The formation of inositol phosphate derivatives by isolated membranes from *Acer pseudoplatanus* is stimulated by guanine nucleotides

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Membranes isolated from *Acer pseudoplatanus* cell suspension cultures that were prelabelled with [^{3}H]inositol are able to release different water-soluble phosphoinositol derivatives in a time-dependent manner. Addition of adenine, cytidine or uridine nucleotide has no effects on the process. In contrast, guanine nucleotide triphosphates were stimulatory in a dose-dependent manner. The non-hydrolyzable derivative guanosine 5′-[γ-thio]triphosphate was the most effective activator while guanosine 5′[β-thio]diphosphate inhibited the action of GTP. It is suggested that GTP-binding proteins exist in the *Acer* membrane and play a regulatory role in the formation of potentially important second messengers.

Phosphoinositide *Phosphoinositol* *Second messenger* *G-protein* *Signal transduction*

1. INTRODUCTION

In animal cells, the binding of certain hormones, neurotransmitters or agonists to their receptor on the plasma membrane is associated with an increase in PI metabolism which leads to the production of DAG and IP_3 [1]. Both compounds are active in signal transmission via either the activation of protein kinase C [2] or the mobilization of calcium ions from internal stores [3]. Plants are known to contain Ca^{2+}-phospholipid-dependent protein kinases [4] and to actively metabolize the membrane-bound PI [5]. Moreover, IP_3 has been reported to induce the efflux of Ca^{2+} from plant microsomes preloaded with ^{45}Ca [6]. Therefore, the metabolism of PI may be part of the transduction system of stimulus effects in plants where calcium has been shown to play a significant role in the mediation of metabolic and developmental events [7]. However, little is known about the role of PI in the production of IP or its role in the coupling of a stimulus to a response.

Such a gap may be due to the fact that some plants contain phytic acid (inositol hexaphosphate) making it difficult to assess the origin of IPs with lower phosphorylation degree.

In this report, we describe experimental data which demonstrate the formation of IPs by isolated membranes of sycamore cell-suspension cultures which have been prelabelled by [^{3}H]-inositol and the control of the IP release by guanosine nucleotides. To the best of our

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Abbreviations: PI, phosphoinositide; IP, inositol phosphate; Gpp[NH]p, guanosine 5′-[β,γ-imido]triphosphate; GTPγS, guanosine 5′-[γ-thio]triphosphate; App[NH]p, adenosine 5′-[β,γ-imido]triphosphate; GDPβS, guanosine 5′-[β-thio]diphosphate; IP_3, inositol triphosphate; DAG, diacylglycerol

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knowledge this paper is the first to describe such a process in plants.

2. MATERIALS AND METHODS

2.1. *Chemicals and biochemicals*

All the chemicals and biochemicals are analytical grade. *myo*-[^{3}H]Inositol (16.5 Ci·mmol^{-1}) was from New England Nuclear; the nucleotides were purchased from Sigma, Dowex 1 × 10 (100–200 mesh, formate form) from Biorad. Other chemicals were from Merck.

2.2. *Labelling of plant material*

For each set of experiments, 400 μCi [^{3}H]inositol were injected into a flask containing 6-day-old *Acer pseudoplatanus* suspension cultures [8] and incubated in the dark for 60 h at 26°C. The labelled cells were collected by filtration and extensively washed with fresh culture medium.

2.3. *Membrane preparation and incubation*

Mixed membranes were prepared from labelled cells [10], the overall procedure was performed at 0–4°C. Briefly, 24 g cells were ground in the presence of 2.4 g acid washed sand and 2.4 g polyvinylpolypyrrolidone into 240 ml of ice-cold 0.3 M sorbitol, 50 mM Tris-Mes, pH 7.5 (buffer A). After filtration through a nylon net (60 μm), the solution was centrifuged 15 min at 10000 × g and the pellet discarded. The supernatant was centrifuged at 50000 × g for 30 min; the pellet was suspended in 50 ml of 25 mM Tris-Mes buffer, pH 6.5, containing 0.3 M mannitol (buffer B) and centrifuged again at 50000 × g for 30 min. The pellet was resuspended in 4 ml buffer B and potterized with a teflon pestle for 3 min.

The isolated membranes (1 mg protein) were incubated for 10 min at 30°C in the absence or presence of 20 mM LiCl. The membranes were divided into aliquots and allowed to stand at 30°C (slowly stirring) in the presence of the specified chemicals.

Aliquots were withdrawn at times 0–90 min and mixed with a stop solution containing 10% perchloric acid and 1 mg·ml^{-1} bovine serum albumin. The insoluble material was removed by centrifugation and the supernatant used for the analysis of IPs. Each measurement was done in triplicate and at least, four independent experiments were performed.

2.4. *Isolation and analysis of IPs*

The neutralized perchloric acid soluble compounds were loaded onto a 1 ml column of Dowex 1 × 10 in the formate form [11]. Free inositol and glycerophosphoinositol were eliminated by successive elution with water (30 ml) and 5 mM sodium tetraborate containing 60 mM ammonium formate. Ensuring that the radioactivity was near background level, the bulk of IPs was then eluted by 30 ml of M ammonium formate/0.1 M formic acid and concentrated. Aliquots were counted for radioactivity or further analyzed by HPLC.

The concentrated IPs were treated by Dowex 50 (in the H^+ form), then 150 μl (1 ml total) were analyzed by HPLC [12]. The chromatography column was a Partisil SAX 10 high-pressure anion-exchange column (Technicol Stockport, Cheshire, England). Markers were cAMP, ADP and ATP (IP_3 generously supplied by J.M. Darbon, Toulouse comigrated with ATP); the experimental conditions were as in [12].

Protein contents were measured as [13].

3. RESULTS AND DISCUSSION

3.1. *Formation of IPs by isolated sycamore membranes*

Cell-suspension cultures of *Acer pseudoplatanus* are a particularly suitable plant material for use in these experiments as the standard culture medium chosen here is free of inositol [8] whereas membranes of *Acer* contain PIs [9]. Therefore, there is no isotopic dilution due to unlabelled exogenous inositol into the membrane-bound PIs and approximately 5% of the added [^{3}H]inositol was incorporated in the membrane preparation.

The absolute amounts and radioactivities of the total IPs varied significantly from one set of experiments to another. However the patterns and the relative changes were highly reproducible (n = 6). Therefore, the data are presented either for a homogeneous series (absolute radioactivity) or as relative variations over appropriate controls.

The data reported in fig.1 show that after the elimination of the free inositol and glycerophosphoinositol by ion-exchange chromatography,

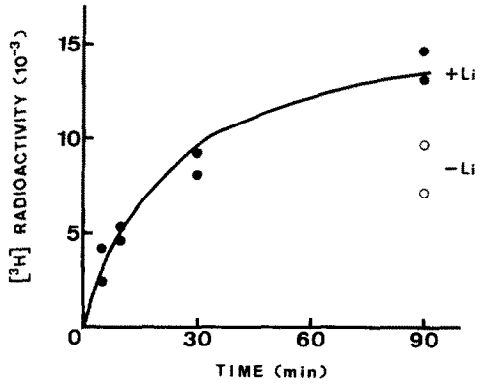

Fig.1. Time-dependent release of inositol phosphates by membranes isolated from sycamore cells (the radioactivity of time 0 was subtracted systematically).

radioactivity was associated with more negatively charged water-soluble inositol derivatives. In the absence of Li^+, radioactivity was significantly lowered suggesting that, like animal systems, Li^+ may inhibit the dephosphorylation of IP_1 [1]. Moreover, the amounts of labelled compounds accumulated are time dependent.

The inositol derived compounds were separated by HPLC and the distribution of tritium was determined. A representative result illustrated in fig.2 shows that, at time zero, the radioactivity is associated only with the less negative (monophosphate) compound(s) whereas at time 90 min, the amount of total water soluble IPs increased with the concomitant formation of compounds with higher degree of phosphorylation. As long incubation times were used (in the presence of lithium) most of the IP fraction is represented by IP_1 type compounds. Moreover, the population was highly heterogeneous making it difficult to assign each individual peak. For example the monophosphate area (AMP) is represented by 3 different peaks and the diphosphate area (ADP) by 2 compounds.

Therefore, it may be concluded that isolated plant membranes are able to release IPs from their endogenous PIs but the spectrum of released compounds is very complex as for certain animal models [14]. Due to the complexity, only the overall changes in the radioactivity of labelled total IPs have been considered in the present work.

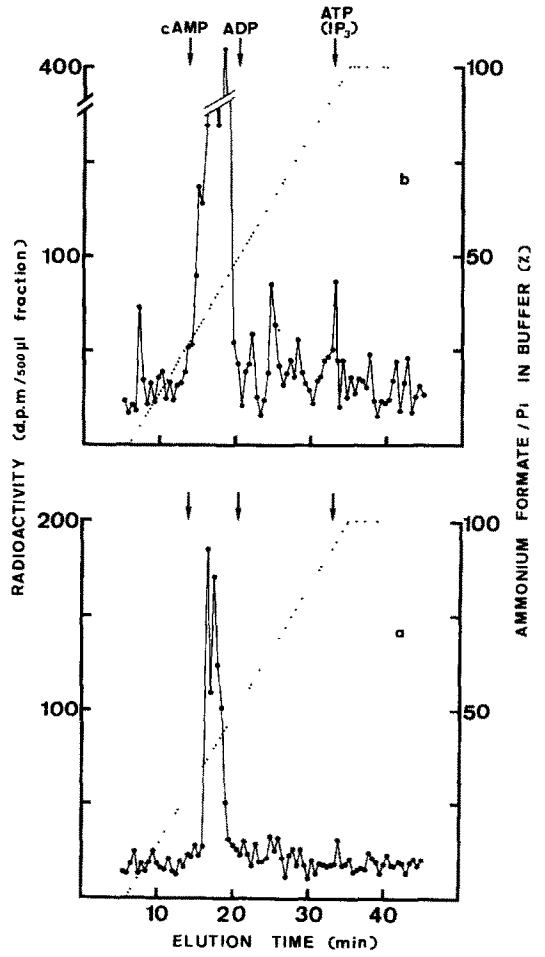

Fig.2. HPLC patterns of the IPs. (a) 0 min incubation; (b) 90 min incubation, using nucleotide markers (arrows). In each case, the membranes were preincubated for 10 min at 30°C for temperature equilibration.

3.2. *Nucleotide dependence of PI release*

The effects of different nucleotides on the release of IPs by membranes have been estimated and compared with controls over an incubation period of 90 min, in the presence of Li^+. When used at concentrations as high as 500 μM final, UTP, CTP and ADP had no effects. ATP by itself has not been tested because it is a substrate for kinase and therefore may lead to the increase of the PI amounts in plant membranes [15] the breakdown of which is measured. However, the non-hydrolyzable derivative AppNHp was not

stimulatory suggesting that the adenyl moiety is not involved. In contrast to the above mentioned compounds, guanine nucleotides were able to promote the release of total IPs in a dose-dependent manner (fig.3). Among the tested derivatives, the GDPβS (a non-hydrolyzable compound) was slightly stimulatory. However, the triphosphates were more active, GTP itself being as effective as its non-hydrolyzable counterpart GppNHp but the best activator was GTPγS. In this case, half-maximal stimulation was observed for a concentration of 30 μM which is consistent with a regulatory effect.

Addition of stoichiometric concentrations of GDPβS in combination or sequentially with GTP led to the inhibition of the guanosine triphosphate effects (fig.4). Such a result suggests that the guanine moiety is recognized by the plant membranes but there is a competition for the same biological target. As GDPβS is non-hydrolyzable, it cannot be displaced by GTP, therefore the stimulatory effect of GTP is prevented [16].

Several lines of evidence have established that plant membranes contain PI [9,17] and are able to phosphorylate PI and PIP when isolated [15]. The data reported in this paper demonstrate that plant membranes are also able to hydrolyze their own phospholipids to release IPs as animal systems [18,19]. Such a property may be of importance in the transduction of signals in relation with calcium and protein kinase C. In this connection, the

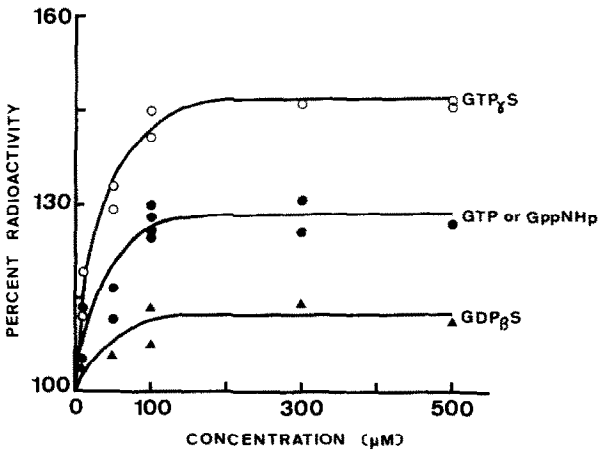

Fig.3. Dose-dependence effects of guanine nucleotides on the release of IPs.

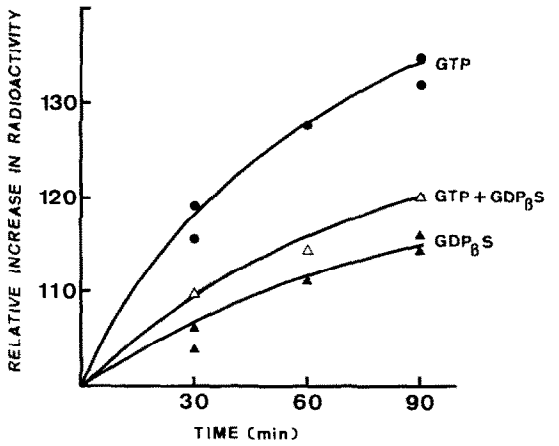

Fig.4. Effects of GDPβS on the GTP-dependent response.

results described here show that the breakdown of PIs is stimulated by nucleotide of the guanine type. Particularly relevant is the effect of GTP which is reversed by the non-hydrolyzable dinucleotide counterpart. Such a result is highly suggestive of the occurrence in plants of GTP-binding proteins that are known to be associated with the stimulus-response coupling in animals [18–21]. Therefore, it may be concluded that plants have the molecular tools that are used by other living cells to adapt themselves to their environment. However, compared to animal systems [18,19] the degree of stimulation is low and the question is now to test how far the process characterized in this work is actually involved in the stimulus-response coupling in plants.

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