Congo red modulates ACh-induced Ca\(^{2+}\) oscillations in single pancreatic acinar cells of mice

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Aim: Congo red, a secondary diazo dye, is usually used as an indicator for the presence of amyloid fibrils. Recent studies show that congo red exerts neuroprotective effects in a variety of models of neurodegenerative diseases. However, its pharmacological profile remains unknown. In this study, we investigated the effects of congo red on ACh-induced Ca\(^{2+}\) oscillations in mouse pancreatic acinar cells in vitro.

Methods: Acutely dissociated pancreatic acinar cells of mice were prepared. A U-tube drug application system was used to deliver drugs into the bath. Intracellular Ca\(^{2+}\) oscillations were monitored by whole-cell recording of Ca\(^{2+}\)-activated Cl\(^{–}\) currents and by using confocal Ca\(^{2+}\) imaging. For intracellular drug application, the drug was added in pipette solution and diffused into cell after the whole-cell configuration was established.

Results: Bath application of ACh (10 nmol/L) induced typical Ca\(^{2+}\) oscillations in dissociated pancreatic acinar cells. Addition of congo red (1, 10, 100 µmol/L) dose-dependently enhanced Ach-induced Ca\(^{2+}\) oscillations, but congo red alone did not induce any detectable response. Furthermore, this enhancement depended on the concentrations of ACh: congo red markedly enhanced the Ca\(^{2+}\) oscillations induced by ACh (10–30 nmol/L), but did not alter the Ca\(^{2+}\) oscillations induced by ACh (100–10000 nmol/L). Congo red also enhanced the Ca\(^{2+}\) oscillations induced by bath application of IP\(_3\) (30 µmol/L). Intracellular application of congo red failed to alter ACh-induced Ca\(^{2+}\) oscillations.

Conclusion: Congo red significantly modulates intracellular Ca\(^{2+}\) signaling in pancreatic acinar cells, and this pharmacological effect should be fully considered when developing congo red as a novel therapeutic drug.

Keywords: Congo red; pancreatic acinar cell; ACh; IP\(_3\); Ca\(^{2+}\) oscillation; whole-cell recording; Ca\(^{2+}\) image

Original Article

Introduction

Congo red is a secondary diazo dye that is usually used as an indicator for the presence of amyloid fibrils and is one of the model compounds that has been extensively studied for its interaction with amyloid beta peptide (Aβ)[1–5]. In addition, it has been found that congo red can block Aβ-formed ion channels[6,7]. Because congo red can stabilize Aβ monomers, markedly decrease the oligomer/monomer ratio, inhibit amyloid fibril formation, and reduce Aβ-amyloid neurotoxicity[8,9], it has been thought to be a candidate for the development of an alternative therapeutic treatment of AD[5]. However, the effects of congo red on cellular biology are not well investigated, and whether congo red modulates intracellular Ca\(^{2+}\) signaling is unknown.

Pancreatic acinar cells play an important role in producing and secreting enzymes for the proper digestion of food. Elevated cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_i\)) is the critical early signal that triggers the release of digestive enzymes from pancreatic acinar cells[10,11]. Acetylcholine (ACh) and cholecystokinin (CCK) are two typical agonists that activate these intracellular Ca\(^{2+}\) oscillations, and such agonist-induced Ca\(^{2+}\) oscillations have been well studied as a classical cellular model of intracellular Ca\(^{2+}\) signaling[12]. The agonist (ACh or CCK) binds to its respective surface membrane receptor to activate second messenger (inositol 1,4,5-trisphosphate [IP\(_3\)])-mediated Ca\(^{2+}\) release from cytoplasmic stores, such
as the endoplasmic reticulum (ER)\(^{13,14}\), and to induce \([\text{Ca}^{2+}]\), elevation. Thereafter, \(\text{Ca}^{2+}\) ATPase pumps on the plasma membrane of the ER are activated to transport \(\text{Ca}^{2+}\) from the cytosol back to the ER. Meanwhile, the depletion of intracellular \(\text{Ca}^{2+}\) stores can activate store-operated or \(\text{Ca}^{2+}\) release-activated \(\text{Ca}^{2+}\) entry (CRAC)\(^{15,16}\) to refill \(\text{Ca}^{2+}\) pools from the extracellular environment. Together, these mechanisms maintain the agonist-induced \(\text{Ca}^{2+}\) oscillations in the presence of persistent extracellular agonists. This is an ideal cell model with which to study the effects of various new drugs on intracellular \(\text{Ca}^{2+}\) signaling.

In the present study, we evaluate the effect of congo red on agonist-induced \(\text{Ca}^{2+}\) oscillations in mouse pancreatic acinar cells using both patch-clamp whole-cell recording (measurement of \(\text{Ca}^{2+}\)-dependent \(\text{Cl}^{–}\) current) and \(\text{Ca}^{2+}\) imaging approaches. We found that congo red modulates \(\text{ACH}\)-induced \(\text{Ca}^{2+}\) oscillations and that this modulation depends on the concentrations of both congo red and \(\text{ACH}\). Congo red itself cannot induce any detectable \(\text{Ca}^{2+}\) responses under patch-clamp whole-cell recording conditions, and congo red modulates \(\text{Ca}^{2+}\) oscillations from extracellular, but not intracellular, targets. Collectively, these data suggest that congo red can modulate intracellular \(\text{Ca}^{2+}\) signaling in pancreatic acinar cells and that this pharmacological effect should be fully considered when developing congo red as a novel therapeutic drug.

### Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Barrow Neurological Institute.

**Acutely dissociated mouse pancreatic acinar cells**

Single pancreatic acinar cells were dissociated as previously described\(^{17-19}\). Briefly, adult (4–6 months old) male CD1 mice were anesthetized with isoflurane, and then the pancreas was rapidly removed and injected with collagenase (200 U/mL) dissolved in standard extracellular solution (see below). The tissue was treated with collagenase (200 U/mL) for 20–25 min at 37°C. Following collagenase digestion, the cell suspension was gently pipetted to further dissociate the cells and then washed three times using oxygenated standard external solution. Next, 150 µL of the suspension was transferred into 2 mL of standard extracellular solution in a 35-mm culture dish. The isolated cells were usually utilized within 3 h after dissociation for patch-clamp recording or \(\text{Ca}^{2+}\) imaging.

**Patch-clamp, whole-cell recording**

\(\text{Ca}^{2+}\)-activated \(\text{Cl}^{–}\) currents were recorded using conventional whole-cell recordings to monitor intracellular \(\text{Ca}^{2+}\) signal oscillations as previously reported\(^{17-19}\). When the recording pipette was filled with the \(\text{K}^{+}\)-containing pipette solution, the resistance was 3–4 MΩ. After the formation of a GΩ seal between the cell membrane and pipette, a whole-cell recording mode was achieved by further brief suction. For perforated-patch recordings, the recording electrodes were filled with amphotericin B (150–200 µmol/L) dissolved in pipette solution (see below). The holding potential (\(V_{\text{h}}\)) for patch recording was -30 mV, and the series-resistance was not compensated. Transmembrane currents were recorded with a patch-clamp amplifier (Axopatch 200B; Molecular Devices; Sunnyvale, CA USA).

**Confocal imaging to measure \(\text{Ca}^{2+}\) oscillations**

Single pancreatic cells were first incubated with the \(\text{Ca}^{2+}\) indicator, Fluo-4-AM (15 µmol/L) (Molecular Probes, Eugene, OR USA) for 15 min, followed by a 10 min incubation period to allow for de-esterification of the indicator. Confocal imaging was performed using an Olympus Fluoview FV1000 microscope (Olympus Corporation) equipped with an argon laser (488 nm) and an UPLSAPO 40×, 0.95 NA objective. X–Y imaging was performed at a rate of 1.644 s per frame, while acquiring 400 frames in total with a resolution of 512×512. The fluorescent Fluo-4 signal was measured using ImageJ v.1.47 (available from the US National Institutes of Health, Bethesda, MD USA; http://image.nih.gov/ij/).

**Solution and chemicals**

The standard extracellular solution contained (in mmol/L) 140 NaCl, 1.0 CaCl\(_2\), 4.7 KCl, 1.13 MgCl\(_2\), 10 glucose, and 10 HEPES, adjusted to pH 7.2 with NaOH. The pipette solution contained (in mmol/L) 140 KCl, 1.13 MgCl\(_2\), 5 Na\(_2\)ATP, 0.24 EGTA, 10 glucose and 10 HEPES, adjusted to pH 7.2. Congo red (Figure 1), \(\text{ACH}\), amphotericin B and IP\(_3\) were purchased from Sigma-Aldrich (St Louis, MO USA). 2-APB was purchased from Tocris Bioscience (Minneapolis, MN USA). Congo red (Supplemental Figure 1) was dissolved with distilled water into a 10 mmol/L stock solution and was diluted with external solution to the final concentrations just before use. During patch recording, the recorded cell was continuously perfused with a stream of standard extracellular solution. A U-tube drug application system was used to deliver drugs via the bath. For intracellular drug applications, the test drug was added to pipette solution, and the establishment of the whole-cell configuration allowed the drug to diffuse into the cell.

![Figure 1. Chemical structure of congo red.](image-url)
over a certain time (usually for 3 min). Control values were measured from the baseline of ACh (or IP3)-induced Ca2+ oscillations for approximately 3 min (normalized to 1) and were compared to the changes induced by congo red exposure. For Ca2+ imaging, the amplitude of the Ca2+ signal was calculated as: ΔR=ΔF/F0, where F0 refers to the background Fluo-4 signal. Student’s t-test was used to analyze paired or unpaired data. A value of P<0.05 was considered statistically significant.

Results
Effects of congo red on ACh-induced Ca2+ oscillations
In the initial experiments, we tested the effects of congo red on ACh-induced Ca2+ oscillations. Bath application of 10 nmol/L ACh induced typical Ca2+ oscillations under patch-clamp whole-cell recordings (Figure 2A). Different concentrations of congo red were applied to the recorded cell in the presence of 10 nmol/L ACh, which potentiated ACh-induced current net charge in a concentration-dependent manner (Figure 2A–2D). These results suggest that congo red significantly enhances Ca2+ oscillations induced by 10 nmol/L ACh in a concentration-dependent manner.

Figure 2. Congo red potentiates ACh-induced Ca2+ oscillations in a concentration-dependent manner. (A–C) Representative traces showing that in the presence of 10 nmol/L ACh, bath-applied congo red enhanced Ca2+ oscillations in a concentration-dependent manner. Traces (A–C) were recorded from the same cell. (D) The bar graph indicates potentiation of the net charge of the normalized current in the ACh-induced response by different concentrations of congo red. In this and the following figures, the numbers inside each column indicate the number of cells tested. The vertical bars represent the Mean±SEM. *P<0.01.

Does congo red alone induce intracellular Ca2+ oscillations?
Next, we addressed this question. Congo red alone at various concentrations did not induce any detectable response under patch-clamp recording conditions (Figure 3A–3C). However, after congo red exposure, application of ACh (10 nmol/L) induced enhanced Ca2+ oscillation responses in a congo red concentration-dependent manner (Figure 3D). These results suggest that congo red does not directly promote intracellular Ca2+ release, but it does modulate ACh-induced Ca2+ oscillations.

Figure 3. Congo red alone fails to induce a detectable current response under patch-clamp recording conditions. (A–C) Bath-applied congo red alone at different concentrations failed to induce any detectable current response. However, when ACh (10 nmol/L) was immediately bath-applied after congo red application, it induced enhanced Ca2+ oscillations in a concentration-dependent manner. (D) The bar graph summarizes the effects of ACh-induced current responses (normalized net charge) with different concentrations of congo red pretreatment. *P<0.01. These results suggest that congo red itself is not able to induce Ca2+ oscillations, but it clearly potentiates ACh-induced Ca2+ oscillations.

Effects of congo red on different concentrations of ACh-induced Ca2+ oscillations
Next, we examined the effects of 10 µmol/L congo red on different concentrations of ACh-induced current responses. At low concentrations of ACh (5–30 nmol/L), congo red potentiated ACh-induced Ca2+ oscillations (Figure 4Aa). However, at higher concentrations of ACh (100 nmol/L) that usually
induced a plateau response with limited oscillations, congo red did not alter the ACh-induced response (Figure 4Ab). Similar effects of congo red on ACh-induced Ca\textsuperscript{2+} responses were also observed using Ca\textsuperscript{2+} imaging (Figure 4Ba, 4Bb), whereby 30 µmol/L congo red enhanced the 30 nmol/L ACh-induced Ca\textsuperscript{2+} response to 420.7%±4.5% (n=65 cells from 5 mice, P<0.01) of the control value, while 30 µmol/L congo red did not alter the 100 nmol/L ACh-induced Ca\textsuperscript{2+} response (95.1%±6.5%; n=44 cells from 3 mice, P>0.05). ACh concentration-dependent responses, suggesting that the 100 nmol/L ACh-induced Ca\textsuperscript{2+} response is not the maximal response (Supplemental Figure 1).

These results suggest that congo red modulation of ACh-induced Ca\textsuperscript{2+} oscillations also depends on ACh concentrations.

**Effects of congo red on IP\textsubscript{3}-induced Ca\textsuperscript{2+} oscillations**

Data presented thus far clearly show that congo red enhances low concentration of ACh-induced Ca\textsuperscript{2+} oscillations. What is the mechanism of this modulation? The first possibility is that congo red may modulate muscarinic receptor function. To test this possibility, we examined the effects of congo red on intracellular IP\textsubscript{3}-induced Ca\textsuperscript{2+} oscillations, because such Ca\textsuperscript{2+} oscillations bypass ACh receptors. IP\textsubscript{3} (30 µmol/L) infusion from the recording electrode into the recorded cell induced pulsatile Ca\textsuperscript{2+} oscillations (Figure 5Aa), and bath-applied congo red (10 µmol/L) dramatically enhanced these Ca\textsuperscript{2+} oscillations (Figure 5Ab). Similar results obtained from the 6 cells tested (Figure 5B) suggest that cell surface ACh receptors appear to be unnecessary for the mediation of the congo red-induced potentiation of Ca\textsuperscript{2+} oscillatory responses.

**Effects of intracellular congo red on ACh-induced Ca\textsuperscript{2+} oscillations**

To determine whether congo red can enhance agonist-induced Ca\textsuperscript{2+} oscillations through intracellular sites, we added 10
µmol/L congo red to the recording electrode and determined the effect of bath-application of ACh (10 nmol/L) after the establishment of a whole-cell recording configuration (infusion of congo red into the cytosol). For this experiment, we initially performed perforated patch recordings to examine the effect of bath-application of congo red on ACh-induced Ca\(^{2+}\) oscillations, and then we produced a brief suction to switch the recording mode to conventional whole-cell recording (Figure 6A). In the absence of congo red in the recording electrode, this switch slightly enhanced the ACh-induced oscillation response due to reduced access resistance (from perforated to whole-cell recording), as shown in the control recording in Figure 6A. Then, we compared the effects of this switch with the effects of congo red in the pipette solution (Figure 6Ba) and found there were no differences between the two conditions (Figure 6A and 6Ba, see the statistical comparison in Figure 6C), suggesting the congo red did not enhance Ca\(^{2+}\) oscillations when it was applied via the recording electrode. To confirm this, we applied the same concentration of congo red via bath application to the same recorded cell (in this cell, 10 µmol/L congo red was already infused via the recording electrode), and found a dramatic enhancement of Ca\(^{2+}\) oscillations (Figure 6Bb, C). These results suggest that congo red potentiates 10 nmol/L ACh-induced Ca\(^{2+}\) oscillations through extracellular, rather than intracellular, targets.

**Comparison of the effects of congo red to 2-APB on ACh-induced Ca\(^{2+}\) oscillations**

To explore the possible role of congo red in modulation of store-operated Ca\(^{2+}\) channels (SOCCs), we compared the effects of congo red to the effects of 2-APB, a SOCC blocker, on ACh-induced Ca\(^{2+}\) oscillations. Figure 7 demonstrates that both congo red (Figure 7A) and 2-APB (Figure 7B) potentiated ACh-induced Ca\(^{2+}\) oscillations in the same manner, suggesting that congo red may modulate ACh-induced Ca\(^{2+}\) oscillations through the blockade of SOCCs.

**Discussion**

Congo red was first synthesized in 1883, and it is commonly used as a histological stain for amyloid detection because congo red binds specifically to fibrillary proteins enriched in the β-sheet conformation. This feature, in addition to its small molecular size and ease of penetration of the blood-brain barrier, make congo red useful as a molecular probe for both the antemortem and in vivo visualization and quantification of brain amyloid\(^1\,2\,5\,6\). Interestingly, emerging studies demon-

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**Figure 6.** Effects of intracellular congo red on ACh-induced Ca\(^{2+}\) oscillations. (A) A representative trace showing the patch recording mode switch from perforated patch to whole-cell recording (without congo red in the pipette solution) in the presence of 10 nmol/L bath ACh. (B) The same recording mode switch with a recording electrode containing 10 µmol/L congo red (a), followed by bath application of 10 µmol/L congo red (b). (C) A summary of pooled data from 6A and 6B showing that, in contrast to extracellular application, intracellular application of congo red did not affect ACh-induced Ca\(^{2+}\) oscillations. Therefore, congo red potentiates ACh-induced Ca\(^{2+}\) oscillations via an extracellular target.

**Figure 7.** Effects of 2-APB, a well-known SOCC blocker, on ACh-induced Ca\(^{2+}\) oscillations. (A) A representative typical trace showing that application of 30 µmol/L congo red enhanced ACh (30 nmol/L)-induced Ca\(^{2+}\) oscillations. (B) A representative typical trace showing that application of 30 µmol/L 2-APB enhanced ACh-induced Ca\(^{2+}\) oscillations. Both congo red and 2-APB potentiated ACh-induced Ca\(^{2+}\) oscillations in the same manner, which suggests that congo red may modulate ACh-induced Ca\(^{2+}\) oscillations through the blockade of SOCCs.
strate that congo red can interfere with the processes of protein misfolding and aggregation and stabilizes native protein monomers or partially folded intermediates while reducing the concentration of more toxic protein oligomers. It has been reported that congo red is able to block Aβ aggregation and toxicity in rat hippocampal neuron culture and HeLa and PC12 cells and in human macrophage culture. In addition, congo red can inhibit amyloid toxicity by the blockade of Ca²⁺ permeable amyloid-formed ion channels. Congo red has demonstrated neuroprotective effects in a variety of models of neurodegenerative disorders, such as Alzheimer’s, Parkinson’s, Huntington’s and prion disease. Therefore, understanding the underlying mechanisms of congo red will be instructive for the design of future compounds to monitor and treat neurodegenerative diseases. However, other than binding to fibrillar proteins in a β-sheet conformation, little is known regarding congo red’s effects on cell biology.

We assessed whether congo red modulates intracellular Ca²⁺ signaling due to its importance as an intracellular signaling molecule that can exert allosteric regulatory effects on many enzymes and proteins. The novel and important finding of this study is that congo red significantly enhances low concentrations of ACh-induced Ca²⁺ oscillations.

The targets that mediate the congo red-induced potentiation of intracellular Ca²⁺ signaling are still unclear. The underlying signaling pathway includes ACh activation of the muscarinic receptor, which then activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to form IP₃ and diacylglycerol (DAG), two classical second messengers. DAG activates protein kinase C (PKC), while IP₃ diffuses into the ER and binds to the IP₃ receptor, a Ca²⁺ channel. This results in Ca²⁺ release from the ER to the cytosolic region in a process called IP₃-induced Ca²⁺ release, which can further trigger Ca²⁺-induced Ca²⁺ release via a ryanodine receptor-sensitive Ca²⁺ pool. The resulting depletion of Ca²⁺ from the ER leads to activation of both ER Ca²⁺ pumps and plasma membrane SOCCs. Thus, ACh induces repetitive Ca²⁺ oscillations in pancreatic acinar cells via these mechanisms, any of which could be a target of congo red activity. We observed that congo red exposure significantly enhanced IP₃-induced Ca²⁺ oscillations, suggesting that muscarinic receptor-PLC signaling is not necessary for the congo red-induced potentiation of Ca²⁺ oscillations. We also used Ca²⁺ imaging to demonstrate that congo red enhanced intracellular Ca²⁺ oscillations, suggesting that Ca²⁺-dependent Cl⁻ channels are not involved. However, intracellular application of congo red failed to alter ACh-induced Ca²⁺ oscillations, which clearly suggests that this effect of congo red involves extracellular, but not intracellular, sites.

If congo red’s effect on Ca²⁺ oscillations in pancreatic acinar cells is extracellular, and neither muscarinic receptors nor Ca²⁺-dependent Cl⁻ channels are necessary, then SOCCs could be a potential target of congo red. In fact, we showed that the SOCC antagonist 2-APB potentiated ACh-induced Ca²⁺ oscillations in a manner similar to congo red (Figure 7). Our results, combined with the evidence that congo red exhibits the ability to block Ca²⁺ permeable channels formed by amyloid, suggest that congo red might block SOCC channels in pancreatic acinar cells, wherein low Ca²⁺ concentration can trigger SOCC channel opening to refill the intracellular Ca²⁺ pool. Thus, under resting conditions in the absence of ACh, when Ca²⁺ pools are full and SOCC channels are mainly closed, congo red would not induce Ca²⁺ responses. However, when Ca²⁺ pools are partially emptied by the persistent presence of low concentrations of ACh and SOCC channels are open, congo red could promote Ca²⁺ release as a homeostatic response. In the presence of high concentrations of ACh, Ca²⁺ pools would be depleted, so congo red would not be able to further enhance the Ca²⁺ response, even though SOCC channels are fully opened.

In summary, we demonstrate the novel finding that congo red significantly potentiates agonist-induced Ca²⁺ oscillations in mouse pancreatic acinar cells. Congo red alone does not induce any detectable Ca²⁺ signaling, but potentiates Ca²⁺ oscillations during ACh activation. Congo red-induced enhancement of Ca²⁺ oscillations occurs at low (10–30 nmol/L), but not high (100–1000 nmol/L), concentrations of ACh, and only when applied extracellularly. Based on these data, we propose that congo red might block the SOCC channels and provide a putative explanation of how congo red modulates agonist-induced intracellular Ca²⁺ signaling. Considering that congo red has great potential to be developed as a novel therapeutic drug for treating various neurodegenerative diseases, our findings provide new insights into and improved understanding of the pharmacological mechanism of congo red, especially on intracellular Ca²⁺ signaling.

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**Author contribution**

Ze-bing HUANG performed patch-clamp experiments and wrote the manuscript; Hai-yan WANG designed research; Na-na SUN performed Ca²⁺ imaging experiments; Jing-ke WANG performed Ca²⁺ imaging experiments; Meng-qin ZHAO performed experiments; Jian-xin SHEN designed experiments, analyzed data and wrote part of manuscript; Ming GAO performed experiments; Ronald P HAMMER Jr designed research and wrote and edited manuscript; Xue-gong FAN designed experiments and edited the manuscript; Jie WU designed experiments, analyzed data and wrote manuscript.

**Supplementary information**

Supplementary figure is available at the Acta Pharmacologica Sinica website.

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