Biochemical and pharmacological studies with KT7692 and LY294002 on the role of phosphatidylinositol 3-kinase in FcεRI-mediated signal transduction

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Wortmannin inhibited phosphatidylinositol 3-kinase (PI3-kinase) and FcεRI-mediated histamine secretion in RBL-2H3 cells to a similar degree, with IC₅₀ values of 3 and 2 nM, respectively. Although PI3-kinase is an acknowledged regulator of intracellular trafficking and secretion, wortmannin has proved to be a difficult drug to use in assessing the role of PI3-kinase because it inhibits another important enzyme, myosin light-chain kinase (MLCK; IC₅₀ = 200 nM). In the present study we synthesized a unique derivative of wortmannin, *O*-acetyl-Δ¹⁶-wortmannin-17-ol (KT7692), that has an inhibitory potency against PI3-kinase one-hundredth that of wortmannin, but retains a similar potency to wortmannin against MLCK. Histamine secretion was influenced 100-fold more by wortmannin

than by KT7692. 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), a structurally different PI3-kinase inhibitor from wortmannin, inhibited PI3-kinase with an IC₅₀ of 2 μM but had little effect on MLCK activity in this concentration range. LY294002 also inhibited histamine secretion in RBL-2H3 cells with an IC₅₀ of 5 μM. These results provide further evidence that PI3-kinase is involved in the signal transduction pathway responsible for histamine secretion after stimulation of FcεRI. Furthermore KT7692 in combination with wortmannin and LY294002 would be a powerful tool for clarifying the involvement of PI3-kinase as distinct from that of MLCK in signal transduction systems of various cellular responses.

INTRODUCTION

PI3-kinase is associated with and activated by a number of proteins containing intrinsic or associated tyrosine kinase activities and by G-proteins such as Ras [1,2]. This enzyme is not in the canonical PI turnover pathway, but catalyses the formation of a family of phosphoinositides with phosphate at the D-3 position of the inositol ring [3,4]. The enzyme's function is somewhat unclear but it has been suggested that PI3-kinase plays an important role in mitogenesis [5], cytoskeletal rearrangement [6,7] and vesicle transportation [8,9].

Mast cells and basophils are granulated cells that play pivotal roles in allergy and inflammation. Rat basophilic leukaemia 2H3 (RBL-2H3) cells are a well-known model for mast cells and basophils. RBL-2H3 cells sensitized with IgE on high-affinity immunoglobulin E receptor (FcεRI) is competent to secrete inflammatory mediators stored in vesicles. The secretory reaction is triggered by cross-linkage of the IgE with specific antibody. By using this cell line, the molecular mechanism of the metabolic events in the course of secretion of inflammatory mediators such as histamine has been extensively studied.

Wortmannin, which was isolated from the culture broth of *Talaromyces wortmannii* KY12420 [10], has been shown to inhibit PI3-kinase (IC₅₀ = 3 nM) with high selectivity and to inhibit production of phosphatidylinositol 3,4,5-trisphosphate (PIP₃) in the cell [11,12]. We were therefore interested in exploring the

effect of wortmannin on PI3-kinase in RBL-2H3 cells. In a previous paper [11] we showed that PI3-kinase was activated in response to FcεRI-mediated stimulation and that wortmannin and its derivatives (WM-4, -25, -12 and -13) inhibited PI3-kinase and histamine secretion in RBL-2H3 cells to a similar degree. However, we have also shown that wortmannin and the WM series of derivatives inhibited MLCK with lower potency and also that there was a positive correlation between the potencies of wortmannin and the above derivatives as inhibitors of histamine secretion and as inhibitors of MLCK, as well as those of PI3-kinase [11].

MLCK is one of the key regulatory enzymes in Ca²⁺-dependent responses. The phosphorylation of myosin light chain by this Ca²⁺-activated enzyme induces contractile force in smooth muscle. MLCK activity has also been detected in non-muscle cells and has been shown to be involved in vesicle transportation [13,14]. Because vesicle transportation is one of the candidates for physiological functions of both PI3-kinase and MLCK, we must treat the data obtained with wortmannin with caution, especially in investigating biological phenomena such as secretion. The important role of MLCK has been shown with wortmannin in transmitter release from neurons, catecholamine release from chromaffin cells and virus release from host cells [15–17]. So wortmannin is still a useful tool as an MLCK inhibitor at higher concentration.

It is desirable to use another derivative of wortmannin that has

Abbreviations used: FcεRI, high-affinity immunoglobulin E receptor; KT7692, *O*-acetyl-Δ¹⁶-wortmannin-17-ol; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; MLCK, myosin light-chain kinase; PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; PIP₂, phosphatidylinositol 3,4-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PI3-kinase, phosphatidylinositol 3-kinase; PI4-kinase, phosphatidylinositol 4-kinase; RBL-2H3 cells, rat basophilic leukaemia 2H3 cells; TNP, 2,4,6-trinitrophenol; TNP-BSA, TNP conjugated with BSA.

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a novel inhibitory profile against PI3-kinase and MLCK to study the possible involvement of PI3-kinase in the mechanism of secretion in RBL-2H3 cells. For this paper we selected KT7692 as such a compound from many wortmannin derivatives. We characterized the inhibitory profile of KT7692 pharmacologically against PI3-kinase, MLCK and histamine secretion. We also used the structurally different PI3-kinase inhibitor LY294002 [18], to clarify the important role that PI3-kinase takes in FcεRI-mediated histamine secretion in RBL-2H3 cells.

MATERIALS AND METHODS

Materials

Wortmannin was purified from the fermentation broth of *Talaromyces wortmannii* KY12420 in our laboratories [10]. Derivatives of wortmannin and LY294002 were chemically synthesized in our laboratories and their structures were confirmed by spectroscopic studies [18–20]. Stock solutions of inhibitors (10 mM) were dissolved in Me₂SO and stored at –20 °C in the dark. 2,4,6-Trinitrophenol conjugated with BSA (TNP-BSA) was prepared according to the method of Schmitt-Verhulst et al. [21]. All other chemicals were of analytical grade.

Proteins and antibodies

PI3-kinase was partly purified from calf thymus essentially as described by Shibasaki et al. [22]. The pooled eluate from the cellulose phosphate column was used as PI3-kinase after it was shown to contain negligible amounts of PI4-kinase activity. PI4-kinase was partly purified from RBL-2H3 cells as described by Moritz et al. [23]. PI4-kinase was extracted from the membrane fraction of the cells and purified by DEAE-cellulose column chromatography. MLCK, other protein kinases, and anti-gizzard 20 kDa myosin light-chain antiserum were prepared as described by Nakanishi et al. [10]. Anti-PI3-kinase p85 monoclonal antibody AB6 was purchased from M.B.L. (Nagoya, Japan). Anti-TNP monoclonal IgE was purified from the culture supernatant of IGEL a2 cells (ATCC/TIB142).

Enzyme assay

The activities of PI3-kinase, PI4-kinase, MLCK, protein kinase C, and cyclic-AMP-dependent protein kinase were measured as previously described [11]. The stock solutions of inhibitors were diluted with Me₂SO just before use. The inhibitor solution was added to the reaction mixture to a final Me₂SO concentration of 2.0% (v/v), a concentration that had no effect on any enzymic activities examined in this paper. Me₂SO in a similar concentration was used in the control. The enzymic activity in the presence of inhibitor was expressed as a percentage of that without inhibitor. We measured each kinase activity for the same preincubation time (10 min), the same reaction time (30 min), and at the same incubation temperature (25 °C). All the experiments were performed in triplicate.

Cell stimulation and assay for histamine secretion

The maintenance of RBL-2H3 cells and the histamine secretion experiments were performed as previously described [11]. Cells were sensitized with TNP-specific IgE. The cells were then incubated with Pipes-buffered saline (25 mM Pipes, pH 7.4, 125 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM CaCl₂ and 0.1% BSA) in the presence of various concentrations of inhibitors at 37 °C for 15 min followed by stimulation with antigen (TNP-BSA) for 45 min.

To prepare cell lysates for immunoprecipitation, the culture medium was removed and 1 ml of NP40 buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP40, 1 mM Na₃VO₄ and 1 mM phenylmethanesulphonyl fluoride) was added to the cells. The resultant lysate was centrifuged for 10 min at 12000 g and the supernatant was used as cell lysates.

For the histamine assay, the medium was collected after stimulation of the cells and extracellular histamine was measured with HPLC [24]. In the absence of inhibitor, the amount of histamine in the culture medium of unstimulated cells was < 5% (spontaneous secretion) and the amount of histamine secreted after antigen stimulation was 42% of the total amount of histamine present in the culture. The actual effect induced by antigen stimulation was corrected for by subtracting the amount of change caused by spontaneous secretion. The response to the stimulant in the presence of inhibitor was expressed as a percentage of that present in the absence of inhibitor. The stock solution of inhibitor was diluted with Pipes-buffered saline just before use. The final concentration of Me₂SO was < 0.1%, a concentration that had no effect on any of the parameters of RBL-2H3 cell function observed here. Me₂SO in a similar concentration was used in the control. At least three cultures were used for each data point in each experiment. Each experiment was performed in triplicate.

Immunoprecipitation

The amounts of cell lysate or enzyme assayed were standardized by protein content in each experiment. The precleared lysate or enzyme was incubated with an appropriate amount of antibody for 1–2 h and then with protein-G-Sepharose for 1 h. The beads containing the immunoprecipitates were washed three times with NP40 buffer, once with LiCl solution (0.1 M Tris/HCl, pH 7.5 and 0.5 M LiCl), once with distilled water and once with washing buffer (20 mM Tris/HCl, pH 7.5, 0.1 M NaCl and 1 mM EDTA), and subjected to PI3-kinase assay.

Phosphorylation of myosin light chain in rat aorta

Phosphorylation of myosin light chain in rat aorta was measured as described by Nakanishi et al. [10]. Briefly, thoracic aorta rings prepared from male Sprague–Dawley rats were allowed to equilibrate in 10 ml of modified Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 12.5 mM NaHCO₃, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 10 mM glucose), and aerated with O₂/CO₂ (95:5, v/v) for 120 min at 37 °C. Various concentrations of inhibitor dissolved in Me₂SO (10 μl) were added to the equilibrated aortic rings. After incubation for 5 min at 37 °C, the rings were stimulated with 40 mM KCl. Phosphorylation of myosin light chain was determined by use of glycerol/urea gel electrophoresis followed by immunoblotting as described by Hathaway and Haeberle [25]. The blotted membranes were treated with anti-myosin light-chain antiserum, biotinylated anti-rabbit IgG, and avidin-conjugated horseradish peroxidase, and the phosphorylated and unphosphorylated myosin light chains were revealed with the ECL Western blotting detection system (Amersham).

RESULTS

Inhibitory effect of wortmannin and its derivatives on PI3-kinase and MLCK *in vitro*

We synthesized many derivatives of wortmannin and measured their inhibitory activities against PI3-kinase and MLCK. The inhibitory potencies of representative derivatives against PI3-

Table 1 Inhibitory effect of wortmannin and its derivatives *in vitro*

Each value has been calculated from the results obtained from an experiment carried out in at least triplicate. The IC_{50} was determined as the concentration of drug that produced 50% inhibition of the enzymic activity or of histamine secretion. PI3-kinase and MLCK activities and histamine secretion from RBL-2H3 cells were determined as described in Materials and Methods.

Compound (μ M)	IC_{50} , PI3-kinase (μ M)	IC_{50} , MLCK (μ M)	$(IC_{50}, \text{PI3-K}) \div (IC_{50}, \text{MLCK})$	IC_{50} values for histidine secretion (μ M)
Wortmannin	0.003	0.2	0.015	0.002
WM-4	0.040	4.0	0.010	0.006
WM-25	0.015	5.0	0.003	0.010
WM-12	10	300	0.033	> 10
WM-13	10	300	0.033	> 10
KT7692	0.2	0.2	1.0	0.1

Figure 1 The structure of KT7692

KT7692 was synthesized in our laboratories. Its structure was confirmed by ^1H NMR and ^{13}C NMR spectra.

kinase and MLCK *in vitro* are listed in Table 1. When the ratio of IC_{50} values for PI3-kinase to those for MLCK are calculated (Table 1), wortmannin and its previous derivatives, WM-4, -25, -12 and -13, give similar ratios ranging from 0.01 to 0.03, as reported previously [11]. By contrast, this ratio for another derivative, KT7692 (Figure 1), is larger value than that for wortmannin and its previous derivatives. So KT7692 has a novel inhibitory profile against PI3-kinase and MLCK. IC_{50} values of wortmannin for PI3-kinase and MLCK were calculated to be 3 nM and 0.2 μ M, respectively. Wortmannin is therefore almost 100 times more potent than MLCK in inhibiting PI3-kinase. In contrast, the IC_{50} of KT7692 for PI3-kinase was calculated to be 0.2 μ M. Although KT7692 has only about one-hundredth the potency of wortmannin against PI3-kinase *in vitro*, its potency against MLCK is similar to that of wortmannin; the IC_{50} of KT7692 for MLCK was calculated to be 0.2 μ M. KT7692 had no effect on cyclic-AMP-dependent protein kinase, protein kinase C and membrane-bound PI4-kinase at concentrations up to 10 μ M (results not shown).

The use of KT7692 in combination with wortmannin would therefore be useful for clarifying the involvement of PI3-kinase in Fc ϵ RI-mediated histamine secretion in RBL-2H3 cells.

Irreversible inhibition of PI3-kinase by KT7692

That the inhibition of PI3-kinase by wortmannin was irreversible was previously shown by an experiment using immobilized PI3-kinase [11]. In the present study, we established the irreversibility of the inhibition of PI3-kinase by KT7692.

The enzyme immunocomplex was treated with various con-

Figure 2 Irreversible inhibition of PI3-kinase by wortmannin and KT7692

Calf thymus PI3-kinase was immobilized on protein-G-Sepharose beads with anti-PI3-kinase p85 antibody AB6. (a) This enzyme immunocomplex was then treated with various concentrations of wortmannin (broken line), or KT7692 (solid line) for 30 min. The enzyme immunocomplex was then washed seven times with washing buffer used in the preparation of immunoprecipitates. Immediately after the final washing (●) or after additional incubation in washing buffer for 60 min (△), PI3-kinase activity in the washed immunoprecipitates was assayed as described in Materials and methods. (b) The enzyme immunocomplex was pre-incubated with various concentrations of wortmannin (broken line), or KT7692 (solid line) for 0 (●), 10 (○), 30 (■), 90 (□) and 270 min (▲). PI3-kinase activity in the immunoprecipitates was then assayed as described in Materials and Methods except that the reaction time was 3 min. The enzymic activity in the presence of a compound is expressed as a percentage of that in the absence of the compound. Data are the means from two independent experiments.

centration of wortmannin or KT7692 and washed thoroughly with a washing buffer. Immediately after washing or after additional incubation in washing buffer, residual PI3-kinase activity in the washed enzyme immunocomplex was measured (Figure 2a). The activity was inhibited with almost the same potency as in Table 1, so the inhibition was not relieved by the extensive washing or additional incubation. These results suggest that KT7692 and wortmannin were not dissociated from the enzyme or decomposed on the enzyme under these conditions.

Next we examined the effect of preincubation time on the inhibitory potency of wortmannin and KT7692 against PI3-kinase (Figure 2b). The inhibition was apparently dependent on the preincubation time. Although the inhibition by wortmannin was saturated after preincubation for 90 min, the inhibition by KT7692 increased over preincubation periods from 0 to 270 min.

Figure 3 Effects of wortmannin and KT7692 on PI3-kinase activity in RBL-2H3 cells

RBL-2H3 cells were pretreated with various concentrations of wortmannin (○) or KT7692 (●) for 15 min. The cells were then stimulated with antigen for 45 min followed by washing four times with PBS. Anti-PI3-kinase p85 immunoprecipitates prepared from the cell lysate were subjected to PI3-kinase assay. PI3-kinase activity in the immunoprecipitates of the cells was determined as described in Materials and methods. The enzymic activity in the immunoprecipitate from drug-treated cells is expressed as a percentage of that from vehicle-treated cells. The same results were obtained in three experiments.

These results suggest that KT7692 associates with the enzyme more slowly than does wortmannin.

Taken together, these results show that KT7692 inhibits PI3-kinase irreversibly and that the difference in inhibitory potency between wortmannin and KT7692 is due to their differences in speed of inhibition.

Inhibitory effect of KT7692 on PI3-kinase in intact cells

Although the levels of phosphatidylinositol 3',4'-bisphosphate (PIP₂) and PIP₃ (the physiological products of PI3-kinase) in RBL-2H3 cells were below the detection limit even under stimulation, we could monitor the inhibitory profile of KT7692 against PI3-kinase in intact cells because of its irreversible inhibitory properties, as described previously [11]. As with wortmannin, activities of PI3-kinase in anti-PI3-kinase p85 immunoprecipitates prepared from RBL-2H3 cells that had been incubated with KT7692 should reflect the actual intracellular activities of PI3-kinase after KT7692 treatment. After sensitization of RBL-2H3 cells with IgE, the cells were incubated with various concentrations of wortmannin or KT7692 at 37 °C for 15 min. The cells were then stimulated with antigen for 45 min and washed four times with PBS. PI3-kinase activities in

anti-PI3-kinase p85 immunoprecipitates from the cells were measured (Figure 3). As described before, wortmannin inhibited PI3-kinase in intact cells with an IC₅₀ of 2 nM [11]. KT7692 inhibited the PI3-kinase in intact cells in a dose-dependent manner at concentrations ranging from 10 nM to 10 μM; the IC₅₀ was calculated as 0.3 μM. Using anti-phosphotyrosine antibody instead of anti-PI3-kinase p85 antibody, we obtained the same results (not shown). KT7692 is therefore equally effective against stimulated PI3-kinase as the total PI3-kinase activities (or their p85-bound isoenzyme) in RBL-2H3 cells. KT7692 also has a weaker inhibitory effect than wortmannin on the production of PIP₃ in PC12 cells stimulated with nerve growth factor (K. Kimura and Y. Fukui, unpublished observations). The inhibitory potency of KT7692 against PI3-kinase in intact RBL-2H3 cells is about one-hundredth that of wortmannin, as shown *in vitro*.

Inhibitory effect of KT7692 on MLCK in rat aorta

The phosphorylation of myosin light chain is known to occur in response to antigen stimulation and is thought to be linked with histamine secretion in RBL-2H3 cells. However, this phosphorylation has been shown to be catalysed by protein kinase C as well as MLCK in this cell line [26,27]. As one of the putative functions of PI3-kinase is activation of one isozyme of protein kinase C, so the pharmacological analysis of wortmannin as an inhibitor of MLCK will be complicated in RBL-2H3 cells. In contrast, in smooth muscle, myosin light chain is known to be phosphorylated by MLCK in a Ca²⁺-dependent manner, and the structures of non-muscle MLCK and smooth muscle MLCK are closely related, especially in their catalytic domains [28]. We therefore examined whether KT7692 would inhibit the phosphorylation of myosin light chain in rat aorta at high KCl concentration.

Phosphorylation was inhibited by KT7692 at concentrations around 0.3–10 μM (Figure 4). The same dose of wortmannin also inhibited phosphorylation, as reported previously [10]. Phosphorylation was almost completely inhibited at around 1.0 μM by both KT7692 and wortmannin. Although the IC₅₀ of KT7692 could not be determined precisely because it was difficult to quantify the density of the immunostained protein bands, we can conclude that KT7692 and wortmannin have similar pharmacological inhibitory effects on MLCK in intact cells or tissues.

Effect of KT7692 on FcεRI-mediated histamine secretion in RBL-2H3 cells

To determine whether KT7692 blocks FcεRI-mediated events in RBL-2H3 cells, we examined the effect of KT7692 on the FcεRI-

Figure 4 Effects of wortmannin and KT7692 on phosphorylation of myosin light chain in rat aorta smooth muscle

Aorta rings were treated with the indicated concentrations of wortmannin or KT7692 for 5 min. Thirty seconds after mock stimulation (—) or K⁺ stimulation (+), the phosphorylation reaction was stopped by quickly immersing the ring in dry ice-cold acetone containing 10% (w/v) trichloroacetic acid and 10 mM dithiothreitol. Phosphorylation of the myosin light chain was determined by glycerol/urea gel electrophoresis and subsequent immunoblotting analysis as described in Materials and methods. A representative blot of three independent experiments is shown. Abbreviations used: MLC, unphosphorylated 20 kDa myosin light chain; MLC-P, phosphorylated 20 kDa myosin light chain.

Figure 5 Effects of wortmannin and KT7692 on antigen-stimulated histamine secretion in RBL-2H3 cells

RBL-2H3 cells were pretreated with various concentrations of wortmannin (○) or KT7692 (●) for 15 min. The cells were then stimulated with antigen for 45 min and the medium was collected. The concentration of histamine in the medium was measured as described in Materials and Methods. The effect induced by antigen has been corrected for by subtracting the amount of change caused by vehicle alone. The response to the stimulant in the presence of inhibitor is expressed as a percentage of that in the absence of inhibitor; 100% secretion corresponds to 42% secretion of the total histamine amount present in the culture. Data are the means $\pm$ S.E.M. (error bars) from an experiment carried out in triplicate.

mediated secretion of histamine. After sensitization of RBL-2H3 cells with IgE, the cells were washed and incubated with various concentrations of KT7692 or wortmannin at 37 °C for 15 min. Antigen was then added to the culture and the incubation was continued for a further 45 min. After this incubation, the amount of histamine in the medium was measured. KT7692 inhibited Fc ϵ RI-mediated histamine secretion by up to 80% in a dose-dependent manner at concentrations ranging from 10 nM to 10 μ M; the IC₅₀ was calculated as 0.1 μ M (Figure 5). It is noteworthy that KT7692 inhibits the Fc ϵ RI-mediated cellular responses with IC₅₀ values almost equal to those for PI3-kinase activity both *in vitro* and in intact cells. As summarized in Table 1, there is a positive correlation between the potencies of wortmannin and its derivatives as inhibitors of histamine secretion and as inhibitors of PI3-kinase, but not as inhibitors of MLCK; IC₅₀ values of these compounds for histamine secretion showed more apparent parallelism with those for PI3-kinase inhibition than for MLCK.

KT7692 and wortmannin itself, even at 10 μ M, did not cause secretion of histamine (results not shown). When RBL-2H3 cells were incubated in the presence of 10 μ M wortmannin or KT7692 for 60 min, extracellular lactic acid dehydrogenase activities in the medium were 0.37% or 0.43%, respectively, of the cellular total activity; these values were almost equal to that in the absence of these compounds (0.25%). Further, wortmannin and KT7692 at 10 μ M did not decrease the respiratory activity of the cells when measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method (results not shown) [29]. These results indicate that the inhibitory effect of wortmannin on histamine secretion is not due to a general cytotoxicity.

Effect of LY294002 on MLCK, PI3-kinase and Fc ϵ RI-mediated histamine secretion in RBL-2H3 cells

LY294002 is a structurally different inhibitor of PI3-kinase from wortmannin. Although LY294002 showed a little effect on MLCK activity (40% at 100 μ M), LY294002 inhibited PI3-kinase in a dose-dependent manner at concentrations ranging from 0.1 to 10 μ M: the IC₅₀ for PI3-kinase was calculated to be

Figure 6 Effect of LY294002 on PI3-kinase and on antigen-induced histamine secretion in RBL-2H3 cells

PI3-kinase activity (○), MLCK activity (□) and IgE-mediated histamine secretion from RBL-2H3 cells (●) were measured in the presence of the indicated concentrations of LY294002 as described in Materials and Methods. The effect induced by antigen has been corrected for by subtracting the amount of change caused by vehicle alone. The response to the stimulant in the presence of LY294002 is expressed as a percentage of that in its absence; 100% secretion corresponds to 42% secretion of the total histamine amount present in the culture. Data are the means $\pm$ S.E.M. (error bars) from an experiment carried out in triplicate.

2 μ M (Figure 6). This is in good agreement with that reported previously [18]. LY294002 is therefore almost 100 times more potent in inhibiting PI3-kinase than MLCK. LY294002 also inhibited Fc ϵ RI-mediated histamine secretion by up to 80% in a dose-dependent manner at concentrations ranging from 0.1 to 10 μ M; the IC₅₀ was calculated as 5 μ M (Figure 6), which is very close to that for PI3-kinase.

LY294002 itself, even at 100 μ M, did not cause secretion of histamine (results not shown). When RBL-2H3 cells were incubated in the presence of 100 μ M LY294002 for 60 min, extracellular lactic acid dehydrogenase activities in the medium were 0.26% of the cellular total activity; these values were almost equal to that seen in the absence of LY294002 (0.25%). Further, LY294002 at 100 μ M did not decrease the respiratory activity of the cells when measured by the MTT method (results not shown) [29]. These results indicate that the inhibitory effect of LY294002 on histamine secretion is not due to a general cytotoxicity.

DISCUSSION

The production of PIP₂ and PIP₃, the reaction products of PI3-kinase in intact cells, correlates well with various cellular functions, such as glucose transporter-4 containing vesicle transport (glucose uptake) in the insulin-target cells [30,31], the respiratory burst and cytoskeletal reorganization in the neutrophils [32,33] and the secretion of granule contents and morphological changes in the platelets [34]. This argues strongly that these molecules are important as messengers in cellular functions [1,5]. To clarify this point, a variety of inhibitors of PI3-kinase would be very useful. Since we and others showed that wortmannin is a potent and selective inhibitor of PI3-kinase *in vitro* and in intact cells [11,12], many papers [35–43] have been published investigating the physiological roles of PI3-kinase using wortmannin as an inhibitor of this enzyme.

Wortmannin inhibits PI3-kinase with an IC₅₀ of 3 nM, but it also inhibits MLCK at 100-fold higher concentrations than those required for the inhibition of PI3-kinase. Although wortmannin derivatives, WM-4, -25, -12 and -13, inhibited both PI3-kinase and MLCK activities to a different degree, there is a positive

correlation between the potencies of these compounds as inhibitors of PI3-kinase and as inhibitors of MLCK. We could not therefore establish more conclusively that PI3-kinase is truly involved in FcεRI-mediated histamine secretion with only wortmannin and the derivatives listed. In the present study we used another type of wortmannin derivative, KT7692, which has one-hundredth of the inhibitory potency of wortmannin against PI3-kinase, but still retains a similar potency against MLCK to wortmannin both *in vitro* and in tissues or cells. KT7692 inhibited the FcεRI-mediated histamine secretion with an IC₅₀ of 0.1 μM, which is almost equal to that for PI3-kinase activity both *in vitro* and in intact cells. As shown in Table 1, there is a positive correlation between the potencies of wortmannin and its derivatives as inhibitors of histamine secretion and as inhibitors of PI3-kinase, but not of MLCK. We also used LY294002, a structurally different inhibitor of PI3-kinase from wortmannin, which is almost 100 times more potent than MLCK in inhibiting PI3-kinase. LY294002 inhibited PI3-kinase *in vitro* and histamine secretion in RBL-2H3 cells to an almost similar degree, with IC₅₀ values of 2 and 5 μM, respectively. It has been reported by Vlahos et al. [18] that LY294002 inhibits *N*-formyl-Met-Leu-Phe-stimulated PIP₂ and PIP₃ formation and the respiratory burst in neutrophils in the same concentration range as shown here. Taken together, these results clearly support the contention that PI3-kinase is involved in the mechanism of histamine secretion in RBL-2H3 cells.

The present results will provide additional support for the physiological significance of this enzyme in intracellular signalling for histamine secretion after stimulation of FcεRI. Furthermore KT7692, in combination with wortmannin and LY294002, will be a powerful control tool in clarifying the involvement of PI3-kinase in cellular signal transduction systems after the stimulation of various membrane receptors.

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