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Plant Inositol Signaling - Biochemical Study of Phospholipase C and D-*myo*-inositol - 1,4,5 - trisphosphate receptor

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ABSTRACT It is now generally accepted that a phosphoinositide cycle is involved in the transduction of a variety of signals in plant cells. In animal cells, the hydrolysis of phosphatidyl-4,5-bisphosphate catalysed by phosphatidylinositol - specific phospholipase C yields to D-*myo*-inositol - 1,4,5-trisphosphate and diacylglycerol, which are well known second messengers. The binding of $InsP_3$ to a receptor located on the endoplasmic reticulum triggers a calcium release from the endoplasmic reticulum. We have detected and partially characterised key components of phosphoinositide signaling. First, tobacco microsomal fraction and plasma membrane PI-PLC. Consecutively, using a radioligand binding assay we have identified a Ca^{2+} - dependent high affinity $InsP_3$ binding site in microsomal membrane fraction vesicle preparation and then we have measured inositol-1,4,5-trisphosphate induced calcium release from tobacco microsomal fraction. These findings suggest that phosphoinositide signaling system is present and operates in the tobacco suspension culture.

Key words: Calcium release, inositol signaling, inositol 1,4,5-trisphosphate receptor, phospholipase C

Abbreviations: PI-PLC: phosphatidylinositol specific phospholipase C, PIP_2: phosphatidylinositol - 4,5-bisphosphate, $InsP_3$: inositol - 1,4,5-trisphosphate, MF: microsomal fraction, PM: plasma membrane, ER: endoplasmic reticulum.

Introduction

In animal cells, the hydrolysis of phosphatidyl - 4,5 - bisphosphate catalysed by phosphatidylinositol - specific phospholipase C yields D-*myo*-inositol - 1,4,5-trisphosphate and diacylglycerol, which are well known second messengers. The binding of $InsP_3$ to a receptor located on the endoplasmic reticulum triggers a calcium release from the ER.

In plant cells, phosphoinositide signaling is one of the major signaling systems thought to operate in the cell. Nevertheless, the physiological significance of phosphoinositide cycle in plant cells is much less known than in case of animal cells.

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The cell suspension culture of the auxin-dependent and cytokinin-independent VBI-0 strain, derived from the stem pith of *Nicotiana tabacum* L., cv. Virginia Bright Italia, was used as experimental material (Opatrny and Opatrna 1976). The strain possesses beneficial properties: high spontaneous friability (no cell clumps), filamentous phenotype, polar growth, time-separated phases of cell division (exponential growth phase) and cell elongation (stationary growth phase) (Opatrny and Opatrna 1976; Zazimalova et al. 1995). Because of these properties, this cell suspension culture may well serve as a model for both physiological and biochemical studies.

In present work the aim was identification and characterisation of the major components of the phosphoinositide cycle: PI specific phospholipase C, D-myo-inositol - 1,4,5-trisphosphate receptor and $InsP_3$ - induced calcium release in membrane fractions of VBI-0 strain.

Material and Methods

Plant material: The tobacco cell strain VBI-0 was routinely cultivated in the liquid medium of standard Heller composition (Heller 1953) supplemented with synthetic auxins naphthalene-1-acetic acid and 2,4-dichlorophenoxyacetic acid (NAA and 2,4-D, respectively, 5.10^{-6} M each).

Microsomal fraction: Filtered suspension culture was homogenised in homogenisation buffer (see Martinec et al. 2000) by sonication. The homogenate was filtered through four layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 6,000 g for 15 min. The supernatant was decanted and centrifuged at 150,000 g for 45 min. The resulting pellet, the microsomal fraction, was resuspended in suspension buffer (see Martinec et al. 2000).

Plasma membrane isolation: Plasma membrane was purified by an aqueous dextran-polyethylenglycol two phase system essentially as described by Kjell and Larson (1984). The purity of plasma membrane fraction was checked by using membrane specific enzyme markers (data not shown). All preparations were carried out at 4°C.

Phosphatidylinositol specific phospholipase C: PI-PLC assay was performed as described in Melin et al. (1987). Assay mixture containing assay buffer, radiolabelled substrate ^{3}H-PIP$_2$ and from 2 to 10 µg of membrane proteins in 50 µl was incubated at 25°C for an appropriate time (usually 5 min.). The reaction product ^{3}H-InsP$_3$ was separated by phase extraction in a chloroform/methanol/water system. The upper water phase was transferred into scintillation vials and the radioactivity was measured.

[^{3}H]InsP$_3$ binding site: The binding of [^{3}H]InsP$_3$ to membrane vesicles was quantified using a radioligand binding assay citace (Hulme and Birdsall 1992; Martinec et al. 2000). The non-specific binding was quantified by parallel experiments that included excess of unlabelled ligand.

InsP$_3$ - induced calcium release: Microsomal fraction vesicles were loaded with $^{45}Ca^{2+}$ in the presence of ATP for about 20 minutes. At given time intervals aliquots were removed and filtered through nitrocellulose membranes. Radioactivity present on the membranes was counted. The same procedure was followed after addition of InsP$_3$ or calcium ionophore A23187.

Results

We have biochemically characterised phosphoinositide specific - phospholipase C activity from the microsomal fraction and plasma membranes of a tobacco suspension culture. In our laboratory the tobacco VBI-0 strain PI-PLC was previously localised on the plasma membrane (Siroky et al. 1996). The pH optimum for PM was 6.9 (data not shown). The optimal concentration of sodium deoxycholate in the assay was 0.5 % (w/v) (data not shown). The enzyme was strictly dependent on Ca^{2+}. The highest activity was observed at free Ca^{2+} concentration of 10^{-5} to 10^{-4} M (Figure 1). The enzyme properties were similar for both the microsomal fraction and plasma membrane (data not shown). The measured properties of PI-PLC are in good correlation with results previously published (for review see Munnik et al. 1998)

The binding site for InsP$_3$ was detected in the microsomal fraction of VBI-0 cells. At pH 9.0 the microsomal fraction binds InsP3 with a specificity of K_d 4.5 × 10^{-7} M (Figure 2). We also identified InsP$_3$-induced calcium release in the same membrane fraction. The maximum degree of calcium release (about 38% - data not shown) was induced with InsP$_3$ level of 5 µM and it was reached within 10 minutes (Figure 3).

We have detected the main components of the phosphoinositide cycle in tobacco cell culture VBI-0. In

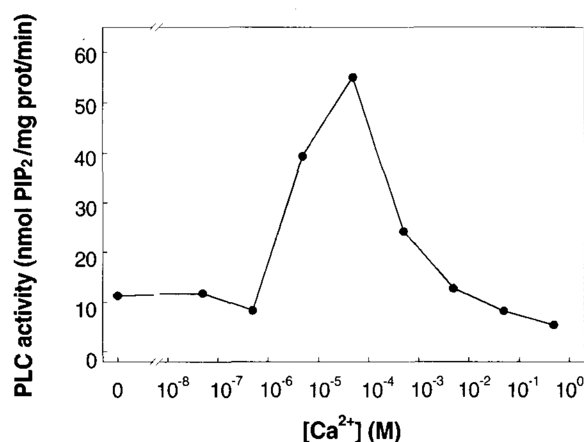

Figure 1. Ca^{2+} dependence of phosphoinositide-specific phospholipase C of tobacco plasma membrane. The maximum activity was found for concentration 10^{-5} to 10^{-4} M of free Ca^{2+}

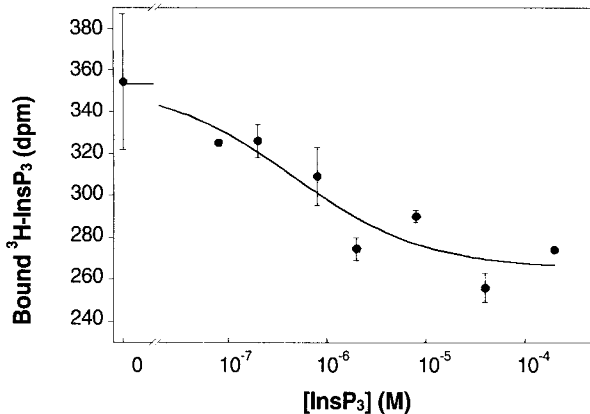

Figure 2. Analysis of binding parameters by competitive displacement assay. Tobacco microsomal membrane vesicles were incubated with ^{3}H-InsP$_3$ (2.32 nM) and the indicated concentrations of nonlabeled InsP$_3$. The resulting K_d = 4.5×10^{-7} M.

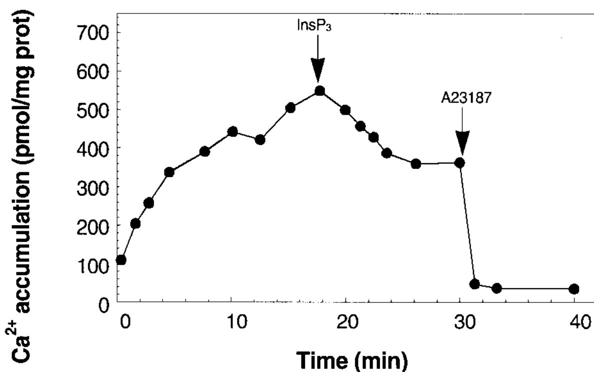

Figure 3. Ca^{2+} accumulation and Ca^{2+} release from tobacco microsomal membrane vesicles. $^{45}Ca^{2+}$ accumulated in the presence of 3 mM ATP into membrane vesicles. After 20 minutes incubation 5 μM InsP$_3$ was added and after another 10 minutes calcium ionophore A23187 (10 μM) was added.

future experiments we are going to characterise and monitor the activities of these components during growth cycle and correlate them with the dynamics of other elements of auxin and possibly cytokinin signaling pathways. The final aim is to elucidate the role of phosphoinositide signaling in auxin and/or cytokinin signal transduction.

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