The Phosphoinositide 3-Kinase-Dependent Activation of Btk Is Required for Optimal Eicosanoid Production and Generation of Reactive Oxygen Species in Antigen-Stimulated Mast Cells

Hye Sun Kuehn, Emily J. Swindle, Mi-Sun Kim, Michael A. Beaven, Dean D. Metcalfe and Alasdair M. Gilfillan

*J Immunol* 2008; 181:7706-7712; doi: 10.4049/jimmunol.181.11.7706
http://www.jimmunol.org/content/181/11/7706

---

**References**  
This article cites 31 articles, 12 of which you can access for free at: http://www.jimmunol.org/content/181/11/7706.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Activated mast cells are a major source of the eicosanoids PGD$_2$ and leukotriene C$_4$ (LTC$_4$), which contribute to allergic responses. These eicosanoids are produced following the ERK1/2-dependent activation of cytosolic phospholipase A$_2$, thus liberating arachidonic acid, which is subsequently metabolized by the actions of 5-lipoxygenase and cyclooxygenase to form LTC$_4$ and PGD$_2$, respectively. These pathways also generate reactive oxygen species (ROS), which have been proposed to contribute to Fc$_\text{eRI}$-mediated signaling in mast cells. In this study, we demonstrate that, in addition to ERK1/2-dependent pathways, ERK1/2-independent pathways also regulate Fc$_\text{eRI}$-mediated eicosanoid and ROS production in mast cells. A role for the Tec kinase Btk in the ERK1/2-independent regulatory pathway was revealed by the significantly attenuated Fc$_\text{eRI}$-dependent PGD$_2$, LTC$_4$, and ROS production in bone marrow-derived mast cells of Btk$^{-/-}$ mice. The Fc$_\text{eRI}$-dependent activation of Btk and eicosanoid and ROS generation in bone marrow-derived mast cells and human mast cells were similarly blocked by the PI3K inhibitors, Wortmannin and LY294002, indicating that Btk-regulated eicosanoid and ROS production occurs downstream of PI3K. In contrast to ERK1/2, the PI3K/Btk pathway does not regulate cytosolic phospholipase A$_2$ phosphorylation but rather appears to regulate the generation of ROS, LTC$_4$, and PGD$_2$ by contributing to the necessary Ca$^{2+}$ signal for the production of these molecules. These data demonstrate that strategies to decrease mast cell production of ROS and eicosanoids would have to target both ERK1/2- and PI3K/Btk-dependent pathways. The Journal of Immunology, 2008, 181: 7706–7712.

Mast cell-derived mediators play a central role in the initiation of the inflammatory reactions associated with atopic asthma and other allergic disorders (1, 2). These mediators, which are released following Ag-dependent aggregation of IgE-occupied high affinity IgE receptors (Fc$_\text{eRI}$) on the mast cell surface (1, 3, 4), are broadly grouped into three main categories: granule-associated mediators, chemokines and cytokines, and eicosanoids. Much is known regarding the signaling events leading to the release of granule-associated mediators and, to a certain extent, cytokine production (reviewed in Refs. 3, 5). However, the signaling events mediating eicosanoid generation are less well defined.

The eicosanoids generated in activated mast cells are primarily represented by leukotriene C$_4$ (LTC$_4$) and PGD$_2$ (1, 3, 6–8). Multiple processes are required for the generation of these mediators; however, the major initiating step is the liberation of arachidonic acid from membrane lipids, primarily, 1-acetyl-2-arachidoyl-phosphatidylcho- line, following hydrolysis catalyzed by cytosolic phospholipase A$_2$ (cPLA$_2$) (9, 10). The liberated arachidonic acid is subsequently metabolized to form LTC$_4$ and PGD$_2$ by the actions of 5-lipoxygenase (5-LO) and LTC$_4$ synthase (11, 12), and cyclooxygenase (COX), respectively (8, 13). Reactive oxygen species (ROS) including hydroperoxides, hydrogen peroxide, and superoxide are formed during the generation of both LTC$_4$ and PGD$_2$ (14, 15). ROS have been proposed to regulate mast cell responses; however, these conclusions remain controversial. In this respect, although it has been suggested that ROS are involved in the signals leading to degranulation and cytokine secretion in mast cells (16, 17), other studies have concluded that Fc$_\text{eRI}$-dependent degranulation and cytokine production is independent of ROS production (14).

The ability of cPLA$_2$ to generate free arachidonic acid requires cPLA$_2$ to be phosphorylated, thus activated, and for it to be translocated to the membrane, allowing access to its phospholipid substrate(s) (9, 11, 18). The phosphorylation of cPLA$_2$ appears to be mediated by the MAPK ERK1/2, whereas its translocation is dependent on Ca$^{2+}$ (19–22). ROS production is also a Ca$^{2+}$-dependent process (17), but the upstream events required for ROS production and cPLA$_2$ activation have not been fully delineated.

In human mast cells (HuMCs), we have previously demonstrated that both PI3K-dependent and -independent pathways contribute to the increase in cytosolic Ca$^{2+}$ concentrations required for mast cell degranulation (23). The PI3K-dependent pathway appears to be mediated through the Tec kinase Btk. We (24) and others (25, 26) have shown that Btk enhances Fc$_\text{eRI}$-mediated PLC$_\gamma_1$ activation in a PI3K-dependent manner. Therefore, it is possible that Btk may similarly contribute to the regulation of the Ca$^{2+}$ signal required for eicosanoid generation and ROS production in activated mast cells. In this study, we show that both ERK1/
2-dependent and -independent pathways regulate FcεRI-mediated LTC₄ and PGD₂ generation and ROS production. The generation of these products was substantially reduced in parallel with the Ca²⁺ signal in Btk-deficient mast cells and in wild-type (WT) mast cells treated with PI3K inhibitors, providing the first evidence that activation of a PI3K-Btk-PLC pathway is required for the Ca²⁺-dependent production of eicosanoids and ROS in FcεRI-mediated activated cells. The recognition of the complementary signaling pathways required for FcεRI-mediated eicosanoid and ROS generation implies that therapeutic approaches to block the generation of these products require a coordinated strategy targeting these pathways.

Materials and Methods

Cell isolation and mast cell culture

The Btk knockout, kindly provided by Dr. Anne B. Satterthwaite (University of Texas Southwestern Medical Center, Dallas, TX), and the WT mice used in this study have been described (24). Mice were backcrossed with C57BL/6J (The Jackson Laboratory) over six generations. The WT mice used in this study have been described (24). Mice were backcrossed with C57BL/6J (The Jackson Laboratory) over six generations. The WT mice of these mice was confirmed by RT-PCR of tail biopsies (data not shown). Mouse bone marrow-derived mast cells (BMMCs) were obtained by flushing bone marrow progenitors from the femurs of the mice then cultured the cells for 4 – 6 wk in RPMI 1640 medium containing IL-3 (30 ng/ml) (Peprotech) as described (23, 28). Experiments were conducted on these cells 7–10 wk after the initiation of culture, at which point, the population was greater than 99% mast cells as assessed by trypan blue staining.

Cell activation

HuMCs or BMMCs were sensitized overnight with an optimal concentration (100 ng/ml) of biotinylated human IgE or mouse SPE-7 (IgE anti-DNP) (Sigma-Aldrich), respectively, in cytokine-free medium. The following day, the cells were washed with HEPES buffer (10 mM HEPES [pH 7.4], 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na₂HPO₄, 7H₂O, 5.6 mM glucose, 1.8 mM CaCl₂·2H₂O, and 1.3 mM MgSO₄·7H₂O) containing 0.04% BSA (Sigma-Aldrich) to remove excess IgE then the cells resuspended in this buffer at the required cell density for a specific assay. The BMMCs or HuMCs were stimulated with DNP-HSA (10 ng/ml) or streptavidin (SA; 10 ng/ml), both also referred to as Ag, at 37°C, respectively.

Intracellular ROS detection by microfluorometry

Intracellular ROS were measured as described (14). Briefly, sensitized mast cells were preincubated with or without indicated inhibitors at 4°C for 10–20 min. After centrifugation, cells were incubated with dichlorofluorescein (DCF)-diacetate (20 μM) (EMD Biosciences) in cell culture medium for 20 min at 4°C. Cells were then washed with HEPES buffer containing 0.04% BSA and seeded at 2 x 10⁶ cells per well in a black opaque 96-well microplate in the presence or absence of inhibitors and/or Ag. DCF fluorescence was monitored at 37°C in 1 min intervals for 20–30 min using a GENios fluorescent plate reader (ReTiSoft) with an excitation wavelength of 492 nm and emission wavelength of 535 nm. Fluorescence was expressed as relative fluorescent units.

LTC₄ and PGD₂ measurements

The release of LTC₄ and PGD₂ from Ag-triggered cells was measured as described (14). Briefly, IgE-sensitized HuMCs or BMMCs were preincubated with or without indicated inhibitors for 20 min before addition DNP-HSA (10 ng/ml, BMMCs) or SA (10 ng/ml, HuMCs) for 20 min, and then cell-free supernatants were analyzed for LTC₄ and PGD₂ by competitive enzyme immunoassay (Cayman Chemicals) according to the manufacturer’s instructions. For comparison, the results are proportionally represented to ng/ml in 100,000 cells/μl.

Immunoblotting

For immunoblot analyses, mast cell lysates were prepared as described (27, 29) and proteins separated by electrophoresis on 4–12% NuPAGE BisTris gels (Invitrogen). Following membrane transfer, proteins were probed using the following Abs: anti-β-actin mAb (clone AC-15) (Sigma-Aldrich); anti-phospho-PLCγ₁ (Tyr(P)-783) pAb, anti-phospho-AKT (Ser(P)-473) pAb, anti-phospho-ERK1/2 (Thr(P)-202, Tyr(P)-204) pAb, anti-phospho-cPLA₂ (Ser(P)-505) pAb, anti-cPLA₂ pAB, anti-ERK pAb, anti-AKT (Cell Signaling); anti-phospho-PLCγ₁ (Tyr(P)-783) pAb (BIOSOURCE; Invitrogen), anti-phospho-Btk (Tyr(P)-551) mAb (BD Biosciences), anti-PLCγ₁,
FIGURE 2. The effect of the ERK1/2 inhibitor on ERK1/2 and cPLA₂ phosphorylation (A and B) and eicosanoid (C–F) and ROS generation (G and H) in Ag-challenged human (A, C, E, and G) and mouse (B, D, F, and H) mast cells. HuMCs and BMMCs were pretreated with or without U0126 for 15–20 min before stimulation with SA (10 ng/ml) or DNP-HSA (10 ng/ml), respectively, for the indicated times. cPLA₂ and ERK1/2 phosphorylation (A and B) was determined by immunoblot analysis as described for Fig. 1. Protein levels were normalized to cPLA₂ or ERK then to the Ag response in the absence of inhibitor to determine the relative intensities presented under each blot. For eicosanoid release, HuMCs or BMMCs were pretreated with or without U0126 for 15–20 min before stimulation with SA (10 ng/ml) or DNP-HSA (10 ng/ml), respectively, for the indicated times. cPLA₂ and ERK1/2 phosphorylation was determined by immunoblot analysis as described for Fig. 1. Protein levels were normalized to cPLA₂ or ERK then to the Ag response responses at 10 min to determine the relative intensities presented under each blot. The blots are representative of three independent experiments.

FIGURE 3. The effects of the PI3K inhibitor Wortmannin (A and B) and the PLC inhibitor U73122 (C and D) on cPLA₂ and ERK1/2 phosphorylation in human (A and C) and mouse (B and D) mast cells. IgE-sensitized HuMCs or BMMCs were pretreated with or without indicated inhibitors (Wortmannin 100 nM, U73122 1 μM) for 15 min before stimulation with SA (10 ng/ml) or DNP-HSA (10 ng/ml), respectively, for the indicated times. cPLA₂ and ERK1/2 phosphorylation was determined by immunoblot analysis as described for Fig. 1. Protein levels were normalized to cPLA₂ or ERK then to the Ag response responses at 10 min to determine the relative intensities presented under each blot. The blots are representative of three independent experiments.

**IP₃ assay**
Sensitized WT or Btk-/- BMMCs (2 × 10⁶) were stimulated with DNP-HSA (10 ng/ml) for 30 s and then cellular IP₃ concentrations were determined as described (27) using a commercially available kit (GE Healthcare) according to the manufacturer’s instructions. The results are expressed as picomoles of IP₃ per 2 × 10⁶ cells.

**Statistical analysis**
Data are represented as the mean ± SE. The statistical analyses were performed by unpaired Student’s t test. Differences were considered significant when p < 0.05. The n values represent experiments from multiple preparations.

**Results**
ERK1/2-dependent cPLA₂ activation and eicosanoid and ROS generation in primary cultured human and mouse mast cells
Since it has been demonstrated that FcεRI-mediated cPLA₂ activation is dependent on phosphorylation of cPLA₂ by ERK in the RBL 2H3 rat mast cell line (20), we examined whether cPLA₂-mediated eicosanoid and ROS generation were similarly regulated in an ERK1/2-dependent manner in primary cultured human and mouse mast cells. SA- or DNP-HSA-induced FcεRI aggregation, as shown in Fig. 1, A and B, respectively, resulted in a rapid increase in PGD₂ and LTC₄ release and ROS production in both HuMCs and mouse BMMCs. As discussed in the figure legend, the apparent delay in ROS production represented a technical artifact due to the time required for the cells to reach 37°C. In HuMCs, PGD₂ was the predominant eicosanoid produced, whereas, in the...
mouse BMMCs, LTC4 was the predominant form. Except for PGD2 release in BMMCs, maximal release in each case was observed within 10 min of cell activation. These observations are consistent with the kinetics observed in RBL 2H3 cells (20). Over this time frame, the phosphorylation of ERK1/2 and cPLA2 was similarly observed to increase following FcγRI aggregation in HuMCs (Fig. 1C) and mouse BMMCs (Fig. 1D). Maximum ERK1/2 phosphorylation appeared to slightly precede the maximum increase in cPLA2 phosphorylation.

To determine whether ERK1/2 participated in cPLA2 activation and eicosanoid and ROS generation in primary cultures of mouse and human mast cells, we next examined the ability of the MEK1/2 inhibitor U0126 to block these responses. As expected, U0126 markedly inhibited FcγRI-dependent ERK1/2 phosphorylation in both HuMCs (Fig. 2A) and BMMCs (Fig. 2B) in a concentration-dependent manner between 1 and 10 μM, while having minimal effect on the phosphorylation of the MAPKs p38 and JNK over this concentration range (data not shown). Similarly, U0126 (10 μM) significantly inhibited FcγRI-mediated cPLA2 phosphorylation (HuMCs: Fig. 2A; BMMCs: Fig. 2B), LTC4 (HuMCs: Fig. 2C; BMMCs: Fig. 2D), and PGD2 (HuMCs: Fig. 2E; BMMCs: Fig. 2F) generation. ROS production was also markedly inhibited by U0126 (HuMCs: Fig. 2G; BMMCs: Fig. 2H), suggesting that ROS production in these cells was also regulated by ERK. HuMCs appeared to be more sensitive to the effects of U0126 on ROS production compared with BMMCs. Thus, both the production of eicosanoids and ROS in primary cultures of mouse and human mast cells is, in part, regulated by an ERK1/2-dependent pathway.

The degree of inhibition of eicosanoid production (LTC4 87%, PGD2 90% inhibition in HuMCs; LTC4 68%, PGD2 57% inhibition in BMMCs, both at 10 μM of U0126), however, was greater than that observed for cPLA2 phosphorylation (52% in both HuMCs and BMMCs at 10 μM of U0126). In addition, the titration curves for the effects of U0126 on LTC4 and PGD2 production in either HuMCs or BMMCs (Fig. 2, C–F) only partially correlated with that for cPLA2 phosphorylation. These data led us to investigate whether other pathways contribute to the regulation of cPLA2 activation leading to eicosanoid generation in activated mast cells.

PI3K- and PLCγ-regulated FcεRI-mediated eicosanoid and ROS generation

PI3K and PLCγ regulate two key intermediary signaling pathways for Ag-mediated mast cell degranulation (23). The effects of respective inhibitors of these enzymes, Wortmannin and U73122, on cPLA2 activation and eicosanoid and ROS generation were thus examined. As shown in Fig. 3, both compounds...
inhibited (~25–40%) FcεRI-mediated of ERK1/2 phosphorylation 10 min after stimulation of both HuMCs (Fig. 3, A and C) and BMMCs (Fig. 3, B). However, neither Wortmannin nor U73122 significantly inhibited cPLA2 phosphorylation. These data show that although ERK is partially regulated by PI3K, FceRI-mediated cPLA2 phosphorylation is independent of PI3K activation. However, these inhibitors as well as LY294002, another PI3K inhibitor, significantly reduced FceRI-mediated LTC4 and PGD2 release and ROS production (HuMCs: Fig. 4, A and B; BMMCs: Fig. 4, C and D). However, at 100 nM and 10 μM, respectively, Wortmannin and LY294002 only partially inhibited these responses. As previously shown (27), and as discussed later, these concentrations completely block PI3K-dependent Akt phosphorylation in mast cells. This is consistent with the conclusion that, although PLCγ is absolutely required for FceRI-dependent eicosanoid generation, a PI3K-independent pathway is involved in addition to a PI3K-dependent pathway.

The role of Btk in PI3K- and PLCγ-dependent eicosanoid and ROS generation

Because Btk is a possible intermediary in the PI3K-dependent regulation of PLCγ1, (30) we next examined whether Btk played a similar role in the regulation of eicosanoid generation following FcεRI aggregation. For these studies, we used BMMCs derived from the bone marrow of Btk−/− and Btk+/+ (WT) mice. Fig. 5 shows that there was a partial attenuation of FcεRI-mediated PGD2, LTC4, and ROS generation in the Btk−/− BMMCs. The degree of inhibition mimicked the partial inhibition of PGD2 and LTC4 production observed in the cells treated with PI3K inhibitors but was less than that achieved in cells treated with the PLCγ inhibitor U73122.

In the Btk−/− BMMCs, there was a significant inhibition of PLCγ1 and PLCγ2 but not AKT phosphorylation (Fig. 6A). Similarly...
PLCγ-dependent IP₃ production (Fig. 6B) and in agreement with previous observations (24, 26), Ca²⁺ mobilization (Fig. 6C) was also attenuated in the Btk⁻/⁻ BMMCs. The defect in Ca²⁺ mobilization in the Btk⁻/⁻ BMMCs mirrored a similar decrease observed in WT BMMCs treated with the PI3K inhibitors Wortmannin and LY 294002 (data not shown). No defects were observed in FcεRI-mediated ERK1/2 and cPLA₂ phosphorylation in the Btk⁻/⁻ BMMCs (Fig. 6D).

The above data suggested that Btk regulated cPLA₂ activation as well as eicosanoid and ROS generation upstream of the PLCγ-mediated Ca²⁺ signal. The unimpaired AKT phosphorylation in Btk⁻/⁻ BMMCs indicated that Btk was downstream of PI3K. This conclusion is supported by the fact that phosphorylation of Btk, PLCγ₁, and PLCγ₂ was reduced following treatment of WT BMMCs with the PI3K inhibitors (Fig. 7A).

To determine whether the observed defect in Ca²⁺ mobilization observed in the Btk⁻/⁻ BMMCs and WT BMMCs treated with the PI3K or PLCγ inhibitor accounts for the inhibition of eicosanoid and ROS generation, we used the Ca²⁺ chelator EDTA and the IP₃ receptor antagonist 2-APB. Both ablated the FcεRI-mediated Ca²⁺ signal in BMMCs (data not shown). These agents also blocked FcεRI-mediated PGD₂, LTC₄, and ROS production to a similar extent to that observed following treatment of the cells with the PLCγ inhibitor U73122 (Fig. 4). These data demonstrated that all three responses were dependent on Ca²⁺. Therefore, it is reasonable to conclude that Btk regulates eicosanoid and ROS generation in part through its regulation of the Ca²⁺ signal.

**Discussion**

In this study, and as summarized in Fig. 8, we have demonstrated that PI3K and Btk are required for optimal FcεRI-mediated cPLA₂ activation leading to COX- and 5-LO-mediated generation of eicosanoids and ROS (Fig. 1) in mast cells. The PI3K-btk axis appears to regulate these responses by the latent regulation of the PLCγ-induced calcium signal, which is essential for cPLA₂ activation and which likely also contributes to the regulation of COX and 5-LO. Rather than leading to cPLA₂ phosphorylation, which is regulated to a certain extent by an ERK1/2-dependent mechanism, the calcium-dependent regulation of eicosanoid and ROS generation by PI3K and Btk may lead to the binding of Ca²⁺ to the C2 domain of cPLA₂, which regulates membrane targeting.

The role of ERK1/2 in cPLA₂ phosphorylation leading to eicosanoid generation previously reported in RBL 2H3 cells (20) was confirmed in human and mouse mast cells by the concurrent Ag-dependent increase in ERK1/2 and cPLA₂ phosphorylation in HuMCs and BMMCs (Fig. 1) and, more specifically, by the ability of the MEK1/2 inhibitor U0126 to attenuate these responses in parallel with the attenuation of PGD₂ and LTC₄ release (Fig. 2, A–F). These studies also revealed a role of ERK1/2 in the production of ROS (Fig. 2, G and H). Nevertheless, the effects of U0126 on the aforementioned responses were partial, suggesting that other pathways are involved in these responses.

The role of PI3K and Btk in the ERK-independent regulation of eicosanoid and ROS generation was indicated by the ability of PI3K inhibitors to reverse FcεRI-mediated PGD₂ and LTC₄ release and ROS production in both HuMCs and BMMCs (Fig. 4), and by the substantial reduction of these responses in Btk⁻/⁻ BMMCs (Fig. 5). These data obtained from the Btk⁻/⁻ BMMCs, however, are in contrast to a previous report of unimpaired FcεRI-mediated LT release in these cells (26). The reason for the disparity is currently unclear. However, the ability of the PI3K inhibitors to block Btk activation (Fig. 7A) and mimic the Btk⁻/⁻ phenotype (Figs. 4 and 5) indicates that PI3K contributes to the control of eicosanoid and ROS generation by regulating the activity of Btk.

It is possible, however, that PI3K also acts independently of Btk. For example, it has been reported that PI3K/Rac/PKC8 acts upstream of ERK and cPLA₂ to regulate FcεRI-mediated cysteinyl leukotriene synthesis in RBL 2H3 cells (31). Regardless, the small differences noted in phosphorylation of cPLA₂ (Figs. 3, A and B, and 6D) implies that PI3K and Btk were regulating eicosanoid and ROS generation other than through control of ERK1/2-mediated cPLA₂ phosphorylation. This conclusion was further supported by the lack of difference in the phosphorylation of ERK1/2 in the Btk⁻/⁻ BMMCs (Fig. 6D).

Although the PI3K inhibitors produced a slight reduction in FcεRI-mediated ERK1/2 phosphorylation (Fig. 3, A and B), this did not translate to reduced cPLA₂ phosphorylation. It may be argued that the degree of inhibition of ERK1/2 phosphorylation produced by the PI3K inhibitors (42% in HuMCs, 39% in BMMCs at 10 min of stimulation) was not of sufficient magnitude to reduce the phosphorylation of cPLA₂. However, it is also possible that the inhibitors of the ERK1/2 pathway used in this and a previous study (19) may be also targeting other kinase pathways leading to cPLA₂ phosphorylation.

The ability of the Ca²⁺-chelator EDTA and the IP₃ receptor antagonist 2-APB to block Ag-induced eicosanoid generation and ROS production (Fig. 7, B–D) demonstrates the requirement for an increase in cytosolic Ca²⁺ concentrations for the liberation of free arachidonic acid, and the subsequent production of PGD₂ and...
PI3K-Btk pathway contributes to the required Ca²⁺ to Ag-mediated generation of eicosanoids and ROS in mast cells. The aforementioned responses were inhibited by the PI3K inhibitors (data not shown), therefore, suggesting of Ca²⁺ signaling of Ca²⁺ also require Ca²⁺ for both the PLC-RI aggregation in mast cells. Therefore, it is likely that PI3K and Btk regulate eicosanoid and ROS generation by this means. This pathway likely involves a latent signal that allows the maintenance of PLCγ activation, which is induced upon FceRI aggregation. In agreement with previous reports (24, 26), we accordingly observed that FceRI-mediated PLCγ phosphorylation and PLCγ-dependent IP₃ production were partially attenuated in Btk⁻/⁻ cells (Fig. 6) and in BMMCs treated with the PI3K inhibitors (Fig. 7A). This level of inhibition was similar to the degree of attenuation of FceRI-mediated eicosanoid and ROS generation observed under similar experimental conditions. The results obtained under Ca²⁺-free conditions and with the IP₃ binding inhibitor 2-APB, however, demonstrate that there is an absolute requirement for both the PLCγ-mediated generation of IP₃ and Ca²⁺ for FceRI-mediated eicosanoid and ROS generation in mast cells (Fig. 7, B–D). The residual calcium signal and eicosanoid and ROS generation observed in Btk⁻/⁻ (Fig. 6C) cells and WT BMMCs treated with the PI3K inhibitors (data not shown), therefore, suggest that other, as yet unidentified, signals may also contribute to the aforementioned responses.

In summary, the results of this study demonstrate that ERK1/2- and PI3K-Btk-dependent pathway (Fig. 8) independently contribute to Ag-mediated generation of eicosanoids and ROS in mast cells. Whereas ERK1/2 regulates cPLA₂ phosphorylation, the PI3K-Btk pathway contributes to the required Ca²⁺ signal for cPLA₂, COX, and 5-LO activation. Therefore, effective therapeutic suppression of eicosanoid and ROS production in activated mast cells may require a combination of strategies to block both ERK- and PI3K/Btk-regulated pathways.

Acknowledgments

We thank Dr. Anne B. Satterthwaite, University of Texas Southwestern Medical Center, for providing Btk⁻/⁻ mice.

Disclosures

The authors have no financial conflict of interest.

References