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Divergent off-target effects of RSK N-terminal and C-terminal kinase inhibitors in cardiac myocytes

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ABSTRACT

P90 ribosomal S6 kinases (RSK) are ubiquitously expressed and regulate responses to neurohumoral stimulation. To study the role of RSK signalling on cardiac myocyte function and protein phosphorylation, pharmacological RSK inhibitors were tested. Here, the ATP competitive N-terminal kinase domain-targeting compounds D1870 and SL0101 and the allosteric C-terminal kinase domain-targeting FMK were evaluated regarding their ability to modulate cardiac myocyte protein phosphorylation. Exposure to D1870 and SL0101 significantly enhanced phospholamban (PLN) Ser16 and cardiac troponin I (cTnI) Ser22/23 phosphorylation in response to D1870 and SL0101 upon exposure to phenylephrine (PE) that activates RSK. In contrast, FMK pretreatment significantly reduced phosphorylation of both proteins in response to PE. D1870-mediated enhancement of PLN Ser16 phosphorylation was also observed after exposure to isoprenaline or noradrenaline (NA) stimuli that do not activate RSK. Inhibition of β-adrenoceptors by atenolol or cAMP-dependent protein kinase (PKA) by H89 prevented the D1870-mediated increase in PLN phosphorylation, suggesting that PKA is the kinase responsible for the observed phosphorylation. Assessment of changes in cAMP formation by FRET measurements revealed increased cAMP formation in vicinity to PLN after exposure to D1870 and SL0101. D1870 inhibited phosphodiesterase activity similarly as established PDE inhibitors rolipram or 3-isobutyl-1-methylxanthine. Assessment of catecholamine-mediated force development in rat ventricular muscle strips revealed significantly reduced EC_{50} for NA after D1870 pretreatment (DMSO/NA: 2.33 μmol/L vs. D1870/NA: 1.30 μmol/L). The data reveal enhanced cardiac protein phosphorylation by D1870 and SL0101 that was not detectable in response to FMK. This disparate effect might be attributed to off-target inhibition of PDEs with impact on muscle function as demonstrated for D1870.

Abbreviations: AR, Adrenergic receptor; ARVM, Adult rat ventricular myocyte; D1870, BI-D1870; CAMK, Calcium/calmodulin-dependent protein kinase; cMyBP-C, Cardiac myosin-binding protein C; CTKD, C-terminal kinase domain; cTnI, Inhibitory subunit of cardiac troponin; DMSO, Dimethylsulfoxide; ECC, Excitation contraction coupling; ECL, Enhanced chemiluminescence; ET-1, Endothelin 1; ERK, Extracellular signal-regulated kinase ½; FRET, Förster resonance energy transfer; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GPCR, G protein-coupled receptors; IB, Immunoblotting; IBMX, 3-isobutyl-1-methylxanthine; IP, Immunoprecipitation; ISO, Isoprenaline; KO, knockout; mTORC1, Mammalian target of rapamycin complex 1; NA, Noradrenaline; NHE1, Na^+/H^+ exchanger 1; NTKD, N-terminal kinase domain; p70 S6K, p70 ribosomal S6 kinase; PDE, Phosphodiesterase; PE, Phenylephrine; PKA, cAMP-dependent protein kinase; PKA RIα, PKA regulatory subunit Iα; PLN, Phospholamban; PLM, Phospholemman; RSK, p90 ribosomal S6 kinase; SDS, Sodium dodecyl sulfate; SERCA, Sarcoplasmic reticulum Ca^{2+}-ATPase; SR, Sarcoplasmic reticulum

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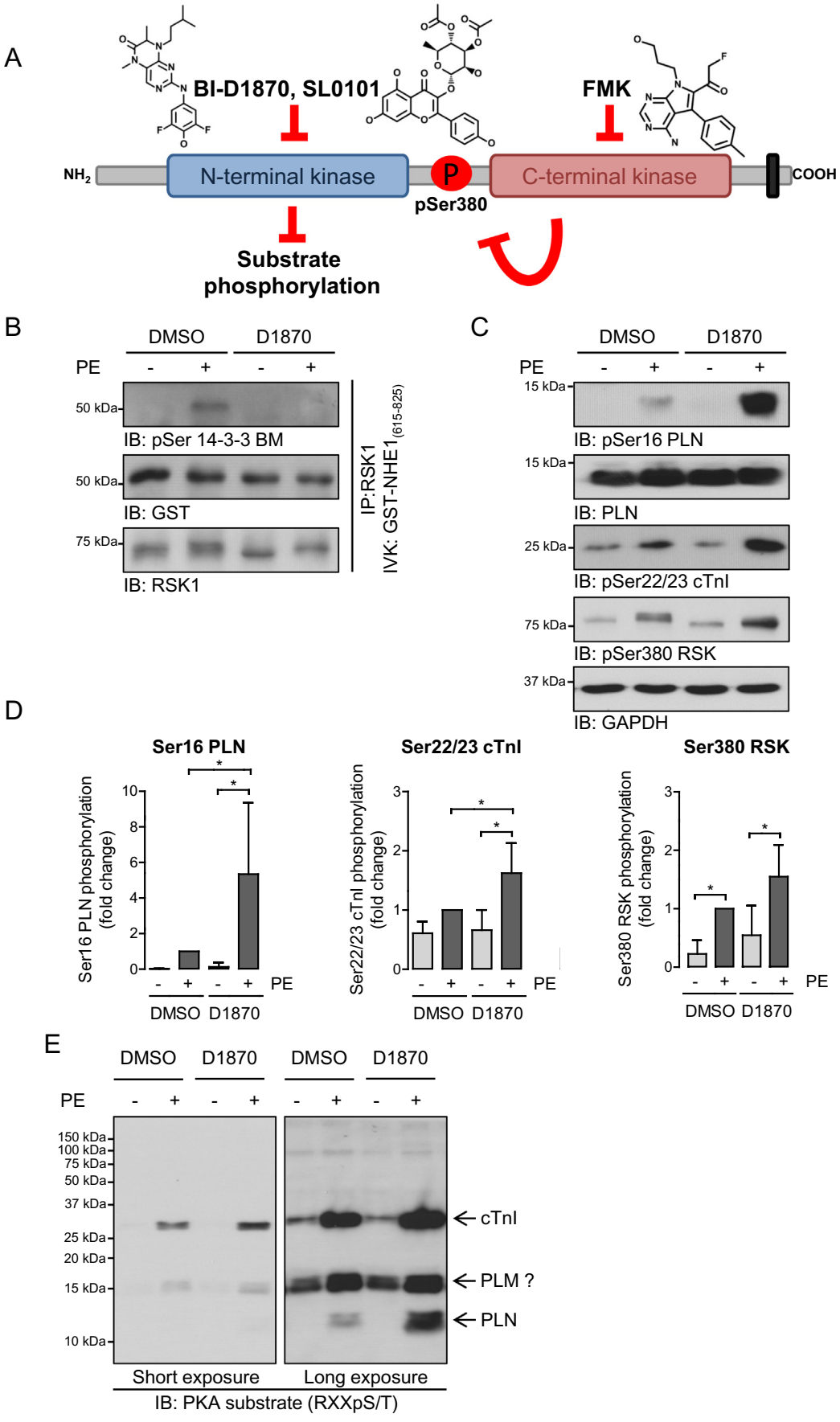

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Fig. 1. Cardiac myocyte protein phosphorylation in response to phenylephrine in ARVMs pretreated with the pharmacological RSK NTKD inhibitor D1870.
(A) Scheme highlighting the RSK structure with the N-terminal and C-terminal kinase domain, the RSK autophosphorylation site at Ser380. D1870 and SL0101 compete with ATP-binding to the N-terminal kinase domain and FMK binds to the C-terminal kinase domain and prevents autophosphorylation and N-terminal kinase activation. (B) Immunoprecipitated RSK1 was used in IVK reactions to phosphorylate recombinant GST-NHE1$_{(615–825)}$. Substrate phosphorylation and loading as well as immunoprecipitated RSK1 were identified by immunoblotting (IB) using anti-phospho-Ser 14-3-3 binding motif (BM), anti-GST or anti-RSK1 antibodies, respectively. (C) ARVMs were pretreated for 1 h with vehicle (DMSO) or 10 μmol/L D1870 and then exposed for 10 min with vehicle (PBS) or 10 μmol/L phenylephrine (PE). Phosphorylation of PLN (pSer16), cTnI (pSer22/23) and RSK (pSer380) was analysed by IB using the phosphospecific antibodies. PLN expression was detected by a total PLN antibody. Protein loading was confirmed by an anti-GAPDH antibody. (D) Bar charts summarize data from 5 to 7 independent experiments. Data were normalized to protein loading and expressed as fold change over DMSO + PE. Results are shown as mean ± S.D. $^{*}P < .05$. (E) Immunoblot analysis of samples using an anti-phospho-Ser/Thr PKA substrate (RXXS/T) antibody. A shorter and a longer exposure of the same representative immunoblot are shown. IP: immunoprecipitation.

1. Introduction

The p90 ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases comprising four isoforms RSK1, RSK2, RSK3 and the atypical longer isoform RSK4 [1,2]. RSKs share a high degree of sequence homology. They contain two distinct functional kinase domains connected by a linker region. The C-terminal kinase domain (CTKD) belongs to the family of calcium/calmodulin-dependent protein kinases (CAMK) and is responsible for RSK autophosphorylation and activation [1,2]. The N-terminal kinase domain (NTKD) belongs to the AGC group of kinases and is responsible for substrate phosphorylation [1,2] (Fig. 1A).

RSKs are activated by the extracellular signal-regulated kinases 1/2 (ERK) and mediate cellular responses to growth and neurohumoral stimulation [1,2]. The biological functions of RSKs include the regulation of transcription [3,4], cell survival [5], protein synthesis [6], cell cycle progression and cell proliferation [7]. RSK expression and activity are increased in cancer where pharmacological inhibition is considered a promising therapeutic strategy [8,9]. However, adverse side effects of pharmacological inhibition of this ubiquitously expressed kinase have not been investigated yet. In the heart, the identification of RSK substrates has only just started to emerge and it has been already shown that RSKs contribute to the regulation of important cardiac functions. In this respect, a large body of evidence has demonstrated the important role of the kinases in the regulation of ion transportation and cellular pH, through phosphorylation of the sarcolemmal Na^+/H^+ exchanger 1 (NHE1) [10–12]. Additionally, a role for RSKs in the regulation of acute contractile function by phosphorylation of cardiac proteins involved in excitation-contraction coupling (ECC) such as cardiac myosin-binding protein C (cMyBP-C) [13] and the inhibitory subunit of cardiac troponin (cTnI) [14] has also been described. Nevertheless, many aspects of the impact of RSK activity on cardiac physiology and pathophysiology, the identification of novel cardiac RSK substrates and the consequences of therapeutic pharmacological RSK inhibition for unperturbed cardiac function remain elusive.

This study performs a detailed characterization of the ATP-competitive inhibitors of the RSK NTKD, D1870 and SL0101 as well as the allosteric RSK CTKD inhibitor FMK [15–17] (Fig. 1A) on the phosphorylation status of proteins that regulate ECC in cardiac myocytes. Among these proteins are the sarcoplasmic reticulum (SR) protein phospholamban (PLN) and the sarcomeric protein cTnI, which are key determinants of cardiac relaxation and inotropy [18,19]. PLN is an endogenous inhibitor of the SR Ca^{2+}-ATPase (SERCA), which is responsible for the transport of cytosolic Ca^{2+} into the SR at the end of each contraction cycle to achieve diastolic relaxation [18,19]. PLN phosphorylation at Ser16 relieves its inhibitory effect and thus enables increased SERCA activity [18,19]. The sarcomeric protein cTnI regulates Ca^{2+}-dependent actin-myosin interaction and sarcomere shortening. The phosphorylation of cTnI at Ser22/23 decreases myofilament Ca^{2+}-sensitivity and increases the rate of crossbridge cycling [20–22]. These phosphorylation events are mainly mediated by cAMP-dependent protein kinase (PKA) in response to β-adrenoceptor (β-AR) stimulation by endogenous catecholamines. Interestingly, the aforementioned phosphorylation sites also match the RSK consensus phosphorylation motif (RXRXXS/T or RRXS/T; X: any amino acid) [2,23]. Therefore, it is possible that RSK activity could also impact on fine tuning ECC in response to AR stimulation by phosphorylating cardiac proteins involved in this process. Unexpectedly, our data reveal disparate effects on the cardiac protein phosphorylation pattern induced by the two different classes of RSK inhibitors. The most striking observation was the potentiation of β_1-AR-mediated PLN phosphorylation at Ser16 by both of the herein tested RSK NTKD inhibitors. This is important as SL0101 at least has been considered as an anti-cancer therapy to treat patients with some type of breast cancer [8]. As a molecular mechanism of this observation, we postulate that enhancement of cardiac protein phosphorylation following pharmacological RSK inhibition by ATP-competitive compounds might occur through off-target inhibition of cardiac phosphodiesterase (PDE) isoforms, leading to a localized increase in cAMP levels that translates functionally into a significant sensitization of catecholamine inotropy of cardiac muscle to endogenous catecholamines, which has not been considered before.

2. Experimental

2.1. Materials

BI-D1870 (D1870) was from Selleckchem (Houston, TX, USA). SL0101-1 was from Cayman Chemical (Ann Arbor, MI, USA). FMK was from Axon Medchem (Groningen, The Netherlands). Phenylephrine (PE), isoprenaline (ISO), noradrenaline (NA), prazosin, atenolol, NKH 477, rolipram and cilostamide were from Sigma-Aldrich (Taufkirchen, Germany). 3-isobutyl-1-methylxanthine (IBMX) and Protein A/G PLUS-agarose were purchased from Santa Cruz Biotechnologies (Dallas TX, USA). We used the following antibodies against: phospho-Ser22/23 cTnI, phospho-Thr202/Tyr204 ERK, phospho-(Ser/Thr) PKA substrate and phospho-Ser 14-3-3 binding motif (#4004, #9101, #9621 and #9606 respectively; Cell Signalling Technology; Leiden, The Netherlands), phospho-Ser16 PLN (#A010-12AP; Badrilla; Leeds, UK), total PLN (#NBP2-19807; Novus Biologicals; Abingdon, UK), phospho-Ser380 RSK (#ab32203; Abcam; Cambridge, UK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #5G4; HyTest; Turku, Finland), calsequestrin (#PA1-913; ThermoScientific; Rockford, IL, USA), RSK1 (#sc-231; Santa Cruz Biotechnology). The enhanced chemiluminescence (ECL) kit, Hyperfilm, polyvinylidene fluoride and nitrocellulose membranes were obtained from GE Healthcare (Buckinghamshire, UK).

2.2. Animals

The study was conducted in accordance with the guidelines for the care and use of laboratory animals as issued by the National Institutes of Health (Publication no. 85-23, revised 1985). Experimental procedures conformed to the German Law for the Protection of Animals. Adult rat ventricular myocytes (ARVMs) were isolated in the Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf from 160 to 240 g male Wistar rats (Charles River Laboratories). The generation of transgenic mice expressing the cAMP Förster resonance energy transfer (FRET) biosensors has been described previously [24,25]. Transgenic *RPS6KA3*-targeted knockout (RSK2 KO)

mice expressing the Epac1-PLN FRET sensor were generated by crossing RSK2 KO mice (described previously in [26,27]) with mice expressing the respective sensor. Adult mouse ventricular myocytes were isolated from transgenic mice in the Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf.

2.3. ARVM isolation and culture

ARVMs were isolated according to the procedure described by Pohlmann et al. [28]. Isolated ARVMs were plated on laminin-coated 6-well plates and cultured for 24 h in modified M199 culture medium (mM199; M199 supplemented with (in mmol/L): taurine 5, creatine 2, L-carnitine 2 and 100 IU/mL penicillin/streptomycin) [13] before pharmacological treatment.

2.4. Pharmacological treatment of ARVM

ARVMs were pretreated with vehicle (dimethylsulfoxide, DMSO) or the RSK inhibitors D1870 (10 μmol/L; 1 h pretreatment), SL0101 (10 μmol/L; 1 h pretreatment) or FMK (3 μmol/L; 1.5 h pretreatment) before stimulation for 10 min with PE (10 μmol/L), ISO (10 nmol/L) or NA (10 nmol/L). When experiments required incubation with the β_1-AR antagonist atenolol (1 μmol/L) or the α_1-AR antagonist prazosin (1 μmol/L), these compounds were added 10 min before stimulation with PE. To inhibit PDE isoforms, ARVMs were incubated 10 min before application of PE with either rolipram (PDE4 inhibitor; 10 μmol/L), cilostamide (PDE3 inhibitor; 10 μmol/L), a combination of inhibitors (D1870 and rolipram; D1870 and cilostamide; rolipram and cilostamide) or IBMX (non-selective PDE inhibitor; 100 μmol/L). After completion of treatments, medium was removed and cells were lysed in 1 × Laemmli sample buffer (62.5 mmol/L Tris-HCl pH 6.8, 2% (*w/v*) sodium dodecyl sulfate (SDS), 10% (*v*/v) glycerol, 3% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue). Samples were then used for SDS-polyacrylamide gel electrophoresis and immunoblot analysis.

2.5. Immunoprecipitation

After treatment, ARVMs were lysed by addition of 250 μL/well lysis/immunoprecipitation buffer (containing in mmol/L: Tris-HCl 50 pH 7.4, EDTA 2, EGTA 2, DTT 2, NaF 2, 1% (*v*/v) Triton X-100 and Complete protease inhibitors). For each intervention, lysates from three wells were pooled together, incubated on ice for 20 min with occasional vortexing and then centrifuged for 10 min at 14,000 × *g*, 4 °C. 450 μL from each cleared lysate were then incubated overnight with 3 μL of anti-RSK1 antibody, at 4 °C and under rotation. This was followed by 1 h incubation at 4 °C with 30 μL protein A/G PLUS-agarose. After centrifugation for 1.5 min at 1000 × *g*, 4 °C, supernatant was removed and beads were washed 4 times with 700 μL lysis buffer. Immunoprecipitated proteins were finally extracted with 80 μL 3 × Laemmli sample buffer, boiled at 95 °C and stored at −20 °C until use.

2.6. In vitro kinase assay

In vitro kinase (IVK) assays to assess RSK activity were performed as described previously [11] using recombinant GST-NHE1$_{(615–825)}$ as substrate. Briefly, RSK was immunoprecipitated from ARVM lysates as presented above. Beads were then washed three times with immunoprecipitation buffer followed by three washes with IVK buffer (mmol/L: Tris-HCl 30 pH 7.4, $MgCl_2$ 15, DTT 1). The immunocomplex was subsequently incubated with 100 pmol GST-NHE1$_{(615–825)}$ and 0.1 mmol/L ATP in 50 μL final volume (in IVK buffer) at 37 °C for 15 min. The assay was stopped by adding 75 μL 3 × Laemmli sample buffer. Substrate phosphorylation was analysed by immunoblotting using a phospho-Ser 14-3-3 binding motif antibody which recognizes RSK-phosphorylated sites in the NHE1 regulatory domain [11,29].

2.7. Immunoblot analysis

Immunoblot analysis was performed as described previously [30] using antibodies against total or phosphorylated forms of the proteins of interest as indicated. Specific protein bands were detected by ECL and band density quantification was performed using GelQuant.NET software provided by *biochemlabsolutions.com*. In each experiment, data were normalized to total protein loading as estimated by immunoblot analysis using anti-GAPDH or anti-calsequestrin antibodies.

2.8. Assessment of intracellular cAMP levels by FRET technology

FRET measurements were performed in adult mouse ventricular myocytes (AMVMs) from transgenic mice expressing cAMP FRET biosensors either targeted to the cytosol (Epac1-camps [24]) or to the SR (Epac1-PLN [25]). AMVMs were freshly isolated by Langendorff perfusion and enzymatic digestion. Cardiac myocytes were then plated onto laminin-coated 25-mm cover glasses and FRET responses assessed as described previously [31]. In brief, after establishment of a stable baseline, AMVMs were exposed to the RSK inhibitors D1870 (10 μmol/L) or SL0101 (10 μmol/L) followed by ISO (100 nmol/L) or a combination of forskolin (FOR; 5 μmol/L)/IBMX (100 μmol/L) to elicit a maximal FRET response. FRET changes were recorded continuously.

Whole organ FRET measurements were performed in hearts isolated from transgenic WT and RSK2 KO animals expressing the cAMP FRET sensor Epac1-PLN as described previously [32,33]. In brief, hearts were excised and Langendorff-perfused at 37 °C with a buffer consisting of (in mmol/L) 118 NaCl, 4.7 KCL, 0.8 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 5.0 Glucose, 110 Na-Pyruvate and 2.5 $CaCl_2$ at pH 7.4. After an equilibration phase of 10 min, changes in the FRET ratio during perfusion with PE (10 μmol/L) followed by the adenylate cyclase activator NKH 477 (10 μmol/L) for a maximal response were recorded. At the end of each experiment, hearts were collected and frozen in liquid N_2. The hearts were then pulverized and homogenised in a buffer consisting of 100 mmol/L Tris pH 7.4, 1% (*v*/v) Triton X-100 and Complete protease inhibitors. The homogenates were analysed by western immunoblot analysis. FRET data presented reflect the FRET changes measured in the left ventricle.

Changes in local cAMP levels in PDE vicinity in response to D1870 treatment were monitored in HEK293A cells using the FRET biosensor Epac1-camps-PDE4A1, which contains a genetic construct encoding PDE4A1 fused to a cAMP sensor [34]. Approximately 5000 cells were plated onto 25-mm glass coverslip and allowed to attach for 24 h in Dulbecco's Modified Eagle's Medium (containing 10% fetal calf serum, 100 U/mL of penicillin/streptomycin) at 37 °C and 4% CO_2. Cells were then transfected with 1 μg of Epac1-camps-PDE4A1 FRET construct plasmid DNA using Lipofectamine® 2000 according to the supplier's instructions (Invitrogen, #11668-019). After 48 h, cells were used for FRET measurements as described before [34]. In brief, cells were exposed to D1870 (10 μmol/L) and then FOR (5 μmol/L)/IBMX (100 μmol/L) or were exposed to ISO (1 μmol/L) followed by treatment with D1870 (10 μmol/L), followed by the PDE4 inhibitor rolipram (10 μmol/L). Alterations in cAMP levels in vicinity to PDE4A1 were determined by changes in the FRET ratio. FRET responses were detected and analysed using the free software MicroManager 1.4 (https://micro-manager.org/). Representative time control FRET traces in HEK293A cells and adult mouse ventricular myocytes are shown in Supplemental Fig. 1.

2.9. Assessment of PDE activity

Freshly isolated AMVMs were pretreated with increasing concentrations of D1870 (0.1; 1; 10 μmol/L), 10 μmol/L rolipram (PDE4) or 100 μmol/L IBMX (unselective), lysed and used for in vitro PDE activity analysis as described previously in the presence of 1 μmol/L cAMP [35–37].

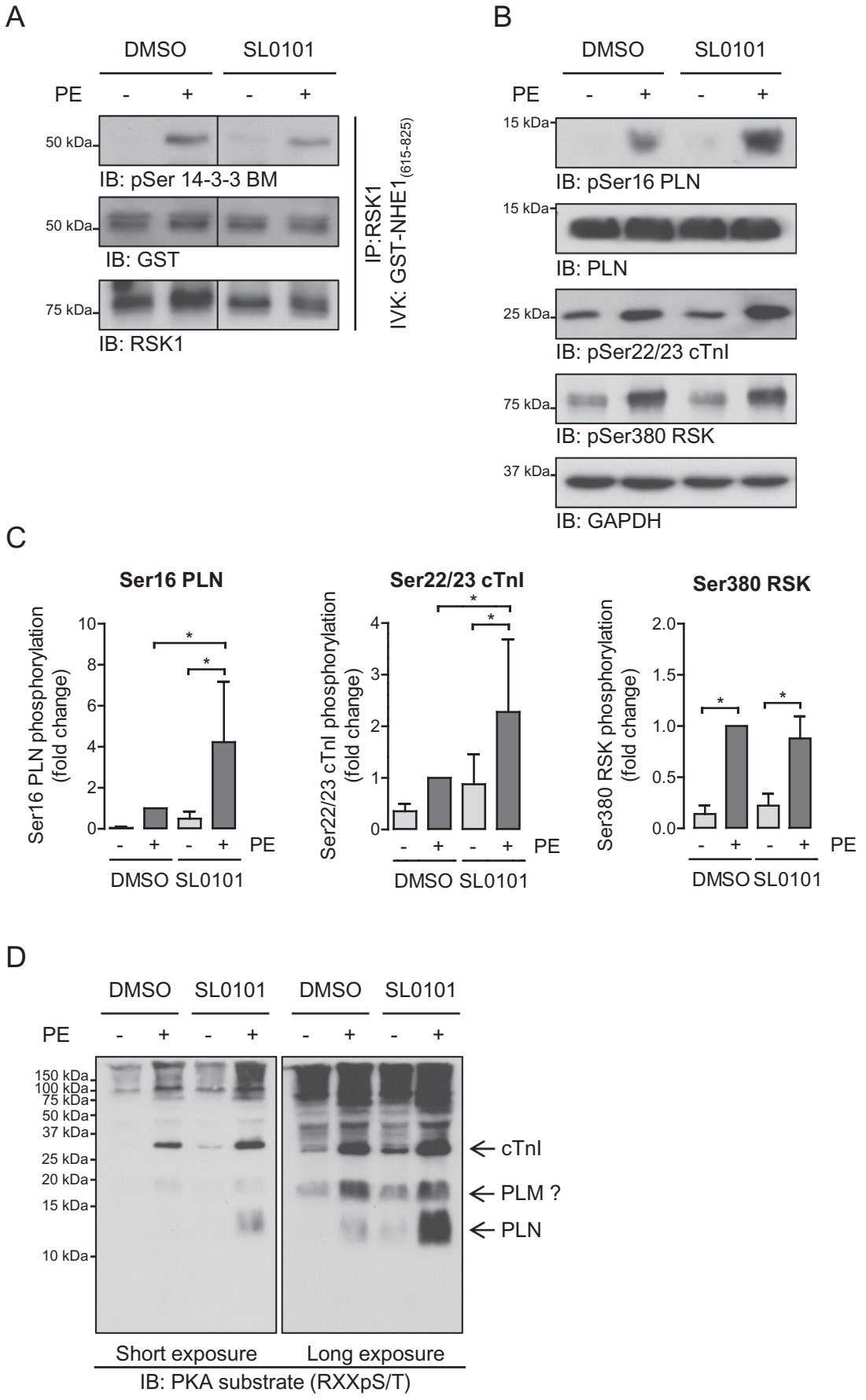

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Fig. 2. Cardiac myocyte protein phosphorylation in response to phenylephrine in ARVMs pretreated with the pharmacological RSK NTKD inhibitor SL0101.
(A) Immunoprecipitated RSK1 was used in IVK reactions to phosphorylate recombinant GST-NHE1$_{(615–825)}$. Substrate phosphorylation and content as well as immunoprecipitated RSK1 were identified by immunoblot (IB) analysis using anti-phospho-Ser 14-3-3 binding motif (BM), anti-GST or anti-RSK1 antibodies, respectively. (B) ARVMs were incubated for 1 h with vehicle (DMSO) or SL0101 (10 μmol/L) and then treated for 10 min with vehicle (PBS) or 10 μmol/L phenylephrine (PE). IB of PLN (total and pSer16), cTnI (pSer22/23) and RSK (pSer380) phosphorylation was performed using the indicated total or phospho-specific antibodies. Protein loading was confirmed by an anti-GAPDH antibody. (C) Bar charts summarize data from 5 to 7 independent experiments. Data were normalized to protein loading and expressed as fold change over DMSO + PE. Results are shown as mean ± S.D. $^{*}P < .05$. (D) Immunoblot analysis of samples using an anti-phospho-Ser/Thr PKA substrate (RXXS/T) antibody. A shorter and a longer exposure of a representative immunoblot are shown. IP: immunoprecipitation.

2.10. Force measurements in rat ventricular muscle strips

Rat ventricular cardiac muscle strips were equilibrated in Tyrode's solution (in mmol/L at pH 7.4 at 5% CO_2/95% O_2: NaCl 126.9, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 22, NaH_2PO_4 0.45, glucose 5.0), pretreated with vehicle (DMSO) or D1870 (10 μmol/L; 1 h) and exposed to cumulatively increasing concentrations of NA (3 nmol/L–100 μmol/L) into the organ bath apparatus. Graphical representation shows concentration-effect curves of NA in the absence ($n = 18$; open signs) or presence of D1870 ($n = 17$; closed signs). Maximum force production was achieved by exposure to 8 mmol/L $CaCl_2$. The NA concentration producing half maximum response (EC_{50}) and the number of strips per condition are indicated in the graphs.

2.11. Statistical analysis

All data were analysed by GraphPad Prism 5 software (GraphPad Software Inc. San Diego, CA, USA) apart from the data concerning FRET measurements, which were analysed by Origin (OriginLab Corporation, Northampton, MA, USA). Data from the immunoblot analysis performed from ARVM lysates were statistically compared by one-way ANOVA following Bonferroni's post-hoc test. Student's *t*-test was used to analyse data obtained from the immunoblot analysis of heart homogenates and to compare changes in cAMP levels in FRET measurements and EC_{50} in force measurements. Quantitative data are given as mean ± S.D. and $P < .05$ was considered as significant.

3. Results

As a prerequisite to study the impact of pharmacological RSK inhibition on cardiac myocyte protein phosphorylation, successful inhibition of RSK NTKD activity by D1870 was confirmed in IVK assays using RSK immunoprecipitated from ARVM samples with GST-NHE1$_{(615–825)}$ as a substrate. Pretreatment of cardiac myocytes with the ATP-competitive RSK NTKD inhibitor D1870 (10 μmol/L; 1 h) prior to RSK activation by phenylephrine (PE) for 10 min (10 μmol/L) inhibited RSK NTKD activity as reflected by abolished substrate phosphorylation detected with a pSer14-3-3 antibody (Fig. 1B). Substrate content in each sample and immunoprecipitation of RSK was demonstrated by immunoblotting for GST and RSK, respectively.

To investigate the contribution of RSK inhibition on the phosphorylation pattern of proteins involved in the regulation of cardiac myocyte ECC, phosphorylation of the SR protein PLN at Ser16 and phosphorylation of the sarcomeric protein cTnI at Ser22/23 were analysed in ARVMs pretreated with D1870 (10 μmol/L; 1 h) and stimulated for 10 min with PE (10 μmol/L). Unexpectedly, compared to incubation with vehicle (DMSO), treatment with D1870 significantly enhanced the PE-induced PLN and cTnI phosphorylation (fold change D1870 + PE vs DMSO + PE: 5.34 ± 4.01 for PLN and 1.62 ± 0.50 for cTnI; Fig. 1C, D). Importantly, there was a trend towards increased autophosphorylation of RSK by the RSK CTKD at Ser380 detectable after pretreatment with D1870, which has been described previously [13] and reflects feedback activation of ERK1/2.

Similar results were observed, when immunoblot analysis was performed with a phospho-(PKA substrate (RXXpS/T) antibody, which recognizes prototypical phosphorylated PKA substrate proteins that contain a phospho-(Ser/Thr) residue with arginine in −3 position (Fig. 1E). Here, increased PE-induced phosphorylation of proteins in response to D1870 pretreatment was detectable migrating at a molecular mass corresponding to PLN at approximately 12 kDa and cTnI at approximately 29 kDa, respectively.

To investigate whether the unexpected potentiation of protein phosphorylation of PLB Ser16 and cTnI at Ser22/23 in response to D1870 is a unique attribute to this compound or a general trait of ATP-competitive RSK inhibitors, experiments were performed using a second, structurally distinct RSK NTKD inhibitor, namely SL0101. Inhibition of RSK NTKD activity by SL0101 was assessed as shown previously in IVK assays using RSK1 immunoprecipitated from ARVMs with GST-NHE1$_{(615–825)}$ as a substrate. Pretreatment of cardiac myocytes with the ATP-competitive RSK NTKD inhibitor SL0101 (10 μmol/L; 1 h) prior to RSK activation by PE for 10 min (10 μmol/L) diminished but did not abolish RSK-mediated substrate phosphorylation detected with a pSer14–3-3 antibody (Fig. 2A). Substrate content in each sample and successful immunoprecipitation of RSK was demonstrated by immunoblotting for GST and RSK, respectively. Importantly, investigation of the cardiac myocyte phosphorylation pattern in response to SL0101 pretreatment revealed comparable enhancement of PE-mediated PLN (fold change SL0101 + PE vs DMSO + PE: 4.23 ± 2.95) and cTnI (fold change SL0101 + PE vs DMSO + PE: 2.28 ± 1.40) phosphorylation as observed with D1870 (Fig. 2B, C). Notably, autophosphorylation of RSK by the RSK CTKD at Ser380 was unaffected by pretreatment with SL0101.

Congruent with the observations made with phosphospecific antibodies, immunoblotting with the phospho-PKA substrate (RXXpS/T) antibody confirmed enhanced phosphorylation of proteins migrating at a molecular mass likely representing PLN and cTnI (Fig. 2D). Interestingly and different to the results obtained with D1870, a cardiac phosphoprotein of a molecular mass corresponding to phospholemman (PLM) at approximately 15 kDa displayed a reduced phosphorylation in response to SL0101 pretreatment.

Taken together, the results from experiments with the ATP-competitive RSK inhibitors D1870 and SL0101 reveal that these two compounds significantly enhance phosphorylation of the cardiac proteins PLN at Ser16 and cTnI at Ser22/23 in response to PE.

To investigate, whether the molecular mechanism behind this observation is indeed a result of pharmacological RSK inhibition, a third inhibitor with a different mode of action, the allosteric inhibitor FMK, was tested for its potential to also modulate cardiac protein phosphorylation. FMK binds irreversibly to a cysteine within the RSK CTKD and subsequently prevents RSK CTKD-mediated activation as reflected by reduced autophosphorylation at Ser380 and inhibition of RSK NTKD activation and activity [17]. Exposure of ARVMs to FMK (3 μmol/L; 90 min) significantly attenuated PE-induced Ser380 RSK autophosphorylation as expected (fold change FMK + PE vs DMSO + PE: 0.46 ± 0.09; Fig. 3A, B). In contrast to the ATP-competitive RSK inhibitors, FMK pretreatment significantly reduced PE-mediated PLN phosphorylation at Ser16 and abolished cTnI phosphorylation at Ser22/23 (fold change FMK + PE vs DMSO + PE: 0.38 ± 0.23; Fig. 3A, B). The latter was anticipated as cTnI is not only substrate of PKA-mediated phosphorylation, but also an established substrate of RSK-mediated phosphorylation [10].

The results reveal antithetic effects of three commercially available

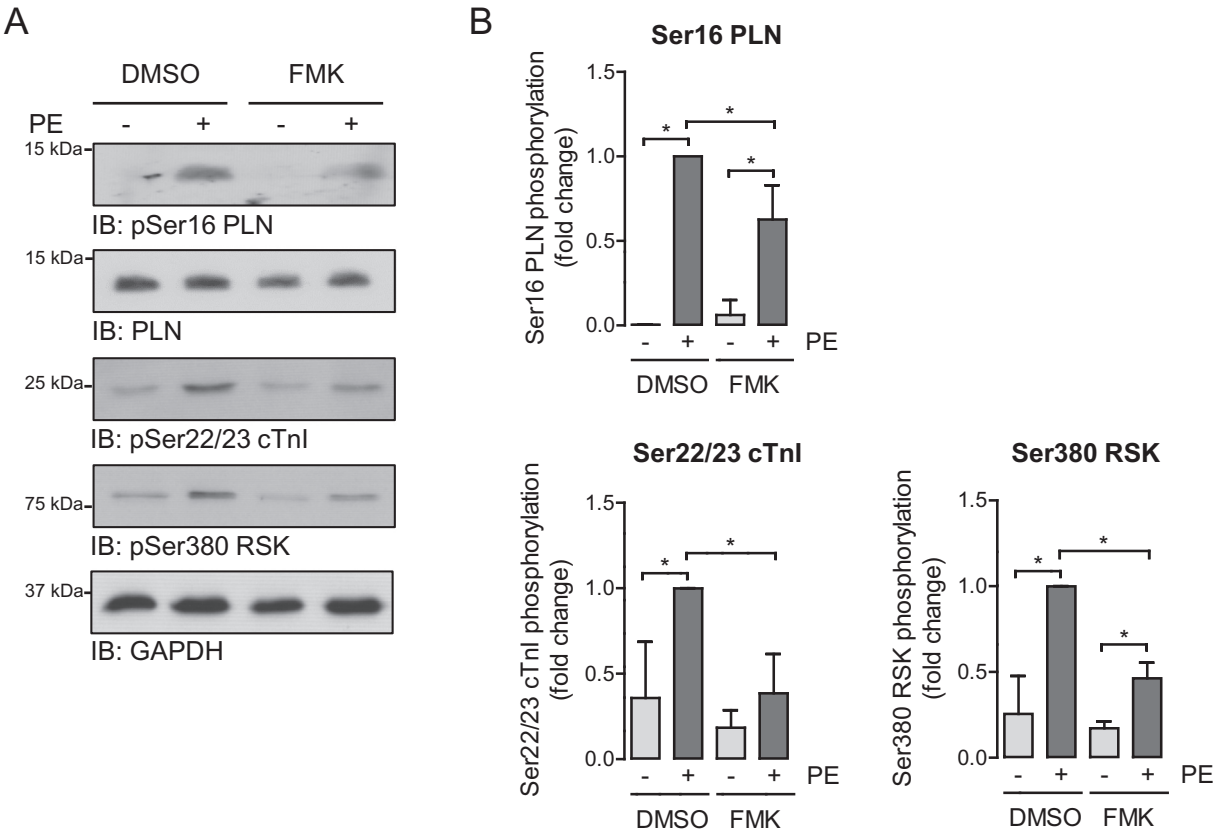

Fig. 3. Cardiac myocyte protein phosphorylation in response to phenylephrine in ARVMs pretreated with the pharmacological RSK CTKD inhibitor FMK. (A) ARVMs were incubated for 1.5 h with vehicle (DMSO) or 3 μmol/L FMK and then treated for 10 min with vehicle (PBS) or with 10 μmol/L PE. Phosphorylation of PLN (pSer16), cTnI (pSer22/23) and RSK (pSer380) phosphorylation was analysed by immunoblotting (IB) using the respective phosphospecific antibodies. Total PLN content and protein loading were confirmed by an anti-PLN and an anti-GAPDH antibody, respectively. (B) Bar charts summarize data from 4 independent experiments. Data were normalized to protein loading and expressed as fold change over DMSO + PE. Results are shown as mean ± S.D. $^{*}P < .05$.

RSK inhibitors on cardiac protein phosphorylation. In all experiments, PE was used as a stimulus to activate RSK in cardiac myocytes. In order to investigate, whether the effect of D1870 on PLN Ser16 and cTnI Ser22/23 phosphorylation may result from interference with the PKA signalling pathway, we measured the effect of D1870 pretreatment on PLN phosphorylation at Ser16 by isoprenaline (ISO; 10 nmol/L; 10 min) and noradrenaline (NA; 10 nmol/L; 10 min). Both stimuli do not activate cellular RSK signalling as revealed by the lack of canonical ERK phosphorylation at Thr202/Tyr204 and subsequent RSK autophosphorylation at Ser380, when compared to PE exposure (Fig. 4A). Despite the lack of RSK activation by ISO and NA, the stimuli tested induced a significantly increased PLN phosphorylation after pretreatment with D1870 (Fig. 4A). This allows the conclusion that potentiated PLN phosphorylation induced by ISO or NA in the presence of D1870 is not attributed to alterations in RSK activity. To further substantiate whether inhibition of RSK is responsible for the observed potentiation of PLN Ser16 phosphorylation, PE-mediated RSK signalling in ARVMs was inhibited by pretreatment with the α_1-adrenoceptor antagonist prazosin (1 μmol/L; 10 min). Exposure to prazosin prevented ERK and RSK activation as demonstrated by reduced canonical ERK phosphorylation and subsequent RSK autophosphorylation (Fig. 4B. Importantly, neither inhibition of α_1-adrenoceptor activation by prazosin pretreatment (Fig. 4B) nor pretreatment of ARVMs with the MEK1/2 inhibitor U0126 (3 μmol/L; 30 min) to inhibit ERK1/2 activation upstream of RSK (Fig. 4C) potentiated the PE-mediated PLN phosphorylation at Ser16 when compared to D1870 pretreated samples.

In contrast, pretreatment of ARVMs with the β-adrenoceptor antagonist atenolol (1 μmol/L; 10 min) prior to exposure to D1870 prevented the PE-induced increase in PLN Ser16 phosphorylation, compared to D1870 pretreatment alone (Fig. 5A). Furthermore, when ARVMs were pretreated with H89 in combination with D1870 to directly inhibit PKA activation, the increase in PE-mediated PLN phosphorylation was abolished, when compared to pretreatment with D1870 alone (Fig. 5B).

To complement the pharmacological experiments, FRET experiments were performed in isolated AMVMs and whole hearts from WT and RSK2 KO mice expressing the Epac1-PLN FRET biosensor in PLN vicinity. The FRET ratio in response to D1870 or PE exposure in AMVMs or left ventricles from WT and RSK2 KO mice was not different between genotypes (Supplemental Fig. 2A, B, C). Immunoblot analysis of heart homogenates from WT or RSK2 KO mice revealed similar phosphorylation levels of PLN and cTnI (Supplemental Fig. 2D).

Taken together, the results suggest that the potentiation in cardiac myocyte protein phosphorylation induced by the ATP-competitive RSK inhibitor D1870 occurs independently of RSK activity, but is most likely due to an off-target effect that requires β-adrenoceptor signalling and PKA activation. A potential explanation for the enhanced protein phosphorylation after treatment with D1870 and SL0101 could be the inhibition of a phosphodiesterase (PDE) that would induce a compartmentalized cAMP elevation and PKA-mediated PLN phosphorylation. This hypothesis was tested in isolated adult ventricular myocytes (AMVMs) isolated from transgenic mice expressing cAMP FRET biosensors that are either cytosolic (Epac1-camps) or targeted to the SR (Epac1-PLN). Thereby, a change in the FRET response indicates an increase in compartmentalized cAMP levels. Exposure of AMVMs to D1870 induced a significant increase in the FRET response in vicinity to the SR when compared to the FRET response detected by the cytosolic sensor, which was approximately 60% of the response induced by ISO

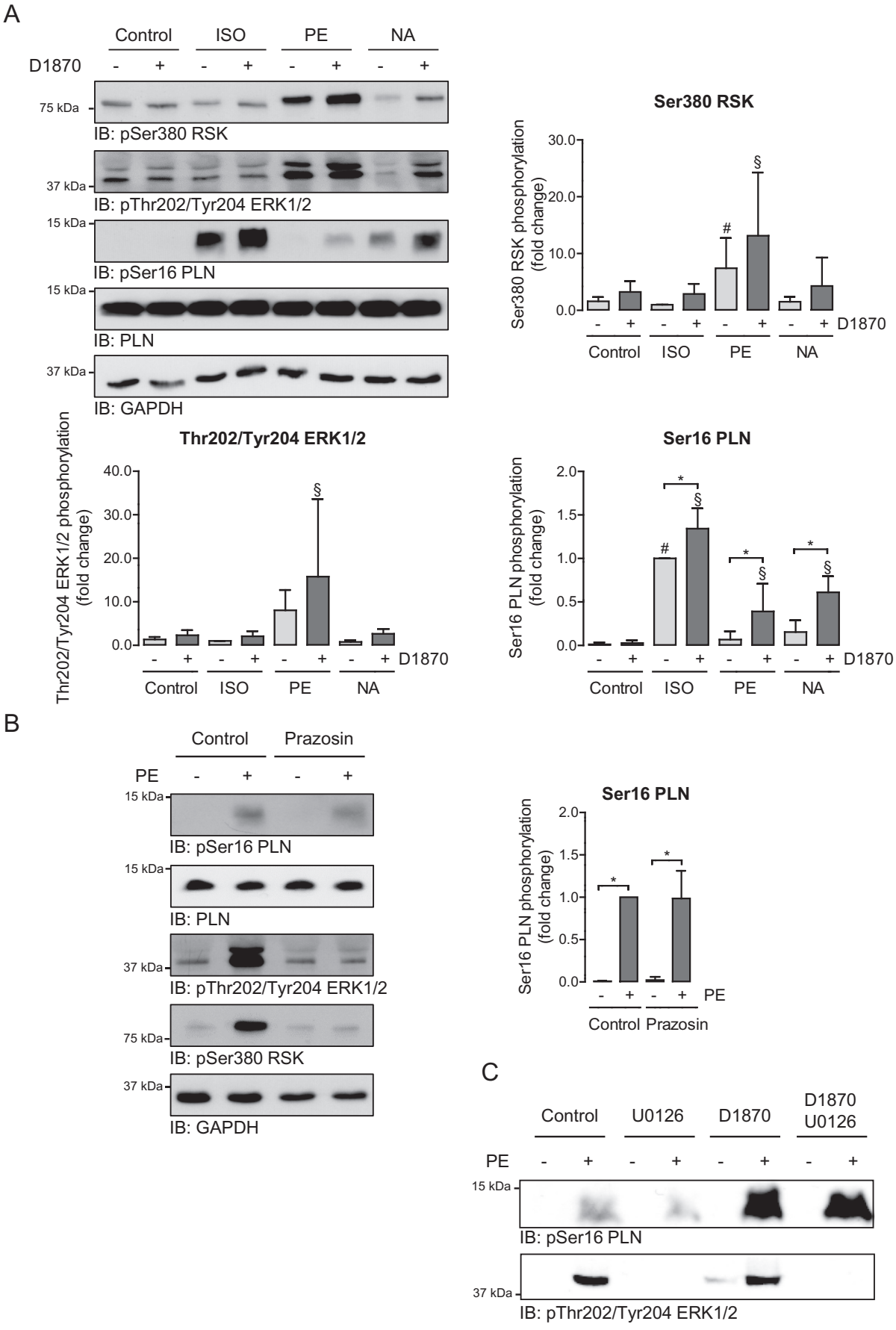

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Fig. 4. Effect of D1870 pretreatment on cardiac myocyte protein phosphorylation in response to adrenergic receptor modulation.
(A) ARVMs were pretreated for 1 h with 10 μmol/L D1870 or vehicle (DMSO) and then treated for 10 min with vehicle (Control; Con; PBS), 10 nmol/L isoprenaline (ISO), 10 μmol/L phenylephrine (PE) or 10 nmol/L noradrenaline (NA). Total PLN levels and phosphorylation of PLN at Ser16, ERK1/2 at Thr202/Tyr204 and RSK at Ser380 were assessed by immunoblot (IB) analysis using the respective antibodies. Equal protein loading was assessed by re-probing the membranes with an anti-GAPDH antibody. Bar charts summarize data from 4 to 6 independent experiments. Data were normalized to protein loading and are expressed as fold change over DMSO + ISO. Results are shown as means ± S.D. $^{*}P < .05$; $^{\#}P < .05$ vs DMSO + Con; $^{\S}P < .05$ vs D1870 + Con. (B) PLN Ser16 phosphorylation was assessed by IB analysis in samples from cultured ARVMs pre-incubated with vehicle (PBS) or prazosin (1 μmol/L; 10 min) and subsequently exposed for 10 min to vehicle (PBS) or 10 μmol/L phenylephrine (PE). The phosphorylation status of ERK1/2 at Thr202/Tyr204 and RSK at Ser380 was also evaluated using respective phosphospecific antibodies. Total PLN levels were evaluated using an anti-PLN antibody. Protein loading was confirmed with an anti-GAPDH antibody. The bar chart summarizes PLN phosphorylation from 5 independent experiments. Data were normalized to protein loading and are expressed as fold change over vehicle + PE. Results are shown as mean ± S.D. $^{*}P < .05$. (C) ARVMs were pre-incubated for 1 h with vehicle (DMSO), D1870 (10 μmol/L), U0126 (3 μmol/L; 10 min) or D1870 and U0126 and subsequently exposed for 10 min to vehicle (PBS) or 10 μmol/L phenylephrine (PE). The phosphorylation status of ERK1/2 at Thr202/Tyr204 and PLN at Ser16 was assessed by IB.

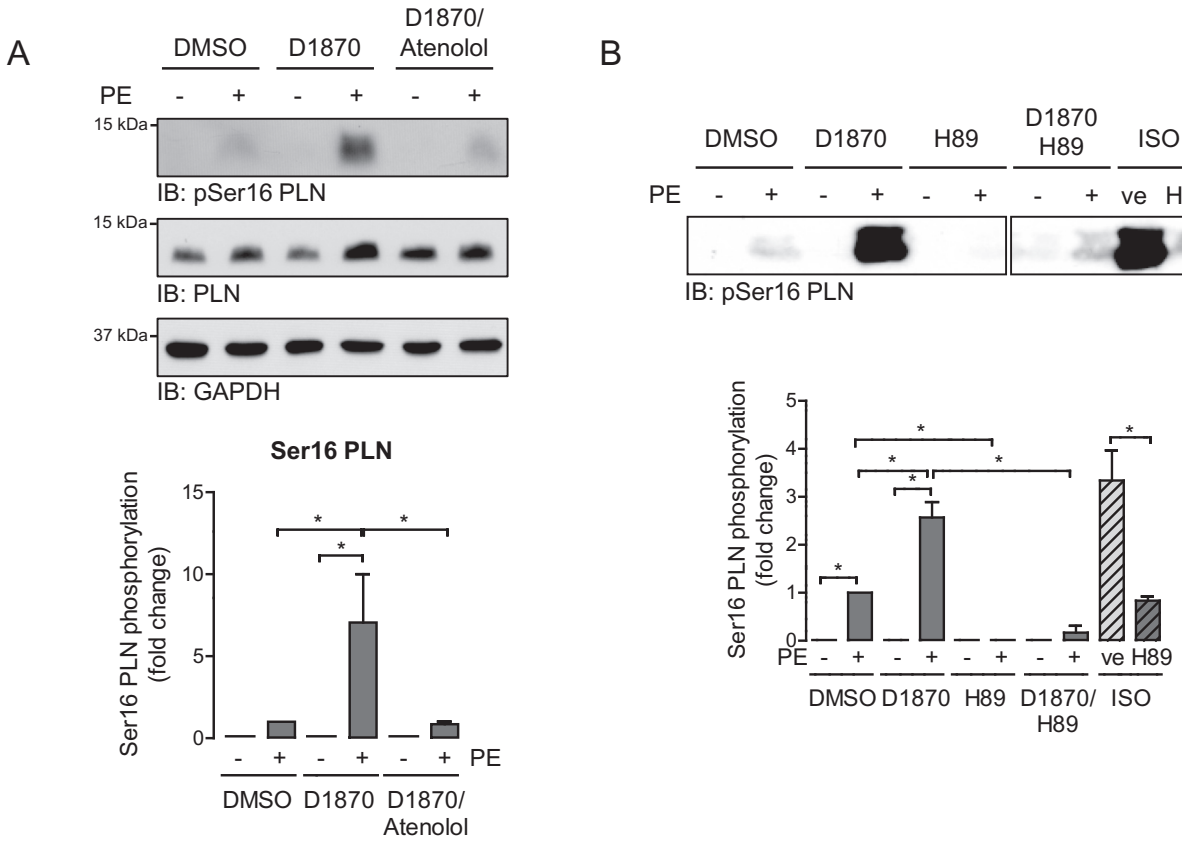

Fig. 5. Enhanced PLN phosphorylation after pretreatment with D1870 depends on β_1-AR receptor activation.
(A) ARVMs were pretreated with vehicle (DMSO), D1870 (10 μmol/L; 1 h), atenolol (1 μmol/L; 10 min) or D1870 and atenolol and then stimulated with vehicle (PBS) or or phenylephrine (PE; 10 μmol/L). Phosphorylation of PLN at Ser16 and total PLN expression were assessed by immunoblot (IB) analysis using the respective antibodies. Protein loading was evaluated with an anti-GAPDH antibody. The bar chart summarizes data from 5 independent experiments. Data were normalized to protein loading and are expressed as fold change over DMSO + PE. Results are shown as mean ± S.D. $^{*}P < .05$. (B) ARVMs were pretreated with vehicle (DMSO), D1870 (10 μmol/L; 1 h), H89 (1 μmol/L; 30 min) or D1870 and H89 and then stimulated with vehicle (PBS), phenylephrine (PE; 10 μmol/L) or isoprenaline (ISO; 10 nmol/L 10 min). Phosphorylation of PLN at Ser16 was assessed by immunoblot (IB) analysis using a phosphospecific antibody. The bar chart summarizes data from 3 independent experiments. Data were normalized to protein loading and are expressed as fold change over DMSO + PE. Results are shown as mean ± S.D. $^{*}P < .05$.

(Fig. 6A). In AMVMs that transgenically express the FRET biosensor Epac1-PLN, SL0101 pretreatment induced a comparable FRET response to D1870 of approximately 40 to 50% of the maximal response induced by exposure to IBMX and forskolin, suggesting that cAMP elevation in SR vicinity is a common feature of both ATP-competitive RSK inhibitors (Fig. 6B).

This type of inhibitor behavior was reminiscent of the effect of the PDE4 selective inhibitor rolipram, which increased local cAMP levels measured with the SR targeted but not with the cytosolic FRET biosensor when applied alone [25]. PDE4 is the main cardiac PDE family that has been reported to regulate SR Ca^{2+} content and cardiac contractility in rodent myocytes [38], which led to the hypothesis that D1870 and SL0101 could inhibit this subfamily of PDE isoforms. HEK293 cells were transfected with the FRET biosensor Epac1-camps-PDE4A1 that contains PDE4A1 fused to the cAMP-binding site of Epac1 and allows visualization of alterations in cellular cAMP levels in vicinity to PDE4A1 [34]. Such biosensors are ideally suited for direct monitoring of PDE inhibitor effects in living cells since they generate a specific FRET signal only when a PDE of interest is inhibited and this happens especially during receptor-stimulated cAMP activation [34]. Pretreatment with D1870 alone did not reveal alterations in the FRET response compared to maximal sensor activation by exposure to FOR/IBMX (Fig. 6C). In contrast, pre-stimulation of cells with ISO (100 nmol/L) prior to exposure to D1870 showed a robust FRET

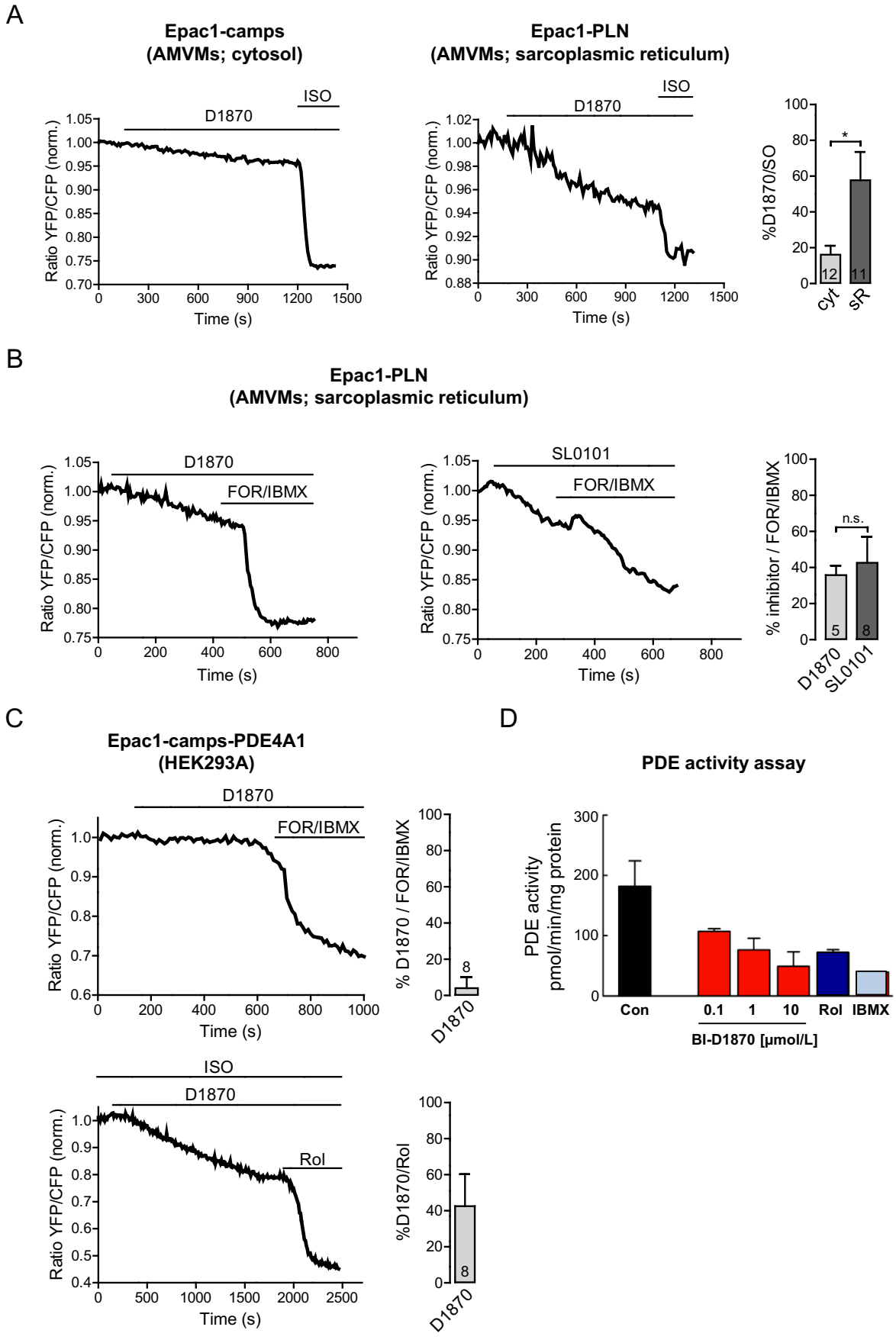

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Fig. 6. Effect of ATP-competitive RSK NTK inhibitors on cellular cAMP levels.
(A) Adult mouse ventricular myocytes (AMVM) transgenically expressing a cAMP-FRET biosensor targeted at the cytoplasm (Epac1-camps) or in vicinity to PLN (Epac1-PLN) were treated with D1870 (10 μmol/L) and the FRET response recorded. The decrease in the YFP/CFP ratio indicates an increase in cellular cAMP levels, which was monitored until a plateau was reached. Addition of isoprenaline (ISO; 100 nmol/L) was used to elicit a maximum cAMP production. Representative recordings of FRET traces are shown. Data are expressed as % of the maximum response induced by ISO and values are expressed as mean ± S.D. Cardiac myocyte numbers are shown in the bars. (B) AMVMs transgenically expressing the Epac1-PLN cAMP-FRET biosensor were treated with D1870 (10 μmol/L) or SL0101 (50 μmol/L). After a plateau was reached, forskolin (FOR; 5 μmol/L)/IBMX (100 μmol/L) was added to induce maximum cAMP production. The bar chart shows quantification of the data expressed as % of the maximum FOR/IBMX response. Values are shown as mean ± S.D. Cardiac myocyte numbers are shown in the bars. (C) Experiments in HEK293A cells transfected to express the PDE4A1-Epac1-camps FRET biosensor. Cells were exposed to D1870 (10 μmol/L) and then to forskolin (FOR; 5 μmol/L)/IBMX (100 μmol/L) to induce maximal cAMP production (left panel). Cells were pre-stimulated with ISO (1 μmol/L) and subsequently exposed to D1870 (10 μmol/L). After the FRET response reached the plateau, they were treated with rolipram (Rol; 10 μmol/L) (right panel). Shown are representative FRET recordings. The bar charts show the quantified FRET responses. Data are expressed as the % responses to D1870/FOR/IBMX (left) or D1870/Rol (right) and values expressed as mean ± S.D. (D) In vitro activity of phosphodiesterase activity was assessed in cardiac myocytes exposed to vehicle (DMSO) or increasing concentrations of D1870 (0.1; 1; 10 μmol/L for 1 h), rolipram (10 μmol/L 30 min) or forskolin (FOR; 5 μmol/L for 30 min)/IBMX (100 μmol/L; for 30 min). PDE activity in the lysates was assessed in the presence of exogenously added substrate cAMP (1 μmol/L). Data are expressed in pmol/min/mg protein.

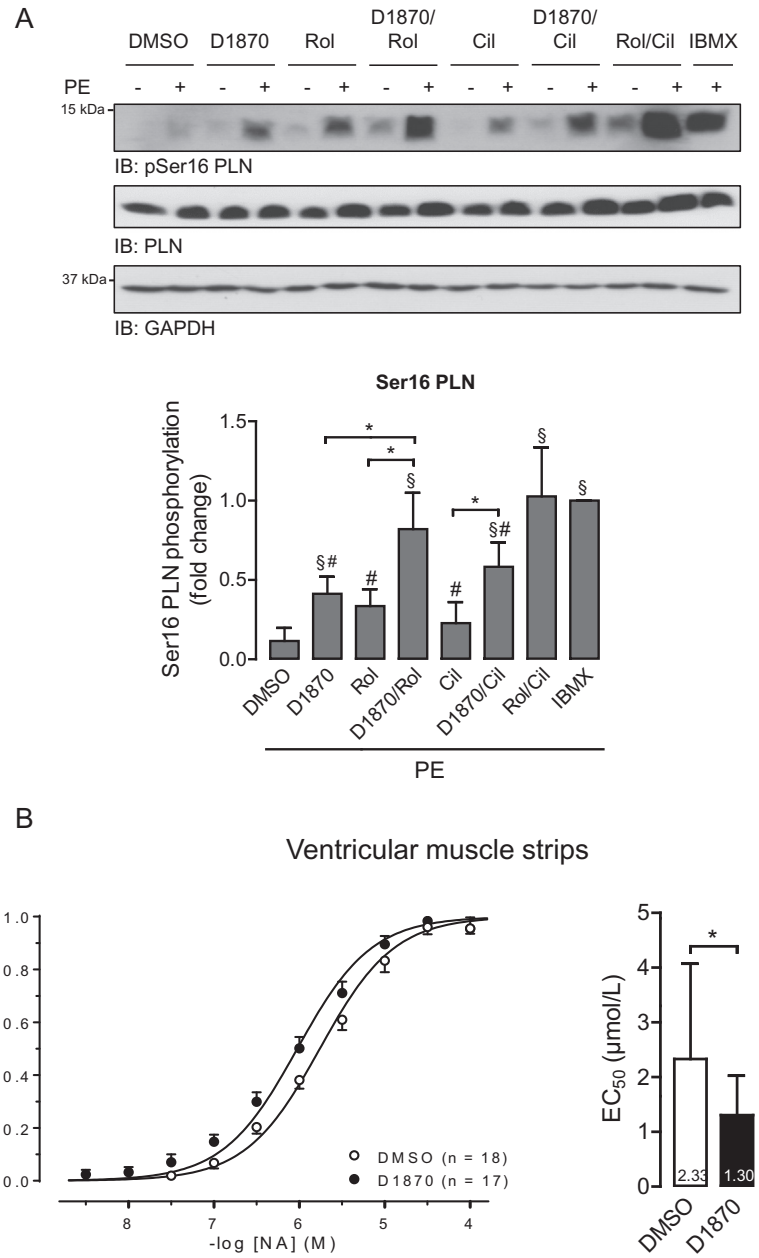

Fig. 7. Functional effects of D1870 in ventricular cardiac preparations.
(A) ARVMs were pretreated with vehicle (DMSO), D1870 (10 μmol/L; 1 h), rolipram (Rol; 10 μmol/L; 10 min), cilostamide (Cil; 10 μmol/L; 10 min), D1870 + Rol, D1870 + Cil, Rol + Cil or IBMX (100 μmol/L; 10 min) and then stimulated with vehicle (PBS) or phenylephrine (PE; 10 μmol/L) for 10 min. Phosphorylation of PLN at Ser16 was assessed by immunoblot (IB) analysis using the phosphospecific Ser16 antibody. An anti-PLN antibody was used to evaluate total PLN levels. Protein loading was assessed using an anti-GAPDH antibody. Bar charts summarize data from 7 individual experiments. Data were normalized to protein loading and expressed as fold change over IBMX-PE. Results are shown as means ± S.D. $^{*}P < .05$; $^{\S}P < .05$ vs D1870 + PE; $^{\#}P < .05$ vs Rol + Cil + PE and IBMX + PE. (B) Rat ventricular muscle strips were pretreated with vehicle (DMSO; $n = 18$, open signs) or D1870 (10 μmol/L; 1 h; $n = 17$, closed signs) and subsequently force development assessed in response to cumulative concentrations of noradrenaline (NA; 3 nmol/L–100 μmol/L). Developed force (F/Fmax) in response to NA is compared to the maximum force production achieved after exposure to 8 mmol/L $CaCl_2$. The NA concentration producing half maximum response (EC_{50}) and the number of strips per condition are indicated in the graphs.

response elicited by the Epac1-camps-PDE4A1 biosensor that measured approximately 40% of the maximal response induced by exposure of cells to the selective PDE4 antagonist rolipram (Fig. 6C). The data suggest that D1870 pretreatment increases cAMP levels in the vicinity of PDE4A1. In vitro PDE activity assays were performed in order to confirm a direct inhibitory effect of D1870 on cardiac PDEs. AMVMs were exposed to increasing concentrations of D1870 (0.1, 1, 10 μmol/L), lysed and PDE activity was tested by assessing the hydrolysis of exogenously added cAMP. This revealed that D1870 pretreatment inhibits PDE activity to a similar extent as achieved by exposure of cells to the established PDE4 inhibitor rolipram (10 μmol/L) or the unselective PDE inhibitor IBMX (100 μmol/L; Fig. 6D). The in vitro assays demonstrated that D1870 directly inhibits PDE activity. To assess PLN phosphorylation evoked by established PDE inhibitors compared to D1870, ARVMs were exposed to D1870 or the PDE inhibitors rolipram and cilostamide (PDE3), alone or in various combinations with subsequent investigation of the phosphorylation status of PLN at Ser16 in response to PE (Fig. 7A). This revealed that D1870 pretreatment significantly increased the PE-mediated PLN phosphorylation (DMSO + PE: 0.12 ± 0.08 vs. D1870 + PE 0.41 ± 0.10). This response occurred to a similar extent as pretreatment with rolipram (rolipram + PE: 0.34 ± 0.10). Interestingly, combined pretreatment with D1870 and rolipram further potentiated PLN phosphorylation (D1870 + Rolipram + PE: 0.82 ± 0.23), suggesting additive effects of the two inhibitors, potentially by combined inhibition of PDE3 and 4. A similar observation was made for cilostamide pretreatment in response to PE, which alone increased PLN phosphorylation (cilostamide + PE: 0.23 ± 0.13), an effect further potentiated in combination with D1870 (D1870 + cilostamide + PE: 0.58 ± 0.15) or rolipram (rolipram + cilostamide + PE: 1.03 ± 0.31), respectively.

To investigate, whether the molecular observations translate into functional consequences, rat ventricular muscle strips were pretreated with vehicle or D1870 and cumulative concentration-response curves to NA (3 nmol/L–100 μmol/L) performed. Under vehicle (DMSO) conditions, the EC_{50} for NA measured 2.33 μmol/L. D1870 pretreatment induced a significant leftward shift of the NA concentration response curve, thereby significantly reducing the EC_{50} for NA to 1.30 μmol/L (Fig. 7B). The data suggest that D1870 pretreatment sensitized force development in response to NA due to PDE inhibition with subsequent cAMP-elevation. The effect magnitude is in accordance with those observed in studies with established PDE inhibitors in rat heart [39].

The results suggest that the potentiation of cardiac myocyte protein phosphorylation in response to pretreatment with the ATP-competitive RSK inhibitors is likely due to off-target inhibition of cardiac myocyte PDE activity leading to compartmentalized cAMP elevation with subsequent catecholamine sensitization of force development (Fig. 8).

4. Discussion

RSKs comprise a family of ubiquitously expressed kinases with ERK being the predominant cellular activator. Multiple stimuli of physiological and pathophysiological significance lead to RSK activation, e.g. oxidative stress [40,41], intracellular acidosis [42], a variety of neurohumoral stimuli including catecholamines, endothelin 1 (ET-1) and angiotensin II through their respective receptors [43–45]. Increased RSK activity has been observed in cancer patients [8,16,46], rendering pharmacological RSK inhibition an attractive therapeutic strategy. However, whether global inhibition of this important kinase leads to detrimental adverse side effects in other organs, remains unexplored. In the heart, RSKs have been demonstrated to phosphorylate the prototypical PKA substrate proteins cTnI [14] and cMyBP-C [13] and thus may contribute to the regulation of acute force development and sensitivity to Ca^{2+}. Importantly, RSK activity was found to increase in patients with dilated cardiomyopathy [47] and also in animal models subjected to experimental heart failure [48] and ischemia/reperfusion injury [10,40]. This suggests that RSK-mediated regulation of contractile function might assume complementary significance under failing heart conditions, when PKA-mediated signalling is perturbed. Therefore, global pharmacological RSK inhibition should be carefully evaluated with determination of the exact contributions of RSKs for maintaining cardiac function.

The present study investigates the outcome of pharmacological RSK inhibition on the phosphorylation of cardiac proteins within two distinct subcellular compartments in cardiac myocytes that are involved in the regulation of acute contractile function. Three commercially available pharmacological RSK inhibitors were compared, namely D1870, SL0101 and FMK, which differ in structure and in their mode of RSK inhibition. D1870 is a dihydropteridinone and acts by competing with ATP-binding to the NTKD of all four RSK isoforms [15]. The kaempferol glycoside SL0101 acts also as an ATP competitor of the NTKD of all RSK isoforms [16] and additionally has been described to induce conformational changes in the kinase domain [49]. In contrast, FMK a fluoromethylketone compound, was rationally designed to bind covalently to cysteine 432 in RSK1 (cysteine 436 in RSK2) localized in the RSK CTKD providing a sterical hindrance on the “gatekeeper” threonine in position 489 (threonine 493 in RSK2) thus preventing access of ATP into the CTKD. Covalent binding to FMK irreversibly inhibits RSK1 and RSK2. Due to a replacement of threonine 486 by methionine, RSK3 is not inhibited by exposure to FMK [17]. Importantly, FMK inhibits the RSK CTKD and consequently prevents autophosphorylation and subsequent activation of the RSK NTKD [50,51]. Our data reveal a differential protein phosphorylation pattern after exposure to the two different classes of inhibitors in response to PE. As expected, FMK pretreatment reduced PE-induced phosphorylation of cTnI at Ser22/23 and this is in accordance with previous reports establishing cTnI as a direct substrate of RSK [14]. The reduction in PLN Ser16 phosphorylation under the same conditions suggests that PLN might be a novel substrate for an RSK-mediated phosphorylation, which is interesting and warrants further investigations. Both observations emphasize a role for RSK in fine tuning PKA activity or as an alternative signal transduction pathway during conditions of perturbed PKA signalling. In sharp contrast are the results obtained with the ATP-competitive compounds D1870 and SL0101, which showed the opposite phosphorylation pattern to that observed with FMK. These contrarious results suggest off-target effects as the underlying molecular mechanism as the different phosphorylation pattern cannot be explained by the inhibitors targeting different RSK isoforms (D1870 and SL0101 inhibit all 4 RSK isoforms; FMK inhibits RSK1, 2 and 4). This is also supported by the observation that enhanced PLN phosphorylation by D1870 pretreatment was also detectable in response to ISO and NA, stimuli that do not activate cellular RSK signalling as reflected by the absence of RSK autophosphorylation, a consensus readout for RSK activity [2]. Whilst modulation of the upstream effectors of the RSK signalling axis by prazosin or U0126 did not induce potentiation of protein phosphorylation as observed by D1870 pretreatment, the activation of the β_1-AR PKA pathway was a prerequisite to unveil the effect of D1870 on PLN phosphorylation. This evidence is supported by the FRET experiments that after D1870 pretreatment showed significantly increased cellular cAMP levels in response to β_1-AR stimulation by ISO. A possible explanation of the unexpected potentiation of protein phosphorylation in response to D1870 was a direct inhibitory effect on cardiac myocyte PDEs, the enzymes responsible for cAMP degradation [52,53]. Off-target effects on PDEs have been observed previously for the phosphatidylinositol 3-kinase inhibitor LY294002 and PDE2 [54]. The data in this study do not conclusively show which PDE isoforms are inhibited, however, experiments performed with the Epac1-camps-PDE4A1 FRET biosensor in HEK293A cells and the in vitro PDE assays reveal that D1870 likely has the potential to inhibit the main PDE isoforms in cardiac myocytes that are responsible for cAMP degradation and the regulation of ECC. Of the PDE4 family members the PDE4D subfamily is known to regulate SR Ca^{2+} content in murine cardiac myocytes [38]. Additionally, PLN phosphorylation is increased in

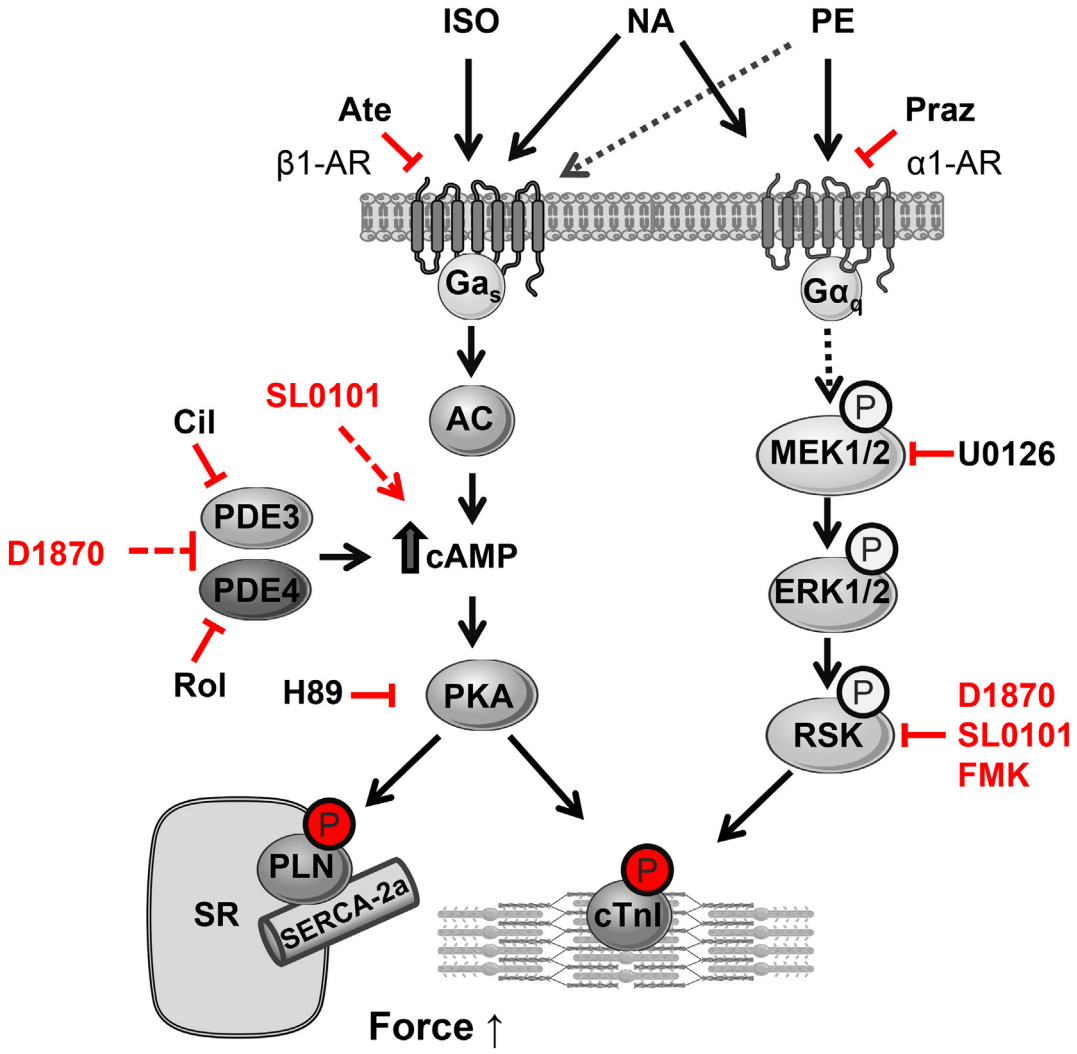

Fig. 8. Scheme summarizing the pharmacological interventions.
AC: adenylate cyclase; AR: adrenergic receptor; Ate: atenolol; Cil: cilostamide; cMyBP-C: cardiac myosin-binding protein C; cTnI: inhibitory subunit of cardiac troponin; ERK: extracellular signal-regulated kinase; ISO: isoprenaline: MEK: mitogen-activated ERK kinase; NA: noradrenaline; PDE: phosphodiesterase; PE: phenylephrine; PKA: cAMP-dependent protein kinase; PLN: phospholamban; Praz: prazosin; Rol: rolipram; RSK: p90 ribosomal-S6 kinase; SERCA: sarcoplasmic reticulum Ca^{2+}-ATPase; SR: sarcoplasmic reticulum.

PDE4D-targeted knockout mice when compared to wildtype littermates [38]. Furthermore, the observation that the cAMP elevation in response to D1870 pretreatment is spatially restricted to specific intracellular compartments rather than uniformly distributed throughout the cytosol, allows to speculate that PDE4D would be a candidate for off-target inhibition by D1870 in cardiac myocytes.

Of note, D1870 did not completely inhibit the PDE4A1 FRET biosensor and concurrent exposure of ARVM to D1870 and rolipram caused additive PE-induced PLN phosphorylation when compared to treatment with each inhibitor alone. Therefore, it is likely that PDE3 isoforms, the other predominant PDE family in rat and especially human cardiac myocytes [55–57] represents another potential D1870 off-target.

Off-target effects of D1870 on other kinases than RSKs have been extensively studied by Sapkota et al. [15]. This showed that D1870 inhibits other kinases of the AGC family from a panel of 54 kinases tested, including Aurora B and Polo-like kinase 1. Moreover, it was recently demonstrated in two glioblastoma cell lines that D1870 pretreatment mediates an RSK-independent increase in p70 ribosomal S6 kinase (p70 S6K) activity [58]. In the same study SL0101 was also shown to cause off-target inhibition of the mammalian target of rapamycin complex 1 (mTORC1) – p70 S6K signalling axis [58]. Furthermore, D1870 partially inhibited insulin-stimulated Akt activation in 3T3-L1 adipocytes [59]. Of the aforementioned kinases, Akt has been shown to phosphorylate PLN at Thr17 [60]. However, it is unlikely that off-target inhibition of a kinase contributes to the observed enhancement of cardiac protein phosphorylation in this study.

Important to mention is that a crosstalk between PKA and RSK has been reported, whereby PKA activity was modulated dependent on the activity status of RSK. Thereby, it was shown that the PKA catalytic subunit is directly inhibited by binding to active RSK1 [61]. Inactive RSK1 can interact with the PKA regulatory subunit Iα (PKA RIα) and this was shown to release the PKA catalytic subunit and enhance substrate phosphorylation [61,62]. These studies represent an interesting aspect with regards to the results presented in the present study, raising the possibility whether D1870-mediated RSK inhibition would enhance the interaction between RSK and PKA RIα, which would subsequently release the PKA catalytic subunit and lead to enhanced cardiac protein phosphorylation. However, we could not experimentally confirm this hypothesis, but cannot exclude the possibility of this kinase crosstalk to occur and its contribution to the observed effects reported in this study.

D1870 has been used previously to demonstrate that Ser282 cMyBP-C is a novel cardiac RSK substrate in response to ET-1 stimulation in ARVMs [13]. The study showed that pretreatment with D1870

abolished ET1-mediated phosphorylation of cMyBP-C at Ser282, suggesting that RSK is a novel kinase that phosphorylates cMyBP-C. Importantly, this observation was validated by various other experiments and independent methodology. In the present study, D1870 did not significantly alter cMyBP-C phosphorylation at Ser282 induced by PE, NA or ISO (Supplemental Fig. 3A) suggesting that following these stimuli, PKA is the main kinase responsible for cMyBP-C phosphorylation. Similarly, pretreatment with SL0101 or FMK did not affect the PE-mediated cMyBP-C phosphorylation (Supplemental Fig. 3B). Whilst D1870 represents a suitable tool to study RSK-mediated phosphorylation events with ET-1 as a stimulus that exclusively activates G_q-coupled signalling pathways, the present study demonstrates that when stimuli are used that activate G_q- and G_s-coupled signalling pathways simultaneously, the obtained results are more difficult to interpret. The discrepant observations could be explained by a crosstalk between signalling pathways converging in the activation of PKA and RSK or by the activation of distinct cellular RSK subpopulations or RSK isoforms. The possibility of different stimuli activating distinct kinase subpopulations has recently been described to play a role in the RSK-mediated regulation of early gene transcription in response to ET-1 and PE in neonatal rat cardiac myocytes. Whilst nuclear RSK2 content was increased by both, ET-1 and PE, only ET-1 induced translocation of RSK1 to the nucleus [44]. Therefore, to conclusively judge on the contribution of individual RSK isoforms or subpopulations on cardiac protein phosphorylation and function in response to diverse stimuli, experiments with pharmacological inhibitors could be paralleled with other approaches such as silencing the expression of individual RSK isoforms such as RSK2 in this study or by expression of RSK isoforms with inactive or modified NTKD or modified substrate proteins. The study emphasizes the problematic nature of routinely applied pharmacological inhibitors that evoke apparent differences in the spectrum of off-targets depending, importantly, on the signal stimulus that is used.

5. Conclusion

The present study demonstrates that two commonly used ATP-competitive RSK NTKD inhibitors potentiate cardiac protein phosphorylation under conditions of β_1-AR stimulation. The data suggest superiority of the rationally designed FMK as a selective and specific kinase inhibitor or also the recently described allosteric CTKD inhibitor dimethyl fumarate [63] over ATP-competitive compounds. This information is important to critically evaluate experiments that are based on the use of the latter class of inhibitors and under the conditions of simultaneous activation of signalling pathways. Additionally, the study highlights the functional contribution of D1870 to heart function that likely results from PDE inhibition and subsequent force production. Considering that development of isoform-selective RSK inhibitors is currently pursued as anti-cancer therapeutics [8,9], it is crucially important that novel compounds that act by competing with ATP-binding to the kinase are tested broadly for unexpected off-targets as consequences on heart function might occur.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109362.

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Declaration of interest

None.

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Author contribution statement

KS performed immunoblot experiments, analysed data and prepared figures.

NIB performed whole organ FRET measurements.

SS performed FRET experiments in HEK293 cells.

JUS performed FRET experiments in cardiac myocytes.

RKP performed in vitro PDE activity assays.

HS performed immunoblot experiments.

BG provided help with the animal care.

JPD kindly provided the RSK2 KO mice.

TC performed experiments in ventricular preparation and organ bath experiments.

VON performed FRET experiments and was a co-applicant for a grant application.

FC designed the study, analysed data, performed immunoblot experiments, obtained funding and wrote the manuscript.

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