Inositol Trisphosphate Metabolism in Carrot (Daucus carota L.) Cells

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ABSTRACT

The metabolism of exogenously added d-my-o-[1-3H]inositol 1,4,5-trisphosphate (IP3) has been examined in microsomal membrane and soluble fractions of carrot (Daucus carota L.) cells grown in suspension culture. When [3H]IP3 was added to a microsomal membrane fraction, [3H]IP3 was the primary metabolite consisting of approximately 83% of the total recovered [3H] by paper electrophoresis. [3H]IP3 was only 6% of the [3H] recovered, and 10% of the [3H]IP3 was not further metabolized. In contrast, when [3H]IP3 was added to the soluble fraction, approximately equal amounts of [3H]IP3 and [3H]IP2 were recovered. Ca2+ (100 micromolar) tended to enhance IP2 dephosphorylation but inhibited the IP2 dephosphorylation in the soluble fraction by about 20%. MoO42− (1 millimolar) inhibited the dephosphorylation of IP3 by the microsomal fraction and the dephosphorylation of IP2 by the soluble fraction. MoO42−, however, did not inhibit the dephosphorylation of IP3 by the soluble fraction. Li+ (10 and 50 millimolar) had no effect on IP3 metabolism in either the soluble or membrane fraction; however, Li+ (50 millimolar) inhibited IP2 dephosphorylation in the soluble fraction about 25%.

It has been firmly established in a wide variety of animal systems that IP3 plays a central role as second messenger for receptor-mediated Ca2+ mobilization (2). In plant cells, the role of inositol phosphates in signal transduction is not well established. The evidence suggesting that IP3 is a second messenger in plant cells comes from three different experimental approaches: (a) identification of polyphosphoinositides isolated from the tissue culture cells (3, 7, 10), and whole plant cells (15); (b) characterization of the kinases involved in the metabolism of PI (21) and of a phospholipase C in the plasma membrane which hydrolyzes PIP2 (14); (c) stimulation of a Ca2+ efflux from tonoplast vesicles (19), isolated vacuoles (16), and isolated proplasts (17) by exogenously applied IP3. There are a few reports that suggest involvement of IP3 in signal transduction in plants (8, 15). However, the connection between the time course of polyphosphoinositide turnover and the elevation of free Ca2+ in the cytosol in response to external stimuli has not been documented.

In mammalian systems, the IP3 response is attenuated by a specific phosphatase which removes the phosphate from the 5-position to yield I(1,4)P2 (6, 11, 20). I(1,4)P2 is further dephosphorylated to IP (13). In previous studies, neither IP2 nor IP3 was detected in carrot suspension culture cells (18). One explanation of these data was that IP3, once formed, was rapidly metabolized to inositol and therefore not detected. The question remained as to whether IP3 and IP2 phosphatases were present in plants. In the present experiment, we studied the rate of IP3 metabolism and the effect of inhibitors on IP3 metabolism by both soluble and microsomal membrane enzymes.

MATERIALS AND METHODS

Plant Material

Carrot cells (Daucus carota L.) were grown in suspension culture and maintained by weekly transferring as previously described (4).

Isolation of Microsomes

Cells (1.6 g) were homogenized at 4°C in a ground glass homogenizer in 3 ml of medium consisting of 10 mm KCl, 1 mm EDTA, 2 mm EGTA, 2 mm DTT (freshly added), 20 mm Hepes buffer (pH 7.0) plus 8% sucrose, 1% PVP. The homogenate was centrifuged at 750g for 4 min, the pellet was discarded, and the supernatant was centrifuged at 7,000g for 10 min. The resulting supernatant was centrifuged at 40,000g for 45 min. The pellet was washed by resuspending in 5 ml of homogenizing medium plus 2 mm DTT followed by centrifuging at 40,000g for 20 min and was resuspended in the same medium. The 40,000g supernatant was used as a source of soluble enzymes.

[3H]IP3 Metabolism

The reaction conditions were similar to those reported by Joseph and Williams (11) who showed a requirement for
Mg\(^{2+}\) and pH optimum of 7.0 for the rat liver enzymes. The total volume of the reaction was 100 \(\mu\)L and consisted of homogenizing medium (pH 7.0) plus 6 mM MgCl\(_2\), 1.5 \(\mu\)M IP\(_3\), 0.45 \(\mu\)Ci/mL d-myoinositol 1,4,5-trisphosphate (New England Nuclear), and approximately 0.2 mg of either microsomal or soluble protein. Na\(_2\)MoO\(_4\) (1 mM), LiCl (10 or 50 mM), or Ca\(^{2+}\)-EGTA to give 100 \(\mu\)M free Ca\(^{2+}\) were added as noted. The reaction was carried out at room temperature and was initiated by adding the microsomes or the 40,000g supernatant to give approximately 206,000 cpm/mg protein for the microsomes and 232,000 cpm/mg protein for the soluble fraction. The reaction was terminated after 15 min by adding 100 \(\mu\)L of ice-cold 20% TCA. The suspension was centrifuged in the Beckman microfuge for 3 min; the supernatant was collected, and the TCA was removed by three ether washes. The aqueous solution was frozen overnight. Aliquots of 70 \(\mu\)L of the reconstituted samples were spotted on Whatman 3MM chromatography paper. The inositol phosphates were separated by electrophoresis at 2000 V for 40 min in a running buffer of 0.1 M formic acid (pH 2.8) as previously described (18). The total \(^3\)H recovered from the initial reaction mixture averaged 91% for all experiments. The amount of \(^3\)H IP\(_3\) hydrolyzed was calculated relative to the buffer controls. The buffer controls were handled as above. Protein was determined by the method of Bradford (5). Each experiment was done in duplicate and the experiments were repeated at least three times.

RESULTS AND DISCUSSION

Microsomal and soluble fractions were isolated from carrot cells grown in suspension culture. These fractions were tested for their ability to metabolize exogenously added \(^3\)H[IP\(_3\)] (Fig. 1; Table I). Approximately 90% of \(^3\)H[IP\(_3\)] was hydrolyzed by the microsomal fraction after 15 min. Of this, 82 to 85% was recovered as \(^3\)H[IP\(_2\)] and 5 to 7% as \(^3\)H[IP\(_1\)] (Tables I and II). With the soluble fraction, \(^3\)H[IP\(_3\)] was more completely metabolized during the 15 min reaction time. Approximately 50 to 60% of the \(^3\)H[IP\(_1\)] was recovered as \(^3\)H[IP\(_2\)] and 40 to 50% as \(^3\)H[IP\(_1\)]. While IP\(_2\) was dephosphorylated to IP only to a minor extent by the microsomal enzymes, it was dephosphorylated readily to IP by the soluble enzymes. These results are consistent with those using rat liver (11, 20) where IP\(_3\) was dephosphorylated by an enzyme located in the plasma membrane that did not dephosphorylate IP\(_2\), and where both IP\(_2\) and IP\(_1\) were degraded by the cytosolic fractions.

Ca\(^{2+}\) (100 \(\mu\)M) slightly enhanced the metabolism of IP\(_2\) and had no significant effect on IP\(_3\) metabolism with the microsomal fraction (Table I). In contrast, 100 \(\mu\)M Ca\(^{2+}\) decreased the metabolism of IP\(_2\) in the soluble fraction about 20%. The Ca\(^{2+}\) effect was observed only in the absence of other inhibitors. Molybdate is a well known phosphatase inhibitor (9), and 1 mM MoO\(_4^{2-}\) effectively inhibited the metabolism of IP\(_3\) by the microsomal fraction and inhibited further metabolism of IP\(_2\) by the soluble fraction (Fig. 1; Table I). However, MoO\(_4^{2-}\) did not inhibit the metabolism of IP\(_2\) by the soluble enzymes. The fact that MoO\(_4^{2-}\) had no effect on the dephosphorylation of IP\(_2\) by the soluble fraction but inhibited the dephosphorylation of IP\(_3\) by the microsomal fraction suggested that two different enzymes were involved. The membrane-associated enzyme, presumably an IP\(_3\) 5-phosphatase, was sensitive to MoO\(_4^{2-}\). Neither enzyme was sensitive to Li\(^+\). The rates of metabolism of \(^3\)H[IP\(_3\)] are summarized in Table III.

The cytosolic IP\(_2\) phosphatase was inhibited by MoO\(_4^{2-}\) and to some extent by Li\(^+\) (Tables I and II). In mammalian tissues, Li\(^+\) does not affect the 5-phosphatase activity (6), but is a potent, noncompetitive inhibitor of IP\(_2\) phosphatases (1, 13). In our system, even 50 mM Li\(^+\) had no significant effect on the IP\(_2\) metabolism in either the microsomal or soluble fractions. Li\(^+\) inhibited IP\(_2\) metabolism in the soluble fraction about 25% (Table II). These results are consistent with those of I-1-P phosphatase activity from pollen (12) which was sensitive to high concentrations of Li\(^+\). In addition, like the pollen cells, no phosphorylation of IP or IP\(_2\) was observed when \([\gamma\text{-}32\text{P}]\)ATP was added to isolated membranes in the presence of 5 mM Mg\(^{2+}\) at pH 7.5 (M Rincon, unpublished work).

![Figure 1](https://example.com/figure1.png)

**Distance From Origin (cm)**

**Figure 1.** Electrophoretic analysis of the products of IP\(_1\), IP\(_2\), and IP\(_3\) metabolism in microsomal and soluble fractions. [\(^{3}\)H]IP\(_3\) (0.45 \(\mu\)Ci/mL; 1.5 \(\mu\)M) was incubated with buffer alone (E) or with the microsomal (A) or soluble (B) fraction (0.2 mg protein) for 15 min as described in "Materials and Methods" in the presence of 6 mM MgCl\(_2\) (pH 7.0) with (E) or without (E) 1 mM Na\(_2\)MoO\(_4\), or with 100 \(\mu\)M CaCl\(_2\) (C). The migration of the radiolabeled compounds was compared to that of authentic standards, myo-inositol, IP, fructose-1, 6-bisP, PI, IP\(_6\), [\(^3\)H]IP\(_2\), and [\(^3\)H]IP\(_3\).
results). This is in contrast to animal cells where IP₃ is phosphorylated to inositol tetrakisphosphate (IP₄) (13).

The rapid rate of metabolism of IP₃ along with the fact that PIP₂ is relatively low in the carrot cells would contribute to the fact that neither IP₂ nor IP₃ was detected (18). Since the soluble fraction contained the highest rate of IP₃ metabolism and since the vacuole has been found to be the IP₃-sensitive calcium store (16, 19) in plant cells, it remains to be proven as to whether or not IP₃ could reach the tonoplast prior to being metabolized in vivo. These data bring into question the potential role of IP₃ as a second messenger and should be an important consideration in studying phosphoinositide metabolism in plant cells.

LITERATURE CITED


2. Berridge MJ (1987) Inositol trisphosphate and diacylglycerol: