

RESEARCH ARTICLE



Inhibition of late sodium current prevents pathological hyperactivation of calcium/calmodulin-dependent protein kinase II δ in a murine model of acute doxorubicin-related cardiotoxicity

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Abstract

Background and Purpose: Doxorubicin (DOX) is a highly effective anthracycline, whose clinical application for cancer is limited by cardiotoxicity. The mechanisms underlying doxorubicin-induced toxic cardiomyopathy (DICM) involve electrophysiological remodelling with intracellular Na^+ overload because of increased late I_{Na} and hyperactivation of CaMKII δ . Increased $[\text{Na}^+]_i$ contributes to CaMKII δ activation through Na-dependent Ca^{2+} overload, and CaMKII δ can further amplify late I_{Na} .

Experimental Approach: We tested whether pharmacological inhibition of the late I_{Na} by either ranolazine (RAN, $10 \mu\text{mol}\cdot\text{L}^{-1}$) or empagliflozin (EMPA, $1 \mu\text{mol}\cdot\text{L}^{-1}$) is sufficient to attenuate DOX-mediated hyperactivation of CaMKII δ in isolated wild-type (WT) ventricular cardiomyocytes. The contribution of reciprocal CaMKII-dependent stimulation of late I_{Na} was tested in transgenic S571A cardiomyocytes lacking the CaMKII-specific phosphorylation site S571A on $\text{Na}_v1.5$. Functional read-outs were obtained using patch-clamp technique, as well as confocal and epifluorescence microscopy.

Key Results: DOX acutely increased late I_{Na} in WT cardiomyocytes by twofold, which was associated with redox- and phospho-dependent activation of CaMKII δ . Hyperactivated CaMKII δ led to acutely impaired Ca^{2+} handling because of diastolic Ca^{2+} loss from the sarcoplasmic reticulum (SR) mediated by phosphorylation of the RyR2 at the CaMKII-specific phosphorylation site serine-2814. Pharmacological inhibition of late I_{Na} by EMPA or RAN, and genetic deletion of the CaMKII-specific phosphorylation-site serine-571 at $\text{Na}_v1.5$ prevented DOX-related stimulation of the

Abbreviations: CaMKII δ , calcium/calmodulin-dependent protein kinase II delta isoform; CaMKII $\delta^{-/-}$, CaMKII δ knockout; DICM, doxorubicin-induced toxic cardiomyopathy; DOX, doxorubicin; EMPA, empagliflozin; late I_{Na} , late sodium current; LV, left ventricular; MEL, melatonin; Mito-T, Mito-TEMPO; $\text{Na}_v1.5$, voltage-gated sodium channel isoform 1.5; RAN, ranolazine; ROS, reactive oxygen species; RyR2, ryanodine receptor isoform 2; SBF1, sodium-binding benzofuran isophthalate.

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late I_{Na} and subsequent CaMKII δ hyperactivation, which functionally preserved intracellular Ca handling.

Conclusion and Implications: Inhibition of late I_{Na} protects cardiomyocytes from pathologic CaMKII δ hyperactivation and impaired Ca^{2+} handling in the setting of acute DOX cardiotoxicity.

KEYWORDS

anthracycline, CaMKII, cardioprotection, cardiotoxicity

1 | INTRODUCTION

Doxorubicin (DOX)-based chemotherapy is used to treat a wide range of cancers, including breast cancer (Dempke et al., 2023). The clinical efficacy of DOX is limited by its cardiotoxic side effects, resulting in dose-dependent left ventricular (LV) dysfunction, with an incidence of approximately 9% that occurred in 98% of cases within the first year after DOX therapy (Cardinale et al., 2015). The pathomechanisms underlying the development of DOX-induced cardiotoxicity are complex, but it is nowadays generally accepted that disturbed calcium (Ca^{2+}) handling is centrally involved (Feder et al., 2026; Lin et al., 2024; Llach et al., 2019; Sag et al., 2011).

In that regard, we have recently reported that DOX disturbs intracellular Ca^{2+} handling by persistent **CaMKII δ** hyperactivation, which induces pathologic hyperphosphorylation of the **ryanodine receptor 2** (RyR2) at the CaMKII-specific phosphorylation site serine-2814 leading to impaired Ca^{2+} handling and systolic dysfunction in vivo (Feder et al., 2026; Lin et al., 2024; Llach et al., 2019; Sag et al., 2011). Disturbed intracellular sodium (Na^+) handling is another key characteristic of heart failure that is associated with the development of systolic and diastolic dysfunction (Bay et al., 2013; Ke et al., 2020; MacLeod 2023; Mustroph et al., 2022; Noble & Noble, 2006; Toischer et al., 2013) and is observed in DOX-induced cardiotoxicity (Cappetta et al., 2017; Maurea et al., 2016; Tocchetti et al., 2014). Maurea and colleagues found that DOX treatment enhances the so-called 'late' sodium current (late I_{Na}) in isolated murine ventricular cardiomyocytes, which is associated with intracellular accumulation of Na^+ (Maurea et al., 2016) (late I_{Na} arises as a consequence of not fully inactivated sarcolemmal Na^+ channels; Noble & Noble, 2006). Increased $[Na^+]_i$ leads to increased $[Ca^{2+}]_i$ because of stimulated Na/Ca exchanger (NCX) reverse-mode-activity and can thus activate Ca-dependent signalling including CaMKII δ -related signalling.

Here, we tested whether DOX-induced late I_{Na} contributes to Na/Ca overload, pathological hyperactivation of CaMKII δ and subsequently impaired intracellular Ca^{2+} handling in a cell model of acute DOX-induced cardiotoxicity. We tested for the first time whether this pathological signalling cascade can be mitigated by i) pharmacological inhibition of late I_{Na} (using **empagliflozin** [EMPA] or **ranolazine** [RAN]) or ii) genetic deletion of the CaMKII-specific phosphorylation site serine-571 at the sarcolemmal sodium channel **Nav1.5** in transgenic S571A myocytes (Mustroph et al., 2022, 2023).

What is already known?

- Electrophysiological remodelling (i.e., impaired calcium and sodium handling) contributes to doxorubicin-induced cardiotoxicity mediated by CaMKII δ .

What does this study add?

- Our study identifies late I_{Na} as an upstream activator of CaMKII δ hyperactivation in doxorubicin-induced cardiotoxicity.
- Pharmacological inhibition of late I_{Na} prevents CaMKII δ -dependent impairment of Ca/Na-handling in doxorubicin-induced cardiotoxicity.

What is the clinical significance?

- Our study suggests late I_{Na} inhibition as a cardioprotective strategy for patients receiving doxorubicin treatment.

2 | METHODS

2.1 | Mice

All experiments are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). In addition, all experiments were conducted in accordance with the guide for care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee. Mice were bred at the animal facility of the University Medical Hospital Regensburg. We used a transgenic mouse model, in which Nav1.5 was made resistant to CaMKII-dependent phosphorylation at serine-571 by knock-in replacement of serine-571 to alanine (S571A), as well as their wild-type (WT) littermates (Glynn et al., 2015; Mustroph et al., 2023).

Transgenic S571A mice and their WT littermates demonstrated equal phenotypes (heart and body weight, as well as survival). Moreover, we used a second transgenic mouse model, in which CaMKII δ was genetically eliminated (CaMKII $\delta^{-/-}$) (Backs et al., 2009). All mouse models were maintained on a C57BL/6J genetic background.

2.2 | Materials

Doxorubicin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at 10 $\mu\text{mol}\cdot\text{L}^{-1}$, reflecting clinically relevant plasma concentrations (Behboudi et al., 2025). In some experiments, 1- $\mu\text{mol}\cdot\text{L}^{-1}$ EMPA or 10- $\mu\text{mol}\cdot\text{L}^{-1}$ RAN was used to inhibit late I_{Na} . EMPA was purchased from Hölzel (Köln, Germany) and RAN from Sigma-Aldrich (St. Louis, MO, USA). The EMPA and RAN concentrations used reflect clinically relevant plasma concentrations (Jerling, 2006; Rascher et al., 2024). EMPA and RAN were added to the experimental solutions 30 min before measurements (and without pacing cardiomyocytes during EMPA or RAN incubation).

2.3 | Mouse cardiomyocyte isolation

Ventricular cardiomyocytes were isolated directly after cervical dislocation from adult mice (12–15 weeks) of both sexes in equal shares according to a well-established procedure as described previously (Feder et al., 2026; Gissibl et al., 2024). In brief, mice were killed by cervical dislocation and hearts were carefully excised and mounted on a Langendorff perfusion apparatus. Hearts were retrogradely perfused with a calcium-free solution containing (in $\text{mmol}\cdot\text{L}^{-1}$): NaCl 113, KCl 4.7, KH_2PO_4 0.6, $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ 0.6, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 12, KHCO_3 10, HEPES 10, Taurine 30, 2,3-butanedione 10, glucose 5.5 and phenol red 0.032, at 37°C (pH 7.4) for approximately 4 min. Afterwards, trypsin 0.6% (Thermo Fisher Scientific Inc. of Waltham, MA, USA), liberase 7.5 mg/ml (Roche diagnostics, Mannheim, Germany) and CaCl_2 0.125 $\text{mmol}\cdot\text{L}^{-1}$ were added to the perfusion solution to allow enzyme digestion of the extracellular matrix and hearts were perfused until they became flaccid. After enzymatic digestion, the ventricular myocardium was dissected from the atria. The ventricular myocardium was collected in perfusion buffer supplemented with 5% bovine calf serum (Sigma-Aldrich, St. Louis, MO, USA) to stop enzyme activity, cut into small pieces and further disintegrated by careful pipetting until no solid tissue was visible. Finally, the received cardiomyocyte cell suspension was filtered using a nylon gaze. For immediate experiments, the cell suspension received a step-wise calcium re-introduction (from 0.1 to 0.8 $\text{mmol}\cdot\text{L}^{-1}$). Afterwards, the received ventricular cardiomyocytes were plated on laminin-coated glass coverslips and allowed to settle for 15 minutes at room temperature to ensure cell adhesion for following experimental procedure. Integrity and viability of isolated cardiomyocytes were ensured by characterisation of morphological criteria, including apoptotic cell rounding and shrinkage as well as presence/absence of membrane bubbles.

2.4 | Measurement of late I_{Na} by whole-cell patch clamp technique

Ruptured-patch whole-cell voltage-clamp was used to measure late I_{Na} with microelectrodes (1.5–3 M Ω) as described previously (Gissibl et al., 2024). Pipettes were filled with pipette solution containing (in $\text{mmol}\cdot\text{L}^{-1}$) CsCl 95, Cs-glutamate 40, NaCl 10, MgCl_2 0.92, Mg-ATP 5, Li-GTP 0.3, HEPES 5, niflumic acid 0.03, nifedipine 0.02, strophanthidin 0.004, EGTA 1 and CaCl_2 0.36, at pH 7.2 by adding CsOH. The bath solution contained (in $\text{mmol}\cdot\text{L}^{-1}$): NaCl 135, tetramethylammonium chloride 5, CsCl 4, MgCl_2 2, glucose 10 and HEPES 10 (at pH 7.4, CsOH). DOX (10 $\mu\text{mol}\cdot\text{L}^{-1}$), EMPA (1 $\mu\text{mol}\cdot\text{L}^{-1}$) or RAN (10 $\mu\text{mol}\cdot\text{L}^{-1}$) were added to the bath solution. Cardiomyocytes were incubated in the bathing solution for 15 min at room temperature before they were mounted on the stage of a microscope (Nikon TE2000-172 U). To measure late I_{Na} , ruptured cardiomyocytes were held at -120 mV and the late I_{Na} was elicited using a train of pulses to -35 mV (1000 ms duration, 10 pulses, BCL 2 s). Access resistance was <7 M Ω . Membrane capacitance and series resistance were compensated after patch rupture. Recordings were initiated 3 to 4 minutes after rupture and were performed at room temperature. The measured current was integrated (between 100 and 500 ms) and normalised to membrane capacitance. For leak calculation, the mean value of the currents between 1.0412 and 1.0492 s was used. The leak is therefore a representation of the current strength at the end of the sweep, indicating the distance of the measured current strength from baseline. The leak was subtracted from each measurement point to make the individual measurements more comparable. In some experiments, cardiomyocytes were incubated with tetrodotoxin (TTX, 2 $\mu\text{mol}\cdot\text{L}^{-1}$) to confirm that the measured current is late I_{Na} -mediated.

2.5 | Assessment of intracellular Na^+ and Ca^{2+} handling using epifluorescence microscopy

In order to investigate intracellular Na^+ and Ca^{2+} handling, cardiomyocytes were loaded with 10- $\mu\text{mol}\cdot\text{L}^{-1}$ SBFI-AM (Thermo Fisher Scientific Inc. of Waltham, MA, USA) or Fura2-AM (Thermo Fisher Scientific Inc. of Waltham, MA, USA) in the presence of 0.02% pluronic acid (Molecular Probes, Eugene, OR, USA) for 90 or 15 min at room temperature in darkness. Afterwards, cardiomyocytes were de-esterified for 15 min in an experimental solution containing (in $\text{mmol}\cdot\text{L}^{-1}$): NaCl 140, KCl 4, MgCl_2 5, HEPES 5, glucose 10, CaCl_2 1, at pH 7.4. The glass coverslips were then plated on the stage of an inverted epifluorescence microscope (Nikon Eclipse TE2000-U). Cardiomyocytes were field-stimulated with platinum bath electrodes at 0.5 Hz under continuous superfusion with experimental solution (at 37°C) until they reached a steady state (usually for ~ 5 min). Despite this period, we generally observed a further time-dependent increase in Ca^{2+} transient amplitudes even after steady-state conditions had been reached in our control cells here (i.e., after 5 min), which should be considered when looking at Δ levels (see below). SBFI-fluorescence and Fura2-fluorescence was recorded at t_0

(baseline) and following 10 min (t_{10}) of suspension with either vehicle or DOX. To avoid interference with fluorescence measurements, cardiomyocytes were transilluminated with red light (wavelength > 650 nm). Excitation light was provided by a 75-W xenon arc lamp (Ushio). Both SBFI and Fura2 were excited at alternating wavelengths (F_{340} nm and F_{380} nm, 250 pairs per second, Ion Optix Hyperswitch System). Emitted fluorescence was recorded at 510 nm for each wavelength using IonWizard software (IonOptix Corporation, Boston, MA). SR Ca^{2+} content was estimated by rapid application of **caffeine** ($10 \text{ mmol}\cdot\text{L}^{-1}$, Sigma-Aldrich, St. Louis, MO, USA). After subtraction of the background fluorescence, intracellular Na^{+} - and Ca^{2+} -mediated fluorescence was analysed. For each recording, Na as well as Ca fluorescence were averaged during steady-state (typically 10 traces) using IonWizard software. Finally, the difference (Δ) in fluorescence between t_0 and t_{10} was calculated and expressed in absolute values. Besides that, sarcomere length of cardiomyocytes was recorded using the Sarcomere Length Detection System (MyoCam, IonOptix Corp.). As with measurement of intracellular Ca^{2+} and Na^{+} handling, cardiomyocyte contractions were averaged over 10 beats and analysed using IonWizard software. Moreover, non-stimulated events (NSEs) that represent spontaneous elevations of Ca fluorescence with subsequent contractions of myocytes (Sag et al., 2009), were counted and the frequency of NSEs per minute was calculated as a surrogate for the arrhythmogenic behaviour of cardiomyocytes.

2.6 | Analysis of SR Ca^{2+} leak and cytosolic ROS formation using confocal microscopy

Diastolic SR Ca^{2+} leak was assessed by confocal microscopy as described previously (Feder et al., 2026; Gissibl et al., 2024). For this study, cardiomyocytes were loaded with Fluo4-AM ($10 \mu\text{mol}\cdot\text{L}^{-1}$, Thermo Fisher Scientific Inc. of Waltham, MA, USA) in the presence of 0.02% pluronic acid (Molecular Probes, Eugene, OR, USA). Afterwards, Fluo4-loaded cardiomyocytes were washed once with experimental solution containing (in $\text{mmol}\cdot\text{L}^{-1}$): NaCl 140, KCl 4, glucose 10, HEPES 5, MgCl_2 1 and CaCl_2 1 and incubated for a further 15 min. Afterwards, cardiomyocytes were placed on the stage of an inverted laser scanning confocal microscope (Zeiss LSM 5 Pascal, Carl Zeiss AG, Oberkochen, Germany). Regular cardiomyocyte contraction was elicited by electrical field stimulation with a platinum bath electrode at 0.5 Hz. As in epifluorescence measurements, cardiomyocytes were continuously superfused with experimental solution (at 37°C) as described above. For the measurements of diastolic Ca^{2+} sparks, line scans (512 pixel of $0.075\text{-}\mu\text{m}$ size, 1309 lines per second, 10,000 lines per scan, 488-nm excitation, 505-nm-long pass emission filter) were acquired immediately after stopping electrical field stimulation. Ca^{2+} spark characteristics were analysed using Image J (Sparkmaster plugin). Ca^{2+} spark frequency was measured as number of sparks per cell volume and time ($100 \mu\text{m}^{-1}\cdot\text{s}^{-1}$).

Intracellular cytosolic ROS formation was monitored in isolated cardiomyocytes stained with CellROX Deep Red ($5 \mu\text{mol}\cdot\text{L}^{-1}$, Thermo Fisher Scientific Inc. of Waltham, MA, USA). CellROX Deep

Red-loaded cardiomyocytes were washed once with experimental solution and excited at 644 nm, whereas emission was collected at 665 nm. CellROX Deep Red fluorescence emission was collected once every minute (over a period of 10 min) and was normalised to initial fluorescence (Feder et al., 2026). In some experiments, CellROX Deep Red fluorescence emission was measured following treatment with the ROS scavenger **melatonin** (MEL; $100 \mu\text{mol}\cdot\text{L}^{-1}$; Sigma-Aldrich, St. Louis, MO, USA). Additionally, Mito-TEMPO (Mito-T; $25 \text{ nmol}\cdot\text{L}^{-1}$; Sigma-Aldrich, St. Louis, MO, USA) was used as a second ROS scavenger to reduce mitochondrial superoxide formation. **NOX2** was pharmacologically inhibited by incubating cardiomyocytes with the NOX2 inhibitor gp91ds-tat ($50 \mu\text{mol}\cdot\text{L}^{-1}$; Sigma-Aldrich, St. Louis, MO, USA).

2.7 | Western blot

For western blot analysis, isolated ventricular cardiomyocytes were used. Isolated cardiomyocytes were plated on culture dishes and incubated with either vehicle or doxorubicin ($10 \mu\text{mol}\cdot\text{L}^{-1}$, Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature. In some experiments EMPA ($1 \mu\text{mol}\cdot\text{L}^{-1}$, Thermo Fisher Scientific Inc. of Waltham, MA, USA) and RAN ($10 \mu\text{mol}\cdot\text{L}^{-1}$, Thermo Fisher Scientific Inc. of Waltham, MA, USA) was added. Harvested cardiomyocytes were frozen immediately. Isolated cardiomyocytes were then lysed in Tris buffer containing (in $\text{mmol}\cdot\text{L}^{-1}$): Tris-HCl 20, NaCl 200, NaF 20, Na_3VO_4 1, Triton X 100 1%, DTT 1, along with a protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (both Roche diagnostics, Mannheim, Germany). In order to quantify the extent of RyR2 cross-linking (referred to Nikolaienko et al., 2023), cardiomyocytes were lysed in Tris buffer containing (in $\text{mmol}\cdot\text{L}^{-1}$): Tris-HCl 20, NaCl 200, Triton X 1001%, Maleimide 1, along with a protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail (both Roche diagnostics, Mannheim, Germany) (Nikolaienko et al., 2023). Cardiomyocytes were denatured (for 5 min at 95°C under reducing conditions for CaMKII δ and pT287 CaMKII δ , for 5 min at 95°C under non-reducing conditions for oxCaMKII, for 30 min at 37°C under reducing conditions for RyR2, pS2814-RyR2, pS2808-RyR2, SERCA2a, PLB, pS16-PLB, pT17-PLB, PLB and for 30 min at 37°C under non-reducing conditions for monomeric and cross-linked RyR2). After denaturation, proteins were separated on a 12% (SERCA2a, PLB, pS16-PLB, pT17-PLB), 8% (CaMKII δ , pT287-CaMKII δ , oxCaMKII) or 5% (RyR2, pS2814-RyR2, pS2808-RyR2, monomeric and cross-linked RyR2) SDS-polyacrylamide gel, transferred wet to a nitrocellulose membrane and blocked with 5% milk. Afterwards, membranes were incubated with primary antibodies: rabbit polyclonal anti-RyR2 (1:10000, Sigma Aldrich, Cat. HPA020028, Lot. C118282), rabbit polyclonal anti-pS2814-RyR2 (1:2000, Badrilla, Cat. A010-31, Lot.A642219), rabbit polyclonal anti-pS2808-RyR2 (1:1000, Thermo Fisher Scientific, Cat. PA5-105712, Lot. 79592370), mouse monoclonal anti-SERCA2a (1:10000, Thermo Fisher Scientific, Cat. MA3-919, Lot. WD320866), mouse monoclonal anti-PLB (1:1000, Thermo Fisher Scientific, Cat. MA3-922, Lot. Y13377836), rabbit polyclonal pS16-PLB (1:1000, Thermo Fisher Scientific, Cat. 07-052, Lot. 4110903), rabbit polyclonal pT17-PLB (Badrilla, Cat. A010-13, Lot.

A642222), rabbit polyclonal anti-pT287-CaMKII δ (1:1000, PhosphoSolutions, Cat. p1005-286, Lot. KS921b), rabbit polyclonal anti-CaMKII δ (1:10000, Thermo Fisher Scientific, Cat. PA5-22168, Lot. AA4599933), rabbit polyclonal anti-oxCaMKII (1:1000, GeneTex, Cat. GTX36254, Lot. 3407316) and mouse monoclonal anti-GAPDH (1:10000, Sigma, Cat. MA5-15738, Lot. AC410117) at 4°C overnight. Secondary antibodies were HRP-conjugated sheep anti-mouse IgG (1:10000, GE Healthcare, Cat. NA931VS, Lot. 18296218) and anti-rabbit IgG (GE Healthcare, Cat. NA934V, Lot. 18111932) and incubated for 1 h at room temperature. For chemiluminescent detection, Immobilon™

Western Chemiluminescent HRP Substrate (Millipore) was used. Values were afterwards normalised to GAPDH values and vehicle.

2.8 | MDA ELISA

Lipid peroxidation was measured by calculating the malondialdehyde (MDA) concentration using the MDA Assay Kit (Signosis, Inc., USA) according to the manufacturer's instructions. In brief, cell lysates were incubated with thiobarbituric acid (TBA) at 95°C for 1 h and

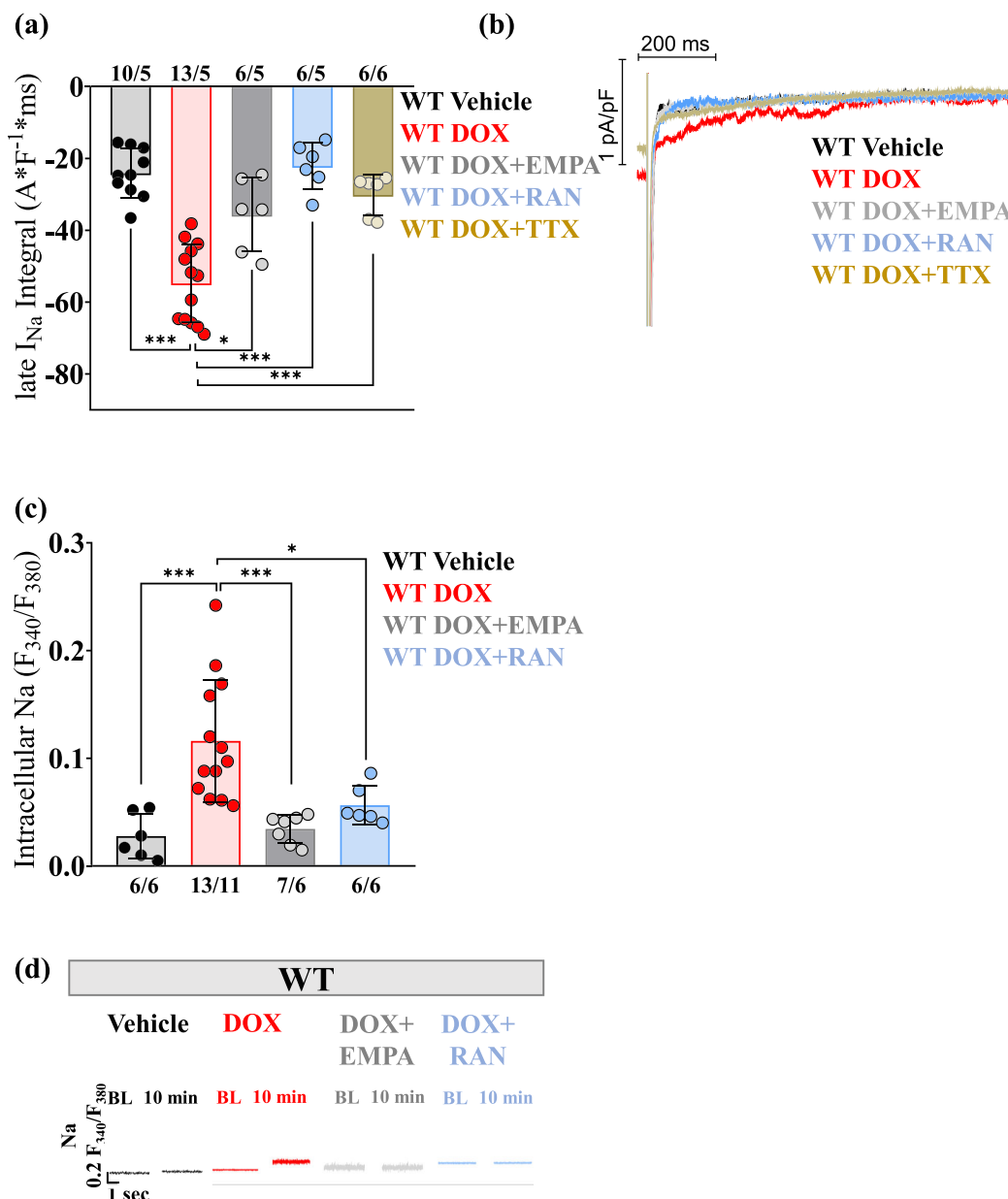


FIGURE 1 EMPA and RAN attenuate the DOX-dependent increase of late I_{Na} . Mean data (a) and original recordings (b) show that empagliflozin (EMPA) and ranolazine (RAN) both attenuate the DOX-dependent increase of late I_{Na} . Mean data (c) and original recordings (d) of SBF1-stained cardiomyocytes show that EMPA and RAN attenuate DOX-dependent Na overload. Data are presented as mean $\pm$ SD. Abbreviations: BL, baseline; DOX, doxorubicin; EMPA, empagliflozin; RAN, ranolazine; SBF1, sodium-binding benzofuran isophthalate; TTX, tetrodotoxin; WT, wildtype.

subsequently cooled to 4°C for 10 min. Afterwards, the absorbance of the TBA-MDA product was measured at 532 nm using a microplate reader. The MDA concentration of each cell lysate was calculated from a standard curve generated with MDA standards provided in the kit by four-parameter logistic regression and corrected for dilution factors where applicable.

2.9 | Troponin I ELISA

Mouse cardiac Troponin I concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) in accordance with the manufacturer's instructions. Briefly, cell lysates were added to ELISA plate wells and incubated for 90 min at 37°C. After removal of the supernatant, wells were incubated with biotinylated detection antibody for 1 h at 37°C, followed by incubation with HRP conjugate for 30 min at 37°C. Following addition of the substrate reagent, absorbance was measured at 450 nm using a microplate reader. Troponin I concentrations were calculated by four-parameter logistic regression and corrected for dilution factors where applicable.

2.10 | Cell survival assessment

Cell survival was evaluated by morphological analysis using light microscopy. For this study, cardiomyocytes were plated on laminin-coated dishes and incubated with either vehicle or DOX for 10 min. In some experiments, cardiomyocytes were treated with EMPA, RAN or MEL for 30 min prior to DOX exposure. Cells were examined for

characteristic features of loss of viability, including plasma membrane alterations (e.g., membrane blebbing and disruption of membrane integrity) and the presence of cellular debris. The proportion of morphologically intact cells relative to damaged or fragmented cells before and after treatment was used as an indicator of cell survival (Figure S10).

2.11 | Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). The n-number as shown in each figure/table depicts n = cells per mouse to show the number of technical replicates (number of cells) and biological replicates (number of mice). Normality was tested using Shapiro-Wilk or D'Agostino-Pearson normality test. Statistical analyses were performed using parametric tests (one-way analysis of variance [ANOVA], and two-way ANOVA for repeated measurements [RM] with Holm-Sidak post hoc test or non-parametric tests [Kruskal-Wallis test and Mann-Whitney test]). Values of $P < 0.05$ were considered as statistically significant. Graphs were created using Graph Pad Prism 8.0.1. The manuscript complies with the recommendations and requirements of the British Journal of Pharmacology on experimental design and analysis (Curtis et al., 2025).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in and are permanently archived in the Concise Guide to PHARMACOLOGY 2025/2026 (Alexander, Fabbro, et al., 2025; Alexander, Striessnig, et al., 2025).

TABLE 1 Pharmacological inhibition of late I_{Na} attenuates DOX-induced hyperactivation of CaMKII δ and subsequent RyR2 phosphorylation.

Protein	Vehicle	n	DOX	n	DOX + EMPA	n	DOX + RAN	n
CaMKII δ	1.000 $\pm$ 0.155	27	1.418 $\pm$ 0.251	26	0.976 $\pm$ 0.185	29	0.466 $\pm$ 0.093	9
pT287/CaMKII δ	1.000 $\pm$ 0.091	23	1.836 $\pm$ 0.223^{aa}	23	1.211 $\pm$ 0.133^b	24	1.011 $\pm$ 0.214^b	11
oxCaMKII	1.000 $\pm$ 0.188	13	1.876 $\pm$ 0.225^a	8	1.039 $\pm$ 0.228^b	14	0.305 $\pm$ 0.043^{bbb}	8
PLB	1.000 $\pm$ 0.065	18	1.043 $\pm$ 0.052	18	0.995 $\pm$ 0.051	18	0.905 $\pm$ 0.060	18
pS16/PLB	1.000 $\pm$ 0.129	6	0.719 $\pm$ 0.123	5	1.118 $\pm$ 0.271	6	0.975 $\pm$ 0.221	6
pT17/PLB	1.000 $\pm$ 0.217	9	1.062 $\pm$ 0.204	9	0.770 $\pm$ 0.270	9	0.804 $\pm$ 0.214	9
RyR2	1.000 $\pm$ 0.170	19	1.190 $\pm$ 0.143	20	0.983 $\pm$ 0.141	20	1.193 $\pm$ 0.112	19
pS2808/RyR2	1.000 $\pm$ 0.206	8	1.039 $\pm$ 0.185	8	1.060 $\pm$ 0.203	8	0.912 $\pm$ 0.141	8
pS2814/RyR2	1.000 $\pm$ 0.184	8	1.839 $\pm$ 0.158^a	9	1.293 $\pm$ 0.159	13	1.224 $\pm$ 0.041^b	7
oxRyR2	1.000 $\pm$ 0.052	8	1.425 $\pm$ 0.147^a	7	1.054 $\pm$ 0.074^b	8	0.941 $\pm$ 0.097^b	8
SERCA2a	1.000 $\pm$ 0.165	13	1.496 $\pm$ 0.248	11	1.208 $\pm$ 0.248	13	1.274 $\pm$ 0.237	13

Note: Significant changes in protein expression levels are indicated by bold emphasis.

Abbreviations: CaMKII δ : calcium/calmodulin-dependent protein kinase II δ isoform; pT287/CaMKII δ : CaMKII autophosphorylation site; oxCaMKII, oxidative-activated CaMKII; PLB, phospholamban; pS16/PLB, PKA-specific PLB phosphorylation site; pT17/PLB, CaMKII-specific PLB phosphorylation site; RyR2, ryanodine receptor; pS2814/RyR2, CaMKII-specific RyR2 phosphorylation site; pS2808/RyR2, PKA/CaMKII-specific RyR2 phosphorylation site; oxRyR2, oxidised RyR2; SERCA2a, SR Ca ATPase.

^aIndicates significance vs. vehicle with $p < 0.05$.

^{aa}Indicates significance vs. vehicle with $p < 0.01$.

^bIndicates significance vs. DOX with $p < 0.05$.

^{bbb}Indicates significance vs. DOX with $p < 0.001$.

3 | RESULTS

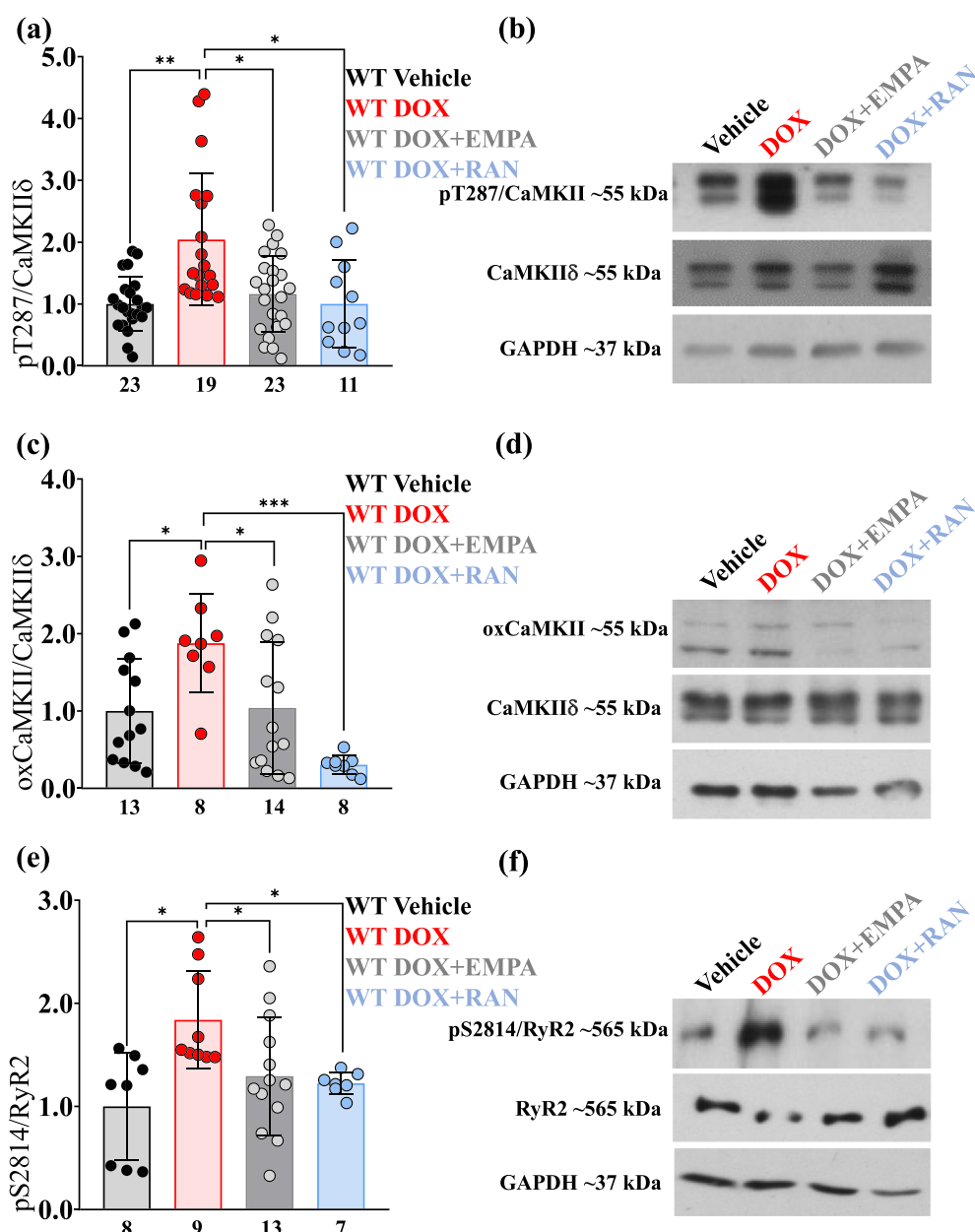
3.1 | DOX acutely enhances late I_{Na} and impairs intracellular Ca^{2+} handling through activated CaMKII δ

DOX ($10 \mu\text{mol}\cdot\text{L}^{-1}$) acutely stimulated late I_{Na} by twofold in our model, which was associated with significant intracellular Na^+ overload (Figure 1) and increased formation of cytosolic reactive oxygen species (ROS) (Figure S1). DOX-dependent ROS formation resulted in increased cross-linking of RyR2 (Figure S2) and redox-dependent hyperactivation of CaMKII δ (Table 1 and Figure 2c,d). Besides redox-dependent activation, WT cardiomyocytes also demonstrated phospho-dependent hyperactivation of CaMKII δ in terms of increased phosphorylation of CaMKII δ at threonine-287 (Table 1 and Figure 2a,b). Accordingly, RyR2 hyperphosphorylation at the CaMKII-specific phosphorylation site

serine-2814 was significantly increased in DOX-exposed WT cardiomyocytes (Table 1 and Figure 2e,f), which was functionally paralleled by increased diastolic Ca^{2+} loss from the SR (Figure 3a,b) which caused decreased SR Ca^{2+} load (Figure 3c). As a functional consequence, DOX-exposed WT cardiomyocytes revealed depressed systolic Ca^{2+} transient amplitudes and severe diastolic Ca^{2+} overload at t_{10} (Figure 3e,f), which was accompanied by significant reduction in cardiomyocyte contractility (Figure S6), reflecting DOX-related impairment in intracellular Ca^{2+} handling. In addition to impaired Ca^{2+} handling, DOX-induced cardiotoxicity was further evident in terms of significantly elevated MDA levels and increased troponin release (Figure S7), along with a trend towards decreased cell survival (Figure S8), supporting direct cardiotoxic effects of DOX.

Next, we tested whether pharmacological inhibition of late I_{Na} is sufficient to attenuate this pathological cascade of late I_{Na} -mediated

FIGURE 2 Pharmacological inhibition of late I_{Na} by RAN and EMPA attenuates DOX-dependent activation of CaMKII δ and subsequent RyR2 hyperphosphorylation. The acute hyperactivation of CaMKII δ by autophosphorylation (a, b) and oxidation (c, d) as induced by DOX is attenuated by empagliflozin (EMPA) and ranolazine (RAN), which is associated with an unaltered phosphorylation status of the RyR2 at the CaMKII-specific phosphorylation site serine-2814 (e, f). Data are presented as mean $\pm$ SD. $n =$ mice. Abbreviations: DOX, doxorubicin; EMPA, empagliflozin; oxCaMKII, oxidised CaMKII; pT287, CaMKII autophosphorylation at threonine-287; pS2814, CaMKII-specific RyR2 phosphorylation at serine-2814; RAN, ranolazine; RyR2, ryanodine receptor isoform 2; WT, wildtype.



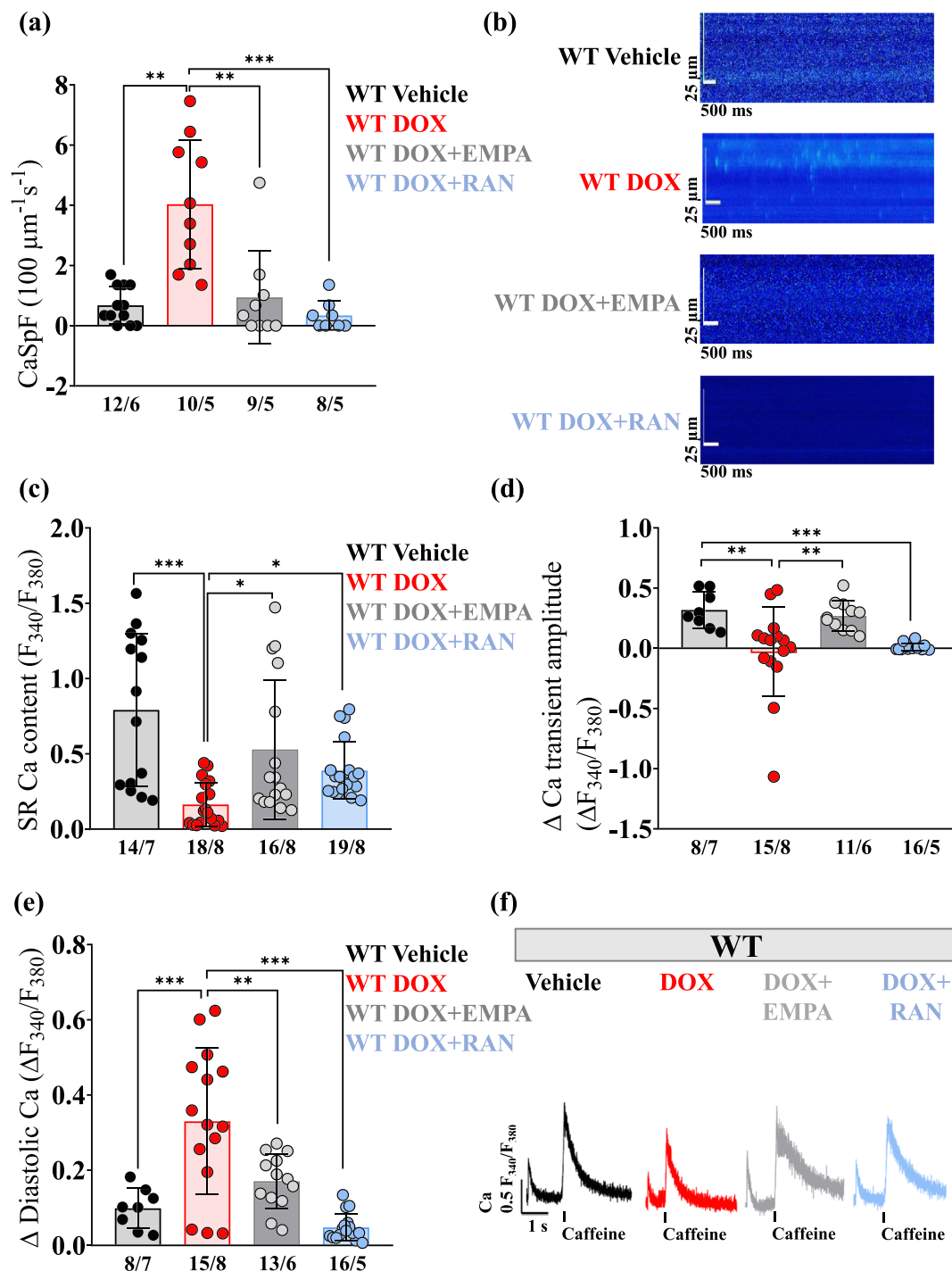


FIGURE 3 Pharmacological inhibition of late I_{Na} preserves intracellular Ca^{2+} handling upon DOX. Mean data (a) and original line scans (b) show that empagliflozin (EMPA) and ranolazine (RAN) both attenuate DOX-dependent SR Ca^{2+} leakage. Consequently, cardiomyocytes demonstrate partly maintained SR Ca^{2+} content (c), systolic Ca^{2+} transient amplitudes (d) and diastolic Ca^{2+} levels (e) upon EMPA and RAN. (f) Original recordings of Fura2-loaded cardiomyocytes depict maintained caffeine-induced Ca transients after late I_{Na} inhibition using EMPA and RAN despite DOX. Data are presented as mean $\pm$ SD. n = cells/mice. Abbreviations: CaSpF, calcium spark frequency; DOX, doxorubicin; EMPA, empagliflozin; RAN, ranolazine; WT, wildtype.

CaMKII δ activation and subsequent SR Ca^{2+} leakage. As depicted in Figure 1, pharmacological inhibition of the late I_{Na} by RAN (10 $\mu\text{mol}\cdot\text{L}^{-1}$) as well as inhibition of CaMKII-dependent stimulation of late I_{Na} by EMPA (1 $\mu\text{mol}\cdot\text{L}^{-1}$; Mustroph et al., 2022) significantly

attenuated DOX-dependent enhancement of late I_{Na} . Similarly, DOX-dependent intracellular Na^{+} overload was prevented by both EMPA and RAN. Consistent with maintained $[\text{Na}^{+}]_{\text{i}}$, EMPA and RAN also prevented DOX-related hyperactivation of CaMKII δ (by both

oxidation and autophosphorylation), which was paralleled by an unaltered phosphorylation status of the RyR2 at the CaMKII-specific phosphorylation site serine-2814 as compared to vehicle treatment (Table 1 and Figure 2). Similarly, EMPA and RAN preserved SR Ca^{2+} content in the face of absent diastolic SR Ca^{2+} leakage. As a consequence, diastolic Ca^{2+} , Ca^{2+} transient amplitudes and cardiomyocyte contractility were largely maintained in the aforementioned groups (Figures 3 and S6). Interestingly, the frequency of non-stimulated events (i.e., NSE), which represent spontaneous, non-stimulated elevations of Ca^{2+} -mediated fluorescence as an indication of cellular arrhythmogenicity, was significantly increased upon DOX, but not elevated upon concomitant treatment with either RAN or EMPA (Figure S9). These anti-arrhythmogenic effects of RAN and EMPA were paralleled by a reduced release of DOX-dependent cellular injury markers. Whereas RAN induced only a minor, non-significant trend towards an attenuation of DOX-induced MDA levels and troponin release, EMPA significantly reduced DOX-induced MDA and troponin release (Figure S7).

3.2 | Scavenging ROS prevents Ca^{2+} handling as a consequence of maintained late I_{Na}

In a next step, we investigated the role of DOX-derived ROS for late I_{Na} stimulation and impaired Ca^{2+} handling in our model of DOX-related cardiotoxicity. First, we assessed the potential contributors and sources of DOX-derived ROS formation by quantifying ROS levels in CaMKII δ knockout cardiomyocytes (CaMKII $\delta^{-/-}$), and following pharmacological inhibition of mitochondrial ROS formation (using the mitochondrial-targeted scavenger Mito-TEMPO [Mito-T]), NOX2-dependent ROS formation (gp91ds-tat) and general ROS formation (using melatonin [MEL]). As shown in Figure S1, CaMKII $\delta^{-/-}$ cardiomyocytes revealed DOX-related ROS formation not less than DOX-treated WT cardiomyocytes, indicating that CaMKII δ is not required for initial DOX-related ROS generation. In contrast, DOX-related ROS formation was significantly reduced by Mito-T, gp91ds-tat and MEL, suggesting that DOX induces ROS in a complex manner in our model that involves all aforementioned sources. Importantly, preventing DOX-related ROS formation (in our study through MEL) attenuated DOX-dependent

stimulation of late I_{Na} (Figure S3) in the face of absent CaMKII hyperactivation and hyperphosphorylation of the RyR2 at serine-2814 (Table 2 and Figure S4). As a consequence, MEL attenuated DOX-dependent SR Ca^{2+} depletion in the face of absent diastolic SR Ca^{2+} leakage, which was associated with maintained Ca^{2+} transient amplitudes and cardiomyocyte contractility (Figures S5 and S6). Moreover, MEL treatment normalised DOX-dependent arrhythmogenic behaviour (Figure S9). Importantly, while EMPA clearly prevented DOX-related ROS formation, there was still robust ROS formation upon concomitant RAN treatment indicating that RAN exerted a rather indirect antioxidative effect here (Figure S1).

3.3 | Prevention of CaMKII-dependent serine-571 phosphorylation at $\text{Na}_v1.5$ attenuates DOX-dependent enhancement of late I_{Na} and reduces DOX-related SR Ca leakage

In the next step, we investigated whether CaMKII-dependent phosphorylation of $\text{Na}_v1.5$ at serine-571 is involved in DOX-dependent stimulation of late I_{Na} and subsequent SR Ca^{2+} leakage by exposing isolated ventricular myocytes from a transgenic S571A mouse model, in which $\text{Na}_v1.5$ was made resistant to CaMKII-dependent phosphorylation, to DOX and in the presence and absence of EMPA or RAN). As shown in Figure 4, DOX-dependent stimulation of late I_{Na} was inhibited in S571A cardiomyocytes, whereas EMPA- and RAN-exposed S571A cardiomyocytes demonstrated no further reduction in DOX-induced late I_{Na} as compared to solely DOX-exposed S571A cardiomyocytes. Similarly, the subsequent increase of $[\text{Na}^+]_i$ was prevented in S571A cardiomyocytes and was not additionally affected by either RAN or EMPA (please note that basal $[\text{Na}^+]_i$ levels were equal in S571A vs. WT myocytes). Likewise, S571A cardiomyocytes showed significantly less DOX-dependent activation of CaMKII δ (by both oxidation and autophosphorylation), which was paralleled by an unaltered phosphorylation status of RyR2 at the CaMKII-specific phosphorylation site serine-2814 (Table 3 and Figure 5), which was not further modified by either RAN or EMPA. Functionally, S571A cardiomyocytes demonstrated maintained SR Ca^{2+} content and systolic Ca^{2+} transient amplitudes

TABLE 2 DOX-dependent hyperactivation of CaMKII δ and RyR2 phosphorylation is attenuated by scavenging ROS.

Protein	WT vehicle	n	WT DOX	n	WT DOX+MEL	n
CaMKII δ	1.000 ± 0.158	8	0.872 ± 0.199	7	0.747 ± 0.124	7
pT287/CaMKII δ	1.000 ± 0.206	7	2.603 ± 0.463^{aa}	7	1.566 ± 0.151	7
oxCaMKII	1.000 ± 0.197	8	2.015 ± 0.240^a	8	1.237 ± 0.259	7
RyR2	1.000 ± 0.201	8	0.912 ± 0.224	6	0.993 ± 0.198	7
pS2814/RyR2	1.000 ± 0.046	8	1.256 ± 0.095^a	6	1.021 ± 0.044^b	7

Note: Significant changes in protein expression levels are indicated by bold emphasis.

Abbreviations: CaMKII δ , calcium/calmodulin-dependent protein kinase II δ isoform; pT287/CaMKII δ ; CaMKII autophosphorylation site; oxCaMKII, oxidative-activated CaMKII; RyR2, ryanodine receptor; pS2814/RyR2, CaMKII-specific RyR2 phosphorylation site.

^aIndicates significance vs. vehicle with $p < 0.05$.

^{aa}Indicates significance vs. vehicle with $p < 0.01$.

^bIndicates significance vs. DOX with $p < 0.05$.

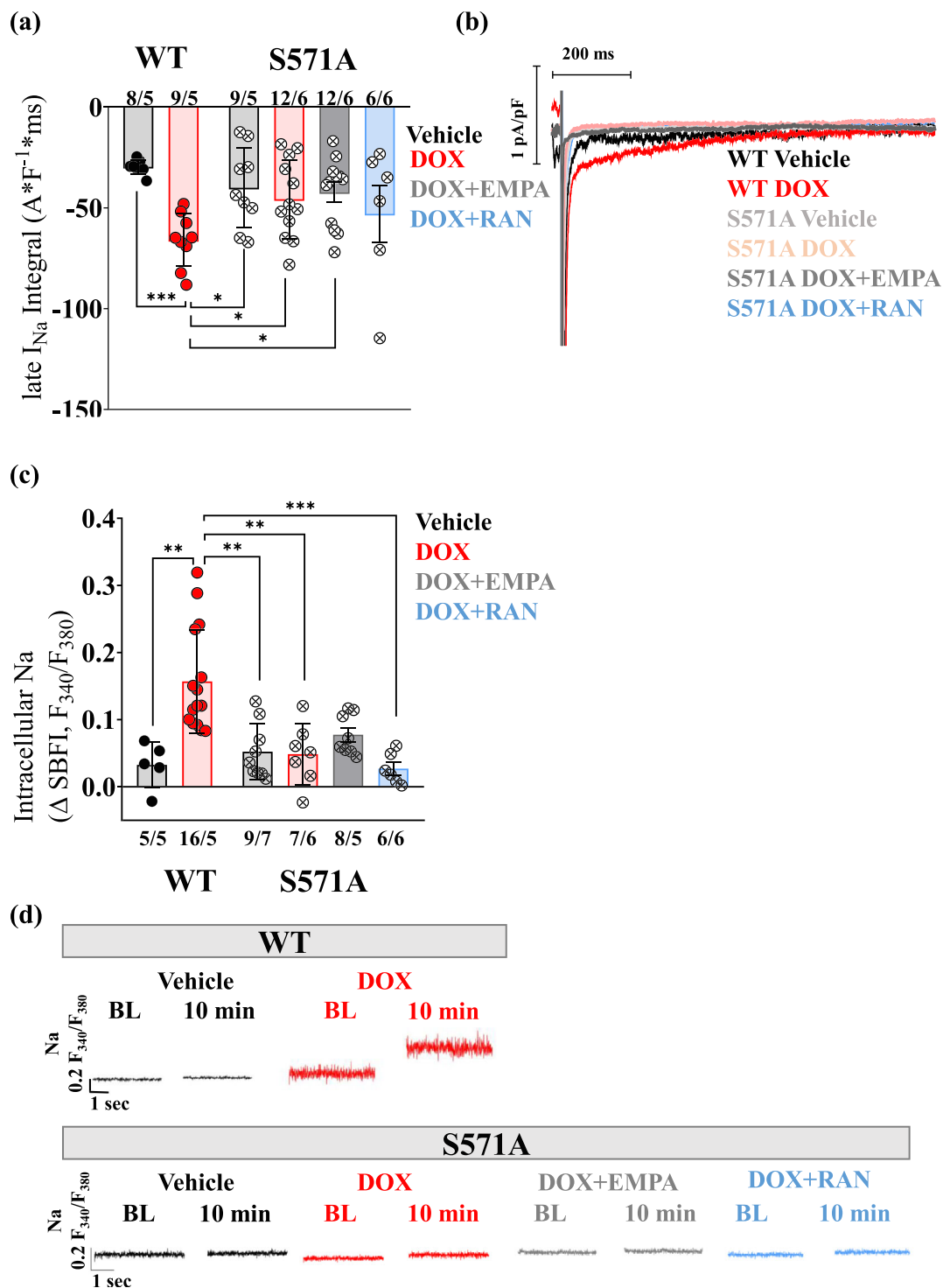


FIGURE 4 DOX-dependent enhancement of late I_{Na} is attenuated in S571A cardiomyocytes. Mean data (a) and original recordings (b) show that S571A cardiomyocytes do not show a DOX-dependent increase of late I_{Na} . Mean data (c) and original recordings (d) of SBFI-stained cardiomyocytes show that S571A cardiomyocytes are protected from DOX-dependent Na^+ overload. Data are presented as mean $\pm$ SD. n = cells/mice. Abbreviations: BL, baseline; DOX, doxorubicin; EMPA, empagliflozin; RAN, ranolazine; S571A, cardiomyocytes lacking CaMKII-dependent phosphorylation of $Na_v1.5$ at serine-571; WT, wildtype.

(please note that Ca^{2+} transient amplitudes were equal under baseline conditions in S571A vs. WT myocytes). This was paralleled by absent SR Ca^{2+} leakage (Figure 6, despite significantly elevated diastolic Ca^{2+} levels in S571A under baseline conditions), and

maintained cardiomyocyte contractility (Figure S6). In summary, this set of data indicates CaMKII-dependent phosphorylation of $Na_v1.5$ at serine-571 as an important amplifier of DOX-related enhancement of the late I_{Na} .

TABLE 3 DOX-induced hyperactivation of CaMKII and RyR2 phosphorylation is attenuated in S571A cardiomyocytes.

Protein	WT vehicle	n	WT DOX	n	S571A vehicle	n	S571A DOX	n	S571A DOX+ EMPA	n	S571A DOX+ RAN	n
CaMKII δ	1.000 $\pm$ 0.154	17	1.064 $\pm$ 0.190	14	0.623 $\pm$ 0.132	8	0.404 $\pm$ 0.077	8	0.570 $\pm$ 0.138	8	0.476 $\pm$ 0.077	8
pT287/ CaMKII δ	1.000 $\pm$ 0.100	14	2.203 $\pm$ 0.287^{aaa}	14	1.368 $\pm$ 0.294	7	1.095 $\pm$ 0.111^b	6	1.080 $\pm$ 0.183^b	7	1.091 $\pm$ 0.163^b	7
oxCaMKII	1.000 $\pm$ 0.151	6	2.690 $\pm$ 0.692^a	7	0.733 $\pm$ 0.197^b	5	1.046 $\pm$ 0.303^b	6	0.598 $\pm$ 0.238^{bb}	6	0.847 $\pm$ 0.274^b	6
RyR2	1.000 $\pm$ 0.167	17	1.157 $\pm$ 0.157	16	1.332 $\pm$ 0.438	9	1.216 $\pm$ 0.319	9	1.734 $\pm$ 0.532	9	2.171 $\pm$ 0.460	8
pS2814/ RyR2	1.000 $\pm$ 0.079	16	1.553 $\pm$ 0.137^{aa}	16	0.943 $\pm$ 0.162^b	9	0.941 $\pm$ 0.160^b	9	0.833 $\pm$ 0.131^{bb}	9	1.187 $\pm$ 0.202^b	8

Note: Significant changes in protein expression levels are indicated by bold emphasis.

Abbreviations: CaMKII δ , calcium/calmodulin-dependent protein kinase II δ isoform; pT287/CaMKII δ , CaMKII autophosphorylation site; oxCaMKII, oxidative-activated CaMKII; RyR2, ryanodine receptor; pS2814/RyR2, CaMKII-specific RyR2 phosphorylation site.

^aIndicates significance vs. vehicle with $p < 0.05$.

^{aa}Indicates significance vs. vehicle with $p < 0.01$.

^{aaa}Indicates significance vs. vehicle with $p < 0.001$.

^bIndicates significance vs. DOX with $p < 0.05$.

^{bb}Indicates significance vs. DOX with $p < 0.01$.

4 | DISCUSSION

The clinical relevance of doxorubicin-induced cardiomyopathy has been known for decades, but guideline-based treatment recommendations have only been available since the publication of the ESC guidelines in 2022 (Lyon et al., 2022). These guidelines currently recommend ACE-inhibitors, β -blockers, and statins for primary prevention of cancer therapy-related cardiovascular toxicity related to anthracycline chemotherapy, but there is still a great need for further cardio-protective adjuncts during anthracycline therapy. DOX-related cardiotoxicity, however, is complex and involves several interacting mechanisms such as oxidative stress and mitochondrial injury, DNA damage (via topoisomerase II β), myocyte apoptosis, cardiac fibrosis and electrophysiological remodelling including impaired Na⁺ and Ca²⁺ handling (Fabiani et al., 2025). Hence, efforts should be made to investigate the therapeutic modifiability of these pathomechanisms to improve the prognosis of affected patients.

In that regard, a growing body of evidence suggests that disturbed intracellular Na⁺ and Ca²⁺ handling represents a relevant disease amplifier of doxorubicin-induced cardiomyopathy rather than a mere consequence of the aforementioned pathomechanisms and is nowadays regarded as a potential therapeutic target to treat DOX-related cardiotoxicity. When looking at electrophysiological remodelling in doxorubicin-induced cardiomyopathy (DICM), a potential vicious cycle between impaired Na⁺ and Ca²⁺ handling can be identified. Several studies have shown that DOX increases [Na⁺]_i because of increased late I_{Na} in both acute and long-term induced cardiotoxicity (Cappetta et al., 2017; Iaffaioli et al., 2014; Maurea et al., 2016). Our group has mainly focussed on CaMKII δ -mediated Ca²⁺ leakage from the SR as a central electrophysiological pathomechanism during the progression of DICM (Feder et al., 2026; Sag et al., 2011), which was later confirmed by others (Lin et al., 2024). It is known that

increased late I_{Na} activates CaMKII δ in several other cardiac conditions, including pressure-induced heart disease or ethanol-induced atrial fibrillation (Mustroph et al., 2023; Sossalla et al., 2008). Vice versa, late I_{Na} is subject to CaMKII-dependent amplification due to CaMKII-specific phosphorylation at serine-571, serine-516 and serine-593 at Nav1.5 (Ashpole et al., 2012). However, what initiates CaMKII activation in the context of DOX *upstream* has remained incompletely defined, and direct mechanistic links between late I_{Na}, CaMKII activation and DICM have been sparse.

In our current study, we show that DOX acutely enhances late I_{Na} and increases cytosolic ROS-formation, which is an effect that we and others have already observed upon specific pharmacological stimulation of late I_{Na} with **ATX-II** (Gissibl et al., 2024; Viatchenko-Karpinski et al., 2014). Whereas ROS might have contributed to redox-dependent CaMKII δ activation upon acute DOX exposure, it is important to note that pharmacological inhibition of late I_{Na} by RAN (Sossalla et al., 2008) largely prevented CaMKII δ activation by both oxidation and autophosphorylation in our model, which identifies late I_{Na} as a potent upstream activator of CaMKII δ in our model as previously suggested by Cappetta and colleagues in an *in vivo* model of DICM in rats (Cappetta et al., 2017). Importantly, our findings are the first to show that the beneficial effects of RAN on cardiac contractility, that Cappetta and colleagues attributed to a reduction in CaMKII activity, are actually accompanied by largely preserved intracellular Ca²⁺ handling and maintained cellular contractility, as evidenced by an unchanged phosphorylation status of RyR2 at the CaMKII-specific phosphorylation site serine-2814 in the presence of RAN (Cappetta et al., 2017). Furthermore, our observation that S571A cardiomyocytes cells lacking the CaMKII-specific phosphorylation site on Nav1.5 are greatly protected from DOX-related CaMKII hyperactivation, suggests a self-reinforcing vicious cycle of late I_{Na}-induced CaMKII δ activation and reciprocal CaMKII δ -induced enhancement of late I_{Na} via

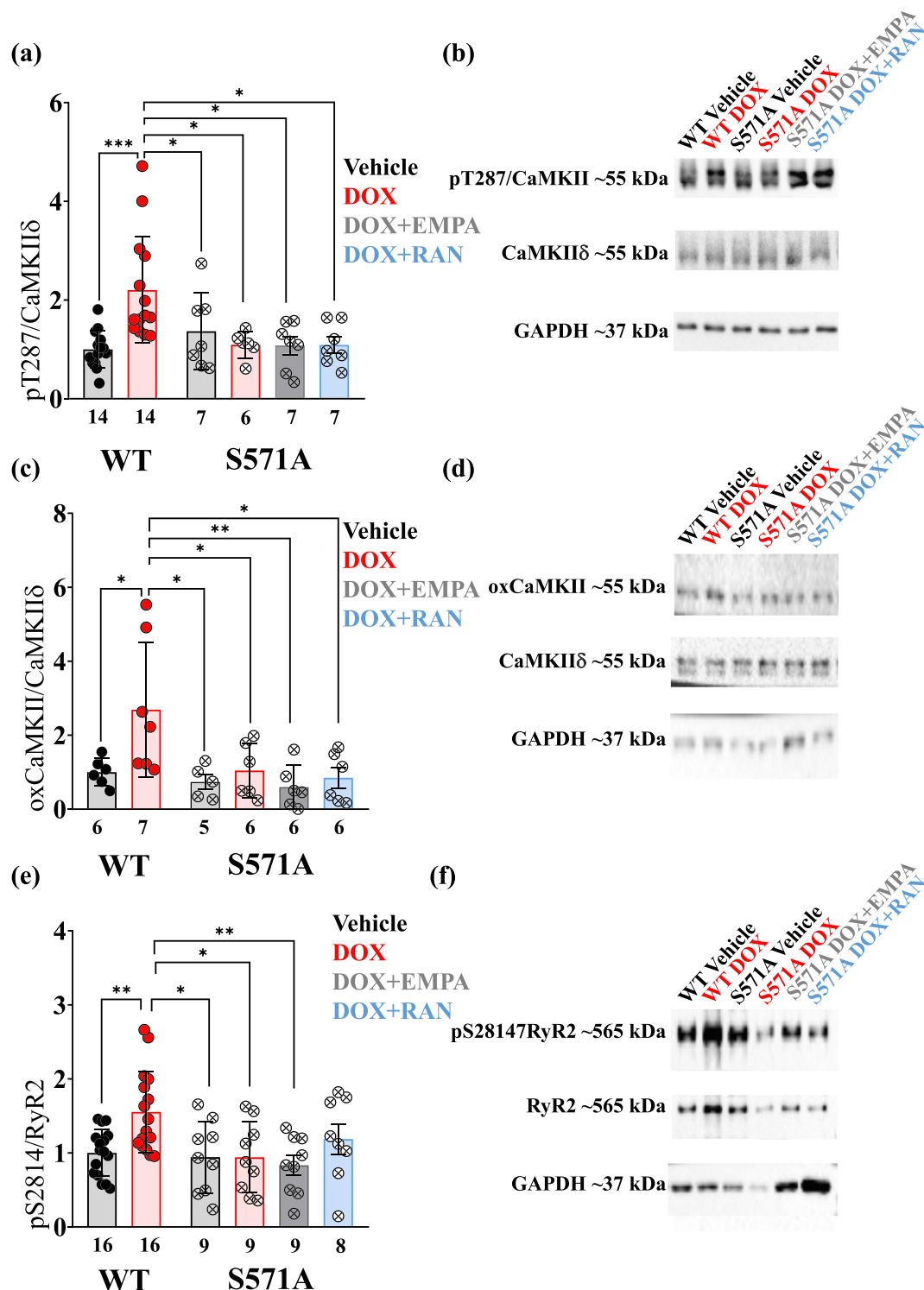


FIGURE 5 DOX-dependent activation of CaMKIIδ and RyR2 hyperphosphorylation is attenuated in S571A cardiomyocytes. The acute hyperactivation of CaMKIIδ by autophosphorylation (a, b) and oxidation (c, d) as induced by DOX is attenuated in S571A cardiomyocytes, which is associated with an unaltered phosphorylation status of the RyR2 at the CaMKIIδ-specific phosphorylation site serine-2814 (e, f). Data are presented as mean ± SD. n = mice. Abbreviations: DOX, doxorubicin; EMPA, empagliflozin; RAN, ranolazine; oxCaMKII, oxidised CaMKII; pT287, CaMKII autophosphorylation at threonine-287; pS2814, CaMKII-specific RyR2 phosphorylation at serine-2814; RyR2, ryanodine receptor isoform 2; S571A, cardiomyocytes lacking CaMKII-dependent phosphorylation of Na_v1.5 at serine-571; WT, wildtype.

phospho-dependent stimulation at serine-571. It is important to note that both EMPA and RAN did not show additional protection against DOX-dependent late I_{Na} stimulation in S571A cardiomyocytes,

suggesting that CaMKIIδ-dependent phosphorylation of Na_v1.5 at serine-571 is a distinct amplifier of this vicious cycle in between enhanced late I_{Na} and hyperactivated CaMKII (Sag et al., 2013).

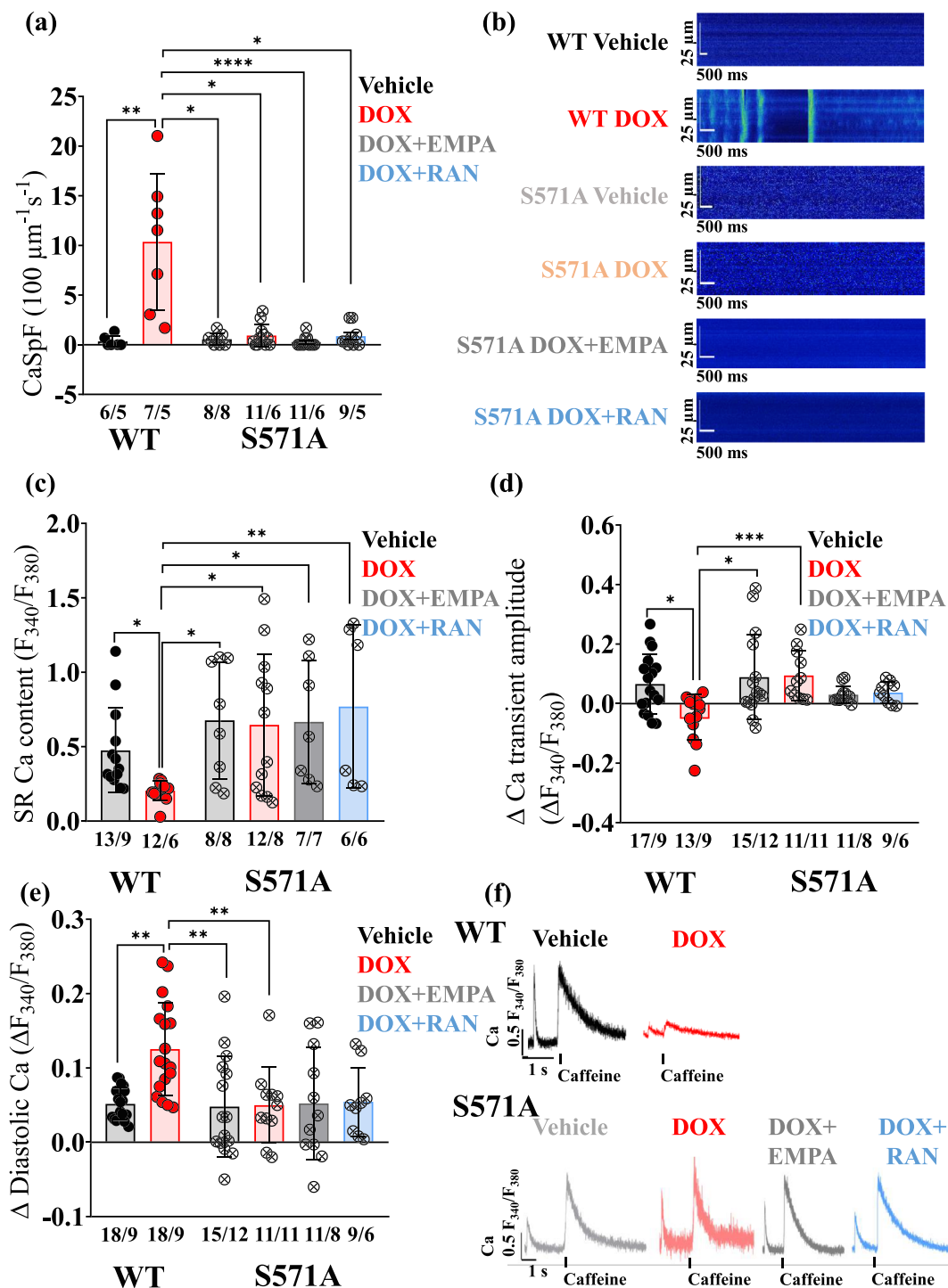


FIGURE 6 DOX-dependent impairment in intracellular Ca^{2+} handling is preserved in S571A cardiomyocytes. Mean data (a) and original line scans (b) show that S571A cardiomyocytes are significantly protected from DOX-dependent SR Ca^{2+} leakage. Likewise, S571A cardiomyocytes demonstrate maintained SR Ca^{2+} content (c), systolic Ca^{2+} transient amplitudes (d) and diastolic Ca^{2+} levels (e). (f) Original recordings of Fura2-loaded cardiomyocytes depict preserved caffeine-induced Ca^{2+} transients in S571A cardiomyocytes despite DOX. Data are presented as mean $\pm$ SD. n = cells/mice. Abbreviations: CaSpF, calcium spark frequency; DOX, doxorubicin; EMPA, empagliflozin; RAN, ranolazine; S571A, cardiomyocytes lacking CaMKII-dependent phosphorylation of $\text{Na}_v1.5$ at serine-571; WT, wildtype.

Hence, our findings are largely in line with previous reports by Hegyi and colleagues, suggesting that late I_{Na} -dependent ROS production and CaMKII δ hyperactivation amplify each other (Hegyi et al., 2021). Our study also shows that DOX-related ROS can directly modulate

RyR2 function through oxidation of redox-sensitive cysteine residues, further amplifying CaMKII δ -dependent SR Ca^{2+} leak and promoting CaMKII δ activation via calcium/calmodulin signalling, which is an observation that transfers the pathophysiological relevance of

oxidised RyR2 (as elegantly shown by the Terentyev group) to the field of doxorubicin-associated pathology (Terentyev et al., 2008). Thus, our present study indicates that inhibition of elevated DOX-related ROS formation should protect cardiomyocytes from DOX-induced SR Ca^{2+} leak by interrupting this pathologic late $I_{\text{Na}}/\text{ROS}/\text{CaMKII}\delta$ signalling cascade, as previously suggested (Hegyí et al., 2021). Our data further show that this beneficial effect of scavenging ROS is accomplished by an attenuation of CaMKII δ hyperactivation (oxidation and autophosphorylation), thereby reducing both SR Ca^{2+} leak and secondary late I_{Na} amplification. Interestingly, Mito-T as well as gp91ds-tat prevented DOX-related ROS formation in our model so that further studies utilising distinct genetic deletion of either mitochondrial or NOX2-derived ROS would be needed to identify a potential primary source of ROS in our model of DOX-related cardiotoxicity. In the context of doxorubicin-induced cardiomyopathy, therapeutic strategies aimed at scavenging ROS may be deleterious, because they could inadvertently diminish the anti-cancer efficacy of DOX that is greatly based on ROS in cancer cells. From a medical perspective, it is therefore interesting to note that Mustroph and colleagues found that the SGLT2 inhibitor EMPA is capable of reducing CaMKII δ -dependent enhancement of late I_{Na} by preventing phosphorylation of $\text{Na}_v1.5$ at serine-571 in aortic stenosis and in human heart failure (Mustroph et al., 2022). In line with this previous report, our current data show that EMPA indeed mitigates the enhancement of late I_{Na} in the context of acute DOX-related cardiotoxicity, by preventing pathologic CaMKII δ hyperactivation and subsequently impaired Ca^{2+} handling, which would support a previous study by Lin and colleagues that attributed preserved intracellular Ca^{2+} handling upon protective EMPA treatment to a reduction in CaMKII activity (Lin et al., 2024).

From a clinical perspective, our data support the idea that inhibition of late I_{Na} by pharmacological means may turn out as an additional strategy to prevent DICM in high risk patients undergoing anthracycline chemotherapy. Such an assumption would be in line with previous clinical trials that have implicated that EMPA and RAN are capable of reducing the risk to develop cardiotoxic side effects during anthracycline-based chemotherapy (Daniele et al., 2024; Minotti, 2013).

4.1 | Conclusions

Our present study shows for the first time that DOX-induced acute cellular cardiotoxicity involves pathologically increased late I_{Na} that initiates a vicious cycle of hampered Na/Ca handling and pathologic CaMKII δ hyperactivation that can be treated with RAN and EMPA.

4.2 | Limitations

Our data come from animal experiments, so any transfer to human pathomechanisms should be done with caution. Nevertheless, our findings indicate that pharmacological inhibition of the late I_{Na} by

RAN and EMPA attenuates DOX-dependent CaMKII δ hyperactivation and subsequently hampered Ca^{2+} handling, pointing to a potential clinical relevance of late I_{Na} inhibitors for the prevention/treatment of DICM. Of note, EMPA and RAN are non-selective inhibitors of late I_{Na} , and other off-target effects, including interaction with Ca/Na-signalling pathways, might participate in the observed cardioprotective phenotype as observed in our cell model. We need to emphasise this, because there are considerable differences regarding the pharmacological effects of RAN versus EMPA in DOX-treated myocytes in our experimental setup, such as *absent* prevention of ROS formation upon RAN, which was paralleled by an only trend-wise reduction of cardiotoxicity markers by RAN as assessed here (i.e., regarding MDA levels and troponin release). Hence, it is very likely that further specific mechanisms of RAN versus EMPA independent of late I_{Na} inhibition exist in the context of acute DOX-related cardiotoxicity, which, however, are beyond the scope of this study. Moreover, we did not assess the long-term effects as well as potential systemic effects of late I_{Na} inhibition during the progression of DICM so that any long-term effects in our model remain elusive. Finally, because there are multiple phosphorylation sites of CaMKII at $\text{Na}_v1.5$ (including serine-516 and serine-593) that we did not investigate here, future studies investigating a potential contribution of these phosphorylation sites to the observed effect are recommended.

AUTHOR CONTRIBUTIONS

Anna-Lena Feder: Validation; software; methodology; data curation; writing—review and editing; writing—original draft; project administration; formal analysis; conceptualization; investigation. **Anna-Maria Pfützenreuter:** Data curation; formal analysis. **Lea Maria Funke:** Data curation; formal analysis. **Maria Johanna Baier:** Data curation; formal analysis. **Daniel Tarnowski:** Writing—original draft; writing—review and editing. **Laura Stengel:** Data curation. **Lars Siegfried Maier:** Writing—review and editing; writing—original draft; project administration; resources. **Can Martin Sag:** Resources; project administration; writing—review and editing; writing—original draft; visualization; supervision; investigation; formal analysis; conceptualization; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design and Analysis](#), [Immunoblotting and Immunochemistry](#) and [Animal Experimentation](#) and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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