Store-operated Cation Entry Mediated by CD20 in Membrane Rafts*

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B cell activation requires sustained elevation of cytoplasmic free calcium, achieved by influx through store-operated calcium (SOC) channels. The molecular identity of these channels is not known. Ectopic expression of the raft-associated tetraspan protein CD20 in Chinese hamster ovary cells introduced a novel SOC entry pathway that was permeable to strontium as well as to calcium. The activity of this SOC pathway was abolished by deletion of a cytoplasmic sequence in CD20 essential for its efficient raft localization. Strontium-permeable SOC channels were detected in B cells, and B cell receptor-stimulated influx was significantly reduced by down-regulation of CD20 expression using short interfering RNA and also by cholesterol depletion. This is the first evidence that raft-associated CD20 constitutes a component of a SOC entry pathway activated by the B cell receptor.

The function of CD20, a tetraspan transmembrane protein expressed in B lymphocytes, is not yet fully elucidated, although electrophysiological evidence of calcium channel activity has been reported. Increased Ca\(^{2+}\) conductance, detected by whole cell patch clamp recordings, was induced by membrane hyperpolarization in a variety of cell types expressing CD20 ectopically and was similar to the native conductance found in B cells (1, 2). The type of calcium channel that is formed or regulated by CD20 and the conditions of its activation are not known.

Intracellular calcium is an essential regulator of cell function (3, 4). Receptor-mediated activation of phospholipase C and consequent inositol 1,4,5-trisphosphate production leads to the efficient raft localization. Strontium-permeable SOC channels were detected in B cells, and B cell receptor-stimulated influx was significantly reduced by down-regulation of CD20 expression using short interfering RNA and also by cholesterol depletion. This is the first evidence that raft-associated CD20 constitutes a component of a SOC entry pathway activated by the B cell receptor.

Electrophysiological studies distinguish several classes of SOC channels, including the highly Ca\(^{2+}\)-selective Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channel described in mast cells and T lymphocytes and others with distinct characteristics (7, 8). Despite intensive research efforts, the molecular identity of SOC channel proteins remains unclear. The mammalian homologues of the Drosophila transient receptor potential (TRP) gene products, now forming a TRP superfamily with more than 20 members in 3 subfamilies (9), provide the only candidate SOC channel proteins currently known. All TRPs apparently form cation channels but with variable selectivity and activation properties (10, 11).

In B lymphocytes, expression of specific genes requires sustained elevation in [Ca\(^{2+}\)]\(_i\) (12). As in other cell types, B lymphocytes activate Ca\(^{2+}\) entry after store-depletion, but the molecular components and the mechanism of activation of the SOC channels are unclear. Recently, TRPC1, for which there is evidence of store-operated, diacylglycerol, or receptor-mediated channel activity in other cell types (10), was genetically disrupted in chicken DT40 B cells and found to reduce but not ablate SOC entry (13). This suggests that TRPC1 may form SOC channels in B cells and also indicates the existence of additional SOC entry pathways. In this report we demonstrate that CD20, when expressed in Chinese hamster ovary (CHO) cells, dramatically enhanced SOC entry through strontium-permeable ion channels, providing the first evidence that CD20 is a component of a SOC entry pathway. In B cells, short interfering RNA (siRNA) reduced both CD20 expression and B cell receptor (BCR)-stimulated calcium entry. Cholesterol depletion or deletion of a 7-residue cytoplasmic CD20 sequence that controls its localization to cholesterol-dependent membrane microdomains, i.e. lipid rafts, inhibited SOC entry, suggesting the involvement of rafts in regulating CD20 SOC activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—Ramos, BJAB, and Molt-4 cells were maintained in RPMI, 10% fetal bovine serum, and CHO cells in e-minimum Eagle’s medium, 10% fetal bovine serum. Antibiotic-antimycotic (Invitrogen) was added to all cell culture media. Molt-4 T cells expressing CD20 or CD20 deletion constructs were described previously (14). CHO cells were transfected with CD20 cDNA or deletion mutants in BCMG-Sne vector or vector alone as described (14). Positive cells were selected by Geneticin (Invitrogen) and further sorted by flow cytometry. For RNA interference experiments, BJAB cells were transfected with 120 pmol of siRNAs in 400 μl of RPMI by electroporation at 300 V and 800 microfarads.

**Reagents and Antibodies**—Fura-2/AM was purchased from Molecular Probes Inc. (Eugene, OR). The abbreviations used are: SOC, store-operated calcium; TRP, transient receptor potential; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\); CHO, Chinese hamster ovary; GMI, Galβ1,3GalNAcβ1,4NeuAcα2,3Gal-β1,4Glcβ1,1-ceramide; siRNA, short interfering RNA; BCR, B cell receptor; ANOVA, analysis of variance; Tg, thapsigargin; MBC, methyl-β-cyclodextrin; SOSr, store-operated Sr\(^{2+}\); 0Ca, Ca\(^{2+}\)-free buffer containing 0.2 mM EGTA.

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lary Probes (Eugene, OR). Thapsigargin, SKF96365, ionomycin, and BAPTA/AM were purchased from Calbiochem, siRNAs against CD20 (CCACCTCTAGGAGGAGTGT) and control (GGCCGCTTTTGAGGATTCG) were purchased from Dharmacon Research Inc. (Lafayette, CO). All other chemicals were from Sigma. Mouse IgG2a isotype control antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), and mouse B1 monoclonal antibody (IgG2a) against human CD20 was from Coulter Corp. (Hialeah, FL). For immunoblotting, anti-Goα antibody was purchased from Oncogene Research Products (Boston, MA), and anti-CD45 and anti-caveolin antibodies were from Transduction Laboratories (Lexington, KY); rabbit antisera generated against a CD20 C-terminal peptide was described previously (15). Cholera toxin conjugated to horseradish peroxidase was purchased from Sigma.

**Calcium Solutions and Fura-2 Imaging**—All buffers contained 125 mM NaCl, 5 mM KCl, 1 mM MgCl2, 20 mM HEPES, and the pH was adjusted to 7.4 with NaOH. CaCl2 and SrCl2 were added to the buffer at 1 mM final concentration as indicated. Ca2+-free buffer contained 0.2 mM EGTA. CHO cells were grown on glass cover slips in 0.05% fetal bovine serum for 2–3 days before the experiment. Ramos and BJAB cells were grown in normal culture medium and adhered to poly-lysine-coated cover slips by low speed centrifugation on the day of the experiment. The cells were loaded with fura-2 by incubation in 5 μM fura-2/AM in HEPES buffer with 1 mM Ca2+ for 40–50 min at room temperature followed by washing and a further 15–20-min incubation to ensure full de-esterification. Digital imaging of fura-2 fluorescence was performed using the ImageMaster system from Photon Technology International, Inc. (Lawrenceville, NJ), essentially as described previously (16). The images were analyzed to give the averaged background-subtracted ratio of fluorescence excited at 340/380 nm (emission at 510 nm) for all the cells in one field (>20 CHO cells or >100 B cells). To quantitatively describe the Ca2+ or Sr2+ entry, the peak ratio value of the Ca2+ or Sr2+ entry was subtracted by the base-line fura-2 ratio value, and this gives Ca’ or Sr’. The Ca’ or Sr’ was then divided by ATP, Tg, or IgM (ATP, Tg, or IgM) are the peak ratio values of Ca2+ release after the stimulation subtracted by the base-line fura-2 ratio value. Statistical significance was tested using Student’s t test or ANOVA as indicated.

**Cell Stimulation and Pretreatment Conditions**—ATP and thapsigargin (Tg) were used at 100 μM and 1 μM final concentration, respectively. Tg was delivered to the chamber by pipetting in a 1-ml volume and was maintained for about 10–20 min. ATP was added to the flow solution and maintained after addition. SKF96365 was added to the EGTA buffer at 50 μM final concentration and maintained for about 9 min. Methyl-β-cyclodextrin (MBC) and cholesterol/MBC (11:181 weight ratio) were used to pretreat cells at 2% (w/v) for 1 h at room temperature. Antibody was used at 1 μg/105 cells.

**Flow Cytometry**—Transfected BJAB or CHO cells were incubated with 1% anti-CD20 monoclonal antibody followed by fluorescein isothiocyanate-conjugated anti-mouse IgG. The data were acquired using a FACScan (BD Biosciences, San Jose, CA) and analyzed using the FlowJo program (Tree Star, Inc., San Carlos, CA).

**Lipid Raft Isolation and Immunoblotting**—Sample preparation for sucrose gradient ultracentrifugation was described previously (17). Briefly, 107 BJAB, Ramos, or Molt4 cells or 5 × 107 CHO cells were lysed in 1% Brij58. Sucrose was added to a final concentration of 40% and overlayed with 5 ml of 30% sucrose and 5 ml of 5% sucrose. Samples were centrifuged for 17 h at 37,000 rpm using a Beckman XL-70 ultracentrifuge with a SW 41 Ti rotor (Beckman Instruments). Eight fractions (1.5 ml each) were collected from top to bottom, and the pellet was dissolved in SDS sample buffer (fraction 9). Equal aliquots of each fraction were loaded on SDS-polyacrylamide gel (PAGE) and run at constant voltage. Proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for immunoblotting, and bound horseradish peroxidase-conjugated secondary antibodies were detected by SuperSignal Chemiluminescent Substrate (Pierce). For GM1 detection, aliquots of each fraction were spotted onto nitrocellulose membranes (Schleicher & Schuell) and blotted with cholera toxin-horseradish peroxidase. The images were acquired and analyzed using a Fluor-S MAX Multimager and Quantity One software, respectively (Bio-Rad).

**RESULTS**

**Novel SOC Entry in CD20-expressing CHO Cells**—CHO cells expressing exogenous CD20 or control CHO cells transfected with vector alone were loaded with the calcium-sensitive dye, fura-2, and the fluorescence values in a field of >20 cells were recorded during the perfusion of various reagents. Intracellular stores were depleted either by receptor-mediated activation of phospholipase C/inositol 1,4,5-trisphosphate pathways using ATP (18) or with Tg, a specific inhibitor of the endoplasmic reticular Ca2+-ATPase (SERCA) (19). We then tested both Sr2+ and Ca2+ entry after store depletion because Sr2+ entry can distinguish endogenous SOC channels from exogenously expressed channels in some cases (20).

As shown in Fig. 1A, no difference in base-line fluorescence

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**FIG. 1.** CHO cells transfected with CD20 have novel permeability to divalent ions after store-depletion. A. CHO cells transfected with CD20 or empty vector were held in Ca2+-free buffer containing 0.2 mM EGTA (0Ca) at the beginning of the experiment. Flow buffer containing 1 mM Sr2+ was then used to perfuse the cells at the time period indicated by the horizontal line under the curve. After switching back to 0Ca buffer, the cells were stimulated by ATP (100 μM in 0Ca buffer) to deplete Ca2+ stores. Sr2+ was then re-introduced at the time period indicated. The images at a, b, and c time points are presented in B and correspond to the fura-2 signal at the resting state, the peak level of ATP-stimulated Ca2+ release, and Sr2+ entry, respectively. C, the protocol was similar to A except 1 ml of 1 mM Tg was in 0Ca buffer was delivered to the flow chamber by pipetting and maintained for about 10 min. Statistical analysis of the results is given in Table 1.
was detected between the control and CD20-expressing CHO cells. ATP sharply increased [Ca\(^{2+}\)] in the absence of extracellular Ca\(^{2+}\) in both cell lines, as expected. [Ca\(^{2+}\)] gradually returned to base line, presumably due to extrusion mechanisms at the plasma membrane and possibly by uptake into internal organelles (21). Subsequent perfusion of Sr\(^{2+}\) induced a large increase in the fluorescence ratio in CD20-transfected cells, suggesting that influx of Sr\(^{2+}\) occurred in these cells in response to store depletion. Vector-transfected cells did not respond significantly to Sr\(^{2+}\), indicating that endogenous SOC channels are relatively impermeable to Sr\(^{2+}\) under the experimental conditions used. The store-operated Sr\(^{2+}\) (SOSr) entry after ATP stimulation in CD20-transfected CHO cells was reproducibly about four times greater than that in vector-transfected CHO cells (Table I, first row). There was no Sr\(^{2+}\) entry before store depletion (Fig. 1A), indicating that CD20 expression does not confer constitutive permeability to Sr\(^{2+}\) in CHO cells. The results were averaged from a relatively uniform response of the majority of cells (\(>85\%\)) as shown in Fig. 1B, which illustrates the fura-2 fluorescence at the time-points indicated as \(a\), \(b\), and \(c\) in Fig. 1A.

Tg was then used to deplete intracellular stores passively. As observed after receptor-mediated store depletion, Sr\(^{2+}\) entered CD20-transfected CHO cells much more efficiently than control cells after Tg-induced store depletion (Fig. 1C and Table I, second row). After Sr\(^{2+}\) was withdrawn and fluorescence values had returned to base line, Ca\(^{2+}\) was introduced into the perfusion chamber. Influx of Ca\(^{2+}\) into vector-transfected cells indicated that these cells maintained a normal Ca\(^{2+}\) entry pathway after Tg treatment. Calcium influx into CD20-transfected cells was greatly increased over that observed in vector-transfected cells (Fig. 1C and Table I, third row). Sr\(^{2+}\) entry into CD20-transfected cells was reduced about 52\% by SKF96356 (Table I, fourth row), a channel blocker frequently used to inhibit receptor-activated Ca\(^{2+}\) entry (22), supporting the conclusion that the enhanced Sr\(^{2+}\) entry obtained in CD20-expressing cells is through SOC ion channels.

**Sr\(^{2+}\)-permeable CD20 SOC Channels in B Cells**—The above results indicated that CD20 has the ability to form and/or organize a SOC entry pathway with some permeability to Sr\(^{2+}\). Because CD20 is normally expressed in B lymphocytes, we then tested whether B cells are permeable to Sr\(^{2+}\) after store depletion. We used Ramos cells, an Epstein-Barr virus-negative, IgM-positive cell line derived from Burkitt’s lymphoma. The SOC channels were activated either with anti-IgM to stimulate the BCR (Fig. 2A) or with Tg (Fig. 2B). Sr\(^{2+}\) entry occurred after stimulation with either reagent and was inhibited by SKF96356 (Fig. 2 and Table II, first and second rows). Ca\(^{2+}\) entry was also inhibited by SKF96356 using similar protocols as in Fig. 2 (Table II, third and fourth rows). Thus, Ramos B cells express SOC entry channels that are permeable to Sr\(^{2+}\) and can respond to BCR stimulation.

Recently, we reported that immediately upon stimulation the BCR translocates into lipid rafts and co-locizes with CD20 (15). These results together with known modulatory effects of anti-CD20 monoclonal antibodies on anti-IgM-stimulated proliferative responses (23) suggest a functional association between CD20 and the BCR. To directly assess the contribution of CD20 to BCR-mediated SOC entry in B cells we used the RNA interference technique (24) to down-regulate CD20 expression in the transfectable human B cell line BJAB. Short interfering RNA was designed to target the region of CD20 mRNA that is 96–115 nucleotides downstream of the translational start site. Two days after transfection, CD20 surface expression was reliably and specifically reduced by 40\% as compared with cells transfected with control siRNA (Fig. 2C). Expression of the BCR was unchanged (Fig. 2C). BCR-mediated SOSr entry was significantly inhibited after down-regulation of CD20 expression with anti-CD20 siRNA, whereas control siRNA had no effect (Fig. 2, D and E). Inhibition of CD20 expression and SOSr entry were both dose-dependent effects of anti-CD20 siRNA (not shown). Inhibition of CD20 expression also inhibited Ca\(^{2+}\) entry after BCR stimulation using the same protocol as in Fig. 2D but substituting Sr\(^{2+}\) with Ca\(^{2+}\) (Table II, fifth row).

**CD20 Is Constitutively Associated with Membrane Rafts**—Previously, we reported that CD20 is soluble in 1% Triton X-100 but becomes insoluble after antibody ligation and floats on sucrose density gradients, consistent with the interpretation that it inducibly associates with lipid rafts (17). However, Brij58, a less stringent detergent than Triton X-100, reveals raft association that may be of lower affinity than observed with Triton X-100. Indeed, we found that when unstimulated B cells were lysed in 1% Brij58, CD20 associated with the low density fractions (Fig. 3A, upper panel), indicating that it may be constitutively raft-associated. CD20 co-fractionated on the gradients with ganglioside GM1 and G0, a subunit of heterotrimeric G proteins that is localized to rafts (Fig. 3A, upper panel). CD45 is excluded from rafts and was not detected in the buoyant fractions of Brij58 lysates, consistent with other reports (25–27) and confirming that the Brij58-insoluble membrane fragments isolated in our experiments did not non-specifically include an abundant non-raft protein. Data derived from BJAB cells are shown in Fig. 3A. Similar data were obtained using Ramos cells (not shown).

Lipid raft microdomains are enriched in cholesterol, which appears to be necessary to maintain their integrity (28). Pretreatment of BJAB cells with the cholesterol-extraction drug MBC reduced the buoyancy of CD20 in the sucrose gradients, demonstrating the cholesterol dependence of CD20-raft association (Fig. 3A, lower panel). Previously, we identified a short membrane-proximal cytoplasmic sequence (residues 219–225) that was critical for antibody-induced CD20 insolubility in 1% Triton X-100 (14). To determine whether this sequence was a determinant of constitutive raft association of CD20, lipid rafts were isolated from Molt-4 T cells transfected with either full-length CD20 cDNA (WT) or the Δ219–225 deletion mutant. The results show that deletion of residues 219–225 largely prevented the association of CD20 with the buoyant fractions of sucrose gradients (Fig. 3B).

### Table I

| Vector / CD20 | n | p |
|--------------|---|---|
| Sr\(^{2+}\)/ATP | 0.083 ± 0.016 | 18 | p < 0.001 |
| Sr\(^{2+}\)/Tg | 0.104 ± 0.016 | 18–20 | p < 0.001 |
| Ca\(^{2+}\)/Tg | 0.736 ± 0.091 | 2.130 ± 0.148 | 18–20 | p < 0.001 |
| Sr\(^{2+}\)/Tg | + SKF | 0.411 ± 0.045 | 7 | |
| Sr\(^{2+}\)/Tg | – SKF | 0.197 ± 0.031 | 7 | p = 0.002 |

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**Data represent the increase in fura-2 ratio during the entry phase after the addition of either Sr\(^{2+}\) or Ca\(^{2+}\) as indicated by the increase in ratio during the Ca\(^{2+}\) release phase induced by either ATP or Tg as indicated.**
**MBC Inhibits SOSr Entry in Ramos Cells**—Depletion of cholesterol by MBC affects various cellular functions, including receptor-mediated signaling events (28), and specific effects of MBC on SOC entry have been observed in other systems (29, 30). Because CD20 was found to be constitutively raft-associated, the effect of MBC on SOSr entry in Ramos B cells was investigated. Cells pretreated with MBC were exposed to F(ab)\textsubscript{2} anti-IgM to stimulate the BCR. Consistent with our previous report that MBC treatment had no inhibitory effect on tyrosine phosphorylation events or on calcium mobilization after BCR stimulation (31), MBC-treated Ramos cells had normal BCR-mediated Ca\textsuperscript{2+} release (Fig. 4A). Sr\textsuperscript{2+} entry, however, was significantly reduced in MBC-treated cells as compared with untreated cells (Fig. 4, A and C) but was normal in cells treated with cholesterol-loaded MBC (Fig. 4, B and C), indicating a cholesterol-specific inhibitory effect of MBC on the entry pathway.

**SOSr Entry in Transfected CHO Cells Is Ablated by Deletion of Residues 219–225**—Because residues 219–225 were found to be required for efficient association of CD20 with lipid rafts, we tested whether these residues altered store-operated cation influx in transfected CHO cells. Constructs encoding CD20(Δ219–225) and CD20(ΔN2–50), for comparison, were expressed in CHO cells. The expression levels of these constructs were similar to one another (Fig. 5A) but were significantly lower than the expression level of native CD20 used for the experiments shown in Fig. 1. Therefore, a second line of CHO cells was derived that expressed full-length CD20 at a similar level to the deletion mutants (Fig. 5A). SOSr entry was compared in the two CHO cell lines expressing different amounts of

### Table II

Store-operated cation entry in B cells reduced by SKF96365 and CD20 down-regulation

| Entry Type | −SKF | +SKF | n  |
|------------|------|------|----|
| Sr\textsuperscript{2+}/IgM | 0.388 ± 0.028 | 0.160 ± 0.027 | 7 | p < 0.001 |
| Sr\textsuperscript{2+}/Tg | 0.710 ± 0.063 | 0.223 ± 0.047 | 7 | p < 0.001 |
| Ca\textsuperscript{2+}/IgM | 1.218 ± 0.138 | 0.479 ± 0.025 | 4 | p = 0.002 |
| Ca\textsuperscript{2+}/Tg | 4.871 ± 0.510 | 1.350 ± 0.251 | 7 | p < 0.001 |
| Ca\textsuperscript{2+}/BCR | 0.068 ± 0.068 | 0.051 ± 0.063 | 7 | p < 0.001 |

Data represent the increase in fura-2 ratio during the entry phase after the addition of either Sr\textsuperscript{2+} or Ca\textsuperscript{2+} as indicated divided by the increase in ratio during the Ca\textsuperscript{2+} release phase induced by either anti-IgM or Tg as indicated.
full-length CD20 (Fig. 5B, top two panels) and was found to be significantly lower in cells with reduced CD20 expression (Fig. 5C). Deletion of the N-terminal cytoplasmic region of CD20 had no significant effect on Sr$^{2+}$ entry when compared with cells expressing a similar level of full-length CD20, whereas the 219–225 deletion abolished CD20-mediated Sr$^{2+}$ entry after store depletion (Fig. 5, B and C). Constitutive raft association of ectopically expressed CD20 in CHO cells was demonstrated (Fig. 5D). Deletion of residues 219–225 significantly reduced buoyancy of CD20 on the density gradient, whereas the N-terminal deletion had little effect (Fig. 5, D and E). Caveolin localization in the raft fractions as a control was not changed by the expression of any of the CD20 constructs.

**DISCUSSION**

This paper is the first report of an integral membrane protein outside of the TRP family functioning as a component of a SOC entry pathway. Like a number of known calcium channel components, CD20 is a phosphoprotein with four transmembrane spans and cytoplasmic termini containing no intrinsic enzymatic activity or recognized protein interaction domains or motifs. The 33-kDa monomer immunoprecipitates as dimers and tetramers after chemical cross-linking of cell-surface proteins (1) and is assembled into ~200-kDa complexes containing CD20 as the major component (32). As shown here, SOSr entry into transfected CHO cells correlated with the level of CD20 expression and was abolished by mutagenesis of CD20. Together, these findings support a direct role for CD20 in ion channel formation.

SOC entry in hematopoietic cells appears to be mediated by $I_{\text{CRAC}}$ channels, as described most clearly in mast cells and Jurkat T cells (6, 7). SOC channels distinct from $I_{\text{CRAC}}$ are known in other cell types (33, 34) but so far have not been described in cells of the hematopoietic lineage. A similarity between $I_{\text{CRAC}}$ and the CD20-mediated current has been noted (1); both are inwardly rectified and voltage-insensitive with similar electrophysiological properties. $I_{\text{CRAC}}$ is highly permeable to Ca$^{2+}$ over Na$^+$ and has selectivity for divalent ions, with Ca$^{2+} >$ Ba$^{2+} =$ Sr$^{2+}$ (6). $I_{\text{CRAC}}$ current has not been described in mammalian B lymphocytes but was reported recently in avian (DT40) B cells (35, 36). Detailed analysis of the ion selectivity of the CD20-mediated current has not been determined, although a previous report showed a well-conducted Ba$^{2+}$ current mediated by CD20 (1). Our results show that the CD20 SOC was permeable to strontium. The fura-2 imaging data show that Sr$^{2+}$ entered the cells more slowly than Ca$^{2+}$; this may reflect greater selectivity of the channel for Ca$^{2+}$ than for Sr$^{2+}$ or it may be attributed to differences between Sr$^{2+}$ and Ca$^{2+}$ with respect to fura-2 binding rates or affinities (37). In summary, our data do not address whether the channel is $I_{\text{CRAC}}$ or a non-$I_{\text{CRAC}}$ SOC but complement previous electrophysiological analyses, supporting the possibility that CD20 might mediate $I_{\text{CRAC}}$ in B cells.

The plasma membrane appears to be structurally compartmentalized to allow separation, aggregation, and cross-talk of signaling events to occur. Lipid raft microdomains currently
FIG. 5. **SOSr entry correlates with CD20 expression level and its localization in lipid rafts.** A, surface expression of wild-type (WT) CD20 and deletion mutants in transfected CHO cells analyzed by flow cytometry. B, CHO cells transfected with wild-type CD20 (high or low expression), N-terminal deletion mutant (ΔN), C-terminal 219–225 deletion mutant (Δ219–225), or vector alone were stimulated by Tg followed by buffer switching to 1 mM Sr²⁺ as indicated. C, statistical analysis of the results from 8–11 experiments using ANOVA. Data shown are the mean ± S.E. The single (*) and triple asterisks (****) indicate significant differences of p < 0.05 and p < 0.001, respectively, compared with cells with low wild-type CD20 expression. D, transfected CHO cells were lysed in 1% Brij58, fractionated by sucrose gradient ultracentrifugation, and analyzed by immunoblotting with anti-CD20 and anti-caveolin antibodies. Statistical analysis of the results from 4–5 experiments are given in E, raft localization is calculated as the percentage of proteins in fractions 3–5 compared with all fractions.
provide the best example of membrane heterogeneity regulating signal transduction. A variety of signaling molecules either constitutively or inducibly associate with membrane rafts, including components of the calcium release pathway (38, 39). Characteristically, proteins associated with rafts are insoluble in the non-ionic detergent Triton X-100 and can be distinguished from the detergent-insoluble cytoskeleton by their low density on sucrose gradients. CD20 is soluble in 1% Triton X-100 but becomes insoluble after antibody ligation and floats on sucrose density gradients (17). Previously, we interpreted this to mean that CD20 inducibly associates with lipid rafts. However, it is apparent from the work described here that CD20 is constitutively raft-associated. Although CD20 is soluble in Triton X-100, it is found in low density insoluble membranes isolated using Brij58 (and other non-ionic detergents; data not shown). Low stringency detergent conditions are also necessary to detect localization of the high affinity IgG receptor, FeCRII, and CD40 to lipid rafts (40, 41). The conclusion that CD20 is constitutively raft-associated is supported by the cholesterol dependence of its buoyancy on sucrose density gradients and by the marked effect of the short membrane-proximal cytoplasmic deletion. The dramatic change in Triton solubility of CD20 that occurs after antibody engagement likely reflects an increased affinity for the raft environment caused by an antibody-mediated conformational change and/or aggregation. BCR-induced SOSr entry was diminished by down-regulation of CD20 expression and by cholesterol depletion, suggesting that SOC entry in B cells required both CD20 and the microenvironment of rafts to function properly. Recently, it was reported that inhibition of Ca$^{2+}$ mobilization in T cells by MBC could be attributed in part to depletion of the intracellular Ca$^{2+}$ stores (42). This was not the case in Ramos B cells because the amount of calcium released from intracellular stores using either ionomycin or BCR stimulation was similar in MBC-treated and untreated cells (Fig. 4 and Ref. 31). Pizzo et al. (42) also report an MBC-induced change in membrane potential in Jurkat cells, manifested by abrogation of MBC-mediated inhibition of calcium entry when experiments were performed in high K$^+$ buffer. In similar experiments using Ramos cells, inhibition of anti-IgM-stimulated SOSr entry by MBC was still observed, indicating that the effects of MBC on BCR-mediated calcium entry cannot be attributed to alterations in membrane potential (data not shown). As suggested previously (43), a fundamental difference in membrane properties and/or antigen receptor signaling of B and T lymphocytes may underlie differences observed in the effects of MBC in these cell populations. Our results are consistent with those from neutrophils and human submandibular gland cells, in which MBC treatment also inhibited SOC entry without affecting calcium release from intracellular stores (29, 30).

Deletion of residues 219–225 diminished both raft localization of ectopically expressed CD20 and its ability to mediate SOSr entry. This deletion does not affect oligomerization of CD20. Taken together with the inhibitory effect of cholesterol depletion on strontium entry in B cells, the most likely interpretation of these data is that localization of CD20 to rafts is necessary for functioning of the channel. However, it is possible that the 219–225 deletion has other effects, and a detailed molecular analysis will be required to distinguish potentially distinct functions of the 219–225 sequence.

CD20 belongs to a family (MS4A) of at least 24 genes sharing sequence homology, particularly in the membrane-spanning regions (44, 45). Most MS4A genes are expressed in cells of the hematopoietic system, and a few are also expressed in other tissues. Other members of this family include the Fe receptor β subunit, expressed in mast cells and basophils (46), and HTM4, which is widely expressed in hematopoietic cells on intracellular membranes and is involved in cell cycle regulation (47).

In summary, we provide here the first evidence of CD20 involvement in store-operated calcium entry. The topology and quaternary structure of CD20 together with the inhibitory effects of the short cytoplasmic deletion are most suggestive of its direct involvement as a component of the channel itself. Raft association of CD20 appears to be necessary for proper functioning of the channel and couples BCR signals to store-dependent calcium influx in cholesterol-dependent membrane microdomains.

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