Br. J. Pharmacol. (1991), **103**, 1237–1241

Demethoxyviridin and wortmannin block phospholipase C and D activation in the human neutrophil

[1]R.W. Bonser, N.T. Thompson, R.W. Randall, J.E. Tateson, G.D. Spacey, *H.F. Hodson & *L.G. Garland

Biochemical Sciences Department and *Research Directorate, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS

1 The fungal metabolite, wortmannin, has recently been shown to inhibit fMet-Leu-Phe-stimulated superoxide production and phospholipase D (PLD) activation in the human neutrophil.

2 We have found that a close structural analogue of wortmannin, demethoxyviridin, has a similar inhibitory profile but in addition blocks phosphatidylinositol 4,5-bisphosphate-specific phospholipase C and hence inositol 1,4,5-trisphosphate (IP_3) formation.

3 Inhibition of fMet-Leu-Phe-stimulated PLD by demethoxyviridin was characteristically non-competitive ($IC_{50} = 31 \pm 10$ nM).

4 Inhibition of fMet-Leu-Phe-stimulated IP_3 formation required concentrations almost 10 times higher ($IC_{50} = 250 \pm 130$ nM).

5 Surprisingly, demethoxyviridin only inhibited fMet-Leu-Phe-induced intracellular calcium mobilization at concentrations 100 times greater than those needed to block IP_3 formation.

6 Demethoxyviridin also inhibited PLD activation induced by sodium fluoride or phorbol myristate acetate (PMA) but the concentrations required were 100 times those needed to block fMet-Leu-Phe-stimulated PLD.

7 These observations support the contention that PLD plays an important role in signal transduction in the human neutrophil and indicate that wortmannin and demethoxyviridin inhibit PLD activation at a common step in the signalling pathway.

8 Furthermore, these results suggest that demethoxyviridin may block the interaction between the chemotactic peptide receptor and a GTP-binding protein that is intimately involved in PLD activation.

Keywords: Neutrophil; phospholipase D; phosphatidylbutanol; phospholipase C; inositol 1,4,5-trisphosphate; diradylglycerol; calcium mobilization; superoxide

Introduction

There is now a considerable amount of evidence suggesting that phospholipase D (PLD) plays an important role in intracellular signal transduction (Pelech & Vance, 1989; Loffelholz, 1989; Exton, 1990). PLD is activated by a variety of stimulatory agonists in many different cell types (Kobayashi & Kanfer, 1987; Bocckino *et al.*, 1987; Pai *et al.*, 1988a; Rubin, 1988; Liscovitch, 1989; Gelas *et al.*, 1989; Ben-Av & Liscovitch, 1989; Qian & Drewes, 1989; Gruchalla *et al.*, 1990). The major substrate for this enzyme appears to be phosphatidylcholine (PC), which is hydrolysed to phosphatidic acid (PA). PA can be further metabolised by phosphatidate phosphatase to yield the second messenger, diacylglycerol, which activates protein kinase C. PA may also serve a second messenger function since it is known (i) to be mitogenic, (ii) to increase intracellular calcium and guanosine 3',5'-cyclic monophosphate (cyclic GMP) concentrations, (iii) to activate phospholipase A_2 and (iv) to inhibit cellular adenylate cyclase (Moolenaar *et al.*, 1986; Yu *et al.*, 1988; Imagawa *et al.*, 1989; Ohsako & Deguchi, 1981; 1983; Harris *et al.*, 1981; Van Corven *et al.*, 1989). The precise mechanism by which PLD is activated is unclear, but it is known that calcium ionophores, phorbol esters, unsaturated fatty acids and guanine nucleotides will increase PLD activity in intact cells and isolated membrane preparations (Bocckino *et al.*, 1987; Qian & Drewes, 1989; Billah *et al.*, 1989; Anthes *et al.*, 1989; Tettenborn & Mueller, 1988). PLD activity can be readily quantitated in intact cells by measuring the formation of phosphatidylalcohols, which are produced by a unique PLD-dependent transphosphatidylation reaction (Kobayashi & Kanfer, 1987; Pai *et al.*, 1988b). Recent work from this laboratory has indicated that in cytochalasin B-primed neutrophils, superoxide production stimulated by the chemotactic peptide, fMet-Leu-Phe, is totally dependent on PLD activation (Bonser *et al.*, 1989).

The fungal metabolite, wortmannin, is a potent inhibitor of neutrophil activation. Wortmannin inhibits fMet-Leu-Phe-stimulated superoxide production without affecting the mobilization of intracellular calcium (Dewald *et al.*, 1988). Further information on its mode of action comes from the studies of Reinhold *et al.* (1990) who have reported that wortmannin blocks PLD activation induced by fMet-Leu-Phe. In this study we have investigated the effects of a close structural analogue of wortmannin i.e. demethoxyviridin, on signal transduction events in the human neutrophil. Like wortmannin, demethoxyviridin potently inhibits the respiratory burst in neutrophils and blocks PLD activation in response to the chemotactic peptide. Furthermore, demethoxyviridin is also able to block fMet-Leu-Phe-stimulated inositol 1,4,5-trisphosphate (IP_3) formation. Evidence is presented which suggests that wortmannin and demethoxyviridin act by a common mechanism that may disrupt the interaction between the chemotactic peptide receptor and a guanosine 5'-triphosphate (GTP)-binding protein(s).

Methods

Human peripheral blood neutrophils were purified (Tateson *et al.*, 1988) and suspended in 30 mM HEPES-buffered Hanks balanced salt solution, pH 7.2 (Hanks & Wallace, 1949). fMet-Leu-Phe- and phorbol ester-stimulated superoxide generation were measured as described by Cohen & Chovaniec (1978). PLD activity was estimated by measuring the incorporation of high specific activity [^{3}H]-butan-1-ol (12 Ci mmol^{-1}, Amersham International, Amersham, Bucks) into [^{3}H]-phosphatidylbutanol (PBut) as described by Randall *et al.* (1990).

[1] Author for correspondence.

Amounts of IP_3 were measured by the method described by Palmer *et al.* (1988) and levels of diradylglycerol (DRG) were determined according to Preiss *et al.* (1986), except that mixed micelles containing 5 mol% of phosphatidylserine in Triton X-100 (0.3%, w/v) were used in place of β-octylglucoside/cardiolipin. Release of intracellular calcium was measured with the fluorescent probe fluo-3AM (Molecular Probes Inc.) Fluo-3AM-loaded neutrophils were stimulated with fMet-Leu-Phe in the presence of 4 mM EGTA and fluorescence was monitored with a Perkin-Elmer MPF2 spectrofluorimeter with monochromators set at 506 nm excitation and 526 nm emission. A K_d value of 4.5×10^{-7} M for fluo-3 was used to quantify the fluorescence (Minta *et al.*, 1989).

Wortmannin was supplied by Sandoz, Basle, Switzerland and demethoxyviridin was a gift from Dr J. Hanson, Sussex University, Brighton, E. Sussex. Inhibitors were dissolved in dimethylsulphoxide and the final vehicle concentration did not exceed 0.2% by volume.

Results

The effects of wortmannin and demethoxyviridin on human neutrophil activation are compared in Figure 1. Wortmannin was found to be a potent inhibitor of fMet-Leu-Phe-stimulated superoxide production, with an IC_{50} value (concentration causing 50% inhibition) of 7 ± 1 nM. Demethoxyviridin was equally potent and blocked the respiratory burst with an IC_{50} of 5 ± 2 nM. At concentrations up to 1 μM, wortmannin and demethoxyviridin had no effect on PMA-stimulated superoxide production (results not shown). Although wortmannin is a potent inhibitor of superoxide production it is reported to have no effect on fMet-Leu-Phe-induced intracellular calcium mobilization, even at concentrations up to 1 μM (Dewald *et al.*, 1988). Figure 1 illustrates the action of demethoxyviridin on chemotactic peptide-stimulated intracellular calcium mobilization. Release of intracellular calcium was inhibited by demethoxyviridin but only at concentrations several thousand times greater than were required to block superoxide production. All of these observations strongly suggest that wortmannin and demethoxyviridin inhibit receptor-effector coupling by a common mechanism.

This mechanism was investigated further by monitoring the effects of wortmannin and demethoxyviridin on fMet-Leu-Phe-stimulated second messenger-diacylglycerol production. Both inhibitors blocked diradylglycerol (DRG) generation stimulated by the chemotactic peptide (Figure 2). Demethoxyviridin was approximately six times more potent than wortmannin as an inhibitor of DRG production (IC_{50} values for

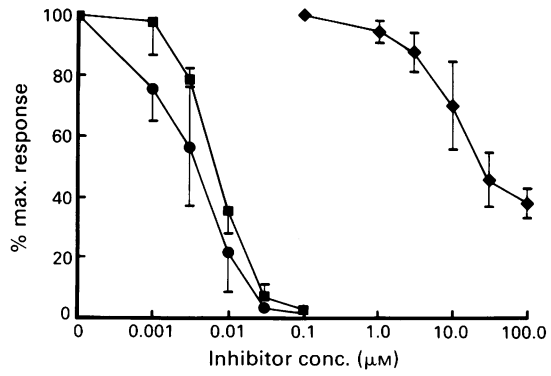

Figure 1 Effect of wortmannin and demethoxyviridin on fMet-Leu-Phe-stimulated superoxide production and intracellular calcium mobilization. Cytochalasin B-treated human neutrophils were stimulated with 100 nM fMet-Leu-Phe in the presence of wortmannin (■) or demethoxyviridin (●, ◆) and superoxide production (●, ■) or intracellular calcium mobilization (◆) measured as described in Methods. Results are the mean from three separate experiments with neutrophils from different donors; vertical bars show s.e.mean.

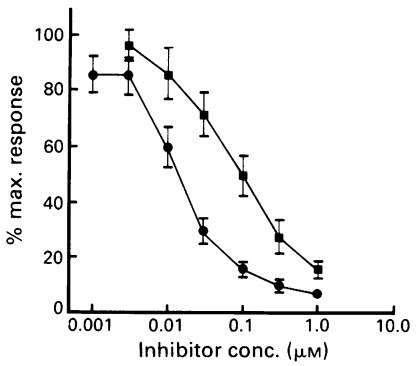

Figure 2 Inhibition of fMet-Leu-Phe-stimulated diradylglycerol (DRG) formation by wortmannin or demethoxyviridin. Human neutrophils pretreated with cytochalasin B were incubated with different concentrations of wortmannin (■) or demethoxyviridin (●) for 5 min at 37°C. fMet-Leu-Phe (100 nM) was then added and DRG production measured by radioenzymatic assay. Results are the mean from three separate experiments with neutrophils from different donors; vertical bars show s.e.mean. Resting DRG levels were 111 ± 10 pmol per 10^7 cells ($n = 36$) and increased after 5 min to 573 ± 44 pmol per 10^7 cells ($n = 36$) in the absence of the inhibitors.

wortmannin and demethoxyviridin were 110 ± 45 nM and 15 ± 3 nM, respectively). Since wortmannin is reported to block fMet-Leu-Phe-induced PLD activation (Reinhold *et al.*, 1990), it was necessary to explore the effect of demethoxyviridin on PLD. Demethoxyviridin inhibited the PLD-dependent formation of phosphatidylbutanol (PBut) in chemotactic peptide-stimulated human neutrophils at concentrations similar to those needed to block DRG formation (Figure 3). This observation provides further evidence that the PLD-phosphatidate phosphatase pathway is the major source of DRG in activated neutrophils. The mode of action and selectivity of demethoxyviridin as an inhibitor of phospholipase activation was examined by measuring its effect on chemotactic peptide-stimulated inositol 1,4,5-trisphosphate (IP_3) production (Figure 3). Surprisingly, phospholipase C-dependent IP_3 formation was blocked by demethoxyviridin at concentrations that were only 10 times higher than those needed to inhibit PLD activation (Figure 3). More importantly, the concentrations of demethoxyviridin that almost totally abolished

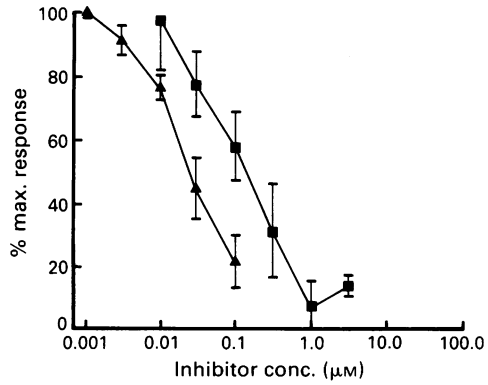

Figure 3 Effect of demethoxyviridin on fMet-Leu-Phe-stimulated 1,4,5-trisphosphate (IP_3) production and [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation. Cytochalasin B-pretreated human neutrophils were incubated with demethoxyviridin for 5 min at 37°C then stimulated with 100 nM fMet-Leu-Phe and phospholipase D (PLD)-dependent [^{3}H]-PBut formation measured after a further 5 min (▲). IP_3 production induced by fMet-Leu-Phe (300 nM) was monitored at 20 s in the absence of cytochalasin B (■). Results are the mean for three separate experiments with neutrophils from different donors; vertical bars show s.e.mean. Basal [^{3}H]-PBut values were 1893 ± 528 d.p.m. ($n = 3$) and increased to 5749 ± 2778 d.p.m. ($n = 3$) in the absence of demethoxyviridin. Resting IP_3 levels were 13.3 ± 2.0 pmol per 10^7 cells ($n = 13$) and reached 50.3 ± 8.1 pmol per 10^7 cells in the absence of the inhibitor.

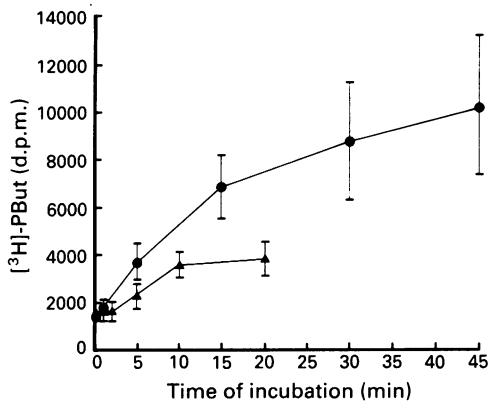

Figure 4 Time courses for phorbol myristate acetate (PMA)- and sodium fluoride-induced [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation in human neutrophils. Neutrophils pretreated with cytochalasin B were incubated with 100 nM PMA (●) or 20 mM sodium fluoride (▲) and phospholipase D-dependent [^{3}H]-PBut formation measured as described in Methods. Results are the mean for three separate experiments with neutrophils from different donors; vertical bars show s.e.mean.

IP_3 formation had no effect on intracellular calcium mobilization (Figures 1 and 3).

The site of interaction of demethoxyviridin with the receptor-linked PLD-dependent signalling pathway in the neutrophil was investigated by monitoring its effects on PMA- and sodium fluoride-stimulated PBut formation. PMA-stimulated transphosphatidylation was slow and still increasing after 45 min (Figure 4). In contrast, PLD activation induced by fluoride displayed a distinct lag period of 1–2 min followed by a rapid response that was complete within 10 min (Figure 4). Demethoxyviridin inhibited PMA-stimulated PLD activity in a concentration-dependent manner ($IC_{50} = 4 \pm 1\,\mu M$) and blocked fluoride-activated PLD at concentrations above 300 nM (Figure 5). It should be noted that these concentrations are much greater than are required to inhibit fMet-Leu-Phe-stimulated PBut formation (Figure 3).

The type of inhibition exhibited by demethoxyviridin in fMet-Leu-Phe-stimulated neutrophils was examined by monitoring its effects on the chemotactic peptide dose-response curve for PBut formation (Figure 6). Increasing concentrations of demethoxyviridin progressively depressed maximum PBut formation in response to fMet-Leu-Phe with little indication of a rightward shift in ED_{50} values, consistent with a non-competitive type of inhibition.

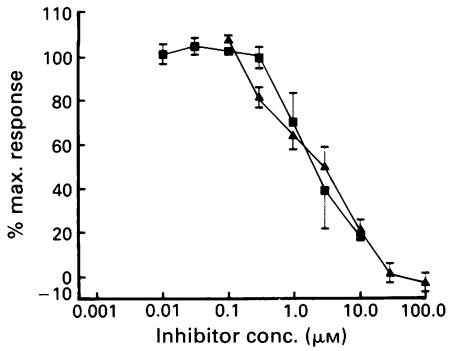

Figure 5 Effect of demethoxyviridin on phorbol myristate acetate (PMA)- and sodium fluoride-stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) production in human neutrophils. Neutrophils (0.5 ml at 2×10^7 cells per ml) were preincubated with cytochalasin B and different concentrations of demethoxyviridin for 5 min at 37°C then treated with 100 nM PMA (▲) or 20 mM sodium fluoride (■) and [^{3}H]-PBut formation quantitated. PMA- and sodium fluoride-induced PBut formation was measured after 30 min and 20 min, respectively. Results are the mean from three experiments with neutrophils from different donors; vertical bars show s.e.mean.

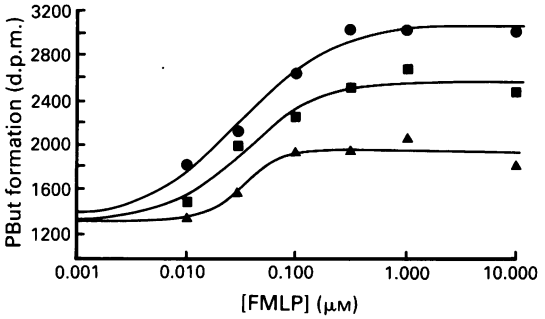

Figure 6 Inhibition of fMet-Leu-Phe (FMLP)-stimulated [^{3}H]-phosphatidylbutanol ([^{3}H]-PBut) formation by demethoxyviridin. Concentration-effect curves for fMet-Leu-Phe-stimulated [^{3}H]-PBut formation were generated in the absence (●) or presence of 20 nM (■) and 50 nM (▲) demethoxyviridin, according to the procedure outlined in the legend to Figure 3. Results are from a single representative experiment.

Discussion

Recent work has suggested that the fungal metabolite, wortmannin, may inhibit chemotactic peptide-stimulated superoxide production by blocking the activation of PLD (Dewald *et al.*, 1988; Reinhold *et al.*, 1990). The formation of phosphatidylalcohols is a specific indicator of PLD activity and provides a simple means of monitoring PLD activity in intact cells (Kobayashi & Kanfer, 1987; Pai *et al.*, 1988b). We have used the formation of [^{3}H]-PBut from high specific activity [^{3}H]-butan-1-ol to investigate the inhibitory action of a close analogue of wortmannin, i.e. demethoxyviridin, in the human neutrophil. We have found that demethoxyviridin and wortmannin inhibit superoxide production at similar concentrations. Furthermore, demethoxyviridin blocked PLD-dependent phosphatidylalcohol formation at concentrations similar to those reported for wortmannin (Reinhold *et al.*, 1990), suggesting that these fungal metabolites act at a common step in the signal transduction pathway. The inhibition of fMet-Leu-Phe-stimulated [^{3}H]PBut formation was characteristically non-competitive, suggesting that demethoxyviridin does not block the interaction of the chemotactic peptide with its receptor. This observation also reinforces the view that these inhibitors block signal transduction events.

Information on the site of action of wortmannin has been obtained from studies that have bypassed receptor events and activated PLD directly. It is reported that wortmannin does not inhibit phosphatidylethanol (PEt) formation stimulated by PMA (Reinhold *et al.*, 1990). In agreement with this, we found that PMA-induced transphosphatidylation was only inhibited by demethoxyviridin at high concentrations. These observations indicate that wortmannin and demethoxyviridin are not active site inhibitors and that inhibition probably occurs at a point along the signalling pathway that lies between the chemotactic peptide receptor and PLD. Interestingly, Reinhold and co-workers (1990) reported that PMA was a weaker stimulus than fMet-Leu-Phe for PLD activation in the neutrophil. It is worthwhile noting that this group measured PMA-induced PEt formation after 5 min. Our data show that this period of incubation would only detect a fraction of the PLD activity stimulated by PMA.

Phorbol esters and fMet-Leu-Phe stimulate the release of large amounts of DRG from cytochalasin B-treated neutrophils (Honeycutt & Niedel, 1986; Bonser *et al.*, 1989; Dougherty *et al.*, 1989). There is now strong evidence indicating that the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC cannot be the sole source of this lipid and that the majority of the DRG released is probably derived through the PLD-phosphatidate phosphatase pathway (Pelech & Vance, 1989; Loffelholz, 1989; Bonser *et al.*, 1989; Exton, 1990). The inhibitory effects of wortmannin and demethoxyviridin on DRG production observed in this study provides further

support for this pathway as the major source of second messenger-diacylglycerol in the neutrophil. It has been reported by Reinhold *et al.* (1990) that wortmannin does not block fMet-Leu-Phe-stimulated diacylglycerol production. We can offer no explanation for their failure to observe any effect, since in our experiments significant inhibition of DRG production (i.e. 40–50%) was observed with wortmannin at the concentrations used by these investigators.

Activation of PLD may involve a GTP-binding protein since non-hydrolysable GTP analogues (e.g. GTPγS) have been shown to stimulate PLD in membranes isolated from brain, liver and HL60 cells (Boccckino *et al.*, 1987; Qian & Drewes, 1989; Tettenborn & Mueller, 1988; Anthes *et al.*, 1989). There are several examples where G-protein activation has been mimicked by high concentrations of fluoride (Eckstein *et al.*, 1979; Sternweis & Gilman, 1982; Bigay *et al.*, 1985; Gabig *et al.*, 1987). Fluoride was able to activate PLD in cytochalasin B-treated neutrophils but this response was not inhibited by demethoxyviridin at concentrations that completely inhibited fMet-Leu-Phe-stimulated PBut formation. These observations suggest that demethoxyviridin does not block GTP-binding protein function but may interfere with the interaction between the GTP-binding protein and the chemotactic peptide receptor.

Mobilization of intracellular calcium is mediated by the second messenger IP_3, produced by a receptor-coupled, phosphatidylinositol 4,5-bisphosphate-specific PLC (Berridge & Irvine, 1989). Wortmannin is reported not to inhibit fMet-Leu-Phe-stimulated calcium release at concentrations which completely block superoxide production, suggesting that it does not inhibit IP_3 formation (Dewald *et al.*, 1988). Demethoxyviridin did inhibit IP_3 formation induced by the chemotactic peptide but the concentrations required were 10–20 times greater than those needed to block PLD activation and DRG production. More surprisingly, inhibition of intracellular calcium mobilization by demethoxyviridin occurred at concentrations 100 times greater than those required to inhibit IP_3 formation. It is possible to envisage several explanations for this discrepancy. The most likely possibility is that only a fraction of the IP_3 that is formed is required to mobilize intracellular calcium. Alternatively, a separate mechanism for mobilizing intracellular calcium may exist that is independent of IP_3 production.

PA is reported to increase cellular calcium concentrations and evoke calcium-mediated physiological responses (Salmon & Honeyman, 1980; Ohsako & Deguchi, 1981; 1983; Harris *et al.*, 1981). It has been suggested that PA may function as an endogenous calcium ionophore, although it is also known to enhance the production of IP_3 (Putney *et al.*, 1980; Jackowski & Rock, 1989). Mobilization of intracellular calcium is unaffected by wortmannin and demethoxyviridin at concentrations that totally inhibit PLD activation. It is clear, therefore, that PA generated by PLD activation does not contribute to the release of intracellular calcium in the neutrophil. It is evident from these studies that demethoxyviridin displays little selectivity for PLD over PLC and consequently blocks both PLC- and PLD-derived second messenger production, yet is able to discriminate between the functional responses that are dependent on these second messengers.

In summary we have found that a close structural analogue of wortmannin, demethoxyviridin, inhibits fMet-Leu-Phe-stimulated superoxide production in the human neutrophil by blocking the activation of PLD. Wortmannin and demethoxyviridin probably act at the same site in the signal transduction pathway and may block the interaction between the receptor for fMet-Leu-Phe and a GTP-binding protein that is intimately involved in PLD activation. Interestingly, demethoxyviridin does not inhibit intracellular calcium mobilization at concentrations that almost completely inhibit IP_3 formation.

We would like to thank Mr C. O'Neil and Miss I. Acevedo for their assistance.

References

ANTHES, J.C., ECKEL, S., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1989). Phospholipase D in homogenates from HL-60 granulocytes: Implications of calcium and G protein control. *Biochem. Biophys. Res. Commun.*, **163**, 657–664.

BEN-AV, P. & LISCOVITCH, M. (1989). Phospholipase D activation by the mitogens platelet-derived growth factor and 12-O-tetradecanoylphorbol 13-acetate in NIH-3T3 cells. *FEBS Lett.*, **259**, 64–66.

BERRIDGE, M.J. & IRVINE, R. (1989). Inositol phosphates and cell signalling. *Nature*, **341**, 197–205.

BIGAY, J., DETERRE, P., PFISTER, C. & CHABRE, M. (1985). Fluoroaluminates activate transducin-GDP by mimicking the gamma-phosphate of GTP in its binding site. *FEBS Lett.*, **191**, 181–185.

BILLAH, M.M., PAI, J-K., MULLMANN, T.J., EGAN, R.W. & SIEGEL, M.I. (1989). Regulation of phospholipase D in HL-60 granulocytes. *J. Biol. Chem.*, **264**, 9069–9076.

BOCCKINO, S.B., WILSON, P.B. & EXTON, J.H. (1987). Ca^{2+}-mobilizing hormones elicit phosphatidylethanol accumulation via phospholipase D activation. *FEBS Lett.*, **255**, 201–204.

BONSER, R.W., THOMPSON, N.T., RANDALL, R.W. & GARLAND, L.G. (1989). Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.*, **264**, 617–620.

COHEN, H.J. & CHOVANIEC, M.E. (1978). Superoxide generation by digitonin-stimulated guinea pig granulocytes. *J. Clin. Invest.*, **61**, 1081–1087.

DEWALD, B., THELEN, M. & BAGGIOLINI, M. (1988). Two transduction sequences are necessary for neutrophil activation by receptor agonists. *J. Biol. Chem.*, **263**, 16179–16184.

DOUGHERTY, R.W., DUBAY, G.R. & NIEDEL, J.E. (1989). Dynamics of the diradylglycerol responses of stimulated phagocytes. *J. Biol. Chem.*, **264**, 11263–11269.

ECKSTEIN, F., CASSEL, D., LEVKOVITZ, H., LOWE, M. & SELINGER, Z. (1979). Guanosine 5'-O-(2-Thiodiphosphate). An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *J. Biol. Chem.*, **254**, 9829–9834.

EXTON, J.H. (1990). Signalling through phosphatidylcholine breakdown. *J. Biol. Chem.*, **265**, 1–4.

GABIG, T.G., ENGLISH, D., AKARD, L.P. & SCHELL, M.J. (1987). Regulation of neutrophil NADPH oxidase activation in a cell-free system by guanine nucleotides and fluoride. *J. Biol. Chem.*, **262**, 1685–1690.

GELAS, P., RIBBES, G., RECORD, M., TERCE, F. & CHAP, H. (1989). Differential activation by fMet-Leu-Phe and phorbol ester of a plasma membrane phosphatidylcholine-specific phospholipase D in human neutrophil. *FEBS Lett.*, **251**, 213–218.

GRUCHALLA, R.S., DINH, T.T. & KENNERLY, D.A. (1990). An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. *J. Immunol.*, **144**, 2334–2342.

HANKS, J.H. & WALLACE, R.E. (1949). Regulation of oxygen and temperature in preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.*, **71**, 196–200.

HARRIS, R.A., SCHMIDT, J., HITZEMANN, B.A. & HITZEMANN, R.J. (1981). Phosphatidate as a molecular link between depolarization and neurotransmitter release in the brain. *Science*, **212**, 1290–1291.

HONEYCUTT, P.J. & NIEDEL, J.E. (1986). Cytochalasin B enhancement of the diacylglycerol response in formyl peptide-stimulated neutrophils. *J. Biol. Chem.*, **261**, 15900–15905.

IMAGAWA, W., BANDYOPADHYAY, G.K., WALLACE, D. & NANDI, S. (1989). Phospholipids containing polyunsaturated fatty acyl groups are mitogenic for normal mouse mammary epithelial cells in serum-free primary cell culture. *Proc. Natl. Acad. Sci., U.S.A.*, **86**, 4122–4126.

JACKOWSKI, S. & ROCK, C.O. (1989). Stimulation of phosphatidyinositol 4,5-bisphosphate phospholipase C activity by phosphatidic acid. *Arch. Biochem. Biophys*, **268**, 516–524.

KOBAYASHI, M. & KANFER, J.N. (1987). Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.*, **48**, 1597–1603.

LISCOVITCH, M. (1989). Phosphatidylethanol biosynthesis in ethanol-exposed NG108-15 neuroblastoma × glioma hybrid cells. *J. Biol. Chem.*, **264**, 1450–1456.

LOFFELHOLZ, K. (1989). Receptor regulation of choline phospholipid hydrolysis. *Biochem. Pharmacol.*, **38**, 1543–1549.

MINTA, A., KAO, J.P.Y. & TSIEN, R.Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.*, **264**, 8171–8178.

MOOLENAAR, W.H., KRUIJER, W., TILLY, B.C., VERLAAN, I., BIERMAN, A.J. & DE LAAT, S.W. (1986). Growth factor-like action of phosphatidic acid. *Nature*, **323**, 171–173.

OHSAKO, S. & DEGUCHI, T. (1981). Stimulation by phosphatidic acid of calcium influx and cyclic GMP synthesis in neuroblastoma cells. *J. Biol. Chem.*, **256**, 10945–10948.

OHSAKO, S. & DEGUCHI, T. (1983). Phosphatidic acid mimicks the muscarinic action of acetylcholine in cultured bovine chromaffin cells. *FEBS Lett.*, **152**, 62–66.

PAI, J.K., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1988a). Activation of phospholipase D by chemotactic peptide in HL-60 granulocytes. *Biochem. Biophys. Res. Commun.*, **150**, 355–364.

PAI J-K., SIEGEL, M.I., EGAN, R.W. & BILLAH, M.M. (1988b). Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. *J. Biol. Chem.*, **263**, 12472–12477.

PALMER, S., HUGHES, K.T., LEE, D.Y. & WAKELAM, M.J.O. (1988). Development of a novel Ins(1,4,5)P3-specific binding assay. Its use to determine the intracellular concentration of Ins(1,4,5)P3 in unstimulated and vasopressin-stimulated rat hepatocytes. *Cell. Signal.*, **1**, 147–156.

PELECH, S.L. & VANCE, D.E. (1989). Signal transduction via phosphatidylcholine cycles. *Trends Biochem. Sci.*, **14**, 28–30.

PREISS, J., LOOMIS, C.R., BISHOP, W.R., STEIN, R., NIEDEL, J.E. & BELL, R.M. (1986). Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes and ras- and sis-transformed normal rat kidney cells. *J. Biol. Chem.*, **261**, 8597–8600.

PUTNEY, J.W., WEISS, S.J., VAN DE WALLE, C.M. & HADDAS, R.A. (1980). Is phosphatidic acid a calcium ionophore under neurohumoral control? *Nature*, **284**, 345–347.

QIAN, Z. & DREWES, L.R. (1989). Muscarinic acetylcholine receptor regulates phosphatidylcholine phospholipase D in canine brain. *J. Biol. Chem.*, **264**, 21720–21724.

RANDALL, R.W., BONSER, R.W., THOMPSON, N.T. & GARLAND, L.G. (1990). A novel and sensitive assay for phospholipase D in intact cells. *FEBS Lett.*, **264**, 87–90.

REINHOLD, S.L., PRESCOTT, S.M., ZIMMERMAN, G.A. & McINTYRE, T.M. (1990). Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB J.*, **4**, 208–214.

RUBIN, R. (1988). Phosphatidylethanol formation in human platelets: Evidence for thrombin-induced activation of phospholipase D. *Biochem. Biophys. Res. Commun.*, **156**, 1090–1096.

SALMON, D.M. & HONEYMAN, T.W. (1980). Proposed mechanism of cholinergic action in smooth muscle. *Nature*, **284**, 344–345.

STERNWEIS, P.C. & GILMAN, A.G. (1982). Aluminum: A requirement for activation of the regulatory component of adenylate cyclase by fluoride. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 4888–4891.

TATESON, J.E., RANDALL, R.W., REYNOLDS, C.H., JACKSON, W.P., BHATTACHERJEE, P., SALMON, J.A. & GARLAND, L.G. (1988). Selective inhibition of arachidonate 5-lipoxygenase by novel acetohydroxamic acids: biochemical assessment *in vitro* and *ex vivo*. *Br. J. Pharmacol.*, **94**, 528–539.

TETTENBORN, C.S. & MUELLER, G.C. (1988). 12-O-tetradecanoylphorbol 13-acetate activates phosphatidylethanol and phosphatidylglycerol synthesis by phospholipase D in cell lysates. *Biochem. Biophys. Res. Commun.*, **155**, 249–255.

VAN CORVEN, E.J., GROENINK, A., JALINK, K., EICHHOLTZ, T. & MOOLENAAR, W.H. (1989). Lysophosphatidate-induced cell proliferation: Identification and dissection of signalling pathways mediated by G proteins. *Cell*, **59**, 45–54.

YU, C-L., TSAI, M-H. & STACEY, D.W. (1988). Cellular ras activity and phospholipid metabolism. *Cell*, **52**, 63–71.

(Received November 16, 1990
Accepted January 15, 1991)