Induction of Cytosolic Calcium Flux by CD20 Is Dependent upon B Cell Antigen Receptor Signaling*

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The anti-CD20 monoclonal antibody (mAb) rituximab is now routinely used for the treatment of non-Hodgkins lymphoma and is being examined in a wide range of other B-cell disorders, such as rheumatoid arthritis. Despite intensive study, the mechanism of action still remains uncertain. In the current study, anti-CD20 mAb-induced calcium signaling was investigated. Previously, we grouped anti-CD20 mAbs into Type I (rituximab-like) and Type II (B1-like) based upon various characteristics such as their ability to induce complement activation and redistribute CD20 into detergent-insoluble membrane domains. Here we show that only Type I mAbs are capable of inducing a calcium flux in B cells and that this is tightly correlated with the expression of the B-cell antigen receptor (BCR). Inhibitor analysis revealed that the signaling cascade employed by CD20 was strikingly similar to that utilized by the BCR, with inhibitors of Syk, Src, and PI3K, but not EGTA, p38, or ERK1/2, completely ablatting calcium flux. Furthermore, binding of Type I but not Type II mAbs caused direct association of CD20 with the BCR as measured by FRET and resulted in the phosphorylation of BCR-specific adaptor proteins BLNK and SLP-76. Crucially, variant Ramos cells lacking BCR expression but with unchanged CD20 expression were completely unable to induce calcium flux following ligation of CD20. Collectively, these data indicate that CD20 induces cytosolic calcium flux through its ability to associate with and “hijack” the signaling potential of the BCR.

CD20 is a 33–37-kDa, non-glycosylated phosphoprotein expressed on the surface of normal B lymphocytes and ~95% of malignant B cells (1,2). This fact has led to CD20-directed immunotherapy, which has been successfully employed in the treatment of over one million patients world-wide with diseases such as non-Hodgkins lymphoma and various autoimmune disorders (3–8). Despite such clinical success and recent progress, we still remain largely ignorant of how anti-CD20 monoclonal antibody (mAb) operates in vivo.

Three main effector mechanisms are available to mAbs to evoke tumor regression: Complement- and antibody-dependent cellular cytotoxicity (CDC and ADCC, respectively) and direct cytotoxic signaling (Refs. 5, 9–14 and reviewed in Ref. 15). The relative contribution of these mechanisms to therapeutic activity remains a controversial issue (15, 16). All anti-CD20 mAbs of the appropriate isotype have the ability to promote efficient ADCC with little evidence that any one mAb is better or worse than any other (17). However, in contrast, they can be separated into two distinct groups: Type I (rituximab, 2F2) and Type II (B1, 11B8), based upon their ability to redistribute CD20 into detergent-insoluble plasma membrane domains, to induce homotypic cellular adhesion, and to evoke CDC (10, 18). Furthermore, it appears that such differences also translate into their effects in vivo with Type I mAb, such as rituximab, being more dependent on CDC for their therapeutic activity (19). Here we have extended these studies to investigate the relative ability of Type I and II CD20 mAbs to evoke intracellular signals, particularly intracellular calcium flux.

Previous work has shown that anti-CD20 mAbs are capable of inducing a range of intracellular signals into target cells (reviewed in Refs. 15, 20), one of which is calcium flux (reviewed in Refs. 15, 21, 22). This is important because relatively few cell surface receptors are able to induce fluctuation in intracellular calcium, and in the B cell this is mainly restricted to the BCR and its co-receptors. It is perhaps more than coincidence that anti-BCR (anti-idiotype) mAb, like anti-CD20, occupies a unique position in immunotherapy for efficacy in treating follicular lymphoma (23, 24) (reviewed in Ref. 25) perhaps indicating the importance of calcium signaling in delivering therapeutic activity by the mAb.

The importance of CD20 in controlling intracellular calcium is known from work showing that transfection of CD20 into a variety of cell lines increases the level of transmembrane calcium conductance, as measured by patch clamp analysis, and also results in higher resting levels of cytosolic calcium (26).

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The abbreviations used are: mAb, monoclonal antibody; BCR, B cell antigen receptor; SOC, store-operated cation; IP3, inositol trisphosphate; siRNA, small interfering RNA; FRET, fluorescence resonance energy transfer; ER, endoplasmic reticulum; PTK, protein-tyrosine kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; STK, Src tyrosine kinases; MaH, mouse anti-human IgG; GaM, goat anti-mouse IgG.
Interestingly, the ability of CD20 to regulate calcium appears to be linked to the generation of calcium flux transduced by ligation of the BCR (27). After BCR ligation, full B cell activation requires sustained elevation of cytoplasmic free calcium, achieved through a combination of its release from intracellular stores and influx of extracellular calcium. Li et al. (27) recently provided good evidence that CD20 is involved in the latter component, where it acts as a capacitance or store-operated cation (SOC) channel, triggered to open when the intracellular stores are depleted. Although it is possible that CD20 facilitates this capacitance calcium flux via an indirect pathway, there is strong evidence to indicate that CD20 itself forms a component of the SOC channel (reviewed in Ref. 15). Together these data indicate that CD20 acts as a SOC channel involved in BCR signal transduction.

In contrast, the situation relating to the calcium flux induced by ligation of CD20 by mAb, such as during immunotherapy, is less clear. Recently, two groups working on this problem have arrived at contrasting conclusions. Although both demonstrate a requirement of Src tyrosine kinases (STK) and raft redistribution in the calcium flux induced after ligation of CD20, one proposes that the calcium flux results from the opening of CD20 as a plasma membrane calcium channel (28), while the other suggests that the calcium flux operates by release of calcium from intracellular stores (21). In the signaling pathway proposed by the latter (21), Lyn kinases become activated after CD20 ligation, resulting in PLCγ2 activation (a known consequence of CD20 ligation (29)), generation of IP₃, and opening of IP₃-sensitive ER calcium stores. A similar pivotal role of STK in inducing calcium flux and apoptosis was suggested by earlier reports (30, 31). It should be noted that in all of the experiments detailed above, the anti-CD20 mAb was hyper-cross-linked with a second polyclonal Ab to generate a calcium flux.

To resolve which model of CD20 calcium flux is correct and to probe more closely how CD20 evokes a calcium flux, we performed a number of new experiments using a range of conditions and different mAbs.

These new experiments demonstrate that the calcium flux triggered through ligation of anti-CD20 mAb is dependent upon several factors. These include the expression level of CD20, the type of anti-CD20 mAb used, the level of hyper-cross-linking required, and the cell type assessed. Surprisingly, the level of calcium flux induced by anti-CD20 mAb was tightly correlated with the expression level of the BCR and appeared to result from a strikingly similar signaling pathway to that used by the BCR to evoke calcium flux. Furthermore, calcium flux correlated with the ability of the anti-CD20 mAb to induce a direct association of CD20 with the BCR. Finally, Ramos cells manipulated to down-regulate or lose expression of the BCR were unable to induce calcium flux following ligation of CD20. Altogether, these data indicate that CD20 ligation induces a calcium flux through its association with the BCR, rather than through any intrinsic calcium channel capability.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Reagents**—Cell lines were obtained from the European Collection of Cell Cultures. NS0-CD20 cells (36) were a kind gift from Saskia Ebeling (Utrecht, Holland). Cells were maintained in antibiotic-free supplemented RPMI (Invitrogen Ltd., Paisley, UK), at 37 °C, 5% CO₂, EGTA, MCD, and cholesterol were purchased from Sigma. Inhibitors of STK (PP2 or SU6656), p38 (SB203580), ERK (PD98059), P13K (LY294002), and Syk (Syk inh) were from Calbiochem.

**Antibodies**—The mAbs used have been described previously (18). B1 and rituximab were kind gifts from Professor T. Illidge (Manchester, UK). Antibodies to pERK1/2 (Cell Signaling Technology), ERK1/2 (Promega), pBLNK, pSLP76 (both BD Biosciences), β-actin (Sigma), anti-pTyr (4G10, Upstate), rabbit F(ab’2) HRP (Pierce, Perbio Sciences), and mouse Ig-HRP (GE Healthcare, UK) were used in Western blotting or flow cytometry. For BCR hyper-cross-linking, goat anti-human IgM F(ab’2) (Jackson Immunoresearch), or M15/8 (in-house) was used (32). If5 and all other mAbs were produced in-house from hybridoma lines, secreting mAb in tissue culture and purified

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**TABLE 1**

| Cell line | Expression of CD20 | Expression of BCR | Calcium flux strength |
|-----------|--------------------|-------------------|----------------------|
| SU-DHL-4  | ++++               | +++               | Strong               |
| Ramos     | ++++               | +++               | Strong               |
| BL60      | +++                | +++               | No                   |
| Normal B cells | +++    | +++               | Moderate-weak        |
| Raji (BCR hi) | +++     | +++               | Moderate            |
| Raji (BCR med) | +++    | +++               | Moderate            |
| Raji (BCR lo) | ++++   | +                | Weak                 |
| ARH77     | +                  |                     | No                   |
| NS0-CD20  | +++++              | −                  | No                   |

**FIGURE 1.** Calcium flux is generated with Type I but not Type II mAb. SU-DHL-4 (A) or Ramos (B) cells were labeled with Indo-1 AM, washed, and then treated as follows. A, samples of SU-DHL-4 cells were assessed by flow cytometry to establish a baseline level of fluorescence, then removed, treated with 10 μg/ml of Type I (rituximab, 1F5) or Type II (B1, 11B8) anti-CD20 mAbs before further flow cytometric analysis. B, mAbs were added to the Ramos cells for 15 min at room temperature before the cells were washed and resuspended. These samples were used to establish a baseline level of fluorescence as before, prior to the addition of a secondary hyper-cross-linking Ab (indicated by the arrow) and subsequent assay by flow cytometry. Depending on the isotype of the primary mAb, either mouse anti-human IgG (MaH) or goat anti-mouse IgG (GaM) (both at 20 μg/ml) was used as a cross-linker. Samples were also treated with 10 μg/ml IgM (M15/8) as a positive control. Data shown are representative of at least three independent experiments.
from culture supernatant using protein A columns (GE Healthcare). The Type II mAb 11B8 (11B8) was produced by cloning the V regions from the 11B8 mAb (Genmab, patent 6737056B1 and described in Ref. 33) into an expression vector (pEE14, Lonza) with Fc regions from Hu Fcγ1, transfecting 293F cells, and purifying the mAb as above. A mouse IgG2a (m2a) chimeric form of rituximab (Ritux m2a) was produced in a similar manner, by cloning the V regions of rituximab into the appropriate expression construct containing the m2a Fc regions. The purity of in-house mAb was assessed by electrophoresis (Beckman EP system; Beckman, FL). F(ab′)2 fragments of IgG were produced by standard pepsin digestions (34). Flow cytometry for surface binding was essentially as described previously (35).

Cytosolic Calcium Flux—Cells were incubated with 10 μM Indo-1-AM with 0.2% pluronic-F127 for 30 min at 37 °C followed by washing and resuspension in RPMI containing 10% fetal calf serum. Cells were maintained in the dark at room temperature before use. Samples were then incubated at 37 °C for 1–2 min and assessed by flow cytometry using an FL5/FL4 ratio to establish a baseline fluorescence for unstimulated cells. Cells were then stimulated with the desired treatment, and the FL5/FL4 ratio recorded over the next 6–10 min. Samples were analyzed using a FACS Vantage flow cytometer with UV excitation at 365 nm. Data were analyzed using CellQuest software (BD Biosciences).

Fluorescence Resonance Energy Transfer (FRET) Analysis—FRET analysis was based on the method described previously (10). Briefly, cells were resuspended at 3 × 10^6 cells/ml in PBS, 0.1% BSA, 1% heat-inactivated mouse and human serum, and equimolar donor (Cy3 or FITC)-conjugated and acceptor (Cy5)-conjugated mAbs were combined and added to the cell suspension (10 μg/ml) for 15 min in the dark at room temperature. Each experiment included cells labeled

FIGURE 2. Requirement for hyper-cross-linking relates to CD20 expression level. SU-DHL-4 cells were transfected with 600 nM siRNA against CD20 (Ambion) or a negative control siRNA and incubated overnight at 37 °C. A, cells were then harvested, and the expression levels of CD20 and (BCR) sIgG were assessed by flow cytometry. Control siRNA samples are shown in the gray-filled histogram and CD20 siRNA samples overlaid in white. B and C, transfected cells were prepared for calcium flux experiments as outlined in the legend to Fig. 1. B, samples were then treated with 10 μg/ml rituximab or anti-BCR (SB2H2) in the absence of cross-linking or C, pretreated with 10 μg/ml ritux 2a, washed, and 20 μg/ml GAM added at the time point indicated by the arrow.
FIGURE 3. Effect of extracellular calcium chelation on the calcium flux induced by CD20 and BCR mAbs. SU-DHL-4 (A) or Ramos cells (B) were prepared for calcium flux experiments as outlined in the legend to Fig. 1. A. SU-DHL-4 cells were treated with either rituximab or anti-BCR (M15/8) (both 10 μg/ml) with or without pretreatment with 1.5 mM EGTA for 15 min at room temperature before addition of the mAb at the time indicated by the first arrow. After 3–4 min, CaCl₂ (3 mM) was added to the sample, indicated by the second arrow. B. Ramos cells were treated as outlined in A except that samples required hyper-cross-linking. As such, after pretreatment with EGTA if required, mAbs were added for 15 min at room temperature, and the samples washed and stimulated with 20 μg/ml MAH at the time indicated by the arrow. CaCl₂ (3 mM) was then added to the sample as indicated by the second arrow. Results shown are representative of two independent experiments.
with donor- and acceptor-conjugated mAbs after preincubation with a 20-fold molar excess of unconjugated mAb, and cells labeled with donor- or acceptor-conjugated mAb in the presence of equimolar unlabeled mAb. FRET was assessed using flow cytometry on a FACSCalibur (BD Biosciences) with calculations as previously described (10). For cholesterol depletion, cells were treated with MCD (10 mM) for 15 min before washing and reconstitution with RPMI, lacking fetal calf serum. Cholesterol repletion was performed by adding cholesterol (12 µg/ml) for 1 h.

**Western Blotting**—For global tyrosine phosphorylation, Ramos cells (2 × 10⁶/ml) were treated with various mAbs (10 µg/ml primary and 50 µg/ml secondary mAbs) at 37 °C for 15 min, and the cells pelletted and lysed on ice for 30 min in lysis buffer (10 mM Tris-HCl, pH 7.4, 200 mM NaCl, Na₃VO₄, NaF, IAA, aprotinin, phenylmethysulfonyl fluoride, with 1% Nonidet P-40 detergent). Samples were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and incubated with 4G10 (anti-pTyr), followed by HRP-conjugated secondary antibody. Blots were visualized using enhanced chemiluminescence reagents (Pierce).

**Assessment of Raft-associated Antigen by Triton X-100 Insolubility**—Triton X-100 insolubility assay was performed as detailed previously (10). Briefly, cells were incubated with 10 µg/ml of FITC-conjugated mAb for 15 min at 37 °C, washed in cold PBS, 1% BSA, 20 mM sodium azide, and then the sample was divided in half. One-half was maintained on ice to allow calculation of 100% surface antigen levels, while the other was treated with 0.5% Triton X-100 for 15 min on ice to determine the proportion of antigens remaining in the insoluble fraction. Cells were then maintained at 4 °C throughout the remainder of the assay, washed once in PBS/BSA/azide, and assessed by flow cytometry as detailed previously.

**Knockdown of CD20**—CD20 expression in cell lines was reduced
using small interfering RNA (siRNA) CD20 siRNA: 5′-CCACUCUCCAGGAGAUUG-3′ (Ambion); 5′-ACGCAGAGCU-UCUCAUGAGUU-3′ or 5′-GAGACGAACUUCCAGAGA-CUU-3′ (Dharmacon); or control siRNA: 5′-GCCGCGCU-UUGAAGAUGUC-3′ (Ambion) were utilized and transfected by Amaxa Nucleofector™ using solution T and program G16 according to the manufacturer’s instructions (Amaxa, Germany). The efficiency of knockdown was determined by flow cytometry.

**Generation of Rx3 and “BCR-low” Ramos Cells**—To generate Ramos cells lacking BCR expression (Rx3 cells), Ramos cells were cultured with suboptimal levels of anti-BCR mAb (M15/8, 1 μg/ml). This level of mAb produced a limited amount of cell death, but allowed a subpopulation of cells to survive and grow through. Following recovery, cells were again cultured in the presence of anti-BCR mAb for another two rounds. At the end of this period, the cells were washed and cultured for another 2 weeks without mAb. BCR-low Ramos cells (expressing reduced levels of BCR) were produced by treating the cells in culture for 24 h with anti-Fdμ mAb (XG9, 10 μg/ml) (32). BCR expression levels were confirmed by staining and flow cytometry for IgM, CD79a, or CD79b.

**Phosphorylation Analysis of BLNK and SLP-76**—Ramos cells were treated for 1, 2, 5, and 15 min with mAb as indicated, and the phosphorylation state of the BCR signaling molecules BLNK and SLP-76 were established utilizing BD Bioscience’s phosphospecific antibodies according to the manufacturer’s protocols. The samples were then washed and analyzed by flow cytometry.

**Generation of FcγRIIb-expressing Ramos Cells**—Cells were transfected with either human FcγRIIb pcDNA3 or empty vector using the Amaxa Nucleofector™ with solution T and program G16 according to the manufacturer’s instructions (Amaxa). Cell lines stably expressing FcγRIIb were selected with neomycin. Surface expression was determined by staining with the specific anti-FcγRII mAb (AT10) and flow cytometry.

**RESULTS**

**Type I but Not Type II Anti-CD20 mAbs Induce Calcium Flux**—In our first experiments, we assessed the ability of a range of Type I and II anti-CD20 mAbs to evoke a calcium flux in the follicular lymphoma cell line SU-DHL-4, using the anti-BCR (anti-IgG) mAb SB2H2 as a positive control. These experiments show clearly that Type I (rituximab and 1F5) but not Type II mAbs (B1 and 11B8) induce a calcium flux in SU-DHL-4 cells without the need for hyper-cross-linking with anti-Ab (Fig. 1A). To establish if this was a cell line-specific phenomenon, we repeated these experiments using the well-known Burkitt’s lymphoma cell line, Ramos. In line with previous observations (21, 29, 30), we found that calcium flux was not evoked in these cells with either Type I or II mAb (Fig. 1B). However, when anti-CD20 mAbs were first bound to the cells and then hyper-cross-linked with anti-Fc reagents, a robust calcium flux was seen, although again only with Type I mAb (Fig. 1B). Importantly, calcium flux was not observed when mAbs to CD52 or MHCII were used, even after hyper-cross-linking, indicating that calcium flux was not simply a consequence of clustering rafts (CD52) or highly expressed cell surface markers (MHCII) (data not shown).

**Requirement for Hyper-cross-linking Is Dependent on the CD20 Expression Level**—In subsequent experiments we assessed a range of other cell lines (Daudi, Raji, BL41, MHH-PREB1, BL60) for their ability to evoke calcium flux through CD20 mAb ligation and found that only SU-DHL-4 cells were capable of calcium flux without the need for hyper-cross-linking (Table 1). To determine the reason for this difference we assessed the CD20 expression level of the various cell lines and revealed that SU-DHL-4 cells expressed ~3–5 times more CD20 than other B cell lines or normal B cells (Table 1). Therefore, we hypothesized that this high expression level negated the requirement for hyper-cross-linking. To address this suggestion directly, we employed CD20-specific siRNA oligos to knockdown the expression of CD20 in the SU-DHL-4 cells. Using this approach ~60% knockdown in CD20 surface expression was achieved with specificity demonstrated by the fact that slgG expression was unaffected (Fig. 2A). Calcium flux was no longer evoked by Type I anti-CD20 mAb in the cells transfected with the CD20 siRNA (Fig. 2B). Importantly, the calcium flux was still evoked in cells transfected with mock siRNA, confirming that the transfection procedure had not caused the lack of calcium flux. Furthermore, hyper-cross-linking of Type I mAb in the CD20 knockdown cells yielded a strong calcium flux, clearly showing that CD20 expression level determines the requirement for hyper-cross-linking to achieve Type I mAb-induced calcium flux (Fig. 2C). (NB: To prevent co-engagement of the BCR and rituximab on these cells (which express IgG as opposed to IgM) a chimeric IgG2a variant of rituximab was used along with anti-mouse IgG cross-linking reagents). These results were confirmed using two additional CD20 siRNA targeting different regions of the CD20 (supplemental Fig. S1 and data not shown).

Given the observation that the CD20 expression level was critical to the calcium flux, we next used murine NS0 cells expressing a range of levels of human CD20 (36) and assessed these for the ability to evoke calcium flux after Type I engagement. Surprisingly, none of the transfectants displayed any calcium flux, in the presence or absence of hyper-cross-linking even when the cells expressed CD20 at levels higher than that reached in the SU-DHL-4 cells (Table 1 and data not shown). These data indicate that CD20 expression

**FIGURE 4. Involvement of intracellular kinases in the generation of calcium flux.** SU-DHL-4 (A) or Ramos (B) cells were prepared for calcium flux experiments as outlined in the legend to Fig. 1. A, SU-DHL-4 cells were treated or not with Syk inhibitor, PI3-kinase inhibitor (LY294002), MEK inhibitor (PD98059), p38 inhibitor (SB203580), STK inhibitor (PP2 or SU6656) (all at 10 μM) or DMSO (0.1%) for 20 min. Baseline fluorescence was established before the addition of rituximab (10 μg/ml) indicated by the arrow. B, Ramos cells were treated as outlined in A except that the cells were incubated for an additional 15 min at room temperature with rituximab, after which samples were washed, baseline fluorescence established, and MAH (20 μg/ml) added at the time point indicated by the arrow. C and D, same inhibitors were tested against SU-DHL-4 (C) or Ramos cells (D) following anti-BCR stimulation using anti-slgG (SB2H2) and anti-slgM (M15/8), respectively. Results shown are representative of three independent experiments.
CD20 Calcium Signaling

A

![Image of a gel showing CD20 Calcium Signaling results]

B

| Time (minutes) | NT | rituximab | B1 | anti-BCR | X-link | rituximab + X-link |
|---------------|----|-----------|----|---------|--------|-------------------|
| 1             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 2             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 5             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 15            | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |

C

| Time (minutes) | NT | rituximab | B1 | anti-BCR | X-link | rituximab + X-link |
|---------------|----|-----------|----|---------|--------|-------------------|
| 1             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 2             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 5             | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
| 15            | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] | ![Graph] |
alone is insufficient for cytosolic calcium flux and support the suggestion that other cofactors required for calcium flux are lacking in the NS0 cells.

Requirement for Extracellular Calcium—Recent evidence suggests that CD20 operates as a SOC calcium channel after BCR ligation, allowing entry of extracellular calcium following depletion of intracellular calcium stores (18). To determine the requirement for extracellular calcium for the calcium flux observed after CD20 ligation, we performed experiments with EGTA in the external medium. As a control, we also assessed the requirement for extracellular calcium during BCR-induced calcium flux. The results shown in Fig. 3 reveal that extracellular calcium chelation had almost no effect on the magnitude or kinetics of the calcium flux induced by CD20 or BCR ligation in either SU-DHL-4 or Ramos cells and indicate that CD20 and BCR mAbs evoke calcium flux predominantly through intracellular calcium release. However, when extracellular calcium was re-introduced (by the addition of CaCl$_2$), later in the assay, a second flux in calcium was observed. These data indicate that a proportion of the total calcium flux observed is due to extracellular calcium influx. This biphasic response is known to occur following BCR ligation, because of intracellular store release of calcium triggering the opening of SOC channels, allowing influx of extracellular calcium. It appears that a similar mechanism accounts for the calcium flux evoked by anti-CD20 mAb. In the case of the BCR, one of the SOC has been shown to be CD20 and so intriguingly the SOC channel opened after anti-CD20 mAb ligation may well be CD20 itself. Taken together, these data indicate that the calcium flux evoked by Type I anti-CD20 mAb results from release of calcium from intracellular stores and precludes a direct channel opening model.

Signaling Cascade Required for Calcium Flux—Release of calcium from intracellular stores is usually preceded by signaling activity. We therefore probed the signaling pathway leading to calcium flux following CD20 stimulation with a panel of well-characterized inhibitors using doses at which target inhibition could be demonstrated (data not shown). These data reveal that the signaling pathway leading to calcium flux evoked by CD20 in both SU-DHL-4 and Ramos cells (after hyper-cross-linking) was the same, being dependent upon Syk, Src-tyrosine kinases (STK), and PI3K but not p38 or ERK1/2 (Fig. 4, A and B).

In the course of these studies, we also noticed that expression of the BCR appeared to correlate with the sensitivity of the cell lines to CD20 calcium flux (Table 1). As such, cell lines expressing no BCR gave no calcium flux (such as the NS0 transfectants), and the cell lines expressing high levels of BCR (SU-DHL-4 and Ramos) gave robust calcium flux, with intermediate-low BCR-expressing lines (BL60, BL41) giving intermediate-low levels of flux. Furthermore, we identified a range of Raji sublines that varied in their expression of BCR (high, mid, or low levels) yet retained similar CD20 expression. The calcium flux induced in these various sublines was then assessed and shown to be directly correlated with the expression of BCR (data not shown and Table 1).

Therefore, in the next series of experiments we assessed the calcium signaling pathway evoked by the BCR in the SU-DHL-4 and Ramos cells. Intriguingly, as shown in Fig. 4, C (SU-DHL-4) and D (Ramos), the inhibitor profile was strikingly similar to that displayed for the CD20 mAb, with the calcium flux inhibited by Syk, STK, and PI3K inhibitors but not p38 or ERK1/2 reagents.

**BCR and CD20 Evoke a Similar Tyrosine Phosphorylation Cascade**—To further explore this apparent link between CD20 and BCR signaling, we explored whether mAb directed to these antigens resulted in similar protein-tyrosine phosphorylation (PTP) patterns. These data shown in Fig. 5A reveal that hyper-cross-linked rituximab or anti-BCR mAb result in essentially the same PTP pattern in Ramos cells, with increased tyrosine phosphorylation in proteins of 28–30, 53–56, and 70–80 kDa sizes (arrows). In contrast, hyper-cross-linking of the Type II mAb 11B8 resulted in no substantial elevation in PTP.

Signaling through the BCR is a tightly regulated process that involves a number of BCR-specific adaptor proteins, which together coordinate activation of downstream pathways. Calcium flux after BCR ligation results from IP$_3$ generated by activation of PLC-$\gamma_2$. PLC-$\gamma_2$ is itself activated by the PTK Syk in combination with the adaptor proteins BLNK and SLP-76. Given the dependence of CD20-calcium signaling on Syk, next we assessed the activation status of these two key BCR-specific adaptor proteins (BLNK and SLP-76), after CD20 ligation. These experiments revealed that phosphorylation of both BLNK and SLP-76 occurs after hyper-cross-linking of Type I (but not Type II) anti-CD20 mAb coincident with calcium flux (Fig. 5, B and C) and to a similar level to that seen with anti-BCR mAb. These data are highly suggestive that ligated CD20 is utilizing the same signaling pathway as the BCR.

**CD20 and BCR Calcium Signaling Is inhibited by Fc$\gamma$RIIb**—The BCR is negatively regulated by the inhibitory Fc$\gamma$R, Fc$\gamma$RIIb. Upon simultaneous engagement with the BCR, the Fc$\gamma$RIIb serves to reduce the duration of calcium flux, as a negative feedback response to high levels of IgG. Therefore, given the similarity between the BCR and CD20 signaling pathways detailed above, we wondered whether Fc$\gamma$RIIb engagement may also serve to reduce CD20-induced calcium signaling. Ramos cells lack Fc$\gamma$RIIb, and so to assess this we generated Ramos cell transfectants (Fig. 6A), which overexpressed Fc$\gamma$RIIb and performed calcium flux experiments in these cells compared with Ramos cells transfected with empty vector alone. As expected, Ramos cells expressing Fc$\gamma$RIIb displayed blunted calcium flux responses after BCR or CD20 ligation compared with mock-transfected cells (Fig. 6B). These data indicate that CD20- and BCR-induced calcium signaling is similarly regulated by Fc$\gamma$RIIb.

**FIGURE 5. Anti-CD20 mAbs induce a similar PTP pattern to anti-BCR mAb with similar downstream targets.** A, Ramos cells (2 × 10$^6$/ml) were treated with various mAbs (10 $\mu$g/ml primary and 50 $\mu$g/ml secondary Abs) at 37 °C for 15 min, and the cells lysed. Samples were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, blocked, and global protein-tyrosine phosphorylation established by Western blot with the anti-pTyr mAb 4G10. B and C, Ramos cells were treated with mAb as indicated for 1, 2, 5, and 15 min, and the phosphorylation state of the BCR-signaling molecules BLNK (B) and SLP-76 (C) were established with phosphospecific antibodies and flow cytometry. Open histograms represent isotype-matched control mAb staining, solid histograms represent staining with the phosphospecific mAb. The blots and histograms shown are representative of three independent experiments.
Type I Anti-CD20 mAbs Induce the Association of CD20 with the BCR—On the basis of these results, we hypothesized that CD20 may be triggering calcium flux through a direct association with the BCR. To investigate this suggestion, we performed FRET experiments using anti-CD20 and anti-BCR mAbs on Ramos cells. FRET allows calculation of the proximity of proteins, and a FRET signal observed here was only generated when the proteins in question (via their associated mAb) were within 10 nm. Using this technique and different combinations of mAbs, the levels of homo- and hetero-association were determined. As such, incubation of FITC-labeled rituximab with Cy5-labeled rituximab allowed us to determine the level of CD20 clustering after a 15-min binding period, whereas incubation of M15/8 FITC (anti-BCR mAb) and rituximab Cy5, allowed us to determine if these antigens (CD20 and the BCR) became associated. Similar experiments were then performed with the Type II mAb 11B8. These data, represented in Fig. 7A, demonstrate that rituximab (Type I) but not 11B8 (Type II) causes CD20 to cluster together after binding, confirming what we have shown previously (10). Furthermore, the data clearly show that Type I, but not Type II, mAb-ligated CD20 associates with the BCR, supporting the hypothesis that Type I mAb may signal for calcium through CD20 association with the BCR. Similar observations were also apparent in SU-DHL-4 and Raji cells, with FRET observed between the BCR and Type I but not Type II mAb (data not shown). Furthermore, the level of FRET observed between the BCR and CD20 was proportional to their expression on these cells. As such, FRET was highest in SU-DHL-4 cells and lowest in Raji cells. Previously, we demonstrated that CD20 homo-association after cross-linking is dependent on its redistribution into lipid microdomains (10), and others have demonstrated that both the BCR and CD20 can redistribute to these microdomains (39). To investigate whether the BCR:CD20 hetero-association was reliant upon cholesterol-dependent membrane lipid microdomains, we repeated these experiments following methyl-β-cyclodextrin (MCD) treatment of the cells. MCD is a cholesterol sequestering agent that removes cholesterol from membranes into the aqueous phase and consequently can be used to deplete plasma membranes of lipid microdomains. Interestingly, these data revealed that hetero-association between Type I mAb-ligated CD20 and the BCR was unaffected by MCD treatment, while homo-association of rituximab was substantially reduced, being restored by subsequent incubation of the cells with cholesterol (Fig. 7B).

The BCR Is Critical for CD20-induced Calcium Flux—Following this demonstration that CD20 and BCR become closely

FIGURE 6. Calcium signaling through CD20 is negatively regulated by FcγRIIb. A, Ramos cells were transfected with FcγRIIb or empty vector and the surface expression of FcγRIIb determined by flow cytometry. Control samples are shown in the filled histogram, and FcγRIIb samples are overlaid as an open histogram. B, these cells were then prepared for calcium flux experiments as outlined in the legend to Fig. 1. As before, cells were incubated with rituximab (10 μg/ml) for 15 min before cross-linking with MAH (50 μg/ml). Anti-BCR mAb was used as a control. The plots shown are representative of two independent experiments.
associated after Type I (but not Type II) anti-CD20 mAb ligation, and that CD20 and the BCR evoke the same signaling cascade leading to calcium flux through a biphasic response, which can be inhibited by FcyRIIb, we next wanted to demonstrate formally that Type I-induced calcium flux was dependent upon BCR expression. For these experiments, we generated a sub-line of Ramos cells that does not express the BCR. These cells, which we term Rx3, were produced by culturing wild-type Ramos cells with anti-BCR mAb for three sequential rounds. At the end of this period, the cells were washed and cultured for an additional 2 weeks without mAb. Following this treatment, the phenotype of the cells was assessed, revealing that all components of the BCR (sIgM, CD79a, and CD79b) were markedly reduced (Fig. 8A) and a range of other surface antigens (data not shown). Importantly, the expression of CD20 (Fig. 8A) and a range of other surface antigens (data not shown) were not substantially affected, and the raft redistribution pattern of the anti-CD20 mAb was identical between wild-type and Rx3 Ramos cells (data not shown). As such, these cells are CD20-positive B cells that lack BCR expression. Calcium flux experiments comparing wild-type Ramos and Rx3 cells, represented in Fig. 8B, clearly demonstrate that in the absence of the BCR, Type I anti-CD20 mAbs were unable to transduce a calcium flux. To further examine whether BCR expression was important for CD20 calcium signaling, we performed experiments where the BCR was modulated from the cell surface using anti-Fdμ mAb to produce BCR-low cells. After 24 h of stimulation, these mAbs reduce surface expression of the BCR to ~20% of the resting level on Ramos cells, but do not cause substantial apoptosis (32) or down-regulation of CD20 (Fig. 8A). Calcium flux experiments were then performed, demonstrating that CD20 calcium flux did not occur in cells where the BCR had been modulated (Fig. 8B, lower panels).

**DISCUSSION**

In this study, we examined the means by which calcium flux is evoked after ligation of CD20. We observed that only Type I anti-CD20 mAbs were capable of evoking a calcium flux, in agreement with recent data (21). Through examining a range of different B cell lines, we further observed that the requirement for hyper-cross-linking to evoke the calcium flux was linked with the expression level of CD20. As such, SU-DHL-4 cells expressing supra-high levels of CD20 did not require hyper-cross-linking, while all other cell lines expressing CD20 at a level akin to that expressed on normal B cells, displayed an absolute requirement for hyper-cross-linking. These experiments and those where CD20 expression was reduced using siRNA demonstrate that CD20 expression level is a key parameter in governing the ability of a cell to evoke calcium flux. Therefore, given that most B cell targets would require hyper-cross-linking for calcium flux generation, this has a significant impact when considering the in vivo effects of anti-CD20 mAbs such as rituximab. The current expectation is that FcγR-expressing effector cells will perform this cross-linking function in vivo, although this has yet to be formally demonstrated.

**Anti-CD20 mAbs Do Not Cause Calcium Flux through CD20 Channel Opening**—Importantly, NS0 cells transfected to ectopically express supra high levels of CD20 did not display any calcium flux, either in the presence or absence of hyper-cross-linking. These data indicate that the calcium flux evoked by CD20 ligation in B cells is not caused by any direct calcium channel activity of CD20 itself and infer that additional cofactors are required for this process to occur. NB: In the context of the NS0 cells this may reflect a simple lack of expression or inability of the mouse cofactor(s) to interact with human CD20.

On initial inspection, these data appear to contradict those reported earlier (26), which showed that transfection of CD20 into non-B cells such as Jurkat, K562, 3T3 caused an increase in calcium conductance. However, Bubien et al. predominantly analyzed calcium conductance using whole cell patch clamp analysis rather than cytosolic calcium changes such as we have here. Furthermore, those authors never examined the increase in calcium flux induced by anti-CD20 mAb in those transfec-tants. Rather, they examined calcium conductance changes induced by mAb binding in Daudi B cells, where they reported that the Type I CD20 mAb, IF5, but not B1 caused an acute increase in calcium conductance. Interestingly, they also reported that ligation of IF5 did not evoke significant increases in cytosolic calcium (in the absence of further cross-linking). As Daudi cells express a similar level of CD20 to Ramos, these data are entirely in line with our current experiments, where hyper-cross-linking is required for generating cytosolic calcium flux. In summary, the data of Bubien et al. (18) indicate that although CD20 operates as a calcium channel, this function is not trig-gered by mAb ligation and is not responsible for the increases in cytosolic calcium flux observed.

Further evidence against the channel opening hypothesis comes from our experiments where extracellular calcium was
FIGURE 8. The BCR is required for CD20-mediated calcium flux generation. A, surface expression level of CD20 (left-hand panel) or BCR (right-hand panel) for the Ramos (black-filled histogram), Rx3 cells (open histogram), and Fδμ-treated BCR-low cells (gray-filled histogram) are shown. B, Ramos, Rx3, or BCR-low Ramos cells were prepared for analysis of calcium signaling as outlined in the legend to Fig. 1. Samples were treated with rituximab (10 μg/ml) to determine their sensitivity to anti-CD20 signaling or with either thapsigargin (100 nM) or anti-BCR (M15/8, 10 μg/ml) as controls.
CD20 Calcium Signaling

Addition of CaCl₂ to replenish the extracellular calcium in these experiments demonstrated that extracellular calcium did provide a component of the cytosolic calcium flux. Interestingly, a similar response to EGTA and CaCl₂ was seen for BCR-induced calcium flux, which is explained by the opening of SOC channels and influx of extracellular calcium. Our data appear to demonstrate that a similar mechanism occurs during CD20-induced calcium flux. In the case of the BCR, one of the SOC has been shown to be CD20 and so intriguingly the SOC opened after anti-CD20 mAb ligation may well be CD20 itself. Therefore, it seems likely that anti-CD20 mAbs induce calcium flux by first triggering release of calcium from intracellular stores, which then triggers the opening of SOC channels (including CD20 itself), providing the influx of extracellular calcium.

**Calcium Flux through Anti-CD20 mAb Is Due To A Signal Transduction Pathway**—In the absence of a direct channel opening model for cytosolic calcium flux generation, we next probed the signaling pathway evoked by CD20 mAb ligation. These data revealed that Syk, Src, and PI3K were central to the signaling pathway leading to calcium flux. Importantly, the signaling pathway involved was the same in both SU-DHL-4 and Ramos cells indicating that hyper-cross-linking does not qualitatively change the signaling pathway. Rather as SU-DHL-4 cells express ~3–5 times more CD20 than Ramos, it hints at a quantitative model, where Ramos cells require hyper-cross-linking to form large CD20 signaling platforms that are formed without hyper-cross-linking in SU-DHL-4 cells.

**CD20-induced Calcium Flux Is Due To Its Association with the BCR**—Based upon this and several key observations, we propose a new model of how anti-CD20 mAb induce calcium flux (Fig. 9). We suggest that Type I anti-CD20 mAb ligation induces a functional association of CD20 with the BCR, and that subsequent clustering of CD20-BCR complexes results in the generation of the calcium flux. In SU-DHL-4 cells, we propose that this occurs without additional hyper-cross-linking due to the supra-high levels of CD20 expressed (and hence higher numbers of CD20-BCR complexes). Conversely, in other cells expressing lower, more typical, levels of CD20 such as Ramos and Raji, hyper-cross-linking is required to bring enough CD20-BCR complexes together to evoke the signaling pathway. We anticipate that following clustering after binding of the Type I anti-CD20 mAb and association with the BCR, two BCR molecules come together, resulting in activation of the BCR ITAM motifs and initiation of the typical BCR signal transduction cascade. We favor this model as it explains all of our available data including the identical signaling pathways, dependence upon BCR expression, lack of an absolute requirement for hyper-cross-linking, and association of Type I but not Type II mAb with the BCR, as well as its regulation by FcγRIIb.

Our inhibitor analysis revealed that Syk and Src PTK were pivotal for the calcium response. These PTK have been shown to be weakly associated with the resting BCR and activated through BCR redistribution following antigen/mAb binding. Therefore, we consider it likely that redistribution of CD20 and the associated BCR results in the favorable orientation of these PTK, initiating the signal transduction cascade leading to calcium flux.

Further evidence for this suggestion was provided by our observations that the pattern of PTP was similar following ligation of anti-BCR and Type I anti-CD20 mAb, and that the BCR-specific adaptors proteins BLNK and SLP76 were equivalently phosphorylated.

A link between the BCR and CD20 has been implied for some time and at multiple levels. First, ligation of CD20 was shown to evoke a similar pattern of phosphotyrosine (pTyr) up-regulation to that induced by BCR ligation (29), and we have confirmed and extended those data here. Second, CD20 ligation can cause down-regulation of BCR expression (37). Third,
hyper-cross-linking of rituximab induces a similar pattern of ERK1/2 activation to BCR ligation (38). More recently, the BCR and CD20 molecules have been shown to be co-localized on the surface of B cells and the proportion of co-localization increased after CD20 ligation (39), and we have confirmed this increase in association after binding of Type I calcium flux-inducing mAb but not Type II anti-CD20 mAb using FRET analysis.

Therefore, in summary, all of our data indicate that CD20 is able to evoke a calcium flux in target cells after ligation with Type I mAb such as rituximab through an ability to associate with and “hijack” the signaling potential of the BCR, rather than through any intrinsic calcium channel capability. We are currently investigating the importance of this signal transduction pathway in evoking immunotherapy by CD20 mAb.

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