Skeletal Muscle Type Ryanodine Receptor Is Involved in Calcium Signaling in Human B Lymphocytes*

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The regulation of intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in B cells remains poorly understood and is presently explained almost solely by inositol 1,4,5-triphosphate (IP\textsubscript{3})-mediated Ca\textsuperscript{2+} release, followed by activation of a store-operated channel mechanism. In fact, there are reports indicating that IP\textsubscript{3} production does not always correlate with the magnitude of Ca\textsuperscript{2+} release. We demonstrate here that human B cells express a ryanodine receptor (Ryr1) that functions as a Ca\textsuperscript{2+} release channel during the B cell antigen receptor (BCR)-stimulated Ca\textsuperscript{2+} signaling process. Immunoblotting studies showed that both human primary CD19\textsuperscript{+} B and DAKIKI cells express a 565-kDa immunoreactive protein that is indistinguishable in molecular size and immunoreactivity from the Ryr1. Selective reverse transcription-polymerase chain reaction, restriction fragment length polymorphism, and sequencing of cloned cDNA indicated that the major isoform of the Ryr1 expressed in primary CD19\textsuperscript{+} B and DAKIKI cells is identical to the skeletal muscle type (Ryr1). Saturation analysis of [\textsuperscript{3}H]ryanodine binding yielded B\textsubscript{max} = 150 fmol/mg of protein and K\textsubscript{D} = 110 nM in DAKIKI cells. In fluo-3-loaded CD19\textsuperscript{+} B and DAKIKI cells, 4-chloro-m-cresol, a potent activator of Ca\textsuperscript{2+} release mediated by the ryanodine-sensitive Ca\textsuperscript{2+} release channel, induced Ca\textsuperscript{2+} release in a dose-dependent and ryanodine-sensitive fashion. Furthermore, BCR-mediated Ca\textsuperscript{2+} release in CD19\textsuperscript{+} B cells was significantly altered by 4-chloro-m-cresol and ryanodine. These results indicate that Ryr1 functions as a Ca\textsuperscript{2+} release channel during BCR-stimulated Ca\textsuperscript{2+} signaling and suggest that complex Ca\textsuperscript{2+} signals that control the cellular activities of B cells may be generated by cooperation of the IP\textsubscript{3} receptor and Ryr1.

In all cells, calcium ions play a critical role in the regulation of diverse cell activities, including gene expression, folding and processing of proteins, exocytosis and endocytosis, cell cycle progression, motility, proliferation, and differentiation (1). The importance of Ca\textsuperscript{2+} signaling has also been demonstrated in the B cells that are responsible for humoral immunity (2). In the process of the humoral immune response, antigen binding to the B cell antigen receptor (BCR) stimulates B cells to proliferate and secrete antigen-specific antibodies. An increase in the intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) is a critical regulatory event in BCR-mediated signal transduction (3, 4). Modulation of the rise in [Ca\textsuperscript{2+}]\textsubscript{i}, during B cell activation has also been suggested to be a key mechanism for controlling BCR-mediated signal transduction by secondary signals produced by B cell accessory molecules (e.g. CD19, CD21, CD22, CD40, etc.) (2, 5, 6). Furthermore, it has recently been demonstrated that the temporal characteristics (i.e. transient or sustained) and the amplitude of the Ca\textsuperscript{2+} signal are important in activating specific transcription factors (i.e. NF-κB, c-Jun N-terminal kinase, NFAT, etc.), which determine the type of gene expression in the B cell system (7). Similarly, sustained calcium signals are often associated with enhanced proliferative responses and secretion of antibodies (4). Therefore, the regulation of Ca\textsuperscript{2+} signaling determines the ultimate responses of B cells, which specifically include proliferation, apoptosis, and secretion of specific antibodies.

Despite the prominent role of Ca\textsuperscript{2+} in B cell activation, the molecular mechanisms responsible for Ca\textsuperscript{2+} movement in B cells are not clearly understood. To date, the regulation of [Ca\textsuperscript{2+}]\textsubscript{i} in the B cell system is still explained almost solely by IP\textsubscript{3}-mediated mechanisms. Antigen binding to the BCR induces a biphasic increase in [Ca\textsuperscript{2+}]\textsubscript{i}, (8, 9). The initial, rapid phase of the BCR-stimulated increase in [Ca\textsuperscript{2+}]\textsubscript{i} is the result of Ca\textsuperscript{2+} release from intracellular stores, whereas the subsequent, sustained elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, results from Ca\textsuperscript{2+} entry through plasma membrane channels, as indicated by its abolition in the absence of extracellular Ca\textsuperscript{2+}, (8, 9). As suggested in other types of non-excitatory cells (10–12), BCR-mediated Ca\textsuperscript{2+} entry is hypothesized to be a consequence of the emptying of the intracellular IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} store (“capacitative” Ca\textsuperscript{2+} entry mechanism) (13). Thus, BCR-stimulation generates IP\textsubscript{3}, which activates Ca\textsuperscript{2+} release from IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores, causing Ca\textsuperscript{2+} channels to open the so-called store-operated channel. However, there are reports indicating that IP\textsubscript{3} production does not always correlate with the magnitude of Ca\textsuperscript{2+} release (9, 14). These findings suggest that an IP\textsubscript{3}-insensitive Ca\textsuperscript{2+} store is involved in BCR-mediated Ca\textsuperscript{2+} signaling in B cells.

This study suggests that the ryanodine receptor (Ryr1) may contribute to the IP\textsubscript{3}-insensitive component of BCR-stimulated Ca\textsuperscript{2+} signaling. The Ryr1 was originally found in the sarcoplasmic reticulum of skeletal muscle (type 1 receptor, Ryr1) and cardiac muscle (type 2 receptor, Ryr2) (15–17). Ca\textsuperscript{2+} release
from the sarcoplasmic reticulum through these receptors plays a central role in regulating the contraction of skeletal and cardiac muscle fibers. A third type of RYR (type 3 receptor, RYR3) has been detected in specific regions of the brain, such as corpus striatum, thalamus, and hippocampus (18, 19), and in human Jurkat T cells (20). The present studies demonstrate that the ryanodine receptor detectable in B cells is identical to the skeletal muscle type (RYR1). Moreover, the receptor was found to function as a Ca$_2^+$ release channel during BCR-stimulated Ca$_2^+$ signaling. Therefore, upon BCR stimulation, B cells utilize at least two types of Ca$_2^+$ release channels, the IP$_3$ receptor and RYR1, to generate highly elaborate Ca$_2^+$ signaling.

**MATERIALS AND METHODS**

**Human B Cells, Cell Lines, and Tissue**—Buffcoats were obtained from healthy blood donors at the National Institutes of Health Blood Bank (Bethesda, MD). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. CD19$^+$ B cells were purified from the peripheral blood mononuclear cells using an antibody-coupled magnetic bead isolation system (Dynal, Oslo, Norway). Cells were first incubated with monoclonal anti-CD19 antibody (Pharmingen, San Diego, CA) for 30 min at 4 °C, followed by a wash with Hanks’ balanced salt solution and incubation with goat anti-mouse IgG-coated M450 Dynabeads (Dynal) for 15 min at 4 °C. Cells attached to the beads were then isolated after three washes with Hanks’ balanced salt solution. Epstein-Barr virus-transformed B cells (DAKIKI cells) (TIB206, American Type Culture Collection, Rockville, MD) (21) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 units of penicillin, and 100 μg/ml streptomycin (Quality Biological, Inc., Gaithersburg, MD). Cell cultures were incubated at 37 °C in a humidified chamber with 5% CO$_2$. Human skeletal muscle from the vastus lateralis muscle, most of which was utilized for histopathology and a caffeine/halothane contracture test for diagnosing susceptibility to malignant hyperthermia, was used to obtain control cDNA and protein for RYR1.

**Western Blot Analysis for RYR1 Protein**—Tissues or purified cells were disrupted in disposable Dounce homogenizers in buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Chaps, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 25 μg/ml p-nitrophenyl guanidinobenzoate were then incubated for 20 min at 4 °C. Following centrifugation at
14,000 × g for 15 min, the supernatants were collected and analyzed for total protein (BCA protein assay kit, Pierce). The protein samples (10–75 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis on a 10% Tris/glycine gel. After separation, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) and then probed with monoclonal anti-RYR antibodies (Affinity Bioreagents Inc., Golden, CO). Alkaline phosphatase-conjugated monoclonal anti-rabbit IgG antibody (Sigma) was used to detect the primary rabbit antibodies. Chemiluminescence detection was performed using the alkaline phosphatase substrate CSPD (Tropix Inc.).

RT-PCR Restriction Fragment Length Polymorphism Analysis—Total RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform method as described previously (22) and reverse transcription performed on the first strand of cDNA using a First-Strand cDNA synthesis kit (Amersham Pharmacia Biotech). Synthesized cDNA was then amplified by RT-PCR using the primers reported by Hakamata et al. (20) (upstream primer, 5'-dTTCATGCTGCTGTTTATAAGGT-3'; and downstream primer, 5'-dCAGATGGAAGGCTCAGCTC-3'). These primers recognize all three isoforms of RYR, producing a 1200-bp product from the 3'-region of RYR1, RYR2, and RYR3 (20). PCR amplifications were carried out with 100 ng of each primer in a total volume of 100 μl. The reaction solution contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.5 mM each dNTP, and 1.5 units of Taq plus Pwo polymerase (Boehringer Mannheim). The PCR amplification conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 2 min, and 68 °C for 3 min, followed by a 7-min extension at 68 °C in a DNA thermal cycler (Perkin-Elmer). The RT-PCR products were then digested with the selected restriction enzymes HgaI, BsmI, and ApaI to identify the RYR isoform. Based on the known sequences of human RYR isoforms, HgaI cuts the amplified 1200-bp RYR1 product into 740-, 349-, and 71-bp fragments, but it does not digest human RYR2 or RYR3. BsmI cuts only RYR2 and produces 810- and 320-bp fragments. ApaI cuts only RYR3 to make 949- and 157-bp fragments. The PCR products were digested at 37 °C for 1 h with 1–5 units of the restriction endonucleases. The restriction fragments were then resolved by electrophoresis on a 1% agarose gel and visualized on a UV transilluminator.

Selective RT-PCR Using an Isoform-specific Primer—The cDNAs obtained from DAKIKI cells, human skeletal muscle, lung (CLONTECH, Palo Alto, CA), and brain (CLONTECH) were amplified by PCR using primer sets that selectively amplify specific isoforms of the RYR. Using the same downstream primer (5'-dCAGATGGAAGGCTCAGCTG-3') and upstream primers JBR1 (5'-dGACATGGAAGGCTCAGCTG-3'), JBR2 (5'-dAAGAGACCCCGGAAAGT-3'), and
JBR3 (5′-dAGGAGGAAAGCGGATTTGTT-3′) amplifies an ~1200-bp product from the 3′-region of RYR1, RYR2, and RYR3, respectively. The PCR amplification conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 2 min, and 68 °C for 3 min, followed by a 7-min extension at 68 °C. The RT-PCR products were resolved by electrophoresis on a 1% agarose gel and visualized on a UV transilluminator.

Cloning of Partial cDNAs from B Cells and Sequencing—Total RNA was isolated from DAKIKI cells, reverse-transcribed, and amplified by PCR. A middle portion of RYR1, which contains a sequence that exhibits marked differences among the RYR isoforms, was amplified using an upstream primer (5′-dTGCCCTCGTATGTA-3′) and a downstream primer (5′-dAAGCCTTTTGCTGTT-3′). A PCR product ~1200 bp in size was purified from a 1% agarose gel with a JETSORB gel extraction kit (GENOMED Inc., Research Triangle Park, NC) and cloned into pGEM-T vector (Promega, Madison, WI). A purified DNA was then sequenced by a method of cycle sequencing using a forward primer (5′-dTGCCTCGTATGTA-3′) and an automated DNA sequencer (Applied Biosystems Model 373A).

Ryanodine Binding—A radioligand binding assay was performed with [3H]ryanodine to determine a profile of ryanodine binding to the RYR in DAKIKI cells using the tissue preparation technique modified from Chen et al. (23). Cells from six 75-cm² culture flasks (~2–3 × 10⁸ cells) were harvested by centrifugation at 500 g for 20 min at 0–4 °C. The pellet was resuspended in 5 ml of buffer A (25 mM Tris/HEPES (pH 7.5), 5 mM dithiothreitol, 1 μg/ml aprotinin, and 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride); transferred to a Potter-Elvehjem homogenizer with a Teflon pestle; and then disrupted with 25 strokes. An additional 1.5 ml of buffer containing buffer A plus 0.5 mM sucrose, 0.3 mM KCl, and 40 μM CaCl₂ was added to the homogenate and processed with another 25 strokes. The homogenate was centrifuged at 20,000 × g for 20 min at 0–4 °C. The supernatant was retained, and the pellet was rehomogenized and recentrifuged. The pellet was retained and kept at 0–4 °C, and the supernatants were combined and centrifuged at 150,000 × g for 90 min. After centrifugation, the P1-2 and P3 pellets were resuspended in assay buffer (10 mM PIPES citrate (pH 7.5), 0.5 mM KCl, 10 mM ATP, and 800 μM CaCl₂). The binding assay was performed in polystyrene multiwell plates (1-ml maximum volume; Beckman Instruments). Assays were performed in duplicate, with each tube containing 50 μl of tissue preparation (500 and 180 μg of protein/tube for P1-2 and P3, respectively), 50 μl of [3H]ryanodine (final concentrations ranging from 50 to 600 nM; specific activity = 61.9 Ci/mmol; NEN Life Science Products), 50 μl of unlabeled ryanodine (for determination of nonspecific binding, final concentration = 10 μM), and sufficient assay buffer to make a final volume of 200 μl. The [3H]ryanodine was isotopically diluted to a specific activity of 6.19 Ci/mmol using unlabeled ryanodine. The assay was incubated in the dark at 37 °C for 90 min. It was then terminated by vacuum filtration onto glass-fiber filters (Schleicher & Schuell No. 32) and washed with 2 ml of wash buffer (5 mM Tris-HCl, 0.5 mM KCl, and 250 μM CaCl₂) at 0–4 °C. Filters were placed into vials with 4 ml of CytoScint scintillation fluid (ICN, Costa Mesa, CA) and counted for 5 min in a scintillation counter (Beckman Instruments Model 6500).

Heavy sarcoplasmic reticulum fragments were prepared from frozen rabbit skeletal muscle (Pel-Freez Biologicals, Rogers, AR) (24). Muscles were ground and then homogenized in 15 ml of pyrophosphate buffer (20 mM sodium pyrophosphate, 20 mM NaH₂PO₄, 1 mM MgCl₂, and 0.5 mM EDTA (pH 7.4) at 0–4 °C) and 10% sucrose using a Polytron (Brinkmann Instruments). The volume of the homogenate was increased to 35 ml with cold buffer and centrifuged at 10,000 × g for 10 min at 0–4 °C. The supernatant was then centrifuged at 10,000 × g for 15 min at 0–4 °C. The pellet was resuspended in 15 ml of buffer and recentrifuged at 27,000 × g for 45 min at 0–4 °C. The final pellet was resuspended in buffer and frozen at ~70 °C overnight. Aliquots (5 ml) were thawed, layered on a discontinuous sucrose gradient (4 ml of 14% (w/v), 12 ml of 25%, 5 ml of 28%, 4 ml of 36%, and 3 ml of 45% sucrose in pyrophosphate buffer), and then centrifuged at 27,000 × g for 60 min.
at 4 °C. The material accumulating at the 36% sucrose layer was collected and resuspended in assay buffer for the radioligand binding assay. [3H]Ryanodine binding to this preparation was performed by adding 50 ml of tissue preparation to 50 ml of [3H]ryanodine (final concentrations of 50–500 nM) and sufficient buffer to yield a 200-ml final volume. The buffer was composed of 40 mM Tris-HCl (pH 7.4), 10 mM ATP, 800 mM CaCl₂, 1.5 M KCl, and 200 mM sucrose (free calcium concentration was set using “Bound and Determined” software, obtained from K. B. Storey (25)). Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine. The assay was incubated for 60 min at 37 °C and terminated by rapid filtration over a Brandel M-24R.

Measurement of Intracellular Calcium—Changes in [Ca²⁺]i were measured directly in human peripheral blood mononuclear cells or DAKIKI cells by measuring the fluorescence intensity of fluo-3-loaded cells (26, 27). Cells (2 × 10⁶/ml) were loaded with 1 μM fluo-3/AM (Molecular Probes, Inc., Eugene, OR) by incubation in subdued light (60 min, 25 °C) and stained with or without phycoerythrin-conjugated monoclonal anti-CD19 antibody. Cells were then washed three times with Hanks’ balanced salt solution and resuspended in 1 ml of Hanks’ balanced salt solution and analyzed by FACSscan (Becton-Dickinson, Palo Alto, CA). The FL-1 signal for fluo-3 was calibrated by transporting in saturating Ca²⁺ with either ionomycin or A23187 (Molecular Probes, Inc.) to obtain the maximum signal (F max) and then adding Mn²⁺ to obtain the minimum signal (F min). [Ca²⁺]i was calculated from the fluo-3 fluorescence intensity using the following formula: [Ca²⁺]i = Kd [(F - F min)/(F max - F)], where [Ca²⁺]i = intracellular ionized calcium concentration and Kd = 400 nM for the intracellular dye. For each experiment, the fluo-3-loaded cells were analyzed to obtain an unstimulated base line. The percentage of responding FL-1⁺ cells was then calculated and analyzed.

RESULTS

The RYR Is Expressed at the Protein Level in Human Primary B Cells and a B Cell Line (DAKIKI)—SDS-polyacrylamide gel electrophoresis immunoblot analysis with monoclonal anti-RYR antibody (clone 34-C) revealed the presence of immunoreactive protein (565 kDa) in human skeletal muscle tissues. An immunoreactive protein of the same size was detected in both CD19⁺ B cells isolated from human mononuclear cells and DAKIKI cells (Fig. 1A).

The RYR mRNA Detectable in Human Primary B Cells and a B Cell Line (DAKIKI) Is Similar to Skeletal Muscle-type RYR1—A PCR-based restriction fragment length polymorphism method was designed to identify isoforms of human RYR (RYR1, RYR2, and RYR3). In this method, cDNA for RYR is synthesized from RNA by reverse transcription and amplified by RT-PCR using the primers reported by Hakamata et al. (20). The primers recognize all three isoforms of RYR, producing an ~1200-bp product from the 3’-region of RYR1, RYR2, and RYR3. To identify which isoforms were produced from B cell...
cDNA, the RT-PCR products were digested with the selected restriction enzymes HgaI, BsmI, and ApaI. Based on the sequences of the human RYR available in the GenBank Data Bank, HgaI, BsmI, and ApaI cut at a unique site in the amplified sequences of RYR1, RYR2, and RYR3, respectively. The majority of the PCR products from human skeletal muscle were digested by HgaI, suggesting that the major isoform of the RYR expressed in skeletal muscle is RYR1. Although the overlapping of any ApaI-digested fragment by excess PCR product prevents detection of possible ApaI digestion of RYR3, incomplete HgaI digestion may be due to the presence of RYR3, as suggested by a selective RT-PCR experiment (Fig. 1C). As clearly shown in Fig. 1B, primary B cells and DAKIKI cells were completely digested by HgaI, but not by ApaI or BsmI. This result suggests that the major isotype of the RYR expressed in primary B cells is RYR1.

Expression of the RYR isoform was further investigated by selective RT-PCR using isoform-specific primers (Fig. 1C). As previously reported (28), RYR1, RYR2, and RYR3 mRNAs were highly expressed in skeletal muscle, lung, and brain, respectively. RYR1 was also expressed in lung, brain, and DAKIKI B cells. RYR2 was also expressed in brain, as previously shown (28). Although at lower levels than RYR1, RYR3 was also detected in skeletal muscle. In human DAKIKI B cells, neither the type 2 nor the type 3 isoform was expressed. The results indicate that RYR1 mRNA is expressed in human B cells.

A middle portion of the RYR cDNA from DAKIKI cells was cloned into pGEM-T vector and sequenced. The cloned cDNA contains an open reading frame of 120 amino acid residues that corresponds to the sequence of RYR1 (amino acids 2298–2417) and has a sequence that exhibits marked differences among the RYR isoforms (human RYR1, amino acids 2379–2417; human RYR2, amino acids 2347–2385; and rabbit RYR3, amino acids 2248–2286) (Fig. 2). The amino acid sequence from DAKIKI cells is identical to that of human RYR1, except for one amino acid (Lys2323/Asn) (Fig. 2). However, Gillard et al. (29) have reported that Lys2323 is an error in the sequence originally reported (17) and corrected it to asparagine. Overall results suggest that RYR1 mRNA is expressed in human B cells.

*[^3H]Ryanodine Binding—[^3H]Ryanodine bound to the P1-2...
fraction of the DAKIKI cell line with $K_d = 130 \pm 24$ nM and $B_{\text{max}} = 170 \pm 31$ fmol/mg of protein ($n = 3$) (Fig. 3). The signal/noise ratio at 50 nM [³H]ryanodine was 1.2:1. There was no specific binding of [³H]ryanodine to the P3 fraction of the cell line at protein concentrations up to 180 µg/tube. In contrast, [³H]ryanodine bound to receptors in rabbit skeletal muscle preparations (in the presence of ATP) with $K_d = 40 \pm 6.9$ nM and $B_{\text{max}} = 3.3 \pm 0.5$ pmol/mg of protein ($n = 5$). The signal/noise ratio at 50 nM [³H]ryanodine was 2.4:1.

4-Chloro-α-m-cresol (4-CmC) induced Ca²⁺ Release in CD19⁺ B Cells in a Dose-dependent and Ryanodine-sensitive Manner—The RYR stimulator (30–32) 4-CmC (100 µM to 1 mM) caused a dose-dependent increase in [Ca²⁺], from ~50 nM to a maximum of 500 nM at 1 mM 4-CmC in CD19⁺ B cells. The rise in [Ca²⁺]i, lasted for ~300–400 s after the addition of 4-CmC (Fig. 4, A and B). This increase was not totally blocked by the addition of excess EGTA (5 mM), indicating that 4-CmC induced Ca²⁺ release and influx (Fig. 4, A and B). Cells treated with ryanodine (200 µM) exhibited significantly higher and longer rises in [Ca²⁺]i, induced by 4-CmC (Fig. 4, A and B). 4-CmC also increased [Ca²⁺]i, in DAKIKI B cells in a dose-dependent and ryanodine-sensitive manner (data not shown).

$F(ab)_2$ Anti-IgM-induced Ca²⁺ Release Is Altered by Ryanodine or 4-CmC in CD19⁺ B Cells—After the addition of EGTA (5 mM), $F(ab)_2$ anti-IgM induced a rapid and short-lasting increase in [Ca²⁺], in CD19⁺ B cells. [Ca²⁺], returned to base-line levels within 120 s (Fig. 5A). Cells treated with ryanodine (200 µM) exhibited significantly larger Ca²⁺ release. The [Ca²⁺], at 60 s after anti-IgM stimulation was significantly higher in cells treated with ryanodine than in control cells (475 ± 43 nM ($n = 5$) and 270 ± 32 nM ($n = 5$), respectively; $p < 0.01$) (Fig. 5C). Following $F(ab)_2$ anti-IgM stimulation, subsequent stimulation with 4-CmC (1 mM) induced a small Ca²⁺ release, and this release was smaller in cells treated with ryanodine (Fig. 5A). In Ca²⁺-free medium, the $F(ab)_2$ anti-IgM-induced increase in [Ca²⁺]i, in CD19⁺ B cells was significantly reduced by pre-exposing cells to 4-CmC (100 µM) (Fig. 4B). The [Ca²⁺], at 60 s after anti-IgM stimulation was significantly lower in cells previously exposed to 4-CmC treatment than in control cells (192 ± 28 nM ($n = 3$) and 305 ± 35 nM ($n = 4$), respectively; $p < 0.05$) (Fig. 5C).

**DISCUSSION**

Although an IP₃-insensitive Ca²⁺ store has been suggested to be involved in BCR-mediated Ca²⁺ signaling in B cells (9, 14), neither the status of expression nor the function of the RYR has been studied in human B cells. In this report, we show that human B cells express a functional RYR that is likely involved in BCR-mediated Ca²⁺ signaling in B cells. Interestingly, unless an alternative splicing variant exists, selective RT-PCR, restriction fragment length polymorphism, cloning, and cDNA sequence analysis indicate that the major isoform of the RYR expressed in B cells is a type 1 isoform of RYR that was originally recognized to be abundantly expressed in skeletal muscle (33).

Considering our current understanding of the functional properties of the isoforms, it is interesting that non-excitable B cells express RYR1 rather than RYR3. RYR1 in skeletal muscle is activated by direct physical coupling to the voltage-sensitive dihydropyridine receptor, whereas RYR3 is activated by cyclic AMP-ribose, which may act as a second messenger for intracellular Ca²⁺ signaling in non-muscle cells (1, 34). However, because the L-type dihydropyridine-sensitive Ca²⁺ channel has recently been suggested to be involved in anti-Ig-induced Ca²⁺ influx in rat B lymphocytes (35), it may be important to investigate whether the dihydropyridine-sensitive Ca²⁺ channel is expressed and associated with RYR1 in human B cells.

Pharmacological studies indicate that the RYR expressed in B cells functions as a Ca²⁺ channel. 4-CmC, a RYR stimulator observed in skeletal muscle (30–32), increased [Ca²⁺], in a dose-dependent fashion in both primary CD19⁺ (or CD21⁺) B cells and DAKIKI cells. That 4-CmC-induced increases in [Ca²⁺], result largely from Ca²⁺ release from intracellular Ca²⁺ stores was indicated by the maintenance of [Ca²⁺], in the absence of extracellular Ca²⁺. Moreover, in the presence of 200 µM ryanodine, 4-CmC induced a higher maximum and slower decay in [Ca²⁺], than in the control. These results may be related to the findings in skeletal muscle that ryanodine keeps the Ca²⁺ release channel of the intracellular Ca²⁺ stores in an open state (36), thus maintaining RYR-mediated elevations in [Ca²⁺]. However, in skeletal muscle, high-affinity ryanodine-binding sites (Kᵣ ≈ 1–5 nM) lock the Ca²⁺ channel in an open state, whereas low-affinity sites (Kᵣ ~ 30–80 nM) inhibit channel opening (34, 37). Accordingly, nanomolar to low micromolar concentrations of ryanodine (<10 µM) tend to activate Ca²⁺ release, whereas higher concentrations (>200 µM) block the Ca²⁺ channel in skeletal muscle (34, 37).

B cells were found to have a single affinity site for [³H]ryanodine with a Kᵣ of 110 nM. Although this appears to be similar to the low affinity site in skeletal muscle, it behaves like the high affinity site. This may be due to differences in stoichiometry, localization of the receptor, or association with endogenous effectors. Indeed, pharmacological properties of RYR1, including [³H]ryanodine binding, vary greatly in different cell types (34, 37). RYR1 in skeletal muscle is well known to be abundant at the triad junction; physically attached to the voltage-sensitive Ca²⁺ channel localized in the transverse tubular membrane; and associated with triadin, calmodulin, and FK506-binding protein (34, 37). However, neither localization of the receptor nor its association with other modulatory proteins is known in B lymphocytes.

Findings by other investigators have already suggested that an IP₃-insensitive Ca²⁺ store is involved in BCR-mediated Ca²⁺ signaling in human B lymphocytes. For example, following BCR stimulation, increases in [Ca²⁺], of 25–35% of control levels were not affected when IP₃ production was abolished by tyrosine kinase inhibition (14). Similarly, inositol monophosphate generation did not correlate well with changes in Ca²⁺ in human B cells when these two parameters were compared over different types of stimulation, i.e., cross-linking membrane IgG, IgD, and IgM (9). Cross-linking membrane IgD induced the largest release of Ca²⁺, but the inositol monophosphate production was the lowest.

In addition to the molecular biological evidence of expression of RYR1 in human B cells, pharmacological studies suggest that the release of Ca²⁺ from stores through the RYR is involved in BCR-mediated Ca²⁺ signaling. Similar to its effects on 4-CmC-induced Ca²⁺ release, $F(ab)_2$ anti-IgM in the presence of ryanodine induced a higher maximum and a slower decay in [Ca²⁺], in Ca²⁺-containing medium (data not shown) and a higher maximum in Ca²⁺-free medium than in the control (Fig. 5, A and C). These results suggest that ryanodine maintains a Ca²⁺ release channel in an open state, as demonstrated in skeletal muscle. Furthermore, depleting Ca²⁺ in the 4-CmC-sensitive store significantly decreased the magnitude of BCR-mediated Ca²⁺ release (Fig. 5, B and C), suggesting that B cells utilize a RYR-operated Ca²⁺ store during BCR-mediated activation perhaps in conjunction with the IP₃ receptor.

B cells appear to decode diverse patterns of Ca²⁺ waves as signals to determine their ultimate responses, such as proliferation, apoptosis, and secretion of specific antibodies (7). Such highly intricate patterns of Ca²⁺ waves may be generated by cooperation of the IP₃ receptor and RYR1 during BCR-stimu-
lated Ca$^{2+}$ signaling. It is also possible that RYR1 may play a specific role in B cell activation. In lymphocytes, IP$_{3}$ and the RYR coexist in murine T lymphoma BW5147 cells (38) and human Jurkat T cells (20). In the BW5147 cells, a RYR that exhibits only a single, low affinity $[^{3}H]$ryanodine-binding site ($K_{d} = 200$ nM) becomes associated with concanavalin A receptor-patched and -capped structures following concanavalin A stimulation (38). Similarly, Ono et al. (39) have reported that the Epstein-Barr virus caused increases in [Ca$^{2+}$i] in a ryanodine-sensitive manner and was temporally associated with Epstein-Barr virus receptor (CD21) capping in human B lymphocytes. Despite the relatively low density of RYR expression, the much larger Ca$^{2+}$ conductance generated through the RYR (compared with the IP$_{3}$ receptor channel) may make it more effective in producing the significant changes in Ca$^{2+}$i required for spatiotemporal phenomena such as movement of cellular components (e.g., patching and capping) and secretion of Ig, hypotheses that are now under investigation.

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