

Old dog, new trick: Charybdotoxin as a novel ryanodine receptor blocker

Zsuzsanna Édua Magyar¹, Judit Hevesi^{2,3}, Frigyes Mészáros⁴, Zsolt Bozsó⁵ , János Szolomájer⁵, Gábor Tóth⁵, György Panyi⁶ , Péter Enyedi⁴, Zoltán Varga⁶ , Zoltán Pethő^{6,7}  and János Almássy^{3,4} 

¹Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

²Department of Orthodontics, Faculty of Dentistry, University of Debrecen, Debrecen, Hungary

³Doctoral School of Molecular Medicine, University of Debrecen, Debrecen, Hungary

⁴Department of Physiology, Semmelweis University, Budapest, Hungary

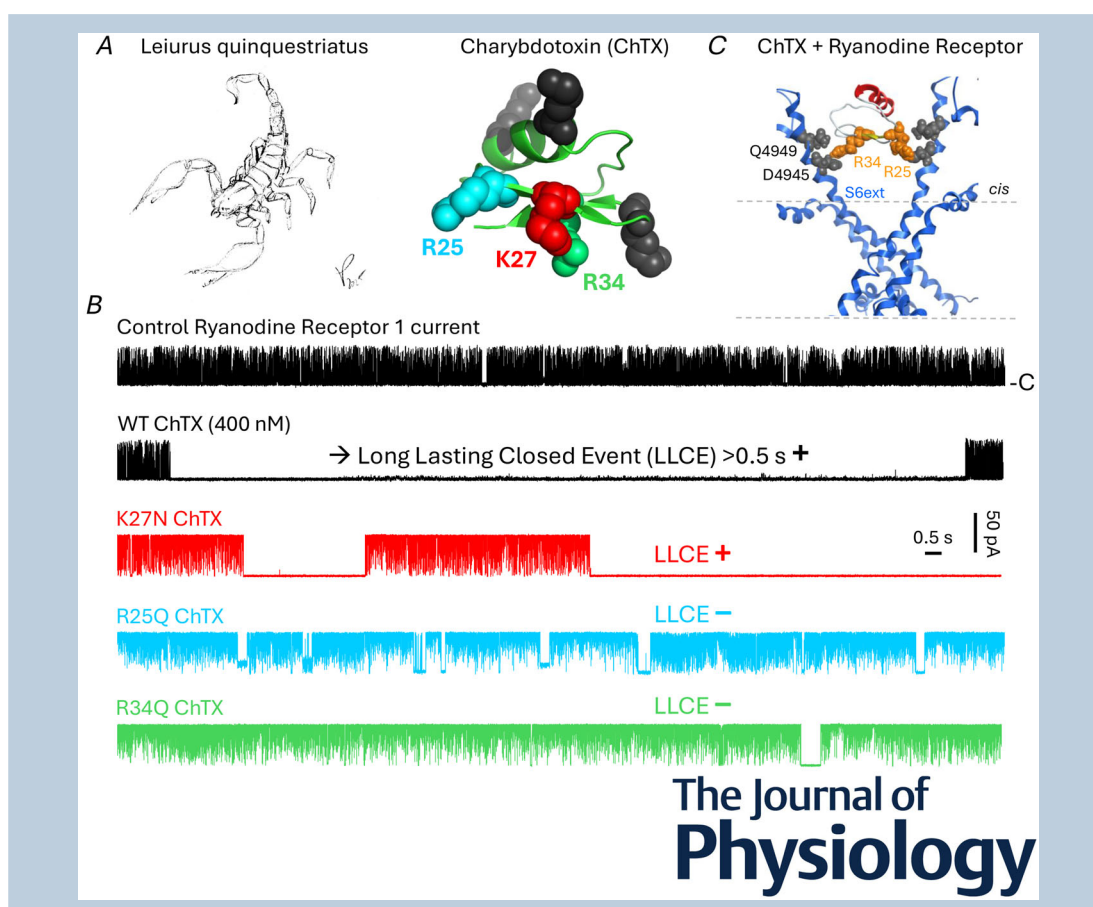
⁵Department of Medical Chemistry, Albert Szent-Györgyi Medical School, MTA-SZTE Biomimetic Systems Research Group, Eötvös Loránd Research Network (ELKH), University of Szeged, Szeged, Hungary

⁶Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

⁷Institute of Physiology II, University of Münster, Münster, Germany

Handling Editors: Eleonora Grandi & Brian Delisle

The peer review history is available in the Supporting Information section of this article (<https://doi.org/10.1113/JP290985#support-information-section>).



Z. É. Magyar and J. Hevesi contributed equally to this work.

Abstract figure legend The peptide toxin charybdotoxin (ChTx) from the scorpion *Leiurus quinquestriatus* was tested on single-channel currents of the ryanodine receptor incorporated into planar lipid bilayers. The structure–function relationship of ChTx was investigated using toxin variants generated by isosteric substitutions of the basic amino acids indicated in (A). Wild-type ChTx induced long-lasting closed events (>0.5 s) (LLCEs) (black trace) in nanomolar concentrations as shown by representative RyR currents in (B). ChTx variants K11N, K31N, R19Q and K27N (red trace) also induced LLCEs, whereas R25Q (cyan) and R34Q (green) did not, suggesting that R25 and R34 are essential for ChTx binding to the channel pore. This conclusion was further supported by *in silico* molecular modelling (C).

Abstract Ryanodine receptors (RyRs) are Ca^{2+} -release channels of the sarcoplasmic reticulum. Because uncontrolled Ca^{2+} -release underlies several muscle and cardiac disorders, pharmacological inhibition of RyRs represents an attractive therapeutic strategy. Natural peptide toxins have provided valuable molecular templates for inhibitor design, with calcins being a particularly promising group. Calcins bind RyRs with high specificity, but, instead of producing the desired full channel block, they stabilize subconductance states that promote Ca^{2+} leak. Given the mechanistic similarity between calcins' action and the well-known charybdotoxin (ChTX)-mediated K^+ channel blockade, we examined whether ChTX showed functional cross-reactivity with RyR channels. Single-channel recordings were performed on skeletal muscle RyRs reconstituted into lipid bilayers. ChTX induced long-lasting (>0.5 s) closed events (LLCEs) in a voltage-dependent manner with an apparent K_d of 85 nM at +60 mV. Under these conditions, channels spent ~ 10 s min^{-1} in the LLCE state. Notably, isosteric substitution of K27, which is essential for K^+ channel block, retained RyR inhibition, indicating a pore interaction mechanistically distinct from that described in K^+ channels. Cardiac RyRs were similarly affected by the K27N variant. Guided by *in silico* docking to the RyR cryo-electron microscopy structure, we mapped the ChTX-RyR interaction surface using five additional ChTX variants, focusing on basic residues. We found that the R25Q and R34Q substitutions abolished LLCE formation, whereas others had no effect or enhanced channel block. These results describe a novel ChTX-RyR interaction mode functionally convergent with calxin peptides, and they also identify K27N ChTX as a selective RyR inhibitor and a promising scaffold for future drug design.

(Received 26 January 2026; accepted after revision 14 July 2026; first published online 10 August 2026)

Corresponding author J. Almássi: Doctoral School of Molecular Medicine, University of Debrecen, Debrecen, Hungary.

Email: almassy.janos@semmelweis.hu

Key points

- Charybdotoxin (ChTX) blocks ryanodine receptor (RyR) single channel currents in bilayer recordings.
- Amino acid residues R25 and R34 are essential for the block.
- K27, which is important for K^+ channel block, is irrelevant in RyR block; thereby, the K27N ChTX variant represents a RyR-selective blocker.
- ChTX shares a common binding site with calxin peptides in the channel vestibule, outside the gate.

Zsuzsanna É. Magyar holds a BSc in Clinical Laboratory Science and an MSc in Molecular Biology. She earned her PhD in Molecular Medicine from the University of Debrecen in 2023. Her research focused on the ryanodine receptor (RyR) ion channel, particularly the Ca^{2+} modulation of the channel and the functional consequences of pathogenic RyR mutations. She is currently doing postdoctoral training at UC Davis. **Judit Hevesi** is a dentist specialized in orthodontics at the Department of Dentistry, Clinical Centre, University of Debrecen, and is also pursuing a PhD at the Doctoral School of Molecular Medicine of the University.



Introduction

Peptide toxins targeting ion channels are regarded as promising leads for the treatment of channelopathies, and their interactions with ion channels are therefore a subject of intensive study. Because these interactions are highly specific and selective, toxins have also been valuable tools for mapping pore architecture before high-resolution ion channel structures became available (Aiyar et al., 1995; Goldstein, 1994; Hidalgo & MacKinnon, 1995; MacKinnon & Miller, 1989; MacKinnon et al., 1990; Naranjo & Miller, 1996; Stampe et al., 1994; Stocker & Miller, 1994). A prototypical example among K^+ channel-targeting peptides is charybdotoxin (ChTX), the founding member of the α -KTx toxin family, designated as α -KTx 1.1.

ChTX is a 37-amino acid peptide originally isolated from the venom of the scorpion *Leiurus quinquestriatus*. ChTX binds with remarkable precision to the extracellular pore region of K^+ channels, in a lock-and-key manner, effectively occluding the pore and preventing ion permeation. The interaction surface of the toxin is critically determined by three positively charged residues: R25, K27 and R34. These side chains establish electrostatic contacts with the outer vestibule of the channel in such a way that the side chain of K27 is positioned into the selectivity filter to interact with the outer K^+ binding site (Fig. 1A) (Goldstein, 1994; Miller et al., 1985; Park & Miller, 1992a, 1992b).

The toxin block exhibits an apparent voltage dependence, even though the receptor site itself does not reside within the transmembrane electric field. Instead, the voltage dependence reflects a dependence on the K^+ efflux. This can be explained by the interference of the K27 side chain with permeating K^+ ions, generating electrostatic repulsion that facilitates toxin dissociation from the pore (i.e. trans-enhanced dissociation). Accordingly, K27 is the sole basic residue responsible for the voltage dependence of toxin block (Banerjee et al., 2013; Goldstein, 1994; MacKinnon & Miller, 1988; Park & Miller, 1992a).

An analogous mechanism has been observed with the peptides of the calxin family, another group of scorpion venom-derived ligands that act on the ryanodine receptor (RyR) (Altafaj et al., 2007; Chi et al., 2019; el-Hayek et al., 1995; Estève et al., 2003; Fajloun et al., 2000; Lee et al., 2004; Lukács et al., 2008; Seo et al., 2006; Tripathy et al., 1998; Valdivia et al., 1992; Xiao et al., 2016).

RyR is a non-selective ion channel abundantly expressed in the sarcoplasmic reticulum (SR) membrane of striated muscles, where it mediates the release of Ca^{2+} required for contraction, under the tight control of the action potential (Bers, 2002; Fill & Copello, 2002; Meissner, 1994). RyRs may carry hundreds of mutations that are associated with malignant hyper-

thermia susceptibility, central core disease, various myopathies or stress-induced arrhythmias and atrial fibrillation (Benkuský et al., 2004; Magyar, Hevesi et al., 2023; Treves et al., 2005; Treves et al., 2008; Zhou H et al., 2006). These RyR-related muscle and cardiac

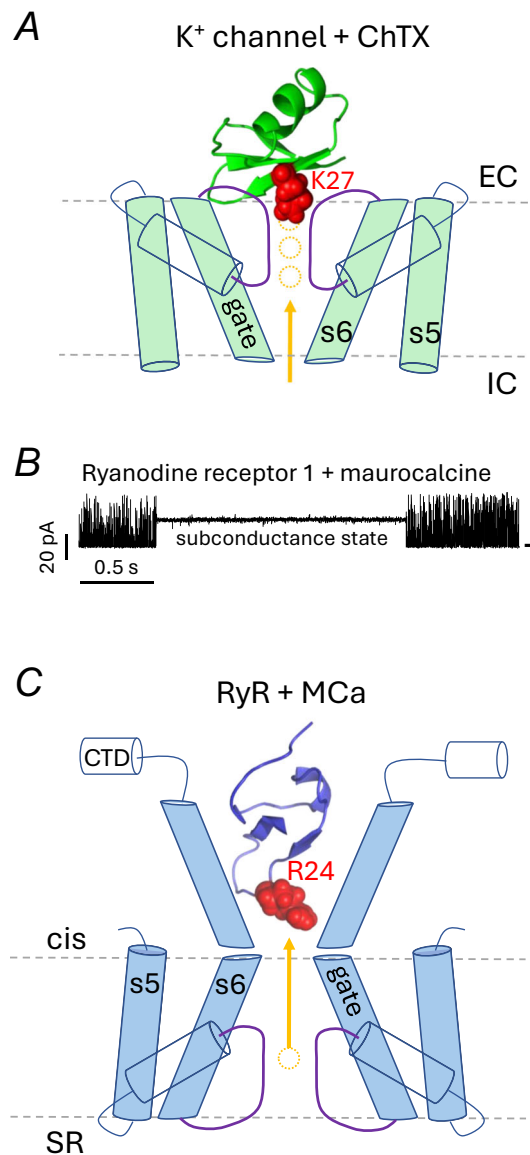


Figure 1. Schematic illustration of ion channel-toxin interactions

A, schematic illustration of charybdotoxin (ChTX) binding to the extracellular (EC) side of a BK channel and blocking it by inserting the side chain of lysine 27 into the ion-conducting pore. The channel gate is formed by the constriction of four S6 transmembrane helices on the intracellular (IC) side. Physiological flow of K^+ (yellow sphere) is shown by the yellow arrow. B, a single RyR1 channel recording in the presence of Mca showing a long-lasting subconductance state. The hyphen on the right indicates the closed state of the channel. C, schematic illustration of maurocalcin (Mca) binding to the cytoplasmic (cis) side of the ryanodine receptor (RyR) vestibule, outside the constriction of the channel gate.

disorders are collectively termed 'ryanopathies', and they share the common feature that the mutant channel escapes the normal regulation by membrane potential leading to spontaneous Ca^{2+} leak at rest or pathological activation in response to specific triggers. Inhibition of such gain-of-function phenotypes would provide an attractive therapeutic strategy, a research direction pioneered by studies on calxin peptides including impercalcin (IpCa; also known as imperatoxin A, IpTx) and maurocalcine (MCA) (Ronjat et al., 2016; Schwartz et al., 2009; Szentandrassy et al., 2022; Xiao et al., 2016).

Calcins are characterized by excellent cell-penetrating properties and high specificity for RyRs (Boisseau et al., 2006; Estève et al., 2005; Poillot et al., 2012; Tisseyre et al., 2013). However, instead of producing a complete channel block as might be therapeutically desirable, they stabilize the channel in a long-lasting subconductance state (LLSS) (Fig. 1B) (el-Hayek et al., 1995; Estève et al., 2003). In earlier work with MCA, we described a mechanism of action strikingly reminiscent of the ChTX mechanism on K^+ channels. Specifically, the duration of LLSS exhibited voltage dependence and substitutional mapping of MCA revealed a positively charged surface patch located on one pole of the toxin as an electrostatic interaction interface, with R24 at its centre, playing an essential role in inducing LLSS (Fig. 1C) (Estève et al., 2003; Lukács et al., 2008).

Although MCA binds to the cytoplasmic side of the RyR, which is structurally analogous to the intracellular face of K^+ channels (as opposed to the extracellular pore targeted by ChTX) (Fig. 1A and C), the similarity between MCA and ChTX in functional architecture and biophysical profile led us to hypothesize that ChTX might also interact with the MCA receptor site on RyR. To test this, we applied ChTX to the cytoplasmic (*cis*) side of purified RyRs reconstituted into planar lipid bilayers and recorded single-channel activity.

We show that nanomolar ChTX induces long-lasting closures of RyR channels in a voltage-dependent manner. We quantitatively characterized the action of ChTX and mapped its active surface. Our analysis revealed that K27 is dispensable for RyR block, whereas R25 and R34 are essential. We also demonstrated competition between MCA and ChTX, supported by molecular modelling showing a perfect fit of ChTX into the MCA-binding site, recently identified by cryo-electron microscopy (cryo-EM) in the channel pore (Haji-Ghassemi et al., 2023).

These data suggest that substitution of K27 with Asn yields a ChTX analogue with reduced potency on K^+ channels, thereby acting as a selective RyR inhibitor. The interaction surface identified by structural modelling in this study reveals a characteristic functional map of the ChTX-RyR complex, which holds promise as a guide for future drug design.

Methods

Ethical approval

All animal procedures were performed in accordance with the ethical guidelines of The Journal of Physiology and complied with the Hungarian Animal Welfare Act and the European Union Directive 2010/63/EU and were approved by the Animal Welfare Committee of the University of Debrecen (22/2012/DEMÁB, 9/2015/DEMÁB).

Animals

RyR1 was purified from rabbit skeletal muscle. Accordingly, 6–10-month-old, male New Zealand White rabbits ($n = 5$) were obtained from an authorized breeder (Juráskó Rabbit Breeding Facility, Debrecen-Józsa, Hungary) and were killed immediately upon arrival at our animal facility; therefore, no husbandry or housing procedures were performed at our institution. Prior to transport, animals had free access to food and water at the breeder facility. The rabbits were anaesthetized with a single i.v. injection of pentobarbital sodium (50 mg kg^{-1}), followed by bilateral thoracotomy.

RyR2 was purified from canine ventricular myocardium ($n = 7$), but no dogs were killed specifically for the purposes of this study, although surplus tissues remaining from an independent animal experiment were collected in accordance with the 3R principles. Young adult (12–20-month-old) mongrel dogs of both sexes were obtained from an authorized distributor and were anaesthetized shortly after arrival to our institution with intramuscular injections of 10 mg kg^{-1} ketamine hydrochloride (Calypsol; Richter Gedeon, Budapest, Hungary) + 1 mg kg^{-1} xylazine hydrochloride (Sedaxylan; Eurovet Animal Health BV, Bladel, The Netherlands). Adequate depth of anaesthesia was confirmed by the loss of the pedal- and righting reflexes and thereafter the heart was surgically removed.

Materials

Charybdotoxin and all the other peptide toxins used in this study were purchased from Alomone Labs (Jerusalem, Israel). Also, charybdotoxin and its variants were synthesized by using a solid phase methodology (see below). Unless otherwise indicated, all other chemicals were obtained from Merck (Sigma-Aldrich, St Louis, MO, USA).

Synthesis of ChTX analogues

ChTX and its analogues (WT, K11N, K31N, K27N, R19Q, R25Q and R34Q) were synthesized using the

standard solid-phase peptide synthesis procedure with an automatic peptide synthesizer (Liberty Blue; CEM, Dublin, Ireland) and TentaGel S RAM resin (Rapp Polymere, Tuebingen, Germany) (loading of amino groups: 0.21 mmol g^{-1}). Fmoc-amino acids were used with the following side-chain protection: Arg(Pbf), Cys(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), His(Trt), Thr(tBu) and Tyr(tBu). The applied chemistry utilized diisopropylcarbodiimide/oxyma coupling with a fivefold excess of reagents. The fluorenyl-9-methoxycarbonyl (Fmoc) group was removed with two cycles of 10% piperazine and 0.1 mol of 1-hydroxybenzotriazole dissolved in a mixture of 10% ethanol and 90% dimethyl carbonate. After synthesis was complete, the peptides were detached from the resin using a 95:5 (v/v) trifluoroacetic acid (TFA)/water mixture containing 3% (w/v) dithiothreitol and 3% (w/v) triisopropylsilane at room temperature for three hours. The resin was then filtered out, and the peptides were precipitated by adding ice-cold diethyl ether. The precipitate was then filtered, dissolved in water and lyophilized. The cyclization reaction to form the disulfide bridge pattern of the molecule was conducted in 20 mM Na_2HPO_4 (pH 7.8) in the presence of 5 mM reduced glutathione, 0.5 mM oxidized glutathione and 0.1 mg mL^{-1} unfolded synthetic ChTX.

High-pressure liquid chromatography (HPLC)

The crude peptides were purified by semi-preparative RP-HPLC using a semi-preparative HPLC system (Shimadzu, Kyoto, Japan) with LC-20AD pumps and an SPD-20A UV-Vis detector. The solvent system consisted of eluent A (0.1% TFA/ H_2O) and eluent B (0.1% TFA/20% H_2O /acetonitrile) on a Luna 10 μm C18(2) 100 Å column (Phenomenex, Torrance, CA, USA) ($10 \times 250 \text{ mm}$). Gradient elution with the following profile was used to elute the peptides from the column: 0% to 30% B over 60 min at a flow rate of 3.0 mL min^{-1} . Peptide signals were detected at 220 nm. The purity of the peptides was confirmed by analytical RP-HPLC using a Luna 5 μm C18(2) 100 Å column ($4.6 \times 250 \text{ mm}$) at a flow rate of 1.0 mL min^{-1} . The appropriate fractions were pooled and lyophilized. The identity of the peptides was verified by electrospray ionization-mass spectrometry using a SQ detector (Waters Corp., Milford, MA, USA). The resulting disulfide patterns were verified by liquid chromatography-mass spectrometry measurements after enzymatic digestion.

ChTX variants also passed a functional quality control performed in a BK (KCa1.1) channel-expressing human embryonic kidney cell line, where their inhibiting effect on whole-cell currents was assessed at 500 nM peptide concentration (data not shown).

SR vesicle isolation

SR vesicles were prepared from rabbit skeletal muscle (musculus longissimus dorsi, musculus spinalis dorsi, musculus multifidus) or canine heart ventricles by differential centrifugation. For this, 50 g of muscle was obtained from a rabbit and used in a single procedure (total of five rabbits, five preparations), whereas cardiac muscle was collected and pooled from multiple dogs (seven dogs, two preparations, 50 g each). All steps were carried out on ice or at 4°C in the presence of protease inhibitors (pefabloc SC, benzamidine, pepstatin A, aprotinin, leupeptin, calpain inhibitor I and II). Muscle homogenates were prepared in $9 \times$ volume of 100 mM NaCl, 20 mM EGTA and 20 mM Na-HEPES (pH 7.5). Cell debris was removed by centrifugation at 3500 g for 35 min, and crude microsomes were collected from the supernatant by centrifugation at $40\,000 \text{ g}$ for 30 min in a Ti45 rotor (Beckman Coulter, Brea, CA, USA). The pellet was resuspended in 120 mL of 600 mM KCl, 10 mM K-Pipes, 250 mM sucrose, 1 mM EGTA and 0.9 mM CaCl_2 (pH 7.0) and incubated for 1 h at 4°C to remove actomyosin. Then, microsomes were centrifuged at $109\,000 \text{ g}$ for 30 min, and the pellet was resuspended in a small volume of 300 mM sucrose, 10 mM K-Pipes (pH 7.0). Vesicles were either used immediately for RyR purification or snap-frozen in liquid nitrogen and stored at -70°C (Diszházi et al., 2019; Magyar, Bauer et al., 2023; Sárközi et al., 2017).

RyR purification

SR vesicles (30 mg of protein) were solubilized for 2 h at 4°C in 1% Chaps, 1 M NaCl, 100 μM EGTA, 150 μM CaCl_2 , 5 mM AMP, 0.45% phosphatidylcholine and 20 mM Na-Pipes (pH 7.2, 6 mL), in a total volume of 9 mL. The solubilized fraction was layered onto a 10–28% linear sucrose gradient and centrifuged for 16 h at $90\,000 \text{ g}$ in a SW27 rotor (Beckman Coulter). RyR-containing fractions were identified, snap-frozen in liquid nitrogen and stored at -70°C in small aliquots (Magyar, Bauer et al., 2023; Sárközi et al., 2017).

RyR single-channel current recording

RyR1s were used from five independent preparations. Purified RyRs were incorporated into planar lipid bilayers formed across a 200 μm diameter aperture of a Delrin cap (Warner Instruments, Hamden, CT, USA) separating two chambers. Both chambers contained 250 mM KCl, 50 μM CaCl_2 and 10 mM HEPES (pH 7.2). In experiments with RyR2, the $[\text{Ca}^{2+}]$ was set to 1 μM . In most recordings, RyRs inserted with their cytosolic foot region facing the recording electrode (*cis*). Transmembrane voltages reported are relative to the other side (*trans*). The bilayer lipid mixture contained phosphatidylethanolamine,

phosphatidylserine and phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) in a 5:4:1 ratio dissolved in *n*-decane at a final lipid concentration of 20 mg mL⁻¹. Currents were obtained with an Axopatch 200 amplifier, filtered at 1 kHz through an eight-pole low-pass Bessel filter, digitized at 3 kHz with a Digidata 1200 interface and recorded with a pClamp, version 6.03 (Axon Instruments, Sunnyvale, CA, USA) (Magyar, Bauer et al., 2023; Sárközi et al., 2017).

Statistical analysis

Total recording time ranged from 13 to 36 min per treatment condition. Dwell-time histograms were generated using Clampfit, version 10.3 (Molecular Devices, Sunnyvale, CA, USA). Statistical analyses of long-lasting closed events (LLCEs) were performed in Origin, version 7.0 (OriginLab, Northampton, MA, USA) and Excel (Microsoft Corp., Redmond, WA, USA). Results were expressed as the mean $\pm$ SD values, collected from three to seven channels. Statistical significance of differences was evaluated by using the Kruskal–Wallis test followed by Dunn's *post hoc* test with comparisons made against RyR channels under control conditions (i.e. in the absence of ChTX). *P* values are provided in Table 1.

Molecular modelling

The C-terminal domain containing the inner and outer vestibules (amino acids 4176–5037) of the rabbit RyR1 cryo-EM structure in an open state (Protein Data Bank ID: 8×4B) was used as the receptor template for the modelling experiments (Wei et al., 2025). The structure of the ligand charybdotoxin was obtained from a published NMR structure (Protein Data Bank ID: 2A9H) (Yu et al., 2005), whereas the ligand iberiotoxin was obtained from the primary amino acid sequence using AlphaFold (Jumper et al., 2021). The parameters of the molecular system were defined using the AMBER 10 forcefield (<https://ambermd.org>), followed by protonation using default ambient parameters (*T* = 300 K, pH 7.0). Molecular docking and subsequent data visualization were performed using the protein-protein docking tool of the Molecular Operating Environment (MOE) software (<https://www.chemcomp.com>), using the cytosolic vestibule of RyR1 as the putative ligand-binding site. Out of 1000 ligand placements, the top 100 scoring positions based on the free enthalpy of ligand binding were selected and further refined, resulting in the final docking output. Specific ligand interactions and fingerprints were obtained via built-in modules in the software.

Results

ChTX induces LLCEs

Single-channel currents were recorded from skeletal muscle RyR1 reconstituted into planar lipid bilayers. ChTX was applied to the cytoplasmic side of the channel at increasing concentrations at a holding potential of +60 mV. Figure 2 shows representative current traces obtained under control conditions and in the presence of ChTX. Notably, ChTX induced LLCEs of the channel, already detectable at concentrations as low as 40 nM, with mean LLCE durations several thousand-fold longer than the mean closed times measured under control conditions. Importantly, these closures were observed only at positive holding potentials, when the current direction was opposite to that of SR Ca²⁺ release. In addition to closures, short-lived subconductance states were occasionally detected, but their low incidence precluded specific statistical analysis (an example with its amplitude histogram is shown in the bottom of Fig. 2). Otherwise, the channel preserved ohmic conductance and normal gating behaviour between LLCE periods without detectable change in channel activity ($P_{\text{O control}} = 0.21 \pm 0.16$, $P_{\text{O 40 nM ChTX}} = 0.24 \pm 0.18$, $P_{\text{O 80 nM ChTX}} = 0.29 \pm 0.13$, $P_{\text{O 240 nM ChTX}} = 0.28 \pm 0.16$).

Quantitative evaluation of closed events and their voltage dependence

Closed events were further evaluated using closed-time histograms generated from pooled recordings under control conditions and at different ChTX concentrations (Fig. 3). ChTX did not increase the amount of time spent in closed states shorter than 500 ms (data not shown), but the histograms clearly revealed a marked increase in the number of events exceeding this threshold (Fig. 3D, inset). Therefore, only closures longer than 500 ms (i.e. LLCEs) were considered for subsequent analyses. Under control conditions, the time spent in LLCEs was 1 s min⁻¹, but it increased in a concentration-dependent manner to 15 s/min (3.7 events min⁻¹ and 4.2 s in mean duration) at 240 nM ChTX (Fig. 3A,D and F). Occasionally, ultra-long (>30 s) closed events also occurred that could only be terminated by switching the holding potential. These events were not included in the analysis because their inclusion would have biased the evaluation. An example of such a long event is shown in Fig. 3C, when a sudden voltage reversal to -60 mV immediately relieved the prevailing LLCE. The concentration-response relationship fitted with a Hill equation yielded a dissociation constant of 85 ± 14 nM ($n_{\text{Hill}} = 0.9 \pm 0.3$) (Fig. 3F). ChTX substantially increased both the frequency and average length of individual LLCEs (Fig. 3F, inset); thus, we conclude that the overall increase in the proportion

Table 1. The frequency, the average duration of individual LLCEs and LLCE-time ratios induced by ChTX (WT) and its variants (K27N, K11N, R19Q, K31N, R25Q and K34Q) at the different concentrations as indicated

ChTX variant	LLCE	[ChTX variant] (nM)				
		0 (0)	50 (40)	100 (80)	200 (240)	500 (400)
WT	Frequency (1 min ⁻¹)	0.65 ± 0.86	2.45 ± 0.89 <i>P</i> = 0.0590	2.5 ± 1.45 <i>P</i> = 0.106	3.76 ± 1.68 <i>P</i> = 0.00364	4.26 ± 2.43 <i>P</i> = 0.00116
	Average duration (s)	1.57 ± 0.91	3.32 ± 1.27 <i>P</i> = 0.045	3.64 ± 0.9 <i>P</i> = 0.0154	4.19 ± 0.34 <i>P</i> = 0.00282	4.11 ± 1.01 <i>P</i> = 0.000638
	Time (s min ⁻¹)	1.06 ± 1.54	7.66 ± 2.5 <i>P</i> = 0.0945	10.43 ± 5.15 <i>P</i> = 0.0129	15.75 ± 7.63 <i>P</i> = 0.00110	16.41 ± 11.1 <i>P</i> = 0.000385
K27N	Frequency (1 min ⁻¹)	1.58 ± 1.82	3.18 ± 0.81 <i>P</i> = 0.366	2.45 ± 0.87 <i>P</i> = 0.591	4.26 ± 3.54 <i>P</i> = 0.261	4.36 ± 3.34 <i>P</i> = 0.187
	Average duration (s)	0.7 ± 0.11	5.72 ± 6.02 <i>P</i> = 0.0652	2.51 ± 0.31 <i>P</i> = 0.170	9.16 ± 12.46 <i>P</i> = 0.0258	7.55 ± 8.5 <i>P</i> = 0.0258
	Time (s min ⁻¹)	1.11 ± 1.31	7.67 ± 6.88 <i>P</i> = 0.154	6.60 ± 0.3 <i>P</i> = 0.253	12.35 ± 3.17 <i>P</i> = 0.0249	25.60 ± 2.49 <i>P</i> = 0.000269
K11N	Frequency (1 min ⁻¹)	1.58 ± 0.62	3.64 ± 1.74 <i>P</i> = 0.635	0.99 ± 0.49 <i>P</i> = 0.102	4.26 ± 0.81 <i>P</i> = 0.332	2.39 ± 0.65 <i>P</i> = 0.948
	Average duration (s)	0.95 ± 0.23	4.66 ± 2.55 <i>P</i> = 0.151	31.7 ± 0.1 <i>P</i> = 0.00239	10.1 ± 3.77 <i>P</i> = 0.0728	20 ± 3.31 <i>P</i> = 0.0129
	Time (s min ⁻¹)	1.58 ± 0.62	17.51 ± 13.7 <i>P</i> = 0.151	39.00 ± 12.4 <i>P</i> = 0.0272	40.97 ± 8.64 <i>P</i> = 0.0189	46.78 ± 5.17 <i>P</i> = 0.00577
R19Q	Frequency (1 min ⁻¹)	1.54 ± 0.91	4.4 ± 4.04 <i>P</i> = 0.233	3.52 ± 1.22 <i>P</i> = 0.194	3.06 ± 1.34 <i>P</i> = 0.291	2.72 ± 0.62 <i>P</i> = 0.538
	Average duration (s)	1.03 ± 0.33	1.47 ± 0.87 <i>P</i> = 0.507	3.28 ± 2.18 <i>P</i> = 0.0329	7.76 ± 3.62 <i>P</i> = 0.000243	3.66 ± 2.46 <i>P</i> = 0.0287
	Time (s min ⁻¹)	1.96 ± 1.44	6.24 ± 5.59 <i>P</i> = 0.212	12.89 ± 9.75 <i>P</i> = 0.0367	16.54 ± 12.3 <i>P</i> = 0.00185	16.35 ± 10.4 <i>P</i> = 0.0205
K31N	Frequency (1 min ⁻¹)	1.61 ± 0.94	2.35 ± 1.63 <i>P</i> = 0.616	1.56 ± 0.66 <i>P</i> = 0.930	1.5 ± 1.13 <i>P</i> = 0.936	1.56 ± 0.71 <i>P</i> = 0.873
	Average duration (s)	1.1 ± 0.29	2.66 ± 0.99 <i>P</i> = 0.215	9.66 ± 6.69 <i>P</i> = 0.00656	35.5 ± 46.5 <i>P</i> = 0.00258	24.6 ± 28.3 <i>P</i> = 0.00332
	Time (s min ⁻¹)	2.12 ± 1.47	6.87 ± 5.96 <i>P</i> = 0.287	18.08 ± 10.7 <i>P</i> = 0.00911	22.31 ± 10.3 <i>P</i> = 0.00457	26.82 ± 23.5 <i>P</i> = 0.00581
R25Q	Frequency (1 min ⁻¹)	1.37 ± 1.27	1.62 ± 1.12 <i>P</i> = 0.831	2.06 ± 0.95 <i>P</i> = 0.427	1.03 ± 0.47 <i>P</i> = 0.509	1.76 ± 0.97 <i>P</i> = 0.698
	Average duration (s)	1.08 ± 0.38	2.23 ± 1.46 <i>P</i> = 0.289	3.15 ± 3.34 <i>P</i> = 0.288	3.57 ± 2.02 <i>P</i> = 0.121	2.56 ± 1.42 <i>P</i> = 0.414
	Time (s min ⁻¹)	1.47 ± 1.27	5.66 ± 7.3 <i>P</i> = 0.218	3.16 ± 1.87 <i>P</i> = 0.187	5.82 ± 5.72 <i>P</i> = 0.0743	5.24 ± 5.48 <i>P</i> = 0.168
K34Q	Frequency (1 min ⁻¹)	0.95 ± 1.04	1.38 ± 0.36 <i>P</i> = 0.197	1.45 ± 1.02 <i>P</i> = 0.519	0.6 ± 0.07 <i>P</i> = 0.292	0.68 ± 0.60 <i>P</i> = 0.639
	Average duration (s)	1.84 ± 0.95	4.26 ± 1.95 <i>P</i> = 0.234	5.57 ± 5.52 <i>P</i> = 0.363	9.49 ± 9.99 <i>P</i> = 0.271	1.59 ± 1.13 <i>P</i> = 0.607
	Time (s min ⁻¹)	1.25 ± 1.74	5.21 ± 1.99 <i>P</i> = 0.0270	5.62 ± 4.38 <i>P</i> = 0.0373	5.27 ± 5.29 <i>P</i> = 0.133	1.04 ± 1.13 <i>P</i> = 0.982

WT ChTX concentrations are shown in parentheses. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. *P* values are reported for comparisons with the untreated control group (0 nM ChTX).

of LLCE was attributable to the simultaneous increase of both parameters. At -60 mV (where ChTX is ineffective), pooled histograms revealed absence of such long closures (Fig. 3B and E) and the LLCE ratio remained at control levels across the entire concentration range (Fig. 3G).

To precisely characterize this pronounced voltage dependence, we examined ChTX efficiency between -100 and $+100$ mV. Representative current traces obtained in the presence of 400 nM ChTX show frequent LLCEs at $+80$ and $+100$ mV (Fig. 4A). The voltage dependence of closed time (Fig. 4B) indicates that the threshold for LLCE

formation is about +40 mV. Between +40 and +100 mV, the LLCE ratio increased steeply from $\sim 3 \text{ s min}^{-1}$ to $\sim 40 \text{ s min}^{-1}$ with a slope of $0.66 \text{ s of LLCE min}^{-1} \text{ mV}^{-1}$. However, ChTX was ineffective below +40 mV.

Because voltage-dependent block often suggests pore binding, experiments were designed to determine whether this is also the case for ChTX. To test whether ChTX shares the MCa binding site -previously mapped to the outer vestibule of RyR- we performed competition experiments. First, a very low concentration of MCa (100 pM) was applied. After a stable subconductance state had developed, ChTX was added at a 1000-fold excess. Shortly thereafter, LLCEs appeared in the recordings, whereas the MCa-induced subconductance states disappeared, indicating direct competition between the two peptides (Fig. 5A). In a subset of experiments, ryanodine was

applied after exposure to 400 nM ChTX (Fig. 5B). Ryanodine induced the characteristic subconductance state, but only when the channel was operating in its normal gating mode (i.e. with no ChTX bound). By contrast, the ryanodine-modified subconductance state failed to develop during ChTX-modified operation (i.e. during LLCEs). However, once established, the ryanodine-induced subconductance state persisted without interruption by LLCEs, indicating that the ChTX-induced closures do not recur in this condition and that the ryanodine-modified channel is unavailable for ChTX binding. These data suggest a mutually exclusive interaction of the two ligands in the outer vestibule of the channel, with two distinct binding sites, being the ryanodine binding-site deeper in the outer vestibule (des Georges et al., 2016).

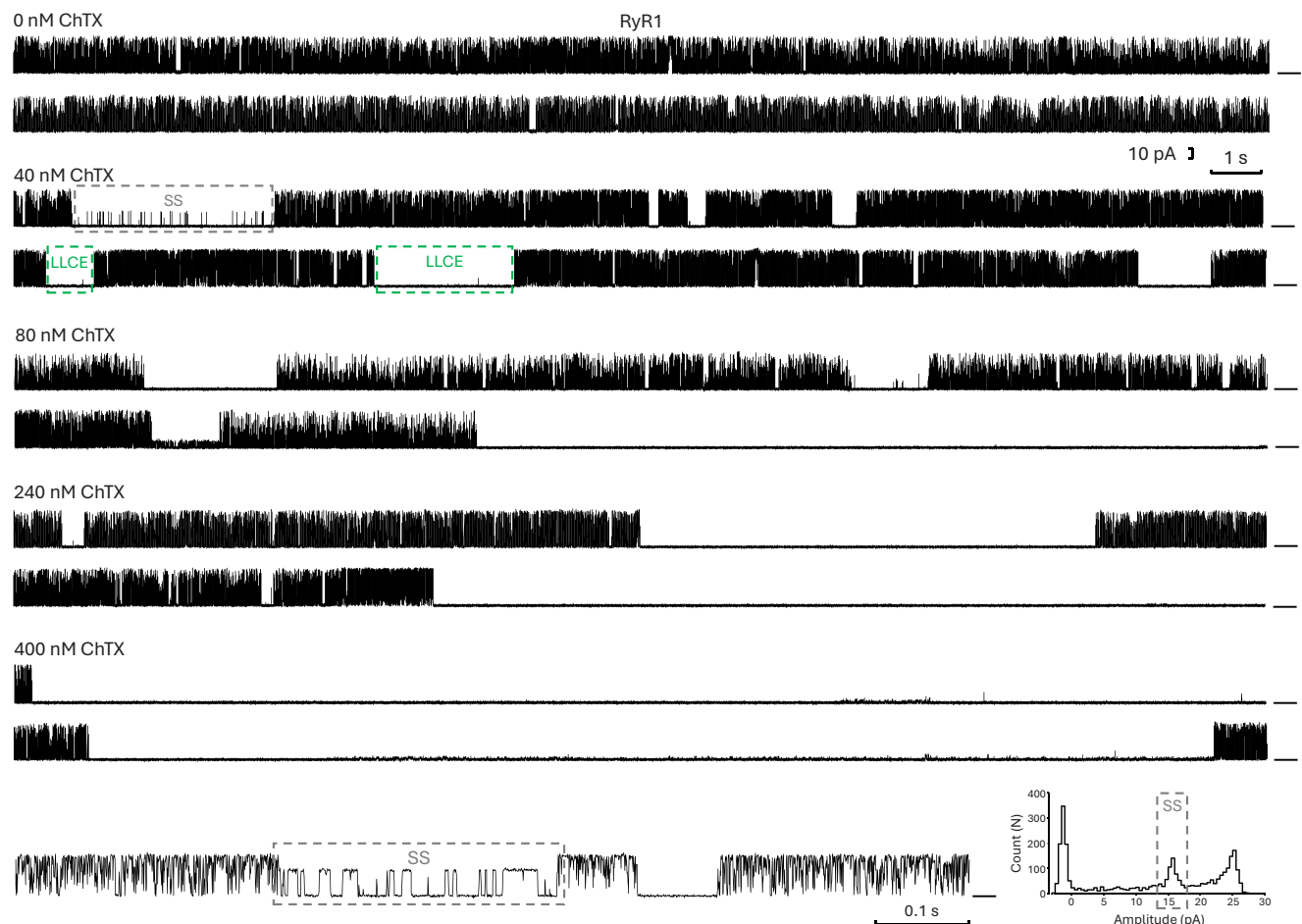


Figure 2. Single-channel recordings of ryanodine receptor 1 (RyR1) under control conditions and in the presence of charybdotoxin (ChTX)

Recording conditions: $50 \mu\text{M Ca}^{2+}$, +60 mV holding potential. Hyphens indicate closed channel states on the right side. Some examples of a subconductance states (SS) and long-lasting closed event (LLCE) induced by 40 nM ChTX are highlighted by a dashed grey or green outlines. Another example of a SS is demonstrated in the bottom trace with higher resolution along with its amplitude histogram.

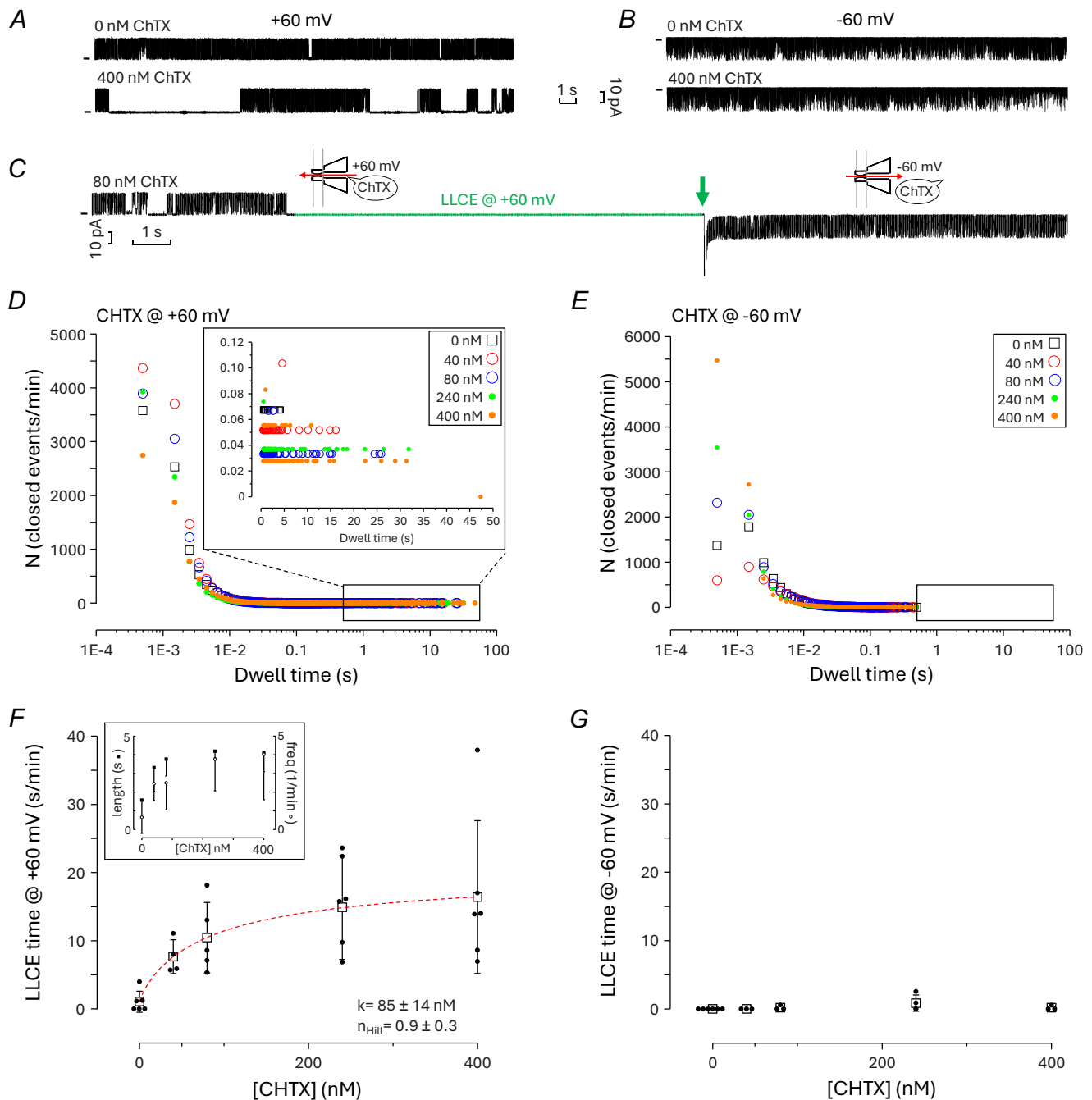


Figure 3. Evaluation of charybdotoxin (ChTX)-induced long lasting closed events (LLCE)

A, representative current recording of a single RyR1 channel at +60 mV membrane potential, showing that 400 nM ChTX induced LLCEs. The closed state current levels are indicated by hyphens on the side. **B**, representative traces under similar conditions, but at -60 mV, demonstrating the absence of LLCEs. **C**, reversibility of LLCE at +60 mV (green) by a rapid change of the membrane potential to -60 mV (indicated by the green arrow). Insets illustrate corresponding current direction at +60 and -60 mV (reverse vs. physiological current direction, respectively). **D**, closed time histograms of RyR1 currents under control conditions and increasing concentrations of ChTX at +60 mV. The inset is an enlargement of the framed detail (closed events >0.5 s), showing that the number and duration of single LLCEs increases with ChTX concentration. **E**, closed time histograms of RyR1 currents under control conditions and increasing concentrations of ChTX at -60 mV, showing the absence of LLCEs at this membrane potential (box). **F** and **G**, average long-lasting closed event (LLCE) times (s min⁻¹) of several channels as the function of ChTX concentration at +60 mV or -60 mV, respectively. Inset: average frequency and duration (length) of LLCEs as the function of ChTX concentration.

Examination of the role of basic amino acids in ChTX-induced LLCE

By analogy with other peptide toxin-channel interactions, the high-affinity ChTX-RyR1 interaction is probably mediated by electrostatic forces. Thus, mutations in the ChTX interaction domain are expected to alter its electrostatic potential and LLCE time. Given that basic residues are critical for both the ChTX-K⁺ channel and the MCa-RyR interactions, we probed the role of positively charged side chains in ChTX using synthetic analogues carrying charge-neutralizing (isosteric) substitutions.

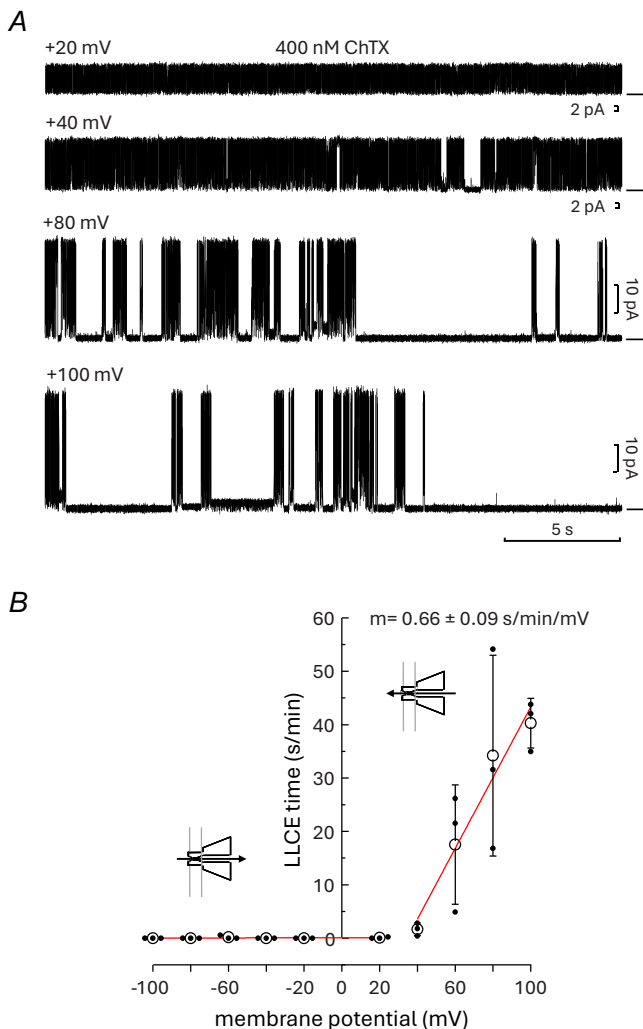


Figure 4. Voltage dependence of charybdotoxin block on RyR1

A, representative single channel current recordings of a ChTX-modified channel at different positive voltages. Closed state current levels are labelled by hyphens on the right side. B, average long-lasting closed events (LLCE) times measured in 400 nM ChTX are plotted as the function of membrane potential (mV). Cartoons indicate current directions through RyR at corresponding membrane potentials: SR lumen-to-cytosolic (i.e. physiological) and reverse current directions at negative and positive voltages, respectively.

We first tested the K27N ChTX variant, which is essentially inactive on K⁺ channels (Park & Miller, 1992b). Unexpectedly, this analogue inhibited RyR1s with potency and efficacy comparable to those of wild-type ChTX (Fig. 6A). This finding indicates that K27N ChTX represents a RyR-selective inhibitory peptide; therefore, we next examined its effect on RyR2. Figure 6B shows representative 50 s long recordings, demonstrating a strong blocking effect of 200 nM K27N ChTX at +60 mV, which is apparently similar to what we observed on RyR1. These results suggest that K27 does not contribute critically to the blocking effect and in conclusion, the RyR binding site differs substantially from that of K⁺ channels. Guided by *in silico* docking results, we next synthesized and examined the K11N, R19Q, R25Q, K31N and R34Q ChTX analogues. In terms of LLCE ratio, the R19Q and K31N variants retained wild-type activity, whereas K11N exhibited markedly enhanced efficacy, reaching 40 s min⁻¹ LLCE-time at 100 nM. By contrast, R25Q and R34Q were essentially inactive, even when tested up to 5 μ M, yielding LLCE ratios below 6 s min⁻¹. The results of the LLCE analysis are summarized in Fig. 7, which also includes a structural model of ChTX highlighting the positions of the mutated residues. The numerical results and statistical analysis of the frequency, average duration of individual LLCEs and LLCE-time ratios (s min⁻¹) are provided in Table 1.

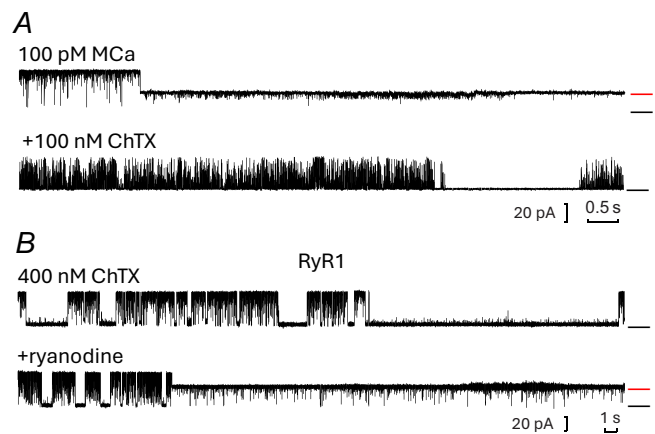


Figure 5. Combined treatment of RyR1 with charybdotoxin (ChTX) and ryanodine or maurocalcine (MCa)

A, top trace shows a Mca-induced long lasting subconductance state (red hyphen), which was followed by ChTX treatment. Bottom trace shows that subconductance states were replaced by long closed events upon ChTX treatment of the Mca-modified channel, indicating competitive binding of the two peptides. B, top trace shows a ChTX-modified channel current with ChTX-induced long lasting closed events. Bottom trace represents the same channel's current after the addition of ryanodine. Ryanodine-stabilized subconductance state is shown by a red hyphen, closed channel state is indicated by black hyphens.

Differential effects of α -KTx toxins on RyR channel gating

ChTX and other, structurally related α -KTx peptides [iberiotoxin (IbTX), kaliotoxin, noxiustoxin and slotoxin] share the conserved cysteine-stabilized α/β scaffold, as well as a standard functional pattern with the pore-occluding lysine (find primary sequence alignment in Fig. 8). These peptides constitute prototypical blockers of K_v channels.

To assess whether RyR modulation is a shared property among closely related α -KTx peptides, we tested several ChTX-related toxins on RyR channels. On the one hand, slotoxin and IbTX did not produce detectable effects. On the other hand, we found that kaliotoxin and noxiustoxin induced LLCEs and subconductive states, consistent with the effects of ChTX. We applied the toxins at progressively increasing concentrations ranging from 250 nM to 8.5 μ M. Representative recordings in Fig. 8. show that kaliotoxin and noxiustoxin induced LLCEs and subconductive states in relatively low concentrations, whereas slotoxin and, unexpectedly, IbTX (which has the greatest homology to ChTX) remained ineffective even at the concentration of 8.5 μ M (Fig. 8).

Molecular modelling of ChTX-RyR1 interaction reveals a 'both feet in the door' blocking mechanism

To gain insight into the ChTX-RyR1 interaction, molecular docking was performed. Docking was restricted to the pore-forming region of the open RyR1, using the NMR structure of ChTX as the ligand. Of 100 docking attempts, 69 predicted ChTX binding within the channel vestibule lined by the S6 extension helices. In the subsequent analysis, we examined the highest-scoring binding orientations and compared them with our electrophysiological results obtained from ChTX variants.

A single binding configuration with the highest predicted docking score was fully consistent with the functional data and provided a mechanistic explanation for the observed effects. Notably, this orientation placed ChTX in the RyR vestibule in a similar orientation that it adopts when engaging K^+ channels. This configuration is shown in the model of Fig. 9A. Importantly, none of the other high-scoring docking orientations outside this symmetry-related family of poses were compatible with, or could satisfactorily explain, our single-channel data.

As highlighted by the interaction maps, corresponding ChTX side chains interact with these RyR1 residues,

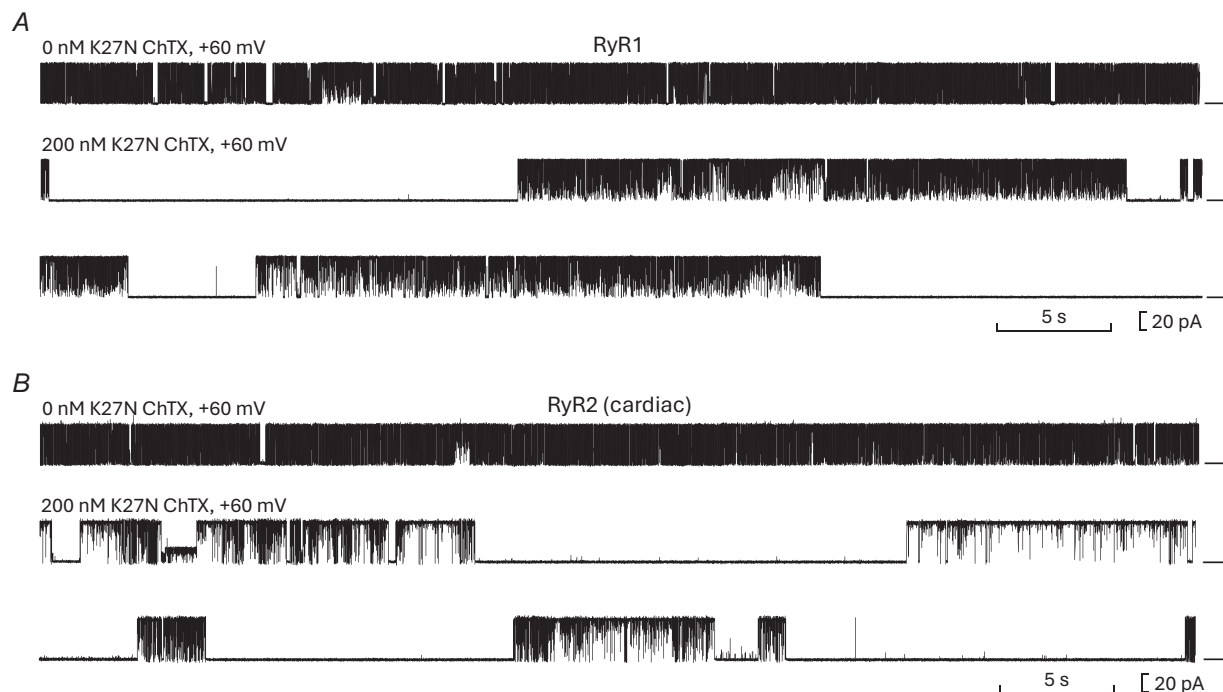


Figure 6. The effect of charybdotoxin (ChTX)-variant K27N on the cardiac ryanodine receptor (RyR2)
Representative recording of single RyR1 (A) and RyR2 (B) channel currents under control conditions and in the presence of 200 nM K27N ChTX at +60 mV holding potential.

by R25 and R34 facing the pore interior and form cross-linking interactions with D4945s of opposite RyR S6ext helices, residues that also play a central role in IpCa-RyR binding (Haji-Ghassemi et al., 2023). Although K27 points toward the pore cavity, it extends into the deep vestibular pocket without finding a binding partner. K11 also interacts with the channel, and its substitution may facilitate stabilization of the remaining contacts, providing a potential explanation for the markedly higher affinity of the K11N variant. Functionally irrelevant residues (such as R19 and K31) project outward. A surface charge map of ChTX, binding frequencies for relevant RyR1 residues and interaction maps of toxin residues were also generated (not shown). These *in silico* results highlight the importance of D4945 in toxin-channel interaction, mapping ChTX to a region that precisely overlaps with the IpCa binding site, supporting our competition experiments shown in Fig. 3B.

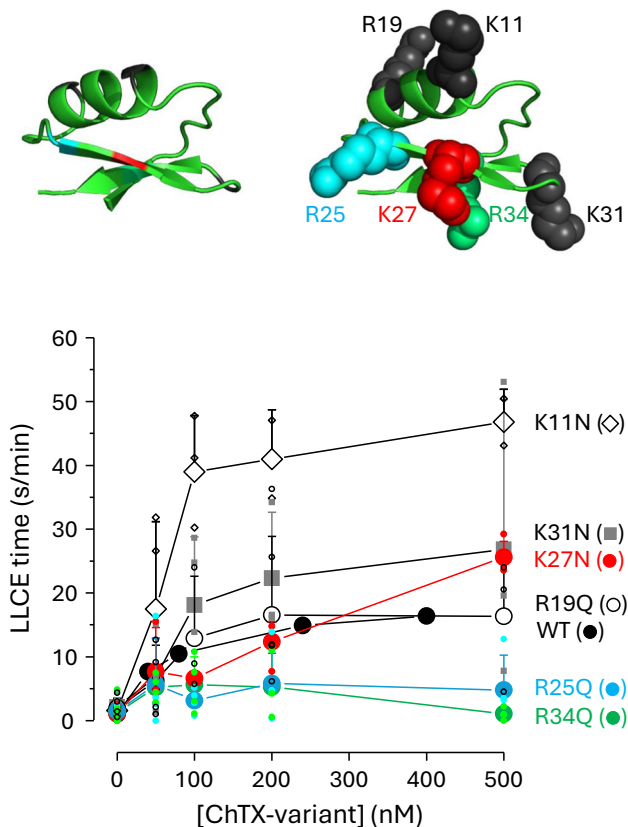


Figure 7. The impact of basic amino acid residues of charybdotoxin (ChTX) on RyR1 block

Ribbon representation of ChTX backbone structure (top left). Amino acid substitutions introduced into the peptide are illustrated with coloured spheres (top right). Average time spent in long lasting closed events (LLCE) ($s \min^{-1}$) was plotted as the function of the concentration of different ChTX-variants. Single channel currents were recorded at +60 mV holding potential. Colours are used consequently.

Discussion

This study was inspired by our realization that MCa and ChTX employ a similar functional logic to modulate RyR or K^+ channels, respectively. Both use a compact, disulfide-bridged, rigid globular scaffold for which the basic surface residues interact with the pore region to stabilize an altered conductance state. These structural and mechanistic analogies motivated us to test whether ChTX exhibits cross-reactivity toward RyRs. We found that ChTX induced long lasting closed states of RyR, in a strong voltage-dependent manner with preference

	1	11	25	27	34
Charybdotoxin	EFTNVSC	TSK	ECWSVC	QRLHNTS	RGKCMNKKRCYS
Kaliotoxin	GVEINV	KCSGSPQCL	KPK	CDAGMRFG	KCMNRKCHCTP
Noxiustoxin	TIINV	KCTSPKQCSK	PK	EKELYGSSAGAK	CMNGKCKCYNN
Slotoxin	TFIDVDCTVS	K	ECWAPCK	AAFGVD	RGKCMGKKCKCYV
Iberitoxin	EFTDVC	SVSK	ECWSVC	KDLFGVDRG	KCMGKKRCYQ

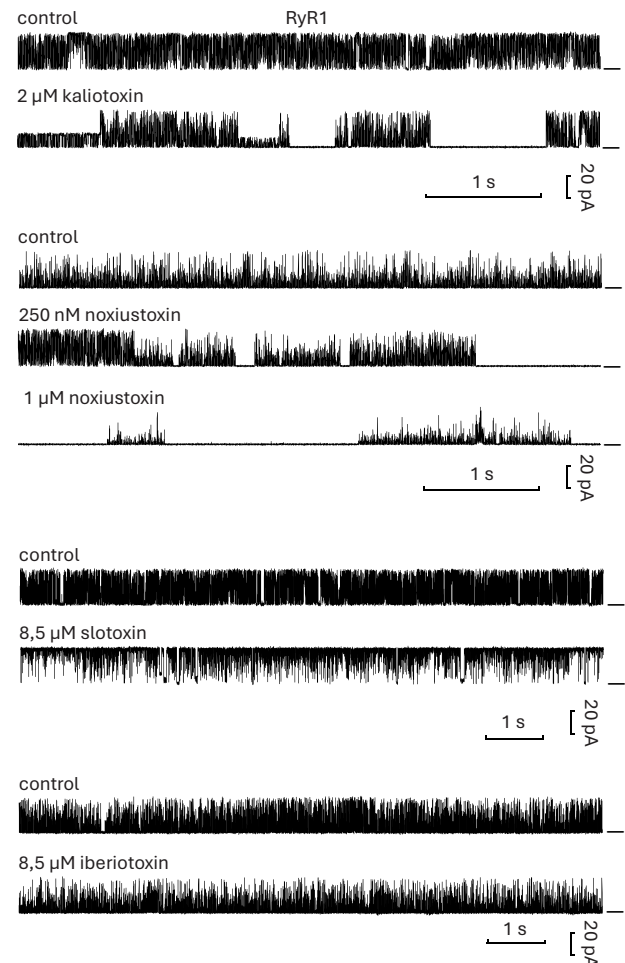


Figure 8. The effect of different α -KTx peptides on the ryanodine receptor (RyR1)

Representative current traces under control conditions and kaliotoxin, noxiustoxin, slotoxin and iberitoxin treatments. Top: primary structures of the peptides.

to positive membrane potentials (that drive current flow opposite to physiological). We hypothesised that, similarly to MCa, a positively charged area on the surface of ChTX is responsible for RyR binding.

Indeed, our main finding was that the basic amino acid residues at positions 25 and 34 are essential for ChTX-RyR interaction, but, unexpectedly, K27 is irrelevant, which we further confirmed using molecular modelling. Together, our findings reveal that the structurally unrelated toxins

ChTX and MCa converge in RyR modulation, driven by shared electrostatic interactions rather than conserved pore-plugging motifs.

To better understand the mechanistic parallels suggested by these results, we need to examine the structural frameworks of ChTX and MCa.

ChTX and MCa are short peptides consisting of 37 and 33 amino acids, respectively. ChTX, as a member of the α -KTx family, shares a characteristic cysteine-stabilized α/β ($CS\alpha\beta$) structural motif. This motif forms a compact fold comprising three antiparallel β -strands (residues 26–35) linked to a short α -helix (residues 10–19). The core structure is stabilized by three disulfide bonds, two of which connect the β -sheet to the α -helix and one that links the β -sheet to an extended segment (Bartok et al., 2015; Bontems et al., 1991). The overall fold adopts a spherical shape of $\sim 25 \times 15$ Å (Bontems et al., 1991; Yu et al., 2005). In complexes with K^+ channels, the β -sheet edge of the toxin faces the pore, while the helical region is oriented away from the channel, resulting in a highly asymmetric binding orientation (Banerjee et al., 2013).

The charge distribution on the surface of ChTX is also highly asymmetric, which is functionally important because it underlies the electrostatic complementarity between ChTX and the negatively charged outer vestibule of K^+ channels. Notably, in K^+ channels, the conserved K27 physically occludes the pore, whereas surrounding basic residues, including R25 and R34, stabilize this interaction electrostatically (Park & Miller, 1992a, 1992b; Stampe et al., 1994). Our data show that ChTX-RyR interaction also relies on R25 and R34 but not on K27, indicating a fundamentally different mode of engagement despite some similarities.

Peptides of the calxin family have similar features to ChTX in that their structure is rigid because it is stabilized by three disulfide bonds too, although the connectivity pattern of the cysteines is different, and thus they show an entirely different fold, called the inhibitory cysteine-knotted (i.e. ICK) structure. This structure consists of two β -strands arranged in an anti-parallel fashion and formed by residues K20–R23 and K30–R33 (Lee et al., 2004; Mosbah et al., 2000). A loop connecting the β -strands (residues 22–30) was shown to be important for inducing the characteristic sub-conductive states of RyR. This loop constitutes a positively charged interaction surface that comprises a well-defined cluster of critical residues, with R24 identified as essential in MCa-induced subconductance state. Neutralization of these residues to alanine resulted in substantial losses of binding affinity, whereas most other charged residues contributed only modestly (Lee et al., 2004; Lukács et al., 2008).

Recent cryo-EM analysis provides a detailed molecular description of the functional surface of IpCa and the mode of interaction with RyR1 (Haji-Ghassemi et al., 2023).

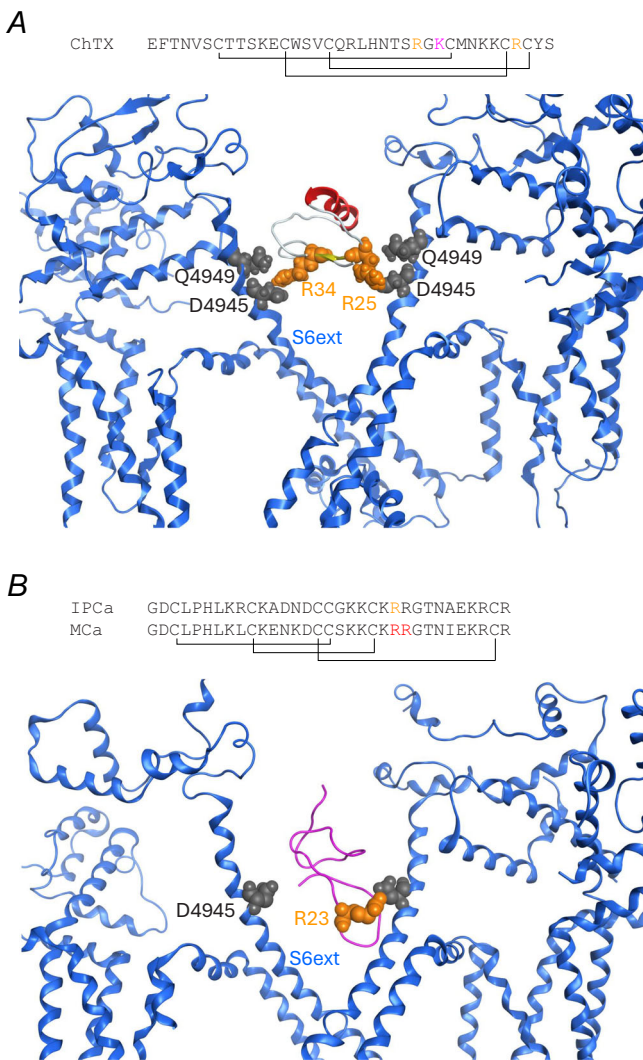


Figure 9. Overview of molecular interactions between RyR1 and ChTX vs. RyR1 and IpCa

A, two oppositely oriented chains of the RyR1 tetramer, highlighting the pore and vestibule, are shown as blue ribbon representations, in complex with ChTX positioned in the vestibule and displayed as a white ribbon with a red α -helix. The key interacting RyR1 residues, D4945 and Q4949 (grey), are highlighted as thick stick representations interacting with R25 and R34 of ChTX (orange). **B**, the same RyR1 region as shown in (**A**) in complex with IpCa positioned in the vestibule and displayed as a purple ribbon. The key interacting residues, D4945 (grey) of RyR1 interacting with R23 of IpCa (orange), are highlighted as thick sticks.

Notably, IpCa inserts deeply into the cytosolic vestibule of the channel with its functional interface formed by the cluster of positively charged residues in its loop. These residues form multiple salt bridges with negatively charged amino acids on RyR1, and importantly with D4945 and E4948. IpCa binds to the open conformation of the channel and induces an asymmetric outward displacement of the four S6 extension helices, further splaying the cytosolic ends of the pore. In this dilated architecture, IpCa fits into the expanded vestibule allowing for the characteristic subconductance state (Haji-Ghassemi et al., 2023). Importantly, this interaction does not involve deep pore occlusion by a single residue but instead relies on an electrostatic anchoring at the cytosolic vestibule. The rare, brief subconductive, flickering block events that we occasionally observed with ChTX may also be the result of a mechanism similar to IpCa, potentially representing a transient, pre-engaged binding configuration that precedes the formation of a fully stable complex.

Our modelling data indicate that ChTX shares a common binding site with IpCa and MCA in the channel vestibule, outside the gate. This space lies outside the transmembrane electrical field, but in the way of permeating ions, which accounts for the apparent voltage-dependent block: permeant ions moving in physiological direction (such as during SR Ca^{2+} release) interfere with ChTX binding and clear the occluding toxin from the channel, whereas opposite ionic flux does not, resulting in a rectification of the ChTX-modified channel. Our *in vitro* and *in silico* data support a mechanistic model such as the classic foot-in-the-door mechanism described for internal quaternary ammonium ion block of K^+ channels (Armstrong, 1969, 1971, 1974; Armstrong & Binstock, 1965). It is similar in a sense that the pore binding site is accessible to ChTX only when the gate is open and stabilizes the open state, but at the same time as occluding the pore. Because two positively charged residues (R25 and R34) act as two legs to wedge the gate open, we refer to this mechanism as 'both feet in the door block'.

Whether the inhibitory activity of ChTX depends on specific arginine residues or reflects a more general requirement for positively charged side chains remains an open question. Future studies involving systematic substitution of arginine residues by lysine or histidine may help distinguish between specific structural recognition and predominantly electrostatic mechanisms of RyR inhibition. However, our *in silico* experiments, designed to answer this question by using R25K or R34K in the modelling. The docking score ($-8.99 \text{ kcal mol}^{-1}$) of the wild-type ChTX was only marginally less negative in an R25K/R34K ($-8.65 \text{ kcal mol}^{-1}$) and R25H/R34H ($-8.48 \text{ kcal mol}^{-1}$) mutants, indicating that electrostatic interactions at these positions are crucial, and that a

generic, positively charged residue suffices to provide the net binding.

Alternatively, the apparent voltage dependence could also result from voltage-dependent conformational changes of RyR that alter ChTX binding within the vestibule, a mechanism previously proposed based on ryanodol experiments.

Unexpectedly, the most closely related peptide toxin, IbTX was ineffective, which could not be fully explained by modelling. Nevertheless, out of 100 docking attempts, only 29 predicted IbTX binding. IbTX is a selective blocker of BK channels, whereas ChTX is non-selective among potassium channels. The broad inhibitory spectrum of ChTX has been attributed to Asn30, as mutation of Gly30 to Asn confers Kv channel-blocking activity to IbTX (Schroeder et al., 2002). Although it would be reasonable to assume that the lack of IbTX activity is also related to the presence of Gly30, our model does not predict an interaction between the wall of RyR vestibule and Gly30. This suggests that the ineffectiveness of IbTX is probably the result of differences in surface electrostatic properties. Despite sharing 68% sequence identity and showing almost identical three-dimensional structures, the two toxins differ mainly in surface residues. These substitutions result in distinct net charges: ChTX is more positively charged (+5), whereas IbTX contains more acidic residues and has a lower net charge (+2), which probably influences their channel-binding properties.

Although our discussion here focuses specifically on pore-blocking peptides and the mechanisms underlying their interaction with RyRs, it should be noted that several other peptides and natural toxins have been investigated previously and have contributed significantly to our understanding of RyR structure–function relationships (Kohn et al., 2020; Morrisette et al., 1995; Olojo et al., 2011; Robinson et al., 2020; Yamamoto & Ikemoto, 2002; Yamamoto et al., 2002). These molecules were also of considerable interest and may serve as valuable templates for the development of novel RyR modulators.

In summary, we propose that ChTX induces LLCE of RyRs by physically occupying the vestibule and establishing high-affinity, slow off-rate toxin-channel interactions through electrostatic complementarity with negatively charged residues in the channel vestibule, suggesting a functional convergence of ChTX and calcins on RyR. Furthermore, we suggest that this mechanistic insight may guide the design of new inhibitory peptides. Therefore, our findings extend beyond pore properties and identify the cytosolic RyR site shared by MCA and ChTX as a common peptide-binding motif. ChTX binds this site through a motif previously described for K^+ channel inhibition, with a key difference in that the critical K27 residue is not required for RyR modulation. This suggests functional convergence between MCA

and ChTX and reveals a shared structural footprint that may guide future peptide design. Both toxins can be viewed as adaptable scaffolds for engineering RyR modulators with tailored effects, at the same time as preserving the geometric features required for binding. Although ChTX may serve as a lead compound, its lack of activity at 0 mV remains a significant limitation. Consistent with this observation, ChTX did not reduce [^3H] ryanodine binding to SR vesicles, did not inhibit spontaneous Ca^{2+} leak from SR vesicles, and, when applied intracellularly, did not reduce the amplitude of depolarization-induced Ca^{2+} release in skeletal muscle fibres either. Thus, the development of ChTX-derived RyR modulators would need to overcome two major limitations of the lead molecule: membrane impermeability and unfavourable voltage dependence. In this effort, MCA represents a promising template because its effects are not restricted to non-physiological current directions and it consistently enhances RyR-mediated Ca^{2+} release. Accordingly, modifying the otherwise membrane-permeable calcins based on the functional map of ChTX may provide a promising strategy for the rational design of novel RyR-targeting peptides.

References

- Aiyar, J., Withka, J. M., Rizzi, J. P., Singleton, D. H., Andrews, G. C., Lin, W., Boyd, J., Hanson, D. C., Simon, M., Dethlefs, B., Lee, C., Hall, J. E., Gutman, G. A., & George Chandy, K. (1995). Topology of the pore-region of a K^+ channel revealed by the NMR-derived structures of scorpion toxins. *Neuron*, **15**(5), 1169–1181.
- Altafaj, X., France, J., Almasy, J., Jona, I., Rossi, D., Sorrentino, V., Mabrouk, K., De Waard, M., & Ronjat, M. (2007). Maurocalcine interacts with the cardiac ryanodine receptor without inducing channel modification. *Biochemical Journal*, **406**(2), 309–315.
- Armstrong, C. M. (1969). Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. *Journal of General Physiology*, **54**(5), 553–575.
- Armstrong, C. M. (1971). Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *Journal of General Physiology*, **58**(4), 413–437.
- Armstrong, C. M. (1974). Ionic pores, gates, and gating currents. *Quarterly Reviews of Biophysics*, **7**(2), 179–210.
- Armstrong, C. M., & Binstock, L. (1965). Anomalous rectification in the squid giant axon injected with tetraethylammonium chloride. *Journal of General Physiology*, **48**(5), 859–872.
- Banerjee, A., Lee, A., Campbell, E., & MacKinnon, R. (2013). Structure of a pore-blocking toxin in complex with a eukaryotic voltage-dependent K^+ channel. *eLife*, **2**, e00594.
- Bartok, A., Panyi, G., & Varga, Z. (2015). Potassium channel blocking peptide toxins from scorpion venom. In *Scorpion venoms* (pp. 493–527). Springer Netherlands, Dordrecht.
- Benkuský, N. A., Farrell, E. F., & Valdivia, H. H. (2004). Ryanodine receptor channelopathies. *Biochemical and Biophysical Research Communications*, **322**(4), 1280–1285.
- Bers, D. M. (2002). Cardiac excitation-contraction coupling. *Nature*, **415**(6868), 198–205.
- Boisseau, S., Mabrouk, K., Ram, N., Garmy, N., Collin, V., Tadmouri, A., Mikati, M., Sabatier, J.-M., Ronjat, M., Fantini, J., & De Waard, M. (2006). Cell penetration properties of maurocalcine, a natural venom peptide active on the intracellular ryanodine receptor. *Biochimica et Biophysica Acta*, **1758**(3), 308–319.
- Bontems, F., Roumestand, C., Boyot, P., Gilquin, B., Doljansky, Y., Menez, A., & Toma, F. (1991). Three-dimensional structure of natural charybdotoxin in aqueous solution by ^1H -NMR. Charybdotoxin possesses a structural motif found in other scorpion toxins. *European Journal of Biochemistry*, **196**(1), 19–28.
- Chi, X., Gong, D., Ren, K., Zhou, G., Huang, G., Lei, J., Zhou, Q., & Yan, N. (2019). Molecular basis for allosteric regulation of the type 2 ryanodine receptor channel gating by key modulators. *Proceedings of the National Academy of Sciences*, **116**(51), 25575–25582.
- Diszházi, G., Magyar, Z. É., Mótýán, J. A., Csernoch, L., Jóna, I., Nánási, P. P., & Almássi, J. (2019). Dantrolene requires Mg^{2+} and ATP to inhibit the ryanodine receptor. *Molecular Pharmacology*, **96**(3), 401–407.
- el-Hayek, R., Lokuta, A. J., Arévalo, C., & Valdivia, H. H. (1995). Peptide probe of ryanodine receptor function. Imperatoxin A, a peptide from the venom of the scorpion *Pandinus imperator*, selectively activates skeletal-type ryanodine receptor isoforms. *Journal of Biological Chemistry*, **270**(48), 28696–28704.
- Estève, E., Mabrouk, K., Dupuis, A., Smida-Rezgui, S., Altafaj, X., Grunwald, D., Platel, J.-C., Andreotti, N., Marty, I., Sabatier, J.-M., Ronjat, M., & De Waard, M. (2005). Transduction of the scorpion toxin maurocalcine into cells. Evidence that the toxin crosses the plasma membrane. *Journal of Biological Chemistry*, **280**(13), 12833–12839.
- Estève, E., Smida-Rezgui, S., Sarkozi, S., Szegedi, C., Regaya, I., Chen, L., Altafaj, X., Rochat, H., Allen, P., Pessah, I. N., Marty, I., Sabatier, J.-M., Jona, I., De Waard, M., & Ronjat, M. (2003). Critical amino acid residues determine the binding affinity and the Ca^{2+} release efficacy of maurocalcine in skeletal muscle cells. *Journal of Biological Chemistry*, **278**(39), 37822–37831.
- Fajloun, Z., Kharrat, R., Chen, L., Lecomte, C., Di Luccio, E., Bichet, D., El Ayeb, M., Rochat, H., Allen, P. D., Pessah, I. N., De Waard, M., & Sabatier, J. M. (2000). Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca^{2+} release channel/ryanodine receptors. *Federation of European Biochemical Societies Letters*, **469**(2–3), 179–185.
- Fill, M., & Copello, J. A. (2002). Ryanodine receptor calcium release channels. *Physiological Reviews*, **82**(4), 893–922.
- des Georges, A., Clarke, O. B., Zalk, R., Yuan, Q., Condon, K. J., Grassucci, R. A., Hendrickson, W. A., Marks, A. R., & Frank, J. (2016). Structural basis for gating and activation of RyR1. *Cell*, **167**, 145–157.e17.

- Goldstein, S. (1994). The charybdotoxin receptor of a Shaker K^+ channel: Peptide and channel residues mediating molecular recognition. *Neuron*, **12**(6), 1377–1388.
- Haji-Ghassemi, O., Chen, Y. S., Woll, K., Gurrola, G. B., Valdivia, C. R., Cai, W., Li, S., Valdivia, H. H., & Van Petegem, F. (2023). Cryo-EM analysis of scorpion toxin binding to ryanodine receptors reveals subconductance that is abolished by PKA phosphorylation. *Science Advances*, **9**(21), eadf4936.
- Hidalgo, P., & MacKinnon, R. (1995). Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. *Science*, **268**(5208), 307–310.
- Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Židek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., ..., & Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, **596**(7873), 583–589.
- Kohn, M., Kobayashi, S., Yamamoto, T., Yoshitomi, R., Kajii, T., Fujii, S., Nakamura, Y., Kato, T., Uchinoumi, H., Oda, T., Okuda, S., Watanabe, K., Mizukami, Y., & Yano, M. (2020). Enhancing calmodulin binding to cardiac ryanodine receptor completely inhibits pressure-overload induced hypertrophic signaling. *Communications Biology*, **3**(1), 714.
- Lee, C. W., Lee, E. H., Takeuchi, K., Takahashi, H., Shimada, I., Sato, K., Shin, S. Y., Kim, D. H., & Kim, J. I. (2004). Molecular basis of the high-affinity activation of type 1 ryanodine receptors by imperatoxin A. *Biochemical Journal*, **377**(2), 385–394.
- Lukács, B., Sztrettye, M., Almássy, J., Sárközi, S., Dienes, B., Mabrouk, K., Simut, C., Szabó, L., Szentesi, P., De Waard, M., Ronjat, M., Jóna, I., & Csernoch, L. (2008). Charged surface area of maurocalcine determines its interaction with the skeletal ryanodine receptor. *Biophysical Journal*, **95**(7), 3497–3509.
- MacKinnon, R., Heginbotham, L., & Abramson, T. (1990). Mapping the receptor site for charybdotoxin, a pore-blocking potassium channel inhibitor. *Neuron*, **5**(6), 767–771.
- MacKinnon, R., & Miller, C. (1988). Mechanism of charybdotoxin block of the high-conductance, Ca^{2+} -activated K^+ channel. *Journal of General Physiology*, **91**(3), 335–349.
- MacKinnon, R., & Miller, C. (1989). Mutant potassium channels with altered binding of charybdotoxin, a pore-blocking peptide inhibitor. *Science*, **245**(4924), 1382–1385.
- Magyar, Z. É., Bauer, J., Bauerová-Hlinková, V., Jóna, I., Gaburjakova, J., Gaburjakova, M., & Almássy, J. (2023). Eu^{3+} detects two functionally distinct luminal Ca^{2+} binding sites in ryanodine receptors. *Biophysical Journal*, **122**(17), 3516–3531.
- Magyar, Z. É., Hevesi, J., Groom, L., Dirksen, R. T., & Almássy, J. (2023). Function of a mutant ryanodine receptor (T4709M) linked to congenital myopathy. *Scientific Reports*, **13**(1), 14659.
- Meissner, G. (1994). Ryanodine receptor/ Ca^{2+} release channels and their regulation by endogenous effectors. *Annual Review of Physiology*, **56**(1), 485–508.
- Miller, C., Moczydlowski, E., Latorre, R., & Phillips, M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+} -activated K^+ channels from mammalian skeletal muscle. *Nature*, **313**(6000), 316–318.
- Morrisette, J., Krätzschar, J., Haendler, B., el-Hayek, R., Mochca-Morales, J., Martin, B. M., Patel, J. R., Moss, R. L., Schleuning, W. D., & Coronado, R. (1995). Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. *Biophysical Journal*, **68**(6), 2280–2288.
- Mosbah, A., Kharrat, R., Fajloun, Z., Renisio, J.-G., Blanc, E., Sabatier, J.-M., Ayeb, M. E., & Darbon, H. (2000). A new fold in the scorpion toxin family, associated with an activity on a ryanodine-sensitive calcium channel. *Proteins*, **40**(3), 436–442.
- Naranjo, D., & Miller, C. (1996). A strongly interacting pair of residues on the contact surface of charybdotoxin and a Shaker K^+ channel. *Neuron*, **16**(1), 123–130.
- Olojo, R. O., Hernández-Ochoa, E. O., Ikemoto, N., & Schneider, M. F. (2011). Effects of conformational peptide probe DP4 on bidirectional signaling between DHPR and RyR1 calcium channels in voltage-clamped skeletal muscle fibers. *Biophysical Journal*, **100**(10), 2367–2377.
- Park, C.-S., & Miller, C. (1992a). Interaction of charybdotoxin with permeant ions inside the pore of a K^+ channel. *Neuron*, **9**(2), 307–313.
- Park, C. S., & Miller, C. (1992b). Mapping function to structure in a channel-blocking peptide: Electrostatic mutants of charybdotoxin. *Biochemistry*, **31**(34), 7749–7755.
- Poillot, C., Bichraoui, H., Tisseyre, C., Bahemberae, E., Andreotti, N., Sabatier, J.-M., Ronjat, M., & De Waard, M. (2012). Small efficient cell-penetrating peptides derived from scorpion toxin maurocalcine. *Journal of Biological Chemistry*, **287**(21), 17331–17342.
- Robinson, K., Culley, D., Waring, S., Lamb, G. D., Easton, C., Casarotto, M. G., & Dulhunty, A. F. (2020). Peptide mimetic compounds can activate or inhibit cardiac and skeletal ryanodine receptors. *Life Sciences*, **260**, 118234.
- Ronjat, M., Feng, W., Dardevet, L., Dong, Y., Al Khoury, S., Chatelain, F. C., Vialla, V., Chahboun, S., Lesage, F., Darbon, H., Pessah, I. N., & De Waard, M. (2016). In cellulo phosphorylation induces pharmacological reprogramming of maurocalcine, a cell-penetrating venom peptide. *Proceedings of the National Academy of Sciences USA*, **113**(17), E2460–E2468.
- Sárközi, S., Komáromi, I., Jóna, I., & Almássy, J. (2017). Lanthanides report calcium sensor in the vestibule of ryanodine receptor. *Biophysical Journal*, **112**, 2127–2137.
- Schroeder, N., Mullmann, T. J., Schmalhofer, W. A., Gao, Y.-D., Garcia, M. L., & Giangiacomo, K. M. (2002). Glycine 30 in ibertoxin is a critical determinant of its specificity for maxi-K versus $K(V)$ channels. *Federation of European Biochemical Societies Letters*, **527**(1–3), 298–302.

- Schwartz, E. F., Capes, E. M., Diego-García, E., Zamudio, F. Z., Fuentes, O., Possani, L. D., & Valdivia, H. H. (2009). Characterization of hadrucalcin, a peptide from *Hadrurus gertschi* scorpion venom with pharmacological activity on ryanodine receptors. *British Journal of Pharmacology*, **157**(3), 392–403.
- Seo, I.-R., Choi, M.-R., Park, C.-S., & Kim, D. H. (2006). Effects of recombinant imperatoxin A (IpTx) mutants on the rabbit ryanodine receptor. *Molecules and Cells*, **22**(3), 328–335.
- Stampe, P., Kolmakova-Partensky, L., & Miller, C. (1994). Intimations of potassium channel structure from a complete functional map of the molecular surface of charybdotoxin. *Biochemistry*, **33**(2), 443–450.
- Stocker, M., & Miller, C. (1994). Electrostatic distance geometry in a K⁺ channel vestibule. *Proceedings of the National Academy of Sciences*, **91**(20), 9509–9513.
- Szentandrassy, N., Magyar, Z. É., Hevesi, J., Bányász, T., Nánási, P. P., & Almássy, J. (2022). Therapeutic approaches of ryanodine receptor-associated heart diseases. *International Journal of Molecular Sciences*, **23**(8), 4435.
- Tisseyre, C., Bahembera, E., Dardevet, L., Sabatier, J.-M., Ronjat, M., & De Waard, M. (2013). Cell penetration properties of a highly efficient mini maurocalcine peptide. *Pharmaceuticals (Basel)*, **6**(3), 320–339.
- Treves, S., Anderson, A. A., Ducreux, S., Divet, A., Bleunven, C., Grasso, C., Paesante, S., & Zorzato, F. (2005). Ryanodine receptor 1 mutations, dysregulation of calcium homeostasis and neuromuscular disorders. *Neuromuscular Disorders*, **15**(9–10), 577–587.
- Treves, S., Jungbluth, H., Muntoni, F., & Zorzato, F. (2008). Congenital muscle disorders with cores: The ryanodine receptor calcium channel paradigm. *Current Opinion in Pharmacology*, **8**(3), 319–326.
- Tripathy, A., Resch, W., Valdivia, H. H., & Meissner, G. (1998). Imperatoxin A induces subconductance states in Ca²⁺ release channels (ryanodine receptors) of cardiac and skeletal muscle. *Journal of General Physiology*, **111**(5), 679–690.
- Valdivia, H. H., Kirby, M. S., Lederer, W. J., & Coronado, R. (1992). Scorpion toxins targeted against the sarcoplasmic reticulum Ca²⁺-release channel of skeletal and cardiac muscle. *Proceedings of the National Academy of Sciences USA*, **89**(24), 12185–12189.
- Wei, R., Chen, Q., Zhang, L., Liu, C., Liu, C., Yin, C.-C., & Hu, H. (2025). Structural insights into transmembrane helix S0 facilitated RyR1 channel gating by Ca²⁺/ATP. *Nature Communications*, **16**(1), 1936.
- Xiao, L., Gurrola, G. B., Zhang, J., Valdivia, C. R., SanMartin, M., Zamudio, F. Z., Zhang, L., Possani, L. D., & Valdivia, H. H. (2016). Structure-function relationships of peptides forming the calxin family of ryanodine receptor ligands. *Journal of General Physiology*, **147**(5), 375–394.
- Yamamoto, T., & Ikemoto, N. (2002). Peptide probe study of the critical regulatory domain of the cardiac ryanodine receptor. *Biochemical and Biophysical Research Communications*, **291**(4), 1102–1108.
- Yamamoto, T., Rodriguez, J., & Ikemoto, N. (2002). Ca²⁺-dependent dual functions of peptide C. *Journal of Biological Chemistry*, **277**(2), 993–1001.
- Yu, L., Sun, C., Song, D., Shen, J., Xu, N., Gunasekera, A., Hajduk, P. J., & Olejniczak, E. T. (2005). Nuclear Magnetic resonance structural studies of a potassium channel–charybdotoxin complex. *Biochemistry*, **44**(48), 15834–15841.
- Zhou, H. (2006). Characterization of recessive RYR1 mutations in core myopathies. *Human Molecular Genetics*, **15**(18), 2791–2803.

Additional information

Data availability statement

The data supporting the findings of this study are available within the article. The original datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Z.É.M., J.H. and Z.P. were responsible for investigations. F.M. and J.A. were responsible for formal analysis and data curation. Z.B. and J.S. were responsible for methodology. Z.É.M., J.A. and Z.P. were responsible for visualization. J.A., Z.P. and Z.V. were responsible for conceptualization and experimental design. J.A., G.P. and G.T. were responsible for funding acquisition and resources. J.A. was responsible for supervision. All authors participated in the interpretation of data, writing the original draft or reviewing and editing the manuscript. All authors approved the final version of the manuscript submitted for publication, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Funding

This work was funded by the National Research, Development and Innovation Fund of Hungary provided to JA (NFKFIH FK144576). Further support was provided to GT by EFOP-3.6.2-16-2017-00006, a project that was co-financed by the European Union and the European Regional Development Fund. This project had been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research Development and Innovation Fund, financed under the TKP2021-EGA funding

scheme (TKP2021-EGA-24). JA was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. ZP received additional funding from Deutsche Forschungsgemeinschaft (PE 3917/2-1).

Acknowledgements

We thank József Orosz for providing technical assistance in the animal facility. We are grateful to Veronika F. S. Pape for creating artwork.

Generative AI statement

OpenAI ChatGPT (GPT-5.5) was used solely for language editing, improvement of English phrasing and translation

of author-generated text during manuscript preparation. The authors reviewed and verified all AI-assisted output and take full responsibility for the content of the manuscript.

Keywords

charybdotoxin, ion channels, ryanodine receptor, single channel

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Peer Review History