Interactions between Protein Kinase C and Pleckstrin Homology Domains

INHIBITION BY PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE AND PHORBOL 12-MYRISTATE 13-ACETATE

(Received for publication, December 31, 1996, and in revised form, March 4, 1997)

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Pleckstrin homology (PH) domains comprised of loosely conserved sequences of ~100 amino acid residues are functional protein motifs found in many signal-transducing and cytoskeletal proteins. We recently demonstrated that the PH domains of Tec family protein-tyrosine kinases Btk and Emt (equal to Itk and Tsk) interact with protein kinase C (PKC) and that PKC down-regulates Btk by phosphorylation. In this study we have characterized the PKC-BtkPH domain interaction in detail. Using pure PKC preparations, it was shown that the Btk PH domain interacts with PKC with high affinity (Kd = 39 nM). Unlike other tested phospholipids, phosphatidylinositol 4,5-bisphosphate, which binds to several PH domains, competed with PKC for binding to the PH domain apparently because their binding sites on the amino-terminal portion of the PH domains overlap. The minimal PKC-binding sequence within the Btk PH domain was found to correspond roughly to the second and third β-sheets of the PH domains of known tertiary structures. On the other hand, the C1 regulatory region of PKCε containing the pseudosubstrate and zinc finger-like sequences was found to be sufficient for strong binding to the Btk PH domain. Phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC that interacts with the C1 region of PKC, inhibited the PKC-PH domain interaction, whereas the bioactive PMA (4α-PMA) was ineffective. The ϵ isozyme of PKC, which has a single zinc finger-like motif instead of the two tandem zinc finger-like sequences present in conventional and novel PKC isoforms, does not bind PMA. Thus, as expected, PH domain binding with PKCε was not interfered with by PMA. Further, inhibitors that are known to attack the catalytic domains of serine/threonine kinases did not affect this PKC-PH domain interaction. In contrast, the presence of physiological concentrations of Ca2+ induced less than a 2-fold increase in PKC-PH domain binding. These results indicate that PKC binding to PH domains involve the β2-β3 region of the Btk PH domain and the C1 region of PKC, and agents that interact with either of these regions (i.e. phosphatidylinositol 4,5-bisphosphate binding to the PH domain and PMA binding to the C1 region of PKC) might act to regulate PKC-PH domain binding.

The importance of functional protein motifs in various signal transduction pathways is well documented. For example, Src homology (SH)1 and SH3 domains play essential roles in signal transduction for cell activation and growth by interacting with phosphotyrosine residues in the context of surrounding sequences (1-3) and short proline-rich stretches (2-4), respectively. Pleckstrin homology (PH) domains are comprised of loosely conserved sequences of approximately 100 amino acid residues (5, 6). Originally recognized as repeated sequences in the platelet protein pleckstrin (a prominent substrate for protein kinase C (PKC)), PH domains have been found in more than 60 proteins (7-9). Most PH domain-containing proteins have been implicated in signal transduction or in cytoskeletal functions and include guanosine triphosphatases (GTPases), GTPase-activating proteins, guanine nucleotide exchange factors, serine/threonine kinases, tyrosine kinases, and phospholipases C (PLCs).

The tertiary structures of four PH domains to date have been determined by nuclear magnetic resonance spectroscopic or x-ray crystallographic techniques (10-16). Basic structural features are shared by the PH domains of pleckstrin, β-spectrin, dynamin, and PLC-δ1. The core of the compact domains is an antiparallel β-sheet consisting of seven strands. The amino-terminal four strands form a pocket-like structure, suggestive of a ligand-binding site. The carboxyl-terminal α-helix follows the last β-sheet. Studies by Lefkowitz and co-workers (17, 18) established that the sequence encompassing the carboxyl-terminal α-helical region of the PH domain was later shown to be important for this interaction (19). The interacting counterpart is the WD40 repeats of the β-subunit of G-protein (20). More recently, several studies showed that various PH domains bind to phosphatidylinositol 4,5-bisphosphate (PIP2) and inositol 1,4,5-trisphosphate (IP3) through their positively charged residues in the amino-terminal four β-sheets (15, 21,
PKC-PH Domain Interactions

PIP₂ interactions with the amino-terminal PH domain of pleckstrin (21) and the PLC-51 PH domain (22) occur with low affinity (Kᵦ = ~30 and 1.7 μM, respectively) while the PLC-51 PH domain binds IP₃ with high affinity (Kᵦ = 210 nM, Ref. 22). Membrane localizing functions have been implicated for the binding of PH domains to G-protein βγ complexes and PIP₂. Indeed, many PH domain-containing proteins are known to be localized at the plasma membrane or other membrane structures.

We recently demonstrated that the PH domains of protein-tyrosine kinases (PTKs) Btk and Emt interact directly with PKC (23). We also presented evidence that PKC phosphorylates Btk and inhibits the kinase activity of the latter enzyme, suggesting that the PH-domain-PKC interaction plays a regulatory role for Btk. Mutations in the gene encoding Btk lead to immunodeficiencies in humans (X-linked agammaglobulinemia, see Refs. 24 and 25) and mice (X-linked immunodeficient mice; xid, see Refs. 26 and 27). Btk and Emt constitute a distinct subgroup (Tec family) of PTKs along with TecII (28), Dsrc28 (29), Bmx (30), and Tsk/Rtk (31, 32). In the present study we have characterized in detail the interaction between the Btk PH domain and PKC using an in vitro binding assay. A high affinity binding between PKC and the Btk PH domain was demonstrated by this assay. The interaction sites were mapped to the amino-terminal β₂-β₃ region of the Btk PH domain and the C1 regulatory region of PKC. In accordance with the mapping data, PIP₂ competes with PKC for binding to the PH domains of Btk and Emt while bioactive PMA that binds to the C1 region of PKC also inhibited the PKC-PH domain interaction.

EXPERIMENTAL PROCEDURES

Reagents—Purified rat brain PKC (>95% pure; a mixture of α, β, and γ isoforms) was purchased from Calbiochem. Anti-glutathione S-transferase (GST) antisera and pGEX-3T (33) were a kind gift from Dr. Wolfgang Northemann (ELIAS Entwicklungslabor). Glutathione-agarose beads, PIP₂, and other chemicals were obtained from Sigma unless otherwise described. Another source of PIP₂ was Calbiochem.

Cells—An immortalized murine mast cell line, MCP-5 (34), was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 U/mL of penicillin, 100 mg/ml streptomycin, and 10 mM HEPES at 37°C in a humidified atmosphere containing 5% CO₂. Aseptic materials were used throughout the experiments. Immediately before use, cells were washed twice with PBS, and attached cells were detached with 0.25% trypsin-EDTA and counted with trypan blue exclusion. Cells were used at less than 1 × 10⁷ cells/mL when the experiment was done.

DNA Constructs—DNA fragments encoding the PH domains of Btk and Emt were amplified with polymerase chain reaction using the cloned murine btk and emt cDNA (39), respectively, as templates. 5′-Polymerase chain reaction primers contain a BamHI recognition sequence attached to the downstream sequence of interest. The polymerase chain reaction products were cloned into pcRII vector (Invitrogen, San Diego). Limited DNA sequence analysis was performed to verify the pcRII clones. The insert sequences released from the vector sequence by digestion with BamHI and EcoRI were ligated to pGEX-3T. GST proteins used in this study were GST-BtkPH (coding for residues 1–139 of Btk), GST-EmtPH (residues 1–109), and truncated Btk PH domain fragments. GST fusion proteins containing the subregions of the Btk PH domain were designated according to the Btk residue numbers at the amino and carboxyl termini, e.g. 28/77, which codes for the region from residue 28 to 77.

Immobilization of Fusion Proteins—pGEX-3T constructs were transformed into an Escherichia coli strain XL1 Blue (Strategene). Fusion proteins were expressed by inducing 1 liter of log-phase bacteria with 0.4 mM isopropyl-β-D-thiogalactopyranoside overnight at 26°C. Cells were collected and sonicated in phosphate-buffered saline, containing 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Lysates were cleared by centrifugation at 12,000 × g for 10 min at 4°C and filtrated through 0.22-μm filter Millex-GS (Millipore, Bedford, MA). Five hundred μl of glutathione-agarose beads were incubated with lysates overnight at 4°C and then washed thoroughly with phosphate-buffered saline, containing 1% Triton X-100 and 0.02% sodium azide. Purities and amounts of fusion proteins bound to beads were assessed after elution with SDS-sample buffer by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Solid-phase Binding Assay with GST Fusion Protein Beads—MCP-5 or HMC-1 cells were used to examine PKC binding activities of GST fusion proteins in vitro. Cells were lysed in 1% Nonidet P-40 buffer (Nonidet P-40 lysis buffer) containing 20 mM Tris-HCl (pH 8.0), 0.15 mM NaCl, 0.1 mM CaCl₂, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 16.5 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM p-nitrophenyl p'-guanidinobenzoate, and 0.1% sodium azide. PKC-PH Domain Interactions

FIG. 1. Kinetics of the interaction between rat brain PKC and the Btk PH domain. A, GST or GST-BtkPH (1 μg each) immobilized onto glutathione-agarose beads was incubated with the indicated amounts of rat brain PKC, and bound PKC was detected by immunoblotting with anti-cPKC (MC5) monoclonal antibody. The enhanced chemiluminescence method was used to visualize the immunoreactive bands (upper panel). Immunoblotting of known amounts of PKC was carried out to generate the standard curve (lower panel). B, amounts of bound PKC were plotted as a function of input PKC. Scatchard analysis based on this experiment gave a Kᵦ value of 39 nM. A representative result out of three similar experiments is presented.
enhanced chemiluminescence kit (Amersham Corp.). Anti-PKC e (Life Technologies Inc.) was used for detection of PKC e holoenzyme and e-epitope-tagged fragments of PKC e or c-Raf-1.

RESULTS
The PH Domain of Btk Interacts with Pure Preparations of PKC with High Affinity—A far Western blotting experiment showed that GST-BtkPH immobilized onto a polyvinylidene difluoride membrane binds to purified rat brain PKC (23), indicating the direct interaction between the PH domain and PKC. The PKC preparations used in the above and following experiments are a pure (107 cells) were mixed with 2 μg each of GST or GST-PH domain fusion proteins immobilized onto glutathione-agarose beads. Bound proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-P membranes. Bound PKC was detected by probing the blots with anti-cPKC (MC5). Bound proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-P membranes. Bound PKC was detected by probing the blots with anti-cPKC (MC5).

FIG. 2. Mapping of the PKC-binding site within the Btk PH domain. A, a summary of the in vitro binding assay data with GST fusion proteins containing the PH domain subregions. The diagram at the top shows the deduced secondary structure of the PH domain of Btk based on the tertiary structural analyses of other PH domains. β1–β7 and α1 indicate β-sheets and an α-helix, respectively. Residue numbers (1 and 139) of the amino and carboxyl terminal are also indicated. Subregion fusion proteins were designated by the residue numbers of the amino- and carboxyl-terminal portions of the PH domains of Btk (39), Emt (39), and pleckstrin (55) are depicted in the single-letter code. Positions of the first residues are indicated in parentheses. Residues forming the β-sheets of the amino-terminal PH domain of pleckstrin (Ple N) are underlined and indicated by β1–β4 at the top (10). Residues in Ple N involved in binding to PIP2, as deduced from chemical shift (21), are shaded. Overlined is the minimal PKC-binding sequence of the PH domain of Btk determined in this study. The asterisk indicates the position of the Btk residue (Arg-28) that is mutated in xid mice and in some X-linked agammaglobulinemia patients. # indicates position 41 (Glu-41) whose substitution with Lys resulted in a constitutively active mutant of Btk (47). The consensus sequences in the second and third β-sheet regions are taken from Ref. 6. Plus signs, basic residues; small phi, hydrophobic residues; large phi, aromatic residues.

Effects of Phospholipids on PKC Binding to the Btk PH Domain—The above mapping results indicated that the minimal PKC-binding sequence overlaps the binding site for PIP2, IP3, and IP6 shown for several PH domains including that of Emt (Fig. 2C). Therefore, we examined possible competition for binding to the Btk PH domain between PKC and various phospholipids. GST-BtkPH beads were incubated with MCP-5 mast cell lysates in the presence of various concentrations of phospholipids. No effects on the interaction between cPKC and GST-BtkPH were found with such lipids as phosphatidylserine (up to 32 μg/ml, data not shown), phosphatidylethanolamine (up to 115 μg/ml), phosphatidylcholine (up to 115 μg/ml, data not shown), phosphatidylinositol (up to 115 μg/ml), and phosphatidylinositol 4-phosphate (up to 115 μg/ml) (Fig. 3). In contrast, PIP2 inhibited cPKC binding to the Btk PH domain in a dose-dependent manner (Fig. 3). The IC50 (~1 μM) estimated by densitometric measurements of the bound PKC bands was similar to the reported dissociation constant between PIP2 and the PLC-5b1 PH domain (1.7 μM).

Mapping of the PH Domain-binding Site within the Regula-
Fig. 3. PIP2 competes with PKC for binding to the Btk PH domain. GST-BtkPH (2 μg) beads were mixed with MCP-8 cell lysates in the presence of 0, 4.6, 23, or 115 μg/ml sonicated lipid vesicles. Bound cPKC was detected as described in Fig. 2B. As a control, a result with GST together with PIP2 is also shown. PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PC, phosphatidylcholine. PIP2 competition experiments were carried out four times with similar results, while experiments with other lipids were repeated twice.

The C1 regulatory Region of PKCe—GST-BtkPH bound to both Ca2+-dependent (α, β, and βII) and Ca2+-independent isoforms (ε and ζ) in mast cell lysates (data not shown). To determine the PH domain-binding region within PKCe we utilized stably transfected NIH/3T3 cell lines that overexpress the epitope-tagged domain fragments of PKCe depicted in Fig. 4 (37, 38). For comparison, we also used NIH/3T3 cells expressing c-Raf-1 fragments tagged with the same epitope (data not shown). These cells were lysed, and the extracts were used in the solid-phase binding assay with GST-BtkPH beads. Bound PKCe fragments were detected by immunoblotting with anti-ε (epitope) antibody. The results showed that the relative band intensities of the GST-BtkPH-bound PKCe fragments normalized to their expression level in cell lysates were high with the PKCe holoenzyme and with fragments ε1 and ε2 (Fig. 4). However, fragments ε3 and ε7, which lack the pseudosubstrate region, exhibited lower but significant binding capacities to GST-BtkPH. GST control beads bound negligible amounts of these PKCe fragments. These data demonstrated that the C1 region of PKCe is sufficient for interaction with the Btk PH domain. Neither RI-ε nor RIΙ-ε fragments derived from the regulatory and catalytic domains, respectively, of c-Raf-1 (38) bound to the PH domain (data not shown). RI-ε contains the zinc finger-like sequence of c-Raf-1 that has a low level homology (~30% identity) with that of the C1 region of PKC. Therefore, the lack of PH domain binding to RI-ε indicates a high specificity of PKC-PH domain interactions. An epitope-tagged rat PKCβ3 construct encompassing residues 312–671, which correspond to the catalytic domain, failed to bind to GST-BtkPH (data not shown).

PIP2 binds to PH domains (21, 22). Since PIP2 also binds to the C2 region of PKC (40), there was a possibility that the observed PIP2 competition with cPKC holoenzyme for binding to PH domains (Fig. 3) could be due to the allosteric effect of PIP2 bound to the C2 region. Therefore, effects of PIP2 on the interactions between GST-BtkPH and the PKCe fragments ε2 and ε3, which have no PIP2-binding region, were examined. The addition of 60 μg/ml PIP2 to the binding mixtures inhibited these interactions by more than 70% (Fig. 4), suggesting that PIP2 binds to the PH domain to prevent ε2 and ε3 from interacting with GST-BtkPH. In contrast, the presence of 60 μg/ml phosphatidylinerse did not inhibit the GST-BtkPH-PKCε fragment interactions (data not shown).

Effects of PKC Activators or Inhibitors on the Interaction between PKC and the Btk PH Domain—Activation of cPKCs requires Ca2+ and diacylglycerol/PMA in addition to phosphatidylinerse (41). Effects of these factors on the PKC-PH domain interaction were examined using GST-BtkPH and mast cell lysates. The addition of 0.1–5 μM Ca2+ resulted in less than a 2-fold increase in cPKC binding compared with the binding in the absence of Ca2+ or in the presence of EGTA (Fig. 5A), although non-physiologically high Ca2+ concentrations (10–1000 μM) induced up to a 5-fold increase in cPKC binding (data not shown). 1 μM Ca2+ did not affect significantly the PH domain binding to recombinant PKCβ1 compared with that in the absence of Ca2+ (data not shown). These data are consistent with the above mapping data that the primary PH domain-binding site is the C1 region.

PMA inhibited cPKC binding to GST-BtkPH in a dose-dependent manner with an IC50 of ~1 μM, while the bioactive PMA (4-α-PMA) did not alter the level of bound cPKC (Fig. 5B). These results are consistent with the mapping results. Thus, PMA (but not 4-α-PMA) binds to the C1 region of conventional and novel PKC isoforms, which are composed of two tandem zinc finger-like motifs. In contrast, the PKCζ and PKCλ isoforms have only one zinc finger-like motif and thus lack the ability to bind PMA (42, 43). The above mapping data strongly suggest that the C1 region of PKC binds to the C-terminal part of the PH domain containing the β2–β3 sheets. If this is the case, the PH domain interaction with PKCζ should not be susceptible to PMA-mediated inhibition due to the lack of interaction between PKCζ and PMA. Indeed, the PKCζ-GST-BtkPH interaction was not affected by increasing concentrations of PMA up to 100 μM (Fig. 5C).

We also examined the effects of inhibitors of PKC and of
other serine/threonine kinases on PKC-PH domain interaction. Staurosporine (a potent PKC inhibitor), KT-5720 (a selective protein kinase A inhibitor), and KT-5926 (a specific inhibitor of myosin light chain kinase), all showed little, if any, effect on the cPKC-PH domain interaction in HMC-1 human mast cell lysates (data not shown).

**DISCUSSION**

In this report we have described a biochemical characterization on PKC-PH domain interactions. Pure PKC preparations interact with the Btk PH domain with high affinity. The interaction sites were mapped to the β2–β3 region of the Btk PH domain and the C1 region of PKCe. This assignment of the interaction sites was supported by several pieces of experimental evidence. First, PIP2, whose binding site within PH domains overlaps that of PKC, competed for PH domain binding with PKC. Second, PMA-mediated inhibition was not observed with PKCe-PH domain interactions. Further, PMA-mediated inhibition was not observed with PKCζ-PH domain interactions.
interactions because of the lack of binding capacity of PMA to the C1 region of PKCβ.

The $K_D$ value (39 nM) obtained for the interaction between rat brain PKC and the Btk PH domain is at least 40-fold lower than the reported $K_D$ values for PIP$_2$ binding to PH domains (~30 μM for the amino-terminal PH domain of pleckstrin and 1.7 μM for the PLC-ε1 PH domain) and 5-fold lower than for IP$_3$ binding to the PLC-ε1 PH domain ($K_D = 210$ nM). The concentrations of PKCo and PKCβ, two major PKC isoforms in a mast cell line, MC-9, were shown to be 26.0 and 63.9 nM, respectively (44). Accordingly, PKCβ is a major Btk-associated isoform of PKC in mast cells (23). The PKC-binding site within PH domains deduced from in vitro binding assays using truncated PH domain fragments is overlapped by the PIP$_2$- and IP$_3$-binding sites determined by nuclear magnetic resonance and x-ray crystallographic methods. In accordance with this, PIP$_2$ competed with PKC for binding to the PH domain of Btk with an IC$_{50}$ of ~1 μM. This is similar to the $K_D$ (1.7 μM) for the PIP$_2$ interaction with the PLC-ε1 PH domain. However, cross-linking of the high affinity IgE receptor, which results in increased levels of PIP$_2$, did not induce significant changes in the levels of PKCβ co-immunoprecipitated with Btk in the cytosolic and membrane compartments for at least 10 min following activation (23). This may reflect the high affinity nature of the PKC-PH domain interaction. The experiments shown in Fig. 1 indicate that only 1 out of every 15 molecules of GST-BtkPH protein in the binding mixture could bind PKC. This low efficient binding could be due to low frequencies of properly folded PH domain, denaturation of PKC preparation, or both.

The minimal PKC-binding sequence was localized to the amino-terminal portion (residues 28–45) of the PH domain of Btk, which overlaps the reported PIP$_2$-binding site on the amino-terminal PH domain of pleckstrin (Fig. 2C). This region corresponds roughly to β-sheets 2 and 3, which form a major part of the β-barrel. It is noteworthy that the hydrophobic residues of the β2–β3 region following the basic residue corresponding to Arg-28 in Btk are well conserved among most of PH domain-containing proteins. Therefore, these conserved residues seem to be major determinants for PKC binding, and most PH domains presumably have PKC-interacting capacity. Mutations of Arg-28 in Btk are reported to be pathogenic for xid mice (substitution of Cys for Arg-28 (26, 27) and for some X-linked agamaglobulinemia patients (substitution of His for Arg-28 in two unrelated cases (45, 46)). Therefore, defects in binding to either PKC or PIP$_2$ might be the mechanism for these immunodeficiencies. A gain-of-function mutation also was obtained by the substitution of residue Guar-28 with Lys in this region of Btk. This mutated form of Btk caused transformation of NIH/3T3 cells and resulted in enhanced membrane localization of Btk (47). Therefore, it will be interesting to determine if this mutant Btk has an altered PKC- or PIP$_2$-binding capacity. The competition for the subregion of the PH domain between PKC and PIP$_2$ raises an interesting question as to a possible regulatory role for this competitive binding. The capacity of PH domains to bind to PIP$_2$ and the G-protein βγ complex is implicated in localizing the PH domain-containing proteins to the membrane compartment. Since PKC can be found located both in the cytosol and membrane compartments, the PKC binding property of PH domains gives more versatility to the subcellular distribution of PH domain-containing signaling molecules. It can be envisioned that a PH domain-containing signaling protein, e.g., Btk, might translocate to the membrane in response to cell stimulation (48) where the PH domain-containing signaling protein could be sorted to either PIP$_2$- or PKC-bound forms and be subjected to distinct regulatory parameters to fulfill specific functions. Cell stimulation, e.g., FceRI cross-linking, which can induce the activation of both G-proteins and tyrosine kinases such as Btk and Emt (48, 49), may complicate the regulation of the PH domain-containing signaling proteins by possible interactions with the βγ subunits of G-protein, PKC, and PIP$_2$ (and other phospholipids). Further experiments are warranted to test these interesting possibilities.

PKC is a large family of serine/threonine kinases implicated in a variety of cellular functions including proliferation, differentiation, and membrane receptor functions (50). Molecular characterization has defined at least three classes of PKCs: conventional PKC isoforms that contain both the C1 and C2 conserved regulatory regions and are dependent for their activation on Ca$^{2+}$; novel PKCs that lack the C2 region and, therefore, are Ca$^{2+}$-independent; and atypical PKCs that contain an atypical C1 region composed of a single zinc finger-like motif instead of the tandem duplicated zinc finger-like motifs found in the other PKC classes. The PH domain binding observed with both Ca$^{2+}$-dependent (conventional; α, βI, and βII) and Ca$^{2+}$-independent (novel ε and atypical δ) isoforms of PKC suggests that the PH domain-binding site of PKC may be localized to the conserved sequences found in various isoforms of PKC. In vitro binding assays using NIH/3T3 cells overexpressing epitope-tagged fragments of PKCe demonstrated that the C1 region of PKCe is sufficient for binding to the Btk PH domain. The sequence around the pseudosubstrate region upstream of the zinc finger-like motifs contributed significantly to strong binding to the PH domain. However, the tandem zinc finger-like region alone was sufficient for weak binding to the PH domain. There is only limited homology around the pseudosubstrate sequence (contained in residues 133–165 of PKCe) among the various PKC isoforms. However, there is a cluster of several conserved residues with hydrophobic side chains following the invariant Ala residue (Ala-157 in PKCe) in the C1 region. The involvement of these hydrophobic residues in interactions with the hydrophobic β2–β3 region of PH domains is implied since PH domain interactions with fragments ε2 and ε3 were found to be resistant to 1 μM NaCl washes (data not shown). In addition to the C1 region, other regions also might play some role in binding to the PH domain. One such candidate is the C2 region. Indeed, non-physiologically high Ca$^{2+}$ concentrations (10–1000 μM) increased PKC-PH domain binding up to 5-fold over that in the absence of Ca$^{2+}$. This effect might be accounted for by allosteric effects on the PH domain-binding C1 region invoked by Ca$^{2+}$ bound to the C2 region. However, the normal physiological fluctuation range of intracellular Ca$^{2+}$ concentrations (less than 1 μM) would lead to only a less than a 2-fold change in the PKC-PH domain binding. Accordingly, we could not detect a significant change in the PKCe levels co-immunoprecipitated with Btk before and after mast cell activation by cross-linking of the high affinity IgE receptor (23). The possible involvement of the catalytic domain of PKC in PKC-PH domain interactions was ruled out by the present study, since the Btk PH domain did not bind to either kinase domains of either PKCaβ or c-Raf-1 (RIII-ε).

Evidence for in vivo PKC-PH domain interactions so far has been reported only for the PH domains of Btk, Emt, and Rac (23, 49, 51). However, this interaction seems to be more generally present. There are various lines of circumstantial evidence that support this possibility. In unstimulated T cells a PH domain-containing protein, spectrin, is colocalized with PKCβII. Upon stimulation with either PMA or anti-T cell antigen receptor antibody, these two proteins are coincidentally translocated to the same focal aggregates in the cytoplasm (52).
The PH domain of spectrin might be involved in this colocalization event. Indeed, the PH domain of β-spectrin has been shown to be capable of associating with brain membranes (53). Both PH domain-containing proteins and PKC are implicated in various signal-transducing pathways. Therefore, it is likely that some of the signaling functions of PKC may be mediated through interactions with PH domain-containing proteins. Physiological relevance of the PH domain-mediated Btk-PKC interaction was supported by a recent study with PKCβ gene knock-out mice (54). The phenotype of PKCβ null mice was shown to be quite similar to those of xid and btk null mice.

Acknowledgments—We thank Dr. Kimishige Ishizaka for encouragement and discussion during the course of this study, Dr. Alexandra C. Newton for kind advice, and Drs. Tomas Mustelin and Katsuji Sugie for critical reading of the manuscript.

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