Chloroquine inhibits Ca$^{2+}$ permeable ion channels-mediated Ca$^{2+}$ signaling in primary B lymphocytes

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Abstract

Background: Chloroquine, a bitter tastant, inhibits Ca$^{2+}$ signaling, resulting in suppression of B cell activation; however, the inhibitory mechanism remains unclear.

Results: In this study, thapsigargin (TG), but not caffeine, induced sustained intracellular Ca$^{2+}$ increases in mouse splenic primary B lymphocytes, which were markedly inhibited by chloroquine. Under Ca$^{2+}$-free conditions, TG elicited transient Ca$^{2+}$ increases, which additionally elevated upon the restoration of 2 mM Ca$^{2+}$. The former were from release of intracellular Ca$^{2+}$ store and the latter from Ca$^{2+}$ influx. TG-induced release was inhibited by 2-APB (an inhibitor of inositol-3-phosphate receptors, IP$_3$Rs) and chloroquine, and TG-caused influx was inhibited by pyrazole (Pyr3, an inhibitor of transient receptor potential C3 (TRPC3) and stromal interaction molecule (STIM)/Orai channels) and chloroquine. Moreover, chloroquine also blocked Ca$^{2+}$ increases induced by the engagement of B cell receptor (BCR) with anti-IgM.

Conclusions: These results indicate that chloroquine inhibits Ca$^{2+}$ elevations in splenic B cells through inhibiting Ca$^{2+}$ permeable IP$_3$R and TRPC3 and/or STIM/Orai channels. These findings suggest that chloroquine would be a potent immunosuppressant.

Keywords: B cells, Ca$^{2+}$, Chloroquine, IP$_3$R, TRPC3 channels, STIM/Orai channels

Background

Chloroquine is a bitter tastant [1–4], which was used to treat malaria [5] and immune-related diseases such as rheumatic disease, systemic lupus erythematosus [6], early-stage AIDS [7] and chronic graft-versus-host disease [8]. Moreover, it inhibits Ia molecule biosynthesis [9] and CpG DNA-induced protection [10] in B cells. These results imply that chloroquine might be an immunosuppressant of B cell activation. Cytosolic Ca$^{2+}$ increases play an important role in B cell development [9], survival [11], activation [12], and differentiation [13, 14], cytokine production [12] and cell death [15]. Ca$^{2+}$ increases in B cells are induced by antigen or anti-B cell receptor (BCR) ligation. BCR antibodies bind to the BCR, resulting in the phosphorylation of tyrosine in phospholinoside-specific phospholipase C (PLC). The phosphorylated PLC catalyzes phosphatidylinositol-4, 5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP$_3$). IP$_3$ binds to IP$_3$Rs located on the surface of the endoplasmic reticulum (ER). The activated IP$_3$Rs then mediate Ca$^{2+}$ release from the ER, leading to increases in intracellular Ca$^{2+}$ [15, 16]. In addition, Ca$^{2+}$ increases can also be induced by thapsigargin (TG) [17]. However, whether and how bitter tastant chloroquine inhibits Ca$^{2+}$ increases in B cells remains unclear.

In this study, we found that chloroquine inhibited Ca$^{2+}$ increases induced by TG and BCR engagement with anti-IgM through inhibiting Ca$^{2+}$ permeable ion channels.

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Results
Chloroquine decreases TG-induced increases of intracellular Ca^{2+}

In this study, we sought to investigate the effect of chloroquine on intracellular Ca^{2+} in primary B lymphocytes from mouse spleens. As shown in Fig. 1a and b, the increases of Ca^{2+} were induced by TG, an inhibitor of the ER Ca^{2+} ATPase, which were inhibited by chloroquine. The dose-relationship of inhibition is shown in Fig. 1c. The IC_{50} was 9.3 ± 0.7 mM (Fig. 1c). These results indicate that chloroquine inhibits TG-induced elevations of cytosolic Ca^{2+}.

Mechanism of chloroquine-caused inhibition on TG-induced Ca^{2+} increases

To study whether chloroquine inhibits extracellular Ca^{2+} entry, we performed the following experiments. Intracellular Ca^{2+} store was first depleted by TG under Ca^{2+}-free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), which resulted in transient increases of Ca^{2+}. Ca^{2+} (2 mM) was then restored in the extracellular solutions, which induced additional increases and were markedly blocked by chloroquine (Fig. 2a). The Ca^{2+} levels in 155 cells were summarized (Fig. 2b). These data indicate that chloroquine inhibits Ca^{2+} influx.

We next investigated whether chloroquine inhibits TG-induced intracellular Ca^{2+} release. Under Ca^{2+}-free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), the incubation of chloroquine abolished TG-induced transient Ca^{2+} increases and the restoration of 2 mM Ca^{2+}-caused additional elevations (Fig. 3a). This phenomenon was observed in 241 cells. These results indicate that chloroquine blocks Ca^{2+} release from the ER.

Next, we investigate the role of RyRs, since which mediate Ca^{2+} release from the ER [21]. Cells were stimulated with caffeine, a selective activator of RyRs, which failed to increase Ca^{2+} (Fig. 4). These results indicate that these cells have no functional RyRs, suggesting that RyRs do not contribute to TG-induced Ca^{2+} release.

Chloroquine inhibits Ca^{2+} influx as shown in Fig. 2, we then studied the underlying mechanism. In previous studies, it has been found that chloroquine blocked TRPC3 and/or STIM/Orai channels, resulting in decreases of Ca^{2+} in airway smooth muscle cells [3, 18] and in murine CD4+ thymocytes [4]. As shown in Fig. 5, TG-induced increases were declined by Pyr3, an inhibitor of TRPC3 and/or STIM/Orai channels, suggesting that chloroquine inhibits TRPC3 and/or STIM/Orai channels-mediated Ca^{2+} influx.

Chloroquine inhibits BCR engagement-induced Ca^{2+} elevations

We finally observed the effect of chloroquine on BCR engagement-induced Ca^{2+} increases. Our previous
results indicate that the engagement of BCR with anti-IgM induces Ca\textsuperscript{2+} elevations in B cells [16]. As shown in Fig. 6, anti-IgM induced Ca\textsuperscript{2+} increases. However, such increases were potently inhibited by the incubation of chloroquine, although that the statistical results show that anti-IgM still induced a significant elevation. These results indicate that chloroquine inhibits BCR engagement-induced Ca\textsuperscript{2+} elevations.

**Discussion**

In the present study, our results indicate that chloroquine depresses Ca\textsuperscript{2+} increases induced by TG and anti-IgM via inhibiting the intracellular Ca\textsuperscript{2+} release mediated by IP\textsubscript{3}R channels and inhibiting the extracellular Ca\textsuperscript{2+} influx mediated by TRPC3 and/or STIM/Orai channels.

The aim of this study was to investigate whether chloroquine inhibits increases of Ca\textsuperscript{2+} and the underlying
mechanism. We found that chloroquine inhibited increases of Ca^{2+} induced by TG (Fig. 1). To define the inhibitory mechanism, we investigated the pathways that mediated TG-induced Ca^{2+} elevations. Intracellular Ca^{2+} increases will be mediated by Ca^{2+} permeation channels on the plasma and the ER membrane [22]. These have been demonstrated by the results that under Ca^{2+}-free conditions, TG induced transient increases and followed by additional elevations upon the restoration of 2 mM Ca^{2+} (Fig. 2), since the former resulted from release and the latter from influx. Moreover, the release was mediated by IP_{3}R in the ER membrane (Fig. 3a, b), because that 2-APB blocked release-induced Ca^{2+} elevations. IP_{3}R3s have three isoforms (IP_{3}R1, IP_{3}R2, and IP_{3}R3), which can be inhibited by 2-APB [20]. While, the influx was mediated by TRPC3 and/or STIM/Orai channels in the plasma membrane. Because that Pyr3 (Fig. 5) blocked TG-induced sustained increases, which will be mediated by influx based on the results shown in Fig. 2. These data indicate that TG-induced intracellular Ca^{2+} elevations were mediated by above described ion channels, and which will be inhibited by chloroquine and then resulting in decreases. These results are consistent with previous findings that chloroquine blocks TRPC3 and/or STIM/Orai channels resulting in decreases in Ca^{2+} levels in smooth muscle cells [1, 3] and in murine CD4^{+} thymocytes [4]. In addition, BCR engagement frequently occurs in vivo, which then results in immunological responses. Therefore, we observed whether the engagement can induce Ca^{2+} increases and are blocked by chloroquine. The results show that chloroquine inhibited BCR engagement-induced Ca^{2+} increases (Fig. 6).

Ca^{2+} is a crucial second messenger that modulates many cellular processes in splenic B cells, such as cell differentiation and activation [14, 23]. Therefore, our data would indicate that chloroquine might be a potent inhibitor for B cells-mediated immunological responses and inflammations.

Conclusions
Chloroquine inhibits Ca^{2+}-permeable ion channels in the plasma and the ER membranes, resulting in decreases of Ca^{2+}. These findings suggest that chloroquine would be an immunosuppressant.

Methods
Animals
6- to 8-week-old BALB/c male mice were purchased from the Hubei Provincial Center for Disease Control and Prevention, Wuhan, China. The mice were housed under controlled temperature (21–23°C) and light (lights on between 08:00 and 20:00) conditions and were provided adequate water and food. All housing and experiments were performed in accordance with the Guide for the Institutional Animal Care and Use Committee of the South-Central University for Nationalities.

Reagents
Fura-2 AM was purchased from Invitrogen (Eugene, OR, USA). Pyrazole-3 (Pyr3) and 2-Aminoethoxydiphenyl borate (2-APB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thapsigargin (TG) was purchased from Cayman (Tallah, Estonia). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). Anti-B220-PE and anti-IgM antibodies were purchased from BD Pharmingen (San Diego, CA, USA). All of the other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Isolation of B cells
B lymphocytes were isolated from mouse spleens as previously described [16]. Briefly, after the animals were killed, the spleens were removed and placed in RPMI 1640 medium containing 10% FBS and 1 mM l-glutamine. The spleens were then gently teased apart with two G27 syringe needles. The non-cellular tissues were removed by filtering this preparation through a 70-μM nylon mesh. The cells were maintained at room temperature.

Measurement of intracellular Ca^{2+}
Intracellular Ca^{2+} was measured using fura-2 AM as previously described [18, 19]. The cells were loaded with 2.5 μM fura-2 AM. Paired 340/380 fluorescence images were acquired using a TILL imaging system (FEI Munich GmbH, Munich, Germany), and the fluorescence ratios represent the intracellular Ca^{2+} levels. The B cells were identified by anti-B220-PE.

Data analysis and statistics
All of the data are presented as the mean ± SEM. The n values represent the number of cells. Unpaired Student’s t tests were performed to identify significant differences between the means. Differences with p < 0.05 were considered statistically significant.

Abbreviations
TG: thapsigargin; IP_{3}Rs: inositol-3-phosphate receptors; TRPC3: transient receptor potential C3; STIM: stromal interaction molecule; BCR: B cell receptor; PLC: phospholnositide-specific phospholipase C; PIP_{2}: phosphatidylinositol-4, 5-bisphosphate; DAG: diacylglycerol; IP3: inositol-1,4,5-trisphosphate; ER: endoplasmic reticulum; 2-APB: 2-aminoethoxydiphenyl borate; Pyr3: pyrazole-3; FBS: fetal bovine serum; RyRs: ryanodine receptors.

Authors’ contributions
YFW, PZ, XL, JCX, QZ and MRX did experiments and analyzed data; JHS, YBP, LX, MY, WWMC and LQM analyzed data; PZ and QHL designed experiments, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.
Acknowledgements
The authors thank Hai-Xia Cheng (College of Life Sciences, South-Central University for Nationalities) for technical assistance. This work is supported by the National Natural Science Foundation of China (31571200, 31140087, and 30971514 to Q-H Liu; 31070744 to P Zhao) and the Fundamental Research Funds for the Central Universities, South-Central University for Nationalities (CZY17009 to P Zhao, CZW15025 and CZW15012 to Q-H Liu).

Competing interests
The authors declare that they have no competing interests.

Compliance with ethical guidelines
All animal housing and experiments were performed in accordance with the Guide for the Institutional Animal Care and Use Committee of the South-Central University for Nationalities.

Data availability
Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Received: 30 December 2016   Accepted: 19 May 2017
Published online: 23 May 2017

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