Functional role of lipid rafts in CD20 activity?

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Abstract

CD20 is a B-lymphocyte-specific integral membrane protein, implicated in the regulation of transmembrane calcium conductance, cell-cycle progression and B-lymphocyte proliferation. CD20 is proposed to function as a SOCC (store-operated calcium channel). SOCCs are activated by receptor-stimulated calcium depletion of intracellular stores. Sustained calcium conductivity across the plasma membrane mediated by SOCC activity is required for long-term calcium-dependent processes, such as transcriptional control and gene expression. Cross-linking of CD20 by antibodies (e.g. Rituxan) has been reported to induce a rapid redistribution of CD20 into specialized microdomains at the plasma membrane, known as lipid rafts. Recruitment of CD20 into lipid rafts and its homo-oligomerization are suggested to be crucial for CD20 activity and regulation. This review outlines recent biochemical studies characterizing the role of CD20 in calcium signalling in B-lymphocytes and evaluates an engagement of lipid rafts in the regulation of CD20-mediated calcium conductivity.

CD20: a B-lymphocyte-specific antigen and its clinical relevance

CD20 belongs to the MS4A (membrane-spanning 4A) gene family, which is expressed in haematopoietic cells, and consists of at least 25 members clustered at human chromosome locus 11q12–13 [1,2]. The MS4A family members [e.g. CD20, FceRIβ (β-subunit of the high-affinity IgE receptor) and HTm4 (haematopoietic transmembrane-4 protein)] have a predicted tetraspanning membrane topology with an N- and C-terminal cytoplasmic domain (Figure 1). Despite their shared chromosomal localization and conservation in protein domain organization, the function of the members is not well characterized and is likely to be diverse [3,4]. CD20 is the best studied member of this family.

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CD20 is specifically expressed on the surface of B-cells and cells from most B-cell lymphoproliferative disorders [5]. Different isoforms of CD20 (33, 35 and 37 kDa) result from multiple phosphorylation of serine and threonine residues in the cytoplasmic domains, implying that CD20 activity is highly regulated by phosphorylation [6,7]. At present, the exact stoichiometry of the cell-surface-expressed CD20 complex has not been determined; however, after chemical cross-linking, CD20 has been observed to be separated as dimers or tetramers by non-reducing SDS/PAGE [8]. Binding of a subset of anti-CD20 antibodies to CD20 is reported to increase calcium conductivity across the plasma membrane, to trigger cell-cycle block at the G1 phase and to inhibit in vitro B-cell differentiation and mitogen-induced immunoglobulin secretion [8,9]. Cross-linking of CD20 by these antibodies is also observed to induce activation of protein kinases and to increase expression levels of several surface proteins [10–14]. Rituxan, a chimaeric anti-CD20 antibody (Rituximab, Mabthera, C2B8), is in clinical use for non-Hodgkin’s B-cell lymphoma, and has also shown excellent efficacy in inducing clinical improvement and remission in rheumatoid arthritis [15–19]. Binding of Rituxan occurs at the extracellular loop between the predicted third and fourth transmembrane domain of CD20, and does not induce internalization of the CD20 after antibody ligation [20].

The antibody-mediated therapy achieves its effectiveness through B-cell depletion. Several mechanisms of anti-CD20-mediated depletion of B-cells have been postulated, including ADCC (antibody-dependent cell cytotoxicity), activation of the complement system, and CD20-mediated regulation of the cell cycle and apoptosis [21–25]. The induction of apoptosis after anti-CD20-antibody treatment is blocked by chelating either extracellular or intracellular calcium, suggesting that variation in calcium levels contribute to the onset of apoptosis. To evaluate which mechanism contributes mainly to the therapeutic outcome is complicated by the fact that the mode of anti-CD20 antibodies

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action on tumour cells and cell lines may differ from that on non-malignant peripheral blood cells and lymphoid organ B-cells [26].

**Store-operated calcium entry in B-lymphocytes**

Intracellular calcium serves as a ubiquitous signal for cellular activation and regulates a broad array of cellular functions ranging from induction of exocytosis, cell proliferation, cell differentiation and cell death [27,28]. In most non-excitable cells, such as lymphocytes, the generation of the receptor-induced increase of intracellular calcium signal involves two phases: ligand binding to a receptor at the plasma membrane triggers a rapid, but transient, mobilization of calcium from intracellular stores such as ER (endoplasmic reticulum) or Golgi apparatus, followed by a slow, but sustained, entry of calcium across the plasma membrane (Figure 2). In B-lymphocytes, the first phase of the calcium response can be initiated by binding of antigen to the BCR (B-cell receptor) resulting in an activation of PLC\(\gamma\) (phospholipase C\(\gamma\)), which then cleaves PtdIns(4,5)\(P_2\) to diacylglycerol, a membrane-integrated messenger, and Ins(1,4,5)\(P_3\) [29]. Ins(1,4,5)\(P_3\) is released from the plasma membrane and activates ligand-dependent channels [Ins(1,4,5)\(P_3\) receptors] in the membrane of the ER, generating an initial calcium signal. Depletion of intracellular stores mediates a signal that activates SOCCs (store-operated calcium channels) at the plasma membrane. The sustained calcium entry phase is required both to refill intracellular calcium pools and to ensure long-term calcium-dependent processes, e.g. transcriptional control, gene expression, developmental transition, maturation and function of cells. The store-operated calcium current is also termed capacitative calcium entry [30]. Although calcium entry across the plasma membrane has been observed experimentally, the molecular identity of the SOCC has not been determined. The coupling mechanism by which the decrease in luminal calcium concentration is transmitted to SOCCs at the plasma membrane is also poorly understood. Despite intensive efforts to understand the coupling mechanism(s), no defined pathways have been identified [31]. One hypothesis includes a low-molecular-mass diffusible factor (calcium influx factor), which is released from the ER after store depletion and activates SOCCs at the plasma membrane [32]. An alternative model favours a direct physical interaction between proteins localized in the plasma membrane with proteins embedded in the membrane of the ER [33,34]. This interaction can be dynamic and induced by store depletion. A further development of the second model proposes an inducible vesicular transfer of proteins from the ER membrane to the plasma membrane after store depletion. This model has been supported by the observation that communication between SOCCs and store depletion is sensitive to the function of a membrane fusion protein SNAP-25 (soluble N-ethylmaleimide-sensitive-factor-attachment protein 25) [35].
Is CD20 operating as a SOCC?

CD20 is proposed to be one of the possible candidate channels mediating capacitative calcium entry in B-lymphocytes. Early electrophysiological studies with cell lines transfected with CD20 cDNA showed that CD20 contributes to transmembrane calcium conductance [8]. The conductive activity of CD20 is proposed to be regulated by a G-protein-dependent mechanism [36]. Recent data demonstrate that CD20, when heterologously expressed in CHO (Chinese-hamster ovary) cells, significantly enhanced calcium entry...
across the plasma membrane [37]. In general, activation of store-operated channels can be achieved experimentally by reducing the calcium content in the lumen of intracellular stores. Inhibitors of SERCAs (sarcoplasmic/endoplasmic-reticulum Ca\(^{2+}\)-ATPases), such as thapsigargin or cyclopiazonic acid, induce passive release of calcium from intracellular stores by blocking calcium re-uptake into the ER. Treatment of B-cells with thapsigargin resulted in a sustained influx of calcium [37]. Thapsigargin did not increase transmembrane calcium conductivity when CD20 expression was down-modulated using siRNA (short interfering RNA), indicating that CD20 is responsible for calcium influx [37]. Furthermore, the sustained calcium influx downstream of the BCR was blocked by use of SKF96365, a known inhibitor of SOCCs. Further evidence supporting CD20 function in calcium entry came from studies with CD20-deficient mice [38]. Murine and human CD20 are well conserved in amino acid sequence (73%). The CD20-deficient mouse did not show an abnormal phenotype in B-cell development and maturation. The most notable observation in these CD20-deficient B-cells was a significant reduction in transmembrane calcium influx following CD19 or BCR ligation. Since receptor-induced calcium response was significantly affected by CD20 deficiency or chelation of extracellular calcium, the authors concluded that CD20 predominantly contributes to conductive calcium responses. The incomplete blockage of calcium transmembrane conductance in CD20-deficient cells is likely to be due to the expression of other MS4A family members which may contribute to transmembrane calcium transport in addition to CD20.

**Association of CD20 with lipid rafts**

Lipid rafts are specialized microdomains of the plasma membrane, which are highly enriched in sphingolipids intercalated by cholesterol. These microdomains have been proposed to provide a mechanism for lateral compartmentalization of the plasma membrane and to be implicated in the organization of numerous membrane-associated signalling pathways [39,40]. The tight packing of sphingolipids with a high degree of saturated fatty acids impart to these microdomains a liquid-ordered phase which is distinguishable from the rather loosely packed, liquid-disordered membrane [41,42]. Owing to their characteristic lipid composition, lipid rafts resist solubilization in non-ionic detergents (classically Triton X-100) at low temperature, and are isolated as DRMs (detergent-resistant membranes) floating on the top of sucrose gradients [43]. This biochemical approach has been extended with the use of new types of detergents, such as Lubrol WX, or Brij 35 or 98, which preserve the structure of rafts that are solubilized by Triton X-100. This allows the isolation of a subset of rafts with an increased population of proteins embedded compared with classical Triton-X-100-resistant rafts [44].

The densely packed environment of lipid rafts excludes most integral membrane proteins from rafts. Raft-associated proteins can be divided into two groups. First, proteins that are tightly anchored in rafts through covalently bound fatty acyl chains [e.g. GPI (glycosylphosphatidylinositol)-linked pro-
teins and dually acylated Src-family kinases] have been shown to be accommodated permanently in lipid rafts. A second class of proteins [e.g. BCR, TCR (T-cell receptor), CD20, CD19, CD40, CD24, FcεRI and PLCγ] associate with lipid rafts in a transient and activation-dependent manner [45–52]. Ligand binding or antibody cross-linking has been shown to trigger lipid raft association of these proteins, accrediting rafts a key role in the initiation and regulation of receptor-mediated signalling. It is attractive to speculate that the ability of certain proteins to associate with lipid rafts in a dynamic manner may effect and modulate proteins’ function [44]. For instance, after antigen presentation, BCR is translocated rapidly into rafts; however, its residence inside the micro-environment is only temporary, and the loss of BCR from rafts is complete within 15 min of stimulation [45]. The duration of BCR raft association can be modulated by the presence of co-receptors, such as CD19, whose raft association is also inducible [53,54]. Interestingly, BCR and CD20 were shown to co-localize on the cell surface of stimulated B-cells for a short period of time before they dissociated into different raft populations. CD20 remains surface-exposed, whereas the antigen-decorated BCR is internalized [55]. Binding of anti-CD20 antibodies, such as 2H7 or Rituxan, to CD20 induces a redistribution of CD20 molecules to lipid rafts, which resist Triton X-100 solubilization [47]. However, recent data provide evidence that, in the absence of cross-linking anti-CD20 antibody, CD20 molecules are constitutively associated with a low affinity to lipid rafts. This association is sensitive to solubilization by Triton X-100, but not by Brij 58 (Figure 2) [56]. Thus the binding of anti-CD20 antibodies increases the affinity of CD20 to lipid rafts as observed by a required insolubility of CD20 to Triton X-100. Furthermore, binding of univalent Fab fragments of 2H7 or Rituxan is sufficient to induce migration of CD20 into Triton-X-100-resistant rafts [56]. This observation suggests that cross-linking of two adjacent epitopes by anti-CD20 antibodies is not essential for clustering of CD20 molecules inside lipid rafts, but binding of the monovalent Fab fragment stabilizes a CD20 conformation, which has an intrinsic high affinity to lipid rafts. Pizzo and Viola [57] proposed a lipid raft model, in which a Triton-X-100-resistant raft nucleus is surrounded by lipid layers with gradually increased solubility towards Triton X-100. We assume that, after antibody binding, CD20 molecules preferentially migrate from the edge of a raft (which is resistant to Brij 58 solubilization) to a nucleus raft area (which is resistant to Triton X-100 solubilization), increasing the local concentration of CD20 molecules in a restricted membrane area (Figure 2). Alternatively, Rituxan binding to CD20 could result in the merger of smaller lipid rafts with larger ones. Thus rafts that coalesce may display a platform for new protein–protein interactions among proteins originally localized to different rafts. CD20 was shown to interact with known raft proteins, such as Src-family kinases and adaptor proteins, CD40, and G-coupled-protein subunits, indicating the formation of a raft-dependent functional complex [10,12,36,58,59]. However, the nature of a physiological intra- or extra-cellular stimulus, which increases CD20 raft association, remains to be identified.
Is recruitment of CD20 into lipid rafts crucial for CD20 activity?

Although antibody-induced CD20 lipid raft association is well documented, only a few studies are available investigating the role of lipid rafts in CD20 activity. Intriguing questions remain whether and why CD20 association with lipid rafts might be critical for CD20 function. Raft association might affect function of a protein in two possible ways: first, accumulation of proteins in rafts might facilitate interactions, and, secondly, the liquid-ordered environment of a raft might affect protein function by altering protein conformation. Polyak et al. [60] generated a truncated CD20 mutant in which seven residues in the C-terminal cytoplasmic sequence were removed (Δ219–225). Deletion of this sequence resulted in an inability of CD20 to associate with lipid rafts. This mutant was shown to be inefficient in mediating sustained calcium entry, demonstrating an involvement of lipid rafts in regulating CD20 channel activity [37]. This deletion did not influence oligomerization of CD20.

A valuable tool in investigating raft-associated signalling is the disruption of lipid raft integrity by reduction of cholesterol concentration in the plasma membrane. Cholesterol can be extracted from the plasma membrane by drugs such as methyl-β-cyclodextrin. This cyclic hydrophilic oligomer of glucose has been demonstrated to extract cholesterol from the plasma membrane without its entry into the membrane [61,62]. Therefore the effect of methyl-β-cyclodextrin differs from those of detergents that first incorporate themselves into membranes and then extract membrane components. Li et al. [37] observed that treatment of B-lymphocytes with methyl-β-cyclodextrin disturbs CD20 raft association, and results in a reduction of capacitative calcium entry after stimulation by cross-linking BCR or by depleting intracellular stores by thapsigargin. In line with these data, we monitored calcium influx in B-lymphocytes stimulated by hypercross-linked Rituxan. We observed in the presence of an increasing concentration of methyl-β-cyclodextrin, a dose-dependent reduction of sustained calcium influx, which correlated with a loss of association of CD20 with lipid rafts (E. Janas, R. Priest and R. Malhotra, unpublished work). Replenishing the pool of plasma membrane cholesterol with a methyl-β-cyclodextrin/cholesterol mixture re-established both calcium influx and association of CD20 with lipid rafts. These data provide evidence that both raft integrity and CD20 raft recruitment are required for CD20-mediated calcium conductivity.

A functional importance of raft association has also been shown for regulation of other channels. For instance, treatment of platelet cells with methyl-β-cyclodextrin inhibited calcium conductance in response to a number of agonists [63]. In these cells, methyl-β-cyclodextrin diminished calcium conductance mediated by TRPC1 (transient receptor potential canonical 1), a putative SOCC, implying a correlation between lipid raft recruitment of TRPC1 and its channel activity [64]. The integrity of lipid rafts is also required for Kv1.3 channel activity, since a change in the composition of rafts by cholesterol extraction was shown to inhibit channel function [65,66].
At present, it is not known how the specific environment of lipid rafts might support channel activity. It is conceivable that the integrity of rafts is required for correct assembly of the channel forming units or for regulation of CD20 activity by raft resident proteins. The targeting mechanism for proteins into lipid rafts is not completely understood. Phosphorylation of CD20 was shown not to be critical for CD20 raft association [56]. BCR raft association is also independent of phosphorylation, suggesting that translocation may rather be the result of alteration in conformation and/or oligomerization of BCR. The oligomer could assume a conformation in the membrane that, unlike the monomer, prefers the environment of liquid-ordered phase of lipid rafts. The formation of CD20 oligomeric complexes in the plasma membrane is regarded as a prerequisite for channel activity, and lipid rafts could provide a convenient environment to stabilize these complexes [8,47]. These conclusions are supported by studies with a unique anti-CD20 antibody (FMC7), which exclusively detects multimeric CD20 structures, which are strongly dependent on cholesterol presence in the plasma membrane. Disturbing raft integrity by methyl-β-cyclodextrin completely abolishes binding of FMC7 to CD20 [67]. Thus the oligomerization of CD20 seems to be a cholesterol-dependent process.

Cragg et al. [68] investigated homo-oligomerization of CD20 at the cell-surface by FRET (fluorescence resonance energy transfer). Binding of Rituxan to CD20 resulted in a high FRET signal, suggesting an antibody-induced oligomerization of CD20 molecules. However, no energy transfer was observed between CD20 molecules after treating the cells with another class of anti-CD20 antibodies, such as B1, demonstrating that binding of B1 did not induce a close proximity of CD20 molecules at the cell surface. In line with this observation, the authors showed by detergent-mediated raft isolation that B1 was inefficient in inducing CD20 raft localization [68]. Electrophysiological studies showed a very low calcium conductance even after 24 h incubation with the B1 antibody [8]. Both Rituxan and , although documented to mediate their therapeutic effect by B-cell depletion, obviously operate through a different mechanism. Treatment by Rituxan and B1 have different impacts on oligomerization of CD20 molecules, CD20 lipid raft association, induction of calcium influx, effects on apoptosis and complement activation [8,24,37]. We conclude that both antibodies stabilize a different conformation of CD20, and that binding of Rituxan to CD20 results in lipid raft association and subsequent calcium influx.

Critical evaluation of the functional role of lipid rafts in CD20 channel activity

At present, it is attractive to speculate that the specific environment of lipid rafts might support CD20 channel function. However, there are conflicting data on the functional role of lipid rafts for protein activity. For instance, Aman and Ravichandran [69], as well as Guo et al. [70], reported that BCR-mediated calcium flux is severely diminished after cholesterol extraction. In line with these observations, disturbance of raft formation by deletion of Raftlin, a raft protein, which was recently demonstrated to play a pivotal role in maintenance
of lipid rafts in B-lymphocytes, resulted in a reduced BCR-mediated calcium mobilization, implying a role for rafts in BCR activation [71]. However, Petrie et al. [72] observed an increase in BCR-induced intracellular calcium mobilization after lipid raft disruption by methyl-β-cyclodextrin, but described a reduced capacitative calcium influx. These observations allow two conclusions: first, lipid rafts are negatively regulating BCR-mediated calcium influx from intracellular stores, and, secondly (and more importantly for this review), the sustained calcium influx across the plasma membrane (partially dependent on CD20) is dependent on lipid raft integrity. Pizzo and Viola [57] re-evaluated studies with methyl-β-cyclodextrin which were used to demonstrate functional role of lipid rafts in T-cell-mediated calcium signalling. The authors observed that treatment with methyl-β-cyclodextrin not only disturbs lipid raft integrity (an effect which most authors tend to obtain using this drug), but also induces depolarization of the plasma membrane [57]. As calcium influx is sensitive to the membrane potential, fluctuations in electrical gradient across the plasma membrane might have considerable effects on calcium influx. In summary, studies using methyl-β-cyclodextrin must be tightly controlled, since depletion of cholesterol by methyl-β-cyclodextrin might cause an intervention to cell integrity. The strongest evidence for the involvement of rafts in protein function is provided when several approaches point to the same conclusion. Mutagenesis of proteins, which resulted in the loss of raft association without altering protein function, is a valuable tool in investigating the functional role of rafts for the protein in focus [60].

Conclusions

The plasma membrane appears to be structurally compartmentalized to allow separation or integration of signalling events. A variety of signalling molecules can associate either constitutively or inducibly to lipid rafts. In this review, we have outlined the functional role of lipid rafts in calcium signalling in B-lymphocytes and we have focused on studies of the B-cell-specific antigen CD20. CD20 is reported to exist in a dynamic association with Triton-X-100-resistant lipid rafts, and the antibody-induced relocalization of CD20 to these microdomains is suggested to be crucial for capacitative calcium entry in B-lymphocytes. Capacitative calcium entry was reduced either by cholesterol depletion or by deletion of a seven-residue cytoplasmic CD20 sequence that controls its localization to lipid rafts. Raft association of CD20 appears to provide a microenvironment for CD20 homo-oligomerization, as well as for protein–protein interaction with regulatory molecules that modulate CD20 activity.

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References


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