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## The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes

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Recent studies indicate that phosphatidylinositol 3-kinase is essential in the regulation of many processes dependent on membrane flow. Autophagy is a complex pathway in which cell material, including proteins, can be degraded. Membrane flow plays a pivotal role in this process. To find out whether phosphatidylinositol 3-kinase is also required for autophagy, we tested the effects on autophagy of two structurally unrelated phosphatidylinositol 3-kinase inhibitors, wortmannin and 2-(4-morpholinyl)-8-phenylchromone (LY294002).

The addition of low concentrations of each of these inhibitors to incubations of hepatocytes in the absence of amino acids resulted in a strong inhibition of proteolysis. The antiproteolytic effect of wortmannin (IC<sub>50</sub> 30 nM) and LY294002 (IC<sub>50</sub> 10 µM) was accompanied by inhibition of autophagic sequestration and not by an increase in lysosomal pH or a decrease in intracellular ATP. No further inhibition of proteolysis by the two compounds was observed when autophagy was already maximally inhibited by high concentrations of amino acids.

3-Methyladenine, which is commonly used as a specific inhibitor of autophagic sequestration, was an inhibitor of phosphatidylinositol 3-kinase, thus providing a target for its action.

It is proposed that phosphatidylinositol 3-kinase activity is required for autophagy. 3-Methyladenine inhibits autophagy by inhibition of this enzyme.

**Keywords:** phosphatidylinositol 3-kinase; lysosome; proteolysis; 3-methyladenine; liver.

Phosphatidylinositol 3-kinase (PtdIns 3-kinase) is one of the first enzymes that becomes stimulated upon activation of cells by insulin, growth factors, and cytokines [1–6]. With the use of inhibitors of PtdIns 3-kinase, wortmannin and 2-(4-morpholinyl)-8-phenylchromone (LY294002), considerable progress has been made in elucidating its role in signal transduction pathways [1–6]. Furthermore, these studies have shown that PtdIns 3-kinase activity is required for many processes involving membrane traffic [7]. These include translocation of GLUT1 and GLUT4 to the plasma membrane [8, 9], endocytosis [10, 11], endosome fusion [12], lysosomal protein sorting [13, 14] and transcytosis [15]. The importance of inositol phospholipids in the control of membrane traffic is also evident from studies in

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**Abbreviations.** PtdIns 3-kinase, phosphatidylinositol 3-kinase; LY294002, 2-(4-morpholinyl)-8-phenylchromone; Lys-Ala-NH(O-Me)Nap, lysyl-alanyl-4-methoxy-2-naphthylamide; Nap(OMe)NH<sub>2</sub>, 4-methoxy-2-naphthylamine; DPP II, dipeptidyl-peptidase II; IC<sub>50</sub>, concentration giving 50% inhibition; Me<sub>2</sub>SO, dimethylsulfoxide; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P<sub>2</sub>, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P<sub>3</sub>, phosphatidylinositol 3,4,5-trisphosphate.

**Enzymes.** 1-Phosphatidylinositol 3-kinase (EC 2.7.1.137); 1-phosphatidylinositol 4-kinase (EC 2.7.1.67); dipeptidyl-peptidase II (EC 3.4.14.2); hexokinase (EC 2.7.1.1); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

yeast. The yeast *Saccharomyces cerevisiae* VPS34 gene product is the analogue of the mammalian catalytic subunit of PtdIns 3-kinase. VPS34 mutants are disturbed in vacuolar protein sorting [16, 17].

Another process involving membrane traffic is autophagy. Autophagy is responsible for accelerated degradation of cell protein during starvation. The process comprises sequestration of cytoplasmic material in autophagosomes, fusion of these vesicles with lysosomes, and degradation of the sequestered material. Important regulators of the process are amino acids. Amino acids inhibit autophagy at the first step of the process, i.e. autophagic sequestration [18].

Because of the importance of membrane traffic in autophagy, both in the formation and fusion of vesicles, it was of interest to find out whether PtdIns 3-kinase is also involved in this process.

In this study we show that wortmannin and LY294002, at concentrations that inhibited PtdIns 3-kinase activity, prevented autophagic sequestration in isolated rat hepatocytes. This is in agreement with a more general requirement of membrane traffic for PtdIns 3-kinase activity. In addition, it is shown that 3-methyladenine, a specific inhibitor of autophagic sequestration [19], is an inhibitor of PtdIns 3-kinase.

### MATERIALS AND METHODS

**Materials.** Wortmannin and phosphatidylinositol were purchased from Sigma. Wortmannin was dissolved in dimethylsulfoxide (Me<sub>2</sub>SO) at 2 mM and stored at –20°C in the dark.

Dilutions in 0.9% NaCl were prepared just before the start of each experiment. LY294002 was obtained from Biomol, and was dissolved in Me<sub>2</sub>SO. The final Me<sub>2</sub>SO concentration in the incubation medium did not exceed 0.25% (by vol.). This concentration of Me<sub>2</sub>SO did not affect the processes that were tested. [<sup>14</sup>C]Sucrose, [<sup>32</sup>P]orthophosphate, and [ $\gamma$ -<sup>32</sup>P]ATP were obtained from Amersham International. The p85 antibody was obtained from Upstate Biotechnology Inc. Sepharose 4B beads were from Pharmacia and TLC plates (silica gel 60) were from Merck. Lysyl-alanyl-4-methoxy-2-naphthylamide [Lys-Ala-NH(OMe)Nap] was obtained from Bachem Feinchemikalien AG.

**Preparation of hepatocytes.** Hepatocytes were isolated from 18–24 h starved male Wistar rats (200–250 g) by collagenase perfusion, as described by Groen et al. [20].

**Measurement of proteolysis.** Proteolysis was measured as production of valine [21] after 90 min of incubation at 37°C in Krebs-Henseleit bicarbonate medium plus 20 mM glucose, 25  $\mu$ M cycloheximide and the additions or omissions indicated in the legends to the figures; the final volume was 2 ml. Cycloheximide was present to prevent simultaneous protein synthesis. At this concentration, cycloheximide did not affect proteolysis [22].

**Measurement of cellular ATP concentration.** For determination of ATP, incubations were terminated by the addition of HClO<sub>4</sub> [final concentration 3% (mass/vol.)] in the cold. After removal of the denatured protein by centrifugation at 12000 g for 1 min, the samples were neutralized to pH 7 with a mixture of 2 M KOH and 0.3 M Mops. ATP was determined fluorimetrically according to Williamson and Corkey [23] using glucose, hexokinase, glucose-6-phosphate dehydrogenase, and NADP<sup>+</sup>.

**Measurement of changes in lysosomal pH.** Changes in lysosomal pH were measured according to a newly described method [24] by monitoring changes in the activity of the lysosomal enzyme dipeptidyl-peptidase II. The activity of this enzyme was determined by the production of fluorescent 4-methoxy-2-naphthylamine [Nap(OMe)NH<sub>2</sub>] from low concentrations of lysyl-alanyl-4-methoxy-2-naphthylamide [Lys-Ala-NH(OMe)Nap]. Cells were incubated at 37°C in Krebs-Henseleit bicarbonate medium plus 20 mM glucose, 20  $\mu$ M Lys-Ala-NH(OMe)Nap, and the additions or omissions indicated in the legend of Table 1. A low cell concentration (0.2 mg dry cells · ml<sup>-1</sup>) was used to avoid depletion of Lys-Ala-NH(OMe)Nap. After 20 min of incubation, the reaction was stopped by diluting samples 20-fold with 0.3 M glycine/NaOH, pH 10.6. The production of Nap(OMe)NH<sub>2</sub> was measured with a Perkin Elmer LS-2 fluorimeter (excitation 340 nm, emission 425 nm).

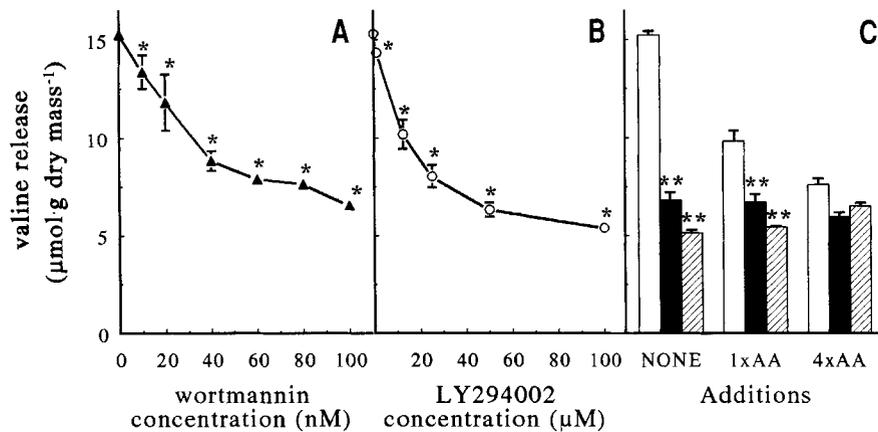
**Electron microscopy.** After 1 h of incubation, hepatocytes were fixed for 1.5 h at room temperature in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate, pH 7.4. Cells were rinsed for 30 min in the same buffer and were postfixed for 1 h at 4°C in 1% OsO<sub>4</sub> in 0.1 M cacodylate, pH 7.4. After transfer to 70% ethanol, aliquots of the cell suspensions were pelleted by centrifugation (10000 g for 8 min). Cell pellets were treated as tissue blocks according to standard procedures, i.e. dehydration by increasing ethanol concentrations and embedding in LX112 via propylene oxide. Ultrathin sections were stained with uranyl acetate and lead citrate. For morphometric analysis, 40 cells/sample were analyzed. A random selection was made of those cell sections that contained a nucleus. For each cell, two micrographs were made at an original magnification of  $\times 4100$ . Volume densities were analyzed on final prints at a magnification of  $\times 30000$ . Area determination of autophagosomes, i.e. all vacuoles containing recognizable cytoplasmic structures, and cell cytoplasm was performed by means of a Kontron MOP Videoplan.

**Composition of the complete mixture of amino acids.** The concentration of each amino acid in this mixture was equal to either one (complete amino acid mixture) or four (4 $\times$  complete amino acid mixture) times its concentration in the portal vein of a starved rat. The composition of the 1 $\times$  mixture was as described in [25], except that the concentration of leucine was 200  $\mu$ M. When proteolysis was measured, valine was omitted from the amino acid mixture because its production was used to monitor proteolytic rates.

**Measurement of [<sup>14</sup>C]sucrose sequestration.** Loading of hepatocytes with [<sup>14</sup>C]sucrose and its autophagic sequestration was carried out with the electroporation procedure as described by Seglen and Gordon [18]. Cells were electroporated and incubated for 1 h with [<sup>14</sup>C]sucrose at 0°C. Cells were then allowed to reseal at 37°C for 30 min. Cells were washed with Krebs-Henseleit medium to remove extracellular [<sup>14</sup>C]sucrose and reincubated for 60 min under the conditions indicated in the legend to Fig. 2. After incubation, cells were lysed by electroshock in a non-ionic medium (10% sucrose) and [<sup>14</sup>C]sucrose in the remaining cell structures was used as a measure of the rate of autophagic sequestration. Amino-acid-resistant [<sup>14</sup>C]sucrose sequestration, which was about 60% of total sequestered sucrose, represents mitochondrial uptake [18].

**Phosphatidylinositol 3-kinase activity measurement.** After 60 min of incubation at 37°C in Krebs-Henseleit bicarbonate medium plus 20 mM glucose in the absence or presence of 100 nM wortmannin, cells were harvested, washed with ice-cold NaCl/P<sub>i</sub> (137 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>; final pH 7.2) and lysed in a buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM NaF, 1% Nonidet-P40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 4  $\mu$ g/ml leupeptin, 1 mM benzamidine, 0.7  $\mu$ g/ml pepstatin and 0.2 mM phenylmethylsulfonyl fluoride, for 15 min at 4°C. Lysates were centrifuged for 15 min at 12000 g. An aliquot of cell lysate was incubated for 1 h at 4°C with a polyclonal antibody raised against the p85 subunit of PtdIns 3-kinase. The immunocomplex was incubated with protein-A–Sepharose 4B for 2 h at 4°C. The Sepharose complex was washed twice with NaCl/P<sub>i</sub> containing 1% Nonidet-P40, twice with Tris/HCl (0.1 M)-buffered LiCl (0.5 M), and twice with a buffer containing 10 mM Tris/HCl, 100 mM NaCl, and 1 mM EDTA. All washing buffers contained 0.1 mM Na<sub>3</sub>VO<sub>4</sub> and the pH was adjusted to 7.4. The PtdIns 3-kinase activity was measured in a final volume of 50  $\mu$ l in a medium containing 20 mM Hepes, 0.4 mM EGTA, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 50  $\mu$ M ATP, 2  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 0.005% Nonidet-P40 and 0.2 mg/ml phosphatidylinositol; the temperature was 23°C. The reaction was initiated with MgATP and was linear for 10 min. The reaction was stopped after 5 min by the addition of 15  $\mu$ l 4 M HCl. Radiolabeled phospholipids were isolated from the reaction mixture by the addition of 130  $\mu$ l 1:1 (by vol.) methanol/chloroform. The lower phase was recovered and after drying the pellet was resuspended in 10  $\mu$ l chloroform and spotted onto a TLC plate. Samples were chromatographed for 1.5 h in 45:35:3:7 (by vol.) methanol/chloroform/25% ammonia/water. Radioactivity incorporated into each spot was measured with a PhosphorImager (Molecular Dynamics).

**Determination of phosphorylation of ribosomal protein S6.** Hepatocytes were incubated at 37°C for 60 min in Krebs-Henseleit bicarbonate medium plus 20 mM glucose, 0.2 mM [<sup>32</sup>P]phosphate (10  $\mu$ Ci/ml) and the additions indicated in the legend to Fig. 6. At the end of the incubations, cells were diluted fivefold with ice-cold Krebs-Henseleit bicarbonate medium and collected by centrifugation (2 min, 50 g). The cell pellets were extracted with 0.6 ml sample buffer and brought to 90°C for 5 min; an amount equivalent to about 100  $\mu$ g protein was analyzed by SDS/PAGE (10% polyacrylamide). Gel slabs were



**Fig. 1. Inhibition of proteolysis by wortmannin and LY294002.** In A and B, hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated in the absence of amino acids, with the indicated concentrations of wortmannin or LY294002. In C, hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated with 0 $\times$ , 1 $\times$  or 4 $\times$  the complete mixture of amino acids, in the absence ( $\square$ ) or presence of either 100 nM wortmannin ( $\blacksquare$ ) or 100  $\mu\text{M}$  LY294002 ( $\text{▨}$ ). Amino acids were added after a 2 min prior incubation of the cells with the inhibitors. After 90 min of incubation, proteolysis was measured as described in the Materials and Methods section. Values are the means ( $\pm$  SE) of 3–7 different hepatocyte preparations. \*, significantly different ( $P < 0.05$ ) from the control in the absence of amino acids. \*\*, significantly different ( $P < 0.05$ ) from the corresponding control in the absence of wortmannin or LY294002.

dried and subjected to autoradiography. Protein phosphorylation was quantified with the PhosphorImager.

**Statistics.** The statistical significance was determined using the Student's *t*-test.

## RESULTS

To ensure maximal proteolytic flux, rat hepatocytes were incubated in the absence of added amino acids. Under these conditions, autophagy accounts for approximately 60% of total proteolysis [26, 27]. To test a possible role of PtdIns 3-kinase in autophagy, increasing concentrations of two structurally unrelated PtdIns 3-kinase inhibitors, wortmannin and LY294002, were added to the incubations. This resulted in a progressive inhibition of overall proteolysis to 40% of the maximal rate (Fig. 1A, B). Half-maximal inhibition of proteolysis was observed at approximately 30 nM wortmannin and 10  $\mu\text{M}$  LY294002. The maximal inhibitory effect of the two PtdIns 3-kinase inhibitors on overall proteolysis was comparable to that obtained with known inhibitors of autophagic proteolysis, i.e. high concentrations of amino acids (4 $\times$  complete amino acids) (Fig. 1C) (compare [18]) and 3-methyladenine [19]. Under conditions where autophagic proteolysis was only partially inhibited, i.e. at low concentrations of amino acids (1 $\times$  complete amino acids), the addition of wortmannin or LY294002 resulted in a further inhibition of proteolysis to the maximal level of inhibition that was observed in the presence of high concentrations of amino acids (Fig. 1C). In the presence of high concentrations of amino acids (4 $\times$  complete amino acids), no significant further inhibition of proteolysis by wortmannin or LY294002 was observed (Fig. 1C).

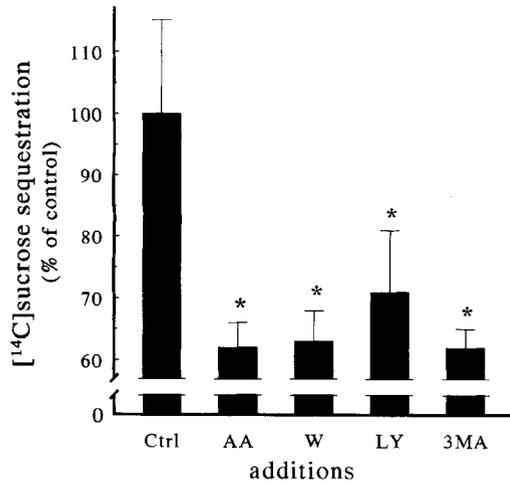
In contrast to their effect on the autophagic-lysosomal proteolytic pathway, amino acids do not inhibit extralysosomal proteolysis [27]. The fact that the anti-proteolytic effects of wortmannin and LY294002 were not additive with that of amino acids indicates that the two compounds also inhibit autophagic-lysosomal proteolysis. However, this finding gives no information about the nature of the step(s) in this process that are inhibited by wortmannin and LY294002. To test the possibility that the antiproteolytic effect of wortmannin and LY294002 was due to an increase in lysosomal pH, the effect of the two compounds on the activity of the lysosomal enzyme dipeptidyl-peptidase II

**Table 1. Effect of wortmannin and LY294002 on the intracellular ATP content and on DPP II activity.** For measurement of ATP, rat hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated for 90 min in Krebs-Henseleit bicarbonate buffer plus 20 mM glucose. For measurement of DPP II activity, hepatocytes were incubated under the same conditions for 20 min at a lower cell concentration ( $0.2 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) (see Materials and Methods section). Values are the means ( $\pm$  SE) with the number of different hepatocyte preparations given in brackets. The rate of Nap(OMe) $\text{NH}_2$  production under control conditions (100%) was  $758 \pm 27 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g dry cells}^{-1}$ . \*, Significantly different ( $P < 0.05$ ) from the control.

Additions	ATP content	DPP II activity
	$\mu\text{mol} \cdot \text{g dry cells}^{-1}$	% of control
None	$12.2 \pm 0.3$ (5)	100 (3)
Wortmannin (100 nM)	$11.8 \pm 0.3$ (5)	$99.3 \pm 4.0$ (3)
LY294002 (100 $\mu\text{M}$ )	$10.0 \pm 0.5$ (3)	$98.0 \pm 5.9$ (3)
Methylamine (10 mM)	$10.6 \pm 0.7$ (3)	$60.2 \pm 4.3$ *(3)
3-Methyladenine (5 mM)	$11.1 \pm 0.8$ (3)	$85.8 \pm 2.1$ *(3)

(DPP II) was studied. The activity of this enzyme is very sensitive to changes in lysosomal pH [24]. The anti-proteolytic effects of wortmannin and LY294002 could clearly not be ascribed to a rise in lysosomal pH, as indicated by their lack of effect on DPP II activity in intact hepatocytes (Table 1). As a control, the effect of the acidotropic agent methylamine is also shown. 10 mM methylamine decreased DPP II activity by 40%. This concentration of methylamine strongly inhibits lysosomal proteolysis [compare 24, 28]. In the same set of experiments, the effect of the autophagic sequestration inhibitor 3-methyladenine [19] was also tested. This compound slightly inhibited DPP II activity, which indicates a slight alkalization of the lysosomes. This is in agreement with a similar conclusion reached previously on the basis of the effect of 3-methyladenine on chloroquine accumulation [29].

Because autophagic proteolysis in hepatocytes is ATP dependent [30], the effect of wortmannin and LY294002 on intracellular ATP was tested. No significant effect on the cellular ATP content was observed (Table 1). Likewise, neither methylamine nor 3-methyladenine significantly affected ATP levels (Table 1).



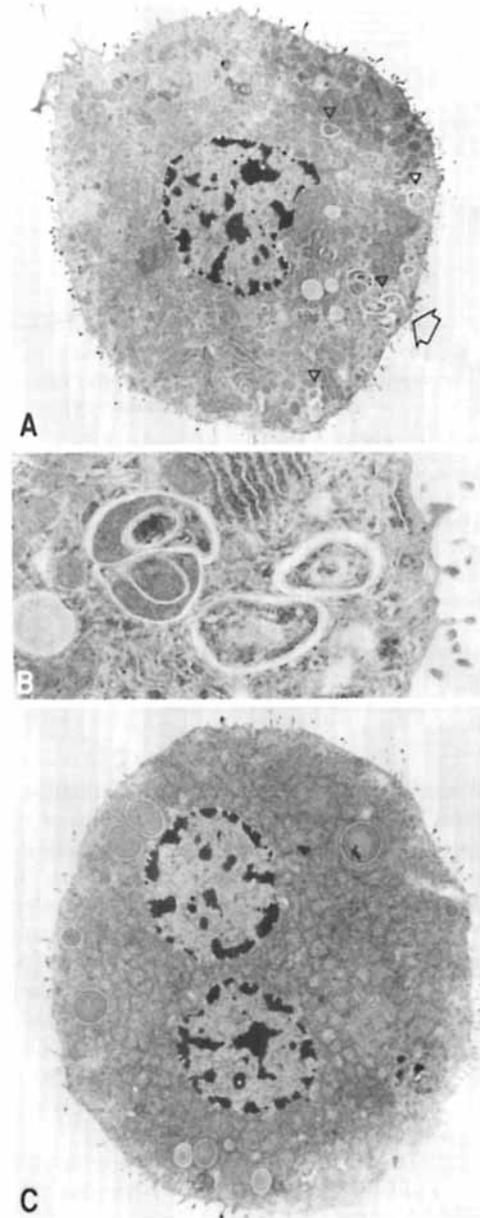
**Fig. 2. Inhibition of sequestration of [ $^{14}\text{C}$ ]sucrose by amino acids, 3-methyladenine, wortmannin and LY294002.** [ $^{14}\text{C}$ ]Sucrose loaded hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated in the absence or presence of either  $100 \mu\text{M}$  LY294002 (LY),  $100 \text{ nM}$  wortmannin (W), the  $4\times$  complete mixture of amino acids (AA) or  $5 \text{ mM}$  3-methyladenine (3MA). After 60 min of incubation, [ $^{14}\text{C}$ ]sucrose sequestration was measured as in [18]. The maximal rate of amino-acid-sensitive sucrose sequestration was 5% of the cytosolic volume in 60 min. Amino-acid-insensitive uptake of [ $^{14}\text{C}$ ]sucrose (60% of total sequestered sucrose) is due to accumulation of sucrose in mitochondria [18]. Values are the means ( $\pm$  SE) of experiments carried out with three different hepatocyte preparations. \*, significantly different from the control condition ( $P < 0.05$ ).

**Table 2. Effect of wortmannin and LY294002 on the volume density of autophagosomes.** Rat hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated for 60 min in Krebs-Henseleit bicarbonate buffer plus  $20 \text{ mM}$  glucose and  $50 \mu\text{M}$  vinblastin. Samples for electron microscopy were prepared and analyzed as described in the Materials and Methods section. Values of control incubations and incubations in the presence of wortmannin ( $100 \text{ nM}$ ) are the means ( $\pm$  SE) of four different hepatocyte preparations. Values of incubations receiving either amino acids ( $4\times$  complete mixture) or LY294002 ( $100 \mu\text{M}$ ) are the means ( $\pm$  range) of two different hepatocyte preparations.

Additions	Volume density of autophagosomes
	% of cytoplasmic volume
None	$2.06 \pm 0.10$
Wortmannin	$0.17 \pm 0.04$
LY294002	$0.15 \pm 0.02$
Amino acids	$0.22 \pm 0.03$

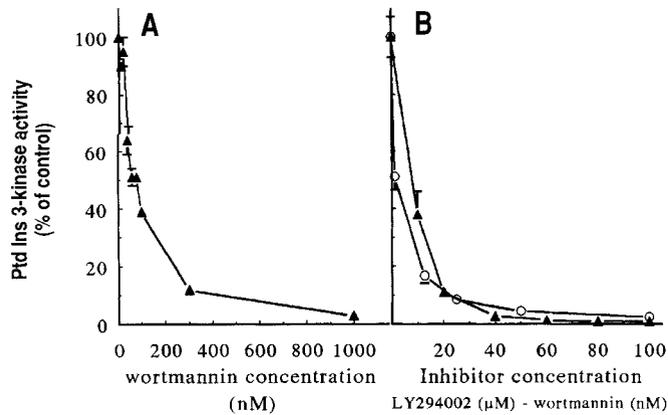
We next investigated the effects of wortmannin and LY294002 on the sequestration step of the autophagic pathway, as measured by sequestration of electroinjected cytosolic [ $^{14}\text{C}$ ]sucrose. In the absence of amino acids, when the rate of autophagic sequestration was maximal, the addition of either  $100 \text{ nM}$  wortmannin or  $100 \mu\text{M}$  LY294002 strongly inhibited sequestration of [ $^{14}\text{C}$ ]sucrose (Fig. 2). Inhibition was comparable to that obtained with 3-methyladenine or with high concentrations of amino acids (Fig. 2).

The results on autophagic sequestration were supported by electron microscopy. The volume density of autophagosomes was measured in cells that were incubated in the absence of amino acids to ensure maximal autophagic flux. To prevent degradation of newly formed autophagosomes, vinblastin was



**Fig. 3. Inhibition of autophagosome formation by wortmannin and LY294002.** Electron micrographs of isolated rat hepatocytes that were incubated for 60 min ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) with  $50 \mu\text{M}$  vinblastin alone (A, B) or in the presence of  $50 \mu\text{M}$  vinblastin plus  $100 \text{ nM}$  wortmannin (C). Autophagosomes in A are indicated by arrowheads. The area in A, indicated by the arrow, is enlarged in B. The vacuole-like structures in C are fat droplets. Magnifications: A and C,  $\times 3200$ ; B,  $\times 15000$ .

added to the incubations. This compound inhibits fusion between autophagosomes and lysosomes without having an effect on autophagic sequestration [31]. Under these conditions, autophagosomes accumulated to up to 2% of the total cytoplasmic volume (Table 2). As illustrated in Fig. 3, autophagosomes could hardly be detected in cells that were incubated in the presence of  $100 \text{ nM}$  wortmannin (Fig. 3C) compared to cells incubated under maximal autophagic conditions (Fig. 3A and B). Morphometric analysis of cells incubated in the absence or presence of either  $100 \text{ nM}$  wortmannin or  $100 \mu\text{M}$  LY294002 (Table 2) revealed a strong inhibitory effect of both compounds on the volume density of autophagosomes. Inhibition was comparable to the effect of high concentrations of amino acids (Table 2).



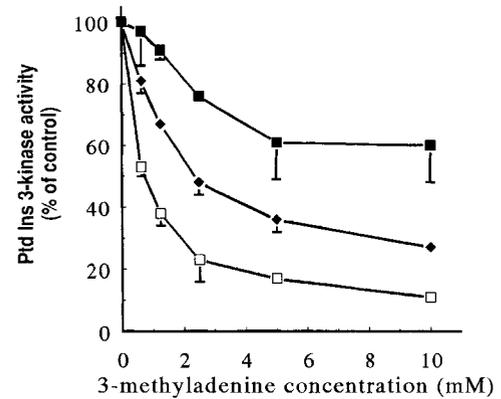
**Fig. 4. Inhibition of PtdIns 3-kinase by wortmannin and LY294002.** In A, hepatocytes ( $5 \text{ mg dry cells} \cdot \text{ml}^{-1}$ ) were incubated for 60 min in the absence or presence of wortmannin at the concentrations indicated. The activity of PtdIns 3-kinase was measured in cell lysates as described in the Materials and Methods section. In B, cells were incubated for 60 min in the absence of inhibitor, lysed, and the activity of PtdIns 3-kinase was tested *in vitro* in the presence of either wortmannin ( $\blacktriangle$ ) or LY294002 ( $\circ$ ) at the concentrations indicated. In A, values are the means ( $\pm$  SE) of experiments carried out with three different hepatocyte preparations. In B, each value is the mean  $\pm$  the range of two experiments with different hepatocyte preparations.

To test whether wortmannin, at the concentrations used, inhibited PtdIns 3-kinase in the cells, the activity of the enzyme was measured in lysates of cells after prior incubation of the cells with different concentrations of wortmannin. Since wortmannin inhibits the enzyme irreversibly by covalent binding [32], its effect on intact cells can still be observed in an *in vitro* assay. The gradual inhibition of proteolysis (Fig. 1A) was accompanied by a parallel inhibition of PtdIns 3-kinase (Fig. 4A). However, inhibition of proteolysis was maximal at a concentration of wortmannin (100 nM) that only inhibited 60% of total intracellular PtdIns 3-kinase activity. In contrast, the addition of wortmannin to PtdIns 3-kinase *in vitro* completely inhibited the enzyme (Fig. 4B) (see also Discussion section).

The effect of LY294002 on PtdIns 3-kinase activity of intact hepatocytes could not be tested directly because inhibition by this compound is not by covalent binding, but is reversible, being competitive with ATP [33]. For this reason, the effect of LY294002 could only be tested *in vitro*. As shown in Fig. 4B, PtdIns 3-kinase was almost completely inhibited at 50  $\mu\text{M}$  LY294002.

Since our data showed that both wortmannin and LY294002 inhibit autophagic sequestration, the possibility was tested whether the anti-sequestration effect of 3-methyladenine could also be ascribed to inhibition of PtdIns 3-kinase. 3-Methyladenine strongly inhibited *in vitro* PtdIns 3-kinase activity (Fig. 5A). Inhibition appeared to be competitive with ATP (Fig. 5B), which is mechanistically similar to that reported for LY294002 [33].

To demonstrate that PtdIns 3-kinase in the intact hepatocyte is also inhibited by 3-methyladenine and LY294002, we tested their effects on phosphorylation of ribosomal protein S6, which is known to require active PtdIns 3-kinase [34]. In the experiment shown in Fig. 6, hepatocytes were incubated in the presence of a complete mixture of amino acids to ensure a high degree of S6 phosphorylation [35]. Both 3-methyladenine and LY294002 almost completely prevented S6 phosphorylation. Likewise, S6 phosphorylation was sensitive to inhibition by wortmannin. This indicates that PtdIns 3-kinase in intact hepatocytes was inhibited by 3-methyladenine and LY294002.



**Fig. 5. Inhibition of PtdIns 3-kinase by 3-methyladenine.** PtdIns 3-kinase activity was measured in cell lysates as described in the legend to Fig. 4B. In the assay, the ATP concentration was either 50  $\mu\text{M}$  ( $\square$ ), 250  $\mu\text{M}$  ( $\blacklozenge$ ) or 1000  $\mu\text{M}$  ( $\blacksquare$ ). 3-Methyladenine was present at the concentrations indicated. Each value is the mean  $\pm$  the range of two experiments with different hepatocyte preparations.

S6								
amino acids	-	-	-	-	+	+	+	+
LY294002	-	+	-	-	-	+	-	-
wortmannin	-	-	+	-	-	-	+	-
3-methyladenine	-	-	-	+	-	-	-	+

**Fig. 6. Inhibition of phosphorylation of ribosomal protein S6 by wortmannin, LY294002 and 3-methyladenine in intact hepatocytes.** Phosphorylation of ribosomal protein S6 was measured as described in the Materials and Methods section in the absence or presence of a complete ( $1\times$ ) amino acid mixture. Wortmannin, LY294002, and 3-methyladenine, if present, were added at concentrations of 100 nM, 100  $\mu\text{M}$  and 5 mM, respectively.

## DISCUSSION

Wortmannin and LY294002 are potent inhibitors of mammalian PtdIns 3-kinase, but their mechanisms of action are different. Wortmannin covalently binds to the enzyme, which is then irreversibly inhibited [32]. By contrast, LY294002 binds to the enzyme in a reversible manner, inhibition being competitive with ATP, presumably because LY294002 and ATP have structural resemblance [33]. Both wortmannin and LY294002 were potent inhibitors of autophagic sequestration in hepatocytes. This suggests that PtdIns 3-kinase activity is required for this process, which is in agreement with the requirement for the enzyme of membrane-flow-dependent processes (see Introduction section).

Although wortmannin is a potent inhibitor of PtdIns 3-kinase, this compound may not be entirely specific. Thus, wortmannin also inhibits PtdIns 4-kinase [36]. It is unlikely, however, that inhibition of PtdIns 4-kinase was responsible for inhibition of autophagic sequestration by wortmannin because LY294002, which either does not affect PtdIns 4-kinase [33] or inhibits the enzyme at concentrations much higher than those used in our study [37], similarly inhibited autophagic sequestration.

Previous work by Seglen and Gordon [18, 19] has resulted in the identification of 3-methyladenine, a now widely used potent inhibitor of autophagic sequestration. However, the mechanism of its action has remained unclear. Our data indicate that PtdIns 3-kinase is a likely target for 3-methyladenine action (Figs 5 and 6). Remarkably, 3-methyladenine also inhibits endocytosis [38, 39], a process requiring PtdIns 3-kinase activity [11, 12, 40]. Even more striking, both 3-methyladenine [39] and wortmannin

[40] appear to inhibit late in the endocytic pathway. This strongly suggests that in endocytosis the 3-methyladenine effect can also be ascribed to its ability to inhibit PtdIns 3-kinase activity.

It must be pointed out that in our experiments hepatocytes were incubated under conditions of maximal autophagic flux, i.e. in the absence of stimuli of signal transduction. Apparently, basal activity of PtdIns 3-kinase, which produces phosphatidylinositol 3-phosphate [PtdIns(3)P] and not phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P<sub>2</sub>] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P<sub>3</sub>] [7, 41], is essential and sufficient to allow maximal autophagic sequestration. A similar situation can be found in yeast where the homologue of the p110 catalytic subunit of the mammalian PtdIns 3-kinase, VPS34p, is a lipid kinase with substrate specificity towards PtdIns [17]. It cannot form PtdIns(3,4)P<sub>2</sub> or PtdIns(3,4,5)P<sub>3</sub> and inactivation of the VPS34 gene results in disturbance of membrane flow [16].

It is noteworthy that in intact hepatocytes wortmannin effectively inhibited autophagic proteolysis at lower concentrations than required for complete suppression of all intracellular PtdIns 3-kinase activity. Thus, 100 nM wortmannin completely prevented autophagy (Figs 1A, 2 and 3) whereas PtdIns 3-kinase activity of intact hepatocytes was only 60% inhibited at this concentration of the inhibitor, as measured by direct enzyme assay (Fig. 4A). Incomplete inhibition of PtdIns 3-kinase in intact cells by wortmannin at these concentrations has also been observed by others [34, 42]. By contrast, 100 nM wortmannin completely inhibited *in vitro* PtdIns 3-kinase activity that was immunoprecipitated from cells incubated in the absence of the inhibitor (Fig. 4B). Apparently, part of the total enzyme activity in intact cells is less affected by the inhibitor. This may be the result of subcellular enzyme distribution. In other cell types, PtdIns 3-kinase activity is both associated with low density membranes and present in the cytosol [41, 43]. It is likely that wortmannin, a highly lipophilic molecule, affects autophagic sequestration at intracellular membrane sites. Possibly, these sites are the ribosome-free parts of the rough endoplasmic reticulum that are considered to be the origin of the autophagosomal membranes [44]. The cytosolic wortmannin concentration can be expected to be low, compared to its concentration in the membranes. This would thus account for the fact that very high concentrations of wortmannin are required to inhibit all PtdIns 3-kinase activity in the cells completely (Fig. 4A).

In summary, three structurally unrelated compounds, wortmannin, LY294002 and 3-methyladenine all inhibit PtdIns 3-kinase and simultaneously inhibit autophagy in hepatocytes at the sequestration step. It is proposed that PtdIns 3-kinase is required for autophagic sequestration.

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