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c-Kit Receptor Signaling through Its Phosphatidylinositide-3′-Kinase-binding Site and Protein Kinase C: Role in Mast Cell Enhancement of Degranulation, Adhesion, and Membrane Ruffling

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In bone marrow-derived mast cells (BMMCs), the Kit receptor tyrosine kinase mediates diverse responses including proliferation, survival, chemotaxis, migration, differentiation, and adhesion to extracellular matrix. In connective tissue mast cells, a role for Kit in the secretion of inflammatory mediators has been demonstrated as well. We recently demonstrated a role for phosphatidylinositide-3′ (PI 3)-kinase in Kit-ligand (KL)-induced adhesion of BMMCs to fibronectin. Herein, we investigated the mechanism by which Kit mediates enhancement of FcεRI-mediated degranulation, cytoskeletal rearrangements, and adhesion in BMMCs. W^{sh}/W^{sh} BMMCs, lacking endogenous Kit expression, were transduced to express normal and mutant Kit receptors containing Tyr → Phe substitutions at residues 719 and 821. Although the normal Kit receptor fully restored KL-induced responses in W^{sh}/W^{sh} BMMCs, Kit^{Y719F}, which fails to bind and activate PI 3-kinase, failed to potentiate degranulation and is impaired in mediating membrane ruffling and actin assembly. Inhibition of PI 3-kinase with wortmannin or LY294002 also inhibited secretory enhancement and cytoskeletal rearrangements mediated by Kit. In contrast, secretory enhancement and adhesion stimulated directly through protein kinase C (PKC) do not require PI 3-kinase. Calphostin C, an inhibitor of PKC, blocked Kit-mediated adhesion to fibronectin, secretory enhancement, membrane ruffling, and filamentous actin assembly. Although cytochalasin D inhibited Kit-mediated filamentous actin assembly and membrane ruffling, secretory enhancement and adhesion to fibronectin were not affected by this drug. Therefore, Kit-mediated cytoskeletal rearrangements that are dependent on actin polymerization can be uncoupled from the Kit-mediated secretory and adhesive responses. Our results implicate receptor-proximal PI 3-kinase activation and activation of a PKC isoform in Kit-mediated secretory enhancement, adhesion, and cytoskeletal reorganization.

INTRODUCTION

c-Kit, a receptor tyrosine kinase belonging to the platelet-derived growth factor (PDGF) receptor subfamily, is encoded at the murine *W* locus and controls diverse cellular processes during development and in the adult animal. Mutations at the *W* locus cause defects in gametogenesis, melanogenesis, and hematopoiesis. The hematopoietic defects include macrocytic anemia and lack of tissue mast cells. The mast cell deficiency of *W* mutant mice can be repaired by transplantation of bone marrow from wild-type animals, indicating that Kit is required for mast cell development in vivo

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(Kitamura *et al.*, 1978). Kit mediates proliferation of mast cells in culture (Nocka *et al.*, 1990; Tsai *et al.*, 1991) and suppresses apoptosis induced by growth factor withdrawal or irradiation (Yee *et al.*, 1994). The Kit receptor also mediates differentiation of bone marrow-derived mast cells (BMMCs) to a more mature connective tissue mast cell phenotype (Tsai *et al.*, 1991).

In addition to growth and maturation, there is evidence that Kit plays a role in regulating mast cell functions. Mast cells release mediators of inflammation such as histamine, serotonin, proteoglycans, and leukotrienes by degranulation in response to cross-linking of their high-affinity IgE receptor (FcεRI). Mast cells are required for most inflammation associated with IgE-triggered reactions in the mouse (Wershil *et al.*, 1991), and mast cells play a role in the immune complex-mediated reverse-arthus reaction (Zhang *et al.*, 1991). Also, Kit receptor signaling has been implicated in in vivo mast cell degranulation (Wershil *et al.*, 1992). In cell culture, KL directly induces a low level of degranulation in peritoneal mast cells (Coleman *et al.*, 1993) and enhances degranulation of lung connective tissue mast cells triggered by cross-linking of the IgE receptor (Bischoff *et al.*, 1992). In addition to secretory effects, KL induces adhesion of murine BMMCs to a fibronectin matrix (Dastych and Metcalfe, 1994; Kinashi and Springer, 1994; Serve *et al.*, 1995). This adhesion is mediated by $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins through an inside-out signaling mechanism (Kinashi and Springer, 1994; Kovach *et al.*, 1995). KL has also been shown to act as a chemotractant for BMMCs (Meininger *et al.*, 1992). Membrane ruffling and cytoskeletal rearrangements are likely to be involved in mast cell motility mediated by Kit. In porcine endothelial cells expressing Kit, KL-induced chemotaxis is accompanied by circular actin reorganization (Blume-Jensen *et al.*, 1991).

Kit receptor signaling involves dimerization of ligand-bound receptor, activation of the intrinsic receptor kinase, autophosphorylation, association of the activated receptor with signaling molecules, and phosphorylation of substrates (Lev *et al.*, 1991). The p85 subunit of phosphatidylinositide-3′- (PI 3) kinase associates with the Kit receptor in vivo (Reith *et al.*, 1991; Rottapel *et al.*, 1991). Mutational analysis of the c-Kit receptor indicates that Tyr-719 in the kinase insert determines binding of the p85 subunit of PI 3-kinase to the activated receptor (Serve *et al.*, 1994). Other cellular proteins known to associate with Kit include phospholipase Cγ-1 (weak association; Reith *et al.*, 1991; Rottapel *et al.*, 1991), the GRB2 adaptor protein (weak association; Blume-Jensen *et al.*, 1994), the c-src protein (Blume-Jensen *et al.*, 1994), the tyrosine protein kinase tec (Tang *et al.*, 1994), and the tyrosine phosphatases PTP1C and Syp (Yi and Ihle, 1993; Tauchi *et al.*, 1994).

In an attempt to dissect the signaling cascades mediated by KL/Kit, W^{sh}/W^{sh} BMMCs lacking expression of endogenous c-*kit* have been reconstituted with mutant versions of the Kit receptor. Introduction of normal murine Kit receptor into W^{sh}/W^{sh} BMMCs restores KL-induced proliferation, survival, and adhesion to fibronectin as well as activation of PI 3-kinase, p21^{ras}, and mitogen-activated protein (MAP) kinase (Serve *et al.*, 1995). We have previously shown that substitution of Tyr-719 with Phe (Y719F) in the kinase insert abolishes PI 3-kinase association and activation at this site and impairs KL-induced adhesion of BMMCs to fibronectin. In addition, the Y719F mutation had partial effects on p21^{ras} activation, cell proliferation, and survival, whereas MAP kinase activation was not appreciably affected (Serve *et al.*, 1994, 1995). On the other hand, a Y821F substitution blocked proliferation and survival without affecting PI 3-kinase, p21^{ras}, or MAP kinase activation. The Y821F mutation had no effect on KL-induced cell adhesion to fibronectin in BMMCs. In agreement with a role for PI 3-kinase in Kit-mediated cell adhesion, wortmannin, a specific inhibitor of PI 3-kinase, blocked Kit-induced adhesion of BMMCs to fibronectin. Therefore, association of Kit with the p85 subunit of PI 3-kinase, and thus with PI 3-kinase activity, is necessary to fully support mitogenesis and adhesion in BMMCs. In contrast, Tyr-821 is essential for Kit-mediated mitogenesis and survival but not cell adhesion.

pp70^{S6kinase} has been placed downstream of PI 3-kinase activation in signaling cascades triggered by both the PDGF and interleukin (IL) 2 receptors (Chung *et al.*, 1994; Monfar *et al.*, 1995). Thus, pp70^{S6kinase} is a candidate signaling molecule for Kit-mediated events involving PI 3-kinase activation. Independently of Kit, protein kinase C (PKC) activation has been linked with degranulation, adhesion, and cytoskeletal rearrangements. Phorbol 12-myristate 13-acetate (PMA) up-regulates peritoneal mast cell secretion (Chakravarty *et al.*, 1990; Koopmann and Jackson, 1990), and PKC is required for IgE receptor-mediated degranulation (Ozawa *et al.*, 1993). PMA, similarly to KL, induces adhesion of BMMCs to fibronectin (Dastych and Metcalfe, 1994; Kinashi and Springer, 1994). Although PMA reduces filamentous (F) actin levels in rat peritoneal mast cells (Koffer *et al.*, 1990), PMA induces membrane ruffling in RBL-2H3 cells (Pfeiffer *et al.*, 1985). Furthermore, inhibition of PKC blocked Kit-mediated circular actin formation in porcine endothelial aortic cells (Blume-Jensen *et al.*, 1993). Therefore, in BMMCs, PKC may play a role in Kit signals influencing degranulation, adhesion, or cytoskeletal rearrangements.

We demonstrate herein that KL stimulates an enhancement of BMMC degranulation triggered by IgE receptor cross-linking or ionomycin treatment. Additionally, we show that Kit mediates BMMC membrane ruffling and actin polymerization. Utilizing a tyrosine to phenylalanine mutation that abolishes PI 3-kinase association with Kit and pharmacological inhibitors of PI 3-kinase, PKC, pp70^{S6kinase}, and actin polymerization, we attempt to dissect BMMC Kit signaling routes leading to secretory enhancement, cytoskeletal rearrangements, and adhesion to fibronectin.

MATERIALS AND METHODS

Mast Cell Cultures

C57BL/6J wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). W^{sh}/W^{sh} mice were provided by Drs. Regina Duttlinger and Katia Manova. BMMCs were obtained by culturing bone marrow in RPMI 1640 supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, 5.5×10^{-5} M 2-mercaptoethanol, 0.075% sodium bicarbonate, 10% fetal bovine serum (RPMI complete), and 10% conditioned medium from IL-3-producing X63 cells (Karasuyama and Melchers, 1988; X63 cells were kindly provided by Dr. Christoph Moroni, University of Basel, Switzerland). Recombinant murine KL (rmKL) was prepared as described previously (Yee *et al.*, 1994). W^{sh}/W^{sh} BMMCs expressing various mutant Kit receptors were obtained as described previously (Serve *et al.*, 1995). Two isoforms of the Kit receptor are known. Control and mutant Kit receptors in this paper are based on the longer isoform (Kit_L), which contains a four-amino acid insert (gNNK) at position 512–513 in the extracellular domain. E86 packaging cells producing kit retroviruses were irradiated with 30 Gy of γ-irradiation and subsequently cocultivated for 2 wk with 3- to 5-wk-old W^{sh}/W^{sh} BMMCs with G418 selection starting at 48 h.

Degranulation Assay

Degranulation was measured by release of serotonin (Coleman *et al.*, 1991). Approximately 10^6 BMMCs/ml in RPMI complete were incubated for 12 to 16 h in 3 μCi/ml [^{3}H]serotonin (5-hydroxy-[G-^{3}H]tryptamine creatine sulfate) with specific activity 8.2 Ci/mmol (Amersham, Arlington Heights, IL). When cross-linking the FcεRI, cells were simultaneously sensitized with 1 μg/ml mouse anti-dinitrophenyl IgE monoclonal antibody (Sigma, St. Louis, MO). BMMCs were then washed in phosphate-buffered saline (PBS) and suspended in RPMI complete without IL-3. Samples (2×10^5 cells/500 μl) were stimulated with rmKL, ionomycin (Calbiochem, San Diego, CA), or 10 ng/ml mouse anti-IgE (Pharmingen, San Diego, CA) as described in the figure legends. Degranulation was allowed to proceed for 15 min at 37°C with gentle rocking. Cells were pelleted at 10,000 × *g* for 6 min at 4°C. The supernatant was saved and the cell pellet was lysed in 1% Triton-X 100 buffer. ^{3}H cpm in supernatant and pellet fractions were determined by scintillation counting in Hydrofluor (National Diagnostics, Atlanta, Georgia), and the percentage of serotonin release was calculated by dividing released serotonin by cell-associated serotonin and subtracting spontaneous release in the absence of any stimulus.

Ca^{2+} Measurements

BMMCs (5×10^5 cells/ml) were loaded with the fluorescent dye Indo-1 (5 μM) in PBS for 30 min at 37°C and 5% CO_2. Cells were washed and suspended at 10^6/ml of RPMI 1640, and fluorescence-activated cell sorting (FACS) analysis with a 410/490-nm wavelength emission ratio was performed after stimulation with 1 μM ionomycin or various concentrations of KL.

Adhesion to Fibronectin

BMMCs (1×10^6 cells) were cultured in the presence of 1.5 mCi of [^{3}H]thymidine in 3 ml of RPMI complete for 36 h at 37°C. Enzyme-linked immunosorbent assay plates (96 wells) were incubated with 20 mg/ml fibronectin in 100 μl of RPMI 640 and 20 mM HEPES (pH 7.4) for 2 h at 37°C, blocked with 3% bovine serum albumin (BSA) in RPMI 1640 and 20 mM HEPES (pH 7.4) for 1 h at 37°C, and washed three times with binding medium (RPMI 1640, 20 mM HEPES, pH 7.4, 0.03% BSA). Then, 50 μl of binding medium were added to the wells containing twice the indicated concentrations of rmKL. The assay was started by adding 10,000 cells in 50 μl of binding medium to each well. After incubation for the indicated times at 37°C, nonadherent cells were collected with the medium, the wells were washed four times with 150 μl of binding medium, and all washes were stored at −20°C. The wells were replenished with 150 μl of binding medium and also stored at −20°C. After thawing, cells in the supernatant with all washes and cells bound to the plate were filtered through glass fiber filters and ^{3}H cpm were measured in a liquid scintillation counter. The percentage of adherent cells was calculated as follows: cpm bound/(cpm bound + cpm supernatant) × 100.

Assay for Membrane Ruffling

BMMCs (2×10^6 cells/ml) were starved from IL-3 and serum in RPMI 1640 containing 0.5% BSA for 16 h. In some cases, cells were treated with inhibitors as described in the figure legends and text. KL (200 ng/ml) or PMA (100 nM) stimulation was for 15 min at 37°C prior to seeding on coverslips that had been coated with 1% poly-L-lysine for 1 h and washed under running distilled water for 1 h. Cells were allowed to settle for 15 min before being fixed in piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) buffer containing 2.5% glutaraldehyde for 30 min at room temperature. Scanning electron microscopy was used to visualize and photograph cells.

Measurement of F-Actin

BMMCs were starved from IL-3 and serum in RPMI 1640 containing 0.5% BSA for 16 h. In some cases, cells were treated with inhibitors as described in the text and figure legends. Stimulation with KL (200 ng/ml) or PMA (100 nM) was for 5 min at 37°C. Cells were then prepared according to the method of Condeelis and Hall (1991). Cells were suspended at 2×10^6/ml of fixation buffer [3.7% formaldehyde, 0.1% Triton-X 100, 20 mM KH_2PO_4, 10 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid), 5 mM EGTA, 2 mM $MgCl_2$, pH 6.2] for 15 min at room temperature. Pelleted cells (10^6 cells) were permeabilized and stained in 50 μl of PBS, 0.1% Triton X-100, and 5 μM fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) for 30 min at room temperature protected from light. The cells then were washed in 4 ml of PBS and cell pellets were suspended in 100 μl of PBS and 1% formaldehyde for FACS analysis. Relative fluorescence values were determined and normalized so that 100% represents unstimulated F-actin levels.

Proliferation Assay

BMMCs were starved of growth factors for 18 h before beginning the proliferation assay. Approximately 5×10^5 BMMCs/ml were grown in 1 ml of RPMI complete containing either 500 ng/ml rmKL or no growth factors at 37°C. Cell viability was determined by the trypan blue exclusion assay. For quantitation of DNA synthesis, 5×10^4 cells were seeded in 100 μl in duplicate in 96-well plates and stimulated with 500 ng/ml rmKL. After 16 h of incubation, 0.5 mCi of [^{3}H]thymidine (2 Ci/mmol) was added and incubation was continued for an additional 8 h. Cells were frozen and thawed and filtered through glass fiber filters, and filter-bound ^{3}H cpm were measured in a liquid scintillation counter.

RESULTS

Kit Up-Regulates BMMC Degranulation

First, the effect of Kit activation on the secretory function of BMMCs was examined. Degranulation was

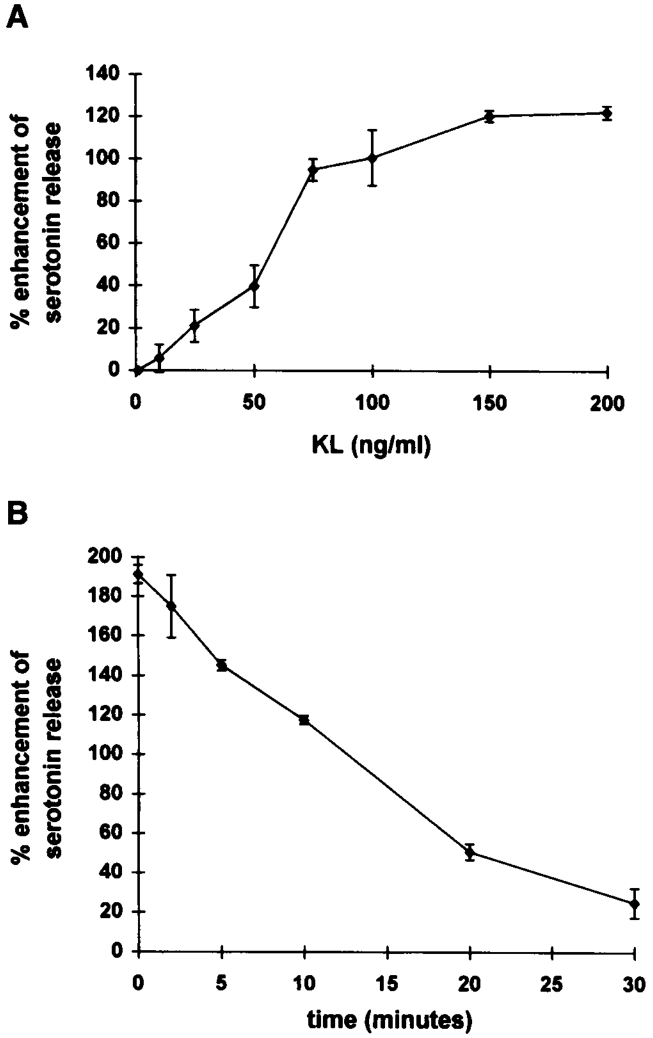


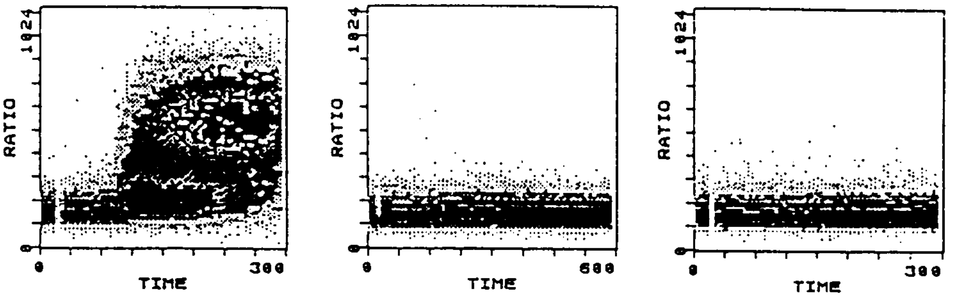
Figure 1. KL enhancement of IgE receptor-mediated degranulation. Wild-type BMMCs were incubated with various concentrations of KL for 2 min prior to FcεRI cross-linking (A) or 200 ng/ml KL for various times before FcεRI cross-linking (B). Degranulation was measured by determining release of [^{3}H]serotonin. Data are the means ± SD of triplicates from representative experiments and are expressed as percentage of enhancement of serotonin release triggered by FcεRI cross-linking alone.

assayed by measuring release of [^{3}H]serotonin (Coleman *et al.*, 1991). Cross-linking of IgE-bound FcεRI with anti-IgE antibody (10 ng/ml) triggered rapid degranulation in BMMCs, causing release of approximately 25% of the intracellular serotonin. Pretreatment of BMMCs with KL for 2 min potentiated FcεRI-mediated serotonin release in a dose-dependent manner (Figure 1A). Maximal secretory enhancement was achieved at a concentration of 200 ng/ml KL, and the EC$_{50}$ was approximately 50 ng/ml. To examine the kinetics of KL potentiation of secretion, BMMCs were pretreated for various time intervals with an optimal concentration of KL (200 ng/ml) before FcεRI receptor cross-linking. The induction of KL secretory enhancement was rapid with a maximal effect achieved by simultaneous addition of KL and cross-linking of the IgE receptor (Figure 1B). There was a linear decline of enhancement levels over a 30-min time course of KL pretreatment. The calcium ionophore ionomycin induces degranulation of mast cells (Bennett *et al.*, 1979). Similar to the potentiation of FcεRI-mediated secretion, KL potentiates secretion triggered by ionomycin (1 μM) (Figure 2A). IL-3 and IL-4, like KL, induce a mitogenic response in BMMCs (Tsuji *et al.*, 1990). However, in contrast to KL, neither IL-3 or IL-4 had any effect on degranulation when used alone or in combination with IgE receptor cross-linking or ionomycin (our unpublished results).

Calcium is an important effector of exocytosis. Cross-linking of the IgE receptor results in a calcium flux that is necessary for degranulation to occur (Lindau and Gomperts, 1991). To determine whether a calcium flux was associated with Kit secretory effects, the fluorescent dye Indo-1 was used to measure changes in intracellular calcium levels upon Kit activation. Although ionomycin (1 μM) induced a rapid increase of intracellular calcium levels, KL at concentrations as high as 1 μg/ml had no discernible effect on calcium levels in BMMCs (Figure 2).

A Role for PI 3-Kinase in the Kit Receptor-mediated Secretory Response in BMMCs

Kit signaling events and their role in distinct downstream responses were investigated. In particular the role of PI 3-kinase activation and events associated with Tyr-821 phosphorylation were examined by using wild-type and mutant Kit receptors with tyrosine to phenylalanine substitutions at positions 719 (Y719F) and 821 (Y821F). Wild-type and mutant c-kit cDNAs were expressed in BMMCs from W^{sh}/W^{sh} mice, which lack endogenous Kit expression as described previously (Serve *et al.*, 1995). The level of Kit receptor expression in the various transduced W^{sh}/W^{sh} BMMC cultures was comparable as determined by FACS analysis, and Kit expression in these BMMCs remained stable throughout their lifetime (at least 2 mo). W^{sh}/W^{sh} BMMCs degranulated normally in response to IgE receptor cross-linking and ionomycin. W^{sh}/W^{sh} BMMC cultures expressing Kit and Kit^{Y821F} receptors supported KL-induced enhancement of secretion, mediating an approximately twofold increase in degranulation upon either IgE receptor cross-linking (5 ng/ml) or ionomycin treatment (1 μM; Figure 3A). In contrast, upon stimulation with KL the Kit^{Y719F} receptor failed to potentiate both FcεRI and ionomycin-triggered serotonin release (Figure 3A). Therefore, loss of PI 3-kinase association with the Kit



10 μM ionomycin

250 ng/ml KL

1000 ng/ml KL

Figure 2. KL does not induce a Ca^{2+} flux in BMMCs. BMMCs loaded with Indo-1 were stimulated with 10 μM ionomycin, 250 ng/ml KL, or 1000 ng/ml KL, and the ratio of fluorescence at 470 nm/490 nm was determined by FACS.

receptor corresponds with a loss of Kit-mediated secretory enhancement.

To exclude the possibility that a protein other than PI 3-kinase binds at Tyr-719 and is responsible for the secretory effect of Kit and to further establish a role for Kit activated PI 3-kinase in degranulation, we examined the effects of PI 3-kinase inhibitors on the Kit-mediated secretory response. Wortmannin binds to the p110 subunit of PI 3-kinase and blocks enzymatic activity (Yano *et al.*, 1993). Although wortmannin blocked IgE-mediated degranulation, as reported previously (Yano *et al.*, 1993), the drug did not affect degranulation induced by ionomycin. Therefore, the effect of wortmannin on KL potentiation of ionomycin-triggered degranulation was investigated. At concentrations known to inhibit PI 3-kinase in vitro and in intact cells (Yano *et al.*, 1993), wortmannin inhibited KL potentiation of ionomycin-triggered secretion in a dose-response manner, with an IC_{50} of about 10^{-7} M (Figure 3B). Another specific inhibitor of PI 3-kinase, LY294002 (Vlahos *et al.*, 1994), had similar inhibitory effects. The loss of Kit enhancement of ionomycin-triggered secretion paralleled the reported inhibitory effects of LY294002 on PI 3-kinase activity in intact cells (Cheatham *et al.*, 1994), with an IC_{50} of about 5 μM (Figure 3B). Therefore, these inhibitor studies support the idea that PI 3-kinase binding and activation is

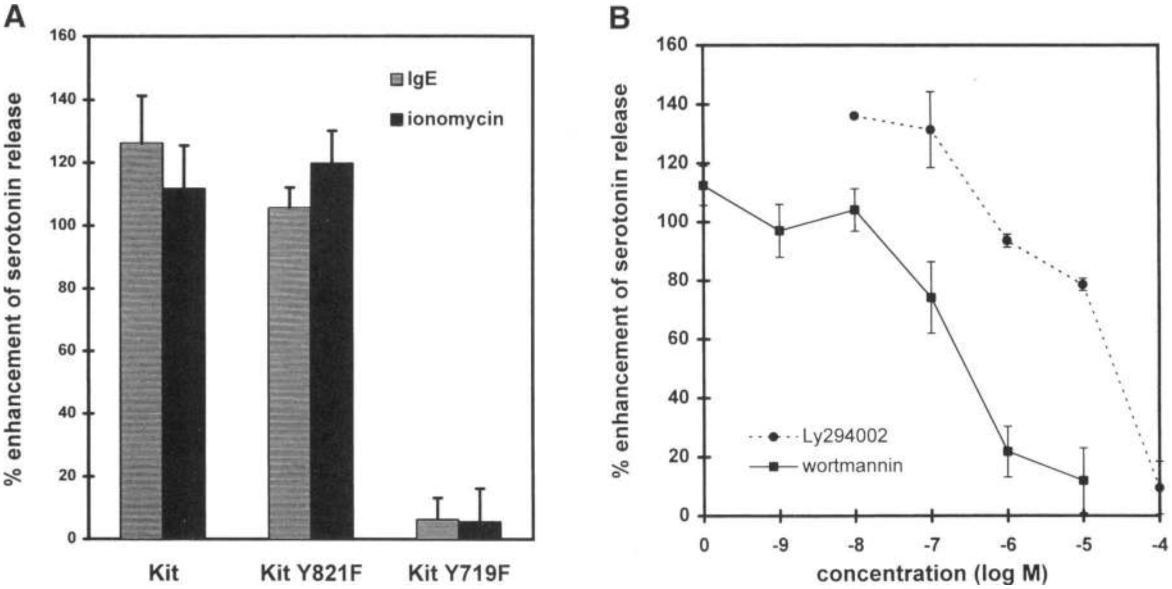


Figure 3. Kit^{Y719F} mutation and pharmacological inhibition of PI 3-kinase block enhancement of degranulation. (A) W^{sh}/W^{sh} BMMCs expressing normal and mutant receptors were incubated for 2 min with 200 ng/ml KL and degranulation was then triggered by either FcεRI cross-linking (shaded bars) or 1 μM ionomycin (solid bars). (B) W^{sh}/W^{sh} BMMCs expressing wild-type Kit receptors were treated with various concentrations of wortmannin or LY294002 for 15 min before KL pretreatment (200 ng/ml for 2 min) and stimulation of degranulation with 1 μM ionomycin. Data are the means ± SD of triplicates from representative experiments and are expressed as percentage of enhancement of serotonin release triggered by FcεRI cross-linking or ionomycin alone.

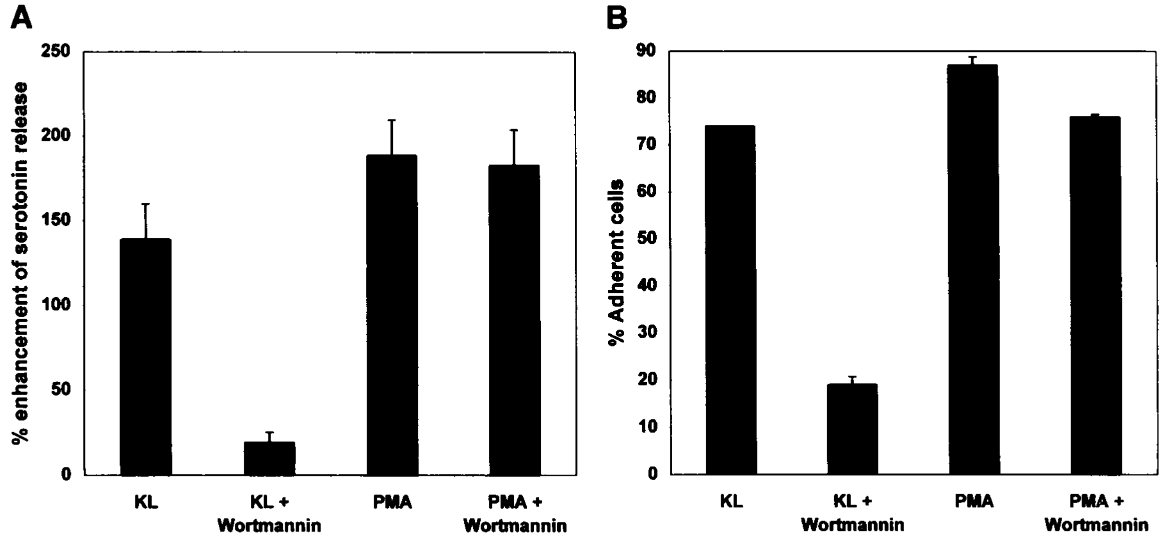


Figure 4. Wortmannin does not block PMA-induced secretory enhancement or adhesion of BMMCs to fibronectin. Wild-type BMMCs were either untreated or treated with 10^{-6} M wortmannin for 15 min prior to measurement of KL (200 ng/ml for 2 min) or PMA (50 nM for 5 min) enhancement of degranulation triggered by 1 μM ionomycin (A) or measurement of KL-induced (50 ng/ml) or PMA-induced (50 ng/ml) adhesion of BMMCs to fibronectin (B). Data are the means ± SD of duplicates from representative experiments and are expressed in A as percentage of enhancement of serotonin release triggered by ionomycin alone or in B as percentage of treated cells adhering to a fibronectin matrix.

specifically required for Kit-mediated secretory enhancement in BMMCs.

PMA-stimulated Secretion and Adhesion in BMMCs Is Independent of PI 3-Kinase

PMA elicits secretory (Chakravarty *et al.*, 1990) and adhesive (Dastych and Metcalfe, 1994) responses in BMMCs that are similar to those triggered by KL, suggesting a potential relationship between PI 3-kinase and PKC in these processes. This led us to determine whether PI 3-kinase activity was required for PMA-induced secretory enhancement or adhesion of BMMCs to fibronectin. In parallel experiments, wortmannin treatment (10^{-6} M) for 15 min, although blocking KL-mediated secretory enhancement and adhesion, did not interfere with PMA-induced potentiation of ionomycin-triggered degranulation (Figure 4A) or adhesion to fibronectin (Figure 4B). Therefore, PI 3-kinase is not required for secretory enhancement or adhesion stimulated directly through PKC.

A PKC Isoform Is Required for Kit-mediated Secretion and Adhesion in BMMCs

PKC activity had been linked with the cellular processes of degranulation (Chakravarty *et al.*, 1990; Ozawa *et al.*, 1993) and adhesion (Dastych and Metcalfe, 1994; Kinashi and Springer, 1994). Although PKC has been shown to phosphorylate and downregulate the Kit receptor (Blume-Jensen *et al.*, 1994), it is required for Kit-mediated chemotaxis (Blume-Jensen *et al.*, 1993). We used the specific PKC inhibitor calphostin C (Kobayashi *et al.*, 1989) to determine whether PKC activity is required for Kit-mediated secretory enhancement and adhesion. Studies on calphostin C inhibition of Kit-mediated secretory enhancement were restricted to potentiation of ionomycin-induced degranulation, since calphostin C blocked IgE receptor-mediated degranulation but not ionomycin-induced degranulation. In a dose-dependent manner, calphostin C treatment for 1 h inhibited both Kit-mediated enhancement of ionomycin-triggered degranulation and adhesion to fibronectin (Figure 5), blocking these responses fully at 0.4 and 0.5 μM, respectively. Positive controls showed that calphostin C blocks both PMA-induced secretory enhancement and adhesion of BMMCs to fibronectin.

A Role for PI 3-Kinase in Kit-mediated Membrane Ruffling

Both KL and PMA induce adhesion to a fibronectin matrix; however, the morphologies of the attached cells obtained by treatment with these two agents are distinct. PMA-treated cells remain rounded on fibronectin, and KL-treated adherent cells assume an angular and more spread out morphology. This observation led us to investigate KL-induced membrane ruffling in BMMCs. Membrane ruffling is observed in many cell types in response to extracellular factors (Stossel, 1993). KL induces circular actin reorganization in Kit-expressing porcine endothelial aortic cells

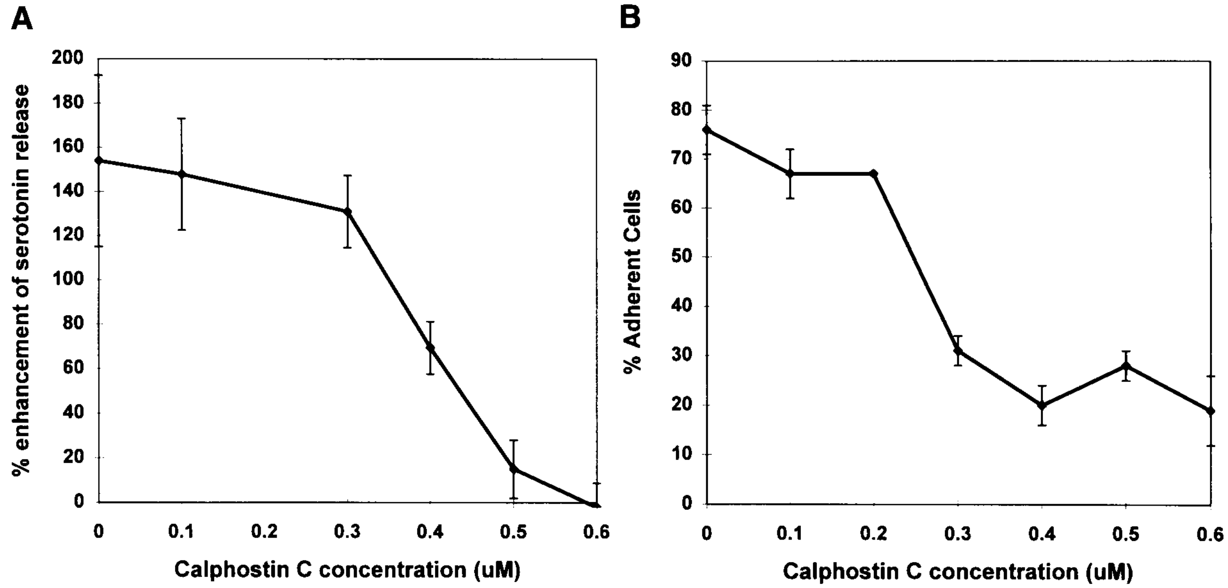


Figure 5. Calphostin C inhibition of Kit-mediated secretory enhancement and adhesion to fibronectin. Wild-type BMMCs were incubated with various concentrations of calphostin C for 2 h prior to measurement of KL (200 ng/ml for 2 min) enhancement of degranulation triggered by 1 μM ionomycin (A) or measurement of KL-induced (50 ng/ml) adherence of BMMCs to fibronectin (B). Data are the means ± SD of triplicates from representative experiments and are expressed in A as percentage of enhancement of serotonin release triggered by ionomycin alone or in B as percentage of treated cells adhering to a fibronectin matrix.

(Blume-Jensen *et al.*, 1991) while acting as a chemotactic agent. Kit mediates directed migration in BMMCs (Meininger *et al.*, 1992), but KL-induced cytoskeletal rearrangements have not been studied in this cell type. With scanning electron microscopy, we examined membrane morphology of BMMCs upon Kit activation. Unstimulated BMMCs appear to be round and compact (Figure 6). KL (200 ng/ml) treatment of these cells for 30 min results in a dramatic structural reorganization, as membrane folds are extended radially and the cell appears to spread (Figure 6). PMA has been reported to have cell-type–specific affects on cytoskeletal rearrangements. For example, in neutrophils, PMA induces membrane ruffling (Downey *et al.*, 1992), but in undifferentiated HL-60 cells, PMA induces a loss of membrane ruffles (Sham *et al.*, 1991). PMA (100 nM) treatment of BMMCs over 30 min did not induce membrane ruffling (Figure 6) but, rather, reduced background ruffling. Treatment of BMMCs for 20 min with 2 μM cytochalasin D, an inhibitor of actin polymerization (Cooper, 1987), blocked Kit-mediated membrane ruffling, demonstrating a requirement for actin polymerization in this response (Figure 6).

Using scanning electron microscopy, we compared the ability of W^{sh}/W^{sh} BMMC cultures expressing either Kit or Kit^{Y719F} receptors to support KL induced membrane ruffling. Photographs of duplicate fields containing >100 cells were used to determine the percentage of cells ruffling in unstimulated and KL-stimulated (200 ng/ml for 30 min) cultures. The percentage of cells ruffling in W^{sh}/W^{sh} BMMC cultures expressing wild-type Kit increased from 17% in unstimulated cells to 51% upon KL treatment (Figure 7A). The Kit^{Y719F}-expressing culture was defective in this response, since KL induced an increase in ruffling from 15% in unstimulated cultures to only 34% (Figure 7A). We next examined the affects of pharmacological inhibition of PI 3-kinase and PKC on Kit-mediated membrane ruffling in BMMCs. Treatment of wild-type BMMCs for 15 min with either wortmannin (10^{-6} M) or LY294002 (10^{-4} M) reduced the percentage of cells ruffling in response to KL from 70% in untreated cultures to less than 30% (Figure 7B). Although PMA does not induce observable membrane reorganization, treatment of BMMCs with the specific PKC inhibitor calphostin C (0.5 μM) for 1 h blocked KL-induced membrane ruffling (Figure 7B). Additionally, inhibition of actin polymerization with cytochalasin D (2 μM) treatment of BMMCs for 20 min completely blocked KL-induced membrane ruffling (Figure 7B).

PI 3-Kinase Is Involved in Kit-mediated F-Actin Formation in BMMCs

The formation of membrane ruffles is accompanied by actin polymerization at the plasma membrane (Stossel, 1993). After observing that KL-induced ruffling was dependent on actin polymerization, we examined Kit-mediated F-actin assembly in BMMCs and the effect of the Y719F mutation on this response. Five minutes after KL (200 ng/ml) addition to W^{sh}/W^{sh} BMMC cultures expressing either Kit or Kit^{Y719F} receptors, relative levels of cellular F-actin were determined by FACS analysis of FITC-labeled phalloidin-stained cells. An increase in relative F-actin of 20% over unstimulated levels was supported by the wild-type Kit receptor. The Kit^{Y719F} receptor was partially defective in this response, as relative F-actin content increased by only 10% over unstimulated levels (Figure 8A). Next, with BMMCs derived from wild-type mice, we examined KL-induced F-actin formation in the presence of pharmacological inhibitors of PI 3-kinase. Although wild-type BMMCs responded to a 5-min KL (200 ng/ml) stimulation with an increase in relative F-actin content of about 40% over unstimulated cells, treatment with the PI 3-kinase inhibitors wortmannin (10^{-6} M) and LY294002 (10^{-4} M) for 15 min strongly inhibited the increase in F-actin content upon KL stimulation (Figure 8B). PI 3-kinase inhibitors also slightly lowered the background F-actin content in unstimulated cells. PMA (100 nM) did not induce increased F-actin levels. Pretreatment of BMMCs with the PKC inhibitor calphostin C (0.6 μM) resulted in a reduction of the cellular F-actin content and this effect could not be overcome by the addition of KL. Cytochalasin D treatment blocked the KL-induced increase in F-actin content (Figure 8B), demonstrating the specificity of the assay.

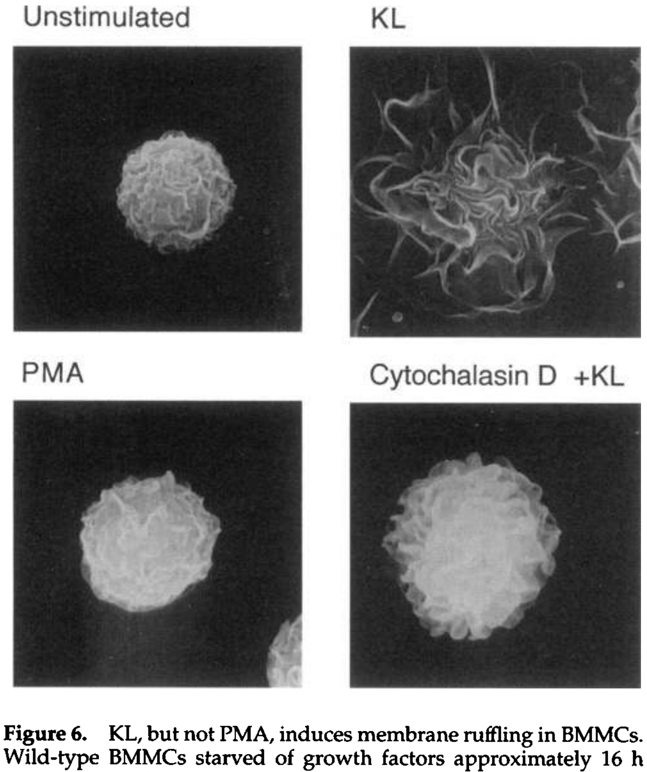


Figure 6. KL, but not PMA, induces membrane ruffling in BMMCs. Wild-type BMMCs starved of growth factors approximately 16 h were untreated (A), treated with KL (200 ng/ml) for 15 min (B), treated with PMA (100 nM) for 15 min (C), or incubated with 2 μM cytochalasin D for 20 min before KL (200 ng/ml) treatment for 15 min (D). Cells were processed for scanning electron microscopy (MATERIALS AND METHODS). Magnification is 6000×.

Secretory Enhancement and Adhesion to Fibronectin Are Independent of Actin Polymerization and Membrane Ruffling

Our results demonstrate that several downstream responses of Kit activation, including secretory enhancement, adhesion to fibronectin, and cytoskeletal rearrangements, share requirements for PI 3-kinase and PKC. A link between cytoskeletal reorganization and secretion has been suggested by experiments showing inhibition of mast cell degranulation by dominant negative mutants of rac and rho, two small G proteins involved in actin rearrangements (Price *et al.*, 1995). Additionally, in the RBL-2H3 mast cell line, actin plaque assembly has been linked to increased cell substrate adhesion (Pfeiffer and Oliver, 1994). Thus, it is possible that KL-induced cytoskeletal rearrangements are functionally coupled to the apparently distinct responses of secretory enhancement or adhesion to fibronectin. To address this possibility, we blocked actin polymerization in BMMCs and examined the ability of these cells to support the Kit downstream responses of secretory enhancement and adhesion to fibronectin. Although cytochalsin D (2 μM) treatment of a wild-type BMMC culture for 20 min blocked KL-induced membrane ruffling and increased F-actin, these cells exhibited normal levels of increased degranulation and adhesion to fibronectin in response to KL (Figure 9). BMMCs adhering to fibronectin in the presence of cytochalasin D lacked the typical spreading normally observed upon KL stimulation. Thus, Kit-mediated cytoskeletal rearrangements that are dependent on actin polymerization can be uncoupled from the downstream responses of secretory enhancement and adhesion to fibronectin.

$pp70^{S6kinase}$ Is Not Required for Kit-mediated Secretion, Adhesion, Membrane Ruffling, and F-Actin Assembly in BMMCs

$pp70^{S6kinase}$, which has been implicated in the mitogenic response, may be activated by PI 3-kinase in signaling arising from both the PDGF and IL-2 receptors (Chung *et al.*, 1994; Monfar *et al.*, 1995). Since PI 3-kinase appeared to mediate the Kit signals influencing secretion and adhesion, we wanted to establish whether $pp70^{S6kinase}$ played a role in the secretory, adhesive, or cytoskeletal responses mediated by Kit in BMMCs. The T cell immunosuppressant rapamycin inhibits activation of $pp70^{S6kinase}$. It has been previously shown that rapamycin inhibits Kit-mediated activation of $pp70^{S6kinase}$ and blocks the mitogenic signal

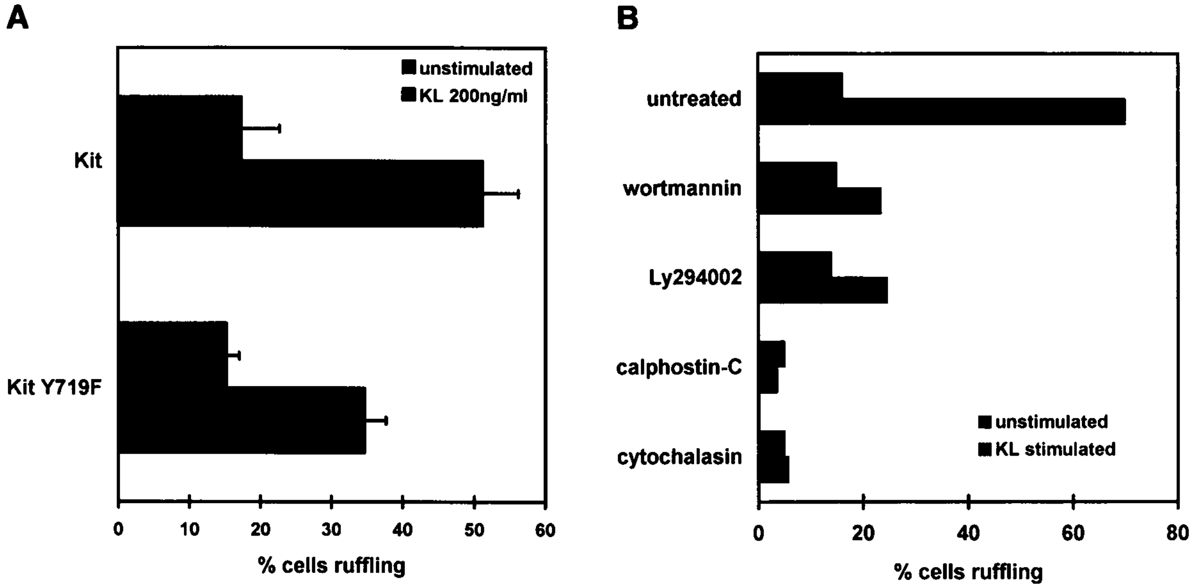


Figure 7. Kit^{Y719F} mutation, wortmannin, and LY294002 inhibit KL-induced membrane ruffling. (A) W^{sh}/W^{sh} BMMCs expressing normal Kit or mutant Kit^{Y719F} receptors. (B) Wild-type BMMCs untreated or treated with wortmannin (1 μM) for 15 min, LY294002 (100 μM) for 15 min, calphostin C (0.6 μM) for 2 h, or cytochalasin D (2 μM) for 20 min were unstimulated or stimulated for 15 min with 200 ng/ml KL before processing for scanning electron microscopy (MATERIALS AND METHODS). Percentage of cells ruffling was determined by counting >100 cells in photographs of representative fields at a magnification of 460×.

in BMMCs (Tsai *et al.*, 1993). At concentrations that fully inhibit pp70^{S6kinase} activation, rapamycin treatment blocked Kit-mediated proliferation of BMMCs but had no effect on Kit-mediated secretory enhancement, adhesion to fibronectin, membrane ruffling, or F-actin assembly (Table 1). In these experiments, rapamycin effectively blocked Kit-mediated activation of pp70^{S6kinase}, because in the presence of the drug, Western blotting failed to show a molecular weight shift due to phosphorylation of pp70^{S6kinase}. Furthermore, cycloheximide inhibition of protein synthesis at concentrations as high as 100 μg/ml had no effect on Kit-mediated secretory enhancement or adhesion, providing additional evidence that posttranslational events are controlling these responses.

DISCUSSION

PI 3-Kinase Is a Critical Second Messenger in Kit Signaling in BMMCs

The Kit receptor tyrosine kinase elicits pleiotropic responses in distinct cell types during embryonic development and in the adult animal. The ability of Kit to produce distinct cellular responses in different cell types may depend in part on the available cellular circuitry. Knowledge gained from studies in a particular cell model should be valuable in understanding Kit-mediated responses in other more elusive cell populations. In the mast cell model, the Kit receptor plays a role in mediating proliferation, survival, adhesion, chemotaxis, and degranulation. We had previously established a role for PI 3-kinase in Kit-mediated cell adhesion to a fibronectin matrix (Serve *et al.*, 1995). Herein, we have defined a role for PI 3-kinase in Kit-mediated secretory enhancement, in membrane ruffling and actin polymerization in BMMCs, by using mast cells expressing the Kit^{Y719F} receptor that lacks the PI 3-kinase-binding site. The ability of the PI 3-kinase inhibitors, wortmannin and LY294002, to interfere with Kit-mediated secretory enhancement, adhesion, membrane ruffling, and F-actin assembly further establishes a specific requirement for PI 3-kinase in these signals. In contrast, Kit^{Y821F} fails to transduce a mitogenic signal but fully supports adhesion of BMMCs to fibronectin (Serve *et al.*, 1995) and mediates secretory enhancement.

Role for PI 3-Kinase in Secretory Processes

PI 3-kinase function has been linked to intracellular vesicle trafficking and secretion. The yeast PI 3-kinase homologue Vps34p is essential for Golgi-vacuole trafficking (Stack *et al.*, 1993; Stack and Emr, 1994). In mammalian cells PI 3-kinase mediates Golgi–lysosome trafficking, PDGF-dependent lysosomal degradation of the activated PDGF receptor, the translocation to the cell surface of vesicles containing glucose transporter upon insulin stimulation (Okada *et al.*, 1994), Fc receptor-stimulated granule release from the basophilic leukemia cell

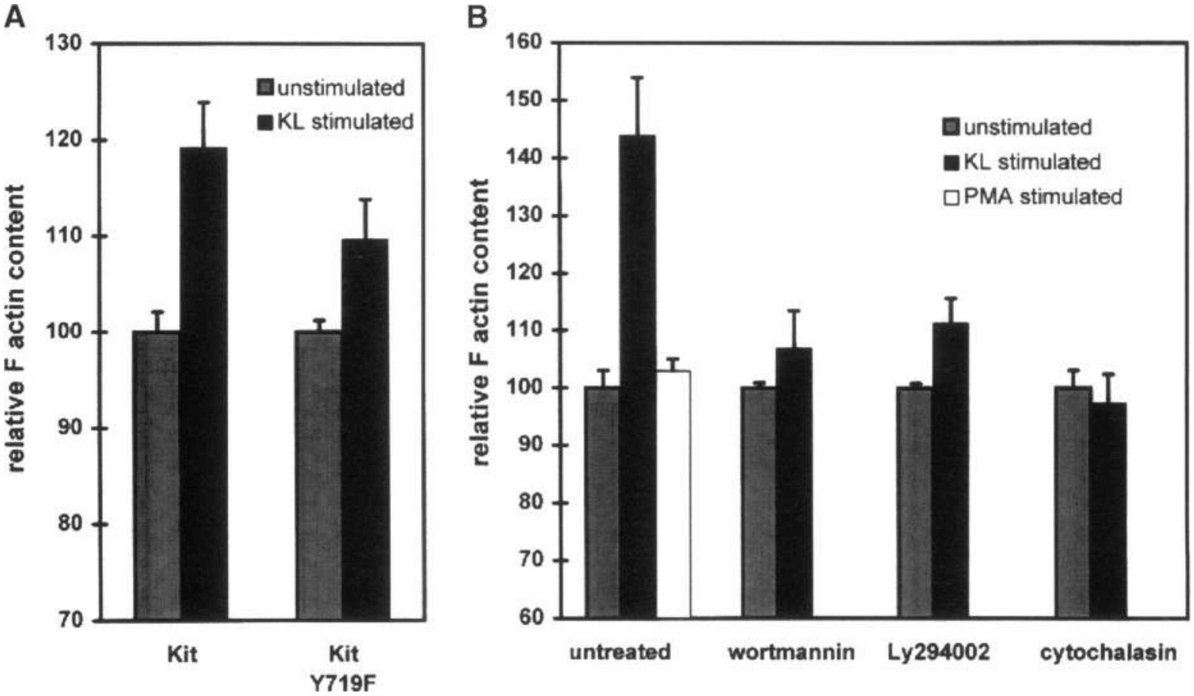


Figure 8. KL induces F-actin polymerization and this response is inhibited by the Kit^{Y719F} mutation, wortmannin, or LY294002 treatment. (A) W^{sh}/W^{sh} BMMCs expressing normal Kit or the Kit^{Y719F} mutant receptor. (B) Wild-type BMMCs untreated or treated with wortmannin (1 μM) for 15 min, LY294002 (100 μM) for 15 min, or cytochalasin D (2 μM) for 20 min were unstimulated or stimulated for 5 min with 200 ng/ml KL and processed for determination of F-actin content (MATERIALS AND METHODS). Data are the means ± SD of duplicates in representative experiments and are expressed as percentage of increase of KL-stimulated F-actin levels over unstimulated.

line (RBL-2H3; Yano *et al.*, 1993), natural killer cells (Bonnema *et al.*, 1994), and BMMCs (our unpublished results). Furthermore, we demonstrate a role for PI 3-kinase in Kit-mediated degranulation in mast cells. Although many of the known PI 3-kinase–mediated functions were investigated by using PI 3-kinase inhibitors, some caution is indicated in the interpretation of data obtained with these inhibitors since it is known that Wortmannin inhibits other enzymes including phosphatidylinosiol-4-kinase (Nakanishi *et al.*, 1995), TOR (Brunn *et al.*, 1996), and DNA PK (Hartley *et al.*, 1995). Mammalian TOR (mTOR) is homologous with the catalytic subunit of PI 3-kinase. Rapamycin acts through inhibition of TOR and this in turn blocks downstream activation of p70^{S6kinase}. Wortmannin and LY294002 have recently been shown to inhibit mTOR (Brunn *et al.*, 1996). Rapamycin selectively blocks KL-mediated proliferation but does not affect the secretory and the adhesive response. Therefore, the inhibition of the secretory and adhesive responses by wortmannin and LY294002 is not a result of the inhibition of mTOR. Importantly, our evidence for a role of PI 3-kinase in Kit-mediated degranulation in BMMCs involved the use of Kit receptor mutants and the use of the inhibitors wortmannin and LY294002.

Although the Kit^{Y719F} mutation abolishes secretory enhancement completely, it has partial effects on adhesion (Serve *et al.*, 1995), cytoskeletal rearrangements, and ruffling. In contrast, pharmacological inhibition of PI 3-kinase abolishes Kit-mediated adhesion and cytoskeletal rearrangements. Interestingly, the level of KL required for half-maximal adhesion of BMMCs to fibronectin is tenfold lower than that required for half-maximal secretory enhancement. Therefore, while Kit^{Y719F} blocks proximal activation of PI 3-kinase, alternative signaling mechanisms mediated by Kit may activate lower levels of PI 3-kinase sufficient for partial adhesion and cytoskeletal rearrangements. Alternative PI 3-kinase activation may occur through src family kinases, because they associate with Kit (Blume-Jensen *et al.*, 1994) and have been linked to activation of PI 3-kinase (Yamanashi *et al.*, 1992; Pleiman *et al.*, 1994). In agreement with this prediction, a Kit receptor mutant in both the PI 3-kinase and the presumptive src-kinase-binding sites abolishes adhesion and F-actin polymerization completely (our unpublished results).

A Role for PKC in Kit/PI 3-Kinase-mediated Responses in BMMCs

Calphostin C inhibition of KL-induced adhesion, secretory enhancement, and membrane ruffling suggests a requirement for PKC in these responses. PKC was previously shown to be required for Kit-mediated chemotaxis and circular actin reorganization in porcine aortic endothelial cells (Blume-Jensen *et al.*, 1993). PKC, in addition to being a downstream mediator of Kit responses, acts in a negative feedback loop that down-regulates Kit receptor activity (Blume-Jensen *et al.*, 1994). Therefore, PKC has a dual role as both a positive and negative regulator of Kit function. Adhesion and secretory enhancement stimulated by PMA are independent of PI 3-kinase, because wortmannin did not inhibit these responses. This is consistent with a model in which PKC functions in a distal step of Kit-mediated secretory enhancement and adhesion. Although it is well established that PKC can be activated by diacylglycerol, it has been demonstrated recently that products of PI 3-kinase, including phosphatidylinositol (PtdIns)-3,4-P_2, and PtdIns-3,4,5-P_3, may activate some Ca^{2+}-independent isoforms of PKC (e.g., PKCδ, PKCϵ, and PKCζ; Nakanishi *et al.*, 1993; Toker *et al.*, 1994). Our observation that KL does not induce a rise in intracellular Ca^{2+} levels in BMMCs is consistent with the idea that a Ca^{2+}-independent isoform of PKC is involved in these Kit-mediated processes. Establishing whether Kit and Kit mutant receptors can activate PKC isoforms should better establish the role of PKC in Kit signaling.

Recently, the PI 3-kinase product PtdIns-3,4,5-P_3 was shown to specifically bind the pleckstrin homology domain of Bruton's tyrosine kinase (Btk), suggesting that Btk is a downstream effector of PI 3-kinase (Salim *et al.*, 1996). Btk is expressed in mast cells, but BMMCs derived from *xid* mice lacking functional Btk were not impaired in Kit-mediated secretory enhancement or adhesion (our unpublished results). Therefore, although Btk may be a target of PI 3-kinase, it is not required for the Kit-mediated adhesion and secretory responses. Also, the serine kinase Akt had been placed downstream of PI 3-kinase (Burgering and Coffer, 1995); however, the Akt pleckstrin homology domain does not bind PtdIns-3,4,5-P_3 (Salim *et al.*, 1996), and it is not known whether Akt lies in the pathway mediating the secretory and adhesion responses.

In BMMCs a calcium flux produced either by IgE receptor cross-linking or by calcium ionophore treatment is required to trigger degranulation. Therefore, our failure to detect a calcium flux in BMMCs upon KL stimulation may explain the inability of KL to directly mediate serotonin release in BMMCs. Therefore, at least two signals are required to trigger degranulation in BMMCs, namely, a calcium flux and activation of PI 3-kinase or a PKC isoform.

Possible Role for PI 3-Kinase in Kit-mediated Cytoskeletal Rearrangements in BMMCs

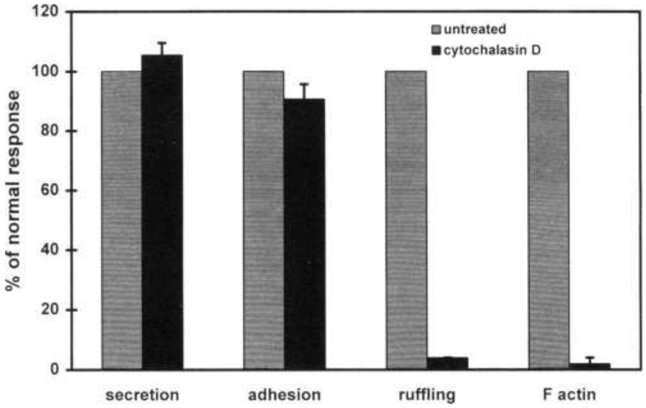


Figure 9. Kit-mediated secretory enhancement and adhesion to fibronectin is independent of membrane ruffling and F-actin assembly. Wild-type BMMCs starved of growth factors for approximately 16 h were untreated or treated with cytochalasin D (2 μM) for 20 min. Responses of membrane ruffling, F-actin assembly, secretory enhancement, and adhesion (MATERIALS AND METHODS) were measured in response to optimal concentrations of KL. Percentage of response of cytochalasin D-treated cells was compared with the response of untreated cells, normalized to 100%.

A role for PI 3-kinase in mediating cytoskeletal rearrangements and membrane ruffling has been proposed previously. In adherent cell types, PDGF, insulin, and KL promote the formation of circular actin ruffling (Blume-Jensen *et al.*, 1991; Kotani *et al.*, 1994; Wennstrom *et al.*, 1994). PDGFβ receptor-mediated circular actin ruffling is blocked by either pharmacological inhibition of PI 3-kinase or by a mutation that abolishes PI 3-kinase association with the receptor (Wennstrom *et al.*, 1994). The morphology of membrane ruffling in KL-treated nonadherent BMMCs is distinct from that in adherent cells: spreading of BMMCs appears to be more pronounced and actin staining is observed in edges of ruffles but not in rings (our unpublished results).

The assembly of filamentous actin is an essential process underlying the formation of filopodia, lamel-

Table 1. Effect of rapamycin on Kit-mediated responses in BMMC

	Untreated	Rapamycin
Proliferation	+	–[a]
Secretion	+	+
Adhesion	+	+
Membrane ruffling	+	+
F-actin assembly	+	+

BMMCs were treated for 1 h with 20 mM rapamycin before assaying KL-induced responses of proliferation ([^{3}H]thymidine incorporation), secretion (enhancement of ionomycin-induced degranulation), adhesion (adhesion to fibronectin), membrane ruffling (electron microscopy), and F-actin assembly (FITC-coupled phalloidin staining) as described in MATERIALS AND METHODS.

[a] 5% of normal [^{3}H]thymidine incorporation 16 h after KL stimulation.

lipodia, and actin stress fibers. A role for PI 3-kinase in agonist-induced F-actin formation, however, has been less clear. Although wortmannin treatment reduces basal F-actin levels in resting neutrophils and RBL 2H3 cells, suggesting a link between PI 3-kinase and F-actin assembly, wortmannin did not affect agonist-induced F-actin assembly in these cell types upon stimulation with fMLP or antigen, respectively (Arcaro and Wymann, 1993; Barker *et al.*, 1995). Similarly, actin assembly was not inhibited by wortmannin in thrombin-stimulated platelets (Kovacsovics *et al.*, 1995). In contrast, KL-induced F-actin assembly in BMMCs is affected both by wortmannin treatment and by eliminating the PI 3-kinase-binding site on the Kit receptor. Also, F-actin assembly mediated by CD2 was recently shown to be inhibited by wortmannin (Shimizu *et al.*, 1995). Therefore, PI 3-kinase activation is not an obligatory step in mediating F-actin assembly and other mechanisms may directly activate components downstream of PI 3-kinase in the pathway leading to F-actin assembly.

The ras-related small G proteins CDC42, rac, and rho have been implicated in growth factor-induced formation of filopodia, lamellipodia, and stress fibers, respectively. It had been suggested that PI 3-kinase may act upstream of rac in growth factor-induced membrane ruffling, because wortmannin inhibits PDGF-stimulated ruffling, but not ruffling induced by an activated mutant form of rac (Ridley and Hall, 1992). Given the involvement of PI 3-kinase in Kit-mediated membrane ruffling in BMMCs, it is possible that rac is downstream of receptor-proximal PI 3-kinase activation in this signaling cascade.

There Is No Role for Cytoskeletal Rearrangements in the Degranulation and Adhesion Responses in BMMCs

Although actin rearrangements have been suggested to play a role in degranulation and cell adhesion (Pfeiffer and Oliver, 1994; Price *et al.*, 1995), we found no connection between Kit-mediated cytoskeletal rearrangements and the distinct responses of secretory enhancement or adhesion to fibronectin. Treatment of BMMCs with cytochalasin D to block actin polymerization was shown to eliminate KL-induced membrane ruffling and F-actin assembly while maintaining both enhanced degranulation and adhesion to fibronectin. The demonstration that PMA fails to induce membrane ruffling or actin polymerization but does enhance degranulation and induce adhesion to fibronectin is consistent with the idea that the secretory and adhesive responses can be triggered independently of actin rearrangements. KL-induced adhesion to fibronectin is facilitated by the induction of a high-affinity state of the $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins on BMMCs. The finding that cytochalasin D does not inhibit this adhesion would suggest that it does not require the formation of new adhesion plaques. Similarly, it has been recently observed that fibronectin binding to activated $\alpha_{IIb}\beta_3$ integrin was unaffected by cytochalasin D treatment in Chinese hamster ovary cells (Wu *et al.*, 1995), and this is in agreement with our observation of uncoupling of actin-polymerization–dependent processes and fibronectin binding to an integrin.

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