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Caffeine-Induced Ca^{2+} Oscillations in Type I Horizontal Cells of the Carp Retina and the Contribution of the Store-Operated Ca^{2+} Entry Pathway

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Abstract

The mechanisms of release, depletion, and refilling of endoplasmic reticulum (ER) Ca^{2+} were investigated in type I horizontal cells of the carp retina using a fluo-3-based Ca^{2+} imaging technique. Exogenous application of caffeine, a ryanodine receptor agonist, induced oscillatory intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) responses in a duration- and concentration-dependent manner. In Ca^{2+}-free Ringer's solution, $[Ca^{2+}]_i$ transients could also be induced by a brief caffeine application, whereas subsequent caffeine application induced no $[Ca^{2+}]_i$ increase, which implied that extracellular Ca^{2+} was required for ER refilling, confirming the necessity of a Ca^{2+} influx pathway for ER refilling. Depletion of ER Ca^{2+} by thapsigargin triggered a Ca^{2+} influx which could be blocked by the store-operated channel inhibitor 2-APB, which proved the existence of the store-operated Ca^{2+} entry pathway. Taken together, these results suggested that after being depleted by caffeine, the ER was replenished by Ca^{2+} influx via store-operated channels. These results reveal the fine modulation of ER Ca^{2+} signaling, and the activation of the store-operated Ca^{2+} entry pathway guarantees the replenishment of the ER so that the cell can be ready for response to the subsequent stimulus.

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Introduction

Ca^{2+} is a ubiquitous intracellular messenger that regulates numerous cellular processes including muscle contraction, transmitter release, apoptosis, and so on [1,2]. In neurons, the basal level of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained very low [3]. When activated by proper stimulation, the opening of the plasma membrane Ca^{2+} channels or the activation of Ca^{2+} release channels on the intracellular Ca^{2+} stores (largely the endoplasmic reticulum, ER) leads to the elevation of $[Ca^{2+}]_i$ [2].

As the primary intracellular reservoir of Ca^{2+} and a major source of $[Ca^{2+}]_i$ elevation, the ER is involved in a wide range of neuronal Ca^{2+}-dependent processes, such as synaptic transmission and plasticity [4,5]. The ER accumulates Ca^{2+} by active transport of Ca^{2+} from the cytoplasm into the ER by Ca^{2+}-ATPase (sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA) pumps expressed on the ER membrane. At the meantime, Ca^{2+} release from the ER is mainly mediated by two families of Ca^{2+} release channels, i.e., the inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) families. While IP_3Rs are gated by IP_3, both IP_3Rs and RyRs can be activated by Ca^{2+}, and such Ca^{2+}-induced Ca^{2+} release (CICR) forms a positive feedback process. RyRs can also be activated by caffeine, which sensitizes RyR's response to Ca^{2+} [6,7].

Type I horizontal cells (H1 HCs) are interneurons in the outer retina of carp, which receive glutamate input from cone photoreceptors. It was found in the fish retina that HCs contained caffeine-sensitive ER [8,9] which was involved in the modulation of synaptic strength [10], GABA transporter currents [11,12], as well as voltage-gated Ca^{2+} channel currents [13]. Despite its functional significance, knowledge about the ER Ca^{2+} dynamics of H1 HCs is still limited.

The present study aims to investigate possible mechanisms of ER Ca^{2+} dynamics of carp H1 HCs, particularly the details of the following aspects: (1) the temporal characteristics of $[Ca^{2+}]_i$ signals initiated by ER Ca^{2+} release, (2) the inter-relationship between the two major Ca^{2+} sources, i.e., the extracellular Ca^{2+} and the ER, and relevant channels underlying their interaction.

To explore the above issues, Ca^{2+} signals elicited by exogenously applied caffeine were recorded from freshly dissociated H1 HCs using a fluo-3-based Ca^{2+} imaging technique. The basic findings are: (1) caffeine induced oscillatory $[Ca^{2+}]_i$ responses in a duration- and concentration-dependent manner, (2) removal of extracellular Ca^{2+} abolished the repeatability of caffeine-induced $[Ca^{2+}]_i$ responses, (3) inhibition of L-type voltage-gated Ca^{2+} channels (L-VGCCs) reduced caffeine-induced $[Ca^{2+}]_i$ oscillations, (4) inhibition of store-operated channels (SOCs) abolished caffeine-induced $[Ca^{2+}]_i$ oscillations. These results reveal the fine modulation of ER Ca^{2+} signaling, and the activation of the store-operated Ca^{2+} entry (SOCE) pathway guarantees the replenishment of the ER so that the cell can be ready for response to the subsequent stimulus.

Materials and Methods

Ethics Statement

The animal experiments were approved by the Ethic Committee, School of Biomedical Engineering, Shanghai Jiao Tong University. All procedures strictly conformed to the humane treatment and use of animals as prescribed by the Association for Research in Vision and Ophthalmology.

Cell Isolation

H1 HCs were enzymatically dissociated from retinas of adult carp (*Carassius auratus*, 15–20 cm body length). After 30 min dark-adaption, the eye was enucleated and hemisected. The retina was then isolated and cut into 8–12 pieces and incubated for 30 min at room temperature of 25°C in 5 ml Hank's solution (see below) containing 25 U/ml papain (E. Merck, Germany) and 1 mg/ml L-cysteine. The retinal pieces were then kept in normal Hank's solution at 4°C until being used (within 4 h). To obtain dissociated H1 HCs, retinal pieces were gently triturated with fire-polished glass pipettes in normal Ringer's solution. The cell suspension was moved to a recording chamber for Ca^{2+} imaging recording.

Solutions

Hank's solution contained (in mM): 137.0 NaCl, 3.0 KCl, 1.0 $MgSO_4$, 1.0 NaH_2PO_4, 0.5 $NaHCO_3$, 2.0 $CaCl_2$, 2.0 Na-pyruvate, 20.0 HEPES and 16.0 Glucose. Ringer's solution contained (in mM): 145.0 NaCl, 5.0 KCl, 1.0 $MgSO_4$, 2.0 $CaCl_2$, 10.0 HEPES and 16.0 Glucose. Ca^{2+}-free Ringer's solution was prepared based on normal Ringer's solution with $CaCl_2$ omitted and 1 mM EGTA added. Caffeine was directly dissolved in Ringer's solution according to the concentration required. Ryanodine (Tocris Bioscience, UK), nifedipine, thapsigargin (TG) and 2-aminoethoxydiphenyl borate (2-APB) were prepared in dimethyl sulfoxide (DMSO) and diluted to their final concentration in Ringer's solution (DMSO <0.5%). The pH value was adjusted to 7.4 with NaOH for Ringer's and Hank's solutions as well as other solutions. All drugs, unless otherwise specified, were purchased from Sigma Aldrich (St. Louis, MO).

Ca^{2+} Imaging

$[Ca^{2+}]_i$ changes were measured using a fluo-3 imaging system. Fluo-3/AM was dissolved in DMSO (1 mM stock solution) and added to the cell suspension at a final concentration of 5 μM (DMSO = 0.5%). Cell suspension was transferred into a perfusion chamber (RC-26, Warner Instruments, USA) with a cover glass bottom (12–548B, Thermo Fisher Scientific Inc., USA) and incubated at room temperature (25°C) for 15 min to allow for cell adherence and fluo-3 loading. The cells were then continuously perfused with Ringer's solution at a flow rate of 1 ml/min for 10 min prior to recording. The volume of the perfusion chamber was about 500 μl. All drugs were applied by perfusion. At the perfusion rate employed, 1 min was needed for the drug to reach the perfusion chamber. Fluorescence measurements were performed on an upright fluorescence microscope (BX51WI, Olympus, Japan). H1 HCs were identified by their characteristic morphology as having a round soma with 4–8 extended, subtle dendrites [14]. Fluo-3 in its Ca^{2+}-bound form was excited at 488 nm, and fluorescence emission at 525 nm was acquired every 2 s by a digital CCD camera (CoolSNAP ES^2, Photometrics, USA). $[Ca^{2+}]_i$ signals were presented by the fluorescence intensity F normalized to the baseline fluorescence value F_0 (F/F_0). For caffeine-induced $[Ca^{2+}]_i$ responses, $[Ca^{2+}]_i$ signals were normalized against the amplitude of the first $[Ca^{2+}]_i$ transient. A transient peak was considered a caffeine-induced $[Ca^{2+}]_i$ transient if it had an amplitude larger than 3×SD of data recorded in the 1 min duration prior to caffeine application.

Statistical Analyses

Statistics were performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Values are presented as mean ± SEM. To determine statistical significance, independent-samples *t* test and one-way analysis of variance (ANOVA) were used for comparing the results between two groups and that among multiple groups respectively. If a significant *p* value was obtained for ANOVA, *post hoc* analyses were performed using Student-Newman-Keuls (SNK) test, $p<0.05$ indicates significant differences.

Results

Caffeine Induced $[Ca^{2+}]_i$ Responses in a Duration- and Concentration-dependent Manner

Caffeine has long been used as a RyR agonist for studying RyR-mediated Ca^{2+} release from intracellular Ca^{2+} stores [15,16]. In our present study, to investigate the temporal characteristics of $[Ca^{2+}]_i$ signal initiated by ER Ca^{2+} release, caffeine was applied at six different concentrations (1, 3, 6, 10, 20, and 40 mM) in combination with four different durations (30, 60, 90, and 240 s). For each of these $6\times4=24$ combinations, one group of independent experiments was conducted (using 5–9 H1 HCs for each experimental condition).

To study the effect of duration of caffeine application, the four groups of experiments with varying durations (i.e., 30, 60, 90, and 240 s) of caffeine application at the same caffeine concentration were compared.

In our experiments, the application of 1 mM caffeine with durations of 30, 60, 90, and 240 s induced no discernible $[Ca^{2+}]_i$ changes in all four groups of H1 HCs tested.

The application of 3 mM caffeine induced $[Ca^{2+}]_i$ transient(s) in a duration-dependent way (Fig. 1A (a–d)). When comparing results from the four duration groups (30, 60, 90, and 240 s), the number of $[Ca^{2+}]_i$ transients elicited by 3 mM caffeine showed an increasing tendency with the duration of caffeine application, which was increased monotonically from 0 to 5.13±0.35 ($p<0.05$, ANOVA, *post hoc* SNK test) when the duration was increased from 30 to 240 s (Fig. 1A (e)).

The application of 6 mM caffeine tended to induce more active $[Ca^{2+}]_i$ transients (Fig. 1B (a–d)). The number of $[Ca^{2+}]_i$ transients elicited by 6 mM caffeine was increased from 0.71±0.18 to 6.00±0.41 ($p<0.05$, ANOVA, *post hoc* SNK test), when the duration of caffeine application was increased from 30 to 240 s (Fig. 1B (e)).

When 10 mM caffeine was applied, duration-dependent $[Ca^{2+}]_i$ transients increment was still observed (Fig. 1C (a–d)). But the increment was very much limited. The number of $[Ca^{2+}]_i$ transients induced by 10 mM caffeine was increased from 1.50±0.19 to 2.88±0.23 ($p<0.05$, ANOVA, *post hoc* SNK test; Fig. 1C (e)) when the duration of caffeine application was increased from 30 to 60 s, and was stabilized around 3 when the duration of caffeine was further increased to 90 and 240 s (3.00±0.27 and 3.13±0.13, respectively), with no statistical significance among the 60, 90, and 240 s groups ($p>0.05$, *post hoc* SNK test; Fig. 1C (e)).

When 20 mM caffeine was applied at various durations (30, 60, 90, and 240 s), the pattern of $[Ca^{2+}]_i$ response was almost uniform among the four groups (Fig. 1D (a–d)), the average number of $[Ca^{2+}]_i$ transients for the four groups were 2.38±0.18, 2.75±0.16, 2.63±0.18, and 2.38±0.26, respectively, with no significant

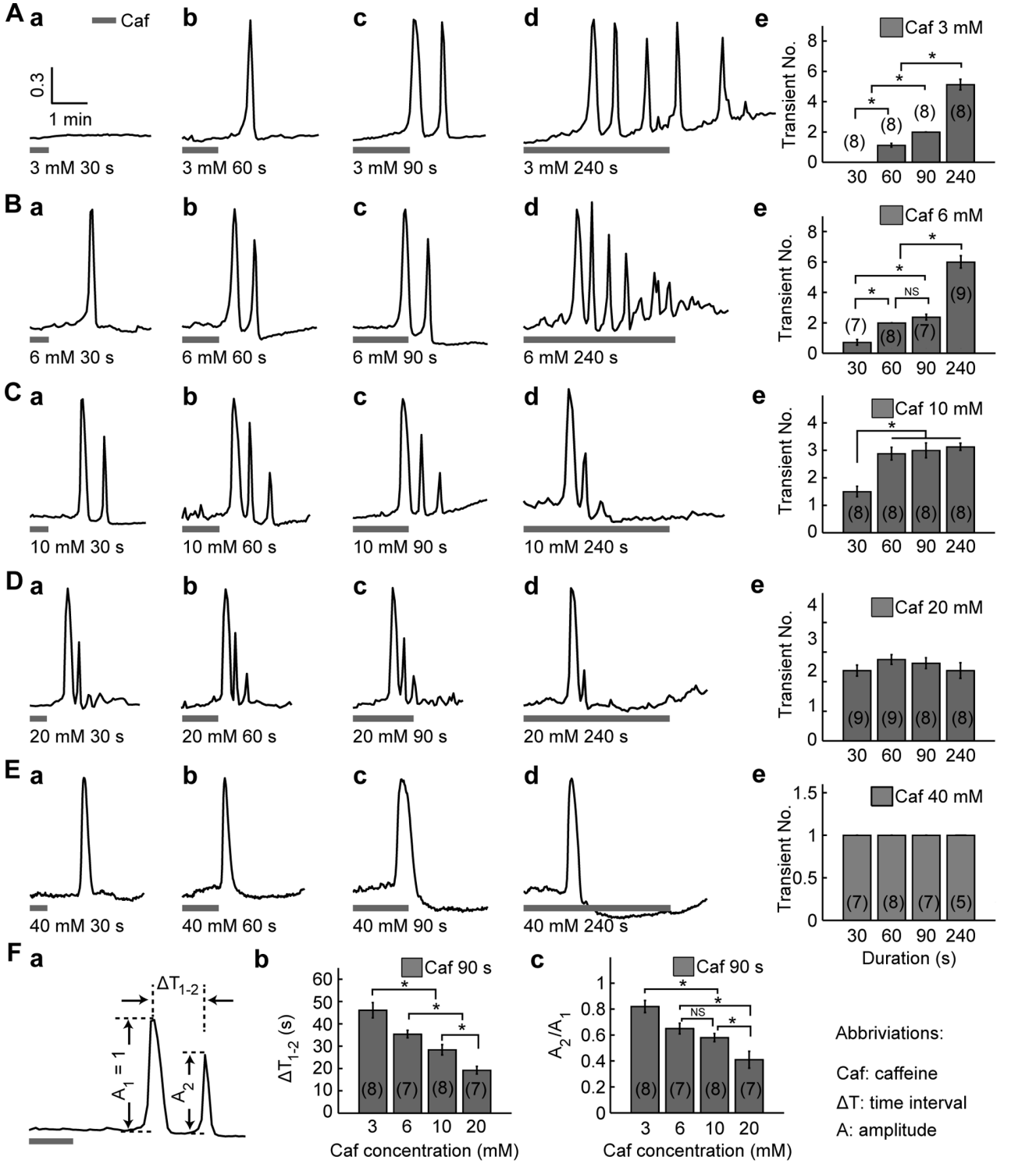

Figure 1. Caffeine induced $[Ca^{2+}]_i$ increase in a duration- and concentration-dependent manner. A–E, $[Ca^{2+}]_i$ transients induced by the application of 3, 6, 10, 20, and 40 mM caffeine, respectively. Columns (a)-(d), $[Ca^{2+}]_i$ transients induced by the application of caffeine for 30, 60, 90, and 240 s, respectively. Column (e), The average number of $[Ca^{2+}]_i$ transients (transient NO.) induced by various durations of caffeine application under each concentration. F, Dependence of caffeine-induced $[Ca^{2+}]_i$ responses (with identical application time of 90 s) on caffeine concentration. (a), Definition of amplitude and time interval between the first and the second Ca^{2+} peaks (ΔT_{1-2}). The amplitude of the second $[Ca^{2+}]_i$ transient (A_2) is normalized against that of the first one (A_1). (b), ΔT_{1-2} decreased with caffeine concentration. (c), A_2/A_1 decreased with caffeine concentration. Horizontal bars bellow the traces indicate the periods of caffeine applications. With our perfusion rate being 1 ml/min, 1 min was needed for the drug to reach the perfusion chamber. In this and subsequent figures, each trace is the representative of a group of independent experiments, and data are presented as mean ± SEM (with sample size in parentheses). * denotes statistical significance of $p<0.05$ by one-way ANOVA followed by *post hoc* SNK test, NS: not significant.

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differences among groups ($p>0.05$, ANOVA, *post hoc* SNK test; Fig. 1D (e)).

During 40 mM caffeine application, only one $[Ca^{2+}]_i$ transient could be induced no matter how long the caffeine application duration was (Fig. 1E).

The above results show that, Ca^{2+} oscillations could be induced by intermediate concentrations of caffeine (3 to 20 mM), with the oscillatory behavior being concentration-dependent. To examine the effect of caffeine concentration on Ca^{2+} oscillations, experiments conducted at varying caffeine concentrations with identical duration (90 s) of caffeine application were compared (Fig.1 column c). When the concentration of caffeine was increased from 3 to 20 mM (with identical application time of 90 s), the time interval between the first and the second Ca^{2+} transients (ΔT_{1-2}; Fig. 1F (a)) decreased from 46.08±3.41 to 19.24±1.68 s ($p<0.05$, ANOVA, *post hoc* SNK test; Fig. 1F (b)).

The amplitude decrement during Ca^{2+} oscillations was also concentration dependent. During the application of 3 mM caffeine, the amplitudes of the oscillatory $[Ca^{2+}]_i$ transients were changing within a limited range (Fig. 1A). However, when caffeine was applied with higher concentrations, a decreasing tendency in amplitudes was observed in the oscillatory transients (Fig. 1B–D). To quantify such amplitude decrement, we normalized the amplitude of the second transient against that of the first one (A_2/A_1; Fig. 1F(a)). When the concentration of caffeine was increased from 3 to 20 mM (with application time of 90 s), the normalized amplitude of the second transient (A_2/A_1) was decreased from 0.82±0.05 to 0.41±0.07 ($p<0.05$, ANOVA, *post hoc* SNK test; Fig. 1F(c)).

Caffeine-induced $[Ca^{2+}]_i$ transient is composed of a rising phase and a decaying phase. In carp HCs, while the $[Ca^{2+}]_i$ increase is caused primarily by Ca^{2+} release from the ER via RyRs and Ca^{2+} influx from the extracellular environment, the decaying phase of the transient depends on the activity of SERCA pumps [3,8,9], Na^+/Ca^{2+} exchangers [17] and plasma membrane Ca^{2+}-ATPase (PMCA) pumps [17]. The balance between these Ca^{2+}-increasing and removal processes determines the pattern of $[Ca^{2+}]_i$ oscillations, which is reflected in the time interval between two adjacent transients, and the amplitude of each transient. To investigate the underlying mechanisms of caffeine-induced $[Ca^{2+}]_i$ oscillations, we looked into the involvement of RyR activation in the initiation of the $[Ca^{2+}]_i$ oscillations.

Ryanodine Application Inhibited the Caffeine-induced $[Ca^{2+}]_i$ Oscillations

Caffeine-induced $[Ca^{2+}]_i$ oscillations can be initiated by Ca^{2+} release from the ER via RyRs. To confirm the contribution of RyRs during caffeine-induced $[Ca^{2+}]_i$ oscillations, high concentration ryanodine (2.5 μM) was used to inhibit the Ca^{2+} release via RyRs [18,19]. The group of experiments conducted with caffeine (10 mM, 60 s) in normal Ringer's solution was taken as control. In normal Ringer's solution, caffeine (10 mM, 60 s) induced three $[Ca^{2+}]_i$ transients in an example HC (Fig. 2A). However, in the presence of ryanodine, the application of caffeine (10 mM, 60 s) induced a single Ca^{2+} transient (Fig. 2B), which is in accordance with the notion that inhibition of RyRs by ryanodine requires the activation of RyRs [20]. Besides, due to the irreversibility of RyR inhibition by ryanodine [20], subsequent application of caffeine induced no $[Ca^{2+}]_i$ increase in the tested HC, even after washing out of ryanodine. Statistical results showed that, the number of $[Ca^{2+}]_i$ transients induced by caffeine (10 mM, 60 s) was 1.00±0.00 for the ryanodine group, which was significantly reduced as compared with 2.88±0.23 ($p<0.05$, independent-samples *t* test) of the control group (Fig. 2C), the absence of subsequent $[Ca^{2+}]_i$ transients after the inhibition of RyRs demonstrated that the generation of subsequent transients was also initiated by Ca^{2+} release via RyRs.

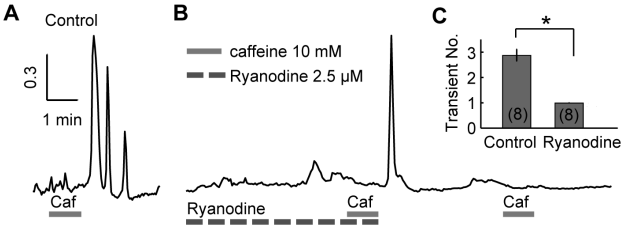

Figure 2. Ryanodine effect on the caffeine-induced $[Ca^{2+}]_i$ oscillations. A–B, Caffeine (10 mM, 60 s) induced $[Ca^{2+}]_i$ responses in: (A) normal Ringer's solution (control), and (B) in the presence of ryanodine (2.5 μM). C, The average number of Ca^{2+} transients induced by caffeine (10 mM, 60 s) in the control group and the ryanodine group. * denotes statistical significance of $p<0.05$ with independent-samples *t* test.
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Extracellular Ca^{2+} was Required for the Oscillatory Caffeine-induced $[Ca^{2+}]_i$ Responses

For each $[Ca^{2+}]_i$ transient, following Ca^{2+} release from the ER via RyR activation, the activation of SERCA pumps, Na^+/Ca^{2+} exchangers, and PMCA pumps brought the elevated $[Ca^{2+}]_i$ back to the basal level. Due to the activity of PMCA pumps and Na^+/Ca^{2+} exchangers in the plasma membrane, Ca^{2+} released from the ER cannot be fully recycled back into the ER by SERCA pumps. To counterbalance this loss of ER Ca^{2+}, Ca^{2+} entry from the extracellular medium is required for the oscillatory caffeine-induced $[Ca^{2+}]_i$ responses. To investigate whether caffeine-induced $[Ca^{2+}]_i$ responses in H1 HCs were dependent on extracellular free Ca^{2+} concentration ($[Ca^{2+}]_o$), Ca^{2+}-free Ringer's solution was used to abolish the putative Ca^{2+} influx across the plasma membrane.

The results show that, in normal Ringer's solution (control), caffeine (10 mM, 60 s) induced three $[Ca^{2+}]_i$ transients in the tested HC, and such response pattern was repeatable (Fig. 3A). In Ca^{2+}-free Ringer's solution, caffeine (10 mM, 60 s) elicited two $[Ca^{2+}]_i$ transients, with the subsequent caffeine application inducing no measurable response (Fig. 3B), implying that the ER was depleted by the first caffeine application. After reintroduction of $[Ca^{2+}]_o$, caffeine (10 mM, 60 s) again induced $[Ca^{2+}]_i$ response, confirming the necessity of Ca^{2+} influx for ER refilling following its depletion for the subsequent Ca^{2+} response.

Of all 12 cells in the Ca^{2+}-free group, caffeine (10 mM, 60 s) induced one transient in 5 cells, and two transients in the rest 7 cells. Hence, in Ca^{2+}-free Ringer's solution, the number of $[Ca^{2+}]_i$ transients induced by caffeine (10 mM, 60 s) was significantly reduced as compared with control (1.58±0.15 and 2.88±0.23, respectively, $p<0.05$, independent-samples *t* test; Fig. 3E). For the 7 cells of the Ca^{2+}-free group in which two transients were elicited by caffeine (10 mM, 60 s), ΔT_{1-2} and A_2/A_1 were calculated. The ΔT_{1-2} interval was similar to that measured in control (25.33±1.10 and 25.75±1.11 for Ca^{2+}-free and control group, respectively, $p>0.05$, independent-samples *t* test; Fig. 3F); however, the A_2/A_1 ratio was significantly smaller than that of control (0.18±0.04 and 0.60±0.06 for Ca^{2+}-free and control group, respectively, $p<0.05$, independent-samples *t* test; Fig. 3G). The reduction of transients number and A_2/A_1 by $[Ca^{2+}]_o$ removal suggests that Ca^{2+} influx contributes to caffeine-induced $[Ca^{2+}]_i$ oscillations.

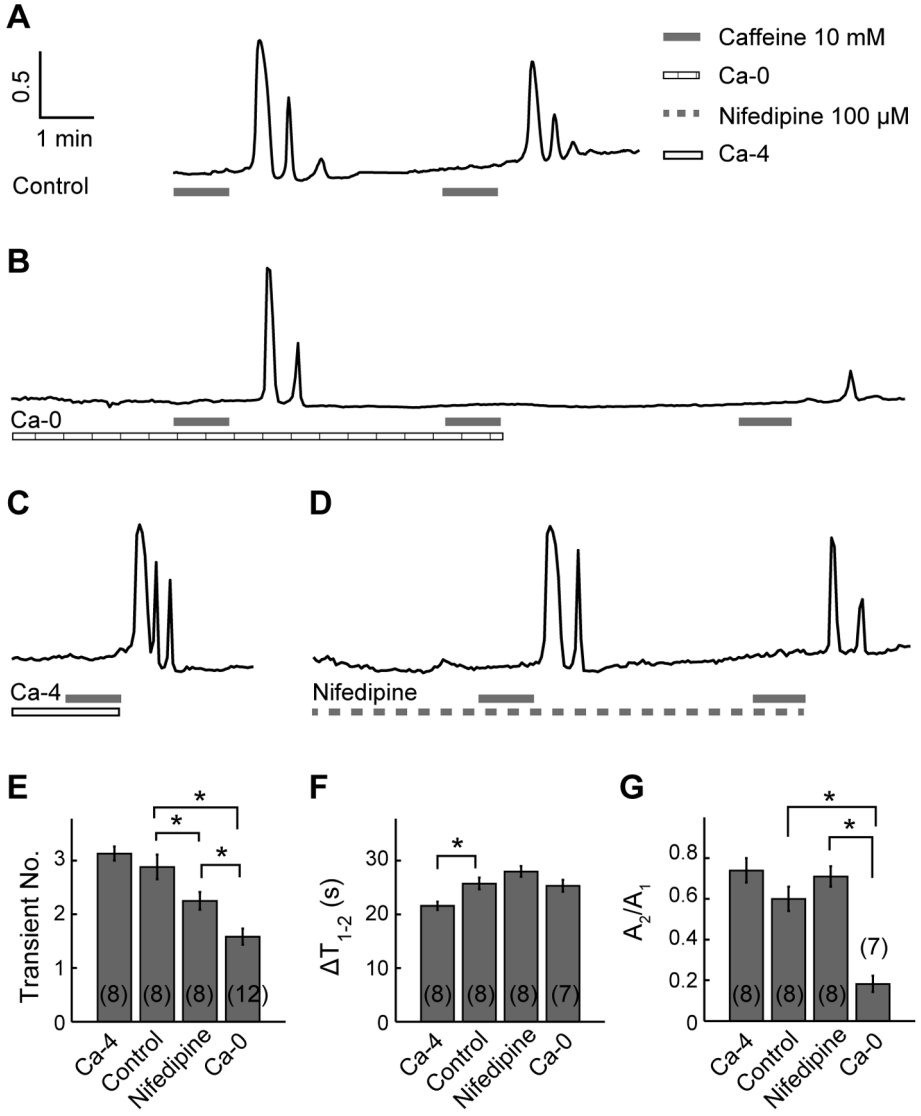

Figure 3. Regulation of $[Ca^{2+}]_o$ entry affects the caffeine-induced $[Ca^{2+}]_i$ oscillations. A–D, caffeine (10 mM, 60 s) induced $[Ca^{2+}]_i$ oscillations in: (A) normal Ringer's solution (control) and Ringer's solution with (B) Ca^{2+}-free, (C) 4 mM $[Ca^{2+}]_o$, and (D) nifedipine (100 μM). E–G, The average number of $[Ca^{2+}]_i$ transients (E), ΔT_{1-2} (F), and A_2/A_1 (G) of caffeine (10 mM, 60 s) induced $[Ca^{2+}]_i$ oscillations for the 4 mM $[Ca^{2+}]_o$ group, the control group, the nifedipine group, and the Ca^{2+}-free group. * denotes statistical significance of $p<0.05$ with independent-samples *t* test. Abbreviations: Ca-0: Ca^{2+}-free; Ca-4:4 mM $[Ca^{2+}]_o$.
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The above results show that caffeine-induced $[Ca^{2+}]_i$ response was significantly reduced by $[Ca^{2+}]_o$ removal. If abolishing Ca^{2+} entry can reduce caffeine-induced $[Ca^{2+}]_i$ response, up-regulation of Ca^{2+} entry should enhance the response. To test this hypothesis, $[Ca^{2+}]_o$ was increase from 2 mM (normal Ringer's solution) to 4 mM to up-regulate Ca^{2+} entry from the extracellular space.

When caffeine (10 mM, 60 s) application was given in the presence of 4 mM $[Ca^{2+}]_o$, three $[Ca^{2+}]_i$ transients were evoked in an example HC (Fig. 3C). Statistical comparison between the high-$[Ca^{2+}]_o$ group and the control group showed that, high $[Ca^{2+}]_o$ resulted in a modest increase in the number of caffeine-induced $[Ca^{2+}]_i$ transients (2 mM $[Ca^{2+}]_o$: 2.88±0.23, 4 mM $[Ca^{2+}]_o$: 3.13±0.13, $p>0.05$, independent-samples *t* test; Fig. 3E); at the meantime, a significant decrease in ΔT_{1-2} (2 mM $[Ca^{2+}]_o$: 25.75±1.11, 4 mM $[Ca^{2+}]_o$: 21.60±0.79, $p<0.05$, independent-samples *t* test; Fig. 3F) and a modest increase in A_2/A_1 (2 mM $[Ca^{2+}]_o$: 0.60±0.06, 4 mM $[Ca^{2+}]_o$: 0.74±0.06, $p>0.05$, independent-samples *t* test; Fig. 3G) were also observed. Hence, up-regulation of Ca^{2+} entry indeed enhanced the oscillatory caffeine-induced $[Ca^{2+}]_i$ signals.

L-VGCCs were Involved in the Caffeine-induced $[Ca^{2+}]_i$ Oscillations

The above results show that the caffeine-induced $[Ca^{2+}]_i$ response was significantly reduced when $[Ca^{2+}]_o$ was removed, which indicates that Ca^{2+} influx is requested for the caffeine-induced $[Ca^{2+}]_i$ oscillations. In carp HCs, Ca^{2+} entry from the extracellular space is known to be mediated by Ca^{2+}-permeable glutamate receptors (GluRs) [8,9,21,22,23,24] and L-VGCCs [23,25,26,27]. In the present study, the activities of GluRs were precluded, because the activation of GluRs requires the binding of glutamate. On the other hand, the membrane potential and

$[Ca^{2+}]_i$ are strongly correlated. Oscillations in $[Ca^{2+}]_i$ may concomitant changes of the membrane potential. Thus, L-VGCCs, which are activated by membrane depolarization, might be involved in the caffeine-induced $[Ca^{2+}]_i$ increase. To test the possibility of L-VGCC involvement, nifedipine, an L-VGCC antagonist, was applied [28]. Previous study in carp retinal H1 HCs suggests that 100 μM nifedipine was sufficient to abolish L-VGCC-mediated Ca^{2+} entry [23]. Hence, 100 μM nifedipine was used for complete inhibition of L-VGCCs in the present study.

In the presence of nifedipine (100 μM), caffeine (10 mM, 60 s) elicited two $[Ca^{2+}]_i$ transients, the subsequent caffeine application also induced two $[Ca^{2+}]_i$ transients (Fig. 3D). Nifedipine (100 μM) significantly reduced the number of $[Ca^{2+}]_i$ transients induced by caffeine (10 mM, 60 s), as compared with the effect of caffeine (10 mM, 60 s) alone (2.25 ± 0.16 and 2.88 ± 0.23, respectively, $p<0.05$, independent-samples t test; Fig. 3E). The ΔT_{1-2} interval was slightly increased (28.00 ± 0.98 and 25.75 ± 1.11 for nifedipine treated and control group, respectively, $p>0.05$, independent-samples t test; Fig. 3F), and the A_2/A_1 ratio was also decreased without statistical significance (0.71 ± 0.05 and 0.60 ± 0.06 for nifedipine treated and control group, respectively, $p>0.05$, independent-samples t test; Fig. 3G).

The nifedipine-induced reduction of $[Ca^{2+}]_i$ transient number suggested that L-VGCCs should be activated by caffeine application, and the resulting Ca^{2+} entry via L-VGCCs contributed to caffeine-induced $[Ca^{2+}]_i$ oscillations. However, in the presence of nifedipine, the number of $[Ca^{2+}]_i$ transients was significantly larger than that in Ca^{2+}-free Ringer's solution ($p<0.05$, independent-samples t test; Fig. 3E). Besides, nifedipine didn't reduce the A_2/A_1 ratio, while the A_2/A_1 ratio was significantly reduced by Ca^{2+}-free Ringer's solution (Fig. 3G). More importantly, different from the result obtained with Ca^{2+}-free Ringer's solution (Fig. 3B), when caffeine was re-applied in the presence of nifedipine (Fig. 3D), $[Ca^{2+}]_i$ responses could be reproduced rather than abolished, which indicated that the ER could still be refilled when L-VGCCs were blocked. These differences between the effects of nifedipine and Ca^{2+}-free Ringer's solution on caffeine-induced $[Ca^{2+}]_i$ oscillations suggest that L-VGCCs weren't the only Ca^{2+} entry pathway activated after caffeine application, there should be other Ca^{2+} entry pathway for ER refilling.

SOCs were Necessary for ER Refilling

It has been reported that in some cell types, Ca^{2+} influx could be triggered by the depletion of ER Ca^{2+}, a process referred to as store-operated Ca^{2+} entry (SOCE) [29,30]. The Ca^{2+} channels mediating SOCE are called store-operated channels (SOCs).

To examine the involvement of SOCs in ER refilling, we tested the existence of SOCs in carp H1 HCs and tried to activate SOCs by depleting the ER. To deplete the ER, HCs were incubated in Ca^{2+}-free Ringer's solution containing 5 μM thapsigargin, an irreversible SERCA inhibitor, which causes passive depletion of ER Ca^{2+} by inhibiting ER Ca^{2+} uptake via SERCA pumps [31]. If SOCs are expressed in carp H1 HCs and can be activated by ER depletion, re-addition of $[Ca^{2+}]_o$ should result in an increase in $[Ca^{2+}]_i$, given that ER Ca^{2+} uptake via SERCA pumps was inhibited. As shown in Figure 4A, after being pre-incubated in thapsigargin-containing (5 μM) Ca^{2+}-free Ringer's solution for 20 min, re-introduction of $[Ca^{2+}]_o$ (2 mM) caused a transient increase of $[Ca^{2+}]_i$ in the tested HC (Fig. 4A). Similar results were obtained from 7 others HCs, demonstrating the existence of SOCE pathways in carp H1 HCs.

To further confirm that this $[Ca^{2+}]_i$ increase activated by ER depletion is mediated by SOCs, 2-APB, a SOC antagonist, was applied at a concentration of 100 μM [32]. As shown in Figure 4B, in the presence of 2-APB (100 μM), $[Ca^{2+}]_i$ increase after $[Ca^{2+}]_o$ re-addition was abolished, however this $[Ca^{2+}]_i$ increase was not eliminated by 100 μM nifedipine (Fig. 4C). Similar results were observed from 7 other HCs for each condition. These results indicate that SOCs rather than L-VGCCs underlay this ER depletion-induced Ca^{2+} entry of HCs.

During the application of 3 mM caffeine, the number of Ca^{2+} transients was increased as the duration of caffeine was increased. At the meantime, the amplitudes of the oscillatory $[Ca^{2+}]_i$ transients were changing within a limited range (Fig. 1A), suggesting the occurrence of ER Ca^{2+} refilling between two adjacent $[Ca^{2+}]_i$ transients. To test whether SOCs underlie ER refilling after caffeine (3 mM) application, 2-APB (100 μM) was applied.

In normal Ringer's solution, $[Ca^{2+}]_i$ oscillations were induced by prolonged application of 3 mM caffeine (7 min; Fig. 5A). However, such caffeine-induced $[Ca^{2+}]_i$ oscillations were abolished when 2-APB (100 μM) was co-applied after 2 min caffeine application (Fig. 5B). For 2-APB treated group, the number of $[Ca^{2+}]_i$ transients induced by caffeine (3 mM, 7 min) was 2.22 ± 0.15, which was significantly reduced as compared with 8.57 ± 0.81 in normal Ringer's solution (control) ($p<0.05$, independent-samples t test; Fig. 5C).

Taken together, these results show that SOCs are expressed in carp H1 HCs, and can be activated by thapsigargin-induced ER depletion; when depleted by caffeine, the ER can be refilled by Ca^{2+} entry via SOCs.

Discussion

In the present study, we investigated the mechanisms of release, depletion, and refilling of ER Ca^{2+} in carp retinal HCs. H1 HCs of carp were stimulated by caffeine under various conditions, $[Ca^{2+}]_i$ changes were recorded using a fluo-3-based Ca^{2+} imaging technique.

Our experimental results demonstrated that exogenous application of caffeine induced $[Ca^{2+}]_i$ increases in a concentration- and duration-dependent manner, with each $[Ca^{2+}]_i$ transient initiated by RyR activation. Extracellular Ca^{2+} was required for ER replenishment in the caffeine-induced $[Ca^{2+}]_i$ oscillations. Ca^{2+} influx via L-VGCCs contributed to the ER refilling, while SOCs expressed on H1 HCs were necessary for the ER refilling.

Temporal Patterns of the Caffeine-induced $[Ca^{2+}]_i$ Responses

Caffeine has long been used as a RyR agonist for studying RyR-mediated Ca^{2+} release from the intracellular Ca^{2+} stores [15,16], and caffeine-induced Ca^{2+} release has been observed in many types of neurons containing the ryanodine-sensitive ER [5]. In general, when the ER is filled with Ca^{2+}, caffeine application can induce $[Ca^{2+}]_i$ increases even in the absence of extracellular Ca^{2+}, and caffeine-induced Ca^{2+} responses can be blocked by high concentration of ryanodine. Such processes were also observed in the present study. However, the temporal characteristics of caffeine-induced Ca^{2+} responses varied with cell types. When exposed to caffeine, transient increase in $[Ca^{2+}]_i$ was observed in neurons such as carp retinal bipolar cells, rat spiral ganglion neurons, and rat primary sensory neurons [33,34,35], while sustained increase in $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ oscillations were observed in honeybee photoreceptors [36] and bullfrog sympathetic neurons [37,38], respectively. Meanwhile, elevation in caffeine concentration (5–30 mM) increased the frequency of caffeine-induced Ca^{2+} oscillations in bullfrog sympathetic neurons. Similar to bullfrog

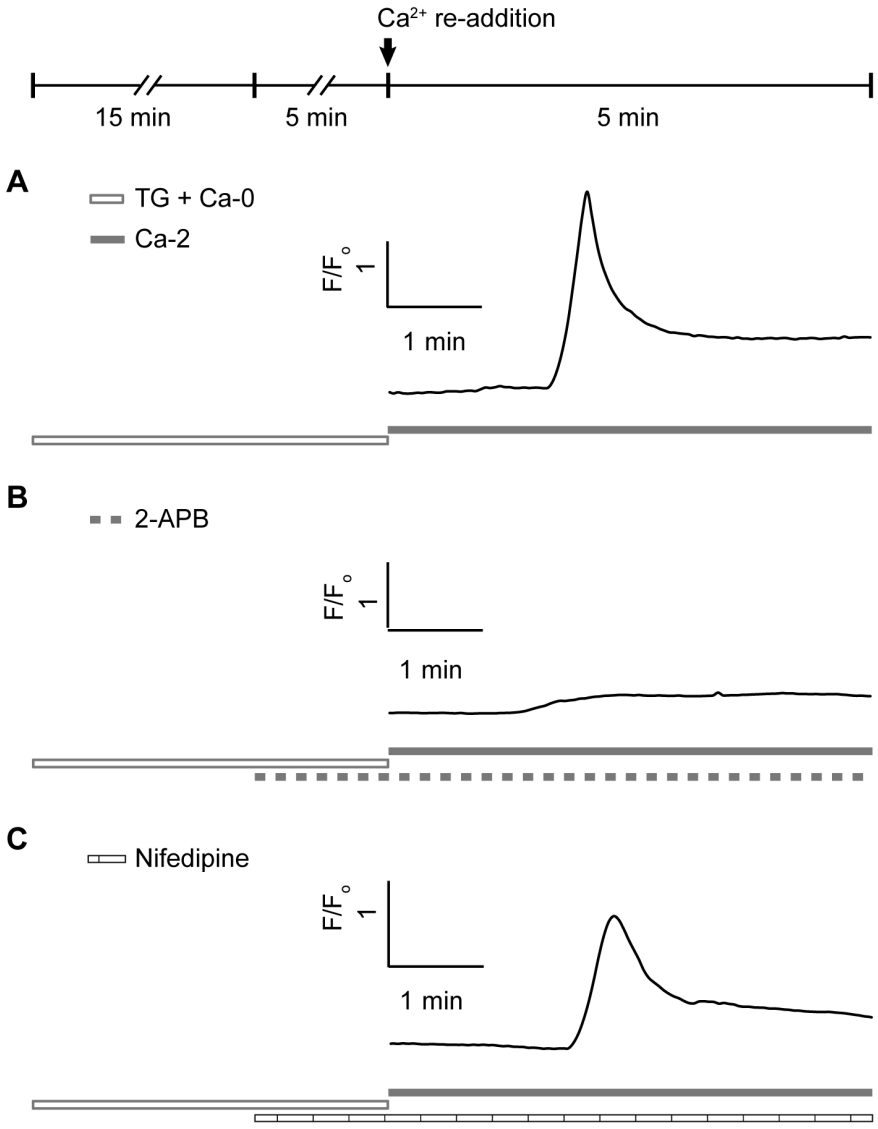

Figure 4. SOCs were nessicary for ER depletion-induced Ca^{2+} entry. A, After pre-application of thapsigargin (TG, 5 μM) in Ca^{2+}-free Ringer's solution for 20 min to deplete ER, reintroduction of $[Ca^{2+}]_o$ (2 mM) elicited a transient increase in $[Ca^{2+}]_i$. B, In the presence of 2-APB (a SOC inhibitor, 100 μM), the $[Ca^{2+}]_i$ increase induced by $[Ca^{2+}]_o$ re-addition was abolished. C, In the presence of nifedipine (a L-VGCC blocker, 100 μM), the $[Ca^{2+}]_i$ increase after $[Ca^{2+}]_o$ re-addition was still observed. Abbreviations: Ca-0: Ca^{2+}-free; Ca-2:2 mM $[Ca^{2+}]_o$. Traces shown are representative of three independent experiments, eight HCs were tested under each condition.
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sympathetic neurons, caffeine (3–20 mM) also induced oscillatory responses in carp H1 HCs in a concentration-dependent manner – when the concentration of caffeine was increased, the time interval between the two adjacent response peaks was decreased. However, the frequency ranges of caffeine-induced $[Ca^{2+}]_i$ oscillations observed in bullfrog sympathetic neurons were much lower when compared with that in carp H1 HCs. On the other hand, while the amplitudes of oscillatory caffeine-induced $[Ca^{2+}]_i$ increases were basically unaltered in bullfrog sympathetic neurons during the time course of caffeine application, a decreasing tendency was observed in the amplitudes of the oscillatory caffeine-induced $[Ca^{2+}]_i$ increases in carp H1 HCs, such differences might result from the differences in frequency ranges. At low oscillation frequencies, depleted ER might be fully refilled, so the amplitudes of the oscillatory caffeine-induced $[Ca^{2+}]_i$ increases were basically unaltered in bullfrog sympathetic neurons, while that measured in carp H1 HCs in our experiments had a decreasing tendency.

The Molecular Mechanisms of SOCE

SOCE is a process by which the depletion of ER Ca^{2+} activates Ca^{2+} influx across the plasma membrane (PM) [30], the concept of which was proposed by Putney in 1986 [29]. Since then, growing evidence revealed that SOCE is a ubiquitous Ca^{2+} influx pathway that exists in a variety of cell types, including neurons [39] and non-excitable cells [40]. In recent years, it was found that stromal interaction molecule (STIM) proteins are sensors of the ER Ca^{2+} content [41,42]. The EF-hand domain of STIM residing in the ER lumen senses the free Ca^{2+} concentration inside the lumen of the ER ($[Ca^{2+}]_{ER}$). Two types of STIM-regulated SOCs have been described: the Orai channels [43,44,45] and transient receptor potential canonical (TRPC) channels [46,47]. Upon store deple-

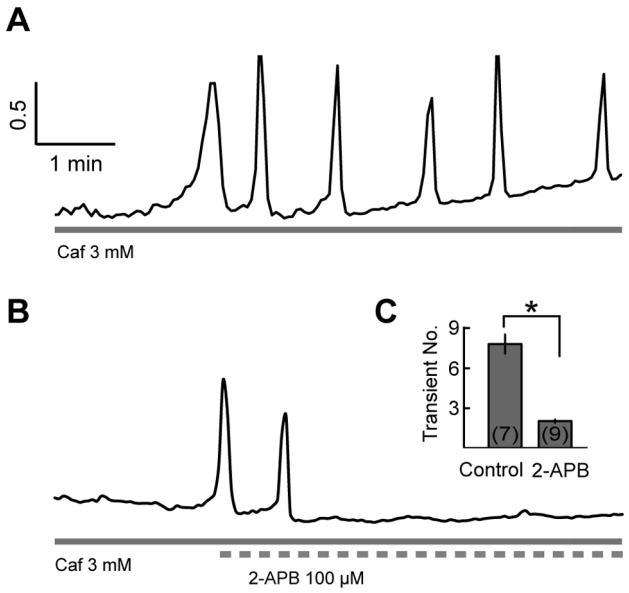

Figure 5. 2-APB effect on the caffeine-induced $[Ca^{2+}]_i$ oscillations. A, $[Ca^{2+}]_i$ oscillations induced by prolonged application of caffeine (3 mM, 7 min). B, When 2-APB (100 μM) was co-applied, $[Ca^{2+}]_i$ oscillations elicited by 3 mM caffeine was abolished. C, The average number of Ca^{2+} transients induced by caffeine (3 mM, 7 min) in the control group and the 2-APB group. * denotes statistical significance of $p<0.05$ with independent-samples *t* test.
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tion, STIM proteins and SOCs translocate and cluster at the PM-ER junctions, leading to the formation of the STIM-Orai [44,48] and STIM-TRPC [49] complexes and SOC activation. Activated SOCs mediateCa^{2+} influx to refill depleted intracellular stores and regulate cellular processes [50,51]. The Orai channels and TRPC channels can both be inhibited by 2-APB [32,52,53]. So based on our experimental results, it's hard to tell whether it is Orai channels or TRPC channels that mediated the store-operated Ca^{2+} entry in HCs.

SERCA is one of the proteins identified as a part of the SOC macromolecular complex [54]. Experiments performed on HEK293T cells and HeLa cells demonstrated that SERCA co-localized with STIM at the PM-ER junctions following store depletion, and co-localization of SERCA with the STIM-Orai complex resulted in a tight coupling between SOCE and ER refilling, so that Ca^{2+} entry via SOCs was mainly transported into the ER [55,56,57]. In our current study, in the process of caffeine-induced $[Ca^{2+}]_i$ oscillations, ER refilling occurred during the interval between two adjacent $[Ca^{2+}]_i$ transients, during which $[Ca^{2+}]_i$ was relatively low, suggesting that the majority of Ca^{2+} entering the cytoplasm via SOCs was transported into the ER. This phenomenon reflected a coupling between the SOCs and SERCA pumps in carp H1 HCs.

In our experiments, 2-APB was used to inhibit SOCE (Fig. 4–5). Although 2-APB has been proven a reliable SOC inhibitor [32], it was also reported that 2-APB can affect hemi-gap-junction (HGJ) channels [58] and IP_3Rs [59].

HGJ channels are expressed in fish retinal HCs, and are involved in negative feedback from HCs to cones [60,61,62]. These channels are gated by factors including membrane potential and $[Ca^{2+}]_o$ [63,64,65]. HGJ channels mediate inward currents at negative membrane potentials and outward currents at positive membrane potentials. Both the inward and outward currents are inhibited by high $[Ca^{2+}]_o$ in a concentration dependent manner [65]. In our experiments, isolated HCs had a resting membrane potential of about −70 mV, and $[Ca^{2+}]_o$ was remained constant at a level of 2 mM, and a small fraction of HGJ channels might be active. However, the application of 2-APB (100 μM) had no significant effect on the basal level of $[Ca^{2+}]_i$ (data not shown), suggesting that even if HGJ channels were active, the inward HGJ channel currents had little contribution to $[Ca^{2+}]_i$ responses observed in the present study. Outward HGJ channel currents with amplitudes larger than the inward currents can be evoked by membrane depolarization (beyond 10 mV) [66]. However, the caffeine-induced Ca^{2+} oscillations were abolished by inhibiting either Ca^{2+} influx or ER Ca^{2+} release. Therefore, it is unlikely that 2-APB abolished Ca^{2+} oscillations by inhibiting outward HGJ channel currents.

In regard to IP_3Rs, no study suggests the expression of IP_3Rs in carp retinal HCs. Besides, in experiments shown in Figure 4, given that the ER was permanently depleted by thapsigargin, IP_3Rs which mediate Ca^{2+} release from the ER were not involved in the $[Ca^{2+}]_i$ increase observed after re-addition of extracellular Ca^{2+}. Therefore, it is unlikely that 2-APB abolished $[Ca^{2+}]_i$ signals observed in our experiments by inhibiting IP_3Rs.

Taken together, despite the fact that 2-APB affects channels other than SOCs, we can still make an inference that there exists a SOCE pathway in carp H1 HCs, which reloads the ER after its depletion.

The Mechanism of the Caffeine-induced $[Ca^{2+}]_i$ Oscillations in H1 HCs

Our results showed that caffeine at low concentration (1 mM) elicited no $[Ca^{2+}]_i$ changes in H1 HCs, and a single $[Ca^{2+}]_i$ transient was evoke by high concentration (40 mM) caffeine, while $[Ca^{2+}]_i$ oscillations were induced by caffeine at intermediate concentrations (3 to 20 mM). The present results indicated that each $[Ca^{2+}]_i$ transient was initiated by RyR activation, and ER refilling, which was required for the generation of subsequent $[Ca^{2+}]_i$ transients, was depending on SOCE. Hence RyRs and SOCs are likely the two major components for caffeine-induced $[Ca^{2+}]_i$ oscillations.

Researches on caffeine effect on single RyR channel found that RyR open probability (P_o) was increased when caffeine level was elevated [6,67]. Low dose of caffeine (<1 mM) increased the P_o by increasing the opening frequency without altering the opening duration. While at high doses, caffeine elevated the P_o by increasing both the frequency and the duration of opening events. The relationship between RyR P_o and caffeine concentration could explain the dependence of the $[Ca^{2+}]_i$ response pattern on caffeine concentration observed in our present study. When caffeine was applied at low level (1 mM), the RyR P_o was very small [6]. With low RyR opening frequency and duration, caffeine was unable to trigger CICR from the ER. On the other hand, upon the application of high dose caffeine (40 mM), with RyR P_o significantly increased and approaching 1.0 [6], ER was constantly leaky, making it impossible to accumulate Ca^{2+} after the initial release, therefore only one Ca^{2+} transient was generated.

In addition to SOCs, Ca^{2+} influx via L-VGCCs also contributed to ER refilling. The involvement of L-VGCCs in caffeine-induced $[Ca^{2+}]_i$ oscillations suggests that the membrane potential of H1 HCs should be depolarized upon caffeine application. The application of caffeine triggered ER Ca^{2+} release and subsequent Ca^{2+} influx. In some cell types, the increase in $[Ca^{2+}]_i$ leads to the activation of several kinds of plasma membrane ion channels, including Ca^{2+}-activated chloride channels, potassium channels, and non-selective channels [68,69,70,71]. Since no evidence suggests that these channels are expressed in retinal horizontal

cells [26], we infer that the main reason for membrane depolarization upon caffeine application was Ca^{2+} currents.

Taking all factors into consideration, we propose the following mechanisms underlying the caffeine-induced $[Ca^{2+}]_i$ oscillations (Fig. 6). When caffeine was applied at intermediate concentrations (3 to 20 mM), RyR P_o was at intermediate levels, which was sufficient for CICR. Upon application, caffeine increased the P_o of the RyR, leading to CICR from the ER. This resulted in an increase in $[Ca^{2+}]_i$ and ER depletion, forming the rapid increasing phase of the $[Ca^{2+}]_i$ transient. The elevation of $[Ca^{2+}]_i$ and the decreasing of $[Ca^{2+}]_{ER}$ further caused the activation of PMCA pumps, Na^+/Ca^{2+} exchangers and SERCA pumps. PMCA pumps and Na^+/Ca^{2+} exchangers extruded Ca^{2+} into the extracellular space, while SERCA pumps transported Ca^{2+} into the ER. The activities of these pumps and exchangers brought $[Ca^{2+}]_i$ back to the baseline level, forming the decay phase of the $[Ca^{2+}]_i$ transient, thus the first Ca^{2+} transient was generated.

Meanwhile, ER depletion activated SOCs on the PM, activated SOC mediated Ca^{2+} influx from the extracellular space. With a tight coupling between SOCE and ER refilling, Ca^{2+} influx via SOCs was mainly transported into the ER by SERCA pumps without disturbing $[Ca^{2+}]_i$.

Due to the activity of SERCA pumps and SOCs, the ER was refilled after the first $[Ca^{2+}]_i$ transient, partially or fully. At this moment, if caffeine was continuously applied, CICR could be re-activated, and a second or more $[Ca^{2+}]_i$ transients could be generated, thereby forming $[Ca^{2+}]_i$ oscillations. The higher the caffeine concentration, the larger the RyR P_o, and the shorter it took for CICR initiation, thus ΔT_{1-2} was decreased when the caffeine concentration was increased.

The amplitudes of the subsequent $[Ca^{2+}]_i$ transients depend on the $[Ca^{2+}]_{ER}$ level. ΔT_{1-2} was decreased when the concentration of caffeine was increased, which means that time duration for ER refilling was decreased when the caffeine concentration was increased, therefore the A_2/A_1 ratio was decreased when the caffeine concentration was increased. For $[Ca^{2+}]_i$ oscillations induced by 3 mM caffeine, the time interval between two adjacent $[Ca^{2+}]_i$ transients was relatively long, which might result in the full refilling of the ER. Thus the amplitudes of Ca^{2+} transients generated during the time course of caffeine application were remained within a limited range. For $[Ca^{2+}]_i$ oscillations induced by higher concentrations of caffeine (6–20 mM), short interval between two adjacent $[Ca^{2+}]_i$ transients resulted in inadequate ER replenishment, thus the amplitudes of the oscillatory Ca^{2+} transients were gradually decreased until the ER was finally running out of Ca^{2+}.

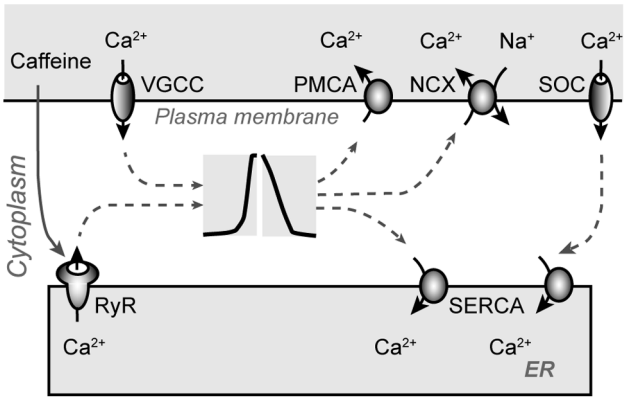

Figure 6. Schematic illustration of Ca^{2+} pathways relevant to the caffeine-induced $[Ca^{2+}]_i$ oscillations in carp retinal HCs. SOC: store-operated channel; VGCC: voltage-gated Ca^{2+} channel; PMCA: plasma membrane Ca^{2+}-ATPase; NCX: Na^+/Ca^{2+} exchanger; RyR: ryanodine receptor; SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase; ER: endoplasmic reticulum.
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Ca^{2+} Signaling in Carp HCs

Similar to other cell types, Ca^{2+} is an important intracellular messenger in carp HCs. $[Ca^{2+}]_i$ increase is involved in many HCs cellular functions, including modulation of GABA transporter currents [11,12], modulation of VGCC currents [13], modulation of synaptic connections [10], as well as the plasticity of spinules at dendrites [21,72], gating of HGJ channels [63], and maintenance of electrical coupling between HCs [73], etc. The universality of Ca^{2+} signaling requires the expression of many Ca^{2+}-related components to create a wide range of spatially and temporally distributed signals [2].

In the vertebrate retina, glutamate is continuously released from photoreceptors in the dark, thus GluRs on HCs can be tonically activated. Activated ionotropic GluRs mediated cation influx (including Ca^{2+}), leading to HC depolarization, VGCC activation, CICR from the ER and an increase in $[Ca^{2+}]_i$ [8,9,23]. A sustained high $[Ca^{2+}]_i$ is cytotoxic [74]. Besides, after carrying out its signaling functions, elevated $[Ca^{2+}]_i$ must be brought back to the basal level so that the cell can be ready for response to the subsequent stimulus. After the initial $[Ca^{2+}]_i$ increase, AMPA receptor desensitization [75], VGCC inhibition via store depletion [13,76], and VGCC inhibition via AMPA receptor activation [13] might help to restrict Ca^{2+} influx, regulating $[Ca^{2+}]_i$ increases and CICR from the ER. Hence, oscillatory $[Ca^{2+}]_i$ responses, such as that observed in the present study, might be one of the strategies that HCs adopt in exposure to tonic glutamate input, so as to prevent cytotoxicity, as well as regulating $[Ca^{2+}]_i$ by the rate and duration of photoreceptor glutamate release.

Oscillations of the membrane potential in response to a brief light flash have been recorded from HCs in the intact retina [77,78]. Since the membrane potential and $[Ca^{2+}]_i$ are strongly correlated, we infer that $[Ca^{2+}]_i$ oscillations can be induced under physiological conditions. But the frequency ranges of the membrane potential oscillations were between 1.5 and 3.5 Hz, much higher than the $[Ca^{2+}]_i$ oscillations recorded in our experiments. In our experiments, bath application of caffeine affects RyRs in isolated H1 HCs, leading to the generation of global intracellular Ca^{2+} signals. While in the intact retina, HCs receive input from synapses of rods and cones, producing localized Ca^{2+} signals. Depletion of the ER in a restricted area takes shorter time to refill, while depletion of the whole ER as observe in the current study takes longer to refill, this might account for the differences in oscillation frequencies observed in HCs in the intact retina and isolated HCs.

Generally, caffeine-induced $[Ca^{2+}]_i$ oscillations in carp H1 HCs is the repetition of the following two steps: ER Ca^{2+} release and subsequent ER refilling. Ca^{2+} release from the ER is mediated by RyRs, while ER refilling depends primarily on Ca^{2+} influx via SOCs. Hence the SOC is an essential component for ER refilling, and its activation is required for the maintenance of $[Ca^{2+}]_i$ oscillations. The ER is the primary intracellular reservoir of Ca^{2+} and a major source of $[Ca^{2+}]_i$ elevation, and it is involved in many cellular processes. The existence of SOCs in H1 HCs and the coupling between SOCE and ER refilling guarantee the efficient replenishment of the ER so that the cell can be ready for response to the subsequent stimulus. Thus SOCs are essential for the fulfilling of normal physiological functions of carp H1 HCs.

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Author Contributions

Conceived and designed the experiments: TL PJL. Performed the experiments: TL HQG. Analyzed the data: TL HQG. Contributed reagents/materials/analysis tools: TL HQG PJL. Wrote the paper: TL PJL.

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