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FcγRIIB1 Inhibition of BCR-Mediated Phosphoinositide Hydrolysis and Ca^{2+} Mobilization Is Integrated by CD19 Dephosphorylation

Keli L. Hippen,*†‡ Anne Mette Buhl,*† Daniele D'Ambrosio,*§ Kazuhiro Nakamura,* Christoph Persin,*‖ and John C. Cambier*
*Division of Basic Sciences
Department of Pediatrics
National Jewish Medical and Research Center
Department of Immunology
University of Colorado Health Sciences Center
Denver, Colorado 80206

Summary

The B cell receptor for immunoglobulin G, FcγRIIB1, is a potent transducer of signals that block antigen-induced B cell activation. Coligation of FcγRIIB1 with B lymphocyte antigen receptors (BCR) causes premature termination of phosphoinositide hydrolysis and Ca^{2+} mobilization and inhibits proliferation. This inhibitory signal is mediated in part by phosphorylation of FcγRIIB1 and recruitment of phosphatases; however, the molecular target(s) of effectors is unknown. Here we report that FcγRIIB1 inhibition of BCR signaling is mediated in part by selective dephosphorylation of CD19, a BCR accessory molecule and coreceptor. CD19 dephosphorylation leads to failed CD19 association with phosphatidylinositol 3-kinase, and this in turn leads to termination of inositol-1,4,5-trisphosphate production, intracellular Ca^{2+} release, and Ca^{2+} influx. The results define a molecular circuit by which FcγRIIB signals block phosphoinositide hydrolysis.

Introduction

Coligation of the B cell's low-affinity receptor for immunoglobulin G (IgG), FcγRIIB1, with the antigen receptor (BCR), as occurs in vivo when B cells encounter immune complexes containing IgG and exposed antigenic epitopes recognized by the BCR, leads to abortive BCR signaling blocking biologic responses to antigen (Chan and Sinclair, 1971; Phillips and Parker, 1983). FcγRIIB1 knockout mice exhibit enhanced antibody production, particularly late in the immune response (Takai et al., 1996). Therefore, FcγRIIB1 appears to play an important negative regulatory function in the humoral immune response.

FcγRIIB1 signaling function is mediated by a 13–amino acid cytoplasmic sequence containing a tyrosine residue that is phosphorylated upon receptor coligation (Amigorena et al., 1992; Fridman et al., 1992; Muta et al., 1994). Phosphorylation of this tyrosine is required for negative signaling (Muta et al., 1994) and mediates binding to and activation of the Src homology domain 2 (SH2)–containing tyrosine phosphatase SHP-1 (also known as PTP1C, HCP, and SHPTP-1) (D'Ambrosio et al., 1995). The FcγRIIB1 phosphotyrosine also mediates receptor binding to SHP-2 (also known as PTP1D, Syp, and SHPTP-2) and SHIP, a phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) 5′- phosphatase (D'Ambrosio et al., 1996; Damen et al., 1996; Ono et al., 1996). Furthermore, SHIP is tyrosine phosphorylated upon BCR–FcγRIIB1 coligation (Chacko et al., 1996). Although the roles of SHP-2 and SHIP in inhibitory signaling are unclear, SHP-1 expression has been shown to be essential for FcγRIIB1 inhibition of BCR-induced B cell proliferation (D'Ambrosio et al., 1995). Recent findings indicate that certain other immune system receptors, notably the killer inhibitory receptors and CTLA-4, also utilize SHP-1 and/or the closely related SHP-2 phosphatase for transduction of inhibitory signals, but interestingly these receptors do not bind SHIP (Burshtyn et al., 1996; Marengere et al., 1996; Olcese et al., 1996; Vely et al., 1997). The logical next step in understanding these inhibitory signaling processes is elucidation of the molecular site of action of the FcγRIIB1 effectors.

FcγRIIB1 has been shown to exercise its effect by inhibiting BCR- mediated phosphoinositide hydrolysis and calcium mobilization (Bijsterbosch and Klaus, 1985; Wilson et al., 1987; Choquet et al., 1993), placing the site of action of Fc receptor effector at or above inositol lipid hydrolysis in the BCR signaling cascade. Some evidence suggests that the calcium influx from the extracellular space may be selectively inhibited by the FcγRIIB1 signal (Wilson et al., 1987; Choquet et al., 1993). The antigen receptor signaling cascade appears to proceed as follows: ligation of the antigen receptor leads to rapid tyrosine phosphorylation of BCR transducer subunits Igα and Igβ (reviewed by Cambier, 1995) and BCR-associated CD19 (Chalupny et al., 1993; Tuveson et al., 1993). Phosphorylation of Igα (tyrosines 182 and 193) and Igβ (tyrosines 195 and 206) (Flaswinkel and Reth, 1994; Cambier, 1995) facilitates recruitment of Src- and Syk-family kinases that bind BCR phosphotyrosines via their SH2 domains and are consequently activated (Clark et al., 1992, 1994; Johnson et al., 1995; Rowley et al., 1995; Shiue et al., 1995). Phosphorylation of Syk mediates its association with phospholipase Cγ (PLCγ), presumably positioning PLCγ for activation by Syk (Sillman and Monroe, 1995). BCR-associated Src-family kinases bind phosphatidylinositol 3-kinase (PI3K) via an SH3 domain–p85 proline-rich region interaction. CD19 phosphorylation at tyrosine residues 484 and/or 515 initiates recruitment and activation of PI3K and perhaps Vav (Tuveson et al., 1993; Weng et al., 1994). Based on this information, we originally hypothesized that FcγRIIB1-coupled phosphatases may terminate BCR-mediated inositol-1,4,5-trisphophate (IP_3) generation and calcium mobilization by dephosphorylating Igα/Igβ, Src- or Syk- family tyrosine kinases, or PLCγ.

Here we describe analysis of the site in the BCR signaling cascade that is disrupted by FcγRIIB1 signaling,

† These authors contributed equally to the study.
‡ Present address: Departments of Medicine and Rheumatology, University of Minnesota, Minneapolis, Minnesota 55455.
§ Present address: Roche Milano Ricerche, Department of Biology and Technology, Hospital S. Raffaele, Via Olgettina 58, Milano, Italy.
‖ Present address: Hoechst AG, Molecular Biology, H528, Rheingaustrasse 190, D-65174 Wiesbaden, Germany.

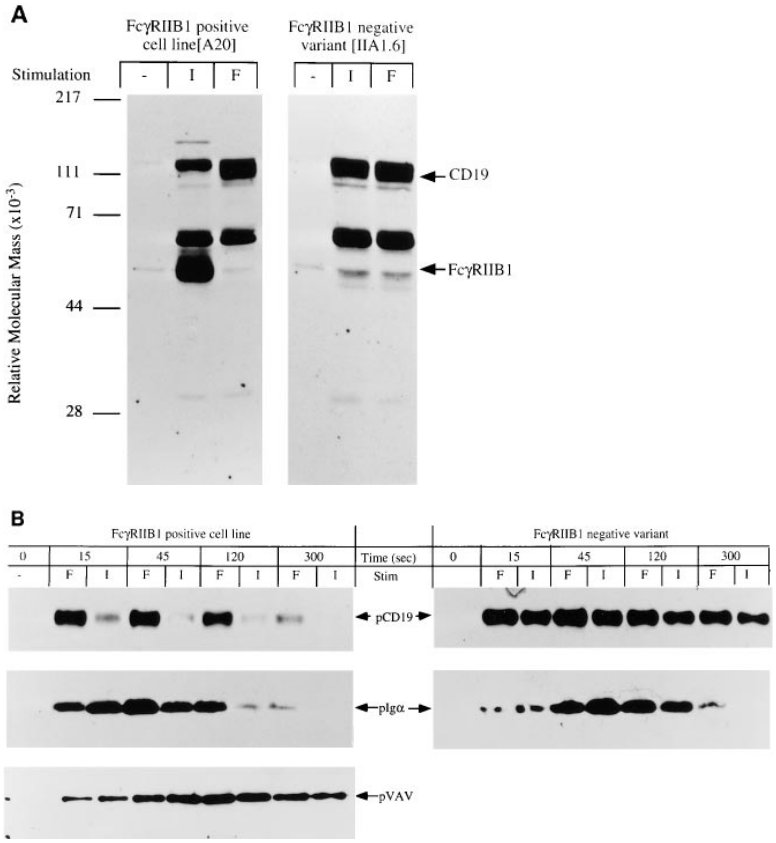


Figure 1. Effect of FcγRIIB1 Coligation on BCR-Mediated Tyrosine Phosphorylation

A20 and IIA1.6 (a FcγRIIB1 negative variant of A20) B lymphoma cells were cultured without stimulus (-) or were stimulated with intact (I) or $F(ab')_2$ (F) fragments of rabbit antibody to mouse immunoglobulin (BCR).

(A) Anti-phosphotyrosine immunoblots of A20 and IIA1.6 lysates (1×10^6 cell equivalents) of cells cultured with and without stimulation for 2 min.

(B) Anti-phosphotyrosine immunoblots of CD19, Vav, and Igα immunoprecipitates from A20 and IIA1.6 cells (20×10^6), stimulated for various time periods (0–300 sec).

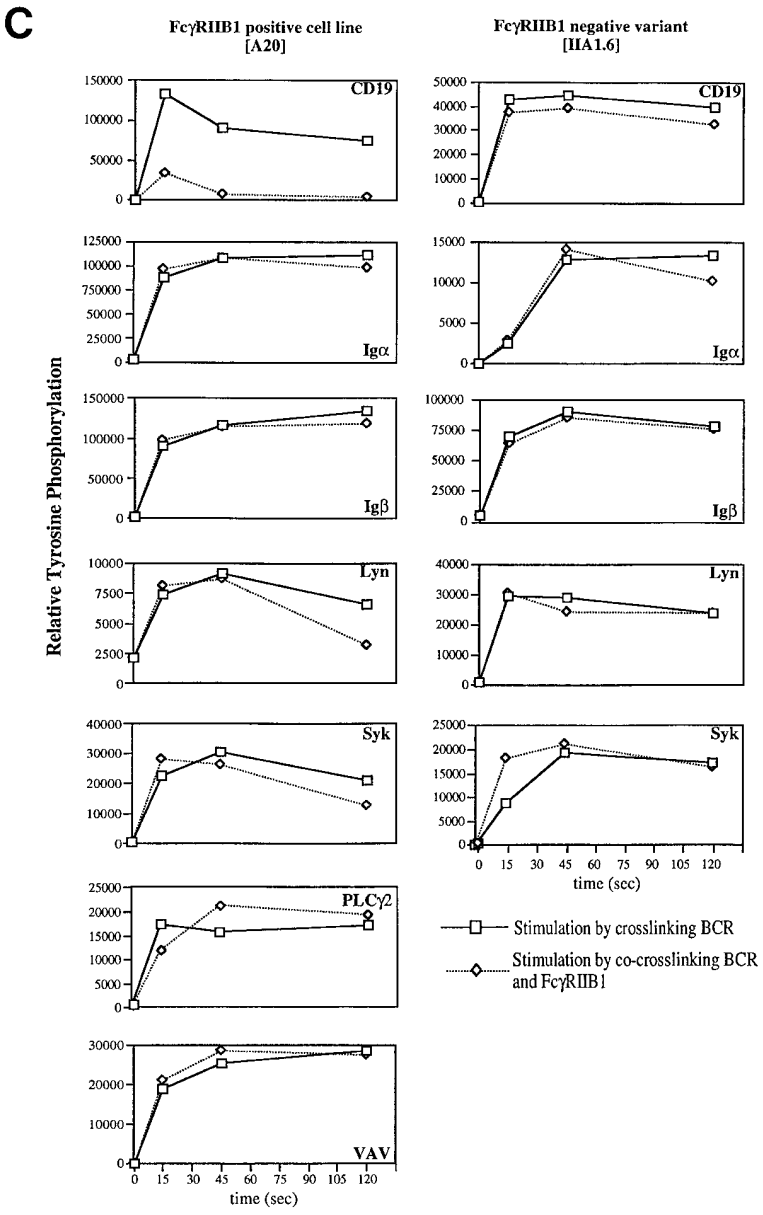
(C) Representation of the tyrosine phosphorylation of CD19, Igα, Igβ, PLCγ2, Lyn, Syk, and Vav isolated from A20 and IIA1.6 cell lysates normalized to the amount of effector present in the sample determined by immunoblotting. Data are representative of three independent experiments.

defining an in vivo substrate of operative effectors, presumably phosphotyrosine phosphatases, and determining the consequences of reduced substrate phosphorylation. Results demonstrate that among major substrates phosphorylated upon BCR ligation, only CD19 exhibits a rapid decrease in phosphorylation when FcγRIIB1 is co-crosslinked with BCR. This apparent dephosphorylation of CD19 occurs between 15 and 45 sec following costimulation and results in failed recruitment of PI3K to CD19. PI3K inhibitors were found to mimic the effect of FcγRIIB1 coligation on both BCR-mediated IP_3 production and Ca^{2+} mobilization, consistent with mediation of Fc receptor function by failed PI3K activation. Finally, results of comparative analysis of B cells from $CD19^{-/-}$ mice and normal littermates support the contention that failed function of CD19 mediates, at least in part, the FcγRIIB1 effect. Specifically, B cells from $CD19^{-/-}$ mice exhibit reduced Ca^{2+} mobilization reminiscent of that seen upon BCR–FcγRIIB1 coligation in $CD19^{+/+}$ cells. Finally, $CD19^{-/-}$ B cells were not sensitive to FcγRIIB1-mediated inhibition of Ca^{2+} mobilization.

Results

Decreased Tyrosine Phosphorylation of CD19 when FcγRIIB1 is Coligated with BCR

To define the target(s) of FcγRIIB1 effectors in the antigen receptor signaling pathway, we compared the phosphorylation of major cellular substrates following crosslinking of BCR alone and co-crosslinking of FcγRIIB1 with BCR. The FcγRIIB1-positive murine B lymphoma cell line A20 and its FcγRIIB1-negative variant IIA1.6 were stimulated with equivalent concentrations of either intact rabbit anti-mouse immunoglobulin to co-crosslink BCR and Fc receptor or with $F(ab')_2$ of the same antibody to crosslink BCR alone, and the spectra of proteins phosphorylated on tyrosine were compared by anti-phosphotyrosine immunoblotting of whole-cell lysates. Initially, 2 min stimulation was used because the inhibitory effects of receptor co-crosslinking on calcium mobilization is evident at this point (D'Ambrosio et al., 1995). As shown in Figure 1A, among the most prominent substrates phosphorylated upon stimulation of BCR alone ($F(ab')_2$), visualized selectively by brief exposure of immunoblots, only one species was not seen upon BCR–FcγRIIB1 coligation. This species, which has an apparent molecular mass of approximately 115 kDa, migrated at a position corresponding to murine CD19. Consistent with previous studies, phosphorylation of species identifiable as FcγRIIB1 (~50 kDa) and the SHIP PI-3,4,5-P_3 5′-phosphatase (~160 kDa) were seen when FcγRIIB1 and BCR were crosslinked (Chacko et al., 1996; D'Ambrosio et al., 1996). Longer exposure of these films did not reveal additional FcγRIIB1-dependent differences in protein tyrosine phosphorylation. Neither failed phosphorylation of CD19 nor phosphorylation of SHIP and FcγRIIB1 was seen when FcγRIIB1-negative cells were stimulated with intact anti-receptor antibody. These data indicate that among substrates that are most strongly tyrosine phosphorylated upon BCR ligation, CD19 may be a substrate for FcγRIIB1-coupled phosphatases.



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To examine directly the effect of FcγRIIB1 coligation on phosphorylation of proximal mediators of BCR signaling, candidate substrates were immunoprecipitated, fractionated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by sequential immunoblotting with anti-phosphotyrosine and anti-effector antibodies. In Figure 1B are shown anti-phosphotyrosine immunoblots of anti-CD19, anti-Igα, and anti-Vav immunoprecipitates. Blotting with anti-CD19 antibodies revealed that equivalent amounts of CD19 were present in each lane. However, to better demonstrate the effects of crosslinking BCR alone or co-crosslinking FcγRIIB1 and BCR on the tyrosine phosphorylation state of CD19, Igα, Igβ, Vav, PLCγ2, Lyn, and Syk, the anti-phosphotyrosine and anti-effector blots were scanned and the pixel density of the anti-phosphotyrosine signal and anti-effector signal determined. Figure 1C shows the anti-phosphotyrosine signal normalized for the amount of effector present in each lane. Surprisingly, among substrates analyzed, CD19 was phosphorylated most rapidly upon BCR ligation. However, CD19 exhibited significantly reduced levels of tyrosine phosphorylation when FcγRIIB1 was coligated with the BCR (>80%), even at the earliest time point tested (15 sec). Conversely, slightly more tyrosine-phosphorylated Igα was detected 15 sec after coligation of FcγRIIB1 and BCR compared to BCR stimulation alone (Figure 1B). At 45–120 sec following FcγRIIB1–BCR coligation, reduced phospho-Igα was seen (Figure 1B). However, this reflected selective loss of Igα from the detergent-soluble fraction in these cells as revealed by anti-Igα immunoblotting and thus was not seen when phosphotyrosine

signals were normalized to the amount of Igα present (Figure 1C). Consistent with their lack of FcγRIIB1 expression, stimulation of IIA1.6 with either intact or F(ab′)$_2$ anti-receptor antibodies induced equivalent levels and durations of protein tyrosine phosphorylation of CD19 and Igα (Figures 1B and 1C). Interestingly, stimulation of IIA1.6 with intact anti-mouse IgG antibody did not cause a decrease in detergent soluble Igα, indicating that cytoskeletal association may be FcγRIIB1 dependent. Findings from parallel analysis of Shc, Cbl, and HS1 phosphorylation (data not shown) revealed no effect of FcγRIIB1 on these substrates. Finally, IIA1.6 cells transfected with wild-type FcγRIIB1 behaved equivalently to A20 (D. Fong and J. C. C., unpublished data). These findings demonstrate that FcγRIIB1 inhibitory signaling involves a selective reduction of CD19 phosphorylation.

The reduced phosphorylation of CD19 seen following FcγRIIB1–BCR co-crosslinking could reflect either dephosphorylation or failure to phosphorylate CD19. However, kinetic analysis showing greater phosphorylation of CD19 at 15 sec than at 45 sec following FcγRIIB1–BCR co-crosslinking (Figures 1B and 1C) supports the action of a phosphotyrosine phosphatase rather than failed phosphorylation of CD19.

FcγRIIB1-Mediated Decreases in CD19 Tyrosine Phosphorylation Prevent its Association with PI3K

Previous studies have shown that phosphorylation of tyrosine residue(s) 484 and/or 515 in CD19 leads to binding of PI3K via the SH2 domains of the PI3K p85 subunit (Tuveson et al., 1993). To investigate whether FcγRIIB1-mediated decreases in CD19 phosphorylation prevented or terminated its association with PI3K, CD19 and PI3K were immunoprecipitated from Nonidet P-40 (NP-40) lysates of intact or F(ab′)$_2$ anti-receptor antibody–stimulated (1 min) A20 cells and coprecipitation assessed. The immunoprecipitates were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted sequentially with anti-CD19 and anti-PI3K p85. Figure 2A shows, in agreement with previously published results (Tuveson et al., 1993), that the amount of p85 associated with CD19 increases 350% (based on densitometric scanning) when cells are stimulated with F(ab′)$_2$ anti-BCR antibodies. In contrast, levels of p85 associated with CD19 increased by only 60% when cells were stimulated with intact antibody that co-crosslinked FcγRIIB1 and BCR. This increased association was demonstrable by immunoprecipitation with either anti-CD19 or anti-p85. Thus FcγRIIB1 coligation with BCR caused CD19 dephosphorylation, and this correlated with reduced association of CD19 with p85.

To assess whether the decreased phosphorylation of CD19 results in decreased recruitment of PI3K activity, reflecting the p85–p110 complex, to the receptor–coreceptor complex, PI3K assays were performed on CD19 immunoprecipitates from the experiment discussed above. CD19 isolated from unstimulated cells contained levels of PI3K activity that were only slightly above the background of the assay. However, following BCR stimulation (F(ab′)$_2$), the amount of PI3K activity associated with CD19 was increased more than 19-fold

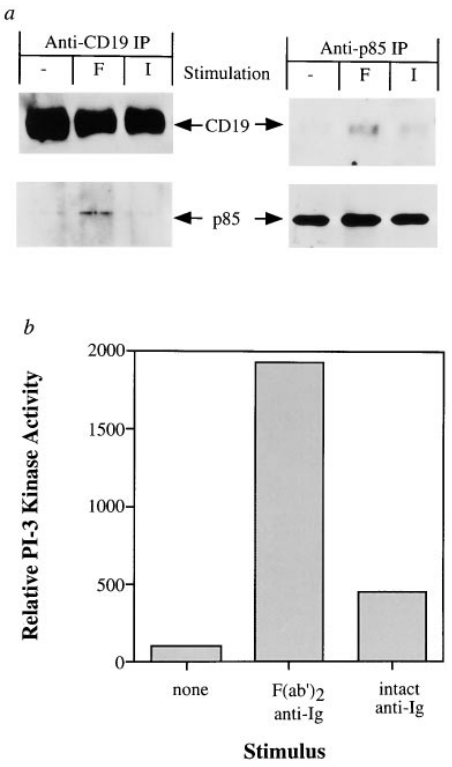

Figure 2. BCR Ligation–Dependent p85 Association with CD19 Is Prevented by FcγRIIB1 Coligation

A20 cells (50×10^6 ml/sample) were cultured without stimulus (-) or were stimulated with intact (I) or F(ab′)$_2$ (F) rabbit antibody to mouse immunoglobulin (BCR) (50 or 32 μg/ml, respectively) for 2 min.
(A) The cells were lysed in 1% NP-40 and CD19 or p85 immunoprecipitated. The immunoprecipitates were washed, eluted with SDS reducing sample buffer, fractionated by SDS-PAGE on a 10% gel, and transferred to Immobilon-P membranes. The membranes were sequentially immunoblotted with anti-CD19 and anti-p85.
(B) Coprecipitation of PI3K activity with CD19 was assessed from A20 cells (10×10^6) that were stimulated and lysed as above. CD19 immunoprecipitates were washed and analyzed for PI3K activity as described. Data are representative of three experiments.

(Figure 2B). Importantly, coligation of FcγRIIB1 reduced BCR-induced CD19 association with PI3K activity to near baseline levels. Consistent with the possibility that binding of p85 to tyrosine-phosphorylated CD19 results in PI3K activation, the BCR-mediated increase in CD19-associated PI3K activity (19-fold) could not be accounted for by increases in coprecipitating p85 (3.5-fold), as determined by immunoblotting. Control experiments with the FcγRIIB1-negative cell line IIA1.6 confirmed the Fc receptor dependence of the decreased association of CD19 and PI3K. Specifically, stimulation of IIA1.6 cells with intact antibody resulted in a strong induction of CD19 phosphorylation and recruitment of PI3K (Figure 1 and data not shown).

Inhibition of BCR-Mediated Ca^{2+} Mobilization, but Not CD19 Tyrosine Phosphorylation, by PI3K Inhibitors

Previously documented effects of FcγRIIB1 coligation with BCR include premature termination of BCR-mediated IP_3 production and calcium mobilization (Figure 3) (Bijsterbosch and Klaus, 1985; Wilson et al., 1987;

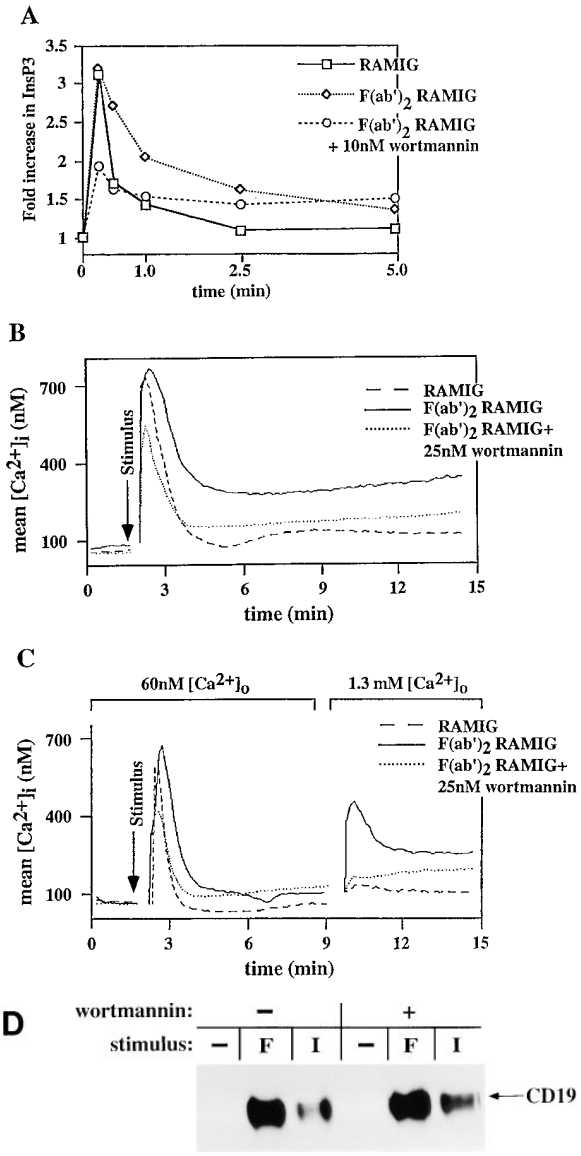

Figure 3. Inhibition of BCR-Mediated IP_3 Production and Ca^{2+} Mobilization, but Not CD19 Tyrosine Phosphorylation, by the PI3K Inhibitor Wortmannin

(A) A20 cells (12×10^6/0.4 ml/sample) were stimulated with F(ab′)$_2$ (12.8 μg) or intact (20 μg) rabbit anti-mouse IgG (RAMIG) in vehicle alone (0.2% dimethylsulfoxide [DMSO]) or with F(ab′)$_2$ plus 10 nM wortmannin in 0.2% DMSO (added 30 min prior to stimulation). At the times indicated, cells were harvested and lysed, and cellular IP_3 concentrations assayed. The results are shown as fold stimulation above resting levels of 1.41 pmol/sample. Results are representative of four replicate experiments.

(B) A20 cells (1×10^6/ml/sample) were loaded with Indo-1 AM and stimulated with either F(ab′)$_2$ (12.8 μg/ml) or intact (20 μg/ml) rabbit anti-mouse IgG. Shown is mean $[Ca^{2+}]_i$ in cells stimulated with intact rabbit anti-mouse IgG antibody, F(ab′)$_2$ fragments after preincubation of cells for 30 min with DMSO (vehicle alone 0.2%), or with 25 nM wortmannin in DMSO. Shown is the mean of the entire population based on analysis of approximately 900 cells/second.

(C) Equivalent analysis of cells as in (B) above after suspension in 60 nM $[Ca^{2+}]_0$ buffered medium followed by repletion of ambient Ca^{2+} to 1.3 mM.

(D) A20 cells (20×10^6/1 ml/sample) were cultured for 30 min with either DMSO alone (− wortmannin) or with 25 nM wortmannin in DMSO (+ wortmannin). Cells were left unstimulated (−) or were stimulated with intact (I) or F(ab′)$_2$ (F) rabbit anti-mouse Ig antibody (50 or 32 μg/ml, respectively) for 2 min and lysed and CD19 immunoprecipitated using specific antibodies. The immunoprecipitates were washed, fractionated by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted sequentially with anti-phosphotyrosine (not shown) and anti-CD19 antibodies.

Data are representative of two independent experiments.

Choquet et al., 1993). These findings and those described above suggest that abortive IP_3 production and Ca^{2+} mobilization may result from failed PI3K activation. To determine whether PI3K is involved in BCR activation of these responses, we assessed the effect of the PI3K inhibitors wortmannin (Yano et al., 1993; Okada et al., 1994) and Ly294002 (Vlahos et al., 1994) on BCR-mediated Ca^{2+} mobilization and IP_3 production. As shown in Figure 3, both wortmannin and FcγRIIB1 coligation inhibited BCR-mediated IP_3 production and Ca^{2+} mobilization. When intracellular calcium release and calcium influx responses were isolated by analyzing the responses in conditions of low extracellular calcium (calcium buffered to 60 nM using EGTA) followed by calcium repletion to 1.3 mM, FcγRIIB1 coligation and wortmannin were found to affect both responses. Doses of wortmannin lower than 1 nM had no effect (data not shown). These results are consistent with previously reported half-maximal inhibitory concentration (IC_{50}) values (~3 nM) for inhibition of PI3K in cell-free systems. Also, in parallel studies, 25 nM wortmannin inhibited PI3K activation following BCR ligation by approximately 80% (A. M. B. and J. C. C., unpublished data). BCR-mediated responses were also sensitive to Ly294002 used at concentrations of 10 mM, slightly greater than its IC_{50} for PI3K (data not shown).

The data presented in Figure 3 also demonstrate that the FcγRIIB1 effect is not specific for extracellular calcium influx. This is consistent with the effect of FcγRIIB1 on IP_3 production. The more selective effect of FcγRIIB1 co-crosslinking than wortmannin on the late Ca^{2+} mobilization response probably reflects the time required to activate BCR-coupled kinases, to coaggregate and phosphorylate FcγRIIB, to recruit and activate FcγRIIB1 effectors, and to dephosphorylate CD19 (Figure 1), before negative signaling is manifest. To rule out the possibility that wortmannin exerts its effect by blocking CD19 phosphorylation, the phosphorylation of CD19 was assessed in A20 cells that had been pretreated with wortmannin (25 nM) and stimulated via BCR crosslinking or BCR–FcγRIIB1 coligation (Figure 3D). Anti-phosphotyrosine immunoblotting of the CD19 immunoprecipitates revealed no effect of wortmannin on the levels of tyrosine phosphorylation. These results indicate that PI3K plays an important role in BCR-mediated IP_3 production and Ca^{2+} mobilization and are consistent with the possibility that FcγRIIB1 mediates its effect by preventing PI3K localization in the antigen receptor complex and/or activation.

CD19 Expression Is Required for Optimal BCR-Mediated Ca^{2+} Mobilization

To address directly whether the function of CD19 and its effectors, such as PI3K, are required for normal Ca^{2+} mobilization following BCR crosslinking, BCR-mediated Ca^{2+} mobilization was analyzed in dense B cells isolated

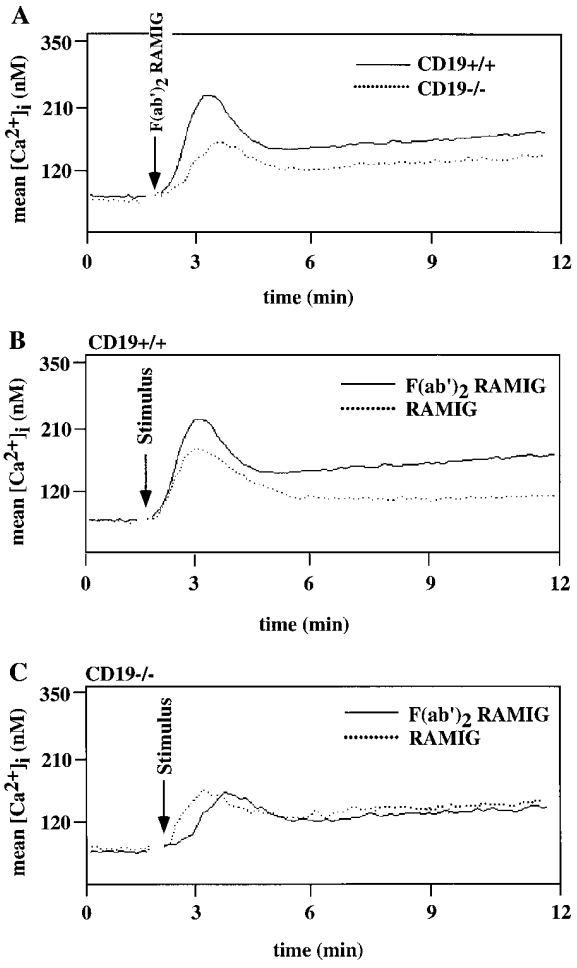

Figure 4. CD19 Expression Is Required for Normal BCR-Mediated Ca^{2+} Mobilization and for FcγRIIB-Mediated Inhibition of BCR-Mediated Calcium Mobilization

Dense B cells from $CD19^{+/+}$ and $CD19^{-/-}$ mice were loaded with Indo-1 AM, and $[Ca^{2+}]i$ was analyzed by flow cytometry under the following conditions: (A) $CD19^{+/+}$ and $CD19^{-/-}$ (1×10^6 cells/ml) cells in 1.3 mM $[Ca^{2+}]_0$ were stimulated with $F(ab')_2$ rabbit anti-mouse IgG (12.8 μg/ml) at the indicated time point (arrow). $CD19^{+/+}$ (B) or $CD19^{-/-}$ (C) B cells (1×10^6/ml) were stimulated with rabbit anti-mouse IgG antibodies (20 μg/ml) or $F(ab')_2$ fragments of the same antibody (12.8 μg/ml) at the indicated time point (arrow). Results are representative of analysis of cells from four individual $CD19^{-/-}$ mice and four individual $CD19^{+/+}$ mice. Shown is the mean $[Ca^{2+}]_i$ based on analysis of approximately 900 cells/sec.

from the spleens of $CD19^{-/-}$ or wild-type littermates (Rickert et al., 1995) (Figure 4). Ligation of the BCR in the $CD19^{-/-}$ B cells resulted in a Ca^{2+} mobilization response that was much reduced compared to that of $CD19^{+/+}$ B cells, consistent with requirement for CD19 and its effector PI3K for optimal BCR-mediated phosphoinositide hydrolysis and Ca^{2+} mobilization. Furthermore, when FcγRIIB1 function was assessed in $CD19^{-/-}$ cells, no FcγRIIB1-mediated inhibition of the residual Ca^{2+} mobilization response was seen (Figure 4C). We also found that intact anti-BCR antibodies induce FcγRIIB1 phosphorylation in B cells from $CD19^{-/-}$ mice (data not shown). These results demonstrate that under conditions in which CD19 does not function, BCR-mediated calcium responses have the appearance of FcγRIIB1-inhibited responses in $CD19^{+/+}$ mice, and FcγRIIB1 coligation does not further suppress this response. Results indicate that CD19 and, indirectly, its effector PI3K are targets of FcγRIIB1-coupled phosphotyrosine phosphatases, and elimination of CD19 function is probably the major mechanism of FcγRIIB1 inhibition of phosphoinositide hydrolysis and Ca^{2+} mobilization in normal B cells.

Discussion

In the presence of appropriate complementary signals, the binding of antigen induces resting B cells to proliferate and differentiate into antibody-secreting plasma cells. Chronic expansion of responding B cells has the potential to cause hyperimmunoglobulinemia and uncontrolled B cell growth. However, negative signaling pathways allow circulating antigen–IgG antibody complexes to control the numbers of antibody-secreting cells not only by inhibiting B cell proliferation (Phillips and Parker, 1983) but also by inducing apoptosis (Ashman et al., 1996). This inhibition is accomplished by immune complex–mediated coligation of the antigen receptors and the B cell's low-affinity receptors for IgG (FcγRIIB1).

Recent studies have shown that FcγRIIB1 function is mediated by phosphorylation of its tyrosine 309 (Muta et al., 1994). This phosphotyrosine has been shown to recruit and activate polyphosphatidylinositide and phosphotyrosine phosphatases, and expression of phosphotyrosine phosphatase SHP-1 was found to be required for FcγRIIB1-mediated inhibition of proliferation (D'Ambrosio et al., 1995). However, the means by which recruitment and activation of these phosphatases or other undefined effectors mediate inhibition of BCR signaling was not well understood.

Here we have identified a primary substrate for FcγRIIB1-activated effectors to be CD19, a BCR accessory molecule and coreceptor. During the revision of this manuscript, Kiener et al. (1997) also reported that crosslinking of the Fc receptor and BCR decreases the tyrosine phosphorylation of CD19. Dephosphorylation of CD19 as indicated by reduced phosphorylation of CD19 15–45 sec following stimulation was found to prevent its recruitment and activation of PI3K. Further experiments showed that expression of CD19 and function of PI3K are required for IP_3 production and calcium mobilization in response to antigen and for the inhibitory function of FcγRIIB1 to be manifest. The specific role of PI3K in the CD19 support of these responses is further demonstrated by studies in a CD19 reconstituted myeloma model, where we have shown that mutation of the PI3K-binding (phospho)tyrosines in CD19 to phenylalanine renders CD19 unable to support BCR-mediated activation of PI3K, IP_3 production, and Ca^{2+} mobilization (A. M. B. et al., unpublished data). In these studies it is also shown that in cells expressing no CD19 or CD19 in which tyrosines 484 and 515 are mutated to phenylalanine, BCR crosslinking does not lead to detectable PI3K activation. Thus, all BCR-mediated activation of PI3K requires phosphorylation of one or both of these residues.

Although the molecular basis of PI3K modulation of phosphoinoside hydrolysis is unclear, findings that FcγRIIB1 coligation does not inhibit BCR-mediated PLCγ phosphorylation (Figure 1C) indicate that a PI3K product may somehow be required for hydrolysis of phosphoinositides by phophorylated PLCγ. Recent observations that the PIP_3 polyphosphorylated inositol lipid product of PI3K binds certain SH2 domains and the pleckstrin homology domain of Bruton's tyrosine kinase (BTK) raise the possibility that PI3K activation leads to translocation of a critical effector to the plasma membrane, where its substrate resides (Rameh et al., 1995; Salim et al., 1996). Thus, PIP_3 may mediate translocation of, for example, BTK, needed for phosphoinositide hydrolysis. Interesting in this regard are recent findings that BTK expression is required for BCR-mediated calcium mobilization (D. Rawlings, personal communication). These results are consistent with the possibility that both aborted IP_3 production and aborted calcium mobilization consequent to FcγRIIB1 coligation with BCR are mediated by failure to generate PIP_3 needed to activate or localize BTK.

As noted earlier, phosphorylated FcγRIIB1 has been shown to bind to only three potential effectors in vitro and in vivo (D'Ambrosio et al., 1995; D'Ambrosio et al., 1996; Ono et al., 1996): SHP-1, SHP-2, and SHIP. Although either phosphotyrosine phosphatases SHP-1 or SHP-2 or downstream effectors of SHIP could mediate the effects described here, it seems most likely given the findings of D'Ambrosio et al. (1995) that FcγRIIB1 is not fully functional in B cells from SHP-1–deficient (motheaten) mice and that SHP-1 is responsible for the observed effect. Consistent with this possibility, it was recently shown that cell-surface expression of SHP-1 leads to reduced TCR-mediated IP_3 production in Jurkat cells but does not affect PLCγ phosphorylation (Musci et al., 1997). It seems likely, however, that SHIP also plays an important role in FcγRIIB1 signaling. In support of this hypothesis are findings that SHIP binds phosphorylated FcγRIIB1 with equal or greater avidity than SHP-1 or SHP-2, and that FcγRIIB1 is capable of transducing signals inhibitory of FcεRI-mediated bone marrow mast cell degranulation even in motheaten mice (Ono et al., 1996). It should be noted, however, that FcγRIIB1 inhibition of FcεRI-mediated Ca^{2+} mobilization in bone marrow mast cells is partially dependent on SHP-1 expression (Fong et al., 1996). We hypothesize that SHIP may provide an inhibitory function that is redundant with SHP-1. Specifically, as a consequence of FcγRIIB1–BCR coligation, SHIP may dephosphorylate PI-3,4,5-P_3, forming PI-3,4-P_2. SHIP, a 5′-inositol phosphate phosphatase, has been shown to prefer PI-3,4,5-P_3 and I-1,3,4,5-P_4 as substrates while being incapable of hydrolyzing I-1,4,5-P_3 (Damen et al., 1996). Thus, while SHP-1 may act to prevent PI-3,4,5-P_3 production, SHIP may act to accelerate its degradation. Both enzymes function to reduce PI-3,4,5-P_3 that is needed for optimal PLCγ-mediated IP_3 production and subsequent calcium release from internal stores and capacitative calcium entry. Consistent with this possibility are recent data that, when placed in an inert chimeric receptor context and crosslinked to BCR, either SHP-1 or SHIP can mediate inhibition of calcium mobilization (Ono et al., 1997). The relative role of SHP-1 and SHIP in inhibitory signals in a particular cell type may depend on a number of parameters such as relative expression of the two enzymes and relative set point in PI-3,4,5-P_3 concentration. Resolution of determinants of the relative roles of these effectors obviously requires further study.

Recent genetic manipulation of CD19 expression using transgenic and gene-ablated animals has allowed studies that demonstrate a role for CD19 in B cell development and immune responses (Zhou et al., 1994; Engel et al., 1995; Rickert et al., 1995; Sato et al., 1995). Overexpression of CD19 leads to impaired B cell development and reduced B cell numbers in the periphery (Zhou et al., 1994). We hypothesize that overexpression of CD19 may lead to enhanced BCR–CD19 signaling in response to selecting ligands. This heightened signaling, mediated by increased PI3K activation, may result in deletion rather than selection of B cells and may be manifest as defective B cell development. Studies of the immune responsiveness of B cells from gene-ablated mice have revealed that CD19 also plays a critical role in BCR-mediated activation as well as germinal center formation and class switching. Thus, CD19 may enhance sensitivity to antigen by enhancing activation of PI3K and other effectors. Consistent with this hypothesis, co-crosslinking of CD19/CD21 with BCR greatly enhances B cell activation (Carter and Fearon, 1992). Based on these findings, it would be predicted that if CD19 is the target of FcγRIIB1-mediated signaling, the consequences of FcγRIIB1–BCR co-crosslinking in normal B cells should be similar to those of BCR crosslinking in $CD19^{-/-}$ B cells. This is in fact the case; stimulation in either situation leads to impaired proliferation and antibody production (Chan and Sinclair, 1971; Engel et al., 1995; Sato et al., 1995). Finally, consistent with a role for SHP-1 as a negative regulator of CD19 signaling are certain phenotypic traits of motheaten ($SHP\text{-}1^{-/-}$) mice. These mice exhibit increased levels of circulating immunoglobulin, as seen in $CD19^{+/+}$ mice relative to $CD19^{-/-}$ mice (Shultz et al., 1993). Lack of SHP-1 in motheaten mice may enhance CD19 signaling, increasing the production of circulating immunoglobulin following immunization by environmental and/or autoantigens.

Finally, it is important to realize that FcγRIIB1 may be involved in the inhibition of signaling by other receptors. For instance, it has recently been reported that coligation of FcγRIIB1 with the chimeric T cell receptor (TCR) inhibits both proliferation and interleukin-2 production, and coligation of FcγRIIB1 with FcεRI results in inhibition of serotonin release (Daeron et al., 1995). The concept that FcγRIIB1 is involved in inhibiting signaling pathways in addition to BCR is supported by the finding that mast cells from FcγRIIB1-knockout mice are hypersensitive to IgG-triggered degranulation, an FcγRIII-mediated event (Takai et al., 1996). This inhibition occurs even though mast cells and T cells do not express CD19, indicating that in these cells a CD19 analog or some other mechanism must be operative. A candidate for the PI3K integrating CD19 analog in T cells is the major TCR-coupled tyrosine kinase substrate pp36/pp38, which has been shown to bind PI3K (Fukazawa et al., 1995). It will be interesting to determine whether the ability of FcγRIIB1 to inhibit signaling in these cells involves failed activation of PI3K.

The studies presented elucidate a molecular mechanism for integration of positive and negative transmembrane signals. The dynamic interplay of these pathways clearly plays an important role in immune regulation but probably have much broader biologic importance.

Experimental Procedures

Reagents and Antibodies

Mice from which CD19 was ablated by homologous recombination were provided by Robert Rickert and Klaus Rajewsky (Cologne, Germany) (Rickert et al., 1995). These mice were used at 8–10 weeks of age as a source of dense splenic B cells ($\rho > 1.066$). The murine B lymphoma cell lines A20 (FcγRIIB1-positive) (Kim et al., 1979) and IIA1.6 an (FcγRIIB1-negative A20 in variant in which the *FcγRIIB* gene contains a mutation) (Jones et al., 1986) and primary B cells were cultured in Iscove's modified Dulbecco's medium supplemented with 5% heat- inactivated fetal calf serum (Hyclone Inc.), 50 U/ml penicillin, and 50 μg/ml streptomicin at 37°C with 7% CO_2. Antibodies to CD19, Igα, Lyn, Syk, Vav, and PI3K were rabbit polyclonals. The immunogens (produced as glutathione S-transferase fusions in bacteria, purified by glutathione–Sepharose chromatography, and cleaved with factor Xa) for CD19 and Igα were the complete cytoplasmic domains, for Lyn residues 1–131, for Syk the linker region as described (Couture et al., 1994), and for p85 the N-terminal SH3 domain. Anti-SHIP was a gift from M. Coggeshall (Ohio State University, Columbus, Ohio). Anti-Vav and anti-PLCγ2 were purchased from Santa Cruz Biotechnology, anti-p85 (blotting) from UBI, anti-phosphotyrosine (Ab-2) from Oncogene Science, and mouse monoclonal to mouse CD19 from Pharmingen. Horseradish peroxidase–conjugated protein A and rat anti-mouse IgG1 (Zymed) were used for detection using an enhanced chemiluminescence detection system (Amersham). Intact and F(ab′)$_2$ rabbit anti-mouse IgG (H+L) (Zymed) were used for stimulation. Na_3VO_4 and H_2O_2 were purchased from Sigma.

Stimulation and Immunoprecipitation

For stimulation, A20 and IIA1.6 cells were harvested, washed once in phosphate-buffered saline and resuspended in Iscove's modified Dulbecco's medium (10^8/ml). After 5 min of incubation at 37°C, the cells were stimulated with F(ab′)$_2$ (12 μg/ml) or intact (20 μg/ml) rabbit anti-mIg, pelleted in a picofuge and stimulating antibody removed. The cells were then lysed in 1% NP-40 lysis buffer (1% NP-40; 10 mM Tris; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 2 μg/ml each of aprotinin, leupeptin, and α-1 antitrypsin; 10 mM NaF: 1 mM Na_3VO_4) and incubated 10 min on ice. Lysates were centrifuged in Eppendorf tubes for 5 min at 14,000 rpm. Cleared lysates were incubated with antibodies to signaling molecules coupled directly to CNBr-Sepharose (Pharmacia), washed three times, eluted with SDS reducing sample buffer, fractionated by SDS-PAGE, transferred, immunoblotted, and developed with an enhanced chemiluminescence detection system (Amersham). For Figure 1, nonspecific protein binding was reduced by adding SDS to the cleared lysates (0.5% final), which were then boiled 5 min and diluted 1:5 in lysis buffer. In Figure 1C, the tyrosine phosphorylation state was determined by sequential immunoblotting for phosphotyrosine and effector; the images were then scanned and the pixel density of the anti-phosphotyrosine signal and anti-effector signal determined. The values shown represent the anti-phosphotyrosine signal normalized for the relative amount of effector present.

Analysis of PI3K Activity

CD19 was immunoprecipitated using affinity purified rabbit polyclonal antibody directly coupled to CNBr-Sepharose, washed three times in lysis buffer and once in assay buffer, assayed using PI as the substrate, and reaction products resolved by thin-layer chromatography (Whitman et al., 1985). The incorporation of $^{32}PO_4$ into PIP was quantitated using a Phosphorimager.

Flow Cytometric Analysis of Ca^{2+} Mobilization

Intracellular free calcium concentration ($[Ca^{2+}]_i$) was determined by preloading the cells with Indo-1 AM (Molecular Probes) and monitoring with a flow cytometer (model 50H, Ortho Diagnostic Systems) as previously described (Cambier et al., 1988; Justement et al., 1989). The mean $[Ca^{2+}]_i$ and percentage of cells that responded was evaluated with an appended data acquisition system and the MultiTIME software (Phoenix Flow Systems). In some cases, intracellular release and extracellular influx components of the calcium mobilziation response were isolated. To detect only intracellular calcium release upon stimulation, cells were suspended in medium in which calcium was buffered to 60 nM (equivalent to $[Ca^{2+}]_i$ in B cells) using EGTA. They were immediately (within 3 min) stimulated and $[Ca^{2+}]_i$ measured. To detect calcium influx, Ca^{2+} in the medium ($[Ca^{2+}]_0$) was repleted to a final concentration of 1.3 mM by addition of $CaCl_2$.

Analysis of Inositol 1,4,5-Trisphosphate Production

Inositol 1,4,5-trisphosphate production was measured using a ^3H radioreceptor binding inhibition assay kit as recommended by the manufacturer (E. I. DuPont).

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