Interaction of Bruton’s Tyrosine Kinase and Protein Kinase Cθ in Platelets

CROSS-TALK BETWEEN TYROSINE AND SERINE/THREONINE KINASES*

Received for publication, September 17, 2001, and in revised form, January 10, 2002
Published, JBC Papers in Press, January 11, 2002, DOI 10.1074/jbc.M108965200

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The nonreceptor Bruton’s tyrosine kinase (Btk) has been previously shown to associate physically and functionally with members of the protein kinase C (PKC) family of serine/threonine kinases in a variety of cell types. Here we show evidence for a novel interaction between Btk and PKCθ in platelets activated through the adhesion receptors GP Ib-V-IX and GP VI. Alboaggregin A, a snake venom component capable of activating both receptors in combination, leads to tyrosine phosphorylation of Btk downstream of Src family kinases. Inhibition of Btk by the selective antagonist LFM-A13 causes a reduction in calcium entry, although secretion of 5-hydroxytryptamine is potentiated. Btk is also phosphorylated on threonine residues in a PKC-dependent manner and associates with PKCθ upon platelet activation by either alboaggregin A or activation of GP Ib-V-IX alone by von Willebrand factor/ristocetin. PKCθ in turn becomes tyrosine-phosphorylated in a manner dependent upon Src family and Btk kinase activity. Inhibition of Btk activity by LFM-A13 leads to enhancement of PKCθ activity, whereas nonselective inhibition of PKC activity by bisindolylmaleimide I leads to reduction in Btk activity. We propose a reciprocal feedback interaction between Btk and PKCθ in platelets, in which PKCθ positively modulates activity of Btk, which in turn feeds back negatively upon PKCθ.

Bruton’s tyrosine kinase (Btk) is a member of the Tec family of nonreceptor tyrosine kinases, which includes Itk, Tec, Txk, and Bmx and is of pathological significance when deficient or functionally mutated in the severe immunodeficiency syndrome X-linked agammaglobulinemia (1). Tec family kinases are characterized by a C-terminal proline-rich region, Src homology 1, 2, and 3 domains, and an N-terminal pleckstrin homology (PH) and Tec homology domains. Btk is expressed in cells of hematopoietic origin including platelets and has been shown to mediate calcium influx in these cells (2–5). Btk has been shown to play an important role in platelet activation by collagen because collagen stimulation induces tyrosine phosphorylation and activation of Btk (6), and in platelets lacking functional Btk, collagen-induced platelet aggregation and calcium influx are impaired significantly (7). The activity of Btk is controlled by several factors including phosphorylation. Btk has been shown to be phosphorylated at tyrosine 551 within the catalytic domain by associated Src family kinases, leading to autophosphorylation at tyrosine 223 within the Src homology 3 domain, which is necessary for full activation (8–11). It has also been shown that for full activation, the PH domain of Btk must interact with the lipid product of phosphatidylinositol 3-kinase, phosphatidylinositol 3,4,5-trisphosphate (12). It has been hypothesized that the function of this interaction is to recruit Btk from the cytosol to the plasma membrane, where it can be subsequently activated by Src family kinases.

Btk has been shown to associate with members of the protein kinase C (PKC) family of serine/threonine kinases through an interaction between the Btk PH domain and PKC C1 domain (13, 14). A constitutive association has been shown between Btk and PKCθ in B cells (15) and between Btk and members of the classical and atypical families of PKC in murine mast cells (13). Btk has been shown in these studies to be a substrate of PKC isoforms and its enzymatic activity to be down-regulated by PKC-mediated phosphorylation (13). In turn, Btk has been shown to regulate the membrane translocation and activation of PKCθ by direct interaction with this kinase (16).

For the initiation of arrest of bleeding and subsequent vascular repair, the ability of platelets to adhere to subendothelial structures is critical. Collagen and von Willebrand factor (vWF) form the two most important structures to which platelets adhere through several surface glycoprotein (GP) receptors. These two adhesion molecules induce signaling events in platelets primarily through GP VI and GP Ib-V-IX, respectively. The initial interaction between platelets and vWF occurs via the platelet glycoprotein receptor complex GP Ib-V-IX (GP Ib). vWF supports not only the initial transient phase of platelet adhesion through GP Ib but also mediates integrin αIIbβ3-based cell arrest (17). The signaling pathways induced by GP Ib and GP VI share a number of similarities including constitutive association with and tyrosine phosphorylation of Fc receptor γ chain and recruitment of multiple components of a tyrosine phosphorylation-dependent signaling pathway. These components include Src family kinases, Syk, and phospholipase Cγ2 (18–20). Activation of GP Ib and GP VI leads to the hydrolysis of phosphoinositides, a rise in cytosolic calcium, activation of PKC, cytoskeletal reorganization, and platelet 5-HT secretion and aggregation (17, 19, 21–25). In this report, combined activation of GP Ib and GP VI is achieved using the snake venom component alboaggregin A (19, 21, 26, 27), and activation of GP Ib alone is achieved by vWF plus ristocetin in the presence of EGTA to block binding to integrin αIIbβ3. It is clear that many aspects of platelet activation are regulated by combined serine/threonine and tyrosine phosphorylation. We were interested in...
investigating points of cross-talk between these two signaling pathways. Here we have investigated the interaction between a member of the novel PKC isoforms, PKCθ, and Btk and show a role for this interaction in terms of functional feedback between the two kinases.

**EXPERIMENTAL PROCEDURES**

**Materials**

Trimeresurus albolabris venom was a kind gift from Professor R. D. G. Theakston (Liverpool, U. K.). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc (TCS Biologicals Ltd., Bucks, U. K.). All anti-PKC antibodies and anti-phosphotyrosine were from Transduction Laboratories (Affiniti Research Products, Exeter, U. K.). Anti-Btk antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Src family kinase inhibitor PP1 was from Alexis Corp (Nottingham, U. K.). PKC inhibitor bisindolylmaleimide I (BIM) was from Toceis (Bristol, U. K.). Btk-PH domain construct was from Dr. C. I. E. Smith (Stockholm, Sweden). LFM-A13 and Raytide were from Calbiochem (La Jolla, CA). Plasma vWF was a kind gift from Prof. J. J. Sixma and Dr. T. Vink (Utrecht, The Netherlands). All other reagents were analytical grade.

**Preparation and Stimulation of Human Platelets**

Human blood was drawn from healthy, drug-free volunteers on the day of the experiment. Acid citrate dextrose (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid, used at 1:7 v/v) was used as an anticoagulant. Platelet-rich plasma was prepared by centrifugation at 200 x g for 20 min. Platelets were then isolated by centrifugation of platelet-rich plasma for 10 min at 1,000 x g, in the presence of 40 ng/ml prostaglandin E1. The pellet was resuspended to a density of 4.10^8 H9262 cells. The reaction was started by the addition of 250 mM KCl, 10 mM HEPES, 1 mM MgCl2, 5 mM glucose, pH 7.3. To this were suspended in 20 mM Na3VO4, 10 mM glucose, 110 mM citric acid, (previously shown to give a near maximal aggregatory response), in the presence of 1:7 v/v acid citrate dextrose (120 mM sodium citrate, 2.9 mM KCl, 10 mM HEPES, 1 mM MgCl2, 5 mM glucose, pH 7.3). This platelet suspension, 10 μg/mL of kinase assay buffer, and 10 μg of Raytide was added to each sample. The reaction was started by the addition of 10 μl of ATP buffer (0.15 mM ATP, 30 mM MgCl2, and 200 μCi/mL [%-32P]ATP in kinase assay buffer). After incubation at 30 °C for 30 min the reaction was terminated by the addition of 10% phosphoric acid. Samples were applied to 2 x 2 cm squares of PS1 ion exchange chromatography paper, washed extensively in 0.5% phosphoric acid followed by a wash in acetone. Papers were then dried, and labeled Raytide was quantified by liquid scintillation counting.

**Measurement of Cytosolic Calcium**

This was performed as described previously (25). Briefly, 3 mM Fura 2-AM was added to platelet-rich plasma and incubated at 30 °C for 45 min in the presence of 10 μM indomethacin. Platelets were isolated as described above. Platelets were stimulated at room temperature in the absence of EGTA. Fluorescence excitation was made at 340 and 380 nm, and emission at 510 nm measured using a PerkinElmer Life Sciences LS5 spectrofluorometer. Data are presented as the excitation fluorescence ratio (340:380 nm).

**Measurement of Released 5-HT**

Platelets were loaded by incubation of platelet-rich plasma with 0.2 μCi/ml 5-[3H]HT for 1 h at 37 °C. Platelets were preincubated with 1 μM EGTA before stimulation to prevent aggregation. Reactions were terminated by brief microcentrifugation, and 5-[3H]HT released into the supernatant was determined by liquid scintillation counting and expressed as a percentage of the total tissue content, as described previously (30).

**Results**

**Alboaggregin A Signaling Induces Tyrosine Phosphorylation of Btk and Associated Proteins—**Previous studies have shown that collagen and collagen-related peptides acting through GP VI induce tyrosine phosphorylation and activation of Btk (6, 7). Fig. 1 shows that upon combined stimulation of GP VI and GP Ib with alboaggregin A, Btk became rapidly tyrosine-phosphorylated and associated with a number of tyrosine-phosphorylated proteins when platelets were lysed into Nonidet P-40 (1%). In contrast, these associated proteins were lost when platelets were lysed in the more stringent RIPA lysis buffer. Fig. 1B shows that upon stimulation of platelets with alboaggregin A, one of the Btk-associated proteins was the Fc receptor γ chain, an accessory signaling molecule that has been shown to associate with both GP VI and GP Ib (19, 31).

**PP1 Inhibits Tyrosine Phosphorylation of Btk and Its Association with Tyrosine-phosphorylated Proteins—**The Src family kinases Fyn and Lyn have been shown to associate with the GP Ib receptor complex and to be essential for signaling downstream of the receptor (19). The Src family kinase inhibitor PP1 is shown in Fig. 2 to inhibit tyrosine phosphorylation of Btk dose dependently, with maximal inhibition by 20 μM. The appearance of tyrosine-phosphorylated associated proteins was also markedly reduced by PP1. Fig. 2 also shows that tyrosine phosphorylation of Btk is receptor-specific; stimulation of platelets with plasma vWF and ristocetin also induces tyrosine phosphorylation of Btk in contrast to a lack of response to the G protein-coupled receptor agonist thrombin.

**Alboaggregin A Signaling Induces Activation of Btk—**Btk has been shown to be activated in platelets after stimulation with collagen and CD32 cross-linking (6, 7). Here, Btk activity was assayed in vitro either as autophosphorylation (Fig. 3A) or
by phosphorylation of an exogenous peptide substrate, Raytide (Fig. 3B). Fig. 3A shows that there is kinase activity in samples containing Btk immunoprecipitated from alboaggregin A-stimulated platelets, and a number of proteins became phosphorylated in vitro. After transfer of these proteins to polyvinylidene difluoride membrane and Western blotting, one of these proteins was identified as Btk. Fig. 3, A(iii) and B, shows that activation of platelets with alboaggregin A induces a marked increase in Btk activity, as assayed by phosphorylation of the tyrosine kinase substrate Raytide. The Btk inhibitor LFM-A13 (40 μM) abolished Btk activity when preincubated with platelets before stimulation. Also, preincubation of platelets with the PKC inhibitor BIM caused ablation of Btk activity. Addition of the Src family kinase inhibitor PP1 to the kinase assay buffer caused a change in the measured activity (data not shown), but addition of LFM-A13 to the kinase assay buffer caused ablation of activity, showing that the tyrosine kinase activity measured was the activity of Btk.

Btk Plays a Role in Calcium Entry and Control of 5-HT Secretion—It has been reported recently that in both platelets and B cells there is a pathway of Ca\(^{2+}\) entry which involves phosphatidylinositol 3,4,5-trisphosphate and Btk but is independent of phospholipase C activity (2-4). Stimulation of platelets with alboaggregin A causes a rise in cytosolic calcium ([Ca\(^{2+}\)]\(_i\)) which is achieved by release of stored calcium and by influx of calcium from the extracellular medium. Fig. 4i shows that upon incubation of platelets with 40 μM LFM-A13, the profile of the [Ca\(^{2+}\)]\(_i\) rise induced by alboaggregin A stimulation was altered markedly. Although the rapid rise in [Ca\(^{2+}\)]\(_i\) was similar to controls, in LFM-A13-treated platelets the [Ca\(^{2+}\)]\(_i\) declined from a peak more rapidly than control platelets. In the presence of 1 mM EGTA to chelate extracellular calcium, LFM-A13 had no effect on alboaggregin A-induced calcium response (Fig. 4ii), supporting the hypothesis that Btk regulated calcium entry. LFM-A13 had no effect on the calcium response to thrombin stimulation (Fig. 4iii). Fig. 4iv shows the secretion of stored 5-HT in response to alboaggregin A stimulation. 5-HT release was increased by preincubation of platelets with the Btk inhibitor LFM-A13 (40 μM) and was decreased by preincubation with the PKC inhibitor BIM (20 μM). In the presence of both inhibitors, secretion was enhanced relative to control samples but less than that in the presence of LFM-A13 alone.

Btk Is Phosphorylated on Threonine and Associates with PKC\(\theta\) upon Alboaggregin A Stimulation—Fig. 5A shows that in addition to tyrosine phosphorylation, alboaggregin A stimulation of platelets induces rapid threonine phosphorylation of Btk, with phosphorylation occurring by 15 s of stimulation and furthermore that this phosphorylation is ablated by preincubation of platelets with 20 μM BIM before stimulation. Preincubation of platelets with BIM had no effect on the tyrosine phosphorylation of Btk (data not shown). An association between Btk and PKC has been demonstrated in mast cells (13), with the α, β1, β2, ε, and ζ isoforms able to bind to Btk in vitro and the β1 isoform associating in vivo. In vitro associations between Btk and PKCs ε, ζ, and μ have also been demonstrated in B cells (14, 16). Using immunoblotting we examined platelet expression of PKC isoforms α, β, γ, δ, η, ζ, ε, μ, and θ, and in Fig. 5B we demonstrate the presence of all isoforms except ζ. Fig. 5B also examines association between Btk and PKC isoforms and shows that only PKC\(\theta\) associates with Btk, in an alboaggregin A stimulation-dependent manner. Fig. 5C shows that PKC \(\theta\) coprecipitates with Btk from alboaggregin A-stimulated...
that PKC/H9258 samples were stimulated, as indicated, after a 10-min preincubation of panel A measured by liquid scintillation counting. Data shown are the mean

**B** stimulation-dependent manner.

**Basal conditions, and association increases in an alboaggregin A** stimulation-dependent manner. vWF/ristocetin (in the presence of 1 mM EGTA to prevent binding to αIIbβ3) also induced phosphorylation of PKCθ (Fig. 7B). In addition to Src family kinase inhibition, Btk inhibition with LFM-A13 was also shown to reduce PKCθ tyrosine phosphorylation dose-dependently (Fig. 7C).

**Alboaggregin A Induces Activation of PKCθ—PKCθ** activity was assayed either as autophosphorylation (Fig. 8A) or as the phosphorylation of MBP, an exogenous PKC substrate (Fig. 8B). Western blotting revealed that this latter procedure induced no tyrosine phosphorylation of MBP (data not shown), i.e. the activity measured in Fig. 8B is a serine/threonine kinase. Using either method, stimulation of platelets with alboaggregin A is shown to induce activation of PKCθ. Addition of 20 μM BIM to the kinase assay buffer caused abolition of the measured activity. However, preincubation of platelets with either BIM or LFM-A13 before stimulation with alboaggregin A caused a marked potentiation of PKCθ activity assayed in vitro.

**DISCUSSION**

Binding of vWF to the GP Ib-V-IX receptor complex and of collagen to GP VI induces a signaling cascade in platelets involving many divergent and convergent pathways including events such as a transient rise in cytosolic calcium levels, hydrolysis of phosphoinositides, synthesis of thromboxane A2, activation of PKC, cytoskeletal reorganization, and the activation of the binding function of integrin αIIbβ3 (22, 24, 33–35). The activity of Btk has been shown not only to be regulated by protein phosphorylation (11) but also to be dependent upon binding to the phospholipid phosphatidylserine trisphosphate, a product of the enzyme phosphatidylinositol 3-kinase (36, 37). It is becoming increasingly clear that tyrosine phosphorylation is a critical element in the process of vWF- and collagen-induced platelet activation and that Btk may represent an important focal point for both the tyrosine phosphorylation and lipid hydrolysis facets of the signaling pathway.

In this study, Btk has been shown to become rapidly tyrosine-phosphorylated downstream of alboaggregin A stimulation or activation of GP Ib by vWF/ristocetin in the presence of EGTA to block interaction of vWF with α1bβ3. To our knowledge this is the first report of involvement of Btk in signaling through GP Ib, although it has previously been shown to be phosphorylated and activated by collagen through GP VI (7). Phosphorylation of Btk was inhibited by FP1 and therefore is downstream of a Src family kinase. This is in agreement with Rawlings et al. (11), who have shown that Btk is transphosphorylated by members of Src family kinases, regulating the activity of Btk. Fig. 1 also shows association of Btk with multiple tyrosine phosphoproteins including Fc receptor γ chain. This is also consistent with previous studies of GP Ib and GP VI signaling which have shown the Src kinases Fyn and Lyn to be recruited to GP Ib and GP VI signaling complexes at an early stage, leading to phosphorylation of the Fe receptor γ chain (18–20). Interestingly, Btk was not tyrosine-phosphorylated upon activation of platelets with thrombin, in contrast to a previous report (38). The difference could be because of the presence of EGTA in our experimental conditions, blocking signaling by α1bβ3 which is likely to be the receptor that couples to activation of Btk under these conditions.

FIG. 3. Alboaggregin A stimulation induces activation of Btk. Panel A(i), Btk was immunoprecipitated from basal platelets or platelets stimulated for 1 min with 3.5 μg/ml alboaggregin A. Samples (transferred to a polyvinylidene difluoride membrane) were subjected to an in vitro autophosphorylation assay as described under "Experimental Procedures," and incorporation was visualized by autoradiography. Panel A(ii) shows a Western blot reprobe with anti-Btk antibody. Panel A(iii), Btk activity as assayed by phosphorylation of exogenous tyrosine kinase peptide substrate Raytide. Btk was immunoprecipitated from unstimulated platelets or platelets stimulated with 3.5 μg/ml alboaggregin A for various times as indicated. Immunoprecipitates were incubated with Raytide, and incorporation of 32P into Raytide was measured by liquid scintillation counting. Data shown are the mean ± S.E. (n = 3). Panel B, effects of Btk and PKC inhibition on Btk activity. The assay was similar to that in panel A(iii) except that samples were stimulated, as indicated, after a 10-min preincubation of platelets with 20 μM BIM or 40 μM LFM-A13. 40 μM LFM-A13 was added to the kinase assay buffer (KAB) in some samples as indicated before the in vitro kinase assay. Incorporation of 32P into Raytide was measured by liquid scintillation counting. Data shown are the mean ± S.E. (n = 3).

platelets lysed in Nonidet P-40 lysis buffer but not with those lysed in RIPA buffer. RIPA buffer has therefore been used in this study to demonstrate definitively PKCθ phosphorylation in gels where Btk has not been coprecipitated. Fig. 6A shows a time course of stimulation-dependent association between Btk and PKCθ, with association occurring by 15 s stimulation and becoming maximal by 30 s. This association is also induced by stimulation of platelet GP Ib-V-IX with vWF/ristocetin in the presence of 1 mM EGTA to block activation of integrin αIIbβ3. PKCθ Associates with Btk via the PH Domain of Btk—It has been shown previously that Btk/PKCθ association is mediated by interaction between the N-terminal PH domain of Btk and the C1 domain of PKC in mast cell lysates (13). Fig. 6B shows that PKCθ associates with the GST-Btk PH domain under basal conditions, and association increases in an alboaggregin A stimulation-dependent manner.

**PKCθ Becomes Tyrosine-phosphorylated upon vWF and Alboaggregin A Stimulation Downstream of Src Family and Btk**

Activity—Activation of T lymphocytes through the T cell receptor leads to rapid tyrosine phosphorylation of PKCθ (32). Consequently, it was decided to investigate the state of tyrosine phosphorylation of PKCθ in platelets, before and after stimulation with alboaggregin A. As can be seen in Fig. 7A, PKCθ is not tyrosine-phosphorylated under basal conditions but becomes so upon platelet activation by alboaggregin A by 15 s. As with Btk, the Src family kinase inhibitor PP1 was found to inhibit tyrosine phosphorylation of PKCθ dose-dependently (Fig. 7B). vWF/ristocetin (in the presence of 1 mM EGTA to prevent binding to αIIbβ3) also induced phosphorylation of PKCθ (Fig. 7B). In addition to Src family kinase inhibition, Btk inhibition with LFM-A13 was also shown to reduce PKCθ tyrosine phosphorylation dose-dependently (Fig. 7C).
Btk has been shown to become enzymically active downstream of collagen stimulation in platelets (6), and here we show that Btk is activated by alboaggregin A also (Fig. 3, A and B). This activation is rapid, with significant activity observed after a 15-s stimulation and maximal by 30 s. Interestingly, the activity of Btk was reduced substantially when platelets were pretreated with BIM, the nonisozyme selective PKC inhibitor. This suggests that in this system, Btk is positively regulated by upstream serine/threonine phosphorylation by PKC. This is not to say that there is a linear pathway from receptor to PKC activation to Btk activation, rather that PKC activity is one of the elements necessary to activate Btk fully. This is a novel finding, but because of the nonselectivity of BIM among the PKC isoforms it is not possible at this stage to assess whether Btk is specifically regulated by associated PKC/H9258. Precedence for the activation of a tyrosine kinase by PKC-mediated phosphorylation has been set by the demonstration that PKCδ phosphorylates and activates the kinase c-Abl in vitro (39), PKCγ activates Fyn (40), and PKCa, β1, β2, and γ are upstream of Syk (41). In other cell types, PKC isoforms have been shown to phosphorylate Btk, leading to an inhibition of Btk activity (13), and therefore our data are in contrast with these reports. In addition our data are in contrast to previous reports that have suggested that (in other cell types) PKC isoforms can be activated by tyrosine phosphorylation by Btk or Src family tyrosine kinases (16, 32). It is possible, however, that Btk and PKC isoforms may be regulated differently by serine/threonine or tyrosine phosphorylation in different cell types and in a PKC isozyme-specific manner, which may explain any discrepancy.

The function of Btk in platelet signaling has yet to be elucidated fully, but there are several possibilities. A role in the regulation of cytosolic calcium concentration has been shown (3). It may accomplish this by activating phospholipase C by tyrosine phosphorylation (2, 42), but it is also possible that Btk may activate a cell surface calcium channel directly or indirectly, leading to calcium influx (2–4). Our data using the Btk inhibitor LFM-A13 support a role for Btk in regulation of a delay phase calcium entry mechanism, without any significant effect on calcium release from internal stores, as indicated by the lack of effect of LFM-A13 on the calcium response in the presence of EGTA (Fig. 4ii). This finding is shown to be receptor-specific because the calcium response elicited by thrombin was unaffected by LFM-A13, in accordance with our finding that Btk is not activated by thrombin. In addition, using a genetic approach, others have shown Btk to have a role in the regulation of aggregation and secretion (6, 7). In X-linked agammaglobulinemia patients, functionally deficient in Btk, no overt bleeding disorders are detected, although there is de-

**FIG. 4.** Alboaggregin A induces a rise in cytosolic calcium and release of stored 5-HT which are modulated by Btk inhibition. Panels i–iii, platelets were loaded by preincubation for 45 min with 3 μM Fura 2-AM, a calcium indicator. Panel i, rise in cytosolic calcium in response to 3.5 μg/ml alboaggregin A in the presence (A + LFM) and absence (A) of 40 μM LFM-A13. Panel ii, as in i but conducted in the presence of 1 mM EGTA. Panel iii, calcium response to 1 unit/ml thrombin in the presence and absence of LFM-A13. All panels show results from one experiment but are representative of at least three repetitions. Panel iv, platelets were loaded by preincubation for 1 h with 0.2 μCi/ml 3H-labeled 5-HT. Platelets were pretreated with either 40 μM LFM-A13, 20 μM BIM, both inhibitors, or dimethyl sulfoxide as control for 10 min before stimulation. Released 3H-labeled 5-HT in response to stimulation with 3.5 μg/ml alboaggregin A for 1 min was measured by liquid scintillation counting. Results shown are the mean ± S.E. (n = 3).
increased platelet aggregation and secretion in response to collagen (7). This is in contrast to our data, which show a marked potentiation of 5-HT secretion induced by alboaggregin A when Btk is inhibited by LFM-A13, although there is no change in platelet aggregation (data not shown). This discrepancy may be the result of functional redundancy between the Tec family kinases because both Btk and Tec are expressed in platelets and have been shown to substitute for each other functionally (6). Our pharmacological approach may differ from selective genetic targeting of Btk because LFM-A13 may affect the function of other Tec family kinases, abolishing the functional redundancy. The potentiation of 5-HT secretion by LFM-A13 may be caused by inhibition of a negative regulation of PKCθ by Btk, as we show in Fig. 8. This leads to increased PKCθ activity, which may in turn lead to increased 5-HT secretion. However, when BIM was also added to LFM-A13-treated platelets, the increase in secretion was attenuated markedly but was not abolished (Fig. 4), so indicating a possible alternative negative regulatory role for Btk in the control of platelet 5-HT secretion.

Previous studies conducted in other hematopoietic cell types have indicated associations between Btk and various isoforms of PKC (13–15). We show here that alboaggregin A stimulation causes rapid threonine phosphorylation of Btk downstream of PKC activity (phosphorylation occurs by 15 s of stimulation...
and is maximal at 30) and an association between Btk and PKC\theta in platelets which is not constitutive but occurs upon platelet activation by alboaggregin A or activation of GP Ib using vWF/ristocetin. We also show that this association occurs via the PH domain of Btk, which is in agreement with previous reports concerning Btk/PKC interaction (14). Interestingly GST-Btk-PH pulls down some PKC\theta even in the absence of cell stimulation. This may indicate that interaction between Btk and PKC\theta is controlled normally by differential compartmentation of the two kinases, which, although capable of interacting, are distributed differentially in the cell under basal conditions. Btk does not associate with other isoforms of PKC which have been shown in other cell types to associate with Btk. A possible explanation for this again may be that other isoforms of PKC are compartmentalized separately from Btk in platelets, or it may be that a conformational change in PKC\theta is necessary before binding to Btk can occur and that alboaggregin A induces the necessary signal to PKC\theta selectively. This report also shows that PKC\theta is tyrosine-phosphorylated subsequent to alboaggregin A stimulation. This is consistent with a previous report showing tyrosine phosphorylation of PKC\theta downstream of T cell receptor activation (32). We show that this phosphorylation is downstream of a Src family kinase, as for tyrosine phosphorylation of Btk, and that although it may be induced by alboaggregin A or vWF/ristocetin, it is not induced by thrombin. In addition, tyrosine phosphorylation of PKC\theta is dependent on Btk activity (Fig. 7B), and it is possible that Btk may therefore phosphorylate PKC\theta directly.

Finally, alboaggregin A was shown to activate PKC\theta (Fig. 8). This activity was increased by pretreatment of platelets with LFM-A13 (indicating that Btk has a negative effect on PKC\theta activity) and intriguingly also by pretreatment with the PKC inhibitor BIM. Inhibition of PKC\theta in platelets may therefore subsequently lead to enhanced activity of immunoprecipitated PKC\theta in vitro, where BIM has been washed out. An explanation for this may be that Btk and PKC\theta are both activated by alboaggregin A and associate with and transphosphorylate each other. Activation of PKC leads to activation of Btk, possibly by direct phosphorylation. Subsequent activation of Btk leads to negative feedback control of PKC\theta, as shown by potentiation of PKC\theta activity after platelet treatment with LFM-A13. So, when PKC\theta is inactivated in platelets by preincubation with BIM, Btk is suppressed as shown in Fig. 3B. Negative feedback control of PKC\theta by Btk is therefore lost such that when PKC\theta is immunoprecipitated and BIM washed out, its activity is greater than control platelets that were not pre-treated with BIM. Fig. 9 illustrates the working model pathway showing reciprocal cross-talk between Btk and PKC\theta. It will be important to determine phosphorylation sites involved in this functional interaction between serine/threonine and tyrosine kinases.

Acknowledgments—We thank Prof. David Theakston, Prof. Edvard Smith, Prof. Jan Sixma, and Dr. Tom Vink for a generous supply of reagents. We are also grateful to Dr. Mark Thomas and Dr. Leo Brady for assistance with purification of alboaggregin A and to Dr. Kanamarlapudi Venkateswarlu for assistance with generating GST-Btk-PH.
REFERENCES

1. Satterthwaite, A. B., Li, Z., and Witte, O. N. (1998) Semin. Immunol. 10, 309–316
2. Fluckiger, A. C., Li, Z., Kato, R. M., Wahl, M. I., Ochs, H. D., Longnecker, R., Kinet, J. P., Witte, O. N., Scharenberg, A. M., and Rawlings, D. J. (1998) EMBO J. 17, 1973–1985
3. Pasquet, J. M., Quek, L., Stevens, C., Bobe, R., Huber, M., Duronio, V., Krystal, G., and Watson, S. P. (2000) EMBO J. 19, 2793–2802
4. Scharenberg, A. M., El-Hillal, O., Fruman, D. A., Beitz, L. O., Li, Z., Lin, S., Gout, I., Cantley, L. C., Rawlings, D. J., and Kinet, J. P. (1998) EMBO J. 17, 1961–1972
5. Genevier, H. C., and Callard, R. E. (1997) Clin. Exp. Immunol. 110, 386–391
6. Oda, A., Ikeda, Y., Ochs, H. D., Druker, B. J., Onuki, K., Handa, M., Ariga, T., Sakiyama, Y., Witte, O. N., and Wahl, M. I. (2000) Blood 95, 1663–1670
7. Quek, L. S., Bolen, J., and Watson, S. P. (1998) Curr. Biol. 8, 1137–1140
8. Wahl, M. I., Fluckiger, A. C., Kato, R. M., Park, H., Witte, O. N., and Rawlings, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11526–11533
9. Park, H., Wahl, M. I., Afar, D. E., Tureck, C. W., Rawlings, D. J., Tam, C., Scharenberg, A. M., Kinet, J. P., and Witte, O. N. (1996) Immunity 4, 515–525
10. Afar, D. E., Park, H., Howell, B. W., Rawlings, D. J., Cooper, J., and Witte, O. N. (1996) Mol. Cell. Biol. 16, 3465–3471
11. Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinet, J. P. (1996) Science 271, 822–825
12. Salim, K., Bottomley, M. A., Querfurth, E., Zelevski, M. J., Gout, I., Scalfi, R., Margulis, R. L., Gigg, H., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996) EMBO J. 15, 6241–6250
13. Yao, L., Kawakami, Y., and Kawakami, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9175–9179
14. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W. B., Kawakami, Y., and Kawakami, T. (1997) J. Biol. Chem. 272, 13033–13039
15. Johannes, F. J., Hausser, A., Storz, P., Truckenmuller, L., Link, G., Kawakami, Y., and Pfizenmaier, K. (1999) FEBS Lett. 461, 69–72
16. Kawakami, Y., Kitaura, J., Hartman, S. E., Lowell, C. A., Siragianian, R. P., and Kawakami, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7423–7428
17. Savage, B., Shattil, S. J., and Ruggeri, Z. M. (1992) J. Biol. Chem. 267, 13030–13036
18. Wu, Y., Suzuki-Inoue, K., Satoh, K., Asazuma, N., Yatomi, Y., Berndt, M. C., and Ozaki, Y. (2001) Blood 97, 3836–3845
19. Falati, S., Edmead, C. E., and Poole, A. W. (1999) Blood 94, 1648–1656
20. Ezumi, Y., Shindoh, K., Tsuji, M., and Takayama, H. (1998) J. Exp. Med. 188, 267–276
21. Andrews, R. K., Lopez, J. A., and Berndt, M. C. (1997) Int. J. Biochem. Cell Biol. 29, 91–105
22. Chow, T. W., Hellums, J. D., Moake, J. L., and Kroll, M. H. (1992) Blood 80, 113–120
23. Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Rabinowitz, I., Salem, H. H., and Mitchell, C. A. (1994) J. Biol. Chem. 269, 27095–27099
24. Kroll, M. H., Harris, T. S., Moake, J. L., Handin, R. I., and Schafer, A. I. (1991) J. Clin. Invest. 88, 1568–1573
25. Poole, A. W., and Watson, S. P. (1995) Br. J. Pharmacol. 115, 101–106
26. Dormann, D., Clemenson, J. M., Navdaev, A., Kehrel, B. E., and Clemenson, K. J. (2001) Blood 97, 929–938
27. Asazuma, N., Marshall, S. J., Berlanga, O., Snell, D., Poole, A. W., Berndt, M. C., Andrews, R. K., and Watson, S. P. (2001) Blood 97, 3989–3991
28. Peng, M., Lu, W., and Kirby, E. P. (1991) Biochemistry 30, 11529–11536
29. Peng, M., Lu, W., and Kirby, E. P. (1992) Thromb. Haemostasis 67, 702–707
30. Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G., Saito, T., Tybuliewicz, V. L., and Watson, S. P. (1997) EMBO J. 16, 2333–2341
31. Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M., and Watson, S. P. (1997) FEBS Lett. 413, 255–259
32. Liu, Y., Witte, S., Liu, Y. C., Doyle, M., Elly, C., and Altman, A. (2000) J. Biol. Chem. 275, 3603–3609
33. Kroll, M., Hellums, J., Guo, Z., Durante, W., Ramdaw, K., Hrbolich, J., and Schafer, A. (1993) J. Biol. Chem. 268, 3520–3524
34. Cunningham, J. G., Meyer, S. C., and Fox, J. E. B. (1996) J. Biol. Chem. 271, 11581–11587
35. Yuan, Y., Dupheide, S. M., Ivanidis, C., Salem, H. H., and Jackson, S. P. (1997) J. Biol. Chem. 272, 21847–21854
36. Li, Z., Wahl, M. I., Egusias, A., Stephens, L. R., Hawkins, P. T., and Witte, O. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13820–13825
37. Saito, K., Scharenberg, A. M., and Kinet, J. P. (2001) J. Biol. Chem. 276, 16201–16206
38. Laffargue, M., Ragah-Thomas, J. M., Ragah, A., Tuceh, J., Missy, K., Monnereau, L., Blank, U., Plantavid, M., Payrastre, B., Raynal, P., and Chap, H. (1999) FEBS Lett. 443, 66–70
39. Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000) J. Biol. Chem. 275, 7470–7473
40. Cabodi, S., Calautti, E., Talora, C., Kuroki, T., Stein, P. L., and Dotto, G. P. (1999) Mol. Cell. Biol. 19, 1121–1129
41. Borowski, P., Heiland, M., Kornetzky, L., Medem, S., and Laufs, R. (1998) Biochem. J. 331, 649–657
42. Takata, M., and Kurosaki, T. (1996) J. Exp. Med. 184, 31–40
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J. Biol. Chem. 2002, 277:9958-9965.
doi: 10.1074/jbc.M108965200 originally published online January 11, 2002

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