Spatiotemporal resolution of Ca\textsuperscript{2+} signaling events by real time imaging of single B cells

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\textbf{A B S T R A C T}

Antigen-induced B cell activation requires mobilization of the Ca\textsuperscript{2+} second messenger. This process is associated with the subcellular relocalization of signal effector proteins of the B cell antigen receptor such as the adaptor protein SLP65. Here we describe a broadly applicable live cell imaging method to simultaneously visualize intracellular Ca\textsuperscript{2+} flux profiles and the translocation of cytosolic signaling proteins to the plasma membrane in real time. Our approach delineated the kinetic hierarchy of Ca\textsuperscript{2+} signaling events in B cells and revealed a timely ordered contribution of various organelles to the overall Ca\textsuperscript{2+} signal. The developed experimental setup provides a useful tool to resolve the spatiotemporal signaling dynamics in various receptor signaling systems.

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\textbf{1. Introduction}

Ligation of the B cell antigen receptor (BCR) induces the mobilization of the second messenger Ca\textsuperscript{2+} that is indispensable for antibody-mediated immune responses [1,2]. The underlying mechanism of Ca\textsuperscript{2+} mobilization has been studied to a great detail by genetic and biochemical approaches that have led to the identification of the responsible BCR signal effector proteins. Based on mutational analyses it is generally accepted that the release of Ca\textsuperscript{2+} ions from the endoplasmic reticulum (ER) is initiated following the assembly and plasma membrane recruitment of a multi-protein complex comprising the SH2 domain-containing leukocyte adaptor protein of 65 kDa (SLP65, also called BLNK) [3,4], the Cbl-interacting protein of 85 kDa (CIN85) [5], Bruton’s tyrosine kinase (Btk) [6,7] and the phospholipase C-\gamma2 (PLC-\gamma2) [8]. Plasma membrane tethered and activated PLC-\gamma2, hydrolyzes membrane phospholipids yielding diacylglycerol and inositol-triphosphate. The latter product activates ligand-gated Ca\textsuperscript{2+} channels in the ER membrane causing the release of Ca\textsuperscript{2+} into the cytosol. Emptying the ER Ca\textsuperscript{2+} store is sensed by the ER membrane residents STIM1 and STIM2 that mediate the opening of Ca\textsuperscript{2+} channels in the plasma membrane [9]. Other cellular organelles such as mitochondria have been described to contribute to the Ca\textsuperscript{2+} response in antigen-stimulated T cells [10,11].

Biphasic Ca\textsuperscript{2+} fluxes can be easily recorded by flow cytometry upon loading of cells with Ca\textsuperscript{2+}-sensitive dyes such as Indol. This approach revealed that Ca\textsuperscript{2+} mobilization in B cells is not an all-or-nothing event but can be modulated in kinetic and quantitative terms depending on the developmental stage of the B cell [2,12]. However, flow cytometric measurements cannot detect Ca\textsuperscript{2+} flux dynamics in individual B cell subcompartments which act in concert to shape the overall Ca\textsuperscript{2+} response profile. Furthermore, it has been a puzzling observation that intracellular Ca\textsuperscript{2+} mobilization already declines before the maximum amount of the SLP65-assembled Ca\textsuperscript{2+} initiation complex becomes recruited to the plasma membrane [2,5]. Recent advances in live cell imaging techniques may offer a solution to this problem, but such approaches are often limited by the temporal and spatial resolution of the microscope. We have now overcome the technical constrains of imaging Ca\textsuperscript{2+}-related signals in single cells by combining fast time-lapsed confocal microscopy with an algorithm to unmix superimposed fluorescence signals. Our real time imaging approach directly visualized the molecular hierarchy of BCR-induced Ca\textsuperscript{2+} signaling events and moreover allowed for the characterization of
individual Ca²⁺ profiles in different subcompartments of the live B cell.

2. Materials and methods

2.1. B cell culture conditions and flow cytometric Ca²⁺ flux measurements

Ramos and DT40 B cells were cultured, stimulated and transfected with expression vectors encoding fluorescently labeled SLP65, pCFP-Golgi or pDsRed-Mito (Clontech) as described [13–15]. For flow cytometric recording of BCR-induced Ca²⁺ flux 10⁶ B cells were loaded with either 1.5 μM Fluo4-AM or 1.5 μM Rhod-2 or 1 μM Indo1 (Molecular Probes) in 700 μl RPMI containing 5% FCS and 0.015% Pluronic F127 (Molecular Probes) at 30 °C for 25 min and subjected to flow cytometric analysis as described [13,14]. For microscopic imaging of B cell stimulation, Ramos or DT40 B cells were settled onto the bottom of a Petri dish and stimulated with goat anti-human IgM (10 μg/ml, Jackson ImmunoResearch) or mouse anti-chicken IgM (2 μg/ml, clone M4, Southern Biotechnologies), respectively. To visualize mitochondria or the Golgi, B cells were transfected with vectors encoding fusion proteins between either CFP and the N-terminal 81 amino acids of human beta 1,4-galactosyl transferase, or DsRed and the mitochondrial targeting sequence from subunit VIII of human cytochrome C oxidase (Clontech), respectively.

Fig. 1. Plasma membrane recruitment of SLP65 precedes Ca²⁺ mobilization. (A) Ramos B cells expressing a citrine-tagged variant of SLP65 were loaded with Rhod-2 and subjected to time-lapsed CLSM to simultaneously visualize BCR-induced Ca²⁺ mobilization (top row) and the subcellular trafficking of SLP65 (middle row) for the indicated time points prior and after BCR ligation. The separation of plasma membrane-derived and cytosolic citrine signals is shown in the bottom row for the zoomed region in the left center image. Image segmentation was carried out automatically for individual frames. Magnification 40×/NA 1.2, frame rate 0.5–1 Hz. For time-lapsed video see supplementary Fig. S1. (B) Time traces of the Rhod-2 signals were averaged for each frame (upper panel, blue line) and are displayed as F/F₀ (F₀ being the time-averaged signal from the first six, pre-stimulation frames). Recalculation of the data according to the fitted functions are shown by the red solid line yielding the onset of Ca²⁺ mobilization (t(onset) depicted as broken vertical line. The subcellular citrine signals that were distinguished in (A) as being either plasma membrane-derived or cytosolic signals were spatially averaged and plotted as blue or magenta line, respectively (F/F₀ middle panel). The ratio of the subcellularly distinct citrine signals (membrane to cytosolic signal) was plotted (lower panel, blue trace) and recalculated according to the fitted functions algorithm (red curve) to determine the onset of SLP65 membrane recruitment t(onset) (dotted vertical line indicates true fit value). Filled circles indicate time points shown in (A). (C) The time difference between onset of the Ca²⁺-signal and onset of the citrine-relocalization was calculated for 38 cells (one value per cell) and plotted as a histogram (bin width 4 s). Δt = 0 and the mean (Δt) = 7.76 s are indicated by the red and green lines, respectively.
2.2. Confocal microscopy and data processing

Imaging was performed on a fast custom-built line-illumination confocal microscope [16] (objective Achromat 100×/NA 1.0 W), except for two-color confocal imaging (Figs. 1 and 3B and C) performed on an Axiovert 100 M/LSM 510 (Zeiss, 63×/NA 1.4 and 40×/NA 1.2). Thickness of optical sections was approximately 1.5 μm for all experiments. All data analyses were performed using custom software written in MATLAB (The MathWorks). For the calculation of cross-correlation values between color channels, color channels had first to be spectrally unmixed in some cases (CFP/Fluo4). This was accomplished using reference data with only one dye present in the imaged cells. The correlation coefficients were calculated after subtle filtering (Gaussian filter, width 1 pixel in x, y, z) across pixels for a number of manually selected cells. Automated image segmentation involved smoothing of data (gaussian blur, width: 0.8 pixels in x/y; 2 points in t) followed by creating a threshold mask using histogram-based automatic thresholding [17]. The cytosolic mask was obtained by eroding the threshold mask by 4 pixels. The membrane mask was defined as the difference of the threshold mask eroded by 1 pixel and the threshold mask eroded by 3 pixels. The segmentation was calculated for each time point individually. Time traces where obtained by averaging the fluorescence signals in the cytosolic and membrane regions for each time point. Fitting of a piecewise defined function was carried out as described [18]. Unmixing of fluorescence time courses was adopted from [19] by replacing the wavelength dimension with the time dimension from our time lapse imaging data. The time courses obtained by the NMF algorithm were further refined using the interactive software tool provided by the authors of the original publication [19] in order to minimize the similarity in the temporal structure of the Ca2+-profiles.

3. Results

3.1. BCR-induced membrane translocation of SLP65 precedes Ca2+ release from the ER

Based on genetic evidence tyrosine phosphorylation and plasma membrane recruitment of the SLP65 adaptor protein are upstream regulatory events for the BCR-induced Ca2+ mobilization. However, the release of Ca2+ ions from the ER can be measured by flow cytometry much earlier than the phosphorylation or the subcellular relocalization of SLP65 monitored by immunoblotting or microscopic imaging techniques, respectively [2,5]. To solve this apparent paradox we set out to simultaneously visualize and quantify the processes of SLP65 targeting and intracellular Ca2+ mobilization. Therefore Ramos B cells expressing a citrine-tagged variant of SLP65 were loaded with the Ca2+-sensitive dye Rhod-2. The fluorescence signals of Rhod-2 and citrine were imaged in the live cell by confocal laser scanning microscopy (CLSM) prior to and after BCR engagement (Fig. 1A, upper and middle row, respectively and supplementary material S1). To determine the time point of SLP65 relocalization we distinguished between cytosolic- and plasma membrane-derived signals (Fig. 1A, lower row, cyan or magenta, respectively). Next we plotted the mean fluorescence intensities of the Rhod-2 and citrine signals in the live cell by confocal laser scanning microscopy (CLSM) prior to and after BCR engagement (Fig. 1A, upper and middle row, respectively and supplementary material S1). To determine the time point of SLP65 relocalization we distinguished between cytosolic- and plasma membrane-derived signals (Fig. 1A, lower row, cyan or magenta, respectively). Next we plotted the mean fluorescence intensities of the Rhod-2 and citrine signals derived from membrane-tethered and cytosolic SLP65.
determine the initiation time point of SLP65 relocalization (Fig. 1B, lower panel). The time difference between the onset of Ca\(^{2+}\) flux and the initiation of SLP65 recruitment (\(\Delta t = t_{\text{Ca}^{2+}} - t_{\text{SLP65}}\)) provided a parameter to delineate the kinetic hierarchy of these signaling events because if \(\Delta t > 0\) membrane recruitment of SLP65 precedes Ca\(^{2+}\) mobilization. Indeed and as shown in Fig. 1C, 29 out of 38 B cells showed \(\Delta t < 0\) with a mean value of \(\Delta t = 7.8\) s. In conclusion, our approach to combine simultaneous imaging of two distinct signaling events in the live cell with mathematical recalculation of the data according to the fitted functions algorithm offers a useful and general tool for the temporal resolution of signaling processes in eukaryotic cells.

### 3.2. Intracellular compartmentalization of Ca\(^{2+}\) mobilization

Having visualized the temporal order of membrane-associated vs. cytosolic Ca\(^{2+}\) signaling events we set out to more precisely characterize the intracellular compartments that participate in Ca\(^{2+}\) mobilization at a given time point. We used Ramos as well as DT40 B cells representing mature and immature B cell stages, respectively. Consistent with previously published results [2,12] the two cell lines responded to BCR ligation with developmental stage-specific Ca\(^{2+}\) flux profiles recorded by flow cytometry (Fig. 2A). Direct monitoring of these responses by time-lapsed CLSM showed that the Ca\(^{2+}\) ions were not equally distributed in-
of the cells but became compartmentalized with a pronounced subcellular dynamics (Fig. 2B and C, images in upper rows). Monitoring the time traces of signals from individually selected regions revealed that the spatiotemporal pattern of the subcellular Ca\(^{2+}\) modulations was similar in Ramos and DT40 B cells (Fig. 2B and C, lower diagrams, respectively). However, the different
4. Discussion

In this study we have visualized early BCR signaling events and the subcellular compartmentalization of Ca\(^{2+}\) mobilization. For the first time we simultaneously imaged the BCR-triggered processes of SLP65 membrane recruitment and Ca\(^{2+}\) mobilization in real time. Our results demonstrate that translocation of SLP65 from the cytosol to the plasma membrane precedes the release of Ca\(^{2+}\) from the ER. This is consistent with genetic and biochemical data previously suggesting that subcellular relocalization of SLP65 in conjunction with its binding partners CIN85, Btk and PLC-\(\gamma\)2 [3–7] is an upstream regulatory event in the Ca\(^{2+}\) signaling pathway. Together with the observation that membrane translocation of the SLP65-assembled complex continues even after Ca\(^{2+}\) fluxing [2,5] our data also show that recruiting minute amounts of the cellular SLP65 pool to the plasma membrane suffices to pass the Ca\(^{2+}\) release threshold. By using three-dimensional time-lapsed CLSM we next showed that the Ca\(^{2+}\) messenger is not equally distributed inside of the B cell but becomes compartmentalized in immature as well as in mature B cells with a high subcellular dynamics that involves various organelles in a discrete time order. The improved spatiotemporal resolution of individual intracellular Ca\(^{2+}\) flux patterns was greatly improved by unmixing of individual fluorescence signals [19]. By localizing individual organelles we generated a multi-label representation (Fig. 4D and H) which was independent on the selected regions of interest, but was solely determined by the specificity of the temporal profiles of the pixels. The Ca\(^{2+}\) profiles of the respective organelles were “spatially cleaned”. Collectively our approach allowed us to determine the kinetic with which the various organelles become engaged in the Ca\(^{2+}\) response. The obtained map of organelle-specific Ca\(^{2+}\) fluxes revealed that following the initial rise of Ca\(^{2+}\) in the cytosol, the decline of cytosolic Ca\(^{2+}\) signals directly correlates with the increase of Ca\(^{2+}\) in the mitochondrion. This result suggests that similar to activated T cells [10,11] but unlike neurons [20] mitochondria potently buffer cytosolic Ca\(^{2+}\) concentrations in B cells, and hence limit the time frame for the initiation of Ca\(^{2+}\)-dependent signaling processes in the cytosol. We also uncovered a marked concentration of Ca\(^{2+}\) in the Golgi that, however, remained almost unaffected by BCR stimulation. This subcellular Ca\(^{2+}\) pool may play a role in late B cell responses that involve vesicle trafficking between the plasma membrane and the Golgi. Collectively, our approaches of processing high-resolution imaging data of early BCR signaling events with mathematical algorithms delineated the spatiotemporal dynamics of Ca\(^{2+}\) mobilization in B cells. Our method is applicable to other cellular signaling systems and together with the development of novel Ca\(^{2+}\) sensing molecules [21] provides a useful tool to overcome the limited spatial resolution of a microscope without affecting the temporal resolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.03.057.

References

[1] Baba, Y. and Kurosaki, T. (2011) Impact of Ca(2+) signaling on B cell function. Trends Immunol. 32, 589–594.
[2] Engelke, M., Engels, N., Dittmann, K., Stork, B. and Wienands, J. (2007) Ca(2+) signaling in antigen receptor-activated B lymphocytes. Immunol. Rev. 218, 235–246.
[3] Fu, C., Turck, C.W., Kurosaki, T. and Chan, A.C. (1998) BLNK: a central linker protein in B cell activation. Immunity 9, 93–103.

[4] Wienands, J., Schweikert, J., Wolfscheid, B., Jumaa, H., Nielsen, P.J. and Reth, M. (1998) SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. J. Exp. Med. 188, 791–795.

[5] Oelerich, T. et al. (2011) The B-cell antigen receptor signals through a preformed transducer module of SLP65 and CIN85. EMBO J. 30, 3620–3634.

[6] Hashimoto, S. et al. (1999) Identification of the SH2 domain binding protein of Bruton’s tyrosine kinase as BLNK–functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. Blood 94, 2357–2364.

[7] Su, Y.W., Zhang, Y., Schweikert, J., Koretzky, G.A., Reth, M. and Wienands, J. (1999) Interaction of SLP adaptors with the SH2 domain of Tec family kinases. Eur. J. Immunol. 29, 3702–3711.

[8] Baba, Y. and Kurosaki, T. (2009) Physiological function and molecular basis of STIM1-mediated calcium entry in immune cells. Immunol. Rev. 231, 174–188.

[9] Schwindling, C., Quintana, A. et al. (2011) Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. EMBO J. 30, 3895–3912.

[10] Hoek, K.L. et al. (2006) Transitional B cell fate is associated with developmental stage-specific regulation of diacylglycerol and calcium signaling upon B cell receptor engagement. J. Immunol. 177, 5405–5413.

[11] Engels, N., Konig, L.M., Heemann, C., Lutz, J., Tsubata, T., Griep, S., Schrader, V. and Wienands, J. (2009) Recruitment of the cytoplasmic adaptor Grb2 to surface IgG and IgM provides antigen receptor-intrinsic costimulation to class-switched B cells. Nat. Immunol. 10, 1018–1025.

[12] Stork, B., Engelke, M., Frey, J., Horejsi, V., Hamm-Baarke, A., Schraven, B., Kurosaki, T. and Wienands, J. (2004) Grb2 and the non-T cell activation linker NTAL constitute a Ca(2+)-regulating signal circuit in B lymphocytes. Immunol. 21, 681–691.

[13] Stork, B., Neumann, K., Goldbeck, I., Alers, S., Kahne, T., Naumann, M., Engelke, M. and Wienands, J. (2008) Subcellular localization of Grb2 by the adaptor protein Dok-3 restricts the intensity of Ca(2+) signaling in B cells. EMBO J. 26, 1140–1149.

[14] Junek, S., Chen, T.W., Alevra, M. and Schild, D. (2009) Activity correlation imaging: visualizing function and structure of neuronal populations. Biophys. J. 96, 3801–3809.

[15] Zack, G.W., Rogers, W.E. and Latt, S.A. (1977) Automatic measurement of sister chromatid exchange frequency. J. Histochem. Cytochem. 25, 741–753.

[16] Junek, S., Klett, E., Wolf, F. and Schild, D. (2011) Olfactory coding with patterns of response latencies. Neuron 67, 872–884.

[17] Neher, R.A., Mitkovski, M., Kirchhoff, F., Neher, E., Theis, F.J. and Zeug, A. (2009) Blind source separation techniques for the decomposition of multiply labeled fluorescence images. Biophys. J. 96, 3791–3800.

[18] Kim, M.S. and Usachev, Y.M. (2009) Mitochondrial Ca(2+) cycling facilitates activation of the transcription factor NFAT in sensory neurons. J. Neurosci. 29, 12101–12114.

[19] Zhao, Y. et al. (2011) An expanded palette of genetically encoded Ca(2+) indicators. Science 333, 1888–1891.