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Inhibition of IgE-mediated Secretion from Human Basophils with a Highly Selective Bruton's Tyrosine Kinase, Btk, Inhibitor

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Abstract

The study of receptor-mediated signaling in human basophils is often limited by the availability of selective pharmacological agents. The early signaling reaction mediated by FcεRI aggregation is thought to require the activity of Bruton's tyrosine kinase (btk), an enzyme that has been identified as important in B cells signaling because mutations lead to X-linked agammaglobulinemia. This study uses the btk selective irreversible inhibitor, PCI-32765, to explore the role of btk in a variety of functions associated with the activation of human basophils. Nine endpoints of basophil activation were examined: induced cell surface expression of CD63, CD203c, CD11b; induced secretion of histamine, LTC₄, IL-4 and IL-13; the cytosolic calcium response; and the induced loss of syk kinase. Four stimuli were examined; anti-IgE antibody, formyl-met-leu-phe (FMLP), C5a and IL-3. For stimulation with anti-IgE, PCI-32765 inhibited CD63, histamine, LTC₄ and IL-4 secretion with an IC₅₀ of 3–6 nM (with 100% inhibition at 50 nM) and it inhibited CD203c and CD11b and the cytosolic calcium response with an IC₅₀ of 30–40 nM. Fifty percent occupancy of btk with PCI-32765 occurred at ~10 nM. Consistent with btk functioning downstream or in parallel to syk activation, PCI-32765 did not inhibit the loss of syk induced by anti-IgE in overnight cultures. Finally, PCI-32765 did not significantly inhibit basophil activation by FMLP or C5a and did not inhibit IL-13 release induced by IL-3. These results suggest that btk is specifically required for IgE-mediated activation of human basophils.

Keywords

Basophils; Atopy; Bruton's Tyrosine Kinase; signal transduction; IgE

1. Introduction

During IgE-mediated secretion in basophils and mast cells, there are several kinases that have been identified as being critical for the initiation of the early reaction. For example, syk, a tyrosine kinase that is related to ZAP-70, is a non-redundant kinase in basophils and mast cells whose activity determines a very large number of downstream events following the aggregation of FcεRI. Pharmacological agents that effectively ablate syk's kinase activities eliminate all mediator release [1]. Likewise, complete inhibition of PI3 kinase, and in particular, PI3 kinase delta, also completely blocks all mediator secretion [2, 3]. The

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kinase btk has a well defined role downstream of the B cell receptor; human mutations that inactivate Btk prevent B cell maturation and cause X-linked agammaglobulinemia. Btk has also been shown to be important in IgE-mediated signaling using rodent mast cell models [4, 5]. However, the tools for clearly determining Btk's relevance to secretion in human basophils and mast cells have not been previously available. For example, dasatinib is an inhibitor that targets several tyrosine kinases. This drug is also a potent and efficacious inhibitor of btk and has been used to study human basophils [6]. However, because dasatinib inhibits a relatively broad range of kinases [7], effects of this inhibitor do not address the specific role of Btk in basophils. Evidence indicates that recruitment of btk to the plasma membrane results from the generation of phosphatidylinositol 3,4,5, triphosphate (PIP3) by PI3 kinase [8]. In some cells, recruited btk is responsible for the phosphorylation and activation of PLC- γ 1/2 which is, in turn, responsible for the generation of inositol, 3,4,5 triphosphate (IP3) and the initiation of the elevation in cytosolic calcium [9]. In human basophils, inhibition of PI3 kinase has only a modest effect on the IgE-mediated cytosolic calcium response [3] so it is unclear whether there is, in fact, an essential role for btk in modulating human basophil function. In recent years, a selective irreversible inhibitor of human btk, PCI-32765, has been developed [10, 11]. This study tests whether btk activity is critical for the expression of multiple indicators of basophil activation in response to a range of physiological stimuli.

2. Materials and Buffers

2.1 Materials, Buffers and Antibodies

The following were purchased: PIPES, bovine serum albumin (BSA), EGTA, EDTA, D-glucose, NaF, Na₃VO₄, 2-ME (2-mercaptoethanol); RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); IMDM, Iscove's modified Dulbecco's Medium (Gibco/Invitrogen, Carlsbad, CA); Percoll (Pharmacia, Piscataway, NJ); Tris(hydroxymethyl)-aminomethane, Tween-20 (Bio-Rad, Hercules, CA); anti-syk mAb(4D10) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p85 (Upstate Biotechnology, Charlottesville, VA); anti-btk, (BD Biosciences, San Jose, CA); HRP-conjugated sheep anti-mouse Ig Ab (Amersham Life Science, Arlington Heights, IL). Mouse anti-human IgE Ab (6061P) (Hybridoma Farms, MD). PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1 % glucose, and 0.003 % HSA. PAGCM was PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂. Countercurrent elutriation and labeling with antibodies for flow cytometry was conducted in PAG containing 0.25 % BSA in place of 0.003 % HSA (elutriation buffer). ESB is Novex electrophoresis sample buffer containing 5 % 2-mercaptoethanol.

2.2 Basophil purification

Two sources of basophils were used for these experiments; enriched basophils obtained by processing blood obtained by venipuncture through two-step Percoll gradients (BEC) and purified basophils obtained from leukopheresis packs. The cells obtained by venipuncture were obtained from donors that were enrolled for these studies by a protocol approved by the Johns Hopkins Institutional Review Board. Basophils were purified to near homogeneity by sequential application of Percoll gradients, countercurrent elutriation and negative selection using the basophil purification kit (Stem Cell Technologies, Vancouver, BC) and columns from Miltenyi Biotec (Auburn, CA) [12]. The average purity of these basophils by alcian blue staining [13] was 99%. Viability of these cells is typically >97%.

2.3 Reaction conditions

For the majority of the outcomes examined (histamine and LTC₄ release, CD11b, CD203c and CD63 expression), the cells were stimulated in PAGCM buffer. They were first treated

with PCI-32765 or vehicle control (DMSO) for 15 minutes at 37°C prior to stimulation with optimal concentrations of either anti-IgE Ab (0.5 µg/ml), FMLP (100 nM), or C5a (100 ng/ml) for 30–45 minutes. These concentrations were determined from extensive prior studies that have used these agents to stimulate human basophils [14, 15]. The stock PCI-32765 concentration was 10 mM in DMSO. If, for example, the maximum PCI-32765 concentration tested was 50 nM, then the vehicle control condition would be 0.0005% DMSO. Histamine and LTC₄ were measured in supernatants by automated fluorimetry [16] and radioimmunoassay [17], respectively. Spontaneous (unstimulated) histamine release was generally less than 5% and was subtracted from stimulated release for the calculation of percent histamine release.

To measure IL-4 secretion, basophils were incubated in IMDM media containing 5% FCS, non-essential amino acids, and 5 µg/ml gentamycin. Cell aliquots of 100 µL (containing 1×10^5 basophils) were added to wells of a 96-well microtiter plate. After pre-equilibrating to 37°C, 5% CO₂ for 15 min., an equal volume of drug (containing twice the final concentration) was then added for 15' before activating with 5× stimulus (50 µl) for 4h. Plates were then removed from incubation (37°C, 5% CO₂), and spun, before removing the top 50 µL of supernatant from each to measure histamine by automated fluorimetry. IL-4 protein in remaining supernatants was determined by ELISA. Concentrations of stimuli were optimal for IL-4 secretion: anti-IgE (20ng/mL); FMLP (1 µM) or A23187 (0.5 µg/ml).

2.4 Flow cytometry

After stimulation of basophils in the presence of PCI-32765 or vehicle control (DMSO), a portion of the cells were fixed by treatment with 1% paraformaldehyde for 10 minutes at room temperature and blocked with elutriation buffer. Cells were then incubated on ice with either anti-CD203c (BioLegend, San Diego, CA), anti-CD63 (BD Pharmingen, San Jose, CA), anti-CD11b (Beckman-Coulter, Sykesville, MD) for 30 minutes, washed once and incubated with secondary antibodies labeled with alexa-647 (Molecular Probes/Invitrogen, Carlsbad, CA). The non-stimulated cells acted as the control; control fluorescence was subtracted from the values obtained for the other conditions. Inhibition was calculated as the fraction of the response in the presence of vehicle control.

2.5 Western blots and btk binding Assay

For the syk loss studies, purified basophils were pelleted and lysed in 20 µl of hot ESB, the tube placed in boiling water for 5 minutes and the samples stored at –80°C until electrophoresis. Samples were run in a 15 well 10% tris-glycine gel with molecular weight markers in the first and last lanes. After blocking, blots were first developed for syk using 4D10 for detection, followed by blotting for p85α levels which act as a lane loading controls. Multiple film exposures were made in order to optimize linearity.

To detect the level of btk occupancy, purified basophils were treated with PCI-32765 at the concentrations used in the reactions described above and after 15 minutes the cells were pelleted and quick frozen for storage at –70°C until probe labeling. Cells were lysed by 3 cycles of freezing/thawing in liquid nitrogen and a 37°C water bath. Protease inhibitors (Sigma) were added to the lysates and the insoluble fraction was removed by centrifugation. The lysates were incubated for 1 hr at 37°C with 2 µM of the probe PCI-33380, a fluorescent derivative of PCI-32765 that binds to Btk at the same site as PCI-32765 [11]. Samples were diluted in LDS Sample Buffer containing Sample Reducing Agent (Invitrogen, Carlsbad, CA) and analyzed by SDS-PAGE and fluorescent gel scanning using a Typhoon scanner (Ex= 532nm and Em= 555nm). The gel was then blotted and total Btk levels detected by standard western blot. Fluorescent probe signal and total Btk levels were quantitated by

densitometry (ImageQuant) to calculate percent Btk occupancy relative to vehicle treated cell.

2.6 $[Ca^{2+}]_i$ measurements

Basophils were labeled with 4 μ M fura-2/AM for 30 min at 37 °C in RPMI 1640 containing 2 % FCS (fetal calf serum)($0.3-0.5 \times 10^6$ cells in 200 μ l). Changes in $[Ca^{2+}]_i$ (cytosolic free calcium concentration) were determined by digital video microscopy [14, 15]. Data were then obtained for 50 – 150 frames at intervals of 1 to 10 s to determine the $[Ca^{2+}]_i$ response.

2.7 Statistics

The data shown in the figures represents averages for multiple experiments and the error bars shown are standard error of the mean (SEM). In experiments where paired data is available, the statistical comparison uses a paired analysis of the results while the figure may show SEM for the group.

3. Results

Seven endpoints of basophil activation and 4 types of stimulation were examined. Figures 1–3 summarize the results. First, in figure 1, purified human basophils were stimulated with an optimal concentration of anti-IgE antibody. A portion of the cells were retrieved for fixation and analysis by flow cytometry for expression of CD203c, CD11b and CD63. Each of these surface proteins are likely regulated by different signaling pathways. A second portion of the cells was pelleted by centrifugation in order to harvest supernatants for the measurement of histamine and LTC4 release. Finally, in two of the four experiments, a portion of the cells was pelleted and the pellet analyzed for the presence of inhibitor bound to btk (see methods). Based on studies in lymphoma cell lines and human B cells, the expected cellular IC_{50} for this drug was ≈ 10 nM. Histamine and LTC4 release showed an IC_{50} of 6–7 nM, CD63 expression showed an IC_{50} of ≈ 10 nM and these curves were consistent with the degree of occupancy of the basophil btk by PCI-32765, with $\sim 50\%$ btk occupancy achieved at 10 nM. In contrast, the inhibition of both CD11b and CD203c expression was shifted to 5 fold higher concentrations relative to histamine release, with an IC_{50} of 25–30 nM.

To explore inhibition of cytokine release, a slightly different format for the experimental design was needed for the longer incubations required. In these experiments, the basophils were incubated in IMDM culture medium for 4 hours and the anti-IgE concentrations optimized for IL-4 secretion (ca. 5–10 fold lower than for optimal histamine release). Supernatants were harvested and analyzed for both histamine and IL-4 release. Figure 1B summarizes results using either partially enriched basophils or purified basophils. The IC_{50} values were consistent with the results shown in figure 1A although it did appear that IL-4 secretion was somewhat more sensitive to this drug.

Figure 2 summarizes the results for stimulation with non-IgE-mediated secretagogues. Figure 2A shows results of experiments where a 50 nM concentration was examined for its effects on the endpoints explored for IgE-mediated release shown in figure 1A; CD63, CD203c, CD11b and LTC4 and histamine release. For either FMLP or C5a, there was no effect on any of these endpoints. In Figure 2B, purified basophils were treated with a broad range of drug concentrations and stimulated with either FMLP or the calcium ionophore, A23187. There was little inhibition of either stimulus at all but the highest concentration of the drug, which was 1,000 fold greater than the expected IC_{50} .

Recent studies have shown that IL-13 secretion that is initiated by IgE-mediated stimulation largely results from the autocrine effects of IL-3 released by aggregating Fc ϵ RI rather than a

direct effect of the signaling cascade initiated by FcεRI [18]. Therefore, on the basis of the studies in figure 1, it was expected that the drug would inhibit any IgE-mediated release of IL-13. The more interesting question was whether it would inhibit the direct effect of IL-3 on IL-13 secretion, which requires a long incubation. To explore the effect of the drug on IL-13 secretion, purified basophils were stimulated for 18 hours in culture medium with IL-3. Figure 3 shows that across a broad range of drug concentrations, there is little direct effect of PCI-32765 on IL-3-induced IL-13 secretion.

Previous studies have noted that recruitment of btk to the plasma membrane is dependent on PIP3 (phosphatidylinositol 3,4,5 trisphosphate), whose presence is dependent on the activity of PI3 kinase. In human basophils, complete inhibition of PI3 kinase activity with either wortmannin or LY294002 results in only ca. 50% inhibition of the cytosolic calcium response [3]. Therefore, one would expect that complete inhibition of btk (which is postulated to influence the activity of PLCγ1 or γ2, [19]) would not completely ablate the cytosolic calcium response. Figure 4 shows that 50 nM PCI-32765 inhibited the IgE-mediated cytosolic calcium by $\approx 65 \pm 1\%$ (a single test of 5 nM PCI-32765 showed no inhibition of the cytosolic calcium response).

Recent studies have noted that signaling events that lie downstream of syk activity do not influence desensitization of basophils [20, 21]. Since PI3 kinase activity appears to lie downstream of syk [20], it would be expected that btk recruitment and activation would also lie downstream of syk. On the other hand, some evidence suggests that Btk can be activated by Src-family kinases that lie upstream of both Syk and Btk. To establish whether Btk inhibition participates in the desensitization of basophils, we measured the loss of syk during stimulation [20, 22]. We found that PCI-32765, at concentrations that completely inhibit histamine and IL-4 release, has no effect on anti-IgE induced loss of syk. (Figure 5).

4. Discussion

Studies in other cell types or species have led to the expectation that inhibition of btk would result in marked inhibition of the IgE-mediated responses of human basophils [4, 5]. The current studies show that 7 outcomes of IgE-mediated stimulation are inhibited at a concentration of PCI-32765 that is consistent with the occupancy of btk as measured in a binding assay. Previous studies have demonstrated that this inhibitor is relatively selective for btk; PCI-32765 is a compound selected from a series of Btk-selective compounds that bind irreversibly to a non-catalytic Cys residue in the active site of Btk [10]. Structural alignment reveals that only 10 kinases have a Cys in a similar position, so this series of inhibitors has the potential for irreversible binding to only a small subset of all kinases. PCI-32765 was selected for the present studies based on its optimized potency (Btk IC₅₀ = 0.5 nM) and its recent clinical advancement into Phase I studies in patients with lymphoma. In cell-based assays, the IC₅₀ values are typically less than 10 nM, [10]. In human basophils, the IgE-mediated reaction is known to be dependent on the activities of the src-family kinase, lyn, the tyrosine kinase, syk, and PI3 kinase delta to the extent that selective inhibition of each of these kinases results in complete inhibition of all mediator release [1, 3, 20, 23]. In this context, it is notable that PCI-32765 is 400-fold selective over Lyn [11] and greater than 10,000 fold selective over syk or PI3 kinases [11]. The src-family kinase member fyn is present in basophils although there is currently no evidence for its activation [1] but regardless of whether fyn is active, fyn is 192 fold less sensitive to PCI-32765 [11]. Several other src-family kinase members, such as hck, fgr and yes are less sensitive to PCI-32765 by about 10 fold [11] but these members have not been observed in basophils. Only two other kinases have been shown to equally sensitive to PCI-32765 [11], blk (a src-family kinase) and bmx (a Tec-family kinase like btk), and neither of these signaling elements is known to participate in the IgE-mediated reaction in basophils (or mast cells).

If this inhibitor is indeed selective for btk in basophils, then the results lead to several insights. First, not all outcomes are equally sensitive to btk inhibition. With respect to CD203c and CD11b, PCI-32765 was 5 fold less potent than expected based on the inhibition of histamine and LTC₄ release. This suggests occupancy of btk by PCI-32765 needed to be more complete to observe inhibition of stimulated CD203c or CD11b expression. In a similar context, inhibition of the cytosolic calcium response was only modest at 50 nM inhibitor. Taken together, these results suggest that CD203c and CD11b inhibition more closely follows the cytosolic calcium elevation and, in support of previous studies, suggests that these two outcomes have different signaling requirements than the other outcomes.

Second, PCI-32765 did not inhibit G-protein coupled receptors like FMLP and C5a. It also did not appear to be required for IL-3-mediated secretion of IL-13. Finally, while PCI-32765 was an efficacious inhibitor of functional outcomes known to be dependent on the activation of syk, it had no effect on post-translationally-mediated down-regulation of syk expression. This result followed previous predictions for desensitization events, i.e., that any signaling event that operated down-stream of syk would not be required to observe signal termination events [20, 21]. Down-regulation of syk is one of these processes and it is dependent on signaling events upstream of syk, such as the activity of src-family kinases. As noted previously, inhibitors that can ablate secretion while not interfering with desensitization may offer a unique advantage for their clinical use. For example, to prevent iatrogenic anaphylaxis during immunotherapy, patients could be pre-loaded with a single dose of the inhibitor after which antigen can be given. No mediator secretion is expected while the mast cells and basophils continue to desensitize so that as the drug is metabolized, any residual antigen present should not be able to induce secretion.

In summary, these studies indicate that btk is a critical signaling component for the secretion of 3 classes of mediators, histamine, LTC₄ and IL-4. Like inhibition of syk or PI3 kinase, complete inhibition of btk activity is accompanied by complete inhibition of secretion.

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Figure 1. PCI-32765 inhibits IgE-dependent functions

Panel A: Purified basophils were stimulated with 0.5 $\mu\text{g}/\text{ml}$ of monoclonal anti-IgE antibody for 30 minutes ($n=4$). A portion of the samples were immediately fixed and stored for analysis by flow cytometry for CD63 (■), CD203c (●), or CD11b (▲). The results were calculated from the net mean fluorescence intensity (above non-stimulated controls). A portion of the supernatants were analyzed for histamine (○) or LTC4 (□) by automated fluorometry or radioimmunoassay, respectively. Histamine release following challenge with anti-IgE in the presence of vehicle control (0.0005% DMSO) averaged $45 \pm 17\%$ and LTC4 release averaged $57 \pm 3 \text{ ng}/\mu\text{g}$ histamine. In parallel, btk occupancy by the inhibitor was analyzed by a fluorescent probe assay (◆) ($n=2$). **Panel B:** IL-4 and histamine secretion in

basophil-enriched (filled symbols) and purified basophil (open symbols) suspensions. Cells were incubated in media for 4 hours and stimulated with 20 ng/ml anti-IgE to optimize IL-4 secretion. Values are the mean $\pm$ SEM of 3 experiments for the basophil enriched suspensions and 2 experiments (mean $\pm$ range) for purified suspensions. Histamine release following challenge with anti-IgE in the presence of vehicle control (a maximum of 0.004% DMSO) from enriched suspensions (■) averaged 50 $\pm$ 12% and IL-4 secretion (●) in enriched suspensions averaged 215 $\pm$ 67 pg/10⁶ basophils, similarly histamine release (□) in purified suspensions averaged 35 $\pm$ 4% and control IL-4 secretion (○) in purified suspensions averaged 31 $\pm$ 2 pg/10⁶ basophils.

Figure 2. PCI-32765 does not inhibit non-IgE-dependent functions

Panel A: Purified basophils were stimulated with 100 nM FMLP or 100 ng/ml C5a in the presence or absence of 50 nM PCI -32765 (n=4). Histamine release in stimulated vehicle control (0.0005% DMSO) samples averaged $92 \pm 7\%$ for FMLP and $100 \pm 5\%$ for C5a, LTC4 release in stimulated vehicle controls samples average d 132 ± 38 ng/ μ g histamine for FMLP and 17 ± 3 ng/ μ gH for C5a. Statistical analysis showed all differences were $p > 0.05$ ($H_0 = 1.0$). Panel B: Purified basophils (n=2) were stimulated with 1 μ M FMLP (○) or 0.5 μ g/ml A23187 (■,●) and supernatants analyzed for histamine (●) or IL-4 secretion (■). Histamine release in vehicle control (a maximum of 0.1% DMSO) samples averaged

64±22% for A23187 and 44±13% for FMLP and IL-4 release averaged 305±38 pg/10⁶ basophils for A23187. FMLP did not induce IL-4 release.

Figure 3. PCI-32765 does not inhibit IL-3-induced IL-13 secretion by human basophils
Purified basophils were cultured in media for 18 hours with 10 ng/ml IL-3 and PCI-32765 at the concentrations shown. Supernatants were harvested after 18h incubation and assayed for IL-13 by ELISA. Shown are the mean $\pm$ SEM values (n=3), statistical analysis showed all differences were $p>0.05$. There was no IL-13 measured in cultures without IL-3 present.

Figure 4. Partial inhibition of the IgE-mediated increase in cytosolic calcium by PCI-32765

Fura-2 loaded purified basophils were incubated with vehicle control (0.0005% DMSO) or 50 nM PCI-32765 for 10 minutes prior to the addition of 0.5 μ g/ml anti-IgE antibody and the cytosolic calcium response monitored. The 350/380 excitation ratio is plotted for the average of two experiments.

Figure 5. PCI-32765 does not inhibit IgE-mediated down-regulation of syk

Purified basophils were incubated in media with the concentrations of PCI-32765 shown or DMSO vehicle (0.0005%) for 10 minutes prior to the addition of 0.5 µg/ml anti-IgE antibody. The samples were then incubated for 18 hours prior to lysis of cell pellets for Western blotting for syk and p85 (which acts as the loading control). The experiment represents one of two experiments using different basophil preparations.