Delineating an extracellular redox-sensitive module in T-type Ca$^{2+}$ channels

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ABSTRACT

T-type (Cav3) Ca$^{2+}$ channels are important regulators of excitability and rhythmic activity of excitable cells. Among other voltage-gated Ca$^{2+}$ channels, Cav3 channels are uniquely sensitive to oxidation and zinc. Using recombinant protein expression in HEK293 cells, patch-clamp electrophysiology, site-directed mutagenesis, and homology modeling, we report here that modulation of Cav3.2 by redox agents and zinc is mediated by a unique extracellular module containing i) a high-affinity metal-binding site formed by the extracellular IS1–IS2 and IS3–IS4 loops of domain I, and ii) a cluster of extracellular cysteines in the IS1–IS2 loop. Patch clamp recording of recombinant Cav3.2 currents revealed that two cysteine-modifying agents, sodium (2-sulfonatoethyl) methanethiosulfonate (MTSES) and N-ethylmaleimide (NEM), as well as a reactive oxygen species–producing neuropeptide, substance P (SP), inhibit Cav3.2 current to similar degrees and that this inhibition is reversed by a reducing agent and a zinc chelator. Pre-application of MTSES prevented further SP-mediated current inhibition. Removal of extracellular cysteines from the IS1–IS2 loop of Cav3.2 reduced its sensitivity to both MTSES and SP. We hypothesize that oxidative modification of IS1–IS2 loop cysteines induces allosteric changes in the zinc-binding site of Cav3.2, such that it become sensitive to ambient zinc.

Introduction

T-type Ca$^{2+}$ channels (CACNA1G, CACNA1H, and CACNA1I genes; Cav3.1, Cav3.2 and Cav3.3 channel α-subunits, respectively) are a family of voltage-gated Ca$^{2+}$ channels (VGCC) with very negative activation threshold (< -60 mV) and fast inactivation kinetics (1,2). The channels are widely distributed in the central and peripheral nervous systems (CNS, PNS), heart and vasculature, as well as in several types of non-excitable cells (3,4). In CNS T-type channels are highly expressed in dendrites of thalamic and hippocampal neurons where they amplify subthreshold postsynaptic potentials and facilitate the spread of depolarization to the cell body (5). Negative activation threshold and fast recovery from inactivation makes T-type Ca$^{2+}$ channels an important contributor to the pacemaker activity in the thalamic, corticothalamic
and other rhythmically-active neurones (6,7). Thalamic T-type currents are enhanced in several rodent models of absence epilepsy, correspondingly, several gain-of-function mutations within the CACNA1 genes were associated with human epilepsies, while T-type channel blockers were shown to suppress seizures and are efficacious in treatment of absent seizures in humans (for review see (4,8)).

In the PNS, T-type Ca\textsuperscript{2+} channels (and Cav3.2 in particular) are abundant in small-diameter, capsaicin-sensitive (presumed nociceptive) dorsal root ganglion (DRG) neurons (9-12), as well as in two distinct types of low-threshold mechanoreceptors innervating skin hair follicles (13). Discovery of relatively high abundance of T-type Ca\textsuperscript{2+} channels in nociceptors led up to establishment of the prominent role of these channels in the peripheral nociceptive transmission. Conditional deletion of Cav3.2 (13) or downregulation in DRG using intrathecal injection of antisense oligonucleotides produced anti-nociceptive effects in rodent pain models of neuropathic and inflammatory pain (14,15) and reduced analgesic efficacy of the T-type channel blocker (11). Conversely, multiple reports found T-type Ca\textsuperscript{2+} currents or Cav3.2 expression being increased in chronic pain conditions, such as diabetic neuropathy (14,16), peripheral nerve injury or inflammation (12,17-19).

Given their clear role in epilepsy and pain, the regulation of T-type Ca\textsuperscript{2+} channel activity received an intense scrutiny. T-type Ca\textsuperscript{2+} channels are regulated by multiple phosphorylation (20), glycosylation (21,22) and ubiquitination (12) mechanisms. In addition to these, Cav3.2 T-type subunit possesses a unique regulatory mode that is targeted by several endogenous regulatory pathways: sensitivity to oxidation and zinc. Thus, Cav3.2 is uniquely sensitive to sub-micromolar concentrations of extracellular zinc (11,23-25). The zinc-sensitive module is located extracellularly, involving interaction of the extracellular loops linking IS1–IS2 and IS3–IS4 transmembrane regions of domain I (23). The histidine H191, which is absent in Cav3.1 and Cav3.3 subunits, is critical to high sensitivity of Cav3.2 to zinc and nickel (11,23,26). In addition, T-type Ca\textsuperscript{2+} currents recorded in native nociceptive neurones and recombinant Cav3.2 currents are enhanced by reducing agents (dithiothreitol (DTT) or L-cysteine) and inhibited by the oxidizing agent 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) and hydrogen peroxide (11,27-30). Moreover, neuropeptide substance P, which induces generation of endogenous reactive oxygen species (ROS) (31), inhibits endogenous T-type Ca\textsuperscript{2+} currents in primary somatosensory neurons and recombinant Cav3.2 current via oxidative mechanism (11). Intriguingly, redox modulation of Cav3.2 also depends on H191 (11,24,32). We have recently shown that oxidative modification of Cav3.2 channels (both recombinant and those endogenously expressed in sensory neurons) induced by substance P enhances channel sensitivity to Zn\textsuperscript{2+} to such an extent that it becomes tonically inhibited by trace amounts of ambient zinc (11). Cav3 channels contain a number of extra- and intracellular cysteines that can be oxidised or reduced, depending on the local redox environment which, in turn, could affect the conformation of zinc binding site. In this study we combined electrophysiology, site-directed mutagenesis and computer modelling to delineate the molecular determinants of the unique redox sensitivity of Cav3.2.

Results

Cysteine-modifying reagents mimic the effect of redox modulation of Cav3.2 by Substance P. Neuropeptide Substance P (SP) induces the production of reactive oxygen species in immune (33) and epithelial (34) cells and sensory neurons (31). In the former cell type, SP inhibits T-type Ca\textsuperscript{2+} current via the oxidative modification of Cav3.2 (11). A mechanism of inhibition was proposed to be via the enhancement of Zn\textsuperscript{2+} sensitivity of Cav3.2 whereby the oxidized channel is inhibited by nanomolar free Zn\textsuperscript{2+} present in extracellular milieu (11). Yet, it is presently unknown how the oxidation of Cav3.2 is translated into higher sensitivity to Zn\textsuperscript{2+}. Cav3 channels have multiple extra- and intracellular...
cysteines, accessible to redox modulation (Fig. 1A). To test if cysteine modification is necessary for the oxidative modulation of Cav3.2 we performed patch clamp experiments testing the effects of the cell-impermeable cysteine-modifying reagent, Sodium (2-Sulfonatoethyl) methanethiosulfonate (MTSES; 2 mM) and cell-permeable cysteine-modifying reagent, N-ethylmaleimide (NEM; 200 μM), on the recombinant Cav3.2 overexpressed in HEK293 cells together with SP receptor, NK1 (Fig 1B-J). Both MTSES (Fig. 1B, C) and NEM (Fig. 1D, E) inhibited the Cav3.2 current to the similar levels and the inhibition was also similar in amplitude to that produced by the NK1-specific agonist [Sar⁹]-Substance P (S9SP; 1 μM; Fig. 1F-H). MTSES, NEM and SP inhibited peak Cav3.2 current amplitude by 40.1±3.5% (n=9, P<0.001), 36.1±5.4% (n = 7, P<0.001) and 40.1 ± 12.3% (n = 6, P<0.05) respectively. Reducing agent, dithioerythritol (DTT, 1 mM) applied in the presence of either MTSES, NEM or SP (Fig. 1E) recovered most of the inhibitory action of either agents, although in the case of NEM the recovery was incomplete (Fig. 1F-H). Interestingly, when S9SP was applied after MTSES, it produced no further inhibition, suggestive of common mechanism of action of both agents (Fig. 1I, J). Neither MTSES, NEM nor S9SP significantly affected activation or inactivation kinetics of recombinant Cav3.2 (Table S1).

Ambient zinc and high-affinity zinc binding site are necessary for Cav3 channel sensitivity to MTSES. It was shown previously that Cav3.2 with H191Q mutation in the high affinity zinc binding site displayed much lower sensitivity to SP (11). To examine if H191 is important for the effect of MTSES, we tested its effect on Cav3.2 (H191Q) mutant overexpressed in HEK293 cells together with NK1 receptors (Fig. 2A, D). MTSES produced only a modest inhibitory effect on Cav3.2 (H191Q) (23.1±5.6%, n=6, P<0.01); the inhibition was significantly reduced as compared to the wild-type Cav3.2 (Fig. 2D, P<0.05), moreover, MTSES-induced inhibition of Cav3.2 (H191Q) was no longer recoverable with DTT (Fig. 2A, D).

Cav3.1 subunit has glutamine instead of histidine in the position equivalent to 191 in Cav3.2 (position 172 in Cav3.1) and, hence, is much less sensitive to zinc (11,23). Thus, we tested the effect of MTSES on the WT CaV3.1 and CaV3.1 (Q172H) mutant. MTSES produced only a very modest inhibition of 9.3±2.8% in the WT Cav3.1 (n=5, P<0.05, Fig. 2B, E). Strikingly, the MTSES effect on Cav3.1 (Q172H) currents was much stronger (inhibition by 36.9±2.8%, n=6, P<0.001, Fig 2C, E). The MTSES inhibition of Cav3.1 (Q172H) was comparable to that of the WT Cav3.2 (cf. Fig 2C, E and Fig. 1B, C). The results further reinforce the hypothesis that oxidative modulation of Cav3 channels depends on the high-affinity zinc binding site.

To further probe the requirement for zinc to the effect of extracellular cysteine modification on Cav3 channel activity, we applied zinc chelator, N, N, N', N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, 10 μM) before or after MTSES. The experiments presented in Fig. 3 demonstrate that TPEN could totally recover the inhibition induced by MTSES on WT Cav3.2; thus 2 mM MTSES inhibited peak Cav3.2 current to 61.2±8.7% of baseline and TPEN applied still in the presence of MTSES recovered the inhibition to 102.4±5.5% of the baseline (n=6, Fig 3A, C). Consistent with previous results, MTSES inhibition of Cav3.2 (H191Q) mutant was much smaller (inhibition to 83.4±1.7% of baseline, n=8, Fig. 3 B, C) and this small inhibition was also recovered by TPEN (Fig. 3 B, C). Application of TPEN on its own (Fig. 3D-F) did not significantly affected the amplitude of either the WT Cav3.2 or the H191Q mutant (some run-up of the current amplitude was observed in some recordings but the effect did not reach significance). Importantly though, in the presence of TPEN, MTSES no longer was able to inhibit currents produced by either of the channels. Interestingly, under these conditions we often observed a small and very transient inhibition by MTSES (could be seen in the examples presented in Fig. 3D and E) which then spontaneously recovered. Clearly, binding of zinc to its high-affinity extracellular binding site is necessary for the full inhibitory action of MTSES. We also tested the effect of TPEN on the WT Cav3.1

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and Cav3.1 (Q172H) mutant currents and there was
no strong effects (Fig. 3G-I). In WT Cav3.1 TPEN
produced small inhibition, which perhaps is a non-
specific effect. In the case of the Cav3.1 (Q172H)
mutant, TPEN had no significant effect (Fig. 3H, I);
as in the case of WT Cav3.2, a small run-up was
often observed but the effect did not reach
significance. Importantly, when applied in the
presence of TPEN, MTSES had no effect on either
the WT Cav3.1 or Cav3.1 (Q172H) mutant (Fig. 3G-
I). This result was in stark contrast to that shown in
Fig. 2C, E, where in the absence of TPEN, MTSES
produced strong inhibition of Cav3.1 (Q172H)
mutant, which has its high-affinity zinc binding site
reintroduced. Mutations H191Q (Cav3.2) and
Q172H (Cav3.1) did not significantly affected
activation and inactivation kinetics and current
densities of the respective channels (Table S2).

In combination, the results presented in Fig. 1-3
point to the following conclusions: i) cysteine
modifying agents and oxidative modification
produce similar inhibitory effect on Cav3 channels;
ii) these effects require zinc and depend on the high-
affinity extracellular zinc binding site formed by the
extracellular loops of domain I; iii) since MTSES is
cell-impermeable, the cysteines mediating the redox
inhibition must be located in the extracellular regions
of the Cav3 channel proteins.

The cysteines in the IS1–IS2 extracellular loop
of T-type Ca\(^{2+}\) channels are necessary and
sufficient for the redox-mediated inhibition of the
channel activity. Earlier studies defined critical
residues of the high-affinity metal binding site of
Cav3.2, including an Asp189-Gly190-His191 motif
in IS3–S4 and an additional Asp residue in IS2 (23)
with His191 being a key residue absent in other Cav3
subunits (and any other VGCC \(\alpha\)-subunits). High-
resolution cryo-EM structure of human Cav3.1 has
been solved recently (35). Since Cav3.1 is not highly
sensitive to zinc or redox-mediated modulation due
to the absence of the critical histidine residue in its
IS3–S4 loop, we obtained Cav3.2 structure using
homology modeling and analyzed putative metal
binding site (Fig. 4, Fig. S1). The "Local quality"
estimate of the model (Fig. 4A) shows that the scores
of all regions except a few loop region are higher
than 0.6, which indicates that the overall structure of
the model is reliable (Fig. 1A).

The putative metal binding site formed by
extracellular IS1-IS2 and IS3-IS4 regions is depicted in
Fig. 1B-E. The electrostatic surface potential (ESP) of the Cav3.2 model indicates that the lower
potential in the extracellular region comprised of
IS1-IS2 and IS3-IS4 loops is favorable for metal
binding (Fig. 1E). Moreover, the model suggests that
ESP near Glu127 and Glu137 is very low, and we
suspect that Glu127 and Glu137 may also contribute
to the metal binding. Interestingly, four cysteines are
located within or near this extracellular region: C114,
C123, C128, C133 (Fig. 4B-D). Although side
chains of several amino acids (e.g. methionines,
arginines and aromatic amino acids) can be modified
by oxidizers, sulfhydryl groups of cysteines are by
far the most susceptible to oxidation protein moieties
(36). C123, C128 and C133 were suggested to be
important for modulation of the T-type channels by
lipoic acid (37) and nitric oxide (38). Thus, we
hypothesized that oxidative modification of some or
all of these residues may introduce allosteric changes
to the metal binding site, favoring channel inhibition.
In order to test this hypothesis, we substituted these
cysteines by alanines and tested the sensitivity of the
mutants to MTSES (Fig. 5). All single mutants
(C114A, C123A, C128A and C133A) showed
significantly reduced sensitivity to MTSES (Fig. 5A-
D, F); C123A displayed the least sensitivity (Fig. 5D,
F). Importantly, the quadruple mutant in which all
the above-mentioned cysteines were substituted by
alanines was largely insensitive to MTSES (Fig. 5E,
F). The quadruple mutant expressed very poorly
though, with current density reduced more than 10
times as compared to WT Cav3.2 (Table S2), hence
only four recordings have been produced.

We also tested the sensitivity of individual
cysteine mutants to SP (Fig. 6) and, again, all four
displayed much reduced sensitivity with inhibition
by 10 μM S9SP in the range of 10-20% (as compared
to 45.1±11.6%, n=7 in the WT Cav3.2). We also
tested one intracellular cysteine in the intracellular
IS2-IS3 linker, C165 as we reasoned it could
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allosterically influence the arrangement of IS1-IS2 and IS3-IS4 loops. However, the C165A mutant still displayed obvious inhibition by SP (26.5±3.9%, n=7), which was not significantly different from the SP-induced inhibition of the WT Cav3.2 (Fig. 6A, B). Neither of cysteine mutants used in this study displayed significantly different activation and inactivation kinetics. Current densities of single mutants also did not change significantly compared to WT Cav3.2. The only exception was the quadruple C-to-A mutant, which displayed much reduced current density (albeit kinetics was not significantly affected; Table S2).

Taken together, the results presented in Fig 4-6 strongly suggest that the oxidative modification of extracellular cysteines in the IS1-IS2 loop increases sensitivity of Cav3.2 to inhibition by trace amounts of extracellular zinc by inducing conformational changes within the high-affinity metal binding site of the channel.

Discussion

Here we report that modulation of T-type Ca\(^{2+}\) channels by redox agents and zinc is structurally coupled and depends on the presence of the high-affinity metal binding site formed by the extracellular IS1-IS2 and IS3-IS4 loops of domain I, as well as on the group of extracellular cysteines present in the IS1-IS2 loop.

A mechanism for convergence of redox- and zinc-dependent modulation of T-type Ca\(^{2+}\) channels has been hypothesized in earlier studies, whereby it was proposed that H191 could be subject to metal-catalysed oxidation (MCO) reaction (28,36). Thus, either binding of Zn\(^{2+}\), or Zn\(^{2+}\)-independent MCO of H191 could result in a similar inhibition of channel activity (24,28,39). Yet, data presented here suggest an alternative mechanism: oxidative modification of extracellular cysteines in the IS1-IS2 loop may allosterically increase the Cav3 channel inhibition induced by binding of zinc to H191. The following observations are in favour of the above hypothesis. i) cysteine-modifying reagents, MTSES and NEM, as well as SP produced similar degree of Cav3.2 inhibition, which was reversible by either the reducing agent, DTT (Fig. 1), or by the zinc chelator, TPEN (Fig. 3); pre-application of MTSES rendered SP ineffective to produce any further current inhibition (Fig. 1I, J). ii) Removal of H191 from Cav3.2 dramatically reduced inhibition of the channel by MTSES (Fig. 2) or SP (11). iii) Introduction of the corresponding histidine into Cav3.1 induced its sensitivity to both, MTSES (Fig. 2) and SP (11). iv) Removal of extracellular cysteines from the IS1-IS2 loop of Cav3.2 dramatically reduced the sensitivity of the channel to both, MTSES (Fig. 5) and SP (Fig. 6); the mutant with cysteine-less IS1-IS2 loop was found to be resistant to MTSES (Fig. 5E, F). Thus, we hypothesize that oxidative modification of extracellular cysteines in the IS1-IS2 loop of Cav3.2 induces allosteric changes in its zinc binding site, such that it become sensitive to ambient zinc. This effect is unique to Cav3.2 since other Cav3 subunits lack critical histidine at positions equivalent to 191 in Cav3.2. Indeed, according to our earlier atomic absorption spectroscopy measurements, total zinc levels in nominally zinc-free laboratory solutions are in the range of 5-10 μM (11). Similar or higher range of zinc concentrations is reported for human plasma (40). Concentrations of free Zn\(^{2+}\) (both in vitro and in vivo) are likely to be much lower as compared to the total zinc, yet, our estimate suggested low nanomolar range (11). This would be still sufficient to produce significant effect on channel activity since the high-affinity zinc binding site in Cav3.2 has a nanomolar zinc affinity (11).

Activation of NK1 receptors was shown to generate endogenous ROS production (31,33,41), which is a necessary step in NK1-mediated modulation of Cav3.2 (11). However it is presently unclear how intracellularly generated ROS act upon extracellular site within Cav3.2 protein. One intriguing possibility is that in response to endogenous ROS release, cells could release some redox-active molecules, such as thioredoxin (TRX). Indeed, TRX can be secreted (42), moreover, it is known to inhibit Cav3.2 channels by interfering with their extracellular zinc-binding site (43). A mechanism of TRPC channel regulation through the

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Breakdown of extracellular disulphide bond by secreted TRX has been reported (42) and it is tempting to hypothesize that a similar mechanism could be at play in the case of NK1-mediated modulation of T-type Ca\(^{2+}\) channels. However, further investigation is required to decipher this intriguing signalling cascade.

The exact structural consequences of the oxidation of IS1-IS2 cysteines are yet to be elucidated. These cysteines may be involved in disulfide bonds or oxidised to cysteine sulfinic (Cys-SO\(_2\)H) or sulfonic (Cys-SO\(_3\)H) acids (44). Cryo-EM structure of Cav3.1 revealed a disulphide bond between the C104 in IS1-IS2 and C889 on IIS5-IIS6 pore loop, which is unique to Cav3 channels (35). This bond was hypothesised to be important for the unique redox sensitivity of T-type Ca\(^{2+}\) channels (35). C104 and C889 in Cav3.1 correspond to C123 and C939 in Cav3.2 (Fig. 1A) and our model of Cav3.2 (Fig. 4, Fig. S1) also predicts a disulphide bond between these residues in Cav3.2, while other cysteines in the IS1-IS2 loop do not form disulfide bonds. Interestingly, C123A Cav3.2 mutant was the least sensitive to MTSES among the all cysteine mutants we have tested (Fig. 5). Thus perhaps this covalent bond, linking high-affinity zinc binding site to the pore region of the channel, is indeed important for coupling of zinc binding to channel activity. Yet, since other IS1-IS2 loop cysteines also affect channel sensitivity to MTSES and SP, the C123-C939 bond is likely not an exclusive determinant.

Oxidative modification of extracellular cysteines in IS1-IS2 loop may produce conformational changes within the high-affinity zinc binding site of the channel that either increases zinc affinity at the binding site or enhances coupling efficiency between the zinc binding and channel inhibition. We believe that the latter is more likely to be the case since SP treatment strongly increased the efficacy of zinc mediated inhibition of Cav3.2 while having no effect on the IC\(_{50}\) (11). Resolving Cav3.2 structures in the presence and absence of zinc and at different states of extracellular cysteine oxidation will shed the light on the exact mechanism of coupling between these two modulatory mechanisms. Nevertheless, the present study clearly demonstrated that the redox and zinc modulation of Cav3.2 is indeed structurally coupled and requires both, the metal-coordinating histidine in the IS3–IS4 loop and extracellular cysteines in the IS1–IS2 loop.

T-type Ca\(^{2+}\) channels are important regulators of excitability and rhythmic activity of excitable cells, the activity of these channels is regulated by multiple physiological signalling pathway, many of which act on the channel targeting its redox/zinc-sensitive module. The examples of these modulatory pathways include nitrous oxide (38,45), carbon monoxide and thioredoxin (43), hydrogen sulphide (46), α-Lipoic acid (37), as well as GABA\(_{A}\) receptors (47) and substance P (11). Hence, elucidation of structural background of T-type channel modulation via the redox/zinc-sensitive module, reported here, sheds new light on the physiological regulation of these channels; moreover, it provides valuable insight for the development of future T-type channel modulators for treatment of excitability disorders, such as epilepsy and pain.

Experimental Procedures

Cell Culture, transfections, cDNA constructs and chemicals. HEK 293 cells were cultured in DMEM supplemented with GlutaMax I, 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) in culture flasks in a humidified incubator (37°C, 5% CO\(_2\)). Cultures were passaged every 2 days (upon reaching 80–90% confluency), one day before transfection the cells were passaged on glass coverslips (1.3 cm × 1.3 cm). All cell culture reagents were purchased from Gibco-BRL (Paisley, UK) unless otherwise stated. Human NK1 receptor (GenBank accession number AY462098) cDNA was purchased from the Missouri Science and Technology cDNA Resource Center. Cav3.1 (GenBank accession number AF027984), Cav3.2 (GenBank accession number AF051946), Cav3.1Q172H and Cav3.2H191Q were kindly provided by Dr E. Perez-Reyes, (University of Virginia, USA). Cav3.2 C114A, Cav3.2 C123A, Cav3.2 C128A, Cav3.2 C133A, Cav3.2 C165A and Cav3.2 (C114A, C123A, C128A, C133A) were
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Custom-made by Sangon Biotech (China). HEK293 cells were transfected using FuGENE HD (Promage, USA) according to the manufacturer’s instructions. MTSES (Sodium(2-sulfonatoethyl)methanethiosulfonate) and Z944 were from Toronto Research Chemicals (Canada). All other chemicals were from Sigma.

**Electrophysiology.** Amphotericin B perforated patch-clamp recordings were used to record Ca\(^{2+}\) currents from transfected HEK293 cells. Recordings were made using Multiclamp 700B amplifier in combination with pCLAMP 10.4 software (Axon Instruments, Union City, CA, USA) as described previously (11). Offline analysis was performed using Clampfit 10.4 (Molecular Devices). Voltage-clamp recordings were sampled at 4 kHz and performed using the Amphotericin B perforated patch clamp method. The standard bath solution contained (in mM): 150 TEA-Cl, 2.5 CsCl, 2.5 CaCl\(_2\); 10 HEPES; 0.5 MgCl\(_2\); 10 glucose (pH 7.4 adjusted with CsOH; 305-310 mosm/kg). The solutions were applied to the bath chamber using 8-channel gravity perfusion system VC3-8 (ALA, USA) in combination with the local perfusion pencil (04-08-250, AutoMate Scientific, USA; inner diameter 250 μm) at ~1 ml/min. The pipette solution contained (in mM): 155 CsCl; 10 HEPES; 1 EGTA; 4 MgCl\(_2\) supplemented with amphotericin B (250 μg/ml), pH 7.4 adjusted with CsOH. Patch electrodes were pulled with a horizontal micropipette puller (P-97, Sutter Instruments, USA) and fire polished. The access resistance was typically within 6-10 MΩ. Cav3 currents were measured by 50 ms square voltage pulses to -40 mV from a holding potential of -90 mV. Series resistance was compensated online by 50-80%. All recordings were performed at room temperature (~22°C).

**Homology modeling.** Structural model of the Cav3.2 channel was constructed using the homology modeling server SWISSMODEL (48) and the Cav3.1 channel structure (PDB: 6kzo) (35) as a template. The sequence identity of transmembrane regions of CaV3.1 and CaV3.2 reached 84.27%. The resolution of the Cav3.1 cryo-EM structure is 3.3 Å. The overall root-mean-square deviation of atomic positions (RMSD) value between the Cav3.2 model and Cav3.1 cryo-EM structure is 0.26 Å (Fig. 1S). QMEANBrane was used for reliable local quality estimation of membrane protein models (49). For the evaluation of the overall protein structure the validation server (SAVES v5.0 Institute of Molecular Biology, University of California: http://nihserver.mbi.ucla.edu/SAVES) has been used. All molecular visualization and structural diagrams were made using Open-Source Pymol (http://pymol.org).

**Statistics.** All mean data are given as mean ± S.E.M. Differences between groups were assessed by Student’s t test (paired or unpaired, as appropriate) or one-way ANOVA with Dunnett’s post-hoc test. The differences were considered significant at P ≤ 0.05. Statistical analyses were performed using Origin 8.6 (OriginLab Corporation, Northampton, CA, USA).
Data availability. All data are available in the main text or the supplementary information section.

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Conflict of interest. The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions. D.H. planed, performed and analyzed experiments, wrote the manuscript; H.A. and S.S. performed computer modeling, analyzed data; C.L. and X.Z. performed some experiments. H.Z., C.P., X.D. helped designing experiments, interpret the data; N.G. designed the study, analyzed data and wrote the manuscript.
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**Abbreviations:**
- ANOVA, analysis of variance
- CNS, central nervous system
- DMEM, Dulbecco's Modified Eagle Medium
- DTT, dithiothreitol
- EGTA, Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
- ESP, electrostatic surface potential
- HEK293, human embryonic kidney 293 cells
- HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
- MCO, metal-catalyzed oxidation
- MTSES, Sodium (2-Sulfonatoethyl) methanethiosulfonate
- NEM, N-ethylmaleimide
- PDB, protein databank
- PNS, peripheral nervous system
- ROS, reactive oxygen species
- RMSD, root-mean-square deviation
- SP, substance P
- TEA, tetraethylammonium
- TPEN, N, N, N', N'-tetrakis(2-pyridylmethyl) ethylenediamine
- TRPC, transient receptor potential cation channel, canonical
- TRX, thioredoxin
- Z944, N-[[1-[2-(tert-butylamino)-2-oxoethyl]piperidin-4-yl]methyl]-3-chloro-5-fluorobenzamide
- VGCC, voltage-gated Ca\textsuperscript{2+} channels
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Figure 1. Cysteine-modifying reagents and substance P inhibit recombinant Cav3.2 currents in a similar mechanism. A, Schematic of Cav3.2 channel; intra- and extracellular cysteines are indicated by spheres. Shown in red are cysteines mutated in the present study; orange square indicates zinc-binding histidine 191; purple area indicates high-affinity zinc binding site. B, Example time course of the effects of 2 mM Sodium (2-Sulfonatoethyl) methanethiosulfonate (MTSES) and 1 mM dithioerythritol (DTT; applied in the presence of MTSES) on the Ca$^{2+}$ current recorded from HEK293 cells transiently overexpressing Cav3.2 and NK1 receptors using perforated patch clamp. Selective T-type channel blocker, Z944 (1 μM) was applied at the end of the experiment. Plotted are peak Ca$^{2+}$ current amplitudes; periods of drug application are indicated by the vertical gray bars. Inset shows example current traces. C, Summary of the effects recorded in experiments exemplified in panel B, individual data points are represented by the colored circles, paired data points from the same experiment are depicted in the same color. D, Example time course of the experiment similar to that shown in panel A but N-ethylmaleimide (NEM; 200 μM) was applied instead of MTSES. E, Summary of the effects recorded in experiments exemplified in panel D. F, Example time course of the experiment similar to that shown in panel B but a selective NK1 receptor agonist [Sar$^9$]-Substance P (S9SP; 1 μM) was applied instead of MTSES. G summarizes these experiments. H, Comparison of the Cav3.2 current inhibition produced by MTSES and S9SP. I, Example time course of the effects of 1 μM S9SP applied after (and in the presence of) MTSES on the recombinant Cav3.2 current, panel J summarizes these experiments. In bar/scatter charts asterisks denote significant difference between the groups indicated by the line connectors with *$P < 0.05$; ***$P < 0.001$ (paired t-test or one-way ANOVA, as appropriate); bars represent mean ± SEM; number of individual recordings is shown within the bars.
Figure 2. MTSES-induced inhibition of Cav3 channels requires intact high-affinity zinc binding site. A, B and C, Example time courses showing the effects of MTSES (2 mM), DTT (1 mM; applied in the presence of MTSES) and Z944 (1 µM) on the Ca\(^{2+}\) current recorded from HEK293 cells transiently overexpressing Cav3.2 H191Q (A), WT Cav3.1 (B) or Cav3.1 Q172H (C). Plotted are peak Ca\(^{2+}\) current amplitudes; periods of drug application are indicated by the vertical gray bars. Inset shows example current traces. D, Summary of the effects recorded in experiments exemplified in panel A, individual data points are represented by the colored circles, paired data points from the same experiment are depicted in the same color; additionally shown is the data set for the MTSES-induced inhibition of the WT Cav3.2 (grey bar, circles) taken from the Fig. 1C. E, Summary of the effects recorded in experiments exemplified in panels B and C. In bar charts asterisks denote significant difference between the groups indicated by the line connectors with \(*P < 0.05; \***P < 0.001\) (paired or unpaired t-test or one-way ANOVA, as appropriate); bars represent mean ± SEM; number of individual recordings is shown within the bars.
Figure 3. MTSES-induced inhibition of Cav3 channels is reversed by zinc chelation. A, B, Example time courses showing the effects of MTSES (2 mM), N, N, N', N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, 10 μM; applied still in the presence of MTSES) and Z944 (1 μM) on the Ca^{2+} currents recorded from HEK293 cells transiently overexpressing WT Cav3.2 (A) or Cav3.2 H191Q (B). C, Summary of the effects recorded in experiments exemplified in panel A and B, individual data points are represented by the colored circles, paired data points from the same experiment are depicted in the same color. D, E, Experiments similar to those shown in panels A and B, but MTSES is applied after (and still in the presence of) the TPEN. F, Summary of the effects recorded in experiments exemplified in panels D and E. G, H, Experiments similar to those shown in panels D and E, but WT Cav3.1 (G) and Cav3.1 Q172H (H) were investigated. I, Summary of the effects recorded in experiments exemplified in panels G and H. In bar/scatter charts asterisks denote significant difference between the groups indicated by the line connectors with *P < 0.05; **P < 0.01; ***P < 0.001 (paired or unpaired t-test or one-way ANOVA, as appropriate); bars represent mean ± SEM; number of individual recordings is shown within the bars.
Figure 4. Homology modeling of redox/metal regulatory module in Cav3.2. Initial structure of the human Cav3.2 channel was obtained by homology modeling (see Fig. S1 for further details) based on the structure of human Cav3.1 (35). A, Local quality estimate of the model obtained with QMEANBrane. B-D, Redox/metal regulatory module shown at various rotation points (protein shown as ribbons and key amino acids are indicated). E, Electrostatic surface potential of the redox/metal regulatory module (-50 kT/e to 50 kT/e, in vacuum).
Figure 5. Extracellular cysteines in the IS1-IS2 loop are necessary for MTSES-mediated inhibition of Cav3.2. A-E, Example time courses showing the effects of MTSES (2 mM) and Z944 (1 µM) on the Ca\(^{2+}\) current recorded from HEK293 cells transiently overexpressing Cav3.2 C114A (A), Cav3.2 C123A (B), Cav3.2 C128A (C), Cav3.2 C133A (D) or a quadruple mutant with C-to-A mutations at positions 114, 123, 128 and 133 (4xC-A; E). F, Summary of the effects recorded in experiments exemplified in panels A-E, individual data points are represented by circles. Asterisks denote significant difference between the groups indicated by the line connectors with *P < 0.05; **P < 0.01; ***P < 0.001 (one-way ANOVA); bars represent mean ± SEM; number of individual recordings is given below each bar.
Figure 6. Extracellular cysteines in the IS1-IS2 loop are necessary for SP-mediated inhibition of Cav3.2. A, Example time courses showing the effects of S9SP (1 μM), DTT (1 mM; applied in the presence of S9SP) and Z944 (1 μM) on the Ca\(^{2+}\) current recorded from HEK293 cells transiently overexpressing Cav3.2 C165A. B, Summary of the effects recorded in experiments exemplified in panel A for HEK293 cells overexpressing Cav3.2 C114A, Cav3.2 C123A, Cav3.2 C128A, Cav3.2 C133A or Cav3.2 C165A; individual data points are represented by circles. Asterisks denote significant difference between the groups indicated by the line connectors with *P < 0.05; ***P < 0.001 (one-way ANOVA); bars represent mean ± SEM; number of individual recordings is given below each bar.
Delineating an extracellular redox-sensitive module in T-type Ca2+ channels
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