Wortmannin and Its Structural Analogue Demethoxyviridin Inhibit Stimulated Phospholipase A2 Activity in Swiss 3T3 Cells

WORTMANNIN IS NOT A SPECIFIC INHIBITOR OF PHOSPHATIDYLINOSITOL 3-KINASE*

(Received for publication, July 10, 1995, and in revised form, August 30, 1995)

Michael J. Cross, Allison Stewart, Matthew N. Hodgkin, David J. Kerr, and Michael J. O. Wakelam†
†To whom correspondence should be addressed: Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT, United Kingdom

Wortmannin and its structural analogue demethoxyviridin (DMV) have been reported to be specific inhibitors of phosphatidylinositol 3-kinase activity. Here we report that these compounds are not as selective as assumed and demonstrate inhibition of bombesin-stimulated androgen receptors of phosphatidylinositol 3-kinase activity. Here we report that these compounds are not as selective as assumed and demonstrate inhibition of bombesin-stimulated phospholipase A2 activity by both wortmannin and DMV with an IC50 (2 mm) which is slightly more potent than the inhibition of insulin-stimulated phosphatidylinositol 3,4,5-trisphosphate generation in these cells (<10 nM). While it has not been possible to fully block in vitro phospholipase A2 activity with wortmannin, inhibition cannot be a consequence of inhibition of PI 3-kinase activity since bombesin fails to generate 3-phosphorylated lipids in the intact cell. Therefore, while wortmannin is indeed a PI 3-kinase inhibitor, it is not as specific as previously reported, and experimental conclusions based solely on its use should be treated with caution.

The fungal metabolite wortmannin and its structural analogue demethoxyviridin (DMV)1 have been demonstrated to have an inhibitory effect upon phosphatidylinositol 3-kinase (PI 3-kinase) activity at nanomolar concentrations (1). Inhibition has been demonstrated upon both PI 3-kinase activity in anti-p85 immunoprecipitates and the stimulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) generation in N-formylmethionylleucylphenylalanine (fMLP)-stimulated neutrophils (1). The binding of 17-[3H]hydroxywortmannin to a number of neutrophil proteins has been demonstrated, and one of these has been identified as the 110-kDa subunit of PI 3-kinase (2).

On the basis of these findings, it has been proposed that wortmannin and DMV are specific inhibitors of PI 3-kinase, and, thus, addition of these compounds to cells will result in the specific inhibition of the PI 3-kinase pathway. Consequently, an increasing number of papers have described the use of wortmannin and, on the sole basis of such experiments, assigned a role for PI 3-kinase in a number of physiological responses (e.g. Refs. 3–5).

Wortmannin has also been reported to inhibit phospholipase D (PLD) (6), myosin light chain kinase, and pleckstrin phosphorylation (see Ref. 1). Although these results could cast doubt upon the specificity of wortmannin, these effects have been shown to occur at concentrations greater than those reported to inhibit PI 3-kinase. However, the specificity of wortmannin for other lipid-metabolizing enzymes has not been examined. In this paper, we have examined the specificity of wortmannin and DMV and show that they inhibit stimulated PIP3-phospholipase C (PI-PLC), PLD, phospholipase A2 (PLA2) as well as PI 3-kinase in Swiss 3T3 cells and, in addition, in vitro PI 3-kinase and PLA2 activities. We also demonstrate that both compounds are more potent inhibitors of PLA2 than PI 3-kinase.

EXPERIMENTAL PROCEDURES

Materials—Radiochemicals were from Amersham International plc, except for the inositol phosphate standards which were from DuPont NEN, all tissue culture media and sera were from Life Technologies, Inc., thin layer chromatography plates and HPLC columns were from Whatman, and lipids were from Sigma, Avanti Polar Lipids, or Lipid Products, Nutley, Surrey, UK. Wortmannin was from Sigma, and DMV was a generous gift from Dr. G. MacAulay, Dept. of Chemistry, Glasgow University, UK. Anti-p85, done U13 was from Serotec. SF9 cell-expressed human cytosolic PLA2 (85-kDa enzyme) was a generous gift from Dr. C. J. Jackson, Fisons Pharmaceuticals, Loughborough, UK.

Culture and Labeling of Cells—Swiss 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% newborn calf serum at 37 °C in a humidified atmosphere of 5% (v/v) CO2 in air, all cells were quiesced in 2% (v/v) serum-containing medium for 24 h, with radiolabel as appropriate, prior to experiment. The cells were labeled in medium containing 2% (v/v) serum with 1 μCi/ml [3H]inositol for PI-PLC experiments, 4 μCi/ml [3H]palmitate for PLD experiments, and 1 μCi/ml [3H]arachidonate for the PLA2 experiments; in each case, labeling was for 24 h and the cells were grown in 24-well plates. For the measurement of 3-phosphorylated lipids, confluent cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium containing 0.1% (v/v) fatty acid-free bovine serum albumin and 20 mM Hepes, pH 7.4, and subsequently labeled for 90 min in 0.25 μCi/ml [3H]inositol phosphate in the same medium.

Phospholipase Assays in Intact Cells—The measurement of PI-PLC activity as the stimulated accumulation of [3H]inositol phosphates in the presence of 10 mM LiCl, PLD activity as the accumulation of [3H]phosphatidylbutanol in the presence of 30 mM butanol, and PLA2 activity by the stimulated increase in [3H]arachidonate generation were as described previously (7–9).

PI 3-Kinase Activity—Confluent quiesced cells were washed in Hanks' buffered saline containing 10 mM glucose and 0.1% bovine serum albumin, stimulated for the required period of time, washed with ice-cold phosphate-buffered saline, and then lysed in 1% (w/v) Nonidet P-40, 10% (v/v) glycerol, 20 mM Tris-HCl (pH 8), 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 0.5 mM Na3VO4 containing 10 μg/ml leupeptin and 0.2 μM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation, and their protein concentrations were determined by the BCA method (Sigma). A solution containing 100 μg of protein was incubated with 15 μl of anti-p85 PI 3-kinase subunit antibody (U13 hybridoma supernatant) for 2 h at 4 °C, and 20 μl of 50% (v/v) Protein G-Sepharose was then added for 2 h at 4 °C. The immunoprecipitates were washed successively at 4 °C as follows: 2 × 1 ml of lysis buffer, 2 × 1 ml of 0.5 M LiCl, 0.1 M Tris-HCl, pH 8.0, 1 × 1 ml of 0.1 M NaCl, 1

* This work was supported by grants from the Wellcome Trust, the Medical Research Council UK, and the Cancer Research Campaign. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Towhomcorrespondenceshouldbeaddressed:InstituteforCancerStudies,UniversityofBirmingham,BirminghamB152TT,UnitedKingdom

2The abbreviations used are: DMV, demethoxyviridin; PLA2, phospholipase A2; PI3-kinase, phosphatidylinositol 3-kinase; PLC, phospholipase C; PLD, phospholipase D; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIP2, phosphatidylinositol 3,4-bisphosphate; PIP1, phosphatidylinositol 3,4,5-trisphosphate; HPLC, high performance liquid chromatography.
Summary of inhibition of bombesin-stimulated phospholipases in Swiss 3T3 cells by DMV

Swiss 3T3 cells were stimulated with 100 nM bombesin, and the activation of the three phospholipases was measured as described under “Experimental Procedures.” The percent inhibition and IC50 values are the means of 3 separate experiments.

| Enzyme            | IC50 (nM) | % inhibition |
|-------------------|-----------|--------------|
| Phospholipase D   | 54.3 ± 12.1 | 55.9 ± 4.6   |
| Phospholipase C   | 27.5 ± 10.6 | 86.8 ± 2.5   |
| Phospholipase A2  | 2.1 ± 0.7   | 96.0 ± 5.3   |

**RESULTS AND DISCUSSION**

In the Swiss 3T3 fibroblast cell line, bombesin stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PI-PLC) and phosphatidylinositol by phospholipase A2 (PLD) and phospholipase D (PLD) and phospholipase A2 (PLA2) (12). Bombesin has been reported to have no effect upon the activation of PI 3-kinase in Swiss 3T3 cells (10); therefore, insulin was used to stimulate PI 3-kinase activity.

DMV inhibited bombesin-stimulated PI-PLC activity, as determined by inositol phosphate accumulation, in Swiss 3T3 cells with an IC50 value of approximately 30 nM (Table I). The wortmannin analogue also inhibited phosphatidylinositol-PLD activity, as measured by phosphatidylalcohol generation, with an IC50 of approximately 50 nM, although maximum inhibition was never greater than 60% (Table I). Similar potency values were reported for FMLP-stimulated neutrophils (6) except that the sensitivity of the PLC response to DMV was greater in the 3T3 cells, and, in the neutrophil, the PLD response was completely inhibited. These differences may reflect different phospholipase isomers in the different cell types.

DMV was an extremely potent inhibitor of bombesin-stimulated phosphatidylinositol-PLA2 activity in Swiss 3T3 cells. Fig. 1 shows that inhibition was observable at concentrations as low as 0.1 nM, and that the IC50 was approximately 2 nM. This inhibition of PLA2 was also observed with wortmannin (Fig. 1), demonstrating the potency of this class of compounds as PLA2 inhibitors. Inhibition of PLA2 was also observed in an in vitro assay. However, inhibition was only partial with approximately 30% inhibition being detected at 0.1 nM (Table II). This inhibition was dependent upon the inclusion of PIP3 in the assay, which has been reported to enhance phospholipase A2 activity (13). Incubation of a Swiss 3T3 cell lysate or an anti-PLA2 immunoprecipitate with wortmannin for 10 min prior to addition of the lipid substrate failed to induce any PLA2 inhibition in vitro.

Bombesin has been reported not to stimulate PI 3-kinase activity in Swiss 3T3 cells (10), and we have confirmed this observation both by assaying the activity of immunoprecipitated kinase (Fig. 2) and by determining changes in PIP3 levels (see Fig. 3). Therefore, we examined the effects of wortmannin and DMV in insulin-stimulated cells. Fig. 2 shows that insulin and platelet-derived growth factor, but not bombesin, increase the PI 3-kinase activity in anti-p85 immunoprecipitates. In the in vitro PI 3-kinase assay, where the insulin-stimulated kinase was immunoprecipitated using an anti-p85 subunit antibody, DMV inhibited enzyme activity with an IC50 value between 1 and 2 nM (Fig. 2). This is similar to the value of 3.4 nM previously reported (14) and compares with reported IC50 values between 1 and 10 nM for PI 3-kinase.
Inhibition of phospholipase A₂ in an in vitro assay by wortmannin

| Wortmannin concentration | Phospholipase A₂ activity (nmol PC hydrolyzed/min/µg enzyme protein) |
|------------------------|---------------------------------------------------------------|
| 0                      | 5.04 ± 0.16                                                  |
| 0.001 mM               | 4.62 ± 0.01                                                  |
| 0.01 mM                | 4.41 ± 0.07                                                  |
| 0.1 mM                 | 3.71 ± 0.20                                                  |
| 1 mM                   | 4.00 ± 0.12                                                  |
| 1 µM                   | 3.91 ± 0.12                                                  |

**Fig. 2. Inhibition of PI 3-kinase activity by DMV.** PI 3-kinase activity was determined in anti-p85 immunoprecipitates prepared from insulin-stimulated cells. Increasing concentrations of DMV were added prior to the addition of the [γ³²P]ATP. Results are presented as percent inhibition ± S.D. of the activity in the absence of DMV which was 0.0129 ± 0.0005 pmol of phosphate incorporated into PI 3-P/mg of cellular protein. The inset shows the generation of PI 3-P in immunoprecipitates prepared from cells stimulated with insulin, platelet-derived growth factor (PDGF) or bombesin.

**Fig. 3. The generation of PIP₃ in Swiss 3T3 cells.** ³²P-Labeled Swiss 3T3 cells were stimulated with insulin or bombesin ± DMV or ± wortmannin, the lipids were isolated, deacylated, and deglycerated, and the Ins(1,3,4,5)P₄ fraction which is generated from PIP₃ was isolated by HPLC. A, generation of PIP₃ in response to insulin (■) and bombesin (○). B, generation of PIP₃ in response to insulin − DMV (□) or + 1 μM DMV (○). C, generation of PIP₃ in response to insulin − wortmannin (□) or + 1 μM wortmannin (○).

PIP₃ generation was inhibited by both DMV and wortmannin (Fig. 3) with DMV being slightly more potent with an approximate IC₅₀ of 10 nM compared to a value greater than 10 nM for wortmannin (Fig. 4). The more potent effect of DMV compared to wortmannin upon 3-phosphorylated lipid generation is similar to that reported for effects upon PI 3-kinase activity in immunoprecipitates (14).

The data presented in Fig. 1 clearly define wortmannin and DMV as potent and complete inhibitors of stimulated phospholipase A₂ activity in Swiss 3T3 cells. However, in vitro inhibition was incomplete when the enzyme was activated by PIP₂, diacylglycerol, and calcium (Table II). The contrast between the in vitro and in vivo inhibition of phospholipase A₂ suggests a complex mode of action for wortmannin and DMV. It is possible that they interact directly with the phospholipase and that the incomplete effect was due to a nonphysiological conformation taken up by the protein in the test tube. An alternative possibility is that inhibition may be due to an interaction with a protein which regulates phospholipase A₂ activity in the stimulated cell. Should this be the case, it is unlikely that the protein involved is PI 3-kinase since in Swiss 3T3 cells bombesin activates phospholipase A₂ without stimulating PI 3-kinase. Additionally, when stimulated phospholipase A₂ and PI 3-kinase activities are inhibited in vivo by wortmannin and DMV, both compounds are more potent against the release of arachidonate than the generation of 3-phosphorylated lipids.
The potency of DMV was greater than that of wortmannin for each of the enzymes examined. However, this difference is offset by the reduced stability of DMV when compared to wortmannin. Indeed, when DMV was incubated in a physiological buffer at 37°C for 10 min prior to addition to cells, it was rendered inactive (results not shown). In view of the instability of these compounds in aqueous solution, their experimental and therapeutic use will remain limited until the development of more stable analogues.

The results presented in this paper demonstrate that wortmannin and its structural analogue DMV are not specific or therapeutic use will remain limited until the development of more stable analogues.

Accordingly, where a role for PI 3-kinase in a particular pathway or cellular function has been assigned on the basis purely of its inhibition by wortmannin, the conclusions may be incorrect and a role for other signaling pathways such as phospholipase A₂ must be considered. A clear example of this is in the regulation of neutrophil responses where a central role for PI 3-kinase has been proposed on the basis of wortmannin sensitivity (1). Other studies have suggested a role for the phospholipase A₂ product arachidonate in the activation of neutrophils (15), and, thus, the nonselective action of wortmannin would suggest a need for a reassessment. Many papers reporting the use of wortmannin have utilized concentrations of 100 nM and above; however, at these levels, as the results presented here demonstrate, there will be inhibition of PLC, PLD, PL₃₂, and PI 3-kinase. Despite these caveats, it is clear that in many cases where wortmannin has been used to identify a role for PI 3-kinase the conclusions are correct, but these studies have utilized further experimental evidence, e.g. dominant negative p85 subunit transfections (16), to confirm their findings. In other cases, it could be phospholipase A₂ that is the enzyme of importance.

Acknowledgments—We thank Dr. C. G. Jackson, Fisons plc, Loughborough, UK, for his assistance with the in vitro phospholipase A₂ assays.

REFERENCES

1. Arcaro, A., and Wymann, M. P. (1993) Biochem. J. 296, 297–301
2. Thelen, M., Wymann, M. P., and Langen, H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4960–4964
3. Nakamura, I., Takahashi, N., Sasaki, T., Tanaka, S., Udagawa, N., Murakami, M., Kimura, K., Kubayama, Y., Kurokawa, T., Suda, T., and Fukui, Y. (1995) FEBS Lett. 363, 79–84
4. Shepherd, P. R., Nave, B. T., and Siddle, K. (1995) Biochem. J. 305, 25–28
5. Kimura, K., Hattori, S., Kubayama, Y., Shizawa, Y., Takayanagi, J., Nakamura, S., Toki, S., Matsuda, Y., Onodera, K., and Fukui, Y. (1994) J. Biol. Chem. 269, 10985–10987
6. Bonser, R. W., Thompson, N. T., Randall, R. W., Tateson, J. E., Spacey, G. D., Hodson, H. F., and Garland, L. G. (1991) Br. J. Pharmacol. 103, 1237–1241
7. Cook, S. J., Briscoe, C. P., and Wakelam, M. J. O. (1991) Biochem. J. 280, 431–438
8. Currie, S. J., Smith, G. L., Critchton, C. A., Jackson, C. G., Hallam, C., and Wakelam, M. J. O. (1992) Biochem. J. 286, 605–6062
9. Plevin, R., Palmer, S., Gardner, S. D., and Wakelam, M. J. O. (1990) Biochem. J. 268, 605–610
10. Jackson, T. R., Stephens, L. R., and Hawkins, P. T. (1992) J. Biol. Chem. 267, 16627–16636
11. Wong, N. S., Bancer, C. J., Morris, A. J., Craxton, A., Kirk, C. J., and Michell, R. H. (1992) Biochem. J. 286, 459–468
12. Wakelam, M. J. O., Briscoe, C. P., Stewart, A., Pettitt, R. T., Cross, M. J., Paul, A., Yule, J. M., Gardner, S. D., and Hodgkin, M. (1993) Biochem. Soc. Trans. 21, 874–877
13. Leslie, C. C., and Channon, J. Y. (1990) Biochim. Biophys. Acta 1045, 261–270
14. Woscholski, R., Kodaki, T., McKinnon, M., Waterfield, M. D., and Parker, P. J. (1994) FEBS Lett. 342, 109–114
15. Dana, R., Malech, H. L., and Levy, R. (1994) Biochem. J. 297, 217–223
16. Wennstrom, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L., and Stephens, L. (1994) Curr. Biol. 4, 385–393