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Immunology

The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C

(mast cell/FcεRI/signal transduction)

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ABSTRACT **Bruton tyrosine kinase (EC 2.7.1.112) [Btk, encoded by *Btk* in mice and BTK in humans (formerly known as *atk*, *BPK*, or *emb*)], which is variously mutated in chromosome X-linked agammaglobulinemia patients and X-linked immunodeficient (*xid*) mice, has the pleckstrin homology (PH) domain at its amino terminus. The PH domain of Btk expressed as a bacterial fusion protein directly interacts with protein kinase C in mast cell lysates. Evidence was obtained that Btk is physically associated with protein kinase C in intact murine mast cells as well. Both Ca^{2+}-dependent (α, βI, and βII) and Ca^{2+}-independent protein kinase C isoforms (ε and ζ) in mast cells interact with the PH domain of Btk *in vitro*, and protein kinase C βI is associated with Btk *in vivo*. Btk served as a substrate of protein kinase C, and its enzymatic activity was down-regulated by protein kinase C-mediated phosphorylation. Furthermore, depletion or inhibition of protein kinase C with pharmacological agents resulted in an enhancement of the tyrosine phosphorylation of Btk induced by mast cell activation.**

Cross-linking of the high-affinity IgE receptor (FcεRI) on mast cells and basophils as well as antigen receptors on T and B lymphocytes induces activation of a variety of intracellular enzymes, including protein-tyrosine kinases and protein kinase C (PKC in humans and rats and Pkc in mice) (1, 2). Cytoplasmic protein-tyrosine kinases closely associated with the signal-transducing subunits of the receptor are activated at early stages of cell activation, leading to tyrosine phosphorylation and activation of phospholipase C_γ. We recently demonstrated that Bruton tyrosine kinase (Btk; EC 2.7.1.112) in mouse mast cells is activated upon cross-linking of FcεRI (34), suggesting a possible role of this enzyme in the receptor-generated signal-transduction pathway. The *Btk* gene in mice (*BTK* in humans) (3–5) encoding the 77-kDa protein is variously mutated in chromosome X-linked agammaglobulinemia patients (3, 4) and X-linked immunodeficient (*xid*) mice (6, 7). The presence of the amino-terminal pleckstrin homology (PH) domains as well as Src homology (SH) domains SH2 and SH3 is characteristic of the Tec subfamily of protein-tyrosine kinases, which include Btk, Emt (=Itk/Tsk) (5), and Tec II (8).

The PH domain, composed of ≈100 loosely conserved amino acids, was originally recognized as comprising repeated sequences in pleckstrin (9), a prominent PKC substrate. The PH domain was found in many proteins, including protein kinases, guanosine triphosphatases, guanosine triphosphatase-activating proteins, nucleotide exchange factors, and phospholipase C (10–12). The distribution of PH domains among signaling proteins raises the possibility that PH domains serve as sequences for protein–protein interactions in the network of signaling proteins. In the light of the observation that cross-linking of FcεRI induces activation of both Btk and PKC, the possibility was considered that Btk may interact with PKC through its PH domain.

The present study was undertaken to determine whether a bacterial fusion protein containing the PH domain (residues 1–137 according to ref. 12) of mouse Btk interacts with PKC and whether Btk is physically associated with PKC in mast cells. Effects of the interaction between Btk and PKC *in vitro* and in mast cells will be described.

MATERIALS AND METHODS

Antibodies. Anti-BtkC antibody was raised in rabbits against the mouse Btk carboxyl-terminal peptide Lys-Ile-Leu-Leu-Ser-Asn-Ile-Leu-Asp-Val-Met-Asp-Glu-Glu-Ser as described (13). A monoclonal anti-bovine PKC antibody (mAb) MC5 reacts with α, β, and γ isoforms and isoform-specific polyclonal antibodies against PKC βI, βII, γ, δ, ε, ζ, η, or θ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-PKC α, β, δ, ε, and θ antibodies were from Transduction Laboratories. Anti-rabbit PKC α and anti-phosphotyrosine 4G10 mAbs were obtained from Upstate Biotechnology (Lake Placid, NY). All the used isoform-specific antibodies react with respective rodent PKC isoforms. Mouse anti-dinitrophenyl (DNP) IgE mAb (14) was provided by K. Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Polyclonal anti-glutathione *S*-transferase (GST) antibody was from W. Northemann (ELIAS Entwicklungslabor).

Cells and Their Activation. Cultures of bone marrow-derived mouse mast cells (BMMC) have been described (15). BMMC and an immortalized BMMC line, MCP-5 (ref. 16; a gift from D. D. Metcalfe, National Institutes of Health, Bethesda, MD), were incubated overnight with 1 μg of anti-DNP IgE mAb per ml, and the sensitized cells were incubated for 2–3 min with a multivalent antigen, DNP derivatives of human serum albumin (15). Mast cell activation was monitored by tyrosine phosphorylation of cellular proteins by immunoblotting and by the measurement of histamine release (35–70% of the total cellular histamine content). Details have been described (15). In some experiments, cells were treated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) during the sensitization period with anti-DNP IgE, with 0.1 μM calphostin C (LC Services, Woburn, MA) for 30 min, or with 2 μM Ro 31-8425 (a gift from K. Yamada, Eisai, Tsukuba) for 10 min prior to antigen stimulation.

***In Vitro* Binding Assay with Immobilized Bacterial Fusion Proteins.** GST fusion proteins were engineered by polymerase chain reaction-assisted cloning. The mouse Btk or Emt

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Abbreviations: Btk, mouse Bruton tyrosine kinase; BMMC, bone marrow-derived mouse mast cells; DNP, dinitrophenyl; FcεRI, high-affinity IgE receptor; mAb, monoclonal antibody; PH, pleckstrin homology; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SH, Src homology; GST, glutathione *S*-transferase.

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cDNA sequences (5), corresponding to the initiation codon through residue 138 (GST-BtkPH) or residue 109 (GST-EmtPH), were amplified and cloned into pGEX-3T vector (17). Amounts and purities of recombinant proteins expressed in *Escherichia coli* were assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and Coomassie brilliant blue staining. The purity of GST fusion proteins was 60–70%. Most contaminants were degradation products of fusion proteins. Two micrograms of fusion proteins immobilized onto glutathione-agarose beads (Sigma) was mixed overnight at 4°C with lysates of BMMC or MCP-5 in 1% Nonidet P-40 (NP-40)/20 mM Tris·HCl, pH 8.0/0.15 M NaCl/0.1 mM $CaCl_2$/0.1 mM sodium orthovanadate/1 mM phenymethylsulfonyl fluoride/16.5 μg of aprotinin per ml/10 μg of leupeptin per ml/25 μM *p*-nitrophenyl *p'*-guanidinobenzoate/0.1% NaN_3 (NP-40 lysis buffer). After extensive washing (eight times) with lysis buffer, bound proteins were eluted with Laemmli sample buffer (35) and subjected to SDS/PAGE. PKC was detected by immunoblotting using anti-PKC (MC5) or antibodies specific for each PKC isoform and the enhanced chemiluminescence kit from Amersham.

PKC Binding Assay on Membrane. To label PKC with ^{32}P, 126 ng of rat brain PKC (α, β, and γ isoforms; >95% pure, Calbiochem) was incubated with 10 μCi (370 kBq) of [γ-^{32}P]ATP in the presence of phosphatidylserine, Ca^{2+}, and PMA (see below). Unincorporated [γ-^{32}P]ATP was removed from the probe by ultrafiltration through Centricon 10 (Amicon). GST or GST-BtkPH proteins were resolved by SDS/PAGE and blotted to a sheet of nitrocellulose. After denaturation and renaturation treatment (18), the membrane was incubated with ^{32}P-labeled rat brain PKC. PKC bound to GST-BtkPH was detected by autoradiography at −70°C for 8 hr.

Immunoprecipitation and Immunoblotting. Cells (2×10^7) were lysed in 300–500 μl of NP-40 lysis buffer. Immunoprecipitation and immunoblotting with anti-BtkC or anti-PKC were performed as described (19).

***In Vitro* Phosphorylation Reactions with PKC and Phosphoamino Acid Analysis.** Btk protein was partially purified as a major protein-tyrosine kinase to phosphorylate a cytoplasmic peptide of FcεRI γ subunit from rat basophilic leukemia RBL-2H3 cells by heparin-agarose, Mono Q, and CM-Sepharose chromatographies (D. J. Price, Y.K., T.K., and B. Rivnay, unpublished data). Partially purified Btk proteins (>80% pure) or affinity-purified GST-BtkPH was incubated with the purified rat brain PKC (α, β, and γ) in the presence of PKC activators (phosphatidylserine at 280 μg/ml, 1 mM $CaCl_2$, and 10 μM PMA) and [γ-^{32}P]ATP. Reaction products were analyzed by SDS/PAGE and blotted onto poly(vinylidene difluoride) membranes (Immobilon-P, Millipore). Phosphorylated proteins were visualized by autoradiography. Phosphoamino acid analysis of ^{32}P-labeled bands was performed as described (20). In some experiments, a PKC substrate, 50 μM Ac-MBP-(4–14) (N-terminally acetylated myelin basic protein-(4–14) hendecapeptide; GIBCO/BRL), was incubated with the purified rat brain PKC in the presence of 1- to 100-fold molar excesses of affinity-purified GST-BtkPH proteins. ^{32}P incorporation into the peptide was measured following the manufacturer's instruction (GIBCO/BRL).

Btk Autophosphorylation Assay. Partially purified rat RBL-2H3-derived Btk proteins were incubated for 30 min at 30°C with cold ATP and PKC activators described above in the presence or absence of the purified rat brain PKC. Btk in the mixtures was recovered as immune complexes with anti-BtkC or anti-PKC MC5 and was incubated with cold ATP (0.1 μM), 10 mM $MnCl_2$, and 2 mM $MgCl_2$ for 3 min at 25°C. Tyrosine autophosphorylation of Btk was detected by immunoblotting with anti-phosphotyrosine mAb 4G10.

RESULTS

PH Domain of Btk Binds Directly to PKC *in Vitro*. To test the possibility that the PH domain of Btk may interact with PKC, detergent lysates of BMMC were incubated with immobilized GST fusion proteins containing the wild-type (GST-BtkPH) or *xid* (GST-BtkPH[*xid*]) PH domain of Btk. Analysis of proteins bound to the beads by immunoblotting with the anti-PKC mAb MC5 showed that PKC bound to GST-BtkPH fusion proteins but not to GST (Fig. 1*A*). Essentially the same results were obtained with MCP-5 cell lysates. Interestingly, the *xid* mouse-derived PH domain of Btk with an amino acid substitution of cysteine for arginine at residue 28 (6, 7) showed a weaker binding capacity to PKC. In more quantitative experiments, 3- to 5-fold more GST-BtkPH(*xid*) proteins over the wild-type counterpart were necessary to give comparable signals of bound PKC, suggesting that residue Arg-28 in the PH domain is involved in PKC binding (data not shown). The GST fusion protein (GST-EmtPH) of the PH domain of the highly related tyrosine kinase Emt (=Itk, Tsk) also bound to PKC in the same assay.

We determined whether the binding of PKC to the PH domain of Btk is direct or indirect. A mixture of highly purified rat brain PKC α, β, and γ isoforms (>95% pure) was labeled with ^{32}P by autophosphorylation and incubated with the blot retaining the purified GST or GST-BtkPH protein. GST-BtkPH proteins, but not GST, bound PKC (Fig. 1*B*), indicating that the PH domain of Btk directly bound PKC. This result also demonstrated that autophosphorylation of PKC did not affect its interaction with the PH domain.

Since several isoforms of PKC are expressed in a rat mast cell model (21), we determined the Btk-interacting isoform in BMMC and MCP-5 by probing the blots retaining the proteins bound to GST-BtkPH beads with PKC isoform-specific antibodies. Both Ca^{2+}-dependent (α, βI, and βII) and Ca^{2+}-independent PKC isoforms (ε and ζ) bound to GST-BtkPH beads, while neither η nor θ, which are expressed in substantial amounts in BMMC and MCP-5, was detected among the proteins bound to GST-BtkPH beads (data not shown).

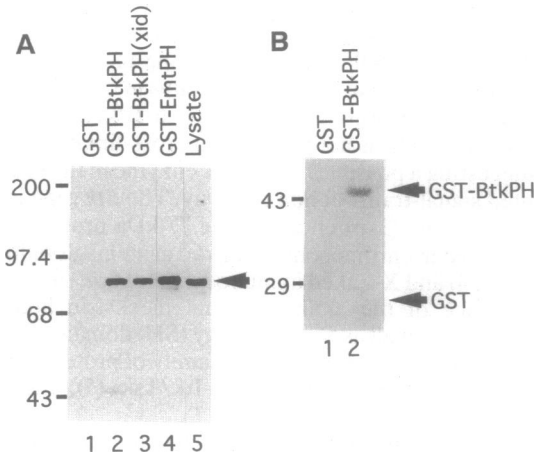

FIG. 1. *In vitro* binding of GST fusion proteins to PKC. (*A*) BMMC lysates (equivalent to 1×10^7 cells) were incubated with GST (lane 1) or GST fusion protein beads (lanes 2–4). PKC bound to beads was detected by immunoblotting with anti-PKC mAb MC5. PKC in the whole-cell lysate (equivalent to 1×10^5 cells) is shown in lane 5. The arrow indicates the position of PKC. Positions of molecular mass markers (in kilodaltons) are shown on the left. (*B*) GST-BtkPH proteins interact directly with PKC. A nitrocellulose membrane retaining 4 μg each of GST (lane 1) or GST-BtkPH (lane 2) was incubated with ^{32}P-labeled PKC. Bound PKC was detected by autoradiography. Positions of GST and GST-BtkPH proteins as determined by immunoblotting with anti-GST antibodies are indicated.

No detectable amount of PKC γ or δ isoform was found in the total cell lysates of BMMC or MCP-5 (data not shown).

Btk Is Physically Associated with PKC in Mast Cells. Experiments were carried out to determine whether PKC is associated with Btk in mast cells. Btk in the lysates of unstimulated or IgE/antigen-stimulated MCP-5 cells was immunoprecipitated with anti-BtkC antibodies, and immune complexes were analyzed by immunoblotting with anti-PKC (MC5). The anti-PKC-reactive ≈80-kDa band was clearly detected in both unstimulated and activated cells (Fig. 2*A*). Cross-linking of FcεRI showed little effect on the level of the coprecipitated PKC. Immunoblotting of the immune complexes with various isoform-specific antibodies revealed constitutive association of only PKC βI with Btk (data not shown). In reciprocal experiments, Btk was detected in the anti-PKC (MC5) immunoprecipitates (Fig. 2*B*). The data illustrated in Fig. 2 also show that anti-PKC mAb MC5 does not cross-react with Btk and that anti-Btk does not bind PKC. These results demonstrate that Btk is constitutively associated with PKC in mast cells.

PKC Phosphorylates and Down-Regulates Btk *in Vitro*. To test the possibility that PKC phosphorylates Btk, the partially purified Btk from rat RBL-2H3 cells or purified GST-BtkPH proteins were incubated with the purified rat brain PKC (α, β, and γ) in the presence of PKC activators and [γ-^{32}P]ATP. PKC phosphorylated both Btk (Fig. 3*A*) and GST-BtkPH proteins. Phosphoamino acid analysis of the phosphorylated GST-BtkPH proteins showed that phosphorylation was found only on serine residues (data not shown).

Since phosphorylation of some tyrosine kinases by PKC affects the enzymatic activity of the former kinases (22), we examined the effects of phosphorylation by PKC on the ability of Btk for autophosphorylation. Btk partially purified from rat RBL-2H3 cells was incubated with cold ATP for 30 min at 30°C in the presence or absence of the purified rat brain PKC under PKC phosphorylation conditions (see *Materials and Methods*). Btk in the mixtures was recovered by precipitation with anti-BtkC, and immune complexes were incubated with cold ATP. Tyrosine autophosphorylation of Btk was then detected by immunoblotting with anti-phosphotyrosine mAb. Phosphorylation of Btk by PKC resulted in a decrease in Btk autophosphorylation by about 60–80% (Fig. 3*B*).

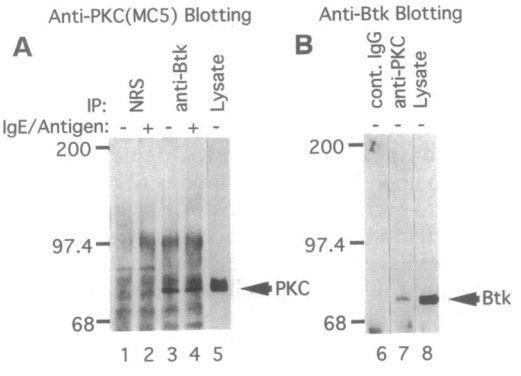

FIG. 2. *In vivo* association between Btk and PKC. (*A*) Anti-PKC blot of anti-Btk immunoprecipitates (IP). PKC coimmunoprecipitated from lysates (equivalent to 2 × 10^7 cells) of unstimulated (lanes −) or IgE/antigen-stimulated (lanes +) MCP-5 cells with normal rabbit serum (NRS, lanes 1 and 2) or anti-BtkC antiserum (lanes 3 and 4) was detected by immunoblotting with anti-PKC (MC5). (*B*) Anti-Btk blot of anti-PKC immunoprecipitates. Btk coimmunoprecipitated from MCP-5 cell lysates (equivalent to 2 × 10^7 cells) with normal mouse IgG (cont. IgG, lane 6) or mouse anti-PKC mAb MC5 (lane 7) was detected by immunoblotting with anti-BtkC serum. Cell lysate (equivalent to 2 × 10^5 cells) lanes (lanes 5 and 8) give the positions of PKC (*A*) or Btk (*B*).

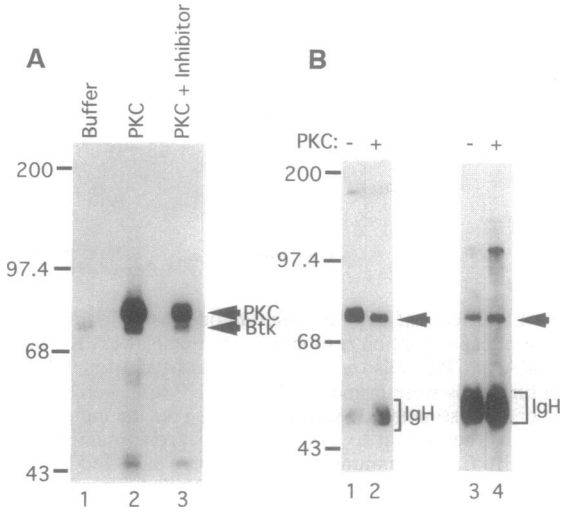

FIG. 3. PKC phosphorylates Btk *in vitro*. (*A*) Partially purified Btk (375 ng) was incubated with buffer alone (lane 1) or 420 ng of purified PKC (lanes 2 and 3) in the presence (lane 3) or absence (lanes 1 and 2) of a pseudosubstrate inhibitor, PKC-(19–36) octadecapeptide (GIBCO/BRL). Phosphorylation products were analyzed by SDS/PAGE and autoradiography. (*B*) Effects of PKC-mediated Btk phosphorylation on the Btk autophosphorylating activity. Partially purified Btk (375 ng) was phosphorylated by treating with 510 ng of purified PKC (lanes +) as described in text. Btk recovered with anti-BtkC was incubated with cold ATP. Tyrosine autophosphorylation of Btk was detected by immunoblotting (*B Left*). The amounts of Btk recovered were measured by reprobing the stripped blot with anti-BtkC (*B Right*). Positions of Btk are indicated by arrows. Three separate experiments of this design gave similar inhibition of Btk tyrosine phosphorylation [60%, 75%, and 80% (this experiment)]. Percent inhibition was calculated by the equation {1 − [(the intensity of the Btk band at lane 2)/(the intensity of the Btk band at lane 4)]/[(the intensity of the Btk band at lane 1)/(the intensity of the Btk band at lane 3)]} × 100.

To confirm that the Btk-associated PKC can regulate the enzymatic activity of Btk by phosphorylation, PKC was immunoprecipitated with anti-PKC mAb MC5 from a mixture of purified rat PKC and partially purified rat Btk. Immune complexes were incubated at 30°C for 20 min with PKC activators in the presence or absence of nonradioactive ATP. After washing, the immune complexes were incubated at 25°C for 3 min with [γ-^{32}P]ATP under Btk autophosphorylation conditions, reaction products were resolved by SDS/PAGE, and blotting was visualized by autoradiography. The results showed that Btk phosphorylation by the associated PKC inhibited the Btk autophosphorylating activity by 80–90% (data not shown).

Effects of PKC Modulation on Tyrosine Phosphorylation of Btk in Response to FcεRI Cross-Linking. We investigated *in vivo* effects of PKC modulation on the FcεRI-mediated Btk tyrosine phosphorylation. Chronic (18 hr) treatment of MCP-5 cells with 100 nM PMA decreased the level of anti-PKC mAb MC5-reactive proteins to <5% of that in untreated cells as measured by immunoblotting (data not shown). Under this condition, IgE-sensitized cells were stimulated with antigen, and lysates were used for immunoprecipitation with anti-BtkC, followed by immunoblotting with anti-phosphotyrosine mAb. Tyrosine phosphorylation on Btk induced by FcεRI cross-linking was higher and more rapid in PMA-treated cells than that in control cells (Fig. 4*A Upper*). Of note is that PMA treatment reduced the Btk protein level to 33–50% (Fig. 4*A Lower*). Augmentation of tyrosine phosphorylation of Btk subsequent to the IgE/antigen stimulation was also observed when MCP-5 cells were pretreated with PKC-specific inhibitors calphostin C for 30 min (Fig. 4*B*) or Ro 31-8425 for 10 min (data not shown).

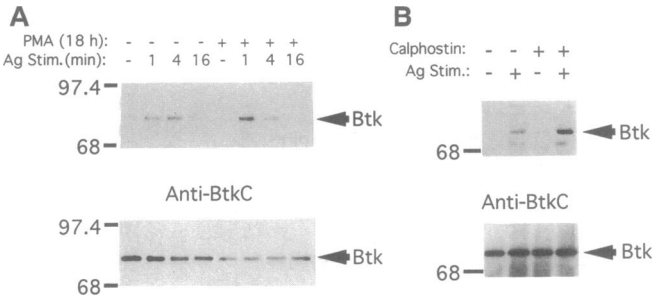

FIG. 4. Effects of PKC modulation on the FcεRI-mediated Btk tyrosine-phosphorylation response in mast cells. (*A*) PKC depletion with PMA. MCP-5 cells treated with 100 nM PMA for 18 hr, during which time cells were primed with anti-DNP IgE, were left unstimulated or were stimulated (Stim.) by antigen for the indicated times. Cell lysates (equivalent to 2×10^7 cells) were immunoprecipitated with anti-BtkC, and immune complexes were analyzed by immunoblotting with anti-phosphotyrosine mAb (*A Upper*) and then with anti-BtkC (*A Lower*) after stripping. (*B*) Inhibition of PKC by calphostin C. MCP-5 cells sensitized with anti-DNP IgE were treated with (lanes +) or without (lanes −) 0.1 μM calphostin C for 30 min prior to antigen stimulation (Stim.) for 3 min. Cell lysates (equivalent to 2×10^7 cells) were immunoprecipitated with anti-BtkC, and immune complexes were analyzed by SDS/PAGE and by immunoblotting with anti-phosphotyrosine mAb (*B Upper*). The stripped, same blot was reprobed with anti-BtkC (*B Lower*). The results shown in *A* and *B* are a representative of three separate experiments.

To test the possibility that the enzymatic activity of PKC might be influenced by interactions with the PH domain of Btk, the phosphorylating activity of the purified rat PKC (α, β, and γ) towards a peptide substrate, N-acetylated-myelin basic protein-(4–14) fragment (GIBCO/BRL), was measured in the absence or presence of 1- to 100-fold molar excesses of purified GST-BtkPH fusion protein. Under the experimental conditions, at least 10% of PKC was physically associated with GST-BtkPH at the highest concentration of GST-BtkPH, as determined by comparing the amount of PKC in anti-GST immunoprecipitates with that of the input PKC measured by immunoblotting with anti-PKC(MC5) (data not shown). However, GST-BtkPH protein failed to affect the PKC activity under the tested conditions.

DISCUSSION

Protein–protein interactions are adopted as a major means for signal transmission in various intracellular signaling systems. SH2 and SH3 domains interact with phosphotyrosine residues in the context of the surrounding sequences (23–25) and short proline-rich sequences (26, 27), respectively. Identification of PKC as a PH domain-binding molecule for the tyrosine kinase Btk implicates PH domain-mediated protein–protein interactions in the elaborate network of signaling molecules. This report shows that PKC associates constitutively with a tyrosine kinase and regulates the enzymatic activity of the latter.

Physical association of PKC with Btk leads to the inhibition of the enzymatic activity of Btk by PKC-mediated phosphorylation *in vitro* and *in vivo*. The stoichiometry of the Btk–PKC association is very small (i.e., 0.2% of the total Btk is associated with PKC) as estimated by comparison of the amount of Btk coprecipitated in the anti-PKC immunoprecipitate with that in the whole-cell extract of MCP-5. Reciprocal immunoprecipitation experiments showed that <0.1% of the total PKC βI isoform is associated with Btk (data not shown). However, PKC depletion or inhibition *in vivo* resulted in a 2- to 3-fold enhancement in tyrosine phosphorylation of Btk induced by FcεRI cross-linking. Considering this observation and the low efficiency of immunoprecipitation with anti-BtkC, it is reasonable to speculate that the true stoichiometry of the Btk–PKC association would be much higher.

On the other hand, purified PKC becomes tyrosine-phosphorylated upon incubation with partially purified Btk. However, there is no indication that PKC in mast cells is tyrosine-phosphorylated by Btk (data not shown). Lack of the effect of GST-BtkPH on the enzymatic activity of PKC suggests that the interaction with the Btk PH domain does not enhance the enzymatic activity of PKC. Given the low efficiency of the PH domain binding, however, the possibility still remains that the PKC activity might be inhibited by the interaction with the PH domain.

The negative regulation of Btk by PKC-mediated phosphorylation can be variously interpreted in the context of the FcεRI signaling system. One interpretation would be that PKC-mediated phosphorylation gives an "off" signal to the activated Btk kinase. However, this is unlikely because PKC depletion or inhibition with calphostin C or Ro 31-8425 augmented the Btk tyrosine-phosphorylation response but did not prolong the duration of the enhanced Btk tyrosine phosphorylation upon receptor cross-linking (Fig. 4). A more plausible explanation is that Btk phosphorylation by PKC keeps in check the basal and peak tyrosine-phosphorylation levels of Btk. Indeed, the basal level of Btk tyrosine phosphorylation prior to antigen stimulation is higher in calphostin C-treated cells than that in untreated cells (Fig. 4*B*). In support of this hypothesis, the Btk-PKC interaction is constitutive. Btk is heavily phosphorylated on serine residues before FcεRI cross-linking and receptor cross-linking enhances not only tyrosine phosphorylation of Btk but also serine and threonine phosphorylation (34), although these data do not necessarily mean that the serine/threonine phosphorylation on Btk is due solely to its associated PKC. The possibility that Btk is associated with multiple PKC isoforms complicates the situation. One PKC isoform might phosphorylate a subset of serine and/or threonine residues on Btk while another phosphorylates a distinct subset. Phosphorylations by different isoforms might result in increases or decreases in the Btk kinase activity.

There is a large body of evidence showing that PKC plays important roles in many signal-transducing systems (28, 29), including the FcεRI signaling pathway (1). Cross-linking of FcεRI leads to degranulation, synthesis, and secretion of lipid mediators and cytokines, and cell proliferation. Most, if not all, of these phenomena are presumed to be mediated by one or more isoforms of PKC. PKC is rapidly translocated to the membrane upon receptor cross-linking (30, 31). PKC depletion with PMA or by washing permeabilized RBL-2H3 cells results in the severe loss of the secretory capability in response to FcεRI cross-linking (32). However, introduction of PKC β or δ isoforms into the PKC-depleted cells restores degranulation upon FcεRI cross-linking (21). Further studies are required to establish the roles of PKC β and Btk in this and other activation-related processes. On the other hand several studies have demonstrated the importance of tyrosine phosphorylation in immune cell activation through antigen receptors and FcεRI (1, 2). Our studies using a variety of protein-tyrosine kinase inhibitors showed that protein-tyrosine kinase activity is required for FcεRI signaling (15). Therefore, the physical association between Btk and PKC gives a biochemical basis for the interaction between the signals transmitted by the two different classes of protein kinases.

The Btk–PKC interaction is not restricted to mast cells. As shown previously (3–5), Btk is expressed not only in mast cells but also in myeloid cells and B lymphocytes. We have evidence that GST-BtkPH binds to PKC in B-cell lysates and that Btk is physically associated with PKC in intact B cells (data not shown). Btk belongs to a recently recognized Tec subfamily of protein-tyrosine kinases including Emt (5) and

TecII (8) as members. These protein tyrosine kinases have PH domains, whose amino acid sequences are highly homologous, especially towards their amino-terminal halves. The GST-Btk(*xid*) protein with the Arg-to-Cys substitution at residue 28 showed the lower PKC-binding capacity, suggesting that residue Arg-28 of Btk may participate in the binding to PKC. It is known that the substitution at residue 28 does not affect enzymatic activity of Btk (6, 7). One might speculate that the lower PKC binding of the mutant Btk in *xid* mice might be involved in immunodeficient status. We expect that other Tec-subfamily protein-tyrosine kinases may be associated with PKC. Indeed, we have evidence that Emt is physically associated with multiple PKC isoforms (Y.K., L.Y., S. Gibson, G. B. Mills, and T.K., unpublished data). Tec II may also interact with PKC. However, the PKC-binding capacity may not be shared by all PH domains. PH domain sequences corresponding to that around residue Arg-28 of Btk are not well conserved among other proteins. The present study, together with the recent observation that carboxyl-terminal portions of various PH domains bind to $\beta\gamma$ subunits of heterotrimeric GTP-binding proteins *in vitro* (33), implicates PH domain-mediated protein–protein interactions in a wide variety of signal-transduction systems.

We cordially dedicate this article to Dr. Teruko Ishizaka on the memorable occasion of her retirement. We thank Dr. Kimishige Ishizaka for his support and helpful suggestions throughout this study and Drs. D. D. Metcalfe, W. Northemann, D. J. Price, and K. Yamada for reagents. This is publication no. 100 from the La Jolla Institute for Allergy and Immunology.

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