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

Stephan Junek^{a,1,2}, Michael Engelke^{b,2}, Detlev Schild^a, Jürgen Wienands^{b,*}

^a Department of Neurophysiology and Biophysics, Georg-August-University of Göttingen, Humboldtallee 23, 37073 Göttingen, Germany

^b Department of Cellular and Molecular Immunology, Georg-August-University of Göttingen, Humboldtallee 34, 37073 Göttingen, Germany

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ABSTRACT

Antigen-induced B cell activation requires mobilization of the Ca^{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^{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^{2+} signaling events in B cells and revealed a timely ordered contribution of various organelles to the overall Ca^{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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1. Introduction

Ligation of the B cell antigen receptor (BCR) induces the mobilization of the second messenger Ca^{2+} that is indispensable for antibody-mediated immune responses [1,2]. The underlying mechanism of Ca^{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^{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- γ 2 (PLC- γ 2) [8]. Plasma membrane tethered and activated PLC- γ 2, hydrolyzes membrane phospholipids yielding diacylglycerol and inositol-trisphosphate. The latter product activates ligand-gated Ca^{2+} channels in the ER mem-

brane causing the release of Ca^{2+} into the cytosol. Emptying the ER Ca^{2+} store is sensed by the ER membrane residents STIM1 and STIM2 that mediate the opening of Ca^{2+} channels in the plasma membrane [9]. Other cellular organelles such as mitochondria have been described to contribute to the Ca^{2+} response in antigen-stimulated T cells [10,11].

Biphasic Ca^{2+} fluxes can be easily recorded by flow cytometry upon loading of cells with Ca^{2+} -sensitive dyes such as Indo1. This approach revealed that Ca^{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^{2+} flux dynamics in individual B cell subcompartments which act in concert to shape the overall Ca^{2+} response profile. Furthermore, it has been a puzzling observation that intracellular Ca^{2+} mobilization already declines before the maximum amount of the SLP65-assembled Ca^{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 constraints of imaging Ca^{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^{2+} signaling events and moreover allowed for the characterization of

Abbreviations: Btk, Bruton's tyrosine kinase; CIN85, Cbl-interacting protein of 85 kDa; ER, endoplasmic reticulum; PLC- γ 2, phospholipase C- γ 2; SLP65, SH2 domain-containing leukocyte protein of 65 kDa

* Corresponding author.

E-mail addresses: jwienan@uni-goettingen.de, jwienan@gwdg.de (J. Wienands).

¹ Current address: Neural Systems and Coding Group, Max-Planck-Institute for Brain Research, Deutschordenstraße 46, 60528 Frankfurt am Main, Germany.

² Equally contributing author.

Fig. 1. Plasma membrane recruitment of SLP65 precedes Ca^{2+} 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 Ca^{2+} 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 $40\times/\text{NA } 1.2$, frame rate $0.5\text{--}1\text{ Hz}$. 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/F_0 (F_0 being the time-averaged signal from the first six, pre-stimulation frames). Recalculation of the data according to the *fitted functions* algorithm is shown by the red solid line yielding the onset of Ca^{2+} mobilization 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/F_0 , 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 (dotted vertical line indicates true fit value). Filled circles indicate time points shown in (A). (C) The time difference between onset of the Ca^{2+} -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). $\Delta t = 0$ and the mean $\langle \Delta t \rangle = 7.76\text{ s}$ are indicated by the red and green lines, respectively.

individual Ca^{2+} profiles in different subcompartments of the live B cell.

2. Materials and methods

2.1. B cell culture conditions and flow cytometric Ca^{2+} 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 Ca^{2+} flux 10^6 B cells were loaded with either $1.5\text{ }\mu\text{M}$ Fluo4-AM or $1.5\text{ }\mu\text{M}$ Rhod-2 or

$1\text{ }\mu\text{M}$ Indo1 (Molecular Probes) in $700\text{ }\mu\text{l}$ RPMI containing 5% FCS and 0.015% Pluronic F127 (Molecular Probes) at $30\text{ }^\circ\text{C}$ 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 ($10\text{ }\mu\text{g/ml}$, Jackson ImmunoResearch) or mouse anti-chicken IgM ($2\text{ }\mu\text{g/ml}$; 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. 2. The spatiotemporal modulations of subcellular Ca^{2+} fluxes are similar in B cells representing different developmental stages. (A) Ramos and DT40 B cells were loaded with Indo1 and Ca^{2+} mobilization was monitored by flow cytometry (red and green lines, respectively). Temporal resolution 1 Hz. BCR-induced Ca^{2+} mobilization of Fluo4-labeled Ramos B cells (B) and DT40 B cells (C) was visualized at the single cell level by CLSM for the indicated time points (upper images). Magnification $100\times/\text{NA } 1.0, 0.9 \text{ Hz}$. Different subcellular regions showed distinct temporal fluctuations. Fluo4 signals of manually selected regions (colored lines) are shown in the lower diagrams. Gray vertical lines represent time points shown in the upper images.

2.2. Confocal microscopy and data processing

Imaging was performed on a fast custom-built line-illumination confocal microscope [16] (objective Achromplan $100\times/\text{NA } 1.0 \text{ W}$), except for two-color confocal imaging (Figs. 1 and 3B and C) performed on an Axiovert 100 M/LSM 510 (Zeiss, $63\times/\text{NA } 1.4$ and $40\times/\text{NA } 1.2$). Thickness of optical sections was approximately $1.5 \mu\text{m}$ 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 were 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 Ca^{2+} -profiles.

3. Results

3.1. BCR-induced membrane translocation of SLP65 precedes Ca^{2+} 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 Ca^{2+} mobilization. However, the release of Ca^{2+} 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 dissolve this apparent paradox we set out to simultaneously visualize and quantify the processes of SLP65 targeting and intracellular Ca^{2+} mobilization. Therefore Ramos B cells expressing a citrine-tagged variant of SLP65 were loaded with the Ca^{2+} -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 signals and fitted a function consisting of a linear baseline and a double-exponential function ([18]; Fig. 1B, upper panel). This fit allowed to determine the onset of Ca^{2+} flux marked by the transition from the linear to the double-exponential function (indicated in Fig. 1B by a vertical broken line). Likewise, the two citrine signals derived from membrane-tethered and cytosolic SLP65 were individually plotted (Fig. 1B, middle panel) and a corresponding function was fitted to the pairwise ratios of these signals to

Fig. 3. Distinct contributions of B cell organelles to BCR-induced Ca^{2+} mobilization. (A) Fluo4-labeled DT40 B cells were subjected to time-lapsed CLSM (12 image planes, stack rate 0.9 Hz) and z-stack images were recorded in single cells in the absence of BCR stimulation (A₁) or following BCR ligation for the indicated time points (A₂ and A₃; 9 out of 12 imaged planes are shown). Numbers indicate the distances of the individual images along the optical axis. White and black arrow heads indicate locations of high and low Ca^{2+} levels before stimulation, respectively. For time-lapsed video see [supplementary Fig. S2](#). Magnification 63 $\times$, NA 1.4; acquisition of image stacks before and 90 s after stimulation; stack acquisition time 8 s. (B and C) DT40 B cells expressing either the CFP-tagged Golgi marker or the DsRed-tagged mitochondrial marker (see [Section 2](#) for details) were loaded with Fluo4 and subjected to two-color time-lapsed CLSM in the absence or presence of BCR ligation. Left and middle panels represent signals of the organelle marker and the Ca^{2+} indicator, respectively, before and 90 s after stimulation (upper left and lower right halves, respectively). Right panel represents overlays. Correlation coefficient 0.72 ± 0.09 vs. 0.04 ± 0.12 90 s after stimulation, measured in six cells. Note the colocalization of Ca^{2+} signals with either the CFP-tagged Golgi marker in activated B cells (B₃) or the DsRed-tagged mitochondrial marker in resting B cells (C₃).

determine the initiation time point of SLP65 relocation (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 $\Delta t \geq 0$ membrane recruitment of SLP65 precedes Ca^{2+} mobilization. Indeed and as shown in Fig. 1C, 29 out of 38 B cells showed $\Delta t \geq 0$ 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-

Fig. 4. Organelle-specific Ca^{2+} signals as revealed by temporal unmixing of individual fluorescence signals over time. BCR-induced Ca^{2+} mobilization of Fluo4-labeled DT40 (A–D) or Ramos B cells (E–H) was analyzed by time-lapsed 3D CLSM. Shown are the results for one single optical section. See [supplementary movie 3](#) for a 3D rendered visualization of the unmixing results from the complete image stack. Twelve image planes, 0.9 Hz stack rate; 100 $\times$, NA 1.0. (A and E) Time traces were obtained from manually selected regions of interest (indicated in B and F). (B and F) Maps of coefficients after linear decomposition of the fluorescent data using the reference traces shown in A and E (same color code). B4 shows the overlay of the three components. (C, D, G, H) Time courses and spatial coefficient maps as a result of time course-optimized unmixing that is independent of the selected regions of interest and that eliminates the “spatial cross-talk” between the time traces.

side 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

developmental B cell stages may account for a distinct subcellular distribution of the signals (see red region).

Next, we imaged single B cells by time-lapsed CLSM in three dimensions (see Fig. 3A and [supplementary material S2](#)). This analysis revealed small and locally restricted areas of high Ca^{2+} concentrations already in resting B cells (Fig. 3A, left images). Furthermore, the early phase of Ca^{2+} mobilization upon BCR ligation affects the central region of the cell encompassing cytosolic areas and the nucleus (Fig. 3A, middle images). Discrete punctuate structures appeared in the peripheral region of the cell approximately 60 s after BCR engagement (Fig. 3A, right images). To identify the compartments of high Ca^{2+} concentrations we used DT40 B cell transfectants expressing either a CFP fusion protein encompassing a Golgi-specific marker peptide, or a DsRed fusion protein encompassing a mitochondrial marker peptide. Cells were loaded with Fluo4 and analyzed by two-color CLSM. Fig. 3B shows that the regions of high Ca^{2+} concentrations in resting B cells colocalized with the CFP signal of the Golgi marker. The punctuate structures of high Ca^{2+} concentrations in the late phase of the response colocalized with the mitochondrial marker (Fig. 3C). Collectively, our single cell analyses of subcellular Ca^{2+} dynamics in resting and activated B cells suggested complex patterns of second messenger generation that involves various organelles in a discrete time order.

3.3. Monitoring of organelle-specific Ca^{2+} flux profiles by “temporal unmixing”

The limited resolution of a light microscope can cause a significant overlap of the fluorescent signals in a given pixel. For example, a pixel located on a mitochondrion might also collect fluorescence signal from the surrounding cytosol (Fig. 4A and B). This phenomenon prevents an exact elucidation of the kinetics with which the various organelles become engaged in the Ca^{2+} response. We attempted to recover the signals of the individual organelles using an approach that was originally developed to separate the fluorescence signals of dyes with unknown spectra [19]. Using time series of image stacks we replaced the spectral dimension in the original approach with the parameter of time, and were thus able to eliminate the cross-contaminations of the various signals (Fig. 4C). This also resulted in a more accurate map of Ca^{2+} -mobilizing organelles during the time course of DT40 B cell activation which does not depend on the selection of regions of interest (Fig. 4D and [supplementary movie S3](#)). Likewise, the spatiotemporal Ca^{2+} flux profiles were monitored in Ramos B cells (Fig. 4E–H). In both types of B cells the initiation of Ca^{2+} mobilization was confined to the central region of the cell before the signal gradually declined. At the same time the Ca^{2+} concentration in the mitochondria markedly increased. In contrast, Golgi-related signals appeared to be independent of BCR ligation. Collectively, our approach to combine fast time-lapsed CLSM with an algorithm to unmix distinct fluorescence spectra [19] provides a useful tool to overcome the technical limitations of microscopic imaging the kinetic and to enable the precise subcellular localization of Ca^{2+} mobilization in the live B cell. The obtained organelle-specific Ca^{2+} flux profiles uncovered the distinct and timely ordered contributions of the various subcellular compartments to the overall Ca^{2+} signal.

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 relocation of SLP65 in conjunction with its binding partners CIN85, Btk and PLC- γ 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 mitochondria. This result suggests that similar to activated T cells [10,11] but unlike neurons [20] mitochondria potentially 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>.

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