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Communication

Inositol Trisphosphate Metabolism in Carrot (*Daucus carota* L.) Cells[1]

Abdul R. Memon[2], Magaly Rincon[3], and Wendy F. Boss*

Department of Botany, Box 7612, North Carolina State University Raleigh, North Carolina 27695

ABSTRACT

The metabolism of exogenously added D-*myo*-[1-^{3}H]inositol 1,4,5-trisphosphate (IP_3) has been examined in microsomal membrane and soluble fractions of carrot (*Daucus carota* L.) cells grown in suspension culture. When [^{3}H]IP_3 was added to a microsomal membrane fraction, [^{3}H]IP_2 was the primary metabolite consisting of approximately 83% of the total recovered [^{3}H] by paper electrophoresis. [^{3}H]IP was only 6% of the [^{3}H] recovered, and 10% of the [^{3}H]IP_3 was not further metabolized. In contrast, when [^{3}H]IP_3 was added to the soluble fraction, approximately equal amounts of [^{3}H]IP_2 and [^{3}H]IP were recovered. Ca^{2+} (100 micromolar) tended to enhance IP_3 dephosphorylation but inhibited the IP_2 dephosphorylation in the soluble fraction by about 20%. MoO_4^{2-} (1 millimolar) inhibited the dephosphorylation of IP_3 by the microsomal fraction and the dephosphorylation of IP_2 by the soluble fraction. MoO_4^{2-}, however, did not inhibit the dephosphorylation of IP_3 by the soluble fraction. Li^+ (10 and 50 millimolar) had no effect on IP_3 metabolism in either the soluble or membrane fraction; however, Li^+ (50 millimolar) inhibited IP_2 dephosphorylation in the soluble fraction about 25%.

It has been firmly established in a wide variety of animal systems that IP_3[4] plays a central role as second messenger for receptor-mediated Ca^{2+} mobilization (2). In plant cells, the role of inositol phosphates in signal transduction is not well established. The evidence suggesting that IP_3 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 PIP_2 (14); (c) stimulation of a Ca^{2+} efflux from tonoplast vesicles (19), isolated vacuoles (16), and isolated protoplasts (17) by exogenously applied IP_3. There are a few reports that suggest involvement of IP_3 in signal transduction in plants (8, 15). However, the connection between the time course of polyphosphoinositide turnover and the elevation of free Ca^{2+} in the cytosol in response to external stimuli has not been documented.

In mammalian systems, the IP_3 response is attenuated by a specific phosphatase which removes the phosphate from the 5-position to yield $I(1,4)P_2$ (6, 11, 20). $I(1,4)P_2$ is further dephosphorylated to IP (13). In previous studies, neither IP_2 nor IP_3 was detected in carrot suspension culture cells (18). One explanation of these data was that IP_3, once formed, was rapidly metabolized to inositol and therefore not detected. The question remained as to whether IP_3 and IP_2 phosphatases were present in plants. In the present experiment, we studied the rate of IP_3 metabolism and the effect of inhibitors on IP_3 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 750*g* for 4 min, the pellet was discarded, and the supernatant was centrifuged at 7,000*g* for 10 min. The resulting supernatant was centrifuged at 40,000*g* for 45 min. The pellet was washed by resuspending in 5 mL of homogenizing medium plus 2 mM DTT followed by centrifuging at 40,000*g* for 20 min and was resuspended in the same medium. The 40,000*g* supernatant was used as a source of soluble enzymes.

[^{3}H]IP_3 Metabolism

The reaction conditions were similar to those reported by Joseph and Williams (11) who showed a requirement for

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[2] Permanent address: Department of Biology, Middle East Technical University, Ankara, Turkey.

[3] Present address: Noble Foundation, P. O. Box 2180, Ardmore, OK 73402.

[4] Abbreviations: IP_3, inositol trisphosphate; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP, inositol phosphate; IP_2, inositol bisphosphate; $I(1,4)P_2$, inositol 1,4-bisphosphate; $I(1,4,5)P_3$, inositol 1,4,5-trisphosphate; IP_4, inositol tetrakisphosphate; IP_5, inositol pentaphosphate; IP_6, inositol hexaphosphate.

Mg^{2+} and pH optimum of 7.0 for the rat liver enzymes. The total volume of the reaction was 100 μL and consisted of homogenizing medium (pH 7.0) plus 6 mM $MgCl_2$, 1.5 μM IP_3, 0.45 μCi/mL D-*myo*[1-^{3}H]inositol 1,4,5-trisphosphate (New England Nuclear), and approximately 0.2 mg of either microsomal or soluble protein. Na_2MoO_4 (1 mM), LiCl (10 or 50 mM), or Ca^{2+}-EGTA to give 100 μM free Ca^{2+} were added as noted. The reaction was carried out at room temperature and was initiated by adding the microsomes or the 40,000*g* 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 μL 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 μL 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 (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_3 was recovered as [^{3}H]IP_2 and 40 to 50% as [^{3}H]IP. 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_3 were degraded by the cytosolic fractions.

Ca^{2+} (100 μM) slightly enhanced the metabolism of IP_3 and had no significant effect on IP_2 metabolism with the microsomal fraction (Table I). In contrast, 100 μM 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_3 by the soluble enzymes. The fact that MoO_4^{2-} had no effect on the dephosphorylation of IP_3 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.

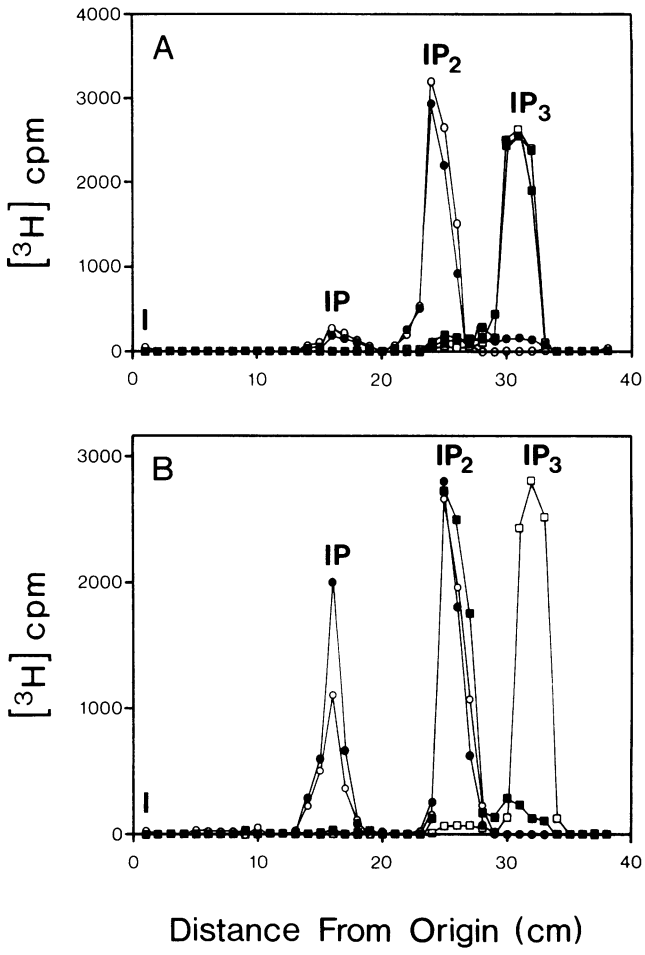

Figure 1. Electrophoretic analysis of the products of I(1,4,5)P_3 metabolism in microsomal and soluble fraction. [1-^{3}H]IP_3 (0.45 μCi/mL; 1.5 μM) was incubated with buffer alone (□) or with the microsomal (A) or soluble (B) fraction (0.2 mg protein) for 15 min as described in "Materials and Methods" in the presence of 6 mM $MgCl_2$ (pH 7.0) with (■) or without (●) 1 mM Na_2MoO_4 or with 100 μM $CaCl_2$ (○). 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.

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_3 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_3 was observed when [γ-^{32}P]ATP was added to isolated membranes in the presence of 5 mM Mg^{2+} at pH 7.5 (M Rincon, unpublished

Table I. *Metabolism of IP by Microsomal and Soluble Enzymes*

Fraction	Inositol	IP	IP_2	IP_3
		cpm/mg protein × 10³ [a]		
Microsomes				
Control	0.76 (0.4 ± 0.1) [b]	13.2 (7 ± 0.5)	160.1 (85 ± 0.7)	14.8 (8 ± 0.4)
MoO_4^{2-} (1 mM)	0.07 (0.03 ± 0.01)	0.7 (0.3 ± 0.02)	12.0 (6 ± 0.07)	185.6 (94 ± 1.0)
Ca^{2+} (100 μM)	1.36 (0.7 ± 0.01)	17.9 (9 ± 0.2)	184.8 (90 ± 1.4)	2.2 (1 ± 0.1)
Ca^{2+} + MoO_4^{2-}	0.05 (0.03 ± 0.01)	0.8 (0.4 ± 0.1)	7.8 (4 ± 0.1)	176.5 (95 ± 0.3)
Soluble				
Control	0.19 (0.08 ± 0.1)	91.4 (40 ± 1.8)	137.1 (60 ± 2.5)	1.5 (0.7 ± 0.14)
MoO_4^{2-} (1 mM)	0.05 (0.02)	4.4 (2 ± 0.5)	177.7 (87 ± 7.0)	21.1 (10 ± 0.3)
Ca^{2+} (100 μM)	0.69 (0.3 ± 0.01)	58.1 (28 ± 0.2)	149.4 (72 ± 0.7)	0.4 (0.2 ± 0.01)
Ca^{2+} + MoO_4^{2-}	0.03 (0.01)	3.0 (2 ± 0.5)	173.8 (86 ± 6)	25.1 (12 ± 0.28)

[a] Values are the average of duplicate samples from one experiment. [b] Numbers in parentheses are percentage of the total radioactivity at time zero ± SD deviation of six values from three separate experiments.

Table II. *Effect of Lithium Ion on IP Metabolism*

Fraction	Inositol	IP	IP_2	IP_3
		cpm/mg protein × 10³ [a]		
Microsomes				
Control	1.25 (0.7 ± 0.07) [b]	8.5 (4.7 ± 0.4)	149 (82 ± 1.4)	24 (13 ± 0.7)
Li^+ (10 mM)	0.36 (0.2 ± 0.07)	9.0 (5 ± 0.7)	167 (85 ± 1.0)	21 (11 ± 1.0)
Li^+ (50 mM)	0.24 (0.1 ± 0.01)	7.8 (4 ± 0.1)	149 (80 ± 2.1)	30 (16 ± 2.0)
Ca^{2+} (100 μM) + Li^+ (10 mM)	0.21 (0.1 ± 0.01)	8.9 (5 ± 0.5)	176 (89 ± 1.4)	12 (6 ± 2.1)
Ca^{2+} (100 μM) + Li^+ (50 mM)	0.04 (0.02 ± 0.01)	8.8 (5 ± 0.5)	155 (85 ± 0.1)	18 (10 ± 0.2)
Soluble				
Control	2.5 (1 ± 0.4)	92 (48 ± 1.4)	88 (47 ± 1.4)	8.2 (4 ± 0.1)
Li^+ (10 mM)	2.4 (1 ± 0.2)	87 (45 ± 0.7)	93 (49 ± 1.0)	9.2 (5 ± 0.7)
Li^+ (50 mM)	0.9 (0.5 ± 0.2)	68 (34 ± 1.4)	114 (58 ± 1.0)	15.0 (8 ± 1.4)
Ca^{2+} (100 μM) + Li^+ (10 mM)	8.3 (4 ± 0.7)	75 (37 ± 6.4)	110 (55 ± 5.7)	7.7 (4 ± 0.07)
Ca^{2+} (100 μM) + Li^+ (50 mM)	6.0 (3 ± 0.7)	59 (31 ± 2.8)	116 (61 ± 2.1)	8.9 (5 ± 0.07)

[a] Values are the average of duplicate samples from one experiment. [b] Numbers in parentheses are percentage of the total radioactivity at time zero ± SD for six values from three separate experiments.

Table III. *Rate of IP_3 Hydrolysis by Microsomal and Soluble Enzymes*

Values are the mean of three experiments ± SD.

Fraction	[³H]IP_3 Hydolyzed
	pmol mg^{-1} protein min^{-1}
Microsomes	
Control	55.3 ± 2.6
MoO_4^{2-} (1 mM)	4.5 ± 0.1
Ca^{2+} (100 μM)	62.8 ± 2.9
Ca^{2+} + MoO_4^{2-}	7.3 ± 0.1
Soluble	
Control	75.6 ± 11.5
MoO_4^{2-} (1 mM)	63.5 ± 5.6
Ca^{2+} (100 μM)	76.1 ± 11.7
Ca^{2+} + MoO_4^{2-}	61.1 ± 4.4

results). This is in contrast to animal cells where IP_3 is phosphorylated to inositol tetrakisphosphate (IP_4) (13).

The rapid rate of metabolism of IP_3 along with the fact that PIP_2 is relatively low in the carrot cells would contribute to the fact that neither IP_2 nor IP_3 was detected (18). Since the soluble fraction contained the highest rate of IP_3 metabolism and since the vacuole has been found to be the IP_3-sensitive calcium store (16, 19) in plant cells, it remains to be proven as to whether or not IP_3 could reach the tonoplast prior to being metabolized *in vivo*. These data bring into question the potential role of IP_3 as a second messenger and should be an important consideration in studying phosphoinositide metabolism in plant cells.

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