PIP_2 in a manner similar to red light irradiation [14], experiments were done to check whether

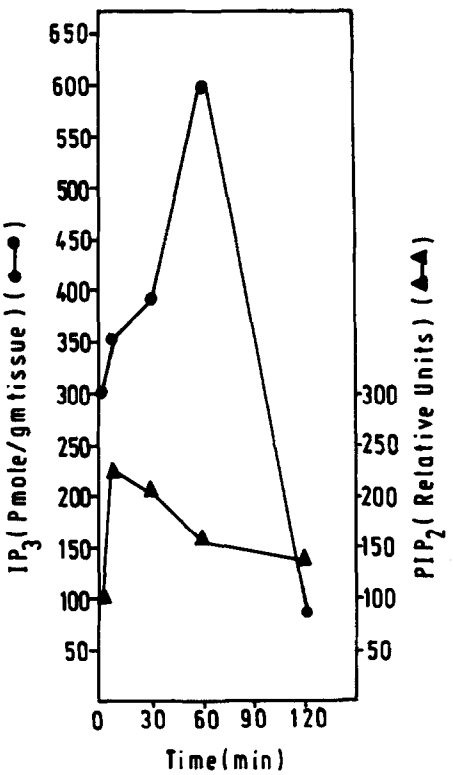

Fig. 3. Effect of 5-HT treatment on PIP_2 and IP_3 levels. Treatments were given as in Fig. 1.

5-HT could replace the requirement of light. To test this, stimulation of nitrate reductase (NR) by light was used as a model system [16]. In this system it was observed that when red light and nitrate were given together stimulation of NRA was less (specific activity, 30 nmol nitrite produced·h^{-1}·mg^{-1} protein) than when nitrate was given after a gap of 2 h of red light irradiation (specific activity, 140 nmol nitrite produced·h^{-1}·mg^{-1} protein). The effect of 5- HT was tested under both conditions and compared to the respective red light controls. Etiolated maize leaves were incubated with different concentrations of 5-HT (together with nitrate): 30 mM was found to stimulate NR to the same extent as was obtained with red light (Fig. 4). This showed that an enzyme that normally requires light for optimal stimulation could be stimulated by a neurotransmitter which has a similar initial action in the PPI cycle as Pfr. Under these conditions, the accumulation of nitrate was not affected by 5-HT (data not given).

3.3. 5-Hydroxytryptamine and Pfr do not stimulate nitrate reductase activity in an identical manner

It was found earlier [16] that the biochemical 'signal' generated by light, after converting Pr to Pfr, is amplified and remains stable for some time and can be utilized by nitrate for the stimulation of NR. When red light was given for 5 min and the plants were returned to darkness for more than 30 min, the red light effect was not reversed by far-red light: it escapes from photoreversibility. We have recently shown that active Pfr is not required for this process; in fact a transmitter with a lifetime of 12 h was produced and the formation of this transmitter was completed within 2 h [21]. As mentioned above, higher NRA was obtained when a 2 h dark period was introduced after red light irradiation and before nitrate addition. Therefore, experiments were done in a similar way with 5-HT to determine the effect of an ensuing dark period on NRA. The leaves were incubated in 5-HT for a short time and left in darkness in the absence of 5-HT for 2 h; subsequently, nitrate was given to stimulate NRA. The kinetics of the stimulation showed that NR was optimally stimulated by 5-HT after 4 h, whereas with red light irradiation the optimal NRA was higher and achieved at 12 h (Fig. 5). However, with 5-HT treatment, NRA also decreased at a faster rate as compared to red light. The stimulation of NRA by 5-HT was not sustained as long as was observed with red light irradiation (Fig. 5). It seems that in the ensuing dark period, the 'signal strength' was lost in 5-HT-treated leaves, NRA was stimulated much less after nitrate addition and it also decreased faster to reach the basal level. This suggests that Pfr may also initiate processes other than those mimicked by 5-HT which keeps the 'signal' stored for a longer period. We have shown earlier that PA levels decreased on exposure of leaves to 5 min of red light [14] whereas with 5-HT treatment (5 min) the PA levels did not decrease (Fig. 1).

3.4. Conclusion

We have shown that a neurotransmitter can affect the turnover of the PPI cycle in plants and induce the release of second messengers. This gives some clues to the functioning of neurotransmitters that have an effect in plants [22]. At the earliest time point at which we have measured the levels of PI, PIP and PIP_2 after short treatments with 5-HT, the level of PIP_2 increased relatively more than the other phosphoinositides and in a manner similar to that obtained after red light irradiation [14]. Since 5-HT stimulated NRA in the absence of light, the present data suggests that one of the initial actions of Pfr could be to initiate the release of messengers through the turnover of the PPI cycle.

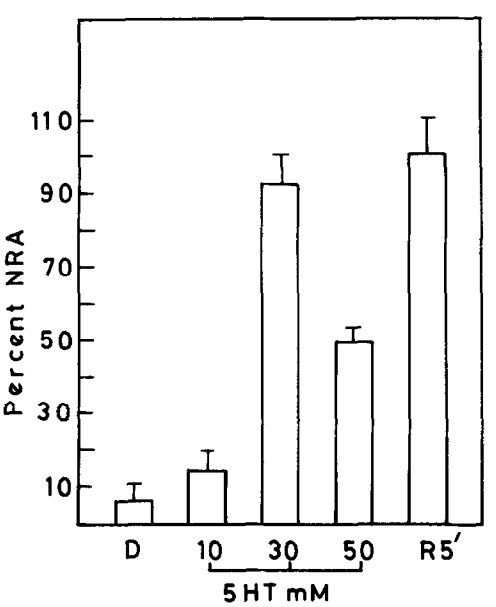

Fig. 4. Effect of different concentrations of 5-HT on NRA. Etiolated maize leaves were incubated in 5-HT together with KNO_3 in darkness and NRA was measured after 12 h. Red light irradiation, given for 5 min together with KNO_3, was taken as 100% control to calculate relative % NRA. The dark value of NRA was 1.1 nmol NO_2·h^{-1}·mg^{-1} protein.

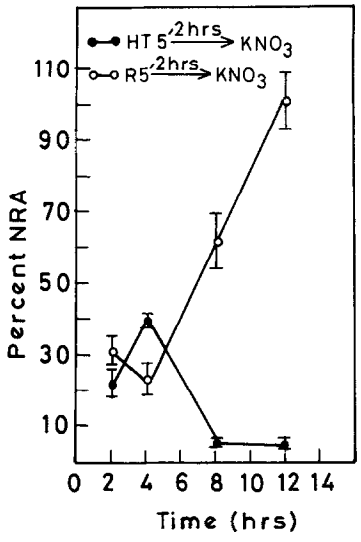

Fig. 5. Effect of introduction of a dark period afer 5-HT treatment and red light irradiation. Leaves were irradiated with red light for 5 min (○) or incubated in 5-HT (30 mM) for 5 min (●). After the treatment the leaves were kept for 2 h in darkness followed by nitrate addition.

In animal systems, phospholipase C activity results in an immediate decrease in PIP_2, resulting in an increase in messengers such as IP_3 and diacyl glycerol (DAG) [23]. Unlike this, the effect of 5-HT in plants and that of Pfr [14] seems to be at the level of PIP kinase/phosphatase to increase the level of PIP_2 which subsequently, through the action of phospholipase C, may lead to a release of second messengers to amplify the signals.

Whether Pfr affects the PPI cycle directly or through the involvement of other proteins, like G proteins [24], remains to be elucidated. Also the exact nature of signals released by 5-HT and Pfr and the mechanism of further signal transduction needs further investigation. We have found that phorbol myristate acetate (PMA), an activator of PKC-type enzymes, also replaces the light effect for the stimulation of NRA [25] and also at the gene expression level [21]. Therefore, besides IP_3, DAG could act as a second messenger in Pfr-mediated responses in plant systems.

Acknowledgements: M.R.C. is thankful to CSIR for a fellowship support. The research was supported through a research grant from the Department of Biotechnology and the Department of Science and Technology, Govt. of India.

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