Original Paper

Chloroquine Inhibits Ca^{2+} Signaling in Murine CD4^{+} Thymocytes

Jin-Chao Xu^a Yong-Bo Peng^a Ming-Yu Wei^a Yi-Fan Wu^a Donglin Guo^{a,b}
Gangjian Qin^c Guangju Ji^d Jinhua Shen^a Qing-Hua Liu^a

^aInstitute for Medical Biology & Hubei Provincial Key Laboratory for Protection and Application of Special Plants in the Wuling Area of China, College of Life Sciences, South-Central University for Nationalities, Wuhan, China; ^bLankenau Institute for Medical Research & Main Line Health Heart Center, 100 Lancaster Avenue, Wynnewood, PA, USA; ^cFeinberg Cardiovascular Research Institute, Department of Medicine-Cardiology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA; ^dNational Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

Key Words

CD4^{+} T cells • Chloroquine • Transient receptor potential C3 (TRPC3) • IP_3 receptor • Thapsigargin

Abstract

Background/Aims: Bitter-tasting chloroquine can suppress T cell activation by inhibiting Ca^{2+} signaling. However, the mechanism of inhibition remains largely unclear. **Methods:** In this study, CD4^{+} T cells were isolated from the thymus, and the calcium content of CD4^{+} thymocytes was measured using fura-2 AM and a TILL imaging system. Pyrazole-3 (Pyr3), thapsigargin (TG), and caffeine were used to assess the effects of chloroquine on the intracellular Ca^{2+} content of CD4^{+} T cells. **Results:** In murine CD4^{+} thymocytes, chloroquine decreased the TG-triggered intracellular Ca^{2+} increase in a dose-dependent manner. In the absence of chloroquine under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), TG induced a transient Ca^{2+} increase. After restoration of the extracellular Ca^{2+} concentration to 2 mM, a dramatic Ca^{2+} increase occurred. This elevation was completely blocked by chloroquine and was markedly inhibited by Pyr3, a selective antagonist of transient receptor potential C3 (TRPC3) channel and stromal interaction molecule (STIM)/Orai channel. Furthermore, the TG-induced transient Ca^{2+} increase under Ca^{2+} -free conditions was eliminated in the presence of chloroquine. Chloroquine also blocked the dialyzed inositol-1,4,5-trisphosphate (IP_3)-induced intracellular Ca^{2+} increase. However, chloroquine was not able to decrease the caffeine-induced Ca^{2+} increase. **Conclusion:** These data indicate that chloroquine inhibits the elevation of intracellular Ca^{2+} in thymic CD4^{+} T cells by inhibiting IP_3 receptor-mediated Ca^{2+} release from intracellular stores and TRPC3 channel-mediated and/or STIM/Orai channel-mediated Ca^{2+} influx.

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J.-C. Xu and Y.-B. Peng contributed equally to this work.

Qing-Hua Liu

Institute for Medical Biology, College of Life Sciences, South-Central University for Nationalities, 182 Min Zu Da Dao, Wuhan 430074, Hubei, (PR China)
Tel. / Fax +86-27-67841906, E-Mail liu258q@yahoo.com; qinghualiu@mail.scuec.edu.cn

Introduction

Bitter-tasting chloroquine and its synthetic analogs have been widely used as antimalarial drugs for several decades [1-3]. The interesting anti-inflammatory effects of chloroquine have made it a treatment option for other immune-related disorders, such as rheumatic disease, chronic graft-versus-host disease, amebic hepatitis, and systemic lupus erythematosus [1, 4-8]. Previous studies have indicated that chloroquine and its analogs can block proliferative responses to T cell mitogens and alloantigens [6, 9]. Furthermore, these drugs have been shown to inhibit the release of various pro-inflammatory cytokines, such as TNF- α and interleukin-6 (IL-6) [2, 9, 10]. These findings imply that chloroquine and its analogs can function as immunosuppressants by blocking one or more steps in the T cell activation pathway [6, 9].

Antigen or anti-T cell receptor (TCR) antibodies can bind to the TCR, resulting in tyrosine phosphorylation of phosphoinositide-specific phospholipase C (PLC). Phosphorylated PLC then breaks down phosphatidylinositol-4,5-bisphosphate (PIP_2) into two components: inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol [11, 12]. IP_3 binds to its receptor (IP_3R), which is located on the surface of the endoplasmic reticulum (ER). Activated IP_3R mediates Ca^{2+} release from the ER, thereby increasing intracellular Ca^{2+} [13]. The amplitude and duration of this increase are crucial for various T cell functions [14, 15], including development [16], survival [17], activation [18], differentiation [19, 20], cytokine production [18], and cell death [13].

However, the Ca^{2+} that is transiently released from the ER is not sufficient to sustain the intracellular levels that are necessary for T cell activation [21]. Therefore, extracellular Ca^{2+} influx is essential [22]. The depletion of intracellular Ca^{2+} stores activates STIM/Orai [21, 23] and CRAC [11, 24] channels, resulting in an influx that sustains the newly elevated Ca^{2+} levels. Transient receptor potential (TRP) channels, voltage-dependent L-type Ca^{2+} channels (VDCCs), and P2X receptor channels can also mediate Ca^{2+} entry [13, 23] and contribute to sustained Ca^{2+} elevation.

In a previous study, we found that bitter-tasting chloroquine can inhibit cytosolic Ca^{2+} elevations by blocking TRPC3 channels [25] and VDCCs in mouse airway smooth muscle cells [25, 26]. In the present study, we explored whether chloroquine could inhibit Ca^{2+} increases in T cells. We demonstrated that chloroquine inhibits Ca^{2+} release from the ER and Ca^{2+} influx, decreasing the intracellular Ca^{2+} concentration in thymic CD4^+ T cells.

Materials and Methods

Animals

Six- 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 08:00-20:00) conditions and were given adequate water and food.

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All the experiments were approved by the Institutional Animal Care and Use Committee at the South-Central University for Nationalities, Wuhan, China. The mice were killed by intraperitoneal injection of sodium pentobarbital (150 mg/kg) before tissue samples were removed.

Reagents

Fura-2 AM was purchased from Invitrogen (Eugene, OR, USA). Pyrazole-3 (Pyr3) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Thapsigargin (TG) was purchased from Cayman (Tallinn, Estonia). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). Anti-CD4 (RM4-4)-FITC and anti-CD8 (8 α 53-6.7)-FITC were purchased from BD Pharmingen (San Diego, CA, USA). All other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Isolation of CD4^+ T cells

CD4^+ T cells were isolated from the thymus as previously described [27]. After the animals were killed, their thymi were removed and placed in RPMI 1640 medium containing 10% FBS and 1 mM L-glutamine. The thymi were then gently teased apart with two G27 syringe needles. Non-cellular tissues were removed by filtering this preparation through a 70 μM nylon mesh. Cells were maintained at room temperature.

Measurement of intracellular Ca^{2+}

Intracellular Ca^{2+} was measured using fura-2 AM as previously described [25, 28]. Cells were loaded with fura-2 AM at 2.5 μM . Paired 340/380 fluorescence images were acquired using a TILL imaging system (FEI Munich GmbH, Munich, Germany), and the fluorescence ratios represented the intracellular Ca^{2+} levels.

Patch-clamp

To dialyze IP_3 into cells, cells were patched in a whole-cell configuration using an EPC10 amplifier (HEAK, Germany), and IP_3 (10 μM) was dialyzed via a glass pipette during current clamping.

Data analysis and statistics

All the data are presented as the mean $\pm$ SEM. The n values represent the number of cells. Unpaired Student's *t*-tests were performed to identify significant differences between means using Origin 9.0 software (OriginLab, Northampton, MA, USA). Differences with $p < 0.05$ were considered statistically significant.

Results

Chloroquine decreases intracellular Ca^{2+}

Our study investigated the ability of chloroquine to alter intracellular Ca^{2+} levels in CD4^+ thymocytes. Intracellular Ca^{2+} levels increased in response to 6 μM TG, an inhibitor of the ER Ca^{2+} ATPase. This effect was completely inhibited by chloroquine (10 mM) (Fig. 1A), as observed in 72 cells (Fig. 1B). The chloroquine-mediated inhibition of the TG-induced intracellular Ca^{2+} increase occurred in a dose-dependent manner (Fig. 1C).

Chloroquine inhibits Ca^{2+} entry

We next studied whether chloroquine can inhibit extracellular Ca^{2+} influx. Under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), TG induced a transient intracellular Ca^{2+} increase (Fig. 2A). Following restoration of the extracellular Ca^{2+} concentration to 2 mM, the intracellular Ca^{2+} concentration increased, and this elevation was sustained; this

Fig. 1. Chloroquine inhibits TG-induced intracellular Ca^{2+} elevation. (A) TG (6 μM) induced a sustained Ca^{2+} increase in primary thymic CD4^+ T cells (thymocytes). This increase was blocked by 10 mM chloroquine. The thick line represents the average. (B) Summary of the average peak changes in intracellular Ca^{2+} levels in 72 cells. (C) Dose-dependent inhibition of the 6 μM TG-induced Ca^{2+} increase by chloroquine. The point values are from 53-81 cells. NS: $p > 0.05$; *** $p < 0.001$. These results indicate that chloroquine inhibits TG-induced Ca^{2+} elevation in a dose-dependent manner.

Fig. 2. Chloroquine blocks extracellular Ca^{2+} influx. (A) Under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), 6 μM TG transiently elevated intracellular Ca^{2+} levels. Following the addition of 2 mM Ca^{2+} , a larger sustained elevation occurred, which was inhibited by 10 mM chloroquine. (B) Summary of the results from 39 cells; *** $p < 0.001$. These results demonstrate that chloroquine inhibits extracellular Ca^{2+} influx.

Fig. 3. Pyr3 inhibits TG-induced Ca^{2+} increases. (A) The Ca^{2+} elevation induced by 6 μM TG was markedly inhibited by 3 μM Pyr3, a selective inhibitor of TRPC3 and STIM/Orai channels. The remaining Ca^{2+} elevation was abolished by 10 mM chloroquine. The averaged values from 108 cells were analyzed, and the results are shown in (B). *** $p < 0.001$. These experiments indicate that the inhibition of TRPC3 and/or STIM/Orai channels plays a key role in chloroquine-induced Ca^{2+} decreases.

Fig. 4. Chloroquine blocks TG-induced Ca^{2+} release. Under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), 6 μM TG failed to elevate intracellular Ca^{2+} levels in the presence of 10 mM chloroquine. The restoration of 2 mM extracellular Ca^{2+} did not increase intracellular Ca^{2+} . This phenomenon was observed in 63 cells. NS: $p > 0.05$. These results demonstrate that chloroquine inhibits Ca^{2+} release from intracellular Ca^{2+} stores.

phenomenon was blocked by chloroquine (Fig. 2A). We analyzed peak Ca^{2+} levels in 39 cells (Fig. 2B). In a previous study, we found that chloroquine can block TRPC3 and/or STIM/Orai channels and thus decrease intracellular Ca^{2+} in airway smooth muscle cells [25]. In the current study, Pyr3 markedly decreased the expected intracellular Ca^{2+} increase, and the addition of chloroquine resulted in complete inhibition of the increase (Fig. 3A). The average peak Ca^{2+} levels in 108 cells are presented in Fig. 3B. All of these data indicate that chloroquine inhibits extracellular Ca^{2+} influx, predominantly by antagonizing TRPC3 and/or STIM/Orai channels.

Fig. 5. Chloroquine inhibits IP_3 -triggered Ca^{2+} elevation. (A) Dialyzed IP_3 increased intracellular Ca^{2+} levels. A summary of the results from 6 cells is shown in (B). (C) In the presence of 10 mM chloroquine, the Ca^{2+} increase induced by dialyzed IP_3 was eliminated. This experiment was performed with 8 cells (D). NS: $p > 0.05$; *** $p < 0.001$. These data indicate that chloroquine blocks IP_3 Rs.

Fig. 6. Caffeine fails to elevate Ca^{2+} . Caffeine (10 mM) did not trigger a Ca^{2+} increase, whereas TG did increase Ca^{2+} levels in 40 cells. This result suggests that thymic CD4^+ T cells lack functional RyRs.

Chloroquine inhibits Ca^{2+} release

To further examine whether chloroquine inhibits TG-induced Ca^{2+} release, we pretreated cells with chloroquine and then observed intracellular Ca^{2+} changes in response to TG under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA). As shown in Fig. 4, TG failed to induce a typical Ca^{2+} increase. This increase did not occur even when the extracellular Ca^{2+} concentration was restored to 2 mM. This phenomenon was noted in 63 cells (Fig. 4), and the data indicate that chloroquine blocks Ca^{2+} release from the ER.

To further understand the underlying mechanism, we performed the following experiments. As shown in Fig. 5A, intracellular Ca^{2+} levels rapidly increased following dialysis of IP_3 into a cell. However, the same dialysis did not induce a Ca^{2+} increase in cells pretreated with chloroquine (Fig. 5B), indicating that chloroquine blocks IP_3 Rs.

We stimulated cells with caffeine, a selective activator of ryanodine receptors (RyRs), to test whether RyRs are involved in the chloroquine-mediated inhibition of the TG-induced Ca^{2+} increase. We found that caffeine failed to increase intracellular Ca^{2+} , although the typical increase in intracellular Ca^{2+} occurred upon addition of TG (Fig. 6). This result suggests that the studied cells did not express functional RyRs. Therefore, the inhibitory effect of chloroquine on intracellular Ca^{2+} is not related to RyRs.

Discussion

In the present study, we demonstrated that chloroquine decreases intracellular Ca^{2+} levels in thymic CD4^+ T cells by blocking Ca^{2+} release from the ER and extracellular Ca^{2+} influx, which suggests that chloroquine might modulate immune cell activation by regulating intracellular Ca^{2+} levels.

Ca^{2+} has been recognized as a critical second messenger that controls numerous cellular processes in T cells, such as cell activation and differentiation [16, 23]. Intracellular Ca^{2+} levels are modulated by several types of Ca^{2+} channels in the ER and plasma membranes. We and others have demonstrated that chloroquine can block these Ca^{2+} channels in smooth muscle [25, 26], skeletal muscle [29] and macrophages [30]. Interestingly, several studies have indicated that chloroquine can inhibit tetanus toxin- and mitogen-induced proliferative responses and cytokine secretion by inhibiting Ca^{2+} signaling [9]. These previous findings imply that through the inhibition of Ca^{2+} signaling, chloroquine affects the physiological function of T cells.

The studied Ca^{2+} increase results from both Ca^{2+} release from the ER and extracellular Ca^{2+} influx. TG, an inhibitor of the ER Ca^{2+} ATPase, inhibits Ca^{2+} re-uptake, which results in a transient Ca^{2+} increase [31]. This process depletes Ca^{2+} stores, which activates Ca^{2+} -permeable channels, such as TRP [11, 32], CRAC and STIM/Orai channels [33]. These channels then open and mediate extracellular Ca^{2+} influx, which sustains the intracellular Ca^{2+} elevation. Thus, we investigated whether these two pathways are involved in the chloroquine-mediated inhibition of TG-induced Ca^{2+} increases.

Ca^{2+} increases in CD4^+ T cells were induced by TG and then completely blocked by chloroquine (Fig. 1A), indicating that chloroquine is a potent inhibitor. We then focused on investigating whether this phenomenon was due to chloroquine-mediated inhibition of the two pathways described above. Chloroquine blocked Ca^{2+} increases induced by treating cells with 2 mM Ca^{2+} (Fig. 2), suggesting that TG induced a Ca^{2+} influx that was completely inhibited by chloroquine. Pyr3, a selective antagonist of TRPC3 and STIM/Orai channels [34], markedly inhibited the TG-induced Ca^{2+} increase (Fig. 3). These findings suggest that the TG-induced Ca^{2+} influx is mediated by TRPC3 and/or STIM/Orai channels; this influx can also be inhibited by chloroquine, ultimately resulting in intracellular Ca^{2+} decrease. Because TRPC3 [35] or STIM/Orai channels alone [36] can increase intracellular Ca^{2+} , at this point, we cannot conclude which channel(s) is involved in chloroquine-induced Ca^{2+} decreases; this will need to be elucidated in future studies. Our recent study observed similar chloroquine-mediated inhibition of Ca^{2+} levels in airway smooth muscle cells [25]. Chloroquine-mediated inhibition of TG-induced Ca^{2+} increases has been previously reported in Jurkat T cells, although the underlying mechanism was not investigated until the present study [9].

Under Ca^{2+} -free conditions, TG induced a transient Ca^{2+} increase (Fig. 2A); however, in the presence of chloroquine, no increase was observed (Fig. 4). This finding indicates that one or more Ca^{2+} release pathways were inhibited by chloroquine. Because the Ca^{2+} ATPase was inhibited by TG, thereby preventing Ca^{2+} from being pumped back into the ER, any Ca^{2+} release would increase intracellular Ca^{2+} . However, we did not observe an increase under these conditions. We inferred that chloroquine might have blocked IP_3Rs and RyRs because these two receptor channels are the main pathways for Ca^{2+} release from the ER [23]. We dialyzed IP_3 , a selective activator of IP_3Rs , into cells and induced a Ca^{2+} increase (Fig. 5A) that was blocked by chloroquine (Fig. 5B), indicating that chloroquine blocks IP_3Rs . This phenomenon has also been observed in airway smooth cells [37]. In addition, because a selective activator of RyRs , caffeine, failed to induce a Ca^{2+} increase (Fig. 6), we concluded that the utilized CD4^+ T cells did not express RyRs in the ER. This result is supported by the previous finding that T cells express no or very few RyRs [23]. Therefore, TG-induced Ca^{2+} release (Fig. 2A) is primarily mediated by IP_3Rs , which can be inhibited by chloroquine.

In summary, chloroquine can inhibit IP_3Rs and Ca^{2+} influx pathways mediated by TRPC3 and/or STIM/Orai channels, resulting in a decrease in TG-induced Ca^{2+} levels. These findings might provide avenues for the development of new methods of immunosuppression.

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Disclosure Statement

The authors declare no conflict of interests.

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