

SHORT COMMUNICATION

Unsaturated fatty acids inhibit MP2C, a protein phosphatase 2C involved in the wound-induced MAP kinase pathway regulation

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Summary

When mechanically injured, plants develop multiple defense systems including the activation of specific genes. These responses are triggered by a complex network of signalling events that include Ca^{2+} fluxes, the production of free fatty acids from membrane lipids, as well as the activation of mitogen-activated protein kinases (MAPK). In the present paper, we address the question of the regulation of the MAPK pathway by wound-induced Ca^{2+} and fatty acid signals. We report that MP2C, a serine/threonine protein phosphatase 2C from alfalfa involved in MAPK pathway inactivation, is inhibited specifically *in vitro* by long-carbon-chain polyunsaturated fatty acids, and α -linolenic acid, the primary product of the octadecanoid pathway, was found to be the most potent inhibitor. Ca^{2+} also inhibits MP2C, but only at high concentrations, and other divalent cations show similar inhibitory effect, making it unlikely that Ca^{2+} is involved in the regulation of MP2C *in vivo*. Overall, our data suggest that cross-talk between wound-induced MAPK and octadecanoid pathways may occur at the level of protein phosphatase 2C and linolenic acid.

Introduction

One of the most severe environmental stresses to which plants can be subjected is wounding, which may come about through such diverse causes as mechanical injury, pathogen or herbivore attack. To cope with such stresses, plants have developed response systems which activate sets of genes that are involved in pathogen defence and wound healing (Bowles, 1993). The signal transduction cascades triggering these responses have been the focus

of recent investigations and have implicated Ca^{2+} ions and fatty acids in wound signalling.

Lipid-based signalling via the octadecanoid pathway, which leads to the production of regulators such as jasmonic acid, plays an important role in this context. Activation of phospholipases D and A_2 upon wounding has been reported (Lee *et al.*, 1997; Ryu and Wang, 1996) and gives rise to rapid increases in free fatty acids that maximally accumulate within the first hour after wounding (Conconi *et al.*, 1996; Ryu and Wang, 1998). Unsaturated fatty acids can subsequently be processed into a large array of compounds including the plant hormone jasmonic acid (Blechert *et al.*, 1995). Calcium signalling has been proposed to participate in free fatty acid production by promoting phospholipase D translocation to membranes and subsequent activation (Ryu and Wang, 1996). This correlates with the cytosolic Ca^{2+} increase that has been observed after wounding in aequorin-transformed transgenic plants (Knight *et al.*, 1993).

Mitogen-activated protein kinases (MAPK), a family of specific serine/threonine protein kinases which can be activated by a large spectrum of extracellular signals in plants and animals, are also activated upon wounding (Jonak *et al.*, 1999). Indeed, a transient stimulation of MAPK has been observed in response to wounding or to wound-related compounds (Bögge *et al.*, 1997; Seo *et al.*, 1995; Stratmann and Ryan, 1997). Moreover, a MAPK that is rapidly activated upon wounding is essential for wound-induced jasmonic acid production (Seo *et al.*, 1995; Seo *et al.*, 1999).

Whereas MAPK activation relies on phosphorylation of the threonine and tyrosine residues of a conserved TXY motif close to sub-domain VIII, inactivation is mediated by dephosphorylation of either of these two residues. Dual-specificity and tyrosine phosphatases, as well as serine/threonine phosphatases such as PP2A and PP2C, have been implicated in the inactivation of MAPK (Keyse, 1998). Recent data have shown the existence of dual-specificity and tyrosine phosphatases in plants, and a role for these factors in MAPK pathway regulation following stresses has been proposed (Gupta *et al.*, 1998; Haring *et al.*, 1995; Xu *et al.*, 1998). We have recently reported the cloning and characterization of a serine/threonine protein phosphatase 2C gene from *Medicago sativa* (MP2C) (Meskiene *et al.*, 1998) which acts as a negative regulator of the stress-

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activated MAPK (SAMK) pathway, which is transiently activated upon cold, touch, drought and wounding (Bögre *et al.*, 1997; Meskiene *et al.*, 1998).

Whereas the activation of MAPK pathways by upstream protein kinases has been thoroughly investigated, much less is known about the regulation of the inactivating principles. In particular, the effects of other pathways and secondary messengers on the negative regulation of MAPKs have hardly been addressed so far. In the present paper, we have investigated the potential role of MP2C as a target for cross-talk between different wound-induced signalling pathways. Our data show that MP2C activity can be regulated by unsaturated fatty acids and Ca^{2+} *in vitro*, suggesting additional mechanisms for regulation of the wound-inducible MAPK pathway.

Results

Inhibition of MP2C by unsaturated fatty acids

Upon wounding, the oxylipin pathway is activated. The first step in this pathway is the activation of a lipase that generates free fatty acids, e.g. linolenic acid, which can be subsequently processed to jasmonates. In addition to their role as precursors of jasmonic acid, free fatty acids can also act as signalling messengers.

To test whether free fatty acids might participate in MP2C regulation, their effect on *Medicago sativa* phosphatase 2C (MP2C) activity was studied. Recombinant GST–MP2C protein was affinity purified. Attempts to cleave the fusion protein were unsuccessful due to the generation of multiple cleavage products. The purified fusion protein showed no contaminant phosphatase activity and was used as the enzyme source.

The activity of MP2C was dramatically affected upon addition of linolenic acid (Figure 1a). An inhibition of casein dephosphorylation was observed, which was dose-dependent and was detected at fatty acid concentrations as low as $10\ \mu\text{M}$. Strong inhibition was observed when $0.5\ \text{mM}$ linolenic acid was added to MP2C, and an IC_{50} of $130 \pm 10\ \mu\text{M}$ was deduced from inhibition curves. No significant change in the linolenic acid inhibition was detected when up to $10\ \mu\text{g}$ of GST were added to the assay, indicating that inhibition of the phosphatase is mediated by the interaction of linolenic acid with MP2C. To further investigate the characteristics of MP2C inhibition by fatty acids, the K_m and V_{max} of the enzyme for casein phosphoserine/phosphothreonine residues were determined in the absence or in the presence of linolenic acid. As shown in Table 1, the K_m and V_{max} values in the absence of inhibitor were $2.45 \pm 0.4\ \mu\text{M}$ and $88.9 \pm 8.3\ \text{pmol min}^{-1}$, respectively. Addition of linolenic acid led to a dose-dependent decrease of the apparent K_m

and V_{max} (Table 1), which was consistent with a mixed-type inhibition mechanism.

In vitro PP2C activity is commonly determined in the presence of artificially high concentrations (approximately $10\ \text{mM}$) of MgCl_2 . However, the cellular Mg^{2+} concentration shows a constant value of $1\ \text{mM}$ *in vivo*. To examine whether the fatty acid inhibition could also be observed at a physiological Mg^{2+} concentration, similar experiments were carried out in the presence of $1\ \text{mM}$ MgCl_2 . Under these conditions, casein dephosphorylation was still strongly inhibited by linolenic acid (Figure 1b), showing a similar dose-dependent inhibition as when high MgCl_2 concentrations were used. These results show that the inhibition of MP2C by linolenic acid was not provoked by an artificially high Mg^{2+} concentration. Instead, the rate of inhibition was even enhanced by lowering the Mg^{2+} concentration, especially for low fatty acid concentrations (Figure 1b).

The structural properties of lipids required for MP2C inhibition were analysed using various fatty acids. Experiments were carried out by preincubating GST–MP2C with $0.5\ \text{mM}$ of each lipid. The phosphatase activity was hardly affected by saturated fatty acids (Table 2). In addition to the strict requirement of double bonds in the lipid molecule, the carbon chain length played a critical role in the ability of the fatty acids to inhibit MP2C activity. Indeed, no inhibition was observed for lipids having carbon chain lengths shorter than 16 carbon atoms. We have compared the ability of different unsaturated C18 analogues to inhibit MP2C. As shown in Table 2, the inhibitory efficiency increased with the number of double bonds displayed by the C18 fatty acid molecule. Overall, maximal inhibition was observed for long-chain polyunsaturated fatty acids, e.g. linolenic and arachidonic acid. As all fatty acids were used above their critical micellar concentration ($30\text{--}100\ \mu\text{M}$), micelle formation alone cannot be responsible for the inhibitory effect observed in this study.

Inhibition of MP2C by calcium

We have previously reported that MP2C activity is strictly dependent upon the presence of Mg^{2+} or Mn^{2+} in the reaction buffer. When Mg^{2+} was substituted by Ca^{2+} , no casein dephosphorylation was observed, providing evidence that Ca^{2+} could not support the phosphatase activity of MP2C. In this study, we addressed the question of whether Ca^{2+} may play a regulatory role. MP2C activity was measured at different Mg^{2+} concentrations, in the absence or presence of various Ca^{2+} concentrations. As shown in Figure 2(a), in the absence of Ca^{2+} , MP2C activity increased with the Mg^{2+} concentration and reached its maximal value at $5\ \text{mM}$ Mg^{2+} . When Ca^{2+} was added to the assay, a dose-dependent inhibition of the activity of the protein phosphatase was observed. In the presence of

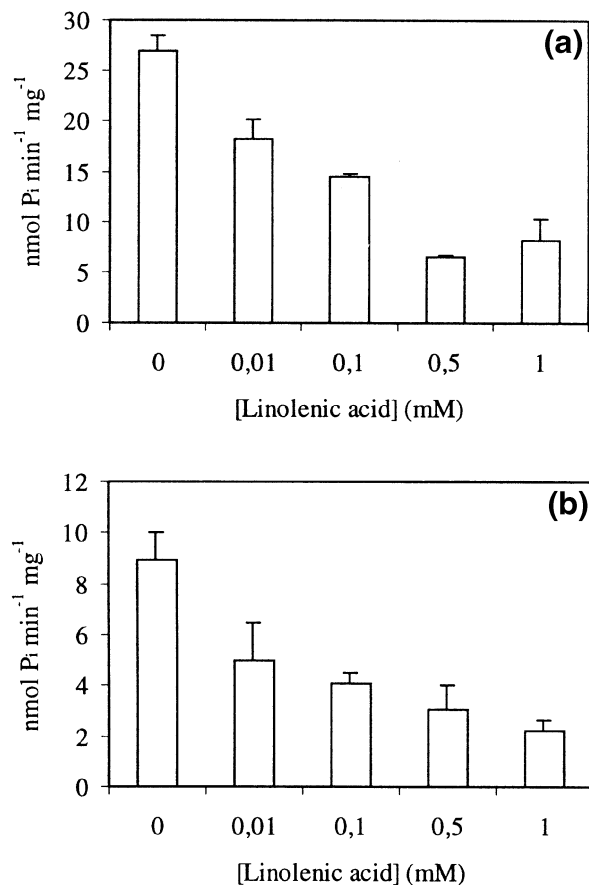


Figure 1. Effect of linolenic acid on MP2C activity. Phosphatase activity of recombinant GST-MP2C was determined in the presence of 10 mM MgCl₂ (a) or 1 mM MgCl₂ (b), in the absence (0) or presence of increasing amounts of α -linolenic acid.

1 mM Mg²⁺, an IC₅₀ of 1.6 ± 0.2 mM was determined from inhibition curves. In experiments in which a constant Ca²⁺ concentration was used, the inhibition was more potent at low Mg²⁺ concentrations.

Additionally, other divalent cations were examined for their effect on MP2C activity. As shown in Figure 2(b), all tested compounds strongly inhibited MP2C activity. In the presence of 1 mM Mg²⁺, most divalent cations resulted in nearly complete inhibition of MP2C, whereas Ca²⁺ addition showed less than 50% inhibition of the enzyme activity.

Discussion

Our data provide evidence that MP2C, a protein phosphatase 2C involved in MAP kinase regulation in alfalfa, is inhibited by fatty acids and divalent cations *in vitro*. The inhibition of MP2C by fatty acids cannot be ascribed to a general effect of lipids on the phosphatase activity nor to the presence of contaminant compounds within the lipid preparation. Indeed, MP2C inhibition is only triggered by

Table 1. Apparent K_m and V_{max} values in the absence or presence of increasing α -linolenic acid concentrations

[α -linolenic acid] (mM)	K_m (μ M)	V_{max} (pmol min ⁻¹)
0	2.46 ± 0.40	88.90 ± 8.30
0.1	2.41 ± 0.26	72.25 ± 3.58
0.2	1.91 ± 0.41	29.33 ± 2.68
0.3	1.59 ± 0.11	20.10 ± 0.45

K_m and V_{max} values for casein phosphoserine/phosphothreonine residues were deduced from Lineweaver-Burk plots, in a range of substrate of 0.8–5 μ M.

polyunsaturated, long-carbon chain fatty acids, whereas the activity is not affected by short carbon chain or saturated fatty acids. These data strongly suggest that particular sterical features of the lipid are required for its ability to inhibit MP2C. In this respect, introduction of a double bond is known to cause bending and shortening as well as an increase in volume, physical properties that are also dependent upon the carbon chain length. Our study also reveals a different behaviour between MP2C and vertebrate PP2Cs. As recently reported, PP2C α and β , two animal PP2Cs, are activated by unsaturated fatty acids (Klump *et al.*, 1998). Although the mechanism by which fatty acids regulate PP2Cs is unknown, the difference in their behaviour could be related to the divergent structures of MP2C and animal PP2Cs. The primary sequence homology between the two types of phosphatases is restricted to their catalytic domains (amino acids 99–380 in MP2C; amino acids 1–300 in human PP2C), which share 30% identity. An additional unique C-terminal region is found in animal PP2Cs, whereas an N-terminal extension with no similarity to animal PP2Cs is present in MP2C. Even though the role of these unique domains of the PP2Cs has not been assessed yet, they could explain why MP2C and animal PP2Cs behave differently when challenged with fatty acids. MP2C also differs from animal PP2Cs as its inhibition by fatty acids is not dependent upon the Mg²⁺ concentration. Crystallography studies have shown that Mg²⁺ plays a crucial role in the coordination of the substrate molecule within the catalytic site of the enzyme (Das *et al.*, 1996). It is unlikely that fatty acids interfere with Mg²⁺ binding in the reactive centre. It is also unlikely that fatty acids inhibit MP2C through substrate competition. Supportive evidence comes from the finding that the apparent V_{max} value is changed upon linolenic acid treatment. As the apparent K_m value is also modified, linolenic acid probably inhibits MP2C by a complex mechanism that affects both the activity of the enzyme and the binding of its substrate, possibly through conformational changes triggered by fatty acid binding.

Previous studies have proposed a regulation of plant PP2Cs by Ca²⁺. This assumption was based on the

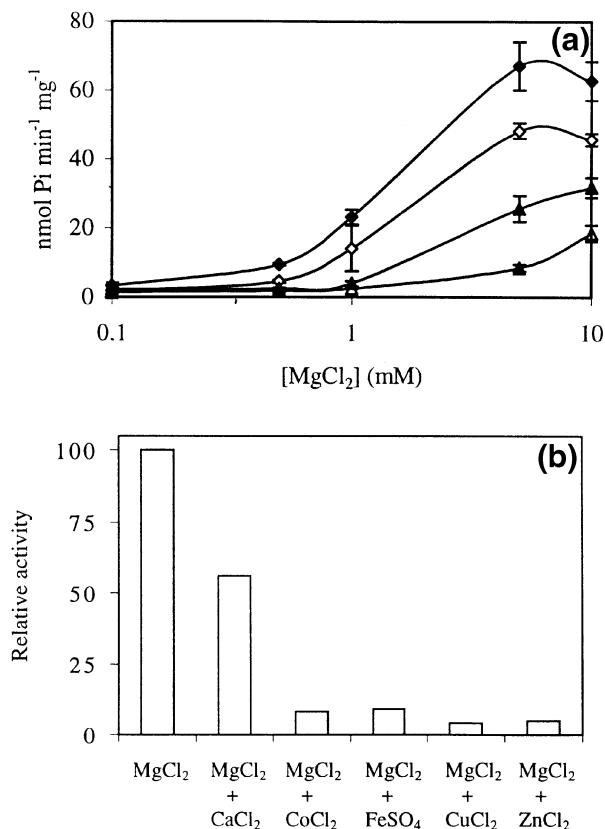
Table 2. Effect of fatty acids on MP2C activity

Fatty acid	Carbon chain	Relative activity (% of the control)
Lauric acid	12:0	98 ± 4
<i>cis</i> -5-dodecenoic acid	12:1	103 ± 8
Myristic acid	14:0	89 ± 3
Myristoleic acid	14:1	87 ± 7
Palmitic acid	16:0	89 ± 13
Palmitoleic acid	16:1	65 ± 3
Stearic acid	18:0	91 ± 8
Oleic acid	18:1	84 ± 11
Linoleic acid	18:2	65 ± 9
α -Linolenic acid	18:3	32 ± 10
Arachidic acid	20:0	74 ± 0
Arachidonic acid	20:4	33 ± 3

Phosphatase activity of GST-MP2C was assayed in the presence of 10 mM MgCl₂ and 0.5 mM fatty acid. Control activity in the absence of fatty acid was 28.9 ± 3.5 nmol Pi min⁻¹ mg⁻¹.

presence of a putative calcium-binding motif within the sequence of the N-terminal domain of the *Arabidopsis* PP2C ABI1 (Leung *et al.*, 1994; Meyer *et al.*, 1994). Further experiments have suggested that, even though calcium inhibits ABI1 activity *in vitro*, the high concentrations required make it unlikely that it is involved in the *in vivo* regulation of ABI1 (Bertauche *et al.*, 1996; Leube *et al.*, 1998). In this report, we show that Ca²⁺ inhibits MP2C *in vitro*, even though the MP2C sequence does not show any putative calcium-binding motif. A likely mechanism for MP2C inhibition by Ca²⁺ could involve competition for Mg²⁺-binding sites. This hypothesis agrees with the strong dependence of the inhibition on the Mg²⁺ concentration and a high IC₅₀ for Ca²⁺, which suggests that no high-affinity calcium-binding site is involved in the inhibition. The high concentration of Ca²⁺ required for efficient inhibition of the enzyme is also above the range observed during the response to wounding. Indeed, Knight *et al.* (1993) suggested that the intracellular calcium increase in response to wounding is in the low micromolar range. Moreover, we show that several divalent cations inhibit MP2C activity. Therefore, the inhibition of MP2C activity by Ca²⁺ is probably not physiologically relevant.

On the other hand, our data support a regulation of the wound-induced MAPK pathway by free fatty acids. In this respect, α -linolenic acid, the major unsaturated fatty acid produced following wounding, was also found to be the most potent inhibitor of MP2C. Although the inhibitory effect of linolenic acid exhibited a high IC₅₀ (approximately 130 μ M), such concentrations can be locally achieved *in vivo* upon wounding (Creelman *et al.*, 1992). Moreover, fatty acids do not inhibit MP2C through competition with its substrate. This makes it possible that the inhibitory effect of linolenic acid is under-estimated *in vitro* and that

**Figure 2.** Effect of divalent cations on MP2C activity.

(a) Phosphatase activity of GST-MP2C was determined as a function of magnesium concentration in the absence of calcium (◆) or in the presence of 1 mM (○), 5 mM (▲) or 10 mM CaCl₂ (△).

(b) Phosphatase activity of GST-MP2C was determined in the presence of 1 mM MgCl₂, with or without 1 mM of each of the divalent cations. Control activity in the presence of MgCl₂ alone was 7.3 nmol Pi min⁻¹ mg⁻¹.

additional regulators could act synergistically together with fatty acids to down-regulate MP2C activity *in vivo*. Ca²⁺ was not found to have any synergistic effect on the inhibition of MP2C by fatty acids (data not shown), and thus the nature of putative additional regulators remains to be established.

MAPK pathway activation upon wounding is a rapid phenomenon, which is observed within the first minutes of stress. In plants, the mechanisms by which MAPK pathways become activated and inactivated are still poorly understood. Based on our data, MP2C is a potential target through which other pathways such as the octadecanoic pathway could control the activity of the wound-activated MAPK pathway *in vivo*. Because wounding activates MAPKs within minutes, whereas free fatty acids generally accumulate between 10 and 30 min after wounding (Ryu and Wang, 1998), it is rather unlikely that fatty acids are involved in triggering MAPK activation. However, fatty acids could affect the total period and maximal strength of

MAPK activity. These parameters have been shown to be major determinants of the biological output of MAPK pathways in mammalian cells (Marshall, 1995). Whereas a transient MAPK activation initiated proliferation of cells, a long-term activation resulted in differentiation (Traverse *et al.*, 1992). It is unlikely that the inhibition of MP2C by free fatty acids represents the complete repertoire by which a cell can regulate the MAPK pathways, but it suggests that different signalling pathways can interact with each other in as yet unforeseen ways.

Experimental procedures

Chemicals

[γ - 32 P]-ATP (specific radioactivity > 185 TBq mmol $^{-1}$) was purchased from Amersham. Dephosphorylated casein, cAMP-dependent protein kinase and fatty acids were purchased from Sigma-Aldrich. Fatty acids were dissolved and diluted in ethanol.

Expression and purification of MP2C

GST-MP2C fusion protein was expressed in *Escherichia coli* as previously described (Meskiene *et al.*, 1998). Following a 2 h induction with 0.1 mM IPTG, cells were harvested by centrifugation. The GST fusion protein was isolated by resuspension of the cells in 10 ml of 50 mM Tris-HCl pH 8 buffer (10 mM MgCl $_2$, 150 mM NaCl and 1 mM PMSF). Following sonication four times for 10 sec, the homogenate was supplemented with 0.1% CHAPS and centrifuged at 10 000 g for 10 min. The supernatant was then applied onto a 1 ml GSH-Sepharose 4B column pre-equilibrated with 50 mM Tris-HCl pH 8 buffer (10 mM MgCl $_2$, 150 mM NaCl, 0.1% CHAPS). Following washing with 50 mM Tris-HCl pH 8 buffer (10 mM MgCl $_2$, 0.1% CHAPS and 10% glycerol), the GST-MP2C fusion protein was eluted by the same buffer containing 10 mM GSH, and stored at -80°C. The protein concentration was determined with the Bio-Rad detection system, using BSA as a standard, and purity of the protein fractions was determined on 10% SDS-PAGE gels.

Measurement of MP2C activity

MP2C activity was measured using 32 P-labelled casein as a substrate (Streuli *et al.*, 1990). Briefly, dephosphorylated casein was 32 P-labelled with bovine heart cAMP-dependent protein kinase. Radiolabelled protein was precipitated with 20% TCA. After extensive washing with 10% TCA, the casein was dissolved in 0.5 M Tris-HCl pH 8.

Phosphatase assays were performed in a total volume of 100 μ l, containing 50 mM Tris-HCl pH 8, 0.1 mM EGTA, 1 mM DTT, 100 nM okadaic acid and 10 mM MgCl $_2$, unless otherwise specified. When the effect of calcium was studied, EGTA was omitted from the reaction buffer. The reaction was started by the addition of 5 μ g labelled casein (approximately 200 000 cpm). MP2C was pre-incubated for 5 min with the tested substances prior to addition of casein. After incubation for 30 min at 30°C, the reaction was stopped by the addition of 700 μ l of an acidic charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH $_2$ PO $_4$, 4% (v/v) NoritA, Merck). Samples were centrifuged (10 min, 10 000 g) and the amount of radioactivity in 600 μ l of supernatant was

determined by scintillation counting. Measurements were done in triplicate and the results presented are the mean of two representative experiments.

Acknowledgements

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