Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 739-745

INOSITOL(1,4,5)TRISPHOSPHATE PRODUCTION IN PLANT CELLS: STIMULATION BY THE VENOM PEPTIDES, MELITTIN AND MASTOPARAN

Bjørn K. Drøbak and Peter A.C. Watkins

Department of Cell Biology
John Innes Centre
Colney Lane, Norwich NR4 7UH,
England

Received October 15, 1994

The ability of the amphiphilic peptides, melittin and mastoparan,to modulate the production of inositol(1,4,5)trisphosphate in cultured plant (Daucus carota L.) cells was investigated. When added to intact cells melittin and mastoparan caused a rapid and dose-dependent increase in inositol(1,4,5)trisphosphate concentrations. In isolated protoplasts, inositol(1,4,5)trisphosphate levels were 12- to 16-fold higher than in the corresponding cells and neither melittin nor mastoparan was able to significantly affect inositol(1,4,5)trisphosphate production. Melittin and mastoparan had a strong inhibitory effect (IC_{50}: 20 μM) on the activity of polyphosphoinositide-specific phospholipase C in purified plasma membranes. These results demonstrate that the plant phosphoinositide system can be activated by amphiphilic peptides in a manner analogous to that observed in specialized mammalian cells but that important functional components are altered, or lost, by the disruption of the intact cell state.

Activation of the phosphoinositide signalling system is now recognized as a primary event following stimulation of many eukaryotic cells. Interaction between specific agonists and their cellular targets results in the activation of phosphoinositide-specific phospholipase C isoforms which leads to the production of the two second messengers: inositol(1,4,5)trisphosphate and diacylglycerol (1). Most components of a phosphoinositide signalling system are present in plant cells (see ref. 2 for review) and Ins(1,4,5)P_3 has been shown to mediate release of Ca^{2+} both from isolated membrane fractions (3) and from intracellular stores when microinjected (4). A highly active polyphosphoinositide-specific phospholipase C (PPI-PLC) is associated

Abbreviations:

Ins(1,4,5)P_3: inositol 1,4,5-trisphosphate, PPI-PLC: polyphosphoinositide-specific phospholipase C.

0006-291X/94 $5.00

with the inner leaflet of the plasma membrane in plant cells (5) but only little is presently known about how this enzyme is activated *in vivo*. Several naturally occurring, amphiphilic polypeptides such as melittin (bee venom toxin) and mastoparan (wasp venom toxin) have been shown to possess a variety of biological activities in mammalian cells, including the ability to modulate membrane-associated signalling enzymes such as phospholipase C, phospholipase A_2, guanylate cyclase and protein kinase C (6-9). The precise mechanisms of action of these peptides are still not fully clarified but it is likely that their ability to take up α-helical configurations upon interactions with membranes is an important determinant for their biological activity. Interest in the use of melittin and mastoparan in signal transduction research has been further stimulated by the recent findings that melittin can inhibit Ca^{2+}-dependent phosphorylase kinase by specifically binding to calmodulin (10) and mastoparan can act as an activator of mammalian G-proteins by mimicking a specific region of activated G-protein-associated receptors (11). Although one study has suggested that the Ca^{2+}-dependent plant PPI-PLC may be regulated by G-proteins (12) there has been a general lack of success in demonstrating effects of non-hydrolysable GTP-analogues, and other G-protein regulators, on the activity of the PPI-PLC in isolated membrane fractions (see 2 and refs. therein).

In this study we have investigated the effect of melittin and mastoparan on the activity of PPI-PLC in intact cells, protoplasts and isolated plasma membranes. We demonstrate that both melittin and mastoparan, in low μmolar concentrations, elicit substantial, and rapid, increases in cellular Ins(1,4,5)P_3 levels when added to intact cells. In contrast, neither peptide has any stimulatory effect on Ins(1,4,5)P_3 production when added to protoplasts, and both peptides inhibit PPI-PLC in isolated plasma membranes. The significance of these findings for the further elucidation of plant signal transduction mechanisms is discussed.

MATERIALS AND METHODS

Cell culture and Ins(1,4,5)P_3 extraction and quantification.

Daucus carota L. cv. "Oxford" suspension cultures were maintained as described by McCann et al. (13). Cell suspensions were used for experiments 4 days after subculturing. Cells were pelleted by gentle centrifugation and adjusted to a concentration of 300-500 mg/ml of culture medium. After temperature equilibration for 5 minutes experiments were initiated by sampling of three 100 μl control samples at 30 seconds interval. Addition of peptides (Mastoparan, Sigma, U.K.; Melittin, Sigma, U.K., 85 % HPLC pure) or appropriate control vehicles was initiated immediately after sampling of the third control. Further aliquots of 100 μl were removed after incubation for the appropriate time and quenched with 100 μl ice-cold $HClO_4$ (10 % w/v). After 15 minutes on ice, protein and cellular debris was sedimented by centrifugation (12000 g) for 7 minutes and 100 μl of the supernatant removed and neutralized by 1.5 M KOH/60 mM Hepes. After removal of precipitated $KClO_4$ by centrifugation the Ins(1,4,5)P_3 concentration was determined in the supernatant by the receptor-binding assay described by Palmer & Wakelam (14). Commercially available preparations of Ins(1,4,5)P_3

binding proteins from bovine adrenal glands (Amersham, U.K., TRK 1000) were used for Ins(1,4,5)P_3 determinations and standard curves were constructed using [2-^{3}H]Ins(1,4,5)P_3 (Amersham, U.K.) as tracer.

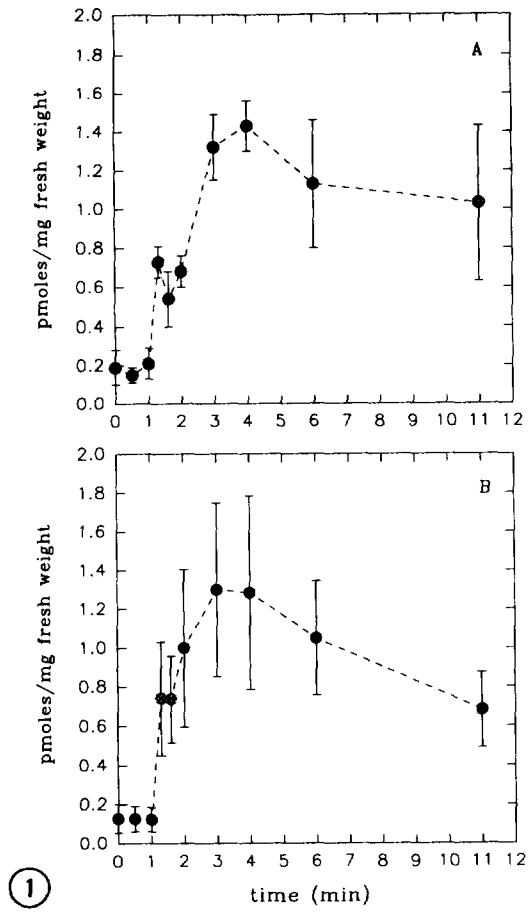
Preparation of protoplasts and plasma membranes.
Protoplasts were prepared as described by Xu et al. (15). Plasma membranes were isolated from suspension cultured carrot cells by aqueous polymer two-phase partitioning essentially as described by Drøbak et al. (16). A 5.7 % PEG/Dextran system, 5 mM KCl was employed and purification was continued to the U_2 fraction. Membrane purity and protein concentration was determined by standard procedures (see ref. 16 for details).

Assay of plasma membrane phospholipase C.
Polyphosphoinositide phospholipase C activity was assayed as described by Drøbak et al. (16) using 142 μM [2-^{3}H]PtdIns(4,5)P_2 micelles as substrate. Radioactivity was determined by liquid scintillation spectrometry (LKB Rack-beta) using Pico-Fluor 15 (Canberra-Packard, U.K.) scintillation fluid.

RESULTS AND DISCUSSION

The effect of melittin and mastoparan on the levels of Ins(1,4,5)P_3 in cultured carrot cells is shown in **figure 1A and 1B**. Prior to peptide addition the concentration of Ins(1,4,5)P_3 is 0.136 +/- 0.077 pmoles/mg fresh weight (n=25). With an average cell diameter of 25 μm and a vacuolar diameter 75 % of this value, an approximate cytoplasmic volume of 4.7 pL/cell can be calculated. Assuming an equal cytosolic distribution, the average resting concentration of Ins(1,4,5)P_3 is 960 +/- 550 nM. Melittin and mastoparan induce a rapid increase in Ins(1,4,5)P_3 levels which reaches a maximum 2-4 minutes after peptide addition. At this point the cytoplasmic concentration of Ins(1,4,5)P_3 is estimated to be around 10 μM. Although these figures are well above the K_d (121 nM) for the putative plant Ins(1,4,5)P_3 receptor (17) these figures are in very good agreement with the levels of Ins(1,4,5)P_3 observed in other eukaryotic cell both at rest and following stimulation (18). In the period of 4 to 10 minutes after peptide addition a gradual, but slow, decrease in Ins(1,4,5)P_3 levels is observed, but even after 10 minutes levels of cellular Ins(1,4,5)P_3 are significantly higher than prior to stimulation. The addition to intact cells of 12 μM of the inactive mastoparan analogue, Mas CP, had no effect on Ins(1,4,5)P_3 production (data not shown). Highly active Ins(1,4,5)P_3 metabolizing enzymes have been demonstrated to be present in plant cells (19) so the prolonged increase in Ins(1,4,5)P_3 levels is likely to be indicative of a sustained activation of the Ins(1,4,5)P_3-producing machinery.

Figure 2 illustrates the dose-dependency of melittin- and mastoparan-induced Ins(1,4,5)P_3 production. Half maximum stimulation of Ins(1,4,5)P_3 production is achieved by approximately 6 μM mastoparan whereas the effect of melittin appears to be linear dependent on doses up to at least 40 μM. Experiments in which melittin and mastoparan were



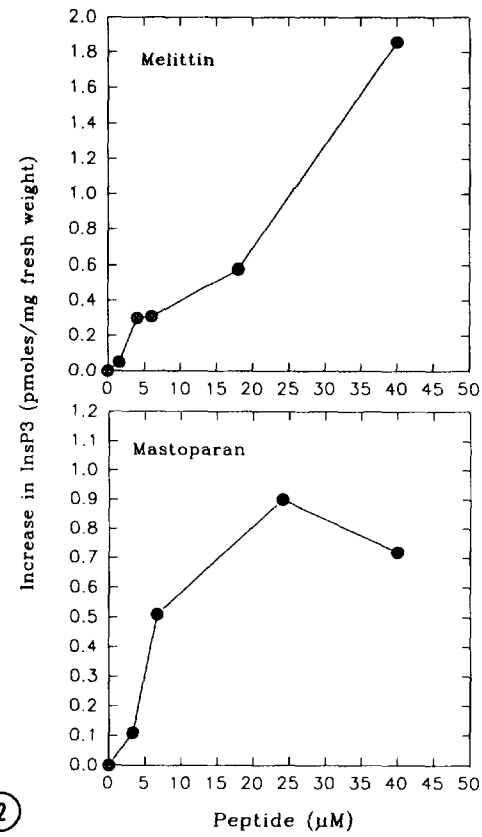


Figure 1. Time dependent effects of melittin (3.5 μM, panel A) and mastoparan (24 μM, panel B) on Ins(1,4,5)P_3 levels in cultured carrot cells. Peptides were added immediately after t = 1 minute. Data are average values obtained from 3-4 independent experiments. Vertical bars indicate range.

Figure 2. Dose-dependency of melittin- and mastoparan-induced Ins(1,4,5)P_3 production in cultured carrot cells. Data are expressed as net increase in cellular Ins(1,4,5)P_3 levels observed 40 seconds after peptide addition.

added to protoplasts are summarized in **figure 3**. It is evident that the pre-stimulation levels of Ins(1,4,5)P_3 are much higher than in the corresponding intact cells - and in contrast to intact cells neither melittin nor mastoparan is able to significantly increase Ins(1,4,5)P_3 production. These findings suggest that the enzymatic removal of the plant cell wall leads to activation of the phosphoinositide system. This hypothesis is in agreement with several sets of recent data showing that: A, cell wall degrading enzymes can affect the activity of phosphoinositide kinases (20); B, cell wall components induce cytosolic Ca^{2+}-increases when added to plant cells (21); and C, that polygalacturonic acid elicitors can promote a transient

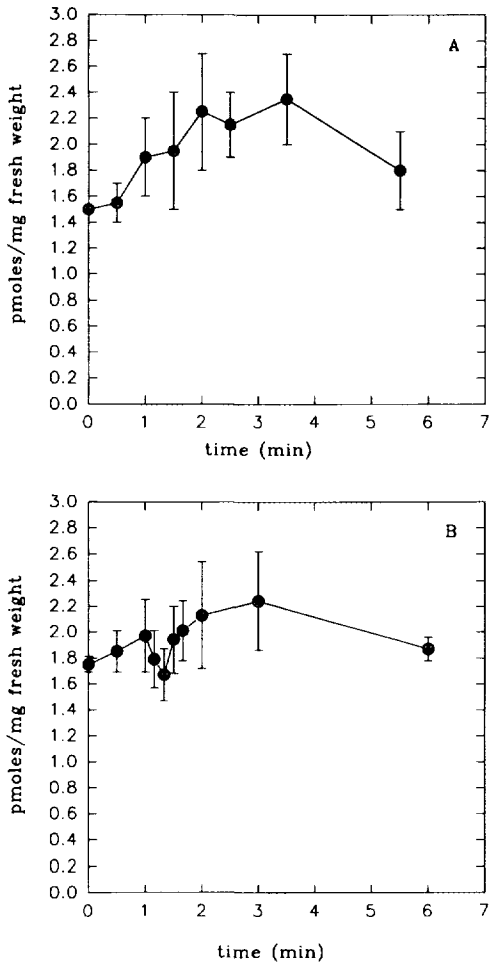


Figure 3. Effect of melittin (3.5 μM, panel A) and mastoparan (24 μM, panel B) on Ins(1,4,5)P_3 levels in carrot cell protoplasts. Peptides were added immediately after t = 1 minute. The solid symbols indicate the mean of Ins(1,4,5)P_3 determinations on two independent protoplast preparations. Vertical bars indicate range.

increase in Ins(1,4,5)P_3 in soybean cells - an effect mimicked by the mastoparan analogue Mas 7 (22). To investigate the possibility of a direct effect of melittin and mastoparan on the plasma membrane polyphosphoinositide phospholipase C these peptides were added to purified carrot cell plasma membranes predominantly with the cytoplasmic side exposed. Results from these experiments are shown in **figure 4** and demonstrate that both mastoparan and melittin inhibit PPI-PLC with an apparent IC_{50} of approximately 20 μM.

Ca^{2+}- and Ca^{2+}-calmodulin dependent protein kinases are established as Ca^{2+}-response elements in plant cells and cyclic GMP has recently been implicated in phototransduction (23). Polypeptides with homology to mammalian G-proteins have also been found in plant

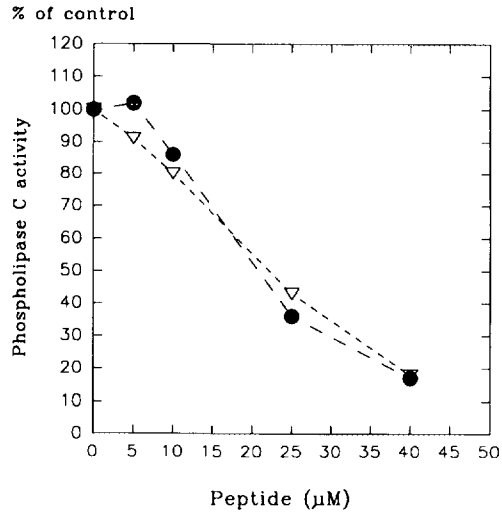


Figure 4. Effect of melittin (open symbols) and mastoparan (closed symbols) on the activity of carrot plasma membrane polyphosphoinositide specific phospholipase C. Data are expressed as percentage of control phospholipase C activity (100 %) measured in the absence of added peptides.

cells both by immunological methods and by cloning and sequencing of cDNAs of plant equivalents of mammalian G-protein α- and β-subunits (24,25). Recent experiments have shown how GTP-γS binding to plant membrane fractions is stimulated by mastoparan, suggesting the possible presence of plant equivalents to the mammalian seven-transmembrane span receptors (11). It also deserves mentioning that melittin and mastoparan have been shown to stimulate growth of zucchini hypocotyls by over 50 % (26).

It is obviously an attractive possibility that the effects of melittin and mastoparan on Ins(1,4,5)P_3 production demonstrated in this study result from their specific interaction with one or more signalling enzyme(s). However, since both peptides are amphiphilic they must be expected to interact with a variety of functional entities of plant membranes and further work is clearly needed to pin-point the specific site(s) of interaction between melittin, mastoparan and components of the Ins(1,4,5)P_3 producing machinery. Irrespective of their precise mode of action there is little doubt that these amphiphilic peptides, will prove valuable tools in the further elucidation of the mechanisms involved in the activation, and regulation, of the plant phosphoinositide system and other signalling pathways.

ACKNOWLEDGMENTS

The generous gift of mastoparan analogues from Drs. P.A. Millner and I.R. White, University of Leeds, U.K.,is gratefully acknowledged and Nicola Stacey (J.I.C., Norwich)

is thanked for her valuable help and advice regarding cell cultures. This work was supported by the Biotechnology and Biological Sciences Research Council, U.K.

REFERENCES

1. Berridge, M.J. (1993) Nature **361**, 315-325
2. Drøbak, B.K. (1992) Biochemical Journal **288**, 697-712
3. Drøbak, B.K. and Ferguson, I.B. (1985) Biochem.Biophys.Res.Commun. **130**, 1241-1246
4. Gilroy, S., Read, N. and Trewavas, A.J. (1990) Nature **346**, 769-771
5. Melin, P.-M., Sommarin, M., Sandelius, A.S. and Jergil, B. (1987) FEBS letters **223**, 87-91
6. Argiolas, A. and Pisano, J.J. (1983) J.Biol.Chem. **258**, 13697-13702
7. Okano, Y., Takagi, H., Tohmatsu, T., Nikashima, S., Kuroda, Y., Saito, K. and Nozawa, Y. (1985) FEBS Letters **188**, 363-366
8. Raynor, R.L., Zheng, B. & Kuo, J.F. (1991) J.Biol.Chem. **266**, 2753-2758
9. Chang, C.-H. and Song, D.-L. (1993) J.Biol.Chem. **268**, 4908-4911
10. Paudel, H.K., Xu, Y.-H., Jarrett, H.W. and Carlsson, G.M. (1993) Biochemistry **32**, 11865-11872
11. White, I.R., Wise, A. & Millner, P.A. (1993) Planta **191**, 285-288
12. Dillenschneider, M., Hetherington, A., Graziana, A., Alibert, G., Berta, P., Haiech, J. and Ranjeva, R. (1986) FEBS Letters **208**, 413-417
13. McCann, M.C., Stacey, N.J., Wilson, R. and Roberts, K. (1993) J.Cell Science **106**, 1347-1356
14. Palmer, S. & Wakelam, M.J.O. (1990) In Methods in Inositide Research (R.F. Irvine, Ed.) pp. 127-134. Raven Press, New York
15. Xu, P., Lloyd, C.W., Staiger, C.J. and Drøbak, B.K. (1992) The Plant Cell **4**, 941-951
16. Drøbak, B.K., Watkins, P.A.C., Valenta, R., Dove, S.K., Lloyd, C.W. and Staiger, C.J. (1994) Plant Journal **6**, 389-400
17. Brosnan, J.M. and Sanders, D. (1993) The Plant Cell **5**, 931-940
18. Bird, G.St.J., Oliver, K.G., Horstman, D.A., Obie, J. and Putney, J.W. (1991) Biochem J. **273**, 541-546
19. Drøbak, B.K., Watkins, P.A.C., Chattaway, J.A., Roberts, K. and Dawson, A.P. (1991) Plant Physiol. **95**, 412-419
20. Chen, Q. and Boss, W.F. (1990) Plant Physiol. **94**, 1820-1829
21. Messiaen, J., Van Cutsem, P. and Trewavas, A.J. (1993) J.Cell Sci. **104**, 365-371
22. Legendre, L., Yueh, Y.G., Crain, R.C., Haddock, N., Heinstein, P.F. and Low, P.S. (1993) J.Biol.Chem. **268**, 24559-24563
23. Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N.H. (1994) Cell **77**, 73-81
24. Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1990) Proc.Natl.Acad.Sci. **87**, 3821-3825
25. Ishida, S., Takahashi, Y. and Nagata, T. (1993) Proc.Natl.Acad.Sci. **90**, 11152-11156
26. Scherer, G.F.E. (1992) Plant Growth Regulation **11**, 153-157