Inositol 1,4,5 trisphosphate is inactivated by a 5-phosphatase in stamen hair cells of *Tradescantia*

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**Abstract** Inositol 1,4,5 trisphosphate [Ins(1,4,5)P₃] is produced from the hydrolysis of phosphatidylinositol 4,5 bisphosphate, and as part of a second-messenger signal transduction mechanism, induces release of Ca²⁺ from internal stores in both plant and animal systems. It is less well established how the active Ins(1,4,5)P₃ is inactivated. Studies in animal cells have demonstrated two separate metabolic pathways. Ins(1,4,5)P₃ can be hydrolyzed by a 5-phosphatase or phosphorylated by a 3-kinase, resulting in the formation of Ins(1,4)P₂ and Ins(1,3,4,5)P₄, respectively, neither of which is able to mobilize intracellular Ca²⁺. Plant cell extracts have been reported to have hydrolytic and kinase activities that produce Ins(1,4)P₂ and Ins(4,5)P₂ and Ins(1,4,5,6)P₄ from Ins(1,4,5)P₃. These results offer little insight into the enzyme activities in the intact plant cell since the observed activities might be confined to intracellular compartments that have little if any impact on the signaling events within the cytosol that require Ins(1,4,5)P₃.

To resolve the mechanism of Ins(1,4,5)P₃ inactivation, we microinjected stamen hair cells of *Tradescantia virginiana* L. with nonhydrolysable analogs of Ins(1,4,5)P₃ that have been previously shown to cause Ca²⁺ release from intracellular stores. Our results indicate a sustained cytosolic [Ca²⁺] increase when cells were injected with the 5-phosphatase-insensitive 5-monophosphorothioate derivative of Ins(1,4,5)P₃, in contrast to a brief transient when injected with the 3-kinase-insensitive 3-fluoro-3-deoxy Ins(1,4,5)P₃ analog. We conclude that the 5-phosphatase pathway is the preferred pathway for Ins(1,4,5)P₃ inactivation in the stamen hair cells of *Tradescantia*.

**Keywords** Calcium ion (imaging) · Inositol 1,4,5 trisphosphate (microinjection) · Stamens hair cells · *Tradescantia* (Ca²⁺, stamen)

**Abbreviations** [Ca²⁺]: calcium ion concentration · Ins(1,4,5)P₃: inositol 1,4,5 trisphosphate

**Introduction**

It is well known that inositol 1,4,5 trisphosphate [Ins(1,4,5)P₃], produced from the hydrolysis of phosphatidylinositol 4,5 bisphosphate, induces release of Ca²⁺ from internal stores in both plant and animal systems (Berridge and Irvine 1989; Berridge 1993; Tucker and Boss 1996). Less well understood is the mechanism that terminates the signal transduction event. Studies in animal cells have demonstrated two separate metabolic pathways; Ins(1,4,5)P₃ can be hydrolyzed by a 5-phosphatase or phosphorylated by a 3-kinase (Irvine et al. 1986; Majerus et al. 1988; Shears 1989) resulting in the formation of products, Ins(1,4)P₂ and Ins(1,3,4,5)P₄, respectively, neither of which is able to mobilize intracellular calcium.

In plants, patch-clamp and vesicular-release experiments demonstrate that Ins(1,4,5)P₃ mediates Ca²⁺ release through specific Ca²⁺ channels in the vacuolar membrane (Schumaker and Sze 1987; Alexandre and Lassalles 1990; Alexandre et al. 1990) whereas Ins(1,4)P₂, Ins(1,3,4,5)P₄ and Ins(1,3,4)P₃, a possible product of Ins(1,3,4,5)P₄ metabolism, failed to cause Ca²⁺ release even at concentrations 20 times higher than that required for release by Ins(1,4,5)P₃ (Alexandre et al. 1990). These studies indicate that, as in animals,
production of either Ins(1,3,4,5)P₄ or Ins(1,4,5)P₂ by Ins(1,4,5)P₃ 3-kinase or Ins(1,4,5)P₃ 5-phosphatase, respectively, would effectively inactivate Ins(1,4,5)P₃.

There is evidence in support of 5-phosphate and 1-phosphate hydrolysis of Ins(1,4,5)P₃ in plant cell extracts. Moreover, the products, Ins(4,5)P₂ and Ins(1,4)P₂, have been shown to be further hydrolyzed to monophosphates, then to inositol (Joseph et al. 1989; Memon et al. 1989; Drobak et al. 1991; Martinoia et al. 1993; Gillaspy et al. 1995). Yet other studies provide support in plants for 6-kinase activity in which Ins(1,4,5)P₃ is converted to Ins(1,4,5,6)P₄ (Chattaway et al. 1992). Although these different enzyme activities have been demonstrated in homogenates, thus far there is no consensus on the preferred pathway of Ins(1,4,5)P₃ inactivation in the intact plant cell. Furthermore, while the findings constitute evidence for the presence of enzyme activities in plant homogenate and/or fractions, they do not indicate that the corresponding enzyme is active in vivo due to considerations of compartmentalization and regulation (Cote and Crain 1993).

To address this question, we conducted a series of experiments using stamen hair cells of *Tradescentia virginiana* that had been microinjected with fura-2-dextran. The cells were then subjected to a second injection with Ins(1,4,5)P₃ or analogs selectively insensitive to 3-kinase or 5-phosphatase activities. Through comparison we find that whereas the Ca²⁺ signal from injected Ins(1,4,5)P₃ rises and falls quickly, that from the 5-monophosphorothioate derivative of Ins(1,4,5)P₃, which is insensitive to the 5-phosphatase pathway of degradation, remains elevated. In contrast, injection of the 3-kinase insensitive 3-fluoro-3-deoxy Ins(1,4,5)P₃ analog resulted in a transient increase of cytosolic [Ca²⁺], similar to that observed with Ins(1,4,5)P₃. These results indicate that the 5-phosphatase is the preferred inactivation pathway in the stamen hair cells of *Tradescentia*.

### Materials and methods

#### Cell culture

*Tradescentia virginiana* L. plants were grown in growth chambers at 22 °C during the 18-h light period and 18 °C during the 6-h dark period; these conditions promoted continuous flowering and thus a constant source of stamen hairs. Stamen hairs removed from young buds were put into a culture medium consisting of 1 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, and 5 mM HEPES buffer at pH 7.0 and then placed in a pre-warmed solution of 1% low-temperature-gelling agarose (Type VII; Sigma, St. Louis Mo., USA) in culture medium with 0.025% Triton X-100 (Sigma). The agarose solution was maintained at 35-40 °C in a microscope slide chamber on a heated stage. The agarose was then gelled at 4 °C for 15 s, immobilizing the stamen hairs. The chamber was then filled with approximately 300 µl of culture medium. During the course of the experiments, distilled water was added to replace evaporated water from the culture medium (Vos et al. 1999).

#### Microinjection

Micropipettes, prepared from borosilicate glass capillaries, (Vos et al. 1999) were loaded with the appropriate test solution using capillary action. The base of the needle shaft was immersed in the test solution until the meniscus was observed near the shaft. The shaft of the needle was then backfilled with water or 0.5 M K₂SO₄, depending on the method of injection. The calcium-sensitive ratiometric dye fura-2-dextran (10 kDa; Molecular Probes, Eugene, Ore., USA) was injected hydraulically and the shafts of those needles were backfilled with water. Test solutions [–Ins(1,4,5)P₃ (40 mM), 1-Ins(1,4,5)P₃ (40 μM), 3-fluoro-3-deoxy Ins(1,4,5)P₃ (40 μM), or the 1,4,5 monophosphorothioate analog of Ins(1,4,5)P₃ (2 μM)] were iontophoretically injected with needles that were backfilled with 0.5 M K₂SO₄. Backfilling was performed using a syringe with a tip fine enough to fit in the shaft of the needle.

The loaded needles were then mounted onto the water-based hydraulic injector or the half-cell used for the iontophoretic injections (Vos et al. 1999). Manipulations were observed with a Nikon Inverted Microscope Diaphot-300 (objective 40×, 1.3 NA), using Nomarski optics or epifluorescence. The microscope slide chamber containing immobilized stamen hair cells, was mounted on the stage of the microscope. Manipulation of the micropipette needle, mounted in the capillary grip holder of the hydraulic injector, was conducted with a Narishige MO-103R micromanipulator (Narishige Scientific Instruments, Tokyo, Japan). Positive pressure in the microinjection system resulted in delivery of fura-2-dextran into the cytoplasm of the cell with an injection volume of less than 1% of the total cell volume. Injection was monitored by observing a visible pressure wave or fluorescence at the point of injection. After hydraulic injection, the needle was left in the cell for 5–10 min to allow for cell recovery, after which it was removed.

Dye-loaded cells were subsequently injected with a test solution using an iontophoretic technique. The microinjection needle was prepared similarly to that for the hydraulic injection in that the test solution was loaded in the tip by capillary action, but the shaft of the needle was backfilled with 0.5 M K₂SO₄. The loaded needle was placed in a half-cell/microelectrode holder (W.P. Instruments, New Haven, Conn., USA) containing an Ag/AgCl pellet at its base and filled with 3 M KCl. The microelectrode holder was connected to the amplification probe of an M-717A measuring W.P. Instruments) electrometer. The return electrode, an AgCl-coated platinum wire, was immersed in the liquid culture medium covering the cells. Introduction of the test species into the cell was achieved by applying a negative current of 6 nA for 10 s (or otherwise specified) while images were being captured for intracellular Ca²⁺ measurements. Further measurements were taken after injection with the needle still in the cell and the current off.

Ratiometric imaging of cytosolic Ca²⁺

Calcium ratio imaging was conducted as described by (Holdaway-Clarke et al. 1997). Excitation was carried out at 340 nm and 360 nm, with emission at 520 nm. Images were captured at approximately 3-s intervals by a thermoelectrically cooled (-45 °C) charge coupled device (CCD) camera (Photometrics, Tucson, Ariz., USA; Type AT200 with Thompson TH7883A CCD chip, 500 kHz data transfer rate). Ratio images (340 nm/360 nm) were calculated from the background-subtracted images of the cells. Intracellular Ca²⁺ concentrations were determined by correlating ratio intensity values with values from BAPTA [1,2-bis(2-aminophenoxy)ethane-N,N,N',N' -tetraacetic acid] standards of zero Ca²⁺, 166 nM (equi-mix equal to the Κₒ of BAPTA) and Ca²⁺-saturated (adjusted to the maximal effective range of the buffer, approximately 2,000 nM), imaged at the same exposures as those for the cell (Pierson et al. 1994).

Ratiometric image pairs, collected at approximately 3-s intervals, were taken beginning at 10–20 s prior to injection through to 2 min after injection. The tested species were introduced into the cells with a negative current of 6 nA (except where noted) for 10 s. Unless otherwise noted, all reported measurements were at points distal to the injection needle to minimize the contribution of Ca²⁺ from the wound site. The basal cytosolic [Ca²⁺] was measured to be 100–200 nM. There was also some increase in [Ca²⁺] associated