Janus kinase 3 (JAK3), a member of the Janus family protein-tyrosine kinases, is expressed in mast cells, and its enzymatic activity is enhanced by IgE receptor/FceRI cross-linking. Selective inhibition of JAK3 in mast cells with 4-(4'-hydroxylphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131) blocked the phospholipase C activation, calcium mobilization, and activation of microtubule-associated protein kinase after IgE receptor/FceRI cross-linking. Treatment of IgE-sensitized rodent mast cells with WHI-P131 effectively inhibited the activation-associated morphological changes, degranulation, and proinflammatory mediator release after specific antigen challenge without affecting the functional integrity of the distal secretory machinery. In vivo administration of the JAK3 inhibitor WHI-P131 prevented mast cell degranulation and development of cutaneous as well as systemic fatal anaphylaxis in mice at nontoxic dose levels. Thus, JAK3 plays a pivotal role in IgE receptor/FceRI-mediated mast cell responses, and targeting JAK3 with a specific inhibitor, such as WHI-P131, may provide the basis for new and effective treatment as well as prevention programs for mast cell-mediated allergic reactions.

Acute allergic reactions, also known as immediate (type I) hypersensitivity reactions, including anaphylaxis with a potentially fatal outcome, are triggered by three major classes of proinflammatory mediators, namely preformed granule-associated bioactive amines (e.g. histamine and serotonin) and acid hydrolases (e.g. β-hexosaminidase), newly synthesized arachidonic acid metabolites (e.g. leukotriene (LT) 1 C4, prostaglandin D2, and platelet activating factor), and a number of proinflammatory vasoactive cytokines (e.g. tumor necrosis factor (TNF) α and interleukin (IL)-6) (1, 2). These proinflammatory mediators are released from sensitized mast cells upon activation through the antigen-mediated cross-linking of their high affinity cell surface IgE receptors/FcεRI (1, 3, 5). IgE receptor/FcεRI is a multimeric receptor with α, β, and homodimeric γ chains (4). Both β and γ subunits of the IgE receptor/FcεRI contain ITAMs (immunoreceptor tyrosine-based activation motifs), which allow interaction with protein-tyrosine kinases (PTK) and PTK substrates via their Src homology 2 domains (4, 6, 7). The engagement of IgE receptors by antigen triggers a cascade of biochemical signal transduction events, including activation of multiple PTK (6, 7). The activation of PTK and subsequent tyrosine phosphorylation of their downstream substrates have been implicated in the pathophysiology of type I hypersensitivity reactions (6, 7). The elucidation of the PTK-dependent signal transduction events that lead to FcεRI-mediated mast cell degranulation and mediator release may provide the basis for the rational design of potent mast cell inhibitors for prevention and treatment of allergic reactions.

Signal transducers and activators of transcription (STAT) are pleiotropic transcription factors that mediate cytokine-stimulated gene expression in multiple cell populations (8, 9). All STAT proteins contain a DNA-binding domain, a Src homology 2 domain, and a transactivation domain necessary for transcriptional activation of target gene expression. Janus kinases (JAK), including JAK1, JAK2, Tyk, and JAK3, are cytoplasmic PTK that play pivotal roles in initiation of cytokine-triggered signaling events by activating the cytoplasmic latent forms of STAT proteins via tyrosine phosphorylation on a specific tyrosine residue near the Src homology 2 domain (10). Tyrosine phosphorylated STAT proteins dimerize through specific reciprocal Src homology 2-phosphotyrosine interactions and translocate to the nucleus where they stimulate the transcription of specific target genes by binding to response elements in their promoters (11). Among the four members of the JAK family, JAK3 is abundantly expressed in lymphoid cells and plays an important role in normal lymphocyte development and function, as evidenced by qualitative and quantitative deficiencies in the B-cell as well as T-cell compartments of the immune system of JAK3-deficient mice (12, 13) and development of severe combined immunodeficiency in JAK3-deficient patients (14, 15). Besides lymphoid cells, nonlymphoid cells (16), including monocytes, megakaryocytes, endothelial cells, cancer cells, and, as we now report, mast cells also express JAK3, but no information is currently available regarding the physiologic function of JAK3 in these nonlymphoid cell populations.

In a recent study, we found that the IgE/antigen-induced degranulation and mediator release are substantially reduced with Jak3'−/− mast cells from JAK3-null mice that were generated by targeted disruption of Jak3 gene in embryonic stem cells (17), indicating that JAK3 plays a pivotal role in IgE receptor/FceRI-mediated mast cell responses both in vitro and in vivo. Here, we report that selective inhibition of JAK3 in mast cells with 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxy-
Janus Kinase 3 in IgE-mediated Mast Cell Responses

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**

- **Mice**—Male BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Animals were caged in groups of five in a pathogen-free environment in accordance with the rules and regulations of U. S. Animal Welfare Act, and the National Institutes of Health. Animal care and the experimental procedures were carried out in agreement with institutional guidelines.

- **Reagents for Mast Cell Culture and in Vitro Mast Cell Assays**—Fetal bovine serum was obtained from HyClone (Logan, UT). Histopaque-1077, calcium ionophore A23187, bovine serum albumin, trolidine blue, hydrogen peroxide, naphthol AS-MX phosphate, fast blue RR, alcian blue, anti-human IgE, piceatannol, and dimethyl sulfoxide were purchased from Sigma. LTC4 ELISA kits were from Cayman Company (Ann Arbor, MI). Histamine ELISA kits were purchased from Immunotech (Westbrook, ME). The preparation of dinitrophenyl (DNP)-BSA (Ann Arbor, MI). Histamine ELISA kits were purchased from Immuno
tech (Westbrook, ME). The preparation of dinitrophenyl (DNP)-BSA (Ann Arbor, MI). Histamine ELISA kits were purchased from Immunotech (Westbrook, ME).

- **Human Mast Cells**—Human mast cell isolation was performed in agreement with institutional guidelines. Animal care and the experimental procedures were carried out in agreement with institutional guidelines.

- **Confluent Microscopy**—Staining of mast cells with primary and secondary antibodies followed by confocal laser scanning microscopy was performed as described previously in detail (26). After staining with appropriate primary and secondary antibodies, cells were washed three times to remove unbound antibody. DNA labeling was performed by incubation of coverslips with Toto-3 (Molecular Probes, Eugene, OR) for 10 min. Excessive dye was washed with PBS plus 0.1% Triton X-100. Cells were visualized under MRC 1024 laser scanning microscope after incubation of coverslips with Toto-3 (Molecular Probes, Eugene, OR) for 10 min. Excessive dye was washed with PBS plus 0.1% Triton X-100.

**FIG. 1.**

**A**

![Image](Image)

**B**

![Image](Image)

**Fig. 1. Expression and activation of JAK3 in mast cells after IgE receptor cross-linking.** A, RBL-2H3 mast cells were stained with a polyclonal anti-JAK3 antibody and labeled with a fluorescein-labeled secondary antibody as well as the DNA-specific dye Toto-3 and visualized using confocal laser scanning microscopy. B, to study IgE/antigen-induced activation JAK3 in mast cells, RBL-2H3 mast cells were sensitized with monoclonal anti-DNP IgE and then challenged with DNP-BSA. Mast cells were lysed using a Nonidet-P40 lysis buffer prior to or 30 min after antigen challenge, and JAK3 immune complexes from these cell lysates were subjected to anti-phosphotyrosine (APT) Western blot (WB) analysis to examine the autophosphorylation of the JAK3 kinase (lanes 1 and 2). In parallel, JAK3 immune complexes were also examined by anti-JAK3 immunoblotting (lanes 3 and 4) to confirm that the increased tyrosine phosphorylation in APT blots was not due to differences in the amount of JAK3 immunoprecipitated (IP).

**Chemical Synthesis of JAK3 Inhibitor, WHI-P131**—The synthesis and chemical characterization of WHI-P131, WHI-P132, WHI-P111, WHI-P112, and WHI-P258 have been previously reported (17–19, 28).

**Stimulation of Mast Cells—**RBL-2H3 cells were sensitized with monoclonal anti-DNP IgE antibody (0.24 mg/ml) for 1 h at 37 °C in a 48-well tissue culture plate. Unbound IgE was removed by washing the cells with phosphate-buffered saline. After washing the PIPES-buffered saline containing 1 mM calcium chloride was added to the monolayers of the RBL-2H3 cells. The cells were challenged with 20 ng/ml DNP-BSA for 30 min at 37 °C. The plate was centrifuged at 200 × g for 10 min at 4 °C. Supernatants were removed and saved. The cell pellets were washed with phosphate-buffered saline and solubilized in PIPES-buffered saline containing 0.1% Triton X-100.

**Fetal Liver-derived human mast cells were resuspended in tyrode buffer containing calcium and magnesium and challenged with anti-FceRI antibody 22E7 for 15 min. In some experiments fetal liver-derived human mast cells were resuspended in culture medium at a cell density of 5 × 10^6/ml and sensitized with IgE (150 μg/ml) for 3 h at 4 °C. After sensitization the cells were washed with tyrode buffer containing calcium and magnesium and challenged with anti-FceRI antibody 22E7 for 15 min. In some experiments fetal liver-derived human mast cells were resuspended in culture medium at a cell density of 5 × 10^6/ml and sensitized with IgE (150 μg/ml) for 3 h at 4 °C.
calcium and magnesium and challenged with mouse monoclonal anti-human IgE (40 μM) for 30 min at 37 °C. To study the effects of the test compounds, mast cells were incubated with WHI-P131, WHI-P111, or WHI-P112 at the indicated concentrations or vehicle for 1 h prior to challenge.

Mediator Release Assays—Histamine content in cell-free supernatants and in the solubilized cell pellets was estimated using a commercially available enzyme immunoassay (29). LTC4 levels were estimated in cell-free supernatants by immunoassay (30). TNFα levels were estimated in cell-free supernatants using a standard cytotoxicity assay (31). In RBL-2H3 cells, β-hexosaminidase release was estimated in cell-free supernatants and Triton X-100 solubilized pellets as described (32). Tryptase levels were quantitated in cell-free supernatants and pellets of fetal liver-derived human mast cells as described previously in detail (24).

Analysis of Stimulation of Inositol Phospholipid Turnover—Inositol-1,4,5-trisphosphate (Ins-1,4,5-P3) levels were measured by using a d-myo-[3H]inositol-1,4,5-trisphosphate assay system purchased from Amersham Pharmacia and Upjohn Co. as reported (33, 34). This highly sensitive assay is based on the competition between nonradiolabeled Ins-1,4,5-P3, in the cellular extracts and a fixed quantity of a high specific activity [3H] Ins-1,4,5-P3 tracer for a limited number of binding sites on a Ins-1,4,5-P3-specific and -sensitive bovine adrenal binding protein (33, 34).

Calcium Measurements—Calcium mobilization assays were performed as described earlier (35). RBL-2H3 cells were loaded with Fluo-3 and stimulated with DNP-BSA in presence and absence of WHI-P131 as described above. Calcium response was measured by an calcium imaging device (Universal Imaging Co., West Chester, PA) mounted onto an inverted microscope. The excitation wavelength was 485 nm, and the emission wavelength for detection was 535 nm. The fluorescent image of an individual cell was acquired by a CCD72 video camera (Dage-MTI Inc., Michigan City, IL) at the speed of 1 frame/s and digitized by computer.
was administered intravenously with 0.5% Evan’s blue (200 mg/kg intraperitoneally) twice at 1-h intervals prior to the antigen challenge. Control mice were treated with an equal volume of vehicle. At 20 h, mice were treated with WHI-P131 (10 or 25 mg/kg) for 2 h, washed with PBS to remove unbound dye, and then mounted in buffered glycerol, 30% triethylenediamine, pH 8.6 (40). For histopathologic evaluation of tissue mast cell degranulation, ears of mice were removed 1 h after the DNP-BSA injection and fixed in 10% buffered formalin. Processed thin sections (3–5 μm) were stained with avidin-FITC (6.25 μg/ml) for 2 h, washed with PBS to remove unbound dye, and then mounted in buffered glycerol, 30% triethylenediamine, pH 8.6 (40). In the murine model for antigen-induced active anaphylaxis, mice were sensitized with 2 mg of BSA in 200 μl of aluminum hydroxide gel (Reheis Inc., Berkeley, NJ). 10 days later, anaphylactic shock was induced by the intravenous challenge of the mice with 200 μg of BSA.

**RESULTS AND DISCUSSION**

**Expression and IgE Receptor/FceRI-mediated Activation of Janus Kinase 3 in Mast Cells**—As shown in Fig. 1A, JAK3 is abundantly expressed in RBL-2H3 mast cells. This finding prompted us to examine the potential involvement of JAK3 in IgE receptor/FceRI-mediated mast cell activation. The baseline tyrosine phosphorylation level of unstimulated RBL-2H3 cells varied from experiment to experiment. Cross-linking of the IgE receptors on RBL-2H3 mast cells that were previously sensitized by a monoclonal anti-DNP-IgE antibody with the specific antigen DNP-BSA resulted in enhanced tyrosine phosphorylation of JAK3 regardless of its baseline phosphorylation state (Figs. 1B and 2A).

**Inhibition of JAK3 in Mast Cells Abrogates IgE Receptor-triggered Biochemical Signaling Events Downstream to Activation of SYK**—We next set out to determine the effects of WHI-P131, a rationally designed JAK3-specific tyrosine kinase inhibitor, on JAK3 activation as well as other biochemical signal transduction events in mast cells. WHI-P131 inhibits JAK3 with an IC_{50} value of 9 μM, but it does not exhibit any detectable inhibitory activity against other protein-tyrosine kinases, including the structurally similar Janus kinases JAK1, JAK2, JAK3, and JAK4.

**Immune Complex Kinase Assays and Western Blot Analyses of Mast Cell Lysates**—RBL-2H3 cells were stimulated as described above in the presence and absence of WHI-P131 for the indicated times. Cells were harvested, lysed (10 mM Tris, pH 7.6, 100 mM NaCl, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 100 μM Na3VO4, 50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and the kinases were immunoprecipitated from the lysates as reported (36, 37). Antibodies directed against JAK3 and SYK used for immunoprecipitations have been described previously (27, 36–38). Commercially available antibodies reactive with tyrosine phosphorylated MAPK (Y-Phos p42MAPK/ERK2; Transduction Labs, Lexington, KY), and actin (Sigma) antibodies were used to examine RBL-2H3 whole cell lysates for evidence of MAPK/ERK2 activation by Western blot analysis. Immunoprecipitations and immunoblotting using the ECL chemiluminescence detection system (Amersham Pharmacia Biotech) were conducted as described previously (36).

**Anaphylaxis Models**—To examine the effect of WHI-P131 on passive cutaneous anaphylaxis in mice, dorsal sides of the ears of BALB/c mice were injected intradermally with 20 mg of DNP-IgE (left ears) or PBS (right ears) in 20 μl of volume using a 30-gauge needle as described previously (39). After 20 h, mice were treated with WHI-P131 (10 or 25 mg/kg intraperitoneally) twice at 1-h intervals prior to the antigen challenge. Control mice were treated with an equal volume of Evan’s blue dye extravasation as a measure of anaphylaxis-associated vascular hyperpermeability, 8-mm skin specimens were removed from the ears of mice, minced in 2 ml of formamide and incubated at 80 °C for 2 h in water bath to extract the dye. The absorbance was read at 620 nm. The data were expressed as plasma exudation indices (i.e. fold increase in optical density over PBS-treated ears at 620 nm).

To induce passive systemic anaphylaxis, BALB/c mice were sensitized with 50 μg of DNP-IgE intravenously. At 24 h DNP-BSA (2 mg) was administered intravenously with 0.5% Evan’s blue (200 μl). For assessment of vascular leak, animals were sacrificed 30 min after the antigen challenge, and their foot pads were examined for blue coloration. Histamine levels in plasma were measured 5 min after the antigen challenge. To this end, blood samples were obtained from the ocular venous plexus by retroorbital venupuncture, and histamine levels were determined by ELISA using a commercial kit (Immunotech, West Brook, ME) (29).

**FIG. 4. Effects of JAK3 inhibition with WHI-P131 on IgE receptor/FceRI-mediated calcium responses in mast cells.** IgE-sensitized RBL-2H3 mast cells were incubated with 1 μM Fluo-3 for 1 h as described under "Experimental Procedures." The cells were then challenged with DNP-BSA or ionomycin, and the change in fluorescence as an indicator of increased intracellular calcium concentration was recorded. The effects of JAK3 inhibition on the calcium responses of mast cells were studied by incubating IgE-sensitized and Fluo-3-loaded RBL-2H3 cells with 3, 12, or 30 μM WHI-P131 for 5 min prior to challenge with 20 ng/ml DNP-BSA or 0.5 μM ionomycin.

**Inhibition of JAK3 in Mast Cells Abrogates IgE Receptor/FceRI-mediated Calcium Responses Downstream to Activation of MAPK/ERK2**—We next set out to determine the effects of WHI-P131, a rationally designed JAK3-specific tyrosine kinase inhibitor, on MAPK/ERK2 activation as a measure of anaphylaxis-associated vascular hyperpermeability. Rushworth et al. have been described previously (27, 36–38). Commercially available antibodies reactive with tyrosine phosphorylated MAPK (Y-Phos p42MAPK/ERK2; New England Biolabs, Beverly, MA), MAPK (p42MAPK/ERK2; Transduction Labs, Lexington, KY), and actin (Sigma) antibodies were used to examine RBL-2H3 whole cell lysates for evidence of MAPK/ERK2 activation by Western blot analysis. Immunoprecipitations and immunoblotting using the ECL chemiluminescence detection system (Amersham Pharmacia Biotech) were conducted as described previously (36).
and JAK2, SRC family tyrosine kinase LYN, ZAP/SYK family tyrosine kinase SYK, TEC family tyrosine kinase BTK, and receptor family tyrosine kinase IRK even at concentrations as high as 350 μM (18, 19). Treatment of the rat mucosal mast cell line RBL-2H3 with WHI-P131 abrogated JAK3 activation after IgE receptor cross-linking (Fig. 2, A and B). Notably, WHI-P131 did not prevent the robust SYK activation signal in RBL-2H3 mast cells after IgE receptor/FcεRI cross-linking (Fig. 2, C and D). Therefore, any biological consequences of JAK3 inhibition in WHI-P131-treated mast cells cannot be attributed to impaired SYK activation. These results also demonstrate that JAK3 in mast cells does not act upstream of SYK in the signal transduction cascade initiated by the engagement of the high affinity IgE receptor/FcεRI, and its activation is not mandatory for activation of SYK. We also noted a slight difference in kinetics of activation of JAK3 versus SYK in that JAK3 tyrosine phosphorylation (Fig. 2A) did not reach its maximum as rapidly as SYK tyrosine phosphorylation (Fig. 2C).

Ozawa et al. (32) have shown that activation of PLCγ and downstream calcium mobilization are essential and sufficient signals for the secretory responses of RBL-2H3 cells to antigen stimulation. Previous studies with SYK-negative variants of RBL-2H3 cells and RBL-2H3 cells expressing truncated SYK proteins with a dominant negative function have clearly shown that SYK activation is essential for activation of PLCγ (41–43). Therefore, we next sought to determine whether JAK3 could act downstream of SYK or cooperate with SYK in activation of PLCγ and calcium mobilization. The PLCγ activation in IgE-sensitized RBL-2H3 mast cells was assayed by measuring the Ins-1,4,5-P3 levels before and after engagement of the IgE receptor/FcεRI with DNP-BSA in two independent experiments. As shown in Fig. 3, the IgE receptor engagement resulted in a rapid increase of the Ins-1,4,5-P3 levels from 4.3 ± 0.6 pmol/10^6 cells to 27.3 ± 5.7 pmol/10^6 cells at 30 s after the stimulation and 31.2 ± 1.3 pmol/10^6 cells at 60 s after the stimulation. In accordance with previous reports, piceatannol, a naturally occurring stilbene that selectively inhibits SYK by competing for its substrate-binding site (44), effectively blocked the Ins-1,4,5-P3 production (Fig. 3). At 30 and 60 s after stimulation, the Ins-1,4,5-P3 levels of piceatannol (30 μM)-treated RBL-2H3 cells were 5.7 ± 1.6 pmol/10^6 cells and 6.8 ± 1.0 pmol/10^6 cells, respectively. Notably, the JAK3 inhibitor WHI-P131 also blocked the Ins-1,4,5-P3 production in RBL-2H3 mast cells.

**Fig. 6.** Effect of JAK3 inhibitor WHI-P131 on IgE receptor/FcεRI-mediated MAPK activity in mast cells. IgE-sensitized RBL-2H3 mast cells were incubated with vehicle or 30 μM WHI-P131 for 30 min prior to challenge with antigen. Cells were lysed at indicated time points, and whole cell lysates were examined for the amounts of tyrosine phosphorylated p42MAPK (upper panel), total p42MAPK (middle panel), and actin (lower panel) by Western blot analysis, as described under “Experimental Procedures.”

**Fig. 5.** Combined effects of the JAK3 inhibitor WHI-P131 and SYK inhibitor piceatannol on IgE receptor/FcεRI-mediated calcium responses in mast cells. IgE sensitized RBL-2H3 cells were loaded with 1 μM Fluo-3 for 1 h as described under “Experimental Procedures.” RBL-2H3 cells were treated with vehicle (Control), WHI-P131, piceatannol, or WHI-P131 + piceatannol (in B and C) for 5 min prior to antigen challenge. The cells were then challenged with DNP-BSA, and the change in fluorescence as an indicator of calcium mobilization was recorded. A, effects of WHI-P131 at 1 and 3 μM concentrations.

B, effects of 1 μM WHI-P131, 200 ng/ml piceatannol, and a combination of 1 μM WHI-P131 plus 200 ng/ml piceatannol. C, combined effects of WHI-P131 and piceatannol.
treated with 30 μM WHI-P131, vehicle, or control compounds WHI-P258 and WHI-P112 prior to challenge with the specific antigen DNP-BSA. Specifically, RBL-2H3 cells were sensitized with IgE and loaded with Fluo-3 prior to stimulation with DNP-BSA. The intracellular calcium ion concentration reached a maximum within 2–3 min after stimulation, which is consistent with previous findings (45, 46). WHI-P131 inhibited the calcium response in a concentration-dependent fashion with abrogation of calcium mobilization at 30 μM (Fig. 4). Thus, JAK3 (similar to SYK) is essential for the IgE receptor/FcεRI-mediated calcium mobilization in mast cells. As shown in Fig. 4, ionomycin-induced IgE receptor-independent calcium responses were not affected by WHI-P131.

We next set out to determine whether JAK3 and SYK might “cooperate” in generation of an optimal calcium signal in mast cells stimulated via IgE receptor/FcεRI by combining WHI-P131 and piceatannol. To this end, we used suboptimal concentrations of WHI-P131 and piceatannol that only partially block the calcium response. As shown in Fig. 5A, WHI-P131 only partially blocked the calcium signal in IgE-sensitized mast cells after DNP-BSA stimulation when it was used at 1 μM or 3 μM concentrations. Similarly, 200 ng/ml piceatannol only partially inhibited the calcium mobilization (Fig. 5B). However, total abrogation of the calcium response was achieved when 1 μM WHI-P131 was combined with 200 ng/ml piceatannol (Fig. 5B). Notably, this combination resulted in complete block of the calcium signal even at a 1:10 dilution (i.e. 0.1 μM WHI-P131 plus 20 ng/ml piceatannol) (Fig. 5C). These findings indicate that JAK3 and SYK promote coincident and potentially cooperative signals in mast cells that both result in the PLCγ activation and calcium mobilization following the engagement of the IgE receptor/FcεRI.

SYK has also been shown to be the initiator of another signaling pathway leading to the tyrosine phosphorylation and activation of MAPK p42MAPK/ERK2 (and subsequently phospholipase A2) in RBL-2H3 mast cells (41). Because of the observed effects of the JAK3 inhibitor WHI-P131 on SYK-dependent signaling events that lead to PLCγ activation and calcium mobilization, we next examined the effects of WHI-P131 on MAPK activation in mast cells following the engagement of the IgE receptor/FcεRI. As shown in Fig. 6, the IgE receptor engagement resulted in enhanced tyrosine phosphorylation of the MAPK p42MAPK/ERK2 (and subsequently phospholipase A2) in RBL-2H3 mast cells blocks the SYK-mediated activation of the PLCγ signaling pathway that follows the stimulation of mast cells via their high affinity IgE receptor/FcεRI. This evidence was further supported by our finding that WHI-P131 blocks the Ins-1,4,5-P3-mediated downstream calcium mobilization in mast cells.

2H3 cells after IgE receptor/FcεRI stimulation. At 30 and 60 s after stimulation, the Ins-1,4,5-P3 levels of WHI-P131-treated RBL-2H3 cells were 3.0 ± 1.2 and 3.1 ± 0.1 pmol/10⁶ cells, respectively, at 30 μM and 3.3 ± 0.4 and 4.0 ± 0.8 pmol/10⁶ cells, respectively, at 100 μM (Fig. 3). These results provided the first experimental evidence that inhibition of JAK3 in mast cells blocks the SYK-mediated activation of the PLCγ signaling pathway that follows the stimulation of mast cells via their high affinity IgE receptor/FcεRI. This evidence was further supported by our finding that WHI-P131 blocks the Ins-1,4,5-P3-mediated downstream calcium mobilization in mast cells. Specifically, RBL-2H3 cells were sensitized with IgE and loaded with Fluo-3 prior to stimulation with DNP-BSA. The intracellular calcium ion concentration reached a maximum within 2–3 min after stimulation, which is consistent with previous findings (45, 46). WHI-P131 inhibited the calcium response in a concentration-dependent fashion with abrogation of calcium mobilization at 30 μM (Fig. 4). Thus, JAK3 (similar to SYK) is essential for the IgE receptor/FcεRI-mediated calcium mobilization in mast cells. As shown in Fig. 4, ionomycin-induced IgE receptor-independent calcium responses were not affected by WHI-P131.

JAK3 inhibitor WHI-P131 on SYK-dependent signaling events that lead to PLCγ activation and calcium mobilization, we next examined the effects of WHI-P131 on MAPK activation in mast cells following the engagement of the IgE receptor/FcεRI. As shown in Fig. 6, the IgE receptor engagement resulted in enhanced tyrosine phosphorylation of the MAPK p42MAPK/ERK2 (and subsequently phospholipase A2) in RBL-2H3 mast cells (41). Because of the observed effects of the JAK3 inhibitor WHI-P131 on SYK-dependent signaling events that lead to PLCγ activation and calcium mobilization, we next examined the effects of WHI-P131 on MAPK activation in mast cells following the engagement of the IgE receptor/FcεRI. As shown in Fig. 6, the IgE receptor engagement resulted in enhanced tyrosine phosphorylation of the MAPK in mast cells, and WHI-P131 blocked this response. Protein expression levels for MAPK or actin were not affected by WHI-P131 (Fig. 6). Thus, JAK3 appears to play an essential role for the IgE receptor-mediated MAPK activation in mast cells as well. Taken together, these in vitro JAK3 inhibitor studies provided biochemical evidence that JAK3 in mast cells is a key regulator of IgE receptor/FcεRI-mediated signal transduction events.

Effects of JAK3 Inhibition on In Vitro Mast Cell Responses—In a systematic effort aimed at elucidating the biologic consequences of JAK3 inhibition in mast cells, we first sought to determine whether the JAK3 inhibitor WHI-P131 could prevent the IgE receptor/FcεRI-mediated activation of mast cells. Because the IgE receptor/FcεRI-mediated activation of mast cells results in a distinct morphologic transformation with marked cell spreading due to membrane ruffling, microtubule formation, and actin polymerization (47), we evaluated the effects of WHI-P131 on the activation-associated transforma-

FIG. 7. Effects of JAK3 inhibition with WHI-P131 on IgE/anti-
gen-induced activation of mast cells. RBL-2H3 cells were cultured overnight on 22 × 22-mm coverslips at a cell density of 0.01 × 10⁶/ml with 0.24 mg/ml DNP-IgE. IgE-sensitized RBL-2H3 cells were then treated with 30 μM WHI-P131, vehicle, or control compounds WHI-P258 and WHI-P112 prior to challenge with the specific antigen DNP-BSA. After stimulation with DNP-BSA for 1 h, cells were fixed in cold methanol for 15 min followed by permeabilization with PBS containing 0.1% Triton X-100. Fixed cells were stained with a monoclonal antibody reactive with α-tubulin (clone B-5-1-2, Sigma) for 40 min at 37 °C. After 3 times wash with PBS plus 0.1% Triton X-100, cells were incubated with a fluorescein-labeled secondary antibody (Zymed Laboratories Inc., San Francisco, CA) for another 40 min. Cells were washed three times to remove the unbound antibody. DNA labeling was performed by incubation of coverslips with Toto-3 (Molecular Probes, Eugene, OR) for 10 min. Excessive dye was washed with PBS plus 0.1% Triton X-100. Cells were visualized under MRC 1024 laser scanning microscope after mounting with Vectashield (Vector Laboratories, Inc., Burlingame, CA), as previously reported (38).

2H3 cells after IgE receptor/FcεRI stimulation. At 30 and 60 s after stimulation, the Ins-1,4,5-P3 levels of WHI-P131-treated RBL-2H3 cells were 3.0 ± 1.2 and 3.1 ± 0.1 pmol/10⁶ cells, respectively, at 30 μM and 3.3 ± 0.4 and 4.0 ± 0.8 pmol/10⁶ cells, respectively, at 100 μM (Fig. 3). These results provided the first experimental evidence that inhibition of JAK3 in mast cells blocks the SYK-mediated activation of the PLCγ signaling pathway that follows the stimulation of mast cells via their high affinity IgE receptor/FcεRI. This evidence was further supported by our finding that WHI-P131 blocks the Ins-1,4,5-P3-mediated downstream calcium mobilization in mast cells. Specifically, RBL-2H3 cells were sensitized with IgE and loaded with Fluo-3 prior to stimulation with DNP-BSA. The intracellular calcium ion concentration reached a maximum within 2–3 min after stimulation, which is consistent with previous findings (45, 46). WHI-P131 inhibited the calcium response in a concentration-dependent fashion with abrogation of calcium mobilization at 30 μM (Fig. 4). Thus, JAK3 (similar to SYK) is essential for the IgE receptor/FcεRI-mediated calcium mobilization in mast cells. As shown in Fig. 4, ionomycin-induced IgE receptor-independent calcium responses were not affected by WHI-P131.

We next set out to determine whether JAK3 and SYK might “cooperate” in generation of an optimal calcium signal in mast cells stimulated via IgE receptor/FcεRI by combining WHI-P131 and piceatannol. To this end, we used suboptimal concentrations of WHI-P131 and piceatannol that only partially block the calcium response. As shown in Fig. 5A, WHI-P131 only partially blocked the calcium signal in IgE-sensitized mast cells after DNP-BSA stimulation when it was used at 1 μM or 3 μM concentrations. Similarly, 200 ng/ml piceatannol only partially inhibited the calcium mobilization (Fig. 5B). However, total abrogation of the calcium response was achieved when 1 μM WHI-P131 was combined with 200 ng/ml piceatannol (Fig. 5B). Notably, this combination resulted in complete block of the calcium signal even at a 1:10 dilution (i.e. 0.1 μM WHI-P131 plus 20 ng/ml piceatannol) (Fig. 5C). These findings indicate that JAK3 and SYK promote coincident and potentially cooperative signals in mast cells that both result in the PLCγ activation and calcium mobilization following the engagement of the IgE receptor/FcεRI.
tion of shape and surface topography of RBL-2H3 mast cells using confocal laser scanning microscopy (48). The vast majority (95%) of unstimulated RBL-2H3 mast cells exhibited a spindle shape with arborized extensions and longitudinally oriented bundles of microtubules (Fig. 7A). Activation of RBL-2H3 mast cells by cross-linking their IgE receptors/FcεRI using IgE/antigen induced a dramatic cell spreading response, and 93% of cells assumed a flattened shape with a generalized microtubule organization throughout their cytoplasm (Fig. 7B).

A 2-h incubation with the JAK3 inhibitor WHI-P131 (but not the unsubstituted parent dimethoxyquinazoline compound WHI-P258, which lacks JAK3 inhibitory activity) at a concentration of 30 μM prevented the IgE/antigen-induced mast cell activation, as evidenced by markedly reduced spreading (23% flattened, 58% spindle shaped) and microtubule organization (Fig. 7, C–F). In contrast to WHI-P131, the bromo-substituted control dimethoxyquinazoline compound WHI-112, which does not inhibit JAK3, was unable to produce any significant effects on antigen-induced mast cell spreading or microtubule organization (Fig. 7, G and H). In parallel, we tested the effect of the compounds on the viability of RBL-2H3 cells (as assessed by trypan blue dye exclusion test) under these experimental conditions and found that they do not affect cell viability at concentrations as high as 300 μM (data not shown).

To further examine the role of JAK3 in IgE receptor/FcεRI-mediated mast cell activation and degranulation, we next as-

Fig. 8. Effects of JAK3 inhibition with WHI-P131 on IgE receptor/FcεRI-mediated mast cell responses. RBL-2H3 cells were sensitized with monoclonal anti-DNP IgE, treated with WHI-P131, vehicle, or control compounds and then challenged with DNP-BSA as described in detail under “Experimental Procedures.” A, mast cell degranulation (β-hexosaminidase release, percentage of total) was assessed by measuring the β-hexosaminidase levels in cell-free supernatants and Triton X-100 solubilized pellets using the formula: β-hexosaminidase release (percentage of total) = 100 × (β-hexosaminidase level in supernatant/β-hexosaminidase level in supernatant + solubilized pellet). Vehicle-treated control RBL-2H3 cells released 45.1 ± 3.1% of their hexosaminidase content after the DNP-BSA challenge. B and C, LTC4 (B) and TNFα (C) levels in cell-free supernatants were measured. Vehicle-treated control cells released 11.3 ± 1.3 ng LTC4 and 160 ± 33.0 pg TNFα/106 mast cells. The results on LTC4 and TNFα release are expressed as percentages of maximum control release from vehicle-treated control mast cells. The data points represent the means ± S.E. obtained from three to six independent experiments. *, p < 0.05 compared with control as determined by Student’s t test; **, p < 0.0001 compared with control as determined by Student’s t test.

Fig. 9. Effects of JAK3 inhibition with WHI-P131 on calcium ionophore A23187-induced degranulation of mast cells. RBL-2H3 cells were stimulated with 0.5 μM A23187 for 30 min. Mast cell degranulation (β-hexosaminidase release, percentage of total) was assessed as described in the legend of Fig. 8. To study the effect of WHI-P131 on A23187-induced mast cell degranulation, RBL-2H3 cells were incubated with WHI-P131 (10 or 30 μM) for 30 min prior to A23187 challenge. The data points represent the means ± S.E. (n = 3). Vehicle-treated control RBL-2H3 cells released 72.0 ± 0.2% of their total cellular β-hexosaminidase content after treatment with A23187. Notably, pretreatment or RBL-2H3 cells with 10 or 30 μM WHI-P131 did not inhibit this calcium ionophore-induced degranulation. In parallel, IgE-sensitized RBL-2H3 cells were also treated with WHI-P131 (3, 10, or 30 μM) and then challenged with specific antigen, as described in the legend to Fig. 8. Vehicle-treated control RBL-2H3 cells released 34.7 ± 5.3% of their total cellular β-hexosaminidase content after IgE receptor cross-linking.
sessed the effects of the JAK3 inhibitor WHI-P131 on mast cell degranulation and mediator release induced by IgE/antigen. WHI-P111 and WHI-P112, which do not inhibit JAK3, were included as control compounds. RBL-2H3 mast cells were pre-incubated with increasing concentrations of the test compounds or vehicle for 1 h before challenge with antigen (DNP-BSA). Stimulation of RBL-2H3 mast cells using IgE/antigen resulted in release of significant amounts of \( \beta \)-hexosaminidase (45.1 ± 3.1% of the total cellular content), LTC4 (11.3 ± 1.3 ng/10⁶ cells), and TNF\( \alpha \) (160 ± 33.0 pg/10⁶ cells). Notably, WHI-P131 prevented mast cell degranulation and release of preformed granule-associated \( \beta \)-hexosaminidase (Fig. 8A) as well as release of the newly synthesized arachidonic acid metabolite LTC4 (Fig. 8B) and the proinflammatory cytokine TNF\( \alpha \) (Fig. 8C) in a dose-dependent fashion with near to complete inhibition at \( \geq 30 \) \( \mu \)M. Unlike these JAK3 inhibitors, the control dimethoxyquinazoline derivatives WHI-P111 and WHI-P112 lacking JAK3 inhibitory activity did not inhibit mast cell degranulation or mediator release after IgE receptor/Fc\( \epsilon \)RI cross-linking (Fig. 8).

The functional integrity of the secretory machinery of mast cells responsible for the release of mediators can be evaluated using ionophores that result in an increase of the intracellular calcium concentration and mediator release independent of the IgE receptor-linked proximal signal transduction events. The observed inhibitory effects of WHI-P131 on IgE receptor/Fc\( \epsilon \)RI-mediated mast cell responses were not due to a functional impairment of the distal secretory machinery in mast cells, because WHI-P131 did not prevent degranulation (as measured by \( \beta \)-hexosaminidase release) of RBL-2H3 mast cells after treatment with the ionophore A23187 (0.5 \( \mu \)M) at concentrations sufficient for inhibition of antigen-induced degranulation of IgE-sensitized RBL-2H3 cells (Fig. 9).

We next set out to examine the effects of WHI-P131 on IgE receptor/Fc\( \epsilon \)RI-mediated degranulation and mediator release from human mast cells. To this end, we cultured fetal liver-derived human mast cells in the presence of stem cell factor and IL-4 for 5 weeks. IgE-sensitized human mast cells were exposed to vehicle or increasing concentrations of WHI-P131 (Fig. 10). Human mast cells store the mast cell-specific protease \( \beta \)-tryptase in their secretory granules (Fig. 10A), and the release of \( \beta \)-tryptase by degranulation is a specific marker for human mast cell activation (49). The Fc\( \epsilon \)RI receptors of fetal liver-derived human mast cells were cross-linked with anti-IgE, and the resulting mast cell degranulation (i.e. \( \beta \)-tryptase release) (24) and LTC4 release (30) were quantitated. WHI-P131 inhibited the release of \( \beta \)-tryptase (Fig. 10B) as well as LTC4 (Fig. 10C) from IgE/antigen stimulated human mast cells in a dose-dependent fashion.

Taken together, these in vitro JAK3 inhibitor studies pro-
provided evidence that JAK3 in mast cells is a key regulator of IgE receptor/FcεRI-mediated responses. The ability of the JAK3 inhibitor WHI-P131 to inhibit mast cell degranulation as well as mediator release after IgE receptor/FcεRI cross-linking prompted us to further evaluate the potential of this compound as an anti-allergic agent.

Effects of the JAK3 Inhibitor WHI-P131 on in Vivo Mast Cell Responses—Increased vascular permeability induced by mast cell mediators, such as histamine and leukotrienes, is a hallmark of anaphylaxis (37, 44). Therefore, we first examined the effect of the JAK3 inhibitor WHI-P131 on vascular permeability in a well characterized murine model of passive cutaneous anaphylaxis (39). WHI-P131 inhibited the IgE/antigen induced plasma exudation (as measured by extravasation of albumin-bound Evan’s blue dye) in mice (n = 12), and the plasma exudation indices were determined for vehicle-treated as well as WHI-P131-treated mice, as described under “Experimental Procedures.” To study the effect of WHI-P131 on anaphylaxis, IgE-sensitized mice were injected with two consecutive doses of 10 or 25 mg/kg WHI-P131 at 90 min before and 30 min before the antigen challenge, respectively. Mice were then challenged with 100 μg of DNP-BSA in 2% Evan’s blue dye, and the plasma exudation indices (fold increase in optical density over PBS-treated ears) were determined. The data points represent the means ± S.E. The mean ΔOD 620 nm, vehicle-treated ears was 0.22 ± 0.04 before and 0.92 ± 0.05 after the IgE/antigen challenge. *, p < 0.05 compared with vehicle-treated controls.

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exudation was blue coloring of foot pads 30 min after the antigen challenge (50). Vehicle-treated control mice showed a marked blue coloring of their foot pads after antigen challenge, but no significant blue coloring was observed in mice pretreated with the JAK3 inhibitor WHI-P131 (Fig. 11B). We also assessed mast cell degranulation in histologic sections of ears by examining their fluorescence intensity after staining with avidin-FITC. Avidin specifically binds to heparin, the major proteoglycan in the granules of connective tissue mast cells (40). The fluorescence intensity of the stained mast cells is proportional to the amount of heparin, and therefore degranulation reduces the fluorescence intensity. Whereas the IgE/antigen challenge resulted in a marked reduction of fluorescence intensity of avidin-FITC-stained tissue mast cells of control mice consistent with degranulation-associated depletion of heparin, no reduction in fluorescence intensity was observed for mast cells from WHI-P131-pretreated mice (Fig. 11C). Because a major vasoactive mediator released from activated mast cells is histamine and systemic anaphylaxis in humans and rodents has been associated with a significant increase in blood histamine levels (39, 50), we obtained blood samples from mice 5 min after the antigen challenge to determine their plasma histamine levels (29). As expected, the antigen challenge resulted in marked elevation of plasma histamine levels, but pretreatment with the JAK3 inhibitor WHI-P131 substantially reduced this histamine response (Fig. 11D). These results demonstrate that WHI-P131 is capable of preventing passive cutaneous and systemic anaphylaxis by blocking mast cell degranulation in vivo.

We next tested the efficacy of WHI-P131 in a model of IgE-antigen-induced active systemic anaphylaxis. To this end, mice were first injected with BSA in an aluminum hydroxide gel to trigger a BSA-specific IgE response. 10 days later, these BSA-sensitized mice were challenged with antigen, resulting in exudation of heparin, no reduction in fluorescence intensity was observed for mast cells from WHI-P131-pretreated mice (Fig. 11). We gratefully acknowledge Dr. Christopher Na-...
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