Regulation of protein kinase CβI by two protein-tyrosine kinases, Btk and Syk


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Two protein-tyrosine kinases, Bruton’s tyrosine kinase (Btk) and Syk, and members of the protein kinase C (PKC) subfamily of serine/threonine kinases play crucial roles in signal transduction through antigen receptors in B lymphocytes and high-affinity IgE receptors (FceRI) in mast cells. The present study provides genetic, biochemical, and pharmacological evidence that, on FceRI stimulation, Syk regulates Btk, and Btk selectively regulates the membrane translocation and enzymatic activity of PKCβI among the conventional PKC isoforms (α, β, and δβ) expressed in mast cells. Syk/Btk-mediated PKCβI regulation is involved in transcriptional activation of the IL-2 and tumor necrosis factor α genes through the JNK pathway induced by FceRI stimulation. Accordingly, FceRI-induced production of these cytokines is inhibited by specific inhibitors of Btk and Syk, as well as broad-specificity inhibitors of PKC and a selective inhibitor of PKCβ. Specific regulation of PKCβI by Btk is consistent with the selective association of Btk with PKCβI. Components of this signaling pathway may represent an attractive set of potential targets of pharmaceutical interference for the treatment of allergic and other immunologic diseases.

Btk (Bruton’s tyrosine kinase) and Syk are protein-tyrosine kinases that play crucial roles in B cell and mast cell activation (1–3). Mutations in the btk gene lead to X-linked agammaglobulinemia in humans (4, 5) and X-linked immunodeficiency (xid) in mice (6, 7). Btk mutations also result in defective cytokine production in the affected mast cells on FceRI stimulation (5). Syk gene inactivation results in profound hematopoietic defects, including B cell development (9, 10). Loss of Syk expression ablates B cell receptor (BCR)- or FceRI-mediated cell activation (11–13). Engagement of BCR and FceRI elicits the enzymatic activation of receptor-bound Src family protein-tyrosine kinases, such as Lyn. These kinases are believed to phosphorylate tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) in signaling subunits of receptor. Tyrosine-phosphorylated ITAMs recruit Src family and Syk kinases through Src homology 2 (SH2) domain-phosphotyrosine interactions and activate these kinases. Both btk and syk mutations impair the Ca2+ response on BCR or FceRI engagement, because of defective activation of phospholipase C (PLC)-γ (11–17). PLC-γ hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP3). Diacylglycerol activates several protein kinase C (PKC) isoforms, and IP3 recruits Ca2+ from intracellular storage sites. PKC is a family of serine/threonine kinases that play crucial roles in a plethora of biological functions, such as proliferation, differentiation, development, and more specialized cellular functions (18–20). Based on cofactor requirements and structure, PKC family members are divided into the Ca2+/diacylglycerol-regulated conventional isoforms (εPKC: α, β, δ, γ, and, γ), the Ca2+-independent but diacylglycerol-regulated novel isoforms (nPKC: δ, ε, η, θ, and, ζ), and the Ca2+/diacylglycerol-independent atypical isoforms (aPKC: θ and ι/λ). In the present study, we provide evidence that Syk regulates Btk and that Btk regulates PKCβI activation. PKCβI is shown to regulate the JNK pathway that leads to transcriptional activation of cytokine genes.

Materials and Methods

Cell Culture and Stimulation. Bone marrow cells derived from wild-type (wt), btk knockout (btk−/−) (21), and lyn knockout (lyn−/−) (22) mice were cultured in IL-3-containing medium for 4–6 wk to generate >95% pure populations of mast cells. Cells were sensitized overnight with anti-dinitrophenyl (DNP) IgE monoclonal antibody and stimulated with antigen, DNP-human serum albumin conjugates. Retroviral transfection of btk- mast cells was done as described (23). Wt, Syk-deficient (syk−/−) variant, and syk cDNA-transfected syk− RBL-2H3 cells (13) were similarly stimulated with IgE and antigen.

Mutagenesis and Transfection. Two-step PCR mutagenesis was performed to generate mutant rat PKCdNAs. COS-7 and bone marrow-derived mast cells were electroporated with plasmid constructs. Luciferase reporter assays were performed as described (24).

Immunoblotting Analysis and Antibodies. Subcellular fractionation was performed as described (25). Otherwise, cells were lysed in 1% Nonidet P-40-containing lysis buffer (20 mM Tris-HCl, pH 8.0/0.15 M NaCl/1 mM EDTA/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/10 μg/ml aprotinin/10 μg/ml leupeptin/25 μM p-nitrophenol p’-guanidinobenzoate/1 μM pepstatin/0.1% sodium azide). Proteins in cleared cell lysates or subcellular fractions were either immunoprecipitated before or directly analyzed by SDS/PAGE followed by immunoblotting. Antibodies used for immunoprecipitation and blotting were anti-Btk (M138), anti-PKCa (C-20), anti-PKCB (C-16), anti-PKCBII (C-18), anti-PKCδ (C-20), anti-PKCε (C-15), anti-PKCa (C-15), anti-PKCb (C-20), anti-Lyn (44), anti-Syk (C-20), anti-JNK1 (C-17), anti-MEKK1 (C-22), anti-MEK4 (K-18) antibodies (all from Santa Cruz Biotechnology) unless otherwise mentioned, and proteins reactive with primary antibody were visualized with a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (NEN Life Science Products).

Kinase Assays. PKC assays were performed by two methods. Cells were lysed and immunoprecipitated with anti-PKCa, PKCβI, or PKCβII antibodies (Santa Cruz Biotechnology). In a protocol,
the immunoprecipitates were subjected to autophosphorylation. Fifty-microliter reactions in kinase buffer (20 mM Tris, pH 7.4/10 mM MgCl₂/10 μM ATP) in the presence of 10 μCi [γ-32P]ATP were analyzed by SDS/PAGE followed by electroblotting and autoradiography. In another protocol, PKCβI immunoprecipitates were incubated in the same buffer with a peptide substrate based on myelin basic protein (EKPRSQ-R-KYL) plus 10 μCi [γ-32P]ATP and cofactors (Ca²⁺, diacylglycerol, and phosphatidyserine). Radioactivity incorporated into phosphorylated peptides that were recovered with SpinZyme separation units (Pierce) was counted. Btk activity was measured by autophosphorylation reactions of immune complexes precipitated by anti-Btk (M138). For JNK assays, anti-HA (12CA5; Roche Molecular Biochemicals) immunoprecipitates were incubated with 3 μg GST-c-Jun (1–79) in 15-min reactions at 30°C in 20 mM Hepes, pH 7.4, 10 mM MgCl₂, 22 mM DTT, 20 mM β-glycerophosphate, 50 μM Na₃VO₄, 20 μM ATP, and 10 μCi [γ-32P]ATP. Reactions were analyzed by SDS/PAGE, blotting, and autoradiography.

**Results**

**Btk Regulates Membrane Translocation and Activation of PKCβI**. In light of the recent studies that Btk regulates the sustained increase of intracellular Ca²⁺ levels induced by BCR stimulation (16, 17), we compared IP₃ production in FceRI-stimulated bone marrow-derived mast cells from wt and btk— mice. As expected, btk— mast cells produced 40% less amounts of IP₃ at their peak (30 s after FceRI stimulation) compared with wt cells (unpublished observations). This data and the defective calcium response in receptor-engaged Btk-deficient B and mast cells (14–17) suggest that the activity of cPKC isoforms may be affected by btk mutation. Therefore, we examined the subcellular locations and activities of these PKC isoforms in mast cells, except for the γ isoform that is not expressed in mast cells. Mast cells were fractionated into the cytosolic and particulate compartments. As reported previously (26), a time-dependent translocation of cPKC isoforms from the cytosol to the particulate (=membrane) compartment was observed on FceRI crosslinking in wt cells. PKCβI levels in the particulate fraction were significantly reduced in both resting and FceRI-stimulated btk— cells compared with wt cells (Fig. 1A). Thus, PKCβI levels in the particulate fraction were higher than those in the cytosol from 1 to 30 min after FceRI stimulation in wt mast cells, whereas the cytosolic PKCβI was more abundant than the particulate PKCβI in btk— cells during the same stimulation period. Surprisingly, however, the translocation of PKCβI was largely intact in btk— cells, whereas that of PKCβII in btk— cells was reduced variably (by 10–30%) from experiment to experiment. These results indicate that the reduced Ca²⁺ response observed in btk— mast cells is strong enough to induce the translocation of the α isoform of PKC. Next, autophosphorylating activities of immunoprecipitated PKC were measured. Six major autophosphorylation sites (27) and three in vivo serine/threonine phosphorylation sites (28, 29) of PKCβII were mapped and are conserved in PKCβI (30, 31). FceRI crosslinking in wt mast cells induced a marked enhancement of PKCβI activity, whereas the activities of PKCα and PKCβII were weakly increased (less than 2-fold over the basal level) at 3–15 min after FceRI stimulation (Fig. 1B). In parallel with the reduced membrane translocation, the activation of PKCβI was drastically reduced in btk— cells, whereas the activation of PKCα and PKCβII was only slightly affected by btk mutation (Fig. 1B). Lyn— cells almost totally lost the autophosphorylating activity of PKCβI, consistent with the previous data that Lyn phosphorylates and activates Btk (32–34). In contrast, Lyn deficiency did not affect the PKCα activity, whereas the PKCβII activity was rather higher in lyn— cells than wt cells. Neither btk nor lyn mutations affected the expression of these PKCs (Fig. 1B). In another type of in vitro PKC assay, phosphorylation of a peptide substrate by PKCβI immunoprecipitated from resting or FceRI-stimulated wt mast cells was higher than that from btk— cells (Fig. 1C), consistent with the data that higher levels of PKCβI were present in the particulate compartment in both resting and FceRI-stimulated wt mast cells than in the corresponding btk— cells (Fig. 1A). We also examined the effect of terriec acid, a specific inhibitor of Btk (35), on PKCs in mast cells. Terriec acid inhibited the autophosphorylating activity of PKCβI, but not PKCβII, in a concentration-dependent manner with a half maximal inhibitory concentration (IC₅₀) of ~8 μM (Fig. 1D), although terriec acid did not directly inhibit the activity of these PKC isoforms (35). This IC₅₀ value for

![Fig. 1. Btk is required for the membrane translocation and activation of PKCβII.](https://www.pnas.org/content/116/11/3924)
PKCβI inhibition is similar to that of Btk inhibition by terreic acid (35). Furthermore, the FcεRI-induced activation of PKCβI was enhanced in btk− mast cells by transfecting with wt btk cDNA, but not empty vector or kinase-dead (K430R) btk cDNA (8) (Fig. 1E). The activities of PKCβII or PKCα in these transfected mast cells were minimally affected by the presence of wt Btk, but K430R Btk not only abrogated FcεRI-induced PKCβI activation but also affected the activation of PKCa and PKCβII, albeit to lesser extents. These last data indicate that the kinase-dead (K430R) mutant of PKCβI works as a dominant negative inhibitor of PKCβI as well as closely related other cPKC isoforms. Collectively, these data demonstrate that the activity of PKCβI among cPKC isoforms is specifically regulated by the enzymatic activity of Btk in mast cells.

Syk Regulates the Activity of Btk and PKCβI. Previous studies showed that Lyn can phosphorylate and activate both Btk and Syk (32–34, 36). We examined whether Btk and Syk independently operate in mast cells. Our previous experiments with btk− mast cells showed that Syk activation on FcεRI crosslinking is not affected by the absence of Btk (unpublished observations). Similar to chicken DT-40 B cells (37), however, basal and FcεRI-induced Btk activities were drastically reduced in syk− RBL-2H3 cells, whereas Lyn activities were intact in these cells (Fig. 2A). Therefore, both Lyn and Syk are required for a full activation of Btk. These results also suggest that PKCβI activation requires both Syk and Btk. To directly examine the role of Syk in PKCβI regulation, the activity of PKCβI was compared between Syk-deficient (syk−) and Syk-sufficient RBL-2H3 rat mast cells (13) (Fig. 2B). The autophosphorylating activity of PKCβI in syk− RBL-2H3 cells was drastically reduced compared with that in wt cells (data not shown). Transfection of syk− RBL-2H3 cells with syk cDNA reconstituted activation of PKCβI. Therefore, we conclude that PKCβI is regulated by Syk.

PKCβI Regulates the JNK Pathway and Cytokine Production. To evaluate the physiological significance of PKCβI regulation by Syk and Btk, we sought the signaling pathways controlled by PKCβI in mast cells. Because btk mutations affect the JNK pathway leading to defective cytokine gene transcription in mast cells (8, 23, 24), we investigated the possibility that PKCβI is involved in the activation of this pathway. First, overexpression of PKCβI in COS-7 cells enhanced the JNK activity (Fig. 3A). However, PKCα and PKCβII also exhibited the same ability, an observation which is consistent with a recent report that PKCβI regulates the JNK pathway by interacting directly with MEKK1 in human myeloid leukemia cells (38). Next, wt or kinase-dead (K371R) PKCβI cDNAs were transfected into mouse mast cells together with a reporter plasmid (HA-tagged JNK1). Activity of anti-HA immunoprecipitated JNK in wt PKCβI-transfected mast cells was higher than that in vector-transfected cells on FcεRI stimulation. K371R PKCβI-transfected cells exhibited a significantly lower JNK activity than the vector control, indicating that JNK is regulated by PKCβI in mast cells (Fig. 3B). PKCβI-dependent
activation of JNK is consistent with the dependence of JNK activation on the PKCβ1 regulators, i.e., Syk and Btk. JNK1 activation was observed in FceRI-stimulated Syk-sufficient RBL-2H3 cells, whereas it was blunted in Syk-deficient cells (Fig. 2B). Similarly, in our previous study (23), we described severe defects in JNK activation in btk mutant mast cells. We also examined effects of PKC overexpression on transcriptional activity of the IL-2 (IL-2Luc) and tumor necrosis factor (TNF-α) (TNF-αLuc) promoters in FceRI-stimulated mast cells. The results clearly demonstrate that PKCβ1, as well as PKCe and PKCβII, can regulate the transcriptional activation of these promoters on FceRI crosslinking (Fig. 3C). These observations are consistent with the recent report that overexpression of wt PKCβ1 enhanced FceRI-induced expression of the IL-2 gene in RBL-2H3 cells (30).

Consistent with the ability of PKCβI to activate the JNK pathway, production of TNF-α and IL-2 in FceRI-stimulated mast cells was sensitive not only to a general PKC inhibitor, RO31-8425, and a cPKC-selective inhibitor, Go 6976, but also to a PKCβ-selective inhibitor, LY379196 (Fig. 4A). Furthermore, TNF-α production from FceRI-stimulated xid mast cells, that was 3–5 times lower than that from wt cells (8), was largely recovered in the presence of phorbol 12-myristate 13-acetate (PMA) (Fig. 4B). These data support the notion that PKC operates downstream of Btk for the cytokine production. We recently reported that terreic acid inhibits production of TNF-α and IL-2 in FceRI-stimulated mast cells (35). As expected from Btk regulation by Syk, two Syk-selective inhibitors, piceatannol and ER-27319, suppressed FceRI-induced transcriptional activation of the TNF-α gene promoter and cytokine secretion (Fig. 4C). Collectively, we conclude that PKCβ1 is involved in cytokine gene activation by means of the JNK pathway in a Btk- and Syk-dependent manner, although the contribution to the cytokine production by other PKC isoforms is not ruled out.

PKCβ1 Specifically Interacts with Btk and Other Upstream and Downstream Signaling Proteins. Signaling proteins form supramolecular complexes to attain the specificity and amplitude required for proper signal transduction. To obtain insight into how PKCβ1, not other cPKC isoforms, is specifically regulated by Btk, we examined whether Btk interacts with these and other PKC isoforms in wt mast cells. Immunoblotting of anti-Btk immunoprecipitates with isomform-specific antibodies revealed the specific and constitutive association of Btk with PKCβ1, not any other PKC isoforms (Fig. 5A), confirming our earlier study (40). We also found the association of PKCβ1 with Btk in WEHI-231 B cells (data not shown). To extend this line of study, we examined whether PKCβ1 interacts with other signaling proteins that operate along the pathway upstream and downstream of PKCβ1. A very low level (~2% of PKCβ1 and 0.3% of constitutive association of PKCβ1 with Syk was detected by reciprocal coimmunoprecipitation (Fig. 5B). A low-stoichiometry, constitutive association of PKCβ1 with MEKK1 (about 2% of PKCβ1) was also detected, whereas no significant association was found between PKCβ1 and either MKK4 (~SEK1/MEK4) or JNK1. These findings are consistent with previous reports that production of IL-2 and TNF-α in mast cells is regulated by MEKK1 (24, 41). However, none of the upstream regulators of PKCβ1, i.e., Lyn, Btk, and Syk, interacts significantly with MEKK1 (data not shown). These results indicate the demarcation of the signaling pathway at the level of PKCβ1 that separates the membrane-proximal signaling proteins, e.g., Lyn, Syk, and Btk, from the distal signaling proteins, e.g., MEKK1, MKK4, JNK1/2, and c-Jun. Consistent with the ability of PKCβII and PKCe to regulate the JNK pathway, these PKC isoforms coimmunoprecipitated with Syk and MEKK1 at low levels (<3%), but not with Btk. Specific physical interactions of Btk with PKCβ1 may reassure the specificity and proper intensity in signal transduction along this pathway.

Discussion

In the present study, we have defined a signaling pathway activated by FceRI crosslinking (Fig. 6): Btk regulates PKCβ1 in
Accumulated studies indicate that tyrosine phosphorylated BLNK recruits PLC-γ2 and Btk by means of SH2-phosphotyrosine interactions and that Btk and Syk phosphorylate and activate PLC-γ2 in a concerted manner (11, 15, 45–47). Similar mechanisms may exist in mast cells. However, BLNK might not be expressed in mast cells because a model mast cell line RBL-1 does not express BLNK (48). Similar functions may be played by other linker proteins, such as SLP-76, a BLNK homologue in T cells, LAT (49, 50), and ClnK (51), which are expressed in mast cells. SLP-76 was shown to be required for FceRI-induced mast cell activation (52). However, tyrosine phosphorylation levels of SLP-76, LAT, and Vav in FceRI-stimulated btk– mast cells were similar to those in wt cells (data not shown), in contrast with the proposed pathway of Vav to Rac1 to JNK (53). Confounding this issue further is the redundant presence of the differentially regulated two PLC-γ isoforms (54) and Btk-related kinases, Emt/Btk (55) and Tec (56), in mast cells. Studies are underway on how PLC-γ1, PLC-γ2, and JNK are regulated by the above linker proteins, Lyn, Syk, Btk, Emt/Btk, and Tec, in mast cells.

The present study focused on the cPKC isoforms expressed in mast cells. It was somewhat surprising that FceRI induces a very robust (3- to 10-fold activity over the baseline level depending on assays) activation of PKCβI compared with a weak (<2-fold) activation of PKCβII and PKCd. Mechanistic basis for this difference remains to be defined. However, direct regulation of PKCβI through tyrosine phosphorylation by Btk was ruled out as a potential mechanism because recombinant Btk or Btk immunoprecipitated from activated mast cells or B cells did not phosphorylate PKCβI in vitro (data not shown). Specific association of PKCβI with Btk might be important for the robust activation of PKCβI. However, it is impossible to explain this phenomenon by the differential sensitivity of PKCβI vs. PKCβII to Ca2+ or diacylglycerol. Thus, PKCβI is 1 order of magnitude more sensitive to Ca2+ than PKCβII (57), and there is no difference in sensitivity to diacylglycerol between PKCβI and PKCβII (58). PKCβI-interacting protein(s), whose expression, activity, or association with PKCβI is under the direct or indirect control of Btk or another upstream signaling molecule, might be involved in differential activation of PKC isoforms. Both Btk and conventional PKC isoforms translocate to membranes in a similar time course (25). Therefore, the putative PKCβI-interacting protein might be involved in membrane translocation of PKCβI in a Btk-dependent manner. In any case, the Lyn/Syk/Btk/PKCβI/JNK signaling pathway defined in this study may be an attractive target of pharmaceutical interference for the treatment of allergic and other immune diseases.

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