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Regulation of cardiac ryanodine receptor function by the cyclic-GMP dependent protein kinase G

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ABSTRACT

Background: The cGMP-dependent protein kinase G (PKG) phosphorylates the cardiac ryanodine receptor (RyR2) *in vitro*. We aimed to determine whether modulation of endogenous PKG alters RyR2-mediated spontaneous Ca^{2+} release and whether this effect is linked to a change in RyR2 phosphorylation.

Methods: & Results: Human embryonic kidney (HEK293) cells with inducible RyR2 expression were treated with the cGMP analogue 8-Br-cGMP (100 μM) to activate endogenous PKG. In cells transfected with luminal Ca^{2+} sensor, D1ER, PKG activation significantly reduced the threshold for RyR2-mediated spontaneous Ca^{2+} release (93.9 ± 0.4% of store size with vehicle vs. 91.7 ± 0.8% with 8-Br-cGMP, $P = 0.04$). Mutation of the proposed PKG phosphorylation sites, S2808 and S2030, either individually or as a combination, prevented the decrease in Ca^{2+} release threshold induced by endogenous PKG activation. Interestingly, despite a functional dependence on expression of RyR2 phosphorylation sites, 8-Br-cGMP activation of PKG did not promote a detectable change in S2808 phosphorylation ($P = 0.9$). Paradoxically, pharmacological inhibition of PKG with KT 5823 (1 μM) also reduced the threshold for spontaneous Ca^{2+} release through RyR2 without affecting S2808 phosphorylation. Silencing RNA knockdown of endogenous PKG expression also had no quantifiable effect on RyR2 S2808 phosphorylation ($P = 0.9$). However, unlike PKG inhibition with KT 5823, PKG knockdown did not alter spontaneous Ca^{2+} release propensity or luminal Ca^{2+} handling.

Conclusion: In an intact cell model, activation of endogenous PKG reduces the threshold for RyR2-mediated spontaneous Ca^{2+} release in a manner dependent on the RyR2 phosphorylation sites S2808 and S2030. This study clarifies the regulation of RyR2 Ca^{2+} release by endogenous PKG and functionally implicates the role of RyR2 phosphorylation.

1. Introduction

The cGMP-dependent protein kinase G (PKG) has been long proposed to influence contractility in the mammalian heart (Shah and MacCarthy, 2000). Mechanistically, extensive research has identified PKG activity as a regulator of several Ca^{2+}-handling processes, including Ca^{2+} flux through voltage-gated channels (Mery et al., 1991; Wahler and Dollinger, 1995), sarcoplasmic reticulum (SR) Ca^{2+} uptake (Cornwell et al., 1991), and myofilament Ca^{2+} sensitivity (Shah et al., 1994; Layland et al., 2002). Despite these findings, a notable gap exists in the understanding of whether PKG also regulates the activity of a key Ca^{2+} handling protein, the cardiac ryanodine receptor (RyR2). It is known that *in vitro* treatment with the catalytic unit of PKG promotes phosphorylation of purified RyR2 (Takasago et al., 1991; Hohenegger and Suko, 1993; Xiao et al., 2006), specifically at serine residues S2808 and S2030; although, to a much greater extent at the former (Xiao et al., 2006). Several studies, including our own, have shown that activation of pathways upstream of PKG increase RyR2 phosphorylation and alter RyR2 function (Gonano et al., 2014; Ho et al., 2016). However, these previous studies were performed in conjunction with stimuli that likely activated a cascade of signalling molecules, many of which are also known to independently alter RyR2 function. Therefore, it is unclear if

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direct activation of endogenous PKG without external stimuli promotes RyR2 S2808 phosphorylation and whether there are changes in RyR2 function specifically due to this PKG-dependent modification.

RyR2 is a multi-subunit channel that mediates Ca^{2+} release from the SR into the cytosol to provide the Ca^{2+} necessary for contraction (Bers, 2002). Typical activation of RyR2 occurs in response to Ca^{2+} entry into the cell as part of the Ca^{2+}-induced Ca^{2+} release process (Bers, 2002). However, in conditions that either overload the SR Ca^{2+} level or modify the sensitivity of RyR2 to Ca^{2+}, Ca^{2+} can be released spontaneously via RyR2 in a process termed store overload-induced Ca^{2+} release (SOICR) (Jiang et al., 2004; Zhang et al., 2015). We and others have shown previously that the propensity for SOICR is enhanced by mutations and post-translational modification of RyR2 that increases the sensitivity of RyR2 to SR and cytosolic Ca^{2+} (Jiang et al., 2005; Jones et al., 2008; Waddell et al., 2016; Zhang et al., 2016). In addition to PKG, RyR2 is also phosphorylated at S2808 and S2030 by the cAMP-dependent protein kinase A (PKA) (Marx et al., 2000; Xiao et al., 2005, 2006). An extensive, albeit controversial, body of research has found that PKA-dependent phosphorylation at S2808 can enhance the luminal (intra-SR) sensitivity of RyR2, leading to increases in RyR2 open probability and promotion of diastolic Ca^{2+} leak (Marx et al., 2000; Wehrens et al., 2006; Voigt et al., 2012; Ullrich et al., 2012). Less well characterized is the effect of S2030 phosphorylation on RyR2 function; however, some show that phosphorylation at S2030 is the primary target of PKA and the major phosphorylation site during adrenergic stimulation of the heart (Xiao et al., 2006). Whether a similar effect of RyR2-mediated Ca^{2+} release occurs following RyR2 S2808 or S2030 phosphorylation by PKG is inconclusive.

Using a HEK293 cell model with inducible expression of RyR2, this study aimed to determine the effect of endogenous PKG activation on SOICR and the link to PKG-mediated phosphorylation of RyR2. These effects were assessed following (1) PKG activation with the cGMP analogue, 8-Br-cGMP, (2) PKG inhibition with KT 5823, and (3) knockdown of endogenous PKG expression using silencing RNA (siRNA). Our results demonstrate that endogenous PKG activation with 8-Br-cGMP promotes spontaneous Ca^{2+} release by reducing the threshold for RyR2-mediated SOICR. This effect was abolished if RyR2 was mutated at the putative PKG phosphorylation sites S2808 or S2030; however, no change in RyR2 phosphorylation could be detected following 8-Br-cGMP treatment using western blotting. In addition, we found that knockdown of endogenous PKG expression has no effect on basal RyR2 function or on RyR2 S2808 phosphorylation. Our study indicates that increased PKG activity can alter RyR2-mediated Ca^{2+} release in a manner dependent on expression of known RyR2 phosphorylation sites, but a lack of effect of knockdown of endogenous PKG expression suggests it plays a limited role at rest.

2. Methods and materials

2.1. Generation of inducible RyR2 expressing HEK293 cells

Human embryonic kidney (HEK293) cells stably expressing wild-type (WT) RyR2 were generated as previously described (Zhang et al., 2016; Aitken-Buck et al., 2021). Separate lines HEK293 cells containing serine-to-alanine RyR2 mutations at serine 2808 (S2808), serine 2030 (S2030), or serine residues 2808, 2030, and 2814 (triple mutant) were also generated. For preparation of cell lysates and live Ca^{2+} imaging, RyR2 expression was induced by tetracycline (0.1 μg/mL) ~16–18 h prior to experimentation.

2.2. Luminal (intra-endoplasmic reticulum) Ca^{2+} imaging

Luminal Ca^{2+} release was measured in HEK293 cells with inducible RyR2 expression transfected with cDNA encoding the Ca^{2+}-sensitive FRET (fluorescence resonance energy-transfer)-based cameleon protein D1ER by Ca^{2+} phosphate precipitation (Palmer et al., 2004; Jones et al., 2008). D1ER transfection was performed 24 h prior to RyR2 induction. Cells were continuously superfused at room temperature with Krebs Ringer HEPES (KRH) buffer containing (in mM): 125 NaCl, 5 KCl, 25 HEPES, 6 glucose, 1.2 $MgCl_2$ and variable $CaCl_2$ (0–2 mM) (pH 7.4). Tetracaine (2 mM) was added to block RyR2 and caffeine (20 mM) to deplete internal Ca^{2+} stores. Cells were treated (10 min pre-incubation each treatment) with PKG activator, 8-Br-cGMP (100 μM), PKG inhibitor, KT 5823 (1 μM), or equivalent volume vehicle controls (dH2O or dimethyl sulfoxide (DMSO)) to modulate endogenous PKG activity. Fluorescent images were captured every 2 s with a 100 ms exposure time and excitation at 436 nm (20 nm bandwidth). Emissions of yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) were captured simultaneously at 535 nm (40 nm bandwidth) and 480 nm (30 nm bandwidth), respectively. Images were collected by a CoolSNAP HQ2 CCD camera (Photometrics, AZ, USA) with a dual-channel imaging system (DV2). Amount of FRET was determined from ratio of 535 and 480 nm emissions (YFP:CFP), as used previously (Zhang et al., 2016). From each cell, the maximum and minimum Ca^{2+} store levels were calculated from the maximal (F_{max}) and minimal FRET signals (F_{min}) induced by tetracaine and caffeine treatment, respectively (Chakraborty et al., 2019). Ca^{2+} store size was calculated from $F_{max} - F_{min}$ and the threshold for SOICR (F_{SOICR}) was measured from the mean of the FRET signal preceding each SOICR event. F_{SOICR} is expressed as percentage of store size. All experiments were performed at room temperature.

2.3. PKG silencing RNA transfection

PKG expression was knocked down using a PKG specific siRNA (Thermo Fisher Scientific, MA, USA). As a negative control, separate HEK293 cells were transfected with the equivalent amount of scrambled siRNA (Cell Signaling Technology, MA, USA). Both transfections were performed using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions.

2.4. Preparation of cell lysate

Cell lysates from induced HEK293 cells were prepared and stored as described previously (Zhang et al., 2016). Briefly, HEK293 cells were harvested with 2 mM EDTA in PBS solution by centrifugation (3000 g, 5 min) after induction of RyR2 expression (~18 h post-induction). After discarding the supernatant, the cell pellet was re-suspended in lysis buffer containing 137 mM NaCl, 25 mM Tris/Hepes, 1% CHAPS, 0.5% soyabean phosphatidylcholine, 1 mM benzamide, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 0.5 mM PMSF and 2.5 mM DTT (pH 7.4). The mixture was then incubated on ice for 1-h before 2x centrifugation (16000 g, 30 min, 4 °C) to remove insoluble materials and the cell lysates. Lysate were stored at −80 °C until required.

2.5. Immunoblotting

Samples were prepared using the Laemmli method. Briefly, 40 μg protein samples were separated by 6% SDS-PAGE, transferred onto a 0.45 μm nitrocellulose membrane (Bio-Rad) and blocked in 5% milk powder in PBS. The membranes were incubated with either anti-RyR2-34C (1:1000, Abcam), anti-RyR2-pS2808 (1:1000, Badrilla), anti-PKG (1:2000, Abcam) or anti-GAPDH (1:3000, GeneTex) antibodies (diluted in 20% FBS in PBS). Membranes were washed in PBST and then incubated in respective anti-mouse (1:10,000) or anti-rabbit (1:10,000) secondary antibody (Abcam). To visualize protein bands, membranes were incubated in SuperSignal West Pico Plus Chemiluminescent Substrate (ThermoFisher Scientific) and images captured using the Chem-iDoc MP Imaging System (Bio-Rad). Band density was semi-quantified using Image J. Total RyR2 and PKG expression was normalized to GAPDH levels. RyR2-pS2808 ratio was normalized to total RyR2 levels.

2.6. Statistical analysis

Results are presented as replicate mean values ± standard error values (SEM) with individual cell data points presented alongside to show data spread. Differences in luminal Ca^{2+} handling parameters and protein expression levels were assessed with unpaired t-tests. At least 2 control experiments (vehicle or scramble) were performed on each imaging day to ensure consistency of cells between days. Statistical analyses were performed on technical replicates (average from all cells in one experiment) only and not individual cell values. Differences were considered significant if $P < 0.05$. All data analyses and plotting were performed using GraphPad 9 software.

3. Results

3.1. PKG activation with 8-Br-cGMP reduces the SOICR threshold with dependency on expression of RyR2 phosphorylation sites

To determine the effect of endogenous PKG activation on SOICR, RyR2-expressing HEK293 cells were transfected with the luminal Ca^{2+} sensor D1ER before treatment with the cGMP analogue, 8-Br-cGMP (100 μM). As shown in Fig. 1, PKG activation with 8-Br-cGMP significantly reduced the threshold for SOICR (Fig. 1A + B) in our RyR2-expressing HEK293 cell model (Vehicle: 93.9 ± 0.4% [of store size], 8-Br-cGMP: 91.7 ± 0.8%, $P = 0.04$) without altering the maximal luminal Ca^{2+} store size (Vehicle: 0.49 ± 0.03 YFP/CFP, 8-Br-cGMP: 0.49 ± 0.04 YFP/CFP, $P = 0.3$) (Fig. 1C). In an *in vitro* setting, PKG has been found to promote phosphorylation of RyR2 (Takasago et al., 1991; Hohenegger and Suko, 1993; Xiao et al., 2006), which in turn could alter RyR2 function, as has been described for other serine/threonine kinases (Marx et al., 2000; Wehrens et al., 2006). To determine whether the functional effects of endogenous PKG activation depends on phosphorylation of RyR2, we then assessed the effect of 8-Br-cGMP in HEK293 cells expressing RyR2 with a triple mutation at serine residues S2808, S2030, and S2814. With mutation of these sites, 8-Br-cGMP had no effect on the SOICR threshold (Vehicle: 94.3 ± 0.7%, 8-Br-cGMP: 93.8 ± 0.9%, $P = 0.6$) (Fig. 1D + E) or Ca^{2+} store size (Vehicle: 0.45 ± 0.04 YFP/CFP, 8-Br-cGMP: 0.43 ± 0.01 YFP/CFP, $P = 0.8$) (Fig. 1F). Together, these findings show that activation of endogenous PKG in cells not exposed to a confounding stimulus reduces the threshold for spontaneous RyR2-mediated Ca^{2+} release in a manner dependent on expression of well-established RyR2 phosphorylation sites.

3.2. Pro-SOICR effect of endogenous PKG activation is prevented by individual RyR2 mutation at S2808 or S2030

Previous *in vitro* studies have found that the catalytic component of PKG can increase RyR2 phosphorylation at the canonical PKA phosphorylation sites, S2808 and S2030. To deductively assess the role of each site in mediating the functional effects of PKG activation, we determined the effect of 8-Br-cGMP in HEK293 cells expressing single mutations at these sites (Fig. 2). As with triple mutation of RyR2, 8-Br-cGMP treatment of cells with a single mutation at S2808 had no effect on the SOICR threshold (Vehicle: 93.3 ± 0.2%, 8-Br-cGMP: 93.6 ± 0.6%, *P*

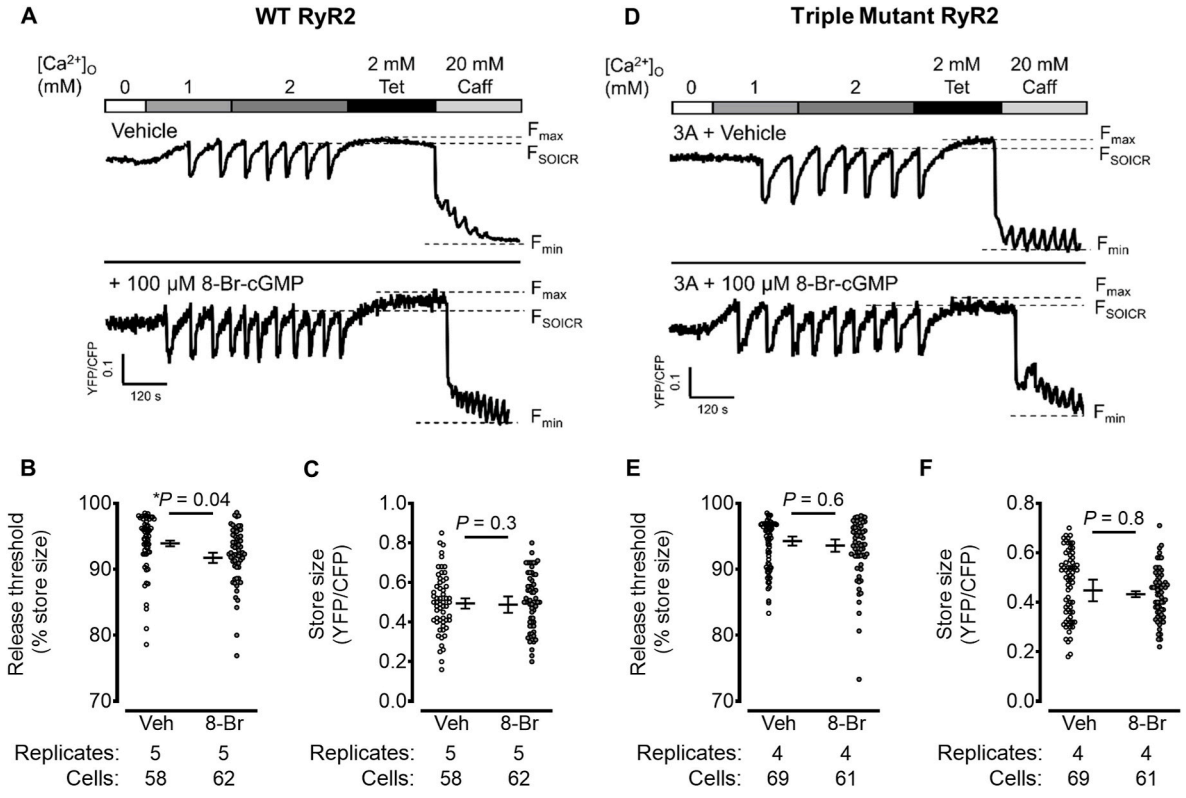

Fig. 1. Effect of protein kinase G (PKG) activation with 8-Br-cGMP on store overload-induced Ca^{2+} release (SOICR) and dependence on RyR2 phosphorylation sites. Human embryonic kidney (HEK293) cells were transfected with luminal Ca^{2+} sensor D1ER and induced to express RyR2 16–18 h prior to experimentation. Cells were treated with 8-Br-cGMP (100 μM, 10 min) or equivalent volume vehicle control before superfusion with increasing extracellular Ca^{2+} concentrations ($[Ca^{2+}]_O$) to stimulate SOICR. Tetracaine (Tet, 2 mM) and caffeine (Caff, 20 mM) were used to block RyR2 and to deplete the internal Ca^{2+} store, respectively. Representative effect of 8-Br-cGMP in HEK293 cells expressing wild-type (WT) RyR2 is shown and panel **A**, with quantification of effect on SOICR threshold and store size shown in panels **B** and **C**, respectively. Panels **D-F** show equivalent results from HEK293 cells expressing RyR2 containing triple mutation (3A) at phosphorylation sites S2808, S2030, and S2814. For **A-C**, vehicle $N = 5$ replicates (from $n = 58$ cells), 8-Br-cGMP $N = 5$ replicates (from $n = 62$ cells). For **D-F**, Triple mutant + vehicle $N = 4$ replicates (from $n = 69$ cells), triple mutant + 8-Br-cGMP $N = 4$ replicates (from $n = 61$ cells). Differences in release thresholds and store sizes were determined by unpaired t-tests of replicate mean values. *P* values are as indicated in the figure. Data are mean ± SEM of replicates with values from all cells presented alongside.

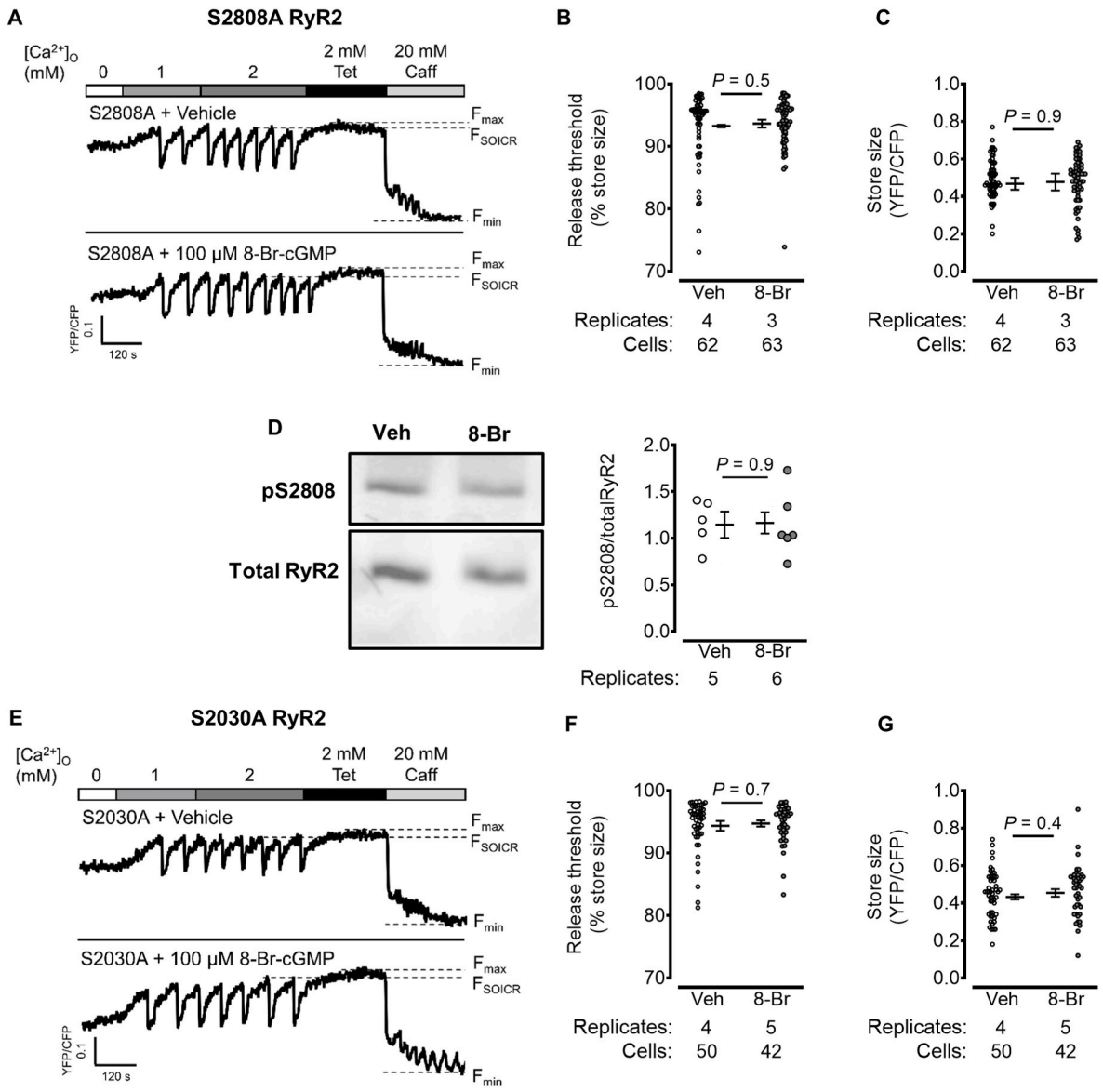

Fig. 2. Effect of single serine 2808 (S2808A) or serine 2030 (S2030A) cardiac ryanodine receptor (RyR2) mutation on response to protein kinase G activation with 8-Br-cGMP. Human embryonic kidney (HEK293) cells were transfected with luminal Ca^{2+} sensor D1ER prior to induction of RyR2 containing a mutation at either S2808A or S2030A. RyR expression was induced 16–18 h prior to experimentation and cells were treated with 8-Br-cGMP (100 μM) or equivalent volume vehicle control. Cells were either superfused with increasing extracellular Ca^{2+} concentrations ($[Ca^{2+}]_O$) to stimulate SOICR (representative traces are shown in panels **A** and **E**) or lysates were harvested for western blotting (**D**). For D1ER imaging Tetracaine (Tet, 2 mM) and caffeine (Caff, 20 mM) were used to block RyR2 and to deplete the internal Ca^{2+} store, respectively. Effects on SOICR threshold and store size of 8-Br-cGMP in S2808A and S2030A cells are shown in panels **A–C** and **E-F**, respectively. For **D**, RyR2 phosphorylation at S2808 following vehicle or 8-Br-cGMP treatment was determined by normalization to total RyR2 expression. For **A-C**, S2808A + vehicle $N = 4$ replicates (from $n = 62$ cells), S2808A + 8-Br-cGMP $N = 3$ replicates (from $n = 63$ cells). For **D**, vehicle $n = 5$ lysates, 8-Br-cGMP $n = 6$ lysates. For **E-F**, S2030A + vehicle $N = 4$ replicates (from $n = 50$ cells), S2030A + 8-Br-cGMP $N = 5$ replicates (from $n = 42$ cells). Differences were determined by unpaired t-tests. *P* values are as indicated in the figure. Data are mean ± SEM of replicates with values from all cells presented alongside.

= 0.5) (Fig. 2B) or the luminal Ca^{2+} store size (Vehicle: 0.47 ± 0.03 YFP/CFP, 8-Br-cGMP: 0.48 ± 0.04, $P = 0.9$) (Fig. 2C). Surprisingly, despite S2808 mutation abolishing the functional effect of endogenous PKG activation, treatment with 8-Br-cGMP did not promote a detectable change in RyR2 phosphorylation at S2808 (Fig. 2D) ($P = 0.9$). Since PKG has been found to also increase S2030 phosphorylation *in vitro*, we then determined the effect of PKG activation in cells expressing RyR2 with S2030 mutation (Fig. 2E). Interestingly, like that found for single S2808 mutation, single S2030 mutation also prevented the reduction in SOICR threshold associated with 8-Br-cGMP treatment (Vehicle: 94.4 ± 0.8%, 8-Br-cGMP: 94.7 ± 0.5%, $P = 0.7$) (Fig. 2F). Similarly, 8-Br-cGMP treatment of these mutant cells also had no effect on luminal Ca^{2+} store size (Vehicle: 0.43 ± 0.01 YFP/CFP, 8-Br-cGMP: 0.45 ± 0.02 YFP/CFP, $P = 0.4$) (Fig. 2G). These data suggest that expression of previously characterised RyR2 phosphorylation targets of PKG (S2808 and S2030) are required to mediate the pro-SOICR effect of endogenous PKG activation. In addition, under the conditions used, this was not accompanied by a detectable change in RyR2 phosphorylation at S2808. A lack of suitable antibodies precluded the measurement of S2030 phosphorylation levels.

3.3. PKG inhibition with KT 5823 also promotes SOICR

We then sought to determine the effect of endogenous PKG inhibition on RyR2 phosphorylation and RyR2-mediated Ca^{2+} leak. We have previously shown that inhibition of endogenous PKG using the class I kinase

inhibitor, KT 5823, prevents RyR2 S2808 phosphorylation in cardiomyocytes stressed in hypotonic solution (Gonano et al., 2014). To determine if pharmacological inhibition of endogenous PKG activity affects SOICR in cells not confounded by external stimuli, we treated our RyR2-expressing HEK293 cells with an equivalent KT 5823 dose (1 μM). Interestingly, KT 5823 inhibition of PKG resulted in a significant decrease in the threshold for SOICR (Vehicle: 90.2 ± 0.5%, KT 5823: 86.6 ± 0.2%, $P = 0.0005$) without changing the luminal Ca^{2+} store size (Vehicle: 0.53 ± 0.03 YFP/CFP, KT 5823: 0.56 ± 0.03 YFP/CFP, $P = 0.5$) (Fig. 3A–C), thereby recapitulating the pro-SOICR effect of PKG activation with 8-Br-cGMP. Also, in line with endogenous PKG activation, inhibition of the kinase with KT 5823 did not change the relative phosphorylation of RyR2 at S2808 ($P = 0.6$) (Fig. 3D + E). These findings suggest that the commonly utilised PKG inhibitor, KT 5823, paradoxically promotes spontaneous Ca^{2+} release through RyR2 without a detectable change in the phosphorylation status of RyR2.

3.4. Endogenous PKG knockdown has no effect on SOICR

Our findings thus far suggest that pharmacological stimulation and inhibition of PKG both promote SOICR. The reason for the analogous effects of the pharmacological manipulations could be related to our recent finding that arrhythmogenic side-effects of therapies comprising class I kinase inhibitors arise due to a direct effect on RyR2 (Chakraborty et al., 2019). Since KT 5823 is a class I kinase inhibitor, it was then pertinent to determine the possibility that this PKG inhibitor promotes SOICR independently of altering PKG activity. To circumvent this potential confounder, we transfected our HEK293 cell model with a PKG-specific siRNA or a scrambled siRNA control. This approach was successful in reducing the expression of endogenous PKG ($P = 0.03$) (Fig. 4A). Endogenous PKG knockdown had no effect on either total RyR2 expression ($P = 0.4$) or basal RyR2 S2808 phosphorylation ($P = 0.9$) (Fig. 4B). However, unlike the pharmacological inhibition of PKG with KT 5823, endogenous PKG knockdown did not reduce the threshold for SOICR (Scramble: 93.7 ± 1.2%, siPKG: 93.1 ± 1.2%, $P = 0.7$), while also not affecting store size (Scramble: 0.47 ± 0.00 YFP/CFP, siPKG: 0.46 ± 0.06 YFP/CFP, $P = 0.9$) (Fig. 4C–E). These results confirm that knockdown of endogenous PKG has no effect on basal RyR2 S2808 phosphorylation or spontaneous RyR2-mediated Ca^{2+} release, suggesting that resting phosphorylation levels at S2808 are not mediated by PKG and that the action of KT 5823 was indeed a pharmacological non-PKG mediated effect.

4. Discussion

This study aimed to determine the effect of endogenous PKG activation on spontaneous RyR2-mediated Ca^{2+} release in recombinant RyR2 expressing HEK293 cells. We show firstly that activation of endogenous PKG promotes SOICR by reducing the threshold for spontaneous Ca^{2+} release. Additionally, we found that this effect depends on expression of the RyR2 phosphorylation sites, S2808 and S2030, which have been described previously as PKG phosphorylation sites on RyR2;

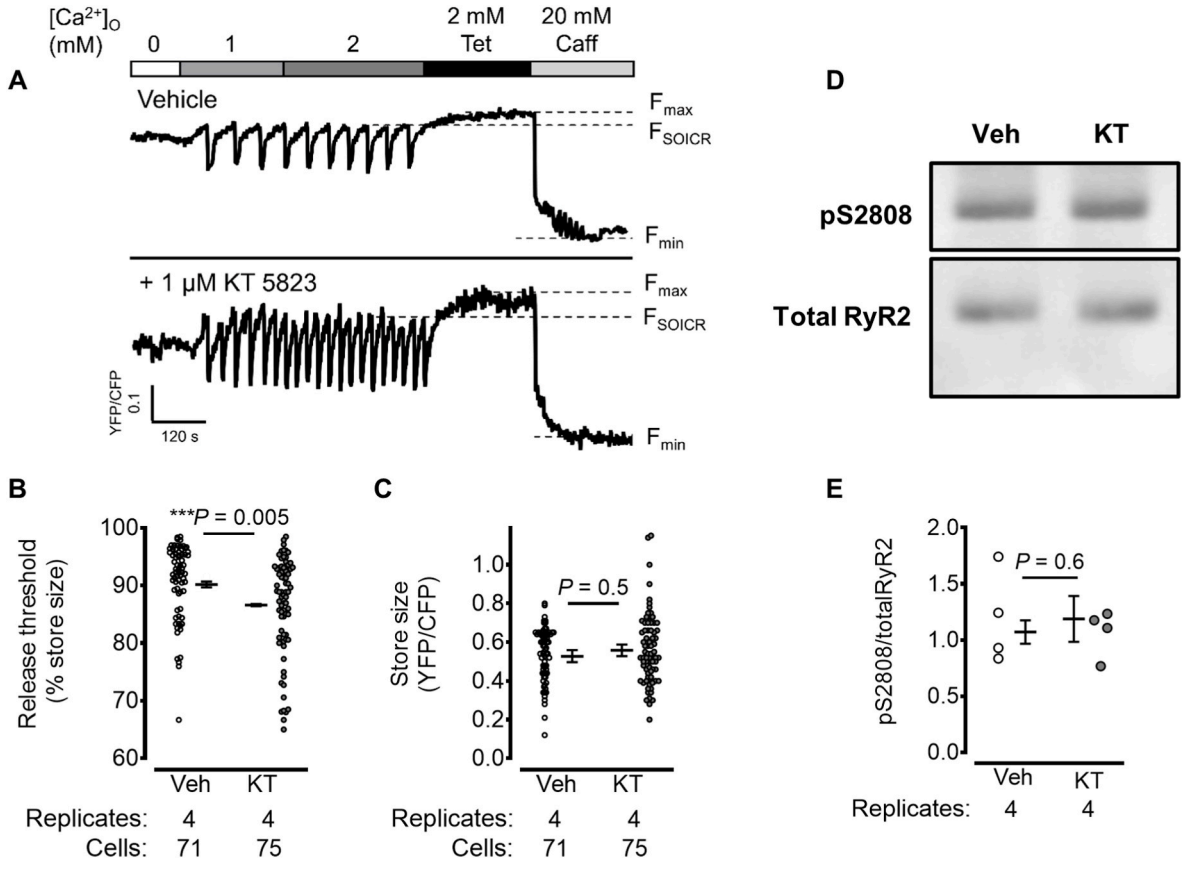

Fig. 3. Effect of PKG inhibition with KT 5823 on store overload-induced Ca^{2+} release (SOICR) and RyR2 S2808 phosphorylation. Human embryonic kidney (HEK293) cells were transfected with luminal Ca^{2+} sensor D1ER and induced to express RyR2 16–18 h prior to experimentation. Cells were treated with PKG inhibitor, KT 5823 (1 μM, 10 min), or equivalent volume DMSO vehicle control before superfusion with increasing extracellular Ca^{2+} concentrations ($[Ca^{2+}]_O$) to stimulate SOICR (representative trace shown in panel **A**). Tetracaine (Tet, 2 mM) and caffeine (Caff, 20 mM) were used to block RyR2 and to deplete the internal Ca^{2+} store, respectively. KT 5823 effects on SOICR threshold and store size and are shown in panels **B** and **C**, respectively. **D**, Lysates were harvested from HEK293 expressing WT RyR2 treated as for A-C and RyR2 phosphorylation at S2808 determined. **E**, Data normalized to total RyR2 expression. For **A-C**, vehicle $N = 4$ replicates (from $n = 71$ cells), KT 5823 $N = 4$ replicates (from $n = 75$ cells). For **E**, vehicle $n = 4$ lysates, KT 5823 $n = 4$ lysates. Differences determined by unpaired *t*-test. *P* values are as indicated in the figure. Data are mean ± SEM of replicates with values from all cells presented alongside.

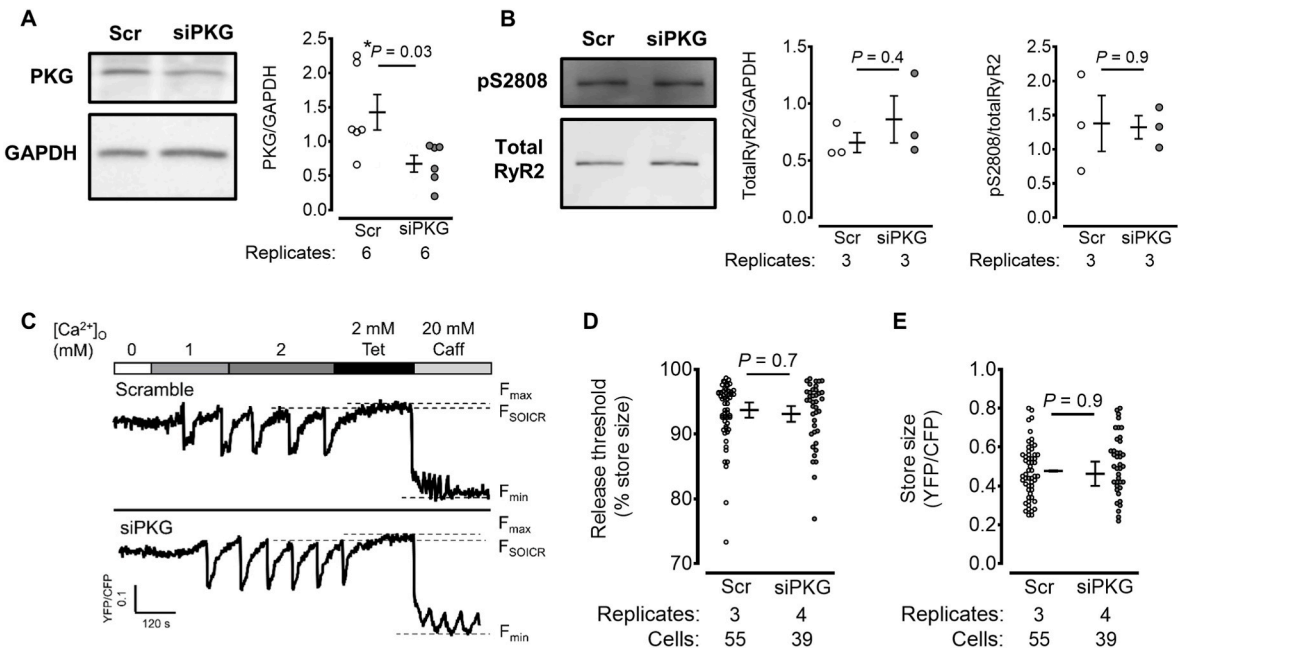

Fig. 4. Effect of endogenous PKG knockdown on RyR2 S2808 phosphorylation and store overload induced Ca^{2+} release (SOICR). RyR2-expressing human embryonic kidney (HEK293) cells were transfected with a silencing RNA targeting endogenous PKG (siPKG) or a scramble control (Scr). Confirmation of PKG knockdown (normalized to GAPDH expression) shown in **A**. **B**, Effect of PKG knockdown on total RyR2 expression (normalized to GAPDH expression) and RyR2 S2808 phosphorylation (normalized to total RyR2 expression). **C**, HEK293 cells with PKG knockdown were transfected with luminal Ca^{2+} sensor D1ER and induced to express RyR2 16–18 h prior to experimentation. Cells were superfused with increasing extracellular Ca^{2+} concentrations ($[Ca^{2+}]_O$) to stimulate SOICR and with tetracaine (Tet, 2 mM) and caffeine (Caff, 20 mM) to block RyR2 and to deplete the internal Ca^{2+} store, respectively (representative trace shown in panel **C**). PKG knockdown effects on SOICR threshold and store size are shown in panels **D** and **E**, respectively. For **A**, scramble and siPKG $n = 6$ lysates each. For **B**, scramble and siPKG $n = 3$ lysates each. For **D** & **E**, scramble $N = 3$ replicates (from $n = 55$ cells), siPKG $N = 4$ replicates (from $n = 39$ cells). Differences were determined by unpaired t-tests. *P* values are as indicated in the figure. Data are mean ± SEM of replicates with values from all cells presented alongside.

although, the effect could not be linked to a detectable change in RyR2 S2808 phosphorylation. Secondly, targeted knockdown of endogenous PKG expression did not affect RyR2 S2808 phosphorylation or RyR2-mediated Ca^{2+} release, suggesting a minimal role for PKG regulation of basal RyR2 phosphorylation and activity. Thirdly, we show that the commonly utilised PKG inhibitor, KT 5823, also promotes SOICR; however, this effect might be due to direct effects on RyR2 independent of PKG modulation. By virtue of the simplicity and absence of confounding stimuli applied to our HEK293 cell model, these findings clarify the direct effect of endogenous PKG activation on spontaneous RyR2 Ca^{2+} release.

4.1. *Activation of endogenous PKG promotes SOICR without a detectable change in RyR2 S2808 phosphorylation*

RyR2 S2808 phosphorylation has been associated with increased RyR2-mediated Ca^{2+} leak in many previous studies (Marx et al., 2000; Wehrens et al., 2006). In cardiomyocytes isolated from patients with chronic arrhythmias, RyR2 S2808 phosphorylation is increased in a manner correlating with enhanced RyR2 open probability and SR Ca^{2+} leak (Voigt et al., 2012). Hyper-phosphorylation of RyR2 at S2808 by PKA has been specifically implicated in triggering RyR2-mediated Ca^{2+} leak in heart failure (Marx et al., 2000; Wehrens et al., 2006); although, the reproducibility of this result is controversial (Eschenhagen, 2010). Outside of disease models, PKA-dependent phosphorylation of RyR2 at S2808 is reported to regulate the luminal Ca^{2+} sensitivity of RyR2 and the propagation velocity of cardiomyocyte Ca^{2+} waves (Ullrich et al., 2012).

Our finding that endogenous PKG activation also reduces the threshold for RyR2 Ca^{2+} release in a manner dependent on S2808, therefore, aligns with these previous investigations (Ullrich et al., 2012). The ~2% decrease in the SOICR threshold induced by endogenous PKG activation is modest relative to the ~5–12% reductions reported in previous studies of RyR2 function (Jones et al., 2008; Chakraborty et al., 2019). However, given that a 5–12% reduction in the SOICR threshold is enough to trigger a large increase in SOICR frequency, Ca^{2+} waves, and is pro-arrhythmic, it is perhaps unsurprising that physiological regulation of RyR2 by PKG results in a more modest change. Indeed, previous studies of pathological modifiers of RyR2, even lethal RyR2 mutations, suggest that only small changes in the threshold for SOICR are required to have dramatic effects on the occurrence of SOICR (Jones et al., 2008; Liu et al., 2013; Chakraborty et al., 2019). It is curious then that the effect observed in our study was not accompanied by an increase in RyR2 phosphorylation at S2808. Previous *in vitro* studies have found that treatment with PKG promotes RyR2 phosphorylation, sometimes markedly, when measured as total phosphorylation or S2808 phosphorylation specifically (Takasago et al., 1991; Hohenegger and Suko, 1993; Xiao et al., 2006). An important distinction to our study is that these previous works treated their RyR2 preparations with cGMP in the presence of an exogenous PKG catalytic unit. Moreover, this form of PKG activation was tested under phosphorylating conditions on either immunoprecipitated RyR2 aliquots (Xiao et al., 2006) or purified RyR2 proteins within cardiac microsomes (Takasago et al., 1991; Hohenegger and Suko, 1993). These conditions would be expected to result in maximal (hyper)-phosphorylation of RyR2 due to the lack of counter acting phosphatases. This contrasts with our HEK293 cell model, in which intact cells were treated with 8-Br-cGMP only. Although these conditions would shift the balance in favour of PKG over phosphatases, it is unlikely to shift it to the same extent as an *in vitro* assay. Additionally, given that the basal level of RyR2 S2808 phosphorylation has been shown to be notably high at rest (Xiao et al., 2006), it is less surprising that an overt change in S2808 phosphorylation was not induced by the method of endogenous PKG activation used in our study. For this reason, it was important to utilise the S2808A RyR2 HEK293 cell model,

which allowed for a more sensitive functional assessment, and subsequent implication, of RyR2 S2808 phosphorylation in mediating the pro-SOICR effects of endogenous PKG activation.

Despite not aligning with the finding of increased S2808 phosphorylation, our results do align with previous *in vitro* studies that the resting level of S2808 phosphorylation by endogenous PKG is minimal (Xiao et al., 2006). Using HEK293 cells transfected with RyR2 cDNA, Xiao et al. demonstrated that treatment with KT 5823 does not change the basal S2808 phosphorylation level (Xiao et al., 2006). Our study replicates this finding using a lower KT 5823 concentration and extends the understanding by showing that knockdown of endogenous PKG also has no effect basal RyR2 phosphorylation at S2808.

4.2. Pro-SOICR effect of endogenous PKG activation also depends on functional S2030 expression

RyR2 is also known to be phosphorylated by PKA at S2030 (Xiao et al., 2005, 2006; Huke and Bers, 2008). One previous study has shown that treatment with exogenous PKG also promotes phosphorylation of immunoprecipitated RyR2 at S2030; although, the level of phosphorylation is notably less than that induced at S2808 (Xiao et al., 2006). Since immunoblot detection of S2030 phosphorylation is now unreliable (Huke and Bers, 2008), we utilised a HEK293 cell expressing RyR2 with a mutation at S2030 (S2030A) to assess the functional importance of the site. Interestingly, we found that mutation of S2030 prevents the effect of 8-Br-cGMP, suggesting that both S2808 and S2030 modification are required to mediate the pro-SOICR effect of endogenous PKG activation. Since no effect of PKG activation on SOICR was observed with either individual S2808 or S2030 mutation, our data also suggest that the CaMKII-specific phosphorylation site, S2814 (Wehrens et al., 2004; Huke and Bers, 2008), does not play a role in mediating the pro-SOICR effect of PKG. This is perhaps not surprising given that PKG shares consensus phosphorylation sites with PKA (S2808 and S2030) and not with sites associated with phosphorylation by CaMKII (Xiao et al., 2006). However, given that the S2814 site on RyR2 was not individually investigated here, we cannot conclusively rule out a role for this phosphorylation site in mediating the effects of PKG on RyR2 function. Overall, our study suggests that expression of RyR2 phosphorylation sites S2808 and S2030 is required to mediate the effects of endogenous PKG activation; although further work is required to determine why the functional effect observed was not accompanied by a measurable change in RyR2 phosphorylation at these sites using western blotting.

4.3. Effect of KT 5823 on RyR2-mediated Ca^{2+} release

A curious finding of our study was that the PKG inhibitor, KT 5823, had a similar effect to PKG activation in that it promoted SOICR. KT 5823 has been used in previous studies to show that changes in cardiac contractility and Ca^{2+} metabolism induced by 8-Br-cGMP can be prevented if the cardiac myocytes are pre-treated with the inhibitor (Shah et al., 1994; Vila-Petroff et al., 1999; Gonano et al., 2014). Our own work shows that KT 5823 pre-treatment prevents RyR2 phosphorylation and contractile changes induced by hypotonic myocyte swelling, suggesting a role for enhanced PKG-mediated RyR2 Ca^{2+} sensitivity as a response to cell swelling (Gonano et al., 2014). Recently, using our RyR2-expressing HEK293 cell model, we showed that class I kinase inhibitors, of which KT 5823 qualifies, promote SOICR by directly increasing the luminal Ca^{2+} sensitivity and the open probability of RyR2 (Chakraborty et al., 2019). Furthermore, these effects occurred without alterations in RyR2 phosphorylation status (Chakraborty et al., 2019). When combined with the absence of RyR2 S2808 phosphorylation and SOICR response to endogenous PKG knockdown (Fig. 4), the resemblance of the KT 5823 effect to that of other class I kinase inhibitors might suggest that the drug acts directly on RyR2 to promote SOICR alongside any modulation of PKG activity. Further study is required to elucidate the direct effects of KT 5823 on RyR2 function and RyR2 phosphorylation at other sites; however, our exploratory finding does question the efficacy of using KT 5823 to investigate the role of PKG in cardiac Ca^{2+} handling.

4.4. PKG phosphorylation of PLB

A key advantage of the HEK293 cell model is the absence of endogenous phospholamban (PLB) expression. This allows the dissection of the impact of PKG activity on RyR2 without altering luminal store Ca^{2+}. In cardiomyocytes, phosphorylation of PLB has been shown to be necessary to promote the SR Ca^{2+} overload required for spontaneous SR Ca^{2+} release, regardless of the RyR2 phosphorylation status (Li et al., 2002). Since RyR2 is intrinsically sensitive to luminal Ca^{2+} (Jones et al., 2017), the expression of PLB can directly modulate the Ca^{2+} store level and thus the threshold for SOICR. Previous findings in our RyR2-expressing HEK293 cell model support this, as an enhancement in luminal Ca^{2+} uptake and impairment in Ca^{2+} buffering promotes SOICR via an increased SR Ca^{2+} load (Zhang et al., 2014). PKG activation using 8-Br-cGMP or NO donation is also known to promote phosphorylation of PLB in cardiomyocytes and aortic smooth muscle cells (Cornwell et al., 1991; Sabine et al., 1995; Sulakhe and Vo, 1995). Furthermore, phosphorylation of PLB has been associated with an increase in SR Ca^{2+} uptake rate, suggesting a role of PKG in mediating SR Ca^{2+}-ATPase activity and luminal Ca^{2+} content (Cornwell et al., 1991). We found that PKG stimulation reduces the SOICR threshold despite the absence of native PLB expression. Therefore, although our cell model allows us to dissect the effect on PKG on the function of RyR2, it is possible that the complete effects of increased PKG activity on SOICR were underestimated in our study.

4.5. Conclusion

In conclusion, activation of endogenous PKG promotes SOICR by reducing the threshold for spontaneous RyR2-mediated Ca^{2+} release in a manner dependent on the presence of known RyR2 phosphorylation sites, S2808, S2030, and S2814. Conversely, knockdown of endogenous PKG expression has no effect on basal RyR2-mediated Ca^{2+} release or RyR2 phosphorylation status, while pharmacological PKG inhibition with KT 5823 paradoxically promotes SOCIR; although, this effect is likely due to direct RyR2 alteration independent of PKG inhibition. This study clarifies the role of PKG in the regulation of RyR2-mediated Ca^{2+} release in a non-stressed cell model.

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CRediT authorship contribution statement

Luis A. Gonano: Conceived and designed experiments, conducted experiments, Formal analysis, result interpretation, Writing – original draft, Writing – review & editing. **Hamish M. Aitken-Buck:** Conducted experiments, Formal analysis, result interpretation, prepared the figures, Writing – original draft, Writing – review & editing. **Akash D. Chakraborty:** Conducted experiments, Formal analysis, Writing – review & editing. **Luke P.I. Worthington:** Conducted experiments, Formal analysis, Writing – original draft, Writing – review & editing. **Tanya R. Cully:** Contributed to experiment design, result interpretation, Writing – review & editing. **Regis R. Lamberts:** Result interpretation, Writing – review & editing. **Martin G. Vila-Petroff:** Result interpretation, Writing – review & editing. **Peter P. Jones:** Conceived

and designed experiments, Formal analysis, result interpretation, Writing – original draft, Writing – review & editing, All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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