Expression of the Ryanodine Receptor Isoforms in Immune Cells

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Ryanodine receptor (RYR) is a Ca\(^{2+}\) channel that mediates Ca\(^{2+}\) release from intracellular stores. We have used RT-PCR analysis and examined its expression in primary peripheral mononuclear cells (PBMCs) and in 164 hemopoietic cell lines. In PBMCs, type 1 RYR (RYR1) was expressed in CD19\(^+\) B lymphocytes, but less frequently in CD3\(^+\) T lymphocytes and in CD14\(^+\) monocytes. Type 2 RYR (RYR2) was mainly detected in CD3\(^+\) T cells. Induction of RYR1 and/or RYR2 mRNA was found after treatment with stromal cell-derived factor 1, macrophage-inflammatory protein-1\(\alpha\) (MIP1\(\alpha\)) or TGF-\(\beta\). Type 3 RYR (RYR3) was not detected in PBMCs. Many hemopoietic cell lines expressed not only RYR1 or RYR2 but also RYR3. The expression of the isoforms was not associated with specific cell lineage. We showed that the RYR-stimulating agent 4-chloro-\(m\)-cresol (4CmC) induced Ca\(^{2+}\) release and thereby confirmed functional expression of the RYR in the cell lines expressing RYR mRNA. Moreover, concordant induction of RYR mRNA with Ca\(^{2+}\) channel function was found in Jurkat T cells. In untreated Jurkat T cells, 4CmC (>1 mM) had no effect on Ca\(^{2+}\) release, whereas 4CmC (<400 \(\mu\)M) caused Ca\(^{2+}\) release after the induction of RYR2 and RYR3 that occurred after treatment with stromal cell-derived factor 1, macrophage-inflammatory protein-1\(\alpha\), or TGF-\(\beta\). Our results demonstrate expression of all three isoforms of RYR mRNA in hemopoietic cells. Induction of RYRs in response to chemokines and TGF-\(\beta\) suggests roles in regulating Ca\(^{2+}\)-mediated cellular responses during the immune response.

Calcium ions play a critical role in the activation of the immune cells that are responsible for cellular and humoral immunity (1–3). In the process of immune responses, Ag binding to the surface receptor stimulates the immune cells to eventually eliminate foreign Ags. Elevation of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is an early and critical event in the biochemical cascade of signal transduction pathways, which include activating specific transcription factors (i.e., NF-kB, JNK, NF-AT, etc.), and thus in a variety of later events in the immune cell activation (4). Therefore, the regulation of Ca\(^{2+}\) signaling determines the ultimate response of an immune cell.

An early manifestation of mitogen- or cell surface receptor-stimulated immune cell activation is a biphasic increase in [Ca\(^{2+}\)]\(_i\), which is the result of rapid Ca\(^{2+}\) release from intracellular stores followed by sustained Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels (SOC) (5, 6). Ca\(^{2+}\) release from intracellular stores is consequent to inositol 1,4,5-trisphosphate (IP\(_3\)) formation. The activation of multiple protein tyrosine kinases occurs immediately after surface receptor ligation (7, 8). Phospholipase C\(\gamma\) is then recruited to an upstream tyrosine kinase via its SH2 domains and activated by phosphorylation. Phospholipase C\(\gamma\) activation leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding IP\(_3\) and diacyl glycerol. IP\(_3\) then mediates the activation of Ca\(^{2+}\) release from stores in the endoplasmic reticulum through the IP\(_3\) receptor. Therefore, calcium mobilization after receptor cross-linking in the immune cells has been explained almost solely by IP\(_3\)-mediated mechanisms.

Although IP\(_3\) is a key messenger regulating [Ca\(^{2+}\)]\(_i\), recent studies have postulated the possibility that the ryanodine receptor (RYR) contributes to the IP\(_3\)-insensitive component of Ca\(^{2+}\) signaling in immune cells (9–12). The RYR was originally found in the sarcoplasmic reticulum of skeletal muscle (type 1 receptor; RYR1) and cardiac muscle (type 2 receptor; RYR2) (13–15). Ca\(^{2+}\) 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, nonmuscle tissues, and also skeletal muscle (16–18). We recently demonstrated that human B cells express a RYR that is identical with skeletal muscle type I by RFLP studies and sequencing analysis of partially cloned cDNA (12). In addition, 4-chloro-\(m\)-cresol (4CmC), a potent activator of the RYR (19), induced Ca\(^{2+}\) release after depleting IP\(_3\)-sensitive Ca\(^{2+}\) pools in B cells (12). These results suggested that human B cells express functional RYR1 that is involved in regulating Ca\(^{2+}\) signaling, perhaps in conjunction with the IP\(_3\) receptor. For T cells, expression of the RYR has been found in human Jurkat T cells (10, 11) and murine T lymphoma cells (9). In both T cell lines, cyclic ADP-ribose increased [3H]ryanodine binding and induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores (9, 10). The isoform of the RYR expressed in Jurkat T cells was identified to be type 3 (10, 11). Therefore, the RYR3 has been proposed to control [Ca\(^{2+}\)]\(_i\) in response to cyclic ADP-ribose.

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3 Abbreviations used in this paper: [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\) concentration; 4CmC, 4-chloro-\(m\)-cresol; IP\(_3\), inositol 1,4,5-trisphosphate; mIg, membrane Ig; RYR, ryanodine receptor; SOC, store-operated Ca\(^{2+}\) channel; SDF-1, stromal cell-derived factor 1; MIP1\(\alpha\), macrophage-inflammatory protein-1\(\alpha\).

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during T cell activation (10). These findings of the RYR in T and B cells allowed us to hypothesize that the RYRs are more widely expressed and responsible for regulating [Ca\(^{2+}\)], in immune cells than currently thought.

In this study, we have investigated expression of all three isoforms of the RYR in human primary T cells, B cells, and monocytes using selective RT-PCR followed by RFLP analysis. We also examined a total of 164 human hemopoietic cell lines (36 T cell, 92 B cell, 19 myelomonocytic, 11 megakaryocytic, 3 erythrocytic, and 3 nonlymphocytic, nonmyelocytic) to determine the lineage and differentiation specificity of the expression of the 3 RYRs. The possibility that any isoform of RYR is induced by stimulation with mitogens, chemokines, and other stimuli were investigated to gain insight of the roles of this Ca\(^{2+}\) release channel in immune function. Finally, to verify the functional expression of the RYRs, Ca\(^{2+}\) release by RYR-stimulating agents was assessed using the cell lines expressing the RYR mRNA. A global view of RYR expression in human immune cells was addressed in this study.

**Materials and Methods**

**Reagents**

Stroma-derived factors 1α and 1β (SDF-1α and -1β), macrophage-inflammation protein-1α (MIP1α), TGF-β, RANTES, nerve growth factor, and 4CmC were obtained from Calbiochem (San Diego, CA). PHA, Con A (type IV), LPS (Escherichia coli; B5W), PMA, and caffeine were from Sigma (St. Louis, MO). Fluoro-3 acetoxyethyl ester was obtained from Molecular Probes (Eugene, OR). Anti-Cyto-D and anti-Cyto-M Abs were from BD PharMingen (San Diego, CA). Total RNA isolated from normal heart and mRNA from brain was purchased from Invitrogen (Carlsbad, CA).

**Primary cells, cell lines, and tissues**

Buffcoo were obtained from healthy blood donors at the National Institutes of Health Blood Bank (Bethesda, MD). PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. CD3\(^+\) T cells, CD19\(^+\) B cells, and CD14\(^+\) monocytes were purified from the PBMCs using an Ab-magnetic bead isolation system (Dynal, Oslo, Norway). Cells (10\(^7\) cells) were first incubated with Dynabeads coated with anti-CD3 mAb for 30 min at 4°C. CD3\(^+\) cells attaching to the beads were isolated after three washes with HBSS. Unattached cells were then incubated with anti-CD19 beads for 30 min at 4°C. CD19\(^+\) cells were isolated after three washes with HBSS, and unattached cells were then incubated with anti-CD14 beads for 30 min at 4°C. CD14\(^+\) cells were then isolated after three washes with HBSS. Jurkat, SupT1, H9, CEM, SKW6.4, DAKIKI, THP-1, and U937 were obtained from American Type Culture Collection (ATCC; Manassas, VA). Other cell lines used in this study were from the repository at Fujisaki Cell Center (Okayama, Japan) (20). Cells were cultured in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mM-glutamine, 100 U penicillin, and 100 g/ml streptomycin (Quality Biological, Gaithersburg, MD). Cell cultures were incubated at 37°C in a humidified chamber with 5% CO\(_2\). Anonymous tissue sample from the vastus lateralis muscle, most of which was used for histopathology and caffeine/halothane contracture testing for diagnosing susceptibility to malignant hyperthermia, was used to obtain control cDNA and protein for the 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% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 10 lM leupeptin, 10 lM aprotonin, and 25 lM p-nitrophenyl-2-2'-dinitrobenzoate and then incubated for 20 min at 4°C. After centrifugation at 14,000 \( \times g \) for 15 min, the supernatants were collected and analyzed for total protein (BCA protein assay kit; Pierce, Rockford, IL). The protein samples (10–75 lM/glane) were separated using SDS-PAGE on a 10% Tris-glycine gel. After separation, the proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and then probed with monoclonal anti-RYR Abs (Affinity Bioreagents, Golden, CO). Alkaline phosphatase-conjugated monoclonal anti-rabbit IgG Ab (Sigma) was used to detect the primary rabbit Abs. Chemiluminescence detection was performed using the alkaline phosphatase substrate CSPD (Tropix, Bedford, MA).

**Selective RT-PCR followed by RFLP analysis**

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI), and reverse transcription was performed to the first strand of cDNA using a cDNA synthesis kit (Promega). Synthesized cDNA was then amplified by RT-PCR using a primer set which selectively amplifies specific isoform of the RYR. Using the same downstream primer, 5'-dAAGTGAAGTTGCTGCTTCAT-3', and an isoform-specific upstream primer: JBR1, 5'-dAG-ACATGAGGGCCTAGCTGCT-3'; JBR2, 5'-dAAGAGGCTCCTCCACGAGAATG-3'; and JBR3, 5'-dAAAAGAGAAGCAGTGTTG-3', an ~1200-bp product was recognized from the 3'-regions of RYR1, RYR2, and RYR3, respectively. PCR amplifications were conducted using the Expand Long PCR system (Boehringer Mannheim, Indianapolis, IN). PCR was performed in a 50-l reaction mixture containing 100 ng DNA, 15 pmol of each primer, 0.5 mM dNTPs, 2.5 U Expand Long polymerase mixture and Expand Long PCR buffer 3 (Boehringer Mannheim). The PCR amplification conditions were 95°C for 2 min, followed by 40 cycles of 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 then digested with selected restriction enzymes, Hgal, BsmI, and HindIII to identify the RYR isoform. Based on the known sequences of human RYR isoforms, Hgal cuts the amplified 1112-bp RYR1 product into 692-, 349-, and 71-bp fragments, but it does not digest human RYR2 or RYR3. BsmI cuts only the 1083-bp RYR2 and produces 762- and 321-bp fragments. HindIII cuts the 1015-bp RYR3 product to make 537- and 478-bp fragments. The PCR products were digested at 37°C for 1 h with 1–5 U of the restriction endonucleases. The restriction fragments were then resolved by electrophoresis on a 2% agarose gel and visualized on a UV transilluminator. As a control of mRNA input, β-actin mRNA levels were determined for each sample in separate RT-PCR. For β-actin amplification, PCR was performed with 25 cycles to ensure that the amplification was completed within the linear range. The sequences of primers for β-actin were 5'-dAAGAGAGCGATCCTCACCCT-3' (sense) and 5'-dTGCTGATCCACATCTGCGA3' (antisense). In some experiments, the signal ratio of RYR to β-actin was determined on the basis of the ratio of the intensity of the PCR product compared with the corresponding β-actin band. The PCR products were imaged, and the relative OD of each band was measured and analyzed using NIH Image software.

**Ca\(^{2+}\) mobilization test using B cells**

Relative changes in [Ca\(^{2+}\)] were derived from changes in the fluorescence intensity of fluo-3-loaded cells (21). Cells (2 × 10\(^3\)ml) were loaded with 1 μM fluo-3 acetoxyethyl ester in subdued light (30 min, 25°C). Cells were then washed once with HBSS, resuspended in 1 ml of HBSS, and analyzed by FACScan (Becton Dickinson). Forward and right angle scatter signals were displayed on a linear scale, with the forward scatter adjusted to gate cells from debris. The fluo-3 fluorescence (excitation at 488 nm with emission at 525 nm) was detected after separation with a 530 (FL-1) band pass filter. FL-1 fluorescence was recorded, amplified, and displayed.
Expression of RYR isoforms in human primary mononuclear cells

Expression of the three isoforms of RYR mRNA was investigated by selective RT-PCR followed by RFLP analysis. In this method, cDNA for RYR is synthesized from mRNA by reverse transcription and amplified by selective PCR using the isoform-specific primers. The isoform-specific PCR primers for amplification of the human RYR1, RYR2, and RYR3 were designed to produce an ~1200-bp product from the 3'-region of the RYR1, RYR2, and RYR3. To further confirm specificity of the RT-PCR products, the PCR products were digested with selected restriction enzymes Hgal, BsmI, and HindIII. Based on the sequences of human RYR1, RYR2, and RYR3 available in the GenBank, Hgal, BsmI, and HindIII cut at a unique site in the amplified sequences for RYR1, RYR2, and RYR3, respectively. We tested our two-step method by examining cDNAs from skeletal muscle, cardiac muscle, and brain which have been shown to express type 1, type 2, and type 3, respectively (Refs. 15 and 17 and Fig. 1). As predicted based on previous observations, RYR1 mRNA was highly expressed in skeletal muscle. RYR3 mRNA was also detected at lower levels than RYR1 in skeletal muscle (Fig. 1A). In cardiac muscle, RYR2 mRNA was dominantly expressed compared with types 1 and 3 (Fig. 1A). In brain, all three isoforms were highly expressed (Fig. 1A). Specificity of the selective RT-PCR products was then examined by RFLP analysis. The RT-PCR products amplified for the RYR1, RYR2, and RYR3 from skeletal muscle, cardiac muscle, and brain, respectively, were digested with restriction enzymes Hgal, BsmI, and HindIII. The PCR product amplified for the RYR1 from skeletal muscle was cut into 692-, 349-, and 71-bp fragments by Hgal, but it was not digested with BsmI or HindIII (Fig. 1B). The amplicon for RYR2 from cardiac muscle was cut into 762- and 321-bp fragments by BsmI (Fig. 1B). The product for RYR3 from brain was cut into 537- and 478-bp fragments only by HindIII (Fig. 1B).

Table I.  RYR expression in primary T and B cells and monocytes and cell lines

| Cell Types         | No. of Individuals (or cell lines) Examined | No. of Positive Individuals (or cell lines) (%) |
|--------------------|---------------------------------------------|-----------------------------------------------|
|                    |                                             | RYR1  | RYR2  | RYR3  | RYR* 1, 2, and 3 |
| Primary cells      |                                             |       |       |       |                  |
| CD3+ T cells       | 9                                           | 1 (11.1) | 4 (44.4) | 0 |
| CD19+ B cells      | 9                                           | 5 (55.5) | 2 (22.2) | 0 |
| CD14+ monocytes    | 9                                           | 1 (11.1) | 0 | 0 |
| Cell lines         |                                             |       |       |       |                  |
| T cell lines       | 36                                          | 2 (5.6) | 3 (8.3) | 2 (5.6) | 1 (2.8)* |
| B cell lines       | 92                                          | 1 (1.1) | 8 (8.7) | 9 (9.3) | 1 (1.1)* |
| Myelomonocytic lines | 19                           | 1 (5.3) | 1 (5.3) | 3 (15.8) | 1 (5.3)* |
| Megakaryocytic lines | 11                                | 0 | 0 | 6 (54.5) | 0 |
| Erythroid lines    | 3                                           | 0 | 1 (33.3) | 1 (33.3) | 0 |
| Non-L, non-M cells | 3                                           | 0 | 0 | 3 (100) | 0 |

* Cell lines expressing multiple isoforms, SupT, SKW6.4, and U937.
Using this selective RT-PCR/RFLP method, we examined the expression of three isoforms of RYR mRNA in T cell, B cell, and monocyte populations purified from nine different donors (Fig. 2 and Table I). Type 1 RYR was detected in freshly isolated CD19+ B lymphocytes from five of nine donors. The RYR1 mRNA was less frequently detected in CD3+ T lymphocytes and CD14+ monocytes. Type 2 RYR was mainly detected in CD3+ T cells. For type 3 amplification, we frequently observed smear without a distinguishable single band of PCR product, whereas RYR3 mRNA expression of three isoforms of RYR mRNA in T cell, B cell, and monocyte populations puriﬁed from the donors that we examined. We performed SDS-PAGE immunoblot analysis to examine the protein expression in mononuclear cells from the donor who showed high levels of RYR1 mRNA in B cells and monocytes. The immunoblot with monoclonal anti-RYR Ab (clone 34-C) revealed the presence of the immunoreactive protein that was similar in size to the protein from skeletal muscle in puriﬁed CD19+ B cells and CD14+ monocytes (Fig. 3). Some immunoreactivity was seen in puriﬁed CD3+ T cell and total mononuclear cell preparation (Fig. 3).

Expression of RYR isoforms in human hemopoietic cell lines

A large array of human hemopoietic cell lines at various stages of differentiation allows us to determine the lineage and differentiation speciﬁcity of the expression of genes and proteins. To investigate speciﬁcity of expression of RYR isoforms, we examined total 164 human cell lines, consisting 36 T and 92 B cell leukemia lines and 19

| Cell Line | Origina | RYR | Cell Line | Origin | RYR |
|-----------|---------|-----|-----------|--------|-----|
| Myeloid   |         |     |           |        |     |
| MR-87     | AUL     | –   | MOLT-10   | ALL    | –   |
| KG-1      | AML     | –   | T-Blast-II| –      | –   |
| ML1       | AML     | –   | RPMI-8402 | ALL    | –   |
| HL-60     | APL     | –   | CCRF-CEM  | ALL    | –   |
| PL-21     | CML     | –   | DND-41    | ALL    | –   |
| KCL-22    | CML     | –   | HPB-ALL   | ALL    | –   |
| GDM-1     | CML     | –   | HD-MAR-2  | HD?    | –   |
| KU-812    | CML     | 2+  | STEER-92  | LY     | –   |
| SCC3      | AML     | 3+/−| MOLT-13   | ALL    | –   |
| HIG       | CML     | 3+/−| ALL-SIL   | ALL    | –   |
| SKH-1     | CML     | –   | T-Blast-III| –    | –   |
| HOR       | CML     | –   | SUPT1     | LY     | 1,2,3+ |
| EOL-1     | EOL     | –   | TALL-1    | ALL    | –   |
| Monocytoid|         |     |           |        |     |
| SUM90-7   | AMMOL   | 3+  | P12/Ichikawa| ALL   | –   |
| P31/Fujoka| AMOL    | 3+/−| KOPT-K1   | ALL    | –   |
| IMS-M1    | AMOL    | –   | MOLT-16   | ALL    | 3+/−|
| MOLM-13   | AMOL    | –   | Jurkat    | LY     | 2,3** |
| THP-1     | AMOL    | 1+  | PF-382    | ALL    | –   |
| U937      | LY      | 1,2,3++| HAY-92N  | LY     | –   |
| Megakaryocytic | | | | | |
| MEG-01    | CML     | 3+  | Willow-89 | ALL    | –   |
| TS9:22    | CML     | 3+  | PEER      | ALL    | –   |
| YS9:22    | CML     | 3+  | T-Blast-IV| –      | –   |
| SS9:22    | CML     | 3+  | SKW-3     | CLL    | –   |
| JURL-MK1  | CML     | 3+/−| A3/Kawakami| ALL   | 3+/−|
| JURL-MK2  | CML     | –   | MAT       | ALL    | –   |
| M-07E     | MEGL    | 3+/−| KARPAS-299| LY     | 2++ |
| UT-7      | MEGL    | 3+/−| T-Blast-V | –      | –   |
| MOLM-1    | CML     | –   | HUT-102   | MF     | –   |
| MOLM-7    | CML     | –   | C5/MJ     | NT     | –   |
| CMK-86    | MEGL    | 3+/−| MT-4      | NT     | –   |
| Erythroid |         |     |           |        |     |
| K562      | CML     | 2+  | ED-S      | ATL    | 2+  |
| HEL       | EL      | 3+/−| H9       | ALL    | –   |
| KMOE      | EL      | –   | SALT-3    | ATL    | –   |
| Non-L, non-M |       |     |           |        |     |
| SU-DHL-1  | LY      | 3+  | MT-1      | ATL    | –   |
| HDLM-2    | HD      | 3+  | HUT-78    | SS     | –   |
| L428      | HD      | 3+  | –         | –      | –   |

*a Classification and deﬁnition of T cell lines (22): T-Blast-I, HLA-DR+, CD10+, TdT−, and T-Ag− (nearly identical with CD3); T-Blast-II, HLA-DR+, CD10+, TdT+ and T-Ag+; T-Blast-III, HLA-DR+, CD10+, TdT+ and T-Ag+; T-Blast-IV, HLA-DR+, CD10+, TdT+ and T-Ag+; T-Blast-V, HLA-DR+, CD10+, TdT+ and T-Ag+; and with human T cell leukemia virus-I. RYR 1, 2 and 3 expression of RYR isoforms, type 1, 2, or 3; +, high level of expression; +/−, moderate level of expression; −/−, no detectable expression; **+, expressed when stimulated with chemokines such as SDF-1β.

b AUL, acute undifferentiated leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; AMOL, acute monocytic leukemia; APL, acute promyelocytic leukemia; AMMOL, acute myelomonocytic leukemia; ATL, adult T cell leukemia; CML, chronic myelogenous leukemia; EL, erythroleukemia; EOL, eosinophilic leukemia; HD, Hodgkin’s disease; HCL, hairy cell leukemia; LY, lymphoma; MEGL, megakaryoblastic leukemia; MF, mycosis fungoides; NKL, natural killer leukemia; NT, normal retrovirus infected; SS, Sézary syndrome.
phocytic, nonmyelocytic cell lines (Tables I–III). In T cell lines, 22% of cell lines expressed type 1, 2, or 3 of the RYR mRNA. All T cell lines expressing detectable levels of RYR mRNA were from either category of blast-III or blast-IV (Table II). T cell lines classified in blast-III and -IV are differentiated and blocked at a more mature stage than cells in blast-I and -II (22). Nine cells classified in blast-I or blast-II did not express RYR mRNA. Similarly, 21% of B cell lines (mainly mature B cell and plasma cell types) expressed type 1, 2, or 3 of the RYR mRNA. None of 26 B cell lines from immature stages, i.e., pro-B, common-B, and pre-B types, expressed the RYR (Table 3 of the RYR mRNA. None of 26 B cell lines from immature stages, i.e., pro-B, common-B, and pre-B types, expressed the RYR (Table III). Many of the nonlymphocytic lines (myelocytic, megakaryocytic, erythroid, and nonlymphocytic, nonmyelocytic) expressed RYR mRNA, mainly type 3 (Tables I and II). Expression of type 3 RYR mRNA was especially manifested in megakaryocytic and nonlymphocytic, nonmyelocytic lines. Some of the cell lines, such as U937, SupT1, and SKW6.4, expressed more than a single isoform (Fig. 4).

Table III. RYR expression in human B cell linesa

| Cell Line | Originb | RYR | Cell Line | Origin | RYR |
|-----------|---------|-----|-----------|--------|-----|
| Pro-B cell | ALL     | –   | ABL-2     | BL     | 2+  |
| NALM-19   | ALL     | –   | BALM-1/2  | ALL    | –   |
| Common-B cell | ALL | –   | BALM6-8   | ALL    | –   |
| NALM-20   | ALL     | –   | MIDDLE-91 | LY     | –   |
| NALM-21   | ALL     | –   | U-698-M   | LY     | –   |
| NALM-24   | ALL     | –   | Tanaka    | LY     | –   |
| NALM-29   | ALL     | –   | MANCA-2   | LY     | –   |
| HAL-01    | ALL     | –   | JOK-1     | HCL    | 3+/–|
| OM9;22    | ALL     | –   | SKW-4     | LY     | 2+/–|
| BV-173    | ALL     | –   | BALM3-5   | LY     | –   |
| KID-92    | ALL     | –   | SU-DHL-4  | LY     | –   |
| REH       | ALL     | –   | MLB-1084  | LY     | –   |
| NALL-1    | ALL     | –   | BAL-KH    | ALL    | –   |
| NALM-16   | ALL     | –   | BOAR-88   | LY     | 2+  |
| NALM-33   | ALL     | –   | BALM-9    | BL     | 2+  |
| PRE-B cell | ALL | –   | BALM-13   | BL     | –   |
| NALM-1    | CML     | –   | BALM-16   | ALL    | –   |
| NALM6-13  | ALL     | –   | BALM-18   | ALL    | –   |
| NALM-17   | ALL     | –   | BALM-19   | ALL    | –   |
| NALM-26   | ALL     | –   | BALM-24   | ALL    | –   |
| INC       | ALL     | –   | BALM-25   | ALL    | –   |
| LAZ-221   | ALL     | –   | BALM-26   | ALL    | –   |
| KOPN-8    | ALL     | –   | Sc-1      | LY     | –   |
| TAHR-87   | ALL     | –   | Ri-1      | LY     | –   |
| P30/Okubo | ALL     | –   | BAL-1     | ALL    | –   |
| KLM-2     | AMOL    | –   | HAIR-M    | HCL    | –   |
| UDD-OCT   | ALL     | –   | JC-1      | HCL    | –   |
| UDD-88    | ALL     | –   | Plasma cell |        | –   |
| B cell | ALL | –   | RPMI-8226 | MM     | –   |
| BAY-91    | ALL     | –   | U-266     | MM     | –   |
| Black-93A | BL     | –   | ARH-77    | MM     | –   |
| EB-3      | BL     | –   | L-363     | MM     | 3+  |
| RAJI      | BL     | –   | OPM-2     | MM     | –   |
| HR1K      | BL     | –   | MOLP-2    | MM     | –   |
| B35M      | BL     | –   | KM-1      | MM     | –   |
| DAUDI     | BL     | 2+  | KM-4      | MM     | 3+  |
| Nalawwa  | BL     | –   | KM-5      | MM     | –   |
| Ramos     | BL     | –   | KM-6      | MM     | –   |
| BJAB      | BL     | –   | KM-7      | MM     | 3+  |
| DG-75     | BL     | –   | KM-11     | MM     | 3+  |
| Chevalier | BL     | –   | ILKM-10   | MM     | 3+  |
| DND-39    | BL     | –   | ILKM-12   | MM     | 3+/–|
| NK-9      | BL     | –   | KMS-18    | MM     | –   |
| B46M      | BL     | –   | KHM-4     | MM     | 3+  |
| OGUN      | BL     | 3+/–| MOLP-5    | MM     | 2+  |
| AL-1      | BL     | 2+  | DAKIKI    | EBV    | 1+  |
| SKW6.4    | BL     | 1,2,3+|         |        |     |

a RYR 1, 2 and 3, expression of RYR isoform type 1, 2, or 3; ++, high level of expression; +, moderate level of expression; +/–, marginal expression; –, no detectable expression.

b ALL, acute lymphoblastic leukemia; AUL, acute undifferentiated leukemia; AMOL, acute monocytic leukemia; CML, chronic myelogenous leukemia; BL, Burkitt’s lymphoma; EBV, EBV transformed; HCL, hairy cell leukemia; LY, lymphoma; MM, multiple myeloma.
three RYRs were examined to gain some insight regarding association of the RYRs with immune function (Table IV). TGF-β (100 pg/ml) treatment for 24 h induced RYR2 mRNA in PBMCs. Although activation of T cells by PHA (1 μg/ml) significantly increased type 1 mRNA, neither Con A (1 μg/ml) nor combination of anti-CD3 plus PMA increased expression of type 1 RYR mRNA. Activation of B cells by the combination of goat F(ab)2, anti-human IgM Ab plus PMA increased expression of type 2 RYR mRNA. LPS (1 μg/ml) caused no significant increase in expression of any isoform of RYR mRNA in PBMCs. Treatment with chemokines, SDF-1α (500 ng/ml), MIP1α (10 ng/ml), and RANTES (100 ng/ml) increased expression of RYR2 mRNA. Type 1 RYR mRNA was also increased 24 h after SDF-1α. SDF-1β (500 ng/ml) had no effect on RYRs mRNA. Type 3 RYR mRNA was not detected in PBMCs after any of the above treatments (Table IV).

The effects of TGF-β and chemokines on the three RYRs were also examined using Jurkat T and U937 cells. Although expression of type 3 RYR in Jurkat T cells has been reported, the Jurkat T cells, which had been purchased from ATCC and maintained in our laboratory, showed no expression of any isoforms of RYR mRNA (Fig. 4). Stimulation with SDF-1β, MIP1α, and TGF-β induced expression of RYR2 and RYR3 mRNA in the Jurkat T cells grown in our laboratory (Fig. 5). SDF-1α induced only RYR3 mRNA. RANTES also induced RYR3 mRNA, but the degree of induction was smaller than that by other chemokines (data not shown). Similar findings were obtained using U937 cells, where the above agents caused enhancements in expression of RYRs; the U937 cells expressed all three types of RYR mRNA before stimulation (data not shown).

Effects of RYR-stimulating agents on [Ca2+]i, using SupT1, Jurkat, DAKIKI, SKW6.4, U937, and THP-1 cell lines

Using the cell lines expressing RYR mRNA, we examined the effects of the RYR-stimulating agents caffeine, 4CmC, and ryanodine on Ca2+ levels. Caffeine (1–50 mM) dose-dependently increased [Ca2+]i, in DAKIKI B cells and SupT1 cells. This increase was totally blocked by the addition of excess extracellular EGTA (5 mM), indicating that caffeine induces Ca2+ influx without eliciting Ca2+ release from the internal Ca2+ store. Caffeine (1–50 mM) caused neither Ca2+ release nor Ca2+ influx in other cell lines. Within a range of 100 μM–1 mM, 4CmC caused a dose-dependent increase in [Ca2+]i in SupT1, DAKIKI, SKW6.4, U937, and THP-1 cells (Fig. 6). The 4CmC-induced increase in [Ca2+]i in the cells was not reduced by excess EGTA and hence involves mainly release from internal stores. In Jurkat T cells, 4CmC (>1 mM) had no effect on [Ca2+]i (Fig. 6A). In contrast, 4CmC (<400 μM) caused Ca2+ release after induction of RYR2 and RYR3 in Jurkat T cells by treatment with SDF-1β, MIP1α or TGF-β (Fig. 6B). None of the above cell lines responded to ryanodine (1 μM–1 mM). There was no clearcut relationship between the kinetics of Ca2+ changes and expression of isoforms. For example, Ca2+ response to 4CmC in the RYR1-expressing line THP-1 was more similar to that in Jurkat cells treated with TGF-β (RYR1 negative) than the Ca2+ response seen in RYR1+ DAKIKI cells. The cell

Table IV. Induction of RYR 1, 2, and 3 mRNA in PMCs

| Treatment   | RYR1 | RYR2 | RYR3 |
|-------------|------|------|------|
| Chemokines  |      |      |      |
| SDF-1α, 0.5 μg/ml | +    | +    | -    |
| SDF-1β, 0.5 μg/ml | -    | -    | -    |
| MIP1α, 10 ng/ml  | -    | -    | -    |
| RANTES, 100 ng/ml | -    | -    | -    |
| Growth factors|      |      |      |
| TGF-β, 100 pg/ml  | +    | -    | -    |
| NGF, 1 μg/ml    | -    | -    | -    |
| Mitogens       |      |      |      |
| Con A, 1 μg/ml  | +    | +    | -    |
| PHA, 1 μg/ml    | +    | +    | -    |
| LPS, 1 μg/ml    | -    | -    | -    |
| Cross-linking receptors|      |      |      |
| Anti-CD3 Ab + PMA, 1 ng/ml | -    | -    | -    |
| Anti-IgM Ab + PMA, 1 ng/ml | -    | -    | -    |

*+, Induction of the transcript; >1.5-fold increase was seen in two independent experiments; -, no detectable induction. Subjects who provided PMCs in this study were unrelated to those presented in Table I or Fig. 2. NGF, Nerve growth factor.
lines expressing all three isoforms, i.e., SKW6.4, U937, and SupT1 cells, tend to show relatively slow but long-lasting increases in [Ca\(^{2+}\)], in response to 4CmC (400 \(\mu\)M) compared with immediate but short-lasting increases in [Ca\(^{2+}\)], seen in THP-1 or Jurkat cells treated with TGF-\(\beta\). In addition to Jurkat T cells, we examined H9 and HL-60 that gave negative RYR expression in RT-PCR experiments. Neither cell line showed an increase in [Ca\(^{2+}\)], in response to 4CmC at concentrations below 1 mM (data not shown).

**Discussion**

In this study, we have investigated expression of three isoforms of the RYR, RYR1, RYR2, and RYR3, in human primary T and B cells and monocytes and a total of 167 hemopoietic cell lines using RT-PCR. In primary mononuclear cells, the isoform of RYR mRNA expressed in CD\(^{3+}\) T cell preparations was either type 1 or type 2. Type 1 RYR was preferentially expressed in CD\(^{19+}\) B cells, but RYR2 mRNA was also detected in some individuals. RYR3 was not detected in T cell, B cell or monocyte preparations under the conditions we used. The expression pattern of the isoforms in cell lines was quite different from that seen in primary cells. Approximately 25% of T or B cell leukemia lines, mostly mature cell types, expressed RYR1, RYR2, or RYR3 mRNA. Through previous screening of B cell lines, we have selected the DAKIKI B cell line as a type 1 RYR-positive line to model RYR1-mediated Ca\(^{2+}\) signaling in primary B cells (12). However, in the present study, we have found that only DAKIKI B cells expressed the RYR1 among 92 B cell lines. Thus, type 1 RYR mRNA, which is preferentially expressed in primary CD19\(^+\) B cells, seems to be down-regulated in other B cell lines. Conversely, type 3 RYR mRNA, which was not at all detected in PBMCs, has been detected in 4 T cell lines and 9 B cell lines. Interestingly, seven of these nine B cell lines were of multiple myeloma origin. Expression of type 3 was also robust in nonlymphocytic cell lines. Although we examined only two individuals, we did not find type 3 in either primary granule cell or platelet preparations (data not shown). Therefore, a shift of the RYR isoform from one type to another may be a noteworthy phenomenon found in hemopoietic cell lines. It is possible that this phenomenon is associated with oncogenesis or transformation of hemopoietic cells.

Type 3 RYR has recently been proposed to be responsible for a novel Ca\(^{2+}\) signaling pathway in T cells (10). This is based on pharmacological and molecular biological findings that include TCR-stimulated increases in cyclic ADP-ribose, cyclic ADP-ribose-mediated Ca\(^{2+}\) release, and expression of RYR3 (10). However, this hypothesis was drawn from observations made in Jurkat T cells, not primary T cells. Because only type 2 or 1 was expressed in primary peripheral T cells, RYR-mediated Ca\(^{2+}\) signaling in T cells must be examined based on genotypic and phenotypic expression of RYR isoforms in primary T cells. Our finding is not surprising in light of the RYR3-deficient animal model in which there is normal proliferation of T and B lymphocytes in response to mitogens or IL-2 (24).

Cloning of type 3 RYR gene has been made by two independent laboratories (23, 25). Induction of type 3 RYR in mink lung epithelial cells (MvILu) by TGF-\(\beta\) treatment led to cloning of the RYR3 gene (previously named \(\beta 4\) gene) by one of these laboratories (23). Similarly, induction of type 1 RYR has also been observed in HeLa cells, murine NIH3T3 fibroblasts, and mammary epithelial cell line HC11 cells (17). Our RYR induction study gave us some important insights as to potential association of the RYRs with immune function. Although which cell subsets responded to each treatment remains to be determined, we found that type 1 and/or type 2 RYR mRNA were inducible by a variety of treatments in PBMCs (Table IV). Substantial increases in RYR1 and RYR2 mRNA expression by PHA, cross-linking surface IgM, and chemokines (SDF-1, MIP1\(\alpha\), and RANTES) suggest functional requirement of RYRs for T cell signaling. B cell receptor-mediated B cell activation and chemotaxis of immune cells. In the immune system, TGF-\(\beta\) antagonizes T cell proliferation and macrophage activation and regulates Ig class switching in B cells. Thus, induction of RYR2 mRNA by TGF-\(\beta\) in PBMCs may be involved in some of the actions of TGF-\(\beta\).

A clear association of RYR expression with Ca\(^{2+}\) channel function was obtained from the studies with Jurkat T cells. At least two laboratories have reported expression of RYR3 mRNA in Jurkat T cells (10, 11), whereas Bennett et al. (26) found no expression. Consistent with the finding by the latter group, Jurkat T cells, which have been obtained from ATCC and maintained in our laboratory, showed no constitutive expression of any RYR isoforms. Nonetheless, Jurkat T cells were able to express not only type 3 but also type 2 RYR when stimulated with SDF-1\(\beta\), MIP1\(\alpha\), and TGF-\(\beta\). Therefore, we suggest that expression of RYR in Jurkat T cells may be clone or culture condition dependent. We have tested the effects of the RYR-stimulating agent 4CmC on Ca\(^{2+}\) response in Jurkat T cells before and after induction of the RYRs. In Jurkat T cells that showed no constitutive expression of any isoform of the RYR, >1 mM 4CmC did not cause any increase in [Ca\(^{2+}\)].
sence of extracellular Ca\(^{2+}\), in Jurkat T cells that expressed RYR2 and RYR3 after the treatment with TGF-\(\beta\) (Fig. 6B). The 4CmC-induced increases in \([Ca^{2+}]_i\) were due to Ca\(^{2+}\) release as shown in the absence of extracellular Ca\(^{2+}\). Jurkat T cells treated with chemokines (SDF-1\(\alpha\), SDF-1\(\beta\), MIP1\(\alpha\), and RANTES) also showed significant Ca\(^{2+}\) release in response to 400 \(\mu\)M 4CmC. Therefore, the appearance of 4CmC-induced Ca\(^{2+}\) release indicated that Jurkat T cells treated with these chemokines or TGF-\(\beta\) indeed expressed functional RYRs. Consistent with that finding, 4CmC also induced Ca\(^{2+}\) release in the RYR-expressing cell lines, U937, THP-1, SKW6.4, DAKIKI, and Sup T cells, suggesting that these cell lines not only express mRNA but also a functional Ca\(^{2+}\) release channel. Contrasting with 4CmC data, the effects of caffeine and ryanodine on cytoplasmic Ca\(^{2+}\) for-}