

# Phosphorylation of RyR2 Ser-2814 by CaMKII mediates $\beta$ 1-adrenergic stress induced $\text{Ca}^{2+}$ -leak from the sarcoplasmic reticulum

Maria J. Baier<sup>1</sup> , Jannis Noack<sup>1</sup>, Mark Tilmann Seitz<sup>2</sup>, Lars S. Maier<sup>1</sup> and Stefan Neef<sup>1</sup>

<sup>1</sup> Department of Internal Medicine II, University Medical Center Regensburg, Germany

<sup>2</sup> Department of Trauma Surgery, Orthopaedics and Plastic Surgery, University Medical Center Göttingen, Germany

## Keywords

adrenergic stress; CaMKII; RyR2; Ser-2814; SR  $\text{Ca}^{2+}$  leak

## Correspondence

S. Neef, Department of Internal Medicine II, University Hospital Regensburg, University Heart Center Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany  
 Fax: +49-941-944 7235  
 Tel: +49-941-944 7207  
 E-mail: stefan.neef@ukr.de

Maria J. Baier and Jannis Noack contributed equally to this work.

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Adrenergic stimulation, while being the central mechanism of cardiac positive inotropy, is a universally acknowledged inductor of undesirable sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  leak. However, the exact mechanisms for this remained unspecified so far. This study shows that  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII)-specific phosphorylation of ryanodine receptor type 2 at Ser-2814 is the pivotal mechanism by which SR  $\text{Ca}^{2+}$  leak develops downstream of  $\beta$ 1-adrenergic stress by increase of the leak/load relationship. Cardiomyocytes with a Ser-2814 phosphoresistant mutation (S2814A) were protected from isoproterenol-induced SR  $\text{Ca}^{2+}$  leak and consequently displayed improved postrest potentiation of systolic  $\text{Ca}^{2+}$  release under adrenergic stress compared to littermate wild-type cells.

Adrenergic activation (the ‘fight or flight’ response) is the central mechanism by which the cardiovascular system responds to stress, resulting in acute positive inotropy due to  $\beta$ 1-adrenergic activation. However,  $\beta$ 1-adrenergic stimulation is also a strong and universally acknowledged inductor of sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  leak [1]. And while  $\beta$ 1-adrenergic stimulation acutely has positive inotropic effects and can be acutely necessary for cardiac decompensation/shock, it is arrhythmogenic and chronic adrenergic stress is even detrimental to cardiac function. It is believed that SR  $\text{Ca}^{2+}$  leak plays a central role in

these detrimental consequences of adrenergic stimulation [2,3].

The molecular mechanisms by which  $\text{Ca}^{2+}$  leak from the SR acutely or chronically develops in the heart are complex and have been subject to controversy and discrepant findings. Activation of the protein kinase A (PKA) pathway downstream of  $\beta$ 1-adrenergic receptor stimulation increases SR  $\text{Ca}^{2+}$  uptake via disinhibition of SR  $\text{Ca}^{2+}$  ATPase type 2a (SERCA2a) due to phospholamban (PLB) phosphorylation by PKA [4] and increased SR  $\text{Ca}^{2+}$  loading has been shown to facilitate  $\text{Ca}^{2+}$  loss from the SR (‘leak/load relationship’) [5]. This

## Abbreviations

A/Ala, alanine; CaMKII,  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II; ISO, isoproterenol; NT, normal Tyrode’s solution; PKA, protein kinase A; PLB, phospholamban; RyR2, ryanodine receptor type 2; S/Ser, serine; SERCA2a, sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase type 2a; SR, sarcoplasmic reticulum; WT, wild-type.

relationship between increased SR  $\text{Ca}^{2+}$  content and induction of SR  $\text{Ca}^{2+}$  leak is exponential [6], with extremely increased SR  $\text{Ca}^{2+}$  content leading to a phenomenon called ‘spillover’ [7]. Another important mechanism for the (patho)genesis of SR  $\text{Ca}^{2+}$  leak is increased open probability of the SR  $\text{Ca}^{2+}$ -release channel ryanodine receptor type 2 (RyR2) due to increased phosphorylation of this protein [8,9]. The RyR2 has multiple phosphorylation sites, the major ones being Ser-2030, Ser-2808, and Ser-2814. Both phosphorylation of the RyR2 by PKA (at Ser-2808) and by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII, at Ser-2814 and possibly also Ser-2808) have been suggested to be important for pathologic RyR2 gating [10,11]. This is important also in cardiac disease, where, for example in heart failure and atrial fibrillation, increased SR  $\text{Ca}^{2+}$  leak has been attributed to RyR2 hyperphosphorylation and appears to play a role in the pathophysiology of both conditions [12,13]. In addition, it was shown that mice with a constitutively unphosphorylatable Ser-2814 phosphorylation site (Ser2814Ala mutation, S2814A mice, [14]) were protected from pressure overload-induced heart failure [12].

An important insight into the development of SR  $\text{Ca}^{2+}$  leak induced by adrenergic stress came from Curran *et al.* [15], who reported that, surprisingly, not PKA but CaMKII is the essential downstream mechanism for SR  $\text{Ca}^{2+}$  leak upon  $\beta$ 1-adrenergic receptor stimulation with isoproterenol (ISO). However, CaMKII is broadly involved in the (dys)regulation of cardiomyocyte  $\text{Ca}^{2+}$  handling and the exact mechanisms by which CaMKII mediates the  $\beta$ -adrenergically induced SR  $\text{Ca}^{2+}$  leak remained unspecified so far. Possible mediators include increased CaMKII-dependent phosphorylation of RyR2, increased SR  $\text{Ca}^{2+}$  loading (via leak/load relationship) due to CaMKII-mediated increased L-type  $\text{Ca}^{2+}$  current influx (upon L-type  $\text{Ca}^{2+}$  channel phosphorylation by CaMKII), or enhanced SR  $\text{Ca}^{2+}$  load as a consequence of CaMKII-dependent phosphorylation of PLB (at Thr-17, thus disinhibiting SERCA2a and increasing SR  $\text{Ca}^{2+}$  uptake).

Importantly, SR  $\text{Ca}^{2+}$  leak is comprised of small fraction of  $\text{Ca}^{2+}$ -spark-mediated and a (quantitatively clearly dominant) nonspark fraction [16–18]. Thus, we used tetracaine-sensitive  $\text{Ca}^{2+}$  shift [5] to assess SR  $\text{Ca}^{2+}$  leak, as this measures global SR  $\text{Ca}^{2+}$  leak (non-spark plus spark-mediated leak) [19].

## Materials and methods

### Animals

S2814A mice and the respective wild-type (WT) littermates were a friendly gift from Prof. Stephan Lehnart (Göttingen,

Germany) and X. Wehrens (Houston, TX, USA), backcrossed to a C57N background at the central animal research facility of the University Medical Center Regensburg. Animals were housed at a 12-h light–dark cycle in cages type IIL. Standard laboratory chow and water were provided ad libitum. Heterozygous breeding resulted in WT and S2814A littermates that were used for the experiments (male and female mice, 12–16 weeks). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the appropriate institutional committees.

### Isolation of cardiomyocytes

Isolation of cardiomyocytes was performed as described previously [20]. Briefly, mice were anesthetized with isoflurane, and hearts were quickly excised. After weighing, hearts were mounted on a Langendorff perfusion apparatus and retrogradely perfused with nominally  $\text{Ca}^{2+}$ -free solution containing (in  $\text{mmol}\cdot\text{L}^{-1}$ ): 113 NaCl, 4.7 KCl, 0.6  $\text{KH}_2\text{PO}_4$ , 0.6  $\text{Na}_2\text{HPO}_4$ , 1.2  $\text{MgSO}_4$ , 12  $\text{NaHCO}_3$ , 10  $\text{KHCO}_3$ , 10 HEPES, 30 taurine, 10 BDM (2,3 butanedione monoxime), 5.5 glucose, 0.032 phenol-red for 4 min (37 °C, pH 7.4). Then, 7.5  $\text{g}\cdot\text{L}^{-1}$  liberase™ (Roche diagnostics, Mannheim, Germany), trypsin 0.6%, and 0.125  $\text{mmol}\cdot\text{L}^{-1}$   $\text{CaCl}_2$  were added to the perfusion solution and perfusion was continued until the heart became flaccid. Ventricular tissue was collected in perfusion buffer containing 5% bovine calf serum, cut into small pieces, dispersed, and filtered, until no solid tissue was left. After  $\text{Ca}^{2+}$  reintroduction by stepwise increasing  $[\text{Ca}^{2+}]$  from 0.1 to 1.4  $\text{mmol}\cdot\text{L}^{-1}$ , cardiomyocytes were plated onto superfusion chambers, which had been coated with laminin to allow cell adhesion.

### Intracellular $\text{Ca}^{2+}$ measurements

Intracellular  $\text{Ca}^{2+}$  measurements were performed as described previously [20] using an epifluorescence detection system (IonOptix Corp, Milton, MA, USA) mounted to a Nikon TE2000U inverted microscope. Isolated cells were loaded with Fluo-4 AM (10  $\mu\text{mol}\cdot\text{L}^{-1}$ ) for 15 min and superfused with normal Tyrode’s solution (‘NT’) consisting of (in  $\text{mmol}\cdot\text{L}^{-1}$ ): 140 NaCl, 4 KCl, 5 HEPES, 1  $\text{MgCl}_2$ , 10 glucose, 2  $\text{CaCl}_2$  (pH 7.4 with NaOH). Excitation of Fluo-4 was at  $480 \pm 15$  nm, emission was collected at  $535 \pm 20$  nm. Myocytes were field-stimulated at 1 Hz until steady-state was achieved. For  $\beta$ 1-adrenergic stimulation, ISO (100  $\text{nmol}\cdot\text{L}^{-1}$ ) was added to the superfusion (starting for 5 min before recording first data). Sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  content was estimated by rapid application of caffeine (10  $\text{mmol}\cdot\text{L}^{-1}$ ).

## SR $\text{Ca}^{2+}$ leak measurements using tetracaine

Tetracaine experiments to measure SR  $\text{Ca}^{2+}$  leak were performed according to the method of Shannon *et al.* [5].  $\text{Na}^+$ - and  $\text{Ca}^{2+}$ -free bath solution ( $0\text{Na}^+/0\text{Ca}^{2+}$ ) was prepared consisting of (in  $\text{mmol}\cdot\text{L}^{-1}$ ): 140 LiCl, 4 KCl, 5 HEPES, 1  $\text{MgCl}_2$ , 10 glucose, 10 EGTA, 2  $\text{CaCl}_2$  (pH 7.4 with LiOH). Tetracaine  $1\text{ mmol}\cdot\text{L}^{-1}$  was added to this solution to prepare the tetracaine solution. The fractional shift in diastolic fluorescence upon tetracaine (an allosteric blocker of ryanodine receptors) under  $0\text{Na}^+/0\text{Ca}^{2+}$  conditions was measured after rapid switching of superfusion solutions using local application immediately at the cell, followed by caffeine application ( $10\text{ mmol}\cdot\text{L}^{-1}$ ) to calculate leak/load relationship.

## Western blot

After isolation of S2814A and WT cardiomyocytes (see above), cells were either incubated in NT or ISO solution ( $100\text{ nmol}\cdot\text{L}^{-1}$ ) for 10 min. Cells were homogenized in Tris buffer containing (in mM): 20 Tris/HCl, 200 NaCl, 20 NaF, 1  $\text{Na}_3\text{VO}_4$ , 1 DTT, 1% Triton X-100 (pH 7.4), complete protease inhibitor cocktail (Roche diagnostics, Mannheim, Germany) and phosphatase-inhibitor mixture (PhosSTOP; Roche). Protein concentration was determined by BCA assay (Sigma-Aldrich Co., St. Louis, MO, USA). Denatured proteins were separated on SDS-polyacrylamide gels (5–12%) and transferred to nitrocellulose membranes (GE Health Care, Chalfont St Giles, UK). Specific proteins were detected using anti-RyR2 (1 : 10 000, Sigma-Aldrich Co.), anti-pS2814-RyR2 (1 : 1000; Badrilla, Leeds, UK), anti-pS2808-RyR2 (1 : 1000; Badrilla), antibodies followed by HRP-conjugated donkey anti-rabbit IgG antibodies (GE Health Care). Chemiluminescent detection was performed with WesternBright™ Chemiluminescent Substrate (Biozym Scientific GmbH, Hess. Oldendorf, Germany). Phosphorylation levels of the proteins were normalized to total protein expression.

## Statistical analysis

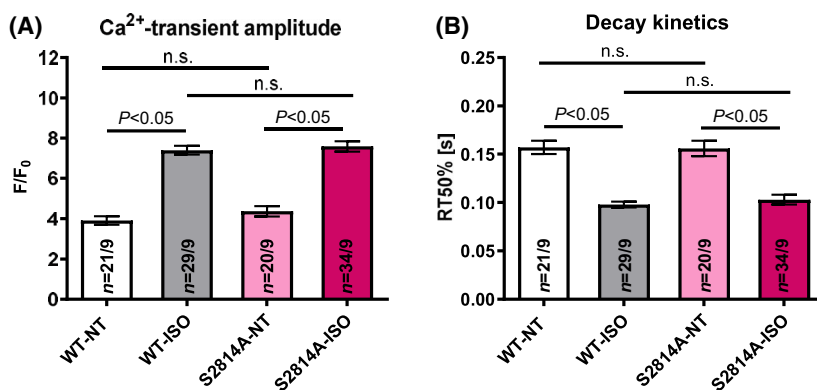
Data are presented as means  $\pm$  SEM. Statistical analyses were performed using one-way ANOVA with Tukey's

correction for multiple comparisons.  $P < 0.05$  was considered statistically significant. Analyses and graphs were generated using GRAPHPAD PRISM 9 Statistical Software (GraphPad, San Diego, CA, USA).

## Results and Discussion

We hypothesized that CaMKII-specific RyR2 Ser-2814 phosphorylation could be the central mechanism by which SR  $\text{Ca}^{2+}$  leak develops in acute  $\beta$ 1-adrenergic stress. We investigated this hypothesis by exposing cardiomyocytes from a mouse model harboring the phosphoresistant S2814A mutation [12,14] (vs littermate WT cells) to ISO ( $100\text{ nmol}\cdot\text{L}^{-1}$ ) as compared to control conditions (NT) and measuring SR  $\text{Ca}^{2+}$  leak using the gold-standard method of tetracaine-sensitive  $\text{Ca}^{2+}$  shift by Shannon *et al.* [5] using  $\text{Ca}^{2+}$ -sensitive epifluorescence microscopy (Fluo-4).

To assess whether Ser-2814 phosphorylation might be relevant for the positive inotropic and lusitropic  $\text{Ca}^{2+}$ -handling responses to adrenergic stimulation, we first compared electrically invoked  $\text{Ca}^{2+}$  transients in the absence vs. presence of ISO-stimulation. We can report that both the positive inotropic increase of  $\text{Ca}^{2+}$  amplitude [WT-ISO:  $7.40 \pm 0.22$  vs WT-NT:  $3.91 \pm 0.21$ ,  $n(\text{WT-ISO}) = 29$ ,  $n(\text{WT-NT}) = 21$ ;  $P < 0.05$ ; S2814A-ISO:  $7.59 \pm 0.26$  vs S2814A-NT:  $4.37 \pm 0.26$ ,  $n(\text{S2814A-ISO}) = 34$ ,  $n(\text{S2814A-NT}) = 20$ ;  $P < 0.05$ ] as well as the positive lusitropic acceleration of  $\text{Ca}^{2+}$ -transient decay (WT-ISO:  $0.098 \pm 0.003\text{ s}$  vs WT-NT:  $0.157 \pm 0.007\text{ s}$ ,  $P < 0.05$ ; S2814A-ISO:  $0.103 \pm 0.005\text{ s}$  vs S2814A-NT:  $0.156 \pm 0.008\text{ s}$ ,  $P < 0.05$ ; numbers as above) are strong in both genotypes and not different between genotypes (Fig. 1A,B). Thus, neither inotropic (transient amplitude) nor lusitropic (transient decay) response to  $\beta$ 1-adrenergic stimulation depend on RyR2 Ser-2814 phosphorylation. (And indeed even mice with knockout of the dominant cardiac isoform of CaMKII, CaMKII $\delta$ , show a normal response to adrenergic stimulation [21].)

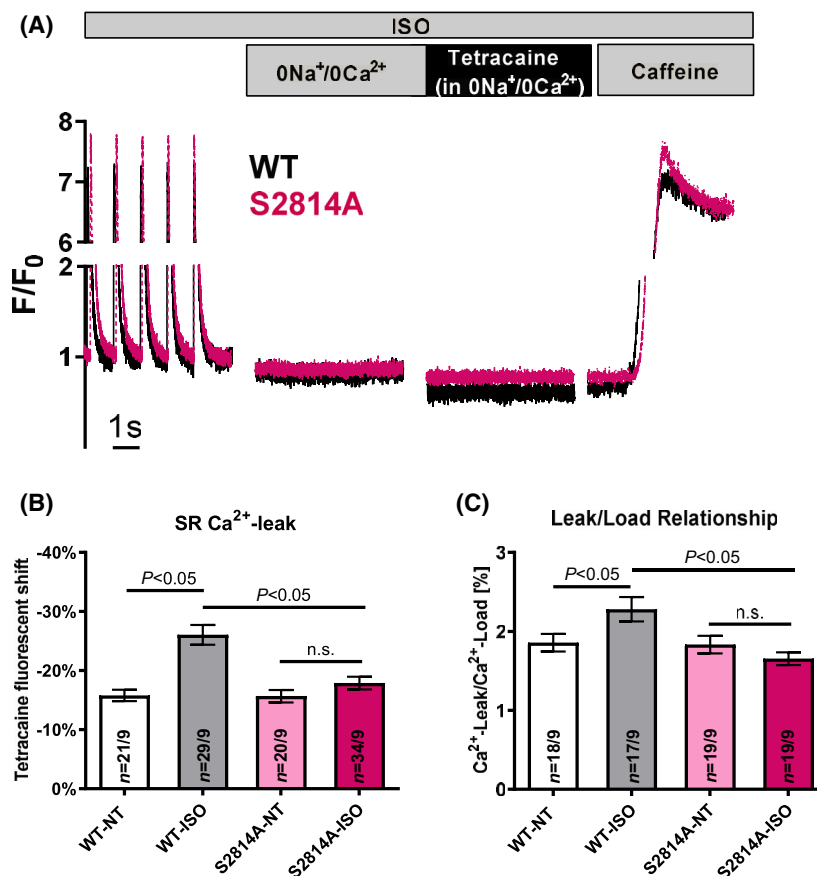


**Fig. 1.**  $\text{Ca}^{2+}$ -transient amplitude and decay kinetics. Mean data for  $\text{Ca}^{2+}$ -transient amplitudes (A) and decay kinetics (rt50%) (B) show that both the increase of  $\text{Ca}^{2+}$  amplitude and the acceleration of  $\text{Ca}^{2+}$  transient decay are strong in both genotypes upon ISO stress and not different between genotypes. Data are shown as means  $\pm$  SEM ( $n = \text{cells/mice}$ ; one-way ANOVA).

Importantly, however, we found that cardiomyocytes with the phosphoresistant RyR2 S2814A mutation were completely protected from  $\beta$ 1-adrenergically induced SR  $\text{Ca}^{2+}$  leak: Under basal conditions (NT), the S2814A mutation had no effect on tetracaine-sensitive SR  $\text{Ca}^{2+}$  leak [fluorescence shift upon tetracaine  $15.78 \pm 0.97\%$  in WT vs.  $15.56 \pm 1.04\%$  in S2814A;  $n(\text{WT}) = 21$  cells,  $n(\text{S2814A}) = 20$  cells; n.s.]. As expected,  $\beta$ 1-adrenergic stimulation with ISO strongly increased the SR  $\text{Ca}^{2+}$  leak in WT cardiomyocytes [WT-ISO: fluorescence shift  $26.05 \pm 1.67\%$ ;  $n(\text{WT-ISO}) = 29$ ;  $P < 0.05$  vs. WT-NT]. In S2814A mice, however, ISO stress did not lead to a significant increase of basal SR  $\text{Ca}^{2+}$  leak [S2814A-ISO: fluorescence shift  $17.88 \pm 1.09\%$ ;  $n(\text{S2814A-ISO}) = 34$ ; n.s. vs. S2814A-NT] and these mice were, consequently, protected from ISO-induced SR  $\text{Ca}^{2+}$  leak as compared to WT ( $P < 0.05$ , means/SEM and numbers as above; Fig. 2A,B). To exclude that possible differences in SR  $\text{Ca}^{2+}$  content between the genotypes were the reason for the protection from ISO-induced  $\text{Ca}^{2+}$  leak rather than the RyR2 function itself, we also measured SR  $\text{Ca}^{2+}$  load in these cells and calculated leak/load

relationship. In contrast to WT myocytes, where ISO significantly increased leak/load relationship, [WT-ISO:  $2.28 \pm 0.15\%$  vs. WT-NT:  $1.86 \pm 0.11\%$ ;  $n(\text{WT-ISO}) = 17$ ;  $n(\text{WT-NT}) = 18$ ,  $P < 0.05$ ], implying increased 'leakiness' of the RyR2 upon this adrenergic stimulation, S2814A cells were completely protected from this worsening of leak/load relationship under  $\beta$ 1-stress [S2814A-ISO:  $1.65 \pm 0.08\%$  vs. S2814A-NT:  $1.83 \pm 0.11\%$ ;  $n(\text{S2814A-ISO}) = 19$ ;  $n(\text{S2814A-NT}) = 19$ , n.s. vs. S2814A-NT,  $P < 0.05$  vs. WT-ISO; Fig. 2C].

Next, we investigated postrest potentiation as an integrative measure for the capacity of the SR to further accumulate  $\text{Ca}^{2+}$  [22] after 10-s cessation of stimulation. In WT mice, postrest potentiation was significantly diminished under  $\beta$ 1-adrenergic stress as compared to basal conditions [WT-ISO:  $+13.0 \pm 0.9\%$  vs. WT-NT:  $+22.5 \pm 2.0\%$ ;  $n(\text{WT-ISO}) = 33$ ,  $n(\text{WT-NT}) = 17$ ;  $P < 0.05$ ], suggesting that the extensively increased SR  $\text{Ca}^{2+}$  load during ISO stimulation has already reached its maximum. However, while in S2814A mice postrest potentiation was also somewhat lower under  $\beta$ 1-adrenergic stress compared to basal



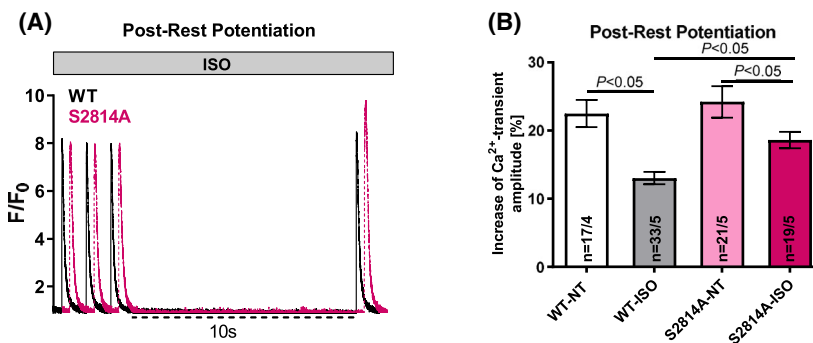
**Fig. 2.** Tetracaine-sensitive SR  $\text{Ca}^{2+}$  leak. Original recordings (A) show tetracaine-sensitive SR  $\text{Ca}^{2+}$  leak in S2814A and WT mice under ISO stimulation. Mean data (B) show protection from ISO-induced SR  $\text{Ca}^{2+}$  leak in phosphoresistant RyR2 S2814A mice as compared to WT mice ( $P = \text{WT-NT vs. WT-ISO}$ ;  $P = \text{WT-ISO vs. S2814A-ISO}$ ). Also, ISO-induced worsening of leak/load relationship (C) was completely prevented in S2814A compared to WT cells. Data are shown as means  $\pm$  SEM ( $n = \text{cells/mice}$ ; one-way ANOVA).

conditions [S2814A-ISO:  $+18.6 \pm 1.2\%$  vs. S2814A-NT:  $+24.2 \pm 2.3\%$ ;  $n(\text{S2814A-ISO}) = 19$ ,  $n(\text{S2814A-NT}) = 21$ ;  $P < 0.05$ ], it was significantly improved compared to WT cells ( $P < 0.05$ ) (Fig. 3A,B). Thus, phosphorylation of RyR2 at Ser-2814 during adrenergic stress impairs the ability of the SR to further accumulate  $\text{Ca}^{2+}$ .

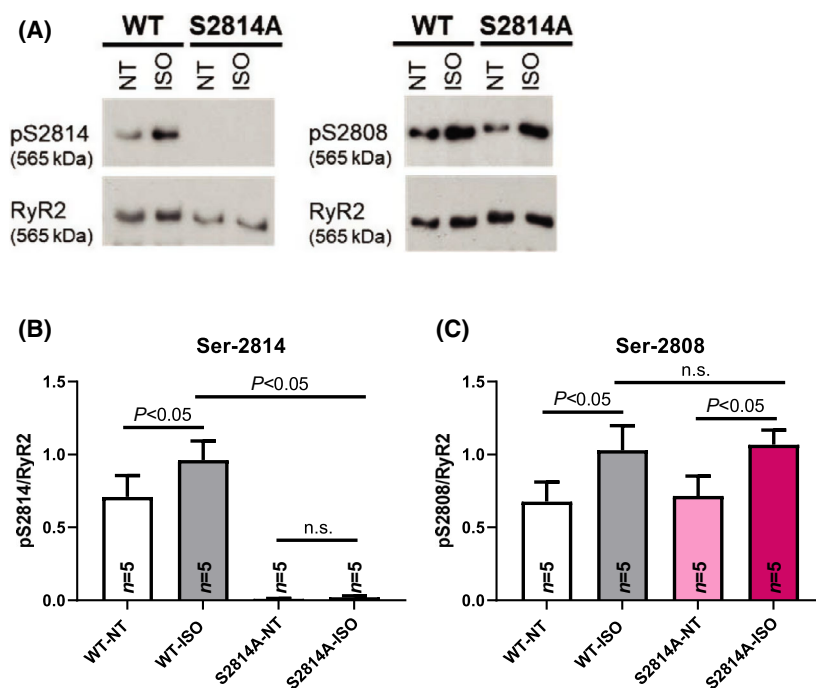
In order to look into phosphorylation of RyR2 at Ser-2808 and Ser-2814 under  $\beta$ -adrenergic stress and to exclude off-target effects of the S2814A mutation on Ser-2808 phosphorylation, we performed western

blots in WT and S2814A cells with or without ISO incubation. We can report that the S2814A mutation indeed completely prevents RyR2 Ser-2814 phosphorylation also under adrenergic stress (Fig. 4A,B). In contrast, phosphorylation of Ser-2808 is significantly increased by ISO stimulation, but neither the basal phosphorylation (NT) nor the phosphorylation level under adrenergic stimulation at this site are different between WT and S2814A (Fig. 4A,C).

In conclusion, our study defines for the first time phosphorylation of RyR2 at the CaMKII-specific



**Fig. 3.** Postrest potentiation of systolic  $\text{Ca}^{2+}$  release. Original recordings (A) show postrest potentiation of S2814A and WT cells under  $\beta$ -adrenergic stimulation with ISO. Original recordings from S2814A are depicted time-shifted vs WT to be visually better discernible. Mean data (B) show that postrest potentiation of systolic  $\text{Ca}^{2+}$  release was significantly better preserved in S2814A mice under ISO stress compared to WT mice. Data are shown as means  $\pm$  SEM ( $n = \text{cells/mice}$ ; one-way ANOVA).



**Fig. 4.** Phosphorylation level of Ser-2814 and Ser-2808. Examples of protein expression (A) in WT and S2814A mice with or without ISO stimulation. Mean data of Ser-2814 (B) and Ser-2808 (C) phosphorylation levels show phosphoresistance at Ser-2814 in S2814A mice, while phosphorylation levels at Ser-2808 were significantly increased upon ISO stress both in WT and S2814A mice. Data are shown as means  $\pm$  SEM ( $n = \text{mice}$ ; one-way ANOVA).

RyR2 Ser-2814 phosphorylation site as the pivotal mechanism mediating  $\beta$ 1-adrenergic induced SR  $\text{Ca}^{2+}$  leak in cardiomyocytes. Phosphoresistance at this site almost prevented ISO-induced SR  $\text{Ca}^{2+}$  leak and increase of leak/load relationship. Furthermore, as SR  $\text{Ca}^{2+}$  leak and leak/load relationship were not different between genotypes under basal conditions, it appears that Ser-2814 only becomes relevant for SR  $\text{Ca}^{2+}$  leak upon (phosphorylation) challenge such as under adrenergic stress.

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## Conflict of interest

The authors declare no conflict of interest.

## Data accessibility

The data that support the findings of this study are available from the corresponding author (stefan.neef@ukr.de) upon reasonable request.

## Author contributions

SN conceived and supervised the study. MJB, JN, and MTS designed and performed experiments and analyzed data. MJB, LSM, and SN interpreted data for the work. MJB and JN wrote the manuscript. MTS, LSM, and SN made manuscript revisions.

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