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 (FcεRI) in mast cells. The present study provides genetic, biochemical, and pharmacological evidence that, on FcεRI stimulation, Syk regulates Btk, and Btk selectively regulates the membrane translocation and enzymatic activity of PKCβI among the conventional PKC isoforms (α, βI, and βII) 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 FcεRI stimulation. Accordingly, FcεRI-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 FcεRI stimulation (8). *Syk* gene inactivation results in profound hematopoietic defects, including B cell development (9, 10). Loss of Syk expression ablates B cell receptor (BCR)- or FcεRI-mediated cell activation (11–13). Engagement of BCR and FcεRI 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 Ca^{2+} response on BCR or FcεRI 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 (IP_3). Diacylglycerol activates several protein kinase C (PKC) isoforms, and IP_3 recruits Ca^{2+} 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 Ca^{2+}/diacylglycerol-regulated conventional isoforms (cPKC: α, βI, βII, and γ), the Ca^{2+}-independent but diacylglycerol-regulated novel isoforms (nPKC: δ, ε, η, θ, and μ), and the Ca^{2+}/diacylglycerol-independent atypical isoforms (aPKC: ζ 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 PKC cDNAs. 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*-nitrophenyl *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-PKCα (C-20), anti-PKCβI (C-16), anti-PKCβII (C-18), anti-PKCδ (C-20), anti-PKCε (C-15), anti-PKCη (C-15), anti-PKCθ (C-18), anti-PKCζ (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-PKCα, PKCβI, or PKCβII antibodies (Santa Cruz Biotechnology). In a protocol,

Abbreviations: BCR, B cell receptor; Btk, Bruton's tyrosine kinase; DNP, dinitrophenyl; PKC, protein kinase C; FcεRI, high-affinity IgE receptors; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; TNF, tumor necrosis factor; wt, wild type; *xid*, X-linked immunodeficiency; PMA, phorbol 12-myristate 13-acetate; HA, hemagglutinin; GST, glutathione *S*-transferase.

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the immunoprecipitates were subjected to autophosphorylation. Fifty-microliter reactions in kinase buffer (20 mM Tris, pH 7.4/10 mM $MgCl_2$/10 μM ATP) in the presence of 10 μCi [γ-^{32}P]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 (EKRPSQRSKYL) plus 10 μCi [γ-^{32}P]ATP and cofactors (Ca^{2+}, diacylglycerol, and phosphatidylserine). 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 $MgCl_2$, 22 mM DTT, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, 20 μM ATP, and 10 μCi [γ-^{32}P]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 Ca^{2+} levels induced by BCR stimulation (16, 17), we compared IP_3 production in FcεRI-stimulated bone marrow-derived mast cells from wt and *btk*− mice. As expected, *btk*− mast cells produced 40% less amounts of IP_3 at their peak (30 s after FcεRI 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 FcεRI crosslinking in wt cells. PKCβI levels in the particulate fraction were significantly reduced in both resting and FcεRI-stimulated *btk*− cells compared with wt cells (Fig. 1*A*). Thus, PKCβI levels in the particulate fraction were higher than those in the cytosol from 1 to 30 min after FcεRI 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 PKCα 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 Ca^{2+} 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). FcεRI 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 FcεRI stimulation (Fig. 1*B*). 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. 1*B*). *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. 1*B*). In another type of *in vitro* PKC assay, phosphorylation of a peptide substrate by PKCβI immunoprecipitated from resting or FcεRI-stimulated wt mast cells was higher than that from *btk*− cells (Fig. 1*C*), consistent with the data that higher levels of PKCβI were present in the particulate compartment in both resting and FcεRI-stimulated wt mast cells than in the corresponding *btk*− cells (Fig. 1*A*). We also examined the effect of terreic acid, a specific inhibitor of Btk (35), on PKCs in mast cells. Terreic acid inhibited the autophosphorylating activity of PKCβI, but not PKCβII, in a concentration-dependent manner with a half maximal inhibitory concentration (IC_{50}) of $\approx$8 μM (Fig. 1*D*), although terreic acid did not directly inhibit the activity of these PKC isoforms (35). This IC_{50} value for

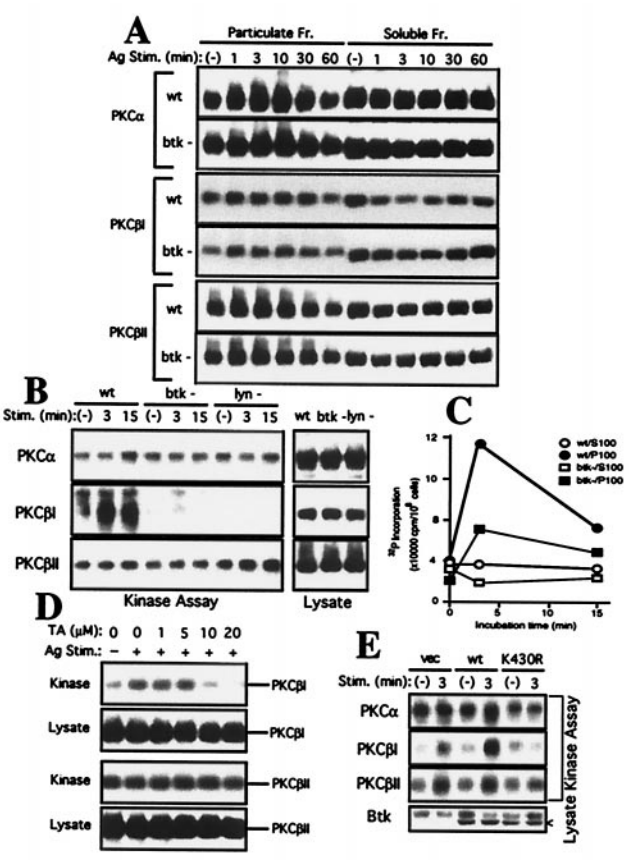

Fig. 1. Btk is required for the membrane translocation and activation of PKCβI. (*A*) Mast cells from wt or *btk*− mice were passively sensitized with anti-DNP IgE antibody, and stimulated by an antigen, 100 ng/ml DNP conjugates of human serum albumin, for the indicated intervals. Cells were fractionated into particulate and cytosolic compartments. These fractions were analyzed by immunoblotting with anti-PKCα, PKCβI, or PKCβII antibodies. (*B*) Mast cells from wt, *btk*−, or *lyn*− mice were stimulated as above. Cells were lysed and immunoprecipitated with anti-PKCα, PKCβI, or PKCβII antibodies. The immunoprecipitates were subjected to autophosphorylation. Reactions were analyzed by SDS/PAGE followed by electroblotting and autoradiography (*Left*). Portions of cell lysates were analyzed by immunoblotting by the above antibodies (*Right*). (*C*) Wt or *btk*− mast cells were fractionated into particulate (P100) and cytosolic (S100) compartments. PKCβI immunoprecipitates were incubated with a peptide substrate in the presence of [γ-^{32}P]ATP and cofactors. Radioactivity incorporated into phosphorylated peptides were counted. (*D*) Wt mast cells sensitized with anti-DNP IgE were treated with various concentrations of terreic acid for 30 min before antigen stimulation for 3 min. Autophosphorylating activities of PKCβI and PKCβII were measured as described above. (*E*) *Btk*− mast cells transfected with an empty retroviral vector (vec) or vectors harboring wt or K430R *btk* cDNAs were stimulated, and autophosphorylating activities of PKCα, PKCβI, or PKCβII were analyzed as above. Expression of transfected Btk is confirmed by immunoblotting of total cell lysates with anti-Btk. Btk is indicated by "<."

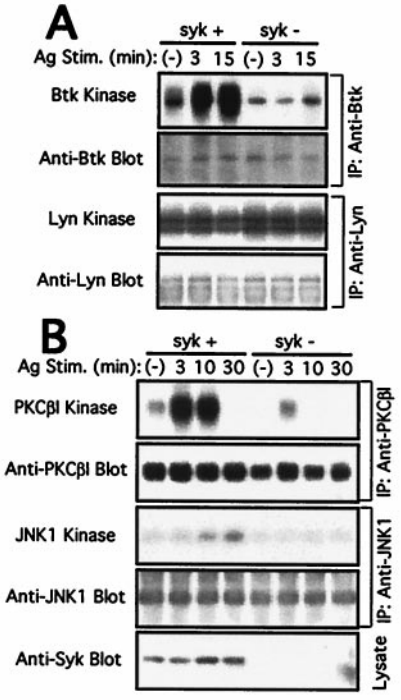

Fig. 2. Activation of Btk and PKCβI depends on Syk. (*A*) *Syk*− or *syk*-reconstituted *syk*− RBL-2H3 cells were sensitized with anti-DNP IgE and stimulated by antigen. Immunoprecipitates with anti-Btk and anti-Lyn were subjected to autophosphorylation (Btk Kinase and Lyn Kinase). The same blots were probed with the respective antibodies (Blots). (*B*) Anti-PKCβI immunoprecipitates were prepared from similarly stimulated *syk*− or *syk*-reconstituted *syk*− RBL-2H3 cells and followed by autophosphorylating reactions (PKCβI Kinase). The same blot was probed with anti-PKCβI antibody (PKCβI Blot). Anti-JNK1 immunoprecipitates were subjected to kinase assays using GST-c-Jun (1–79) as substrate. Reaction products were analyzed by SDS/PAGE, electroblotting, and autoradiography (JNK1 Kinase). The same blot was probed with anti-anti-JNK1 antibody (Anti-JNK1 Blot). Syk deficiency of *syk*− cells was confirmed by immunoblotting of total cell lysates (Anti-Syk Blot).

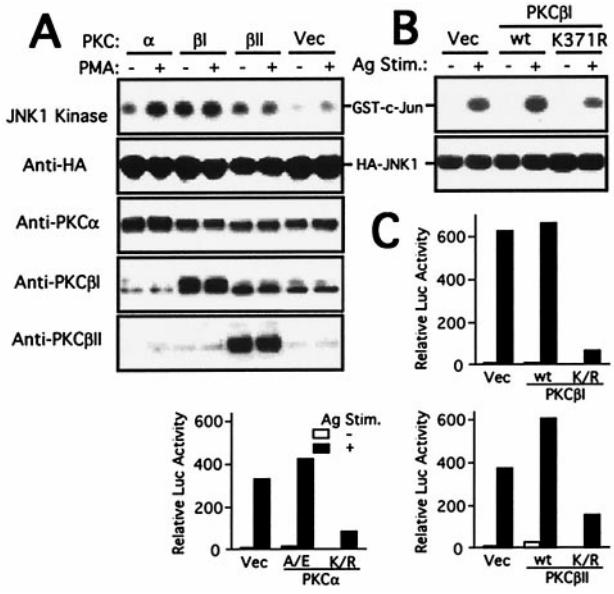

Fig. 3. PKCβI regulates the JNK pathway and cytokine gene expression. (*A*) COS-7 cells were transfected with HA-JNK1 plasmid together with an empty vector (vec), wt PKCα, wt PKCβI, or wt PKCβII vectors. Forty-eight hours later, cells were left unstimulated or stimulated with 100 nM PMA for 10 min. Cell lysates were immunoprecipitated with anti-HA, and immunoprecipitates were incubated with GST-c-Jun (1–79) in the presence of [γ-^{32}P]ATP. Reactions were analyzed by SDS/PAGE and autoradiography (*Top*). Expression of HA-JNK1, PKCα, PKCβI, or PKCβII was detected by immunoblotting of total cell lysates. (*B*) Bone marrow-derived mast cells from wt mice were transfected with wt or kinase-dead (K371R) PKCβI cDNAs together with HA-JNK1 plasmid. Forty-eight hours later, cells were stimulated with IgE and antigen. Anti-HA immunoprecipitates were subjected to kinase assays. (*C*) Wt mast cells were transfected with IL-2Luc plasmid (8 μg) together with wt or mutant cPKC cDNAs (20 μg each). Cells were stimulated with IgE and antigen for 8 h. Luciferase activity was measured. PKCα A/E is a constitutively active form of PKCα. Similar data were obtained with TNF-αLuc plasmid (not shown).

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. 1*E*). 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 PKCα 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. 2*A*). 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. 2*B*). 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. 3*A*). However, PKCα and PKCβII also exhibited the same ability, an observation which is consistent with a recent report that PKCβII 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. 3*B*). PKCβI-dependent

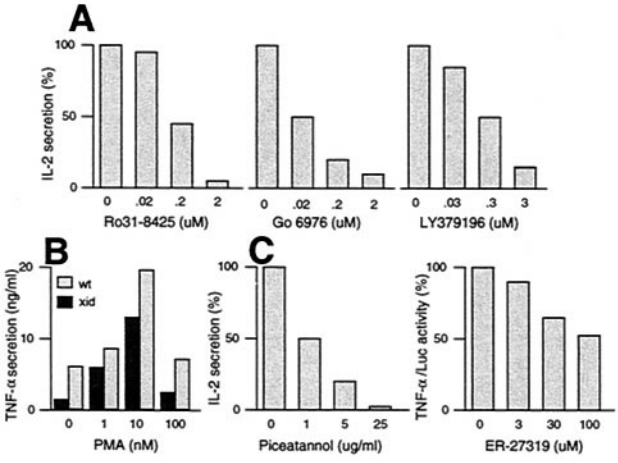

Fig. 4. Pharmacological regulation of cytokine response to FcεRI stimulation in mouse mast cells. IgE-sensitized wt mast cells were pretreated with the indicated concentrations of PKC inhibitors [Ro 31-8425, Go 6976, and LY379196 (*A*)] or Syk inhibitor [piceatannol (*C, Left*)] for 30 min and stimulated by antigen for 20 h before sampling supernatants for IL-2 ELISA assays. (*B*) Mast cells from wt and *xid* mice were similarly sensitized with IgE and stimulated by antigen in the presence or absence of PMA for 24 h before sampling supernatants for TNF-α assays (*C, Right*). Wt mast cells were transfected with TNF-αLuc plasmid. Twenty-four hours later, cells were overnight sensitized with IgE and stimulated by antigen in the presence or absence of ER-27319 for 8 h before luciferase assays.

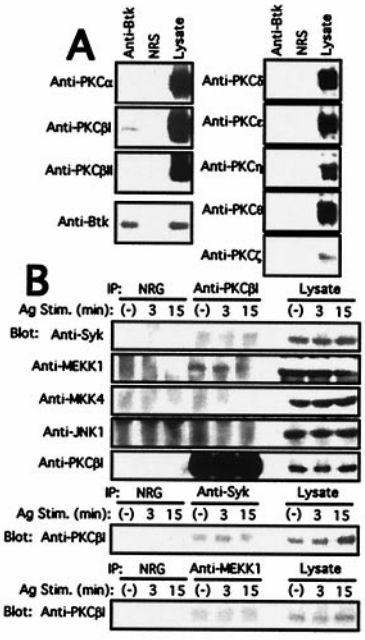

Fig. 5. Association of PKCβI with upstream and downstream signaling molecules. (*A*) Bone marrow-derived mast cells from wt mice were immunoprecipitated with anti-BtkC antibody (40) or normal rabbit serum (NRS). Immune complexes were analyzed by SDS/PAGE and immunoblotting with PKC isoform-specific antibodies as indicated. (*B*) MCP-5 mast cells were stimulated with IgE and antigen. Cell lysates were precipitated with antibodies specific for PKCβI, Syk, MEKK1, or MKK4. Immunoprecipitates were analyzed by immunoblotting with the indicated antibodies.

activation of JNK is consistent with the dependence of JNK activation on the PKCβI regulators, i.e., Syk and Btk. JNK1 activation was observed in FcεRI-stimulated Syk-sufficient RBL-2H3 cells, whereas it was blunted in Syk-deficient cells (Fig. 2*B*). 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 FcεRI-stimulated mast cells. The results clearly demonstrate that PKCβI, as well as PKCα and PKCβII, can regulate the transcriptional activation of these promoters on FcεRI crosslinking (Fig. 3*C*). These observations are consistent with the recent report that overexpression of wt PKCβI enhanced FcεRI-induced expression of the IL-2 gene in RBL-2H3 cells (39).

Consistent with the ability of PKCβI to activate the JNK pathway, production of TNF-α and IL-2 in FcεRI-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. 4*A*). Furthermore, TNF-α production from FcεRI-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. 4*B*). 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 FcεRI-stimulated mast cells (35). As expected from Btk regulation by Syk, two Syk-selective inhibitors, piceatannol and ER-27319, suppressed FcεRI-induced transcriptional activation of the TNF-α gene promoter and cytokine secretion (Fig. 4*C*). Collectively, we conclude that PKCβI 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.

PKCβI 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 PKCβI, 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 isoform-specific antibodies revealed the specific and constitutive association of Btk with PKCβI, not any other PKC isoforms (Fig. 5*A*), confirming our earlier study (40). We also found the association of PKCβI with Btk in WEHI-231 B cells (data not shown). To extend this line of study, we examined whether PKCβI interacts with other signaling proteins that operate along the pathway upstream and downstream of PKCβI. A very low level (≈2% of PKCβI and 0.3% of Syk) of constitutive association of PKCβI with Syk was detected by reciprocal coimmunoprecipitation (Fig. 5*B*). A low-stoichiometry, constitutive association of PKCβI with MEKK1 (about 2% of PKCβI) was also detected, whereas no significant association was found between PKCβI 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 PKCβI, 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 PKCβI 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 PKCα 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 PKCβI 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 FcεRI crosslinking (Fig. 6): Btk regulates PKCβI in

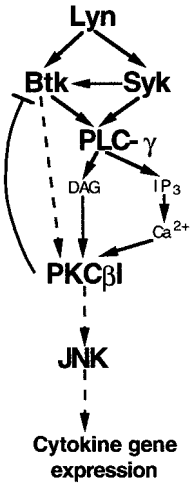

Fig. 6. The signaling pathway from the upstream protein-tyrosine kinases, Lyn/Syk/Btk, to cytokine gene expression through intermediate signaling proteins, PKCβI/JNK. For the sake of simplicity, potential contribution of PKCα and PKCβII to JNK activation is omitted here. Signaling proteins are depicted in bold letters and nonprotein messengers in plain letters. Direct signal transmission by catalytic function is indicated by solid lines and indirect transmission by dashed lines.

a Lyn- and Syk-dependent manner. PKCβI activation in turn leads to the activation of JNK and eventually results in the transcriptional activation of cytokine genes. Activation of this pathway should be important in the late phase response in IgE/allergen-dependent anaphylactic reactions because this phase depends in part on TNF-α secreted from activated mast cells (42). This pathway seems to operate in B cells as well, because BCR-mediated JNK1 activation, which also depends on Syk and Btk, depends on intracellular calcium and PMA-sensitive PKCs (43). Therefore, Btk-mediated PKCβI activation is probably the underlying mechanism by which identical phenotypic defects in humoral immunity were found in both *btk* mutant (*xid* and *btk*$-$) and PKCβ knockout mice (44). Given our previous findings that PKC phosphorylates Btk and down-regulates the Btk activity (40), Btk and PKCβI may form a negative feedback loop in the FcεRI signaling system: First, Btk, which is activated by Lyn and Syk, activates PKCβI, and then active PKCβI down-regulates the Btk activity. This may represent an important mechanism for maintaining the dynamic homeostasis of Btk activity in the signaling network. However, it is noteworthy that PKCα and PKCβII may also contribute to JNK activation leading to cytokine gene activation on FcεRI stimulation.

In B cells, the activation of PLC-γ2 and JNK involves the tyrosine phosphorylation of a linker protein BLNK by Syk (45). 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 FcεRI-induced mast cell activation (52). However, tyrosine phosphorylation levels of SLP-76, LAT, and Vav in FcεRI-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/Itk (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/Itk, and Tec, in mast cells.

The present study focused on the cPKC isoforms expressed in mast cells. It was somewhat surprising that FcεRI 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 PKCα. 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 Ca^{2+} or diacylglycerol. Thus, PKCβI is 1 order of magnitude more sensitive to Ca^{2+} than PKCβII (57), and there is no difference in sensitivity to diacylglycerol between PKCβI and PKCβII (58). A 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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