



Sigma-1 receptor modulation fine-tunes K_v1.5 channels and impacts pulmonary vascular function

Alba Vera-Zambrano^{a,b,c,*}, Maria Baena-Nuevo^b, Susanne Rinné^d,
Marta Villegas-Esguevillas^{c,e,f}, Bianca Barreira^{c,e,f}, Gokcen Telli^g, Angela de Benito-Bueno^b,
José Antonio Blázquez^h, Belén Climentⁱ, Francisco Pérez-Vizcaino^{c,e,f}, Carmen Valenzuela^{b,j},
Niels Decher^d, Teresa Gonzalez^{a,b,i}, Angel Cogolludo^{c,e,f,*}

^a Department of Biochemistry, School of Medicine, Universidad Autónoma de Madrid, Madrid, Spain

^b Instituto de Investigaciones Biomédicas "Alberto Sols" CSIC-UAM, Madrid, Spain

^c Department of Pharmacology and Toxicology, School of Medicine, University Complutense of Madrid, Madrid, Spain

^d Institute of Physiology and Pathophysiology, Vegetative Physiology, University of Marburg, 35043 Marburg, Germany

^e Ciber Enfermedades Respiratorias (CIBERES), Spain

^f Instituto de Investigación Sanitaria Gregorio Marañón (IISGM), Madrid, Spain

^g Hacettepe University, Department of Pharmacology, Faculty of Pharmacy, Ankara, Turkey

^h Departamento de Cirugía Cardíaca, Hospital Universitario La Paz, Madrid, Spain

ⁱ Department of Physiology, Faculty of Pharmacy, University Complutense of Madrid, Madrid, Spain

^j Spanish Network for Biomedical Research in Cardiovascular Research (CIBERCV), Instituto de Salud Carlos III, Madrid, Spain

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ABSTRACT

K_v1.5 channels are key players in the regulation of vascular tone and atrial excitability and their impairment is associated with cardiovascular diseases including pulmonary arterial hypertension (PAH) and atrial fibrillation (AF). Unfortunately, pharmacological strategies to improve K_v1.5 channel function are missing. Herein, we aimed to study whether the chaperone sigma-1 receptor (S1R) is able to regulate these channels and represent a new strategy to enhance their function. By using different electrophysiological and molecular techniques in *X. laevis* oocytes and HEK293 cells, we demonstrate that S1R physically interacts with K_v1.5 channels and regulate their expression and function. S1R induced a bimodal regulation of K_v1.5 channel expression/activity, increasing it at low concentrations and decreasing it at high concentrations. Of note, S1R agonists (PRE084 and SKF10047) increased, whereas the S1R antagonist BD1047 decreased, K_v1.5 expression and activity. Moreover, PRE084 markedly increased K_v1.5 currents in pulmonary artery smooth muscle cells and attenuated vasoconstriction and proliferation in pulmonary arteries. We also show that both K_v1.5 channels and S1R, at mRNA and protein levels, are clearly downregulated in samples from PAH and AF patients. Moreover, the expression of both genes showed a positive correlation. Finally, the ability of PRE084 to increase K_v1.5 function was preserved under sustained hypoxic conditions, as an in vitro PAH model. Our study provides insight into the key role of S1R in modulating the expression and activity of K_v1.5 channels and highlights the potential role of this chaperone as a novel pharmacological target for pathological conditions associated with K_v1.5 channel dysfunction.

1. Introduction

K_v1.5 channels play a key role in the pulmonary circulation. They are responsible for setting or regulating the resting membrane potential and

thereby, for controlling pulmonary vascular tone, cell apoptosis, proliferation, and survival of pulmonary artery smooth muscle cells (PASMC) [1–5]. Moreover, K_v1.5 dysfunction is a common feature in pulmonary arterial hypertension (PAH) [4–6]. In the cardiovascular

Abbreviations: AF, atrial fibrillation; BrdU, 5-bromo-2-deoxyuridine, Dulbecco's modified Eagle's medium; PAH, pulmonary arterial hypertension; S1R, sigma-1 receptor; PA, intrapulmonary arteries; PASMC, pulmonary artery smooth muscle cells; Phe, Phenylephrine; TEVC, two-microelectrode voltage-clamp technique.

* Corresponding authors at: Department of Pharmacology and Toxicology, School of Medicine, University Complutense of Madrid, Ciudad Universitaria S/N, 28040 Madrid, Spain.

E-mail addresses: avera04@ucm.es (A. Vera-Zambrano), acogolludo@med.ucm.es (A. Cogolludo).

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system, $K_v1.5$ channels also participate in the cardiac atrial repolarization of the action potentials [7] and their dysregulation is a common feature in atrial fibrillation (AF). Therefore, $K_v1.5$ channels are considered potential therapeutic targets in these pathological conditions, but unfortunately, pharmacological strategies to rescue the reduced expression and activity of $K_v1.5$ channels are still lacking.

Sigma-1 receptor (S1R) is a 25-KDa chaperone which plays a central role in protein folding and quality control [8]. In a resting state, it resides in the mitochondrion-associated membrane (MAM) where it forms a complex with a chaperone called immunoglobulin protein (BiP) [8]. When activated, S1R interacts with different client proteins in the ER or other organelles [9] and, under chronic stress, it can translocate to the plasma membrane to regulate the activity of different plasma membrane proteins such as ion channels [10–13].

S1R can interact with itself to form oligomers and their distribution can be modified by different ligands. In this regard, agonists (e.g., SKF10047, PRE084) bias the equilibrium to lower molecular weight states while antagonists (e.g., BD1047, haloperidol, and sphingosine) may shift the equilibrium to higher molecular weight states [14,15]. Moreover, those ligands that induce hyperlocomotion or other physiological responses through binding to S1R were considered as agonists, and those blocking or blunting these responses were considered as antagonists [9,16].

Due to its role in the human physiology, the function of S1R has been studied in different pathologies and, nowadays, it is considered as a potential therapeutic target for several disorders including neurodegenerative pathologies such as Alzheimer's and Parkinson's diseases [17], stroke [18] and cancer [19]. During the last decade, several groups have suggested that S1R represents a novel molecular target in cardiovascular diseases [20]. S1Rs are expressed in cardiomyocytes [21,22], and their ligands have shown to alter contractility, calcium flow and rhythmic activity [23,24]. Previous studies suggest that S1R associates to and regulates several K^+ channels, including hERG, $K_v1.2$, $K_v1.3$, $K_v1.4$ and $K_v1.5$ [10,12,13,25,26]. Few *in vitro* studies in heterologous systems suggest that $K_v1.5$ channels can be targeted by S1R ligands, but it is still unknown whether S1R regulates K^+ channels, in particular, $K_v1.5$ in the cardiovascular system and whether this interaction can be modulated pharmacologically.

Therefore, the aim of this study was to analyse the potential modulation of $K_v1.5$ channels by S1R and its possible physiological, pathophysiological and pharmacological significance. Our data identify S1R as a potential novel pharmacological target in pathological conditions associated with $K_v1.5$ impairment.

2. Material and methods

2.1. Human lung and cardiac samples

Lung samples of the right upper lobe from patients with (8 samples, 3 females and 5 males) or without (10 samples, 5 females and 5 males) PAH were obtained from the Pulmonary Hypertension Biobank of CIBERES (Barcelona, Spain). The control samples belonged to patients without any other cardiovascular disease and that were discarded for lung transplantation. Patients were between 30 and 62 years old. Cardiac samples were provided by the Cardiac Surgery Service of Hospital Universitario La Paz (Madrid, Spain). 8 atrial samples (40–80 years old) from patients under SR as controls (4 females and 4 males) and 12 from patients with AF (5 females and 7 males). Human PASMC were isolated from human PA derived from non-PAH patients undergoing lobectomy or pneumonectomy during resection of lung carcinoma (60–80 years old, 4 men and 1 N/A). Fully written informed consent was obtained from all the donors and the Human Research Ethics Committees of the Hospital Universitario de Getafe (Madrid, Spain), the Hospital General Universitario Gregorio Marañón (Madrid, Spain) and Hospital Universitario La Paz approved the use of these samples (Refs. A04/16, 11/216 and PI-2550, respectively). All the investigation was conducted in

accordance with the Declaration of Helsinki.

2.2. Animal samples

All procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH Guide for the Care and Use of Laboratory Animals. The animals (rats and *Xenopus laevis*) were handled according to animal welfare regulations and the protocols were approved by the local ethics committee of the *Universidad Complutense* Review Boards (PROEX-301/16) and the *Regierungspräsidium Giessen* (MR 20/28 Nr. A 23/2017).

2.3. Rat vessel isolation

Male Wistar (230–280 g) rats from Janvier Labs (Mayenne, France) were sacrificed by exposition to CO_2 . The left lungs were removed and immediately immersed in Krebs solution (118 NaCl, 4.75 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11 glucose) for vascular reactivity, or external solution for patch-clamp experiments. Later, left lungs were dissected to isolate the intrapulmonary arteries (PA). For vascular reactivity, PA rings (1.8–2 mm length and approximately 0.4–0.8 mm of internal diameter) were carefully dissected in Krebs solution.

2.4. Cell culture and transfection

Human embryonic kidney 293 derived cell line (HEK293) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Life Technologies) at 37 °C, 99 % of humidity and 5 % CO_2 . PA were randomly assigned to treatment (1, 20 or 50 μ M PRE084) or control (DMSO) groups and incubated during 24 h or 48 h under normoxic (21 % O_2) or hypoxic (3 % O_2) conditions in DMEM-high glucose supplemented with 1 % non-essential amino acids and 1 % antibiotics. Thereafter, the arteries were treated with an enzymatic solution at 37 °C during ~ 10 min to dissociate the PASMC for the electrophysiological studies. The enzymatic solution contained the following (mg/ml): 1 papain, 0.8 dithiothreitol (DTT), and 0.7 albumin. All from Sigma-Aldrich. In some experiments, PA were cultured for 48 h in normoxia, hypoxia or hypoxia plus PRE084 (20 μ M). Information on cell transfection is provided in the [supplementary material](#).

2.5. *Xenopus laevis* oocyte injection

Oocytes (at stages IV and V) were injected each of them with 50 nl of cRNA. For TEVC experiments, the cRNA concentrations used were: 150 ng/ μ l of $K_v1.5$ together with 30, 150, 300 or 450 ng/ μ l of S1R. 2 ng/ μ l for $K_v2.1$ and $K_{ir}2.1$, alone or together with 300 ng/ μ l of S1R.

2.6. Western-blot assay

The primary antibodies were incubated overnight: anti-GFP (1:1000, #11814460001 Roche), anti-S1R (1:1000, #sc-137075 Santa Cruz), anti- $K_v1.5$ (1:1000, #APC-004 Alomone), anti-vinculin (1:80000, #sc-25336 Santa Cruz). Species-specific horseradish peroxidase-conjugated were used as secondary antibodies (dilution 1:10000, Santa Cruz). Immunoblot signals were visualized by chemiluminescence on an image acquisition system (Vilber Lourmat) and quantified using Image J software.

2.7. Co-immunoprecipitation

The Co-Immunoprecipitations (Co-IPs) were performed using Dynabeads™ Protein A and following the manufacturer's instructions (Invitrogen). Briefly, the corresponding antibody (30 μ l of anti-S1R or 2.4 μ l anti-GFP) was added to 50 μ l of dynabeads. During the short incubation,

the antibody binds to the dynabeads via their Fc-region. The tube is then placed on a magnet, where the beads migrate to the side of the tube facing the magnet and allow for easy removal of the supernatant. After several washing steps, the bead-bound antibody was mixed with the protein extract prior to western blot. Primary antibodies were anti-GFP (1:100, #Ab290 Abcam) and anti-S1R (1:500, #sc-166392 Santa Cruz). Species-specific horseradish peroxidase-conjugated from Thermo Fisher Scientific: anti-mouse (1:5000) and anti-rabbit (1:8000).

2.8. Chemiluminescence assay

pcDNA3-K_v1.5-HA plasmid had the nine-amino-acid-residue HA tag into the coding sequence of K_v1.5 between the extracellular residues 307 and 308 [27]. Surface expression of HA-tagged K_v1.5 channel construct was analysed in *X. laevis* oocytes using the ELISA technique. Two days after cRNA injection, oocytes were incubated for 30 min with 1 % BSA/ND96 at 4 °C to block non-specific binding of antibodies. Subsequently, oocytes were incubated with 1 µg/µl rat monoclonal anti-HA antibody, (Roche) for 60 min at 4 °C, washed six times with 1 % BSA/ND96 at 4 °C, and incubated with 2 µg/µl peroxidase-conjugated affinity purified F(ab)2 fragment goat anti-rat IgG antibody (Dianova) in 1 % BSA/ND96 for 60 min. Oocytes were washed again thoroughly, initially in 1 % BSA/ND96 (at 4 °C for 60 min) and then in ND96 without BSA (at 4 °C for 15 min). Individual oocytes were placed in 20 µl SuperSignal Elisa Femto solution (Pierce), and chemiluminescence was quantitated in a luminometer (Promega). The luminescence produced by non-injected oocytes was used as a reference signal (negative control).

2.9. Flow cytometry

For the whole-cell patch-clamp experiments in HEK293 cells, 48 h after transfection, positive transfected cells were trypsinised and selected by sorting (FACSVantage SE; BD Biosciences) in the interdepartmental investigation service of the UAM. Cells transfected with K_v1.5 alone (GFP⁺) or K_v1.5 co-transfected with S1R (GFP⁺cherry⁺) were selected. A 488-nm laser was used, and cell debris and doublets were excluded for the selection.

To measure the average intensity of GFP⁺/cherry⁺ fluorescence in transfected cells, a FACSCanto™ II cytometer (Becton Dickinson) was used, illuminating with 488-nm laser, and excluding cell debris and doublets for the analysis. BD FACSDiva™ and FlowJo software were used for fluorescence signal quantification and data visualization.

Cell death in human PSMC, was determined 24 h after treatment with vehicle, 20 µM PRE084 or 10 % DMSO that was included as a positive control of cell death. It was evaluated using annexin V and propidium iodide staining (ANXVDY-100 T, Immunostep) on a FACSCanto™ II cytometer (Becton Dickinson) illuminating with 488-nm and 633-nm lasers and excluding cell debris and doublets from the analysis. Cells were classified as viable (annexin V⁻ propidium iodide⁻), early apoptotic (annexin V⁺ propidium iodide⁻), necrotic (annexin V⁻ propidium iodide⁺) and late apoptotic (annexin V⁺ propidium iodide⁺). All data were quantified using BD FACSDiva™ and FlowJo software.

2.10. Whole-cell patch-clamp

The electrophysiological experiments were performed using the whole-cell configuration of the patch-clamp technique, with a patch-clamp amplifier Axopatch-200B (Molecular Devices) and were stored on a personal computer with a Digidata 1440 A analog-to-digital converter (Molecular Devices). pCLAMP 10 software (Molecular Devices) was used for both data acquisition and analysis. The electrophysiological protocols are explained in detail in the [supplementary methods](#).

2.11. Two-electrode voltage-clamp

X. laevis oocytes measurements were recorded with the two-microelectrode voltage-clamp technique (TEVC) at RT with a TurboTEC 10 CD amplifier (npi electronic) and a Digidata 1200 Series A/D converter (Molecular Devices). Micropipettes were made from borosilicate glass capillaries (Science Products, GB 150TF-8P) and pulled with a DMZ-Universal Puller (Zeitz). Recording pipettes had a resistance of 0.5 – 1.2 MΩ when filled with 3 M KCl solution. ND96 was the recording solution. Voltage protocols and data analysis were the same than in whole-cell patch-clamp (see [supplementary methods](#)).

2.12. Vascular reactivity

Rat PA rings were mounted in a wire myograph (model 610 M or 620 M, Danish Myo Technology) in Krebs buffer, maintained at 37 °C and continuously bubbled the chamber with 21 % O₂ + 5 % CO₂ + 74 % N₂. [28] Vessels were stretched to give an equivalent transmural pressure of 30 mmHg. The wire myograph is connected to a digital system to record the vessel tension (PowerLab, ADINSTRUMENTS). To study the effects of S1R modulation on vascular reactivity, PA were incubated for 24 h in the absence or in the presence of the S1R agonist, PRE084 (1, 20 or 50 µM). Thereafter, preparations were first stimulated with 80 mM KCl. Afterwards, the contractile responses induced by phenylephrine (Phe, 1 nM - 3 µM) were examined. The contraction to Phe was also tested after 15 min incubation with DPO-1 (1 µM). In another set of experiments, PA were incubated under hypoxic conditions (3 % O₂, 48 h) and then the contractile responses to 80 mM KCl, DPO-1 (1 µM) or serotonin (5-HT, 10 nM - 30 µM) were tested. Data were represented as active effective pressure (AEP; mN/mm²) or as a percentage of KCl response. Concentration-response curves were fitted to a logistic equation. The maximal drug effect (Emax) and the negative log molar drug concentration producing 50 % of the Emax (pD₂) were calculated from the fitted curves for each ring.

2.13. Cell proliferation

Details on human PSMC isolation and culture are provided in [Supplementary Methods](#). Cell proliferation was measured by a colorimetric immunoassay based on the measurement of the thymidine analogue 5-bromo-2-deoxyuridine (BrdU), during DNA synthesis (Roche Applied Science).

2.14. Transcriptomic analysis

The data were downloaded from the publicly available Gene Expression Omnibus GEO- dataset GSE113439 and the specific methods are described in Mura et al [29]. The expression in the control (*N* = 11) and the PAH (*N* = 14) samples was compared for the whole transcriptome by the GEO2R analysis tool.

2.15. Immunofluorescence

For S1R microscopy assays, as HeLa cells were transfected with proteins fused to fluorescence proteins, cells were directly visualized in a confocal microscopy (LSM710, Zeiss).

2.16. Drugs

All drugs and reagents were obtained from Sigma-Aldrich except PRE084 (Cayman), and SKF10047 and BD1047 (Tocris Bioscience).

2.17. Statistical analysis

The data are presented as mean ± SEM of *n* measurements, where *n* identifies the number of independent experiments (in the case of patch-

clamp experiments, the number of cells or oocytes registered). N identifies the number of independent animals or human samples. Data were tested for normal distribution using the Shapiro-Wilk test and parametric or non-parametric statistics were used as appropriate. Comparisons were performed by a student t test or Mann-Whitney test (paired in the case of comparing control condition vs. drug effect in the same cell)

or with one-way ANOVA to compare more than two groups with a Dunnett or a Dunn-test as post-hoc analysis. All the statistical analysis was done using GraphPad Prism 8. Statistical significance was set at $P < 0.05$.

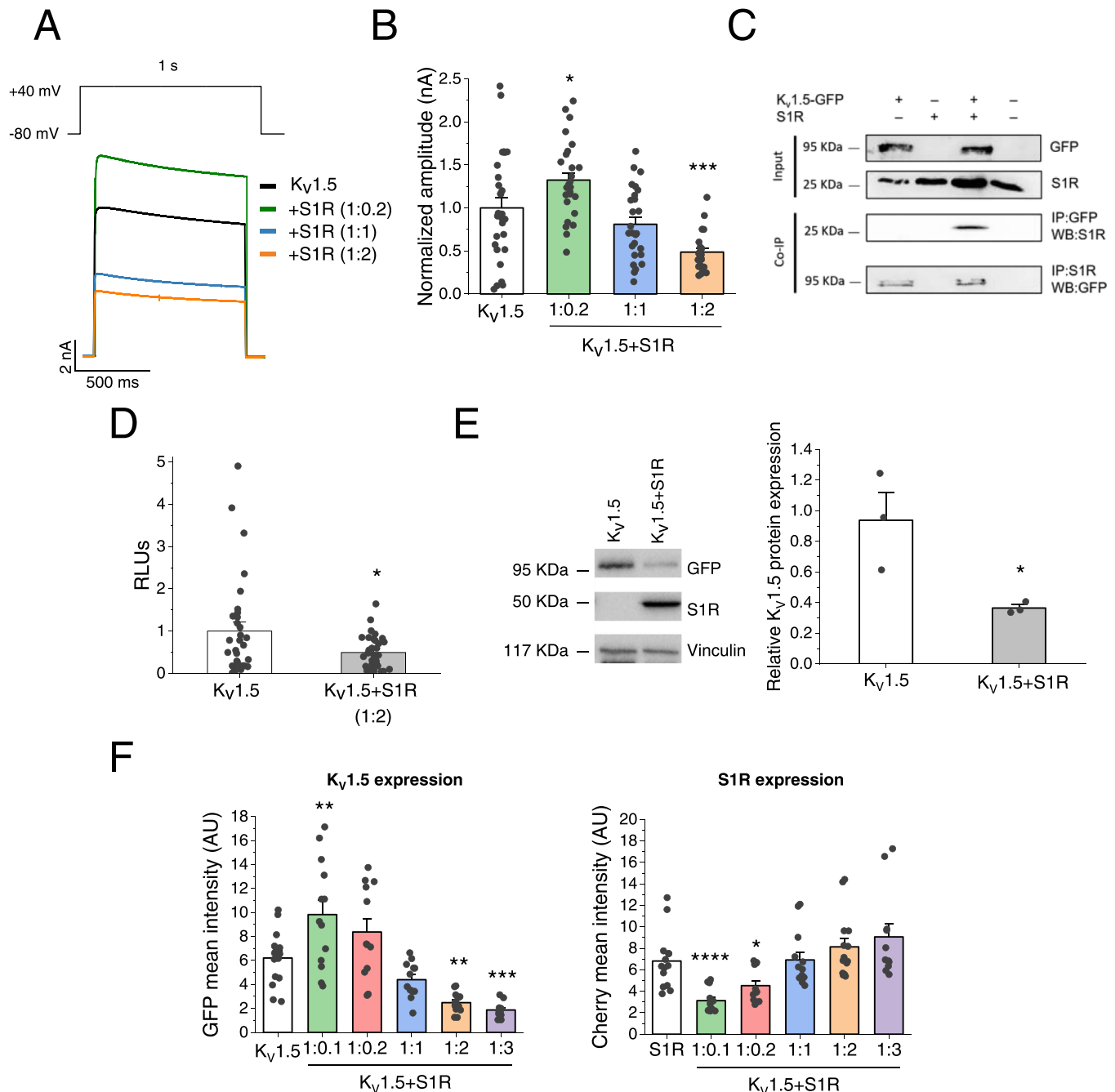


Fig. 1. Modulation of $K_v1.5$ by S1R. (A) Representative current traces of $K_v1.5$ and $K_v1.5 + S1R$ at different $K_v1.5:S1R$ ratios (1:0.2, 1:1, 1:2) recorded in *X. laevis* oocytes. (B) Maximum current amplitude of $K_v1.5$ and $K_v1.5 + S1R$ at different ratios, 48 h after injection. Maximum current at +40 mV (nA) in all conditions was normalized to $K_v1.5$ amplitude ($n = 22-28$ oocytes, from $N = 3$ *X. laevis*). (C) Protein extracts were prepared from HeLa cells (input). The Co-IP was performed by immunoprecipitating with an anti-GFP antibody and probing with an anti-S1R antibody ($n = 2$). To confirm the interaction, a reverse Co-IP experiment immunoprecipitating with an anti-S1R antibody and probing with an anti-GFP antibody was performed ($n = 1$). (D) Protein expression of $K_v1.5^{HA}$ in the plasma membrane of *X. laevis* oocytes injected with $K_v1.5^{HA}$ or $K_v1.5^{HA} + S1R$ (1:2 ratio). RLU: relative light units ($n = 32$ oocytes, from $N = 2$ *X. laevis*). (E) Relative protein expression of $K_v1.5$ (GFP⁺) and S1R in $K_v1.5$ and $K_v1.5 + S1R$ conditions, analysed by western blot in HEK293 cells and normalized by vinculin expression ($n = 3$). (F) Mean intensity of the GFP and cherry signals (arbitrary units: AU) by flow cytometry in HEK293 cells at different $K_v1.5:S1R$ ratios ($n = 12-15$). * : $P < 0.05$, ** : $P < 0.01$, *** : $P < 0.001$ and **** : $P < 0.0001$ vs. $K_v1.5$. In panel B and F right vs. S1R. In panels B and D, data were analysed by a 1-way ANOVA followed by a post-hoc Dunnett test, in D and E, by a two-tailed unpaired student t-test.

3. Results

3.1. Bimodal modulation of $K_{V1.5}$ channels by S1R

To start the characterization of the potential interaction between S1R and the $K_{V1.5}$ channel we first studied the potassium currents recorded by TEVC in *X. laevis* oocytes transfected with a single $K_{V1.5}$ and different S1R cRNA concentrations. Fig. 1A shows representative overlapping current traces at different S1R concentrations. Interestingly, we found that low S1R concentrations ($K_{V1.5}$:S1R ratio 1:0.2) increased, while higher concentrations (1:2) decreased $K_{V1.5}$ current amplitude (Fig. 1B).

Next, we searched for potential evidence of physical protein-protein interaction between $K_{V1.5}$ and S1R by Co-IP studies. To this end, HeLa cells co-transfected with $K_{V1.5}$ and S1R were immunoprecipitated with the anti-GFP antibody and probed with the anti-S1R antibody, consistent with the Co-IP of the two proteins. This protein-protein interaction was confirmed by a reverse immunoprecipitation with the anti-S1R antibody and by probing with the anti-GFP antibody (Fig. 1C).

Since changes in current amplitude may be essentially due to alterations in gating and/or expression of $K_{V1.5}$ channels in the plasma membrane, we studied this second possibility in *X. laevis* oocytes injected with $K_{V1.5}$, now harbouring an extracellular HA-epitope, alone or with S1R (ratio $K_{V1.5}$:S1R 1:2). Then, the surface expression was probed with ELISA assays, exclusively detecting channels at the plasma membrane. Our data revealed that the decreased current amplitude at that ratio was linked to a decreased surface expression of $K_{V1.5}$ channels (Fig. 1D). A similar downregulation of total $K_{V1.5}$ channel expression was observed in HEK293 cells transfected with a 1:2 $K_{V1.5}$:S1R ratio by Western blot (Fig. 1E) and in HeLa cells by fluorescence microscopy (see Supplementary material online, Fig. S1). Interestingly, S1R did not affect any electrophysiological feature apart from the referred decrease in current amplitude (see Supplementary material online, Table S1) suggesting that this chaperone mainly affects the expression rather than the gating of $K_{V1.5}$ channels. To analyse whether S1R also exerts a bimodal regulation of $K_{V1.5}$ channel expression, $K_{V1.5}$ -GFP and S1R-cherry plasmids were transfected at different ratios in HEK293 cells, and the mean fluorescence signal of both proteins was determined by flow cytometry. Our data confirmed that overexpression with low S1R levels was associated with increased total $K_{V1.5}$ channel expression while higher S1R concentrations led to downregulation of $K_{V1.5}$ channels (Fig. 1F).

Finally, to elucidate whether the modulation by S1R was specific for $K_{V1.5}$ channels, we also analysed its effects on $K_{V2.1}$ and $K_{ir2.1}$ channels in *X. laevis* oocytes. We found that current amplitude recorded at +40 mV was similar for $K_{V2.1}$ (2.48 ± 0.30 nA, $n = 14$) and $K_{V2.1}$ +S1R (2.41 ± 0.26 nA, $n = 14$; $P > 0.05$) (see Supplementary material online, Fig. S2A, C). Similarly, $K_{ir2.1}$ current amplitude recorded at -140 mV (1.40 ± 0.13 nA, $n = 14$) was not significantly affected by S1R (1.22 ± 0.11 nA, $n = 17$; $P > 0.05$) (see Supplementary material online, Fig. S2B-C).

3.2. Incubation with S1R ligands modulate $K_{V1.5}$ channels

Next, we studied whether S1R ligands could modulate $K_{V1.5}$ channel expression and activity. We found that acute exposure (~10 min) to the agonist PRE084 led to a reduction (< 20 %) of the potassium current amplitude that was similar in HEK293 cells transfected with $K_{V1.5}$ or $K_{V1.5}$ +S1R (see Supplementary material online, Fig. S3A, B). Notably, a similar inhibitory effect was observed after acute challenge to the S1R antagonist BD1047, which was also unaltered by S1R expression (see Supplementary material online, Fig. S3C, D).

Since our data suggested that S1R affects mainly the expression rather than the gating of $K_{V1.5}$ channels we then focused on the sustained (24 h) effects of PRE084. $K_{V1.5}$ channel activity assessed by whole-cell patch-clamp markedly augmented following the incubation

with PRE084 in HEK293 cells transfected with $K_{V1.5}$ +S1R (2.55 ± 0.26 nA, $n = 36$ vs. 3.44 ± 0.28 nA, $n = 33$ at +40 mV, in the absence and in the presence of PRE084, respectively; $P < 0.05$) (Fig. 2A, B). Of note, this enhancing effect of PRE084 was not observed in cells only transfected with the channel (i.e. in the absence of S1R; see Supplementary material online, Fig. S4A-B).

Protein expression analysis revealed that 24 h incubation with the S1R agonists PRE084 or SKF10047 led to a marked increase in $K_{V1.5}$ expression (Fig. 2C left). Conversely, chronic (24 h) but not acute (2 h) incubation with the S1R antagonist BD1047 reduced $K_{V1.5}$ expression (Fig. 2D left). Neither of these modulators significantly affected the S1R expression (Fig. 2C-D, right panels). Of note, the effect of PRE084 on $K_{V1.5}$ expression was not observed in cells not transfected with S1R (see Supplementary material online, Fig. S4C) strongly suggesting that PRE084 effect was through S1R.

3.3. The S1R agonist PRE084 increases $K_{V1.5}$ current and decreases contraction in pulmonary arteries

Since $K_{V1.5}$ channels play an important role in the pulmonary circulation we aimed to study the impact of S1R modulation on these channels in rat isolated PASM. Whole-cell patch-clamp recordings from PASM revealed that the incubation during 24 h with PRE084 dramatically increased the total K^+ current (Fig. 3A). To analyse whether the $K_{V1.5}$ current, which represents the main component of the total K_V current in the pulmonary vasculature, was affected by PRE084, cells were perfused with DPO-1 (1 μ M), a selective $K_{V1.5}$ channel inhibitor. DPO-1-sensitive currents, which reflect the $K_{V1.5}$ channel component, were markedly higher in PASM following PRE084 (20 μ M) treatment (54.15 ± 10.03 pA/pF at +40 mV, $n = 9$) in comparison with those observed in PASM treated with vehicle (27.61 ± 2.91 pA/pF, $n = 8$; $P < 0.05$) (Fig. 3B left). A further increase in $K_{V1.5}$ currents was observed when PA were incubated with a higher concentration of PRE084 (50 μ M), yielding values of 128.29 ± 18.97 pA/pF at +40 mV ($n = 6$; $P < 0.01$ vs Control; see Supplementary material online, Fig. S5). On the other hand, PRE084 (20 μ M) did not significantly affect the DPO-1-insensitive current confirming that the increase in K^+ current was mainly due to $K_{V1.5}$ channels (Fig. 3B right). PRE084 did not modify the voltage-dependence activation (Fig. 3C) but induced a lower degree of inactivation in the total current than that observed in control PASM (3.53 ± 1.74 % and 17.08 ± 5.82 %, respectively, $n = 10$; $P < 0.05$) (Fig. 3D).

As mentioned in the introduction, $K_{V1.5}$ channel activity/expression critically regulates PA contractility. Therefore, the effects of 24 h incubation with PRE084 (20 μ M) on the contractility of these vessels were studied. The vasoconstrictor response to KCl (80 mM), which is regarded as an index of the contractile capacity of the vessel, were unaffected by incubation with 20 μ M PRE084 (1.84 ± 0.22 mN/mm², $n = 12$ vs. 1.64 ± 0.23 mN/mm², $n = 9$). On the other hand, PRE084 decreased PA contraction to Phe (Fig. 4A-B). In particular, PRE084 reduced the maximal contraction (E_{max}), but not the potency, of Phe in a concentration-dependent manner (see Supplementary material online, Table S2). Moreover, the attenuated Phe contraction by PRE084 (20 μ M) was not observed in the presence of DPO-1 (Fig. 4C-D), strongly suggesting that the attenuation of pulmonary vasoconstriction induced by PRE084 was mainly due to an increase in $K_{V1.5}$ channel activity. In addition, to test whether PRE084 influences cell proliferation or apoptosis, human PASM were incubated with increasing concentrations of PRE084 (1, 20 and 50 μ M) for 24 h. Our results showed that PRE084 decreased PASM proliferation in a concentration-dependent manner (Fig. 4E). On the other hand, this drug did not significantly affect cell viability as assessed by flow cytometry analysis of annexin-V and propidium iodide staining of human PASM (see Supplementary material online, Fig. S6).

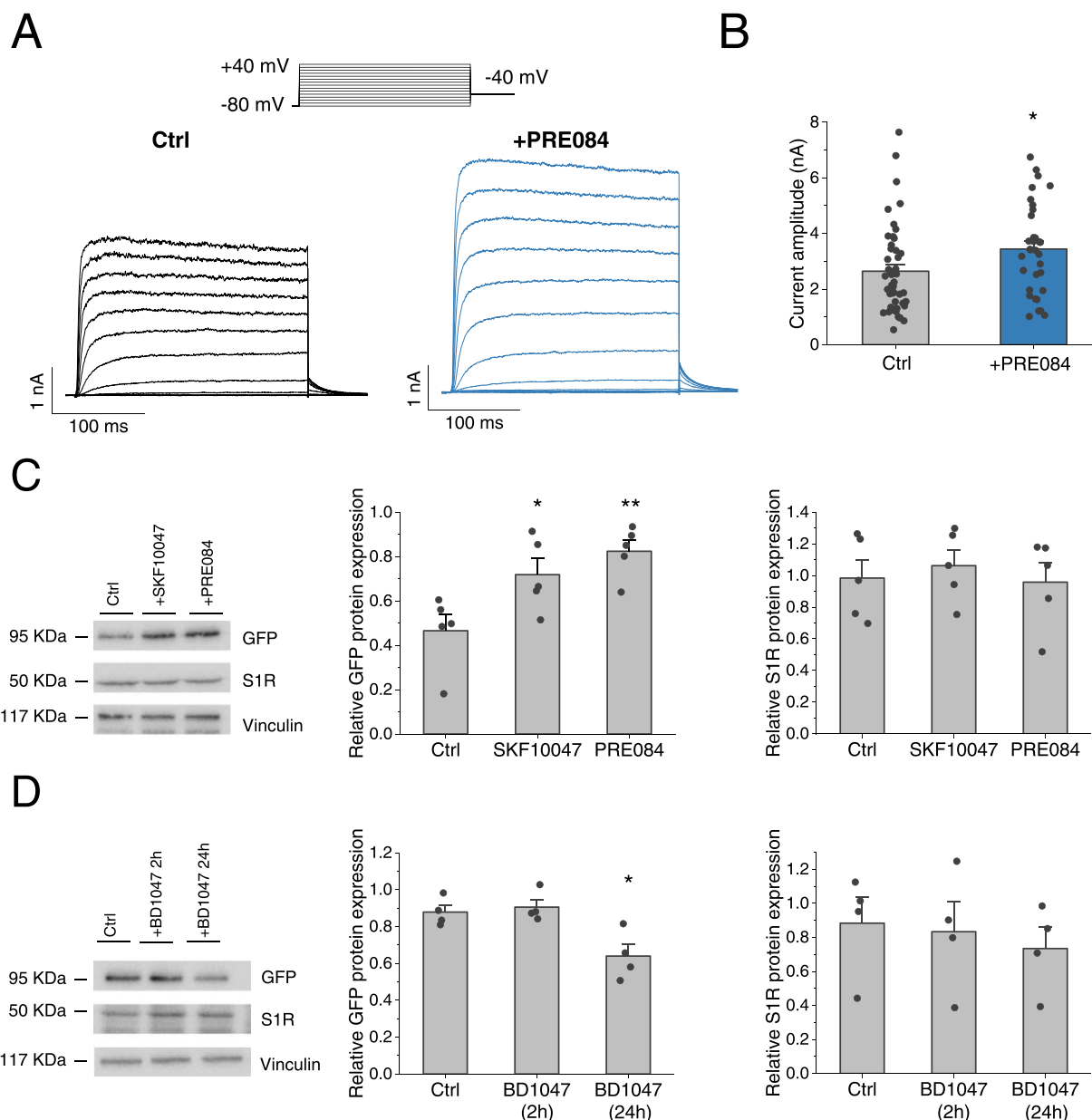


Fig. 2. Modulation of $K_v1.5$ activity and expression by S1R ligands in HEK293 cells. **(A)** Representative I-V recordings and **(B)** average data of the maximum $K_v1.5$ current amplitude at +40 mV was analysed in cells transfected with $K_v1.5$ and S1R after incubation for 24 h with ($n = 33$) or without PRE084 (10 μ M) ($n = 47$). **(C)** Cells transfected with $K_v1.5$ +S1R were incubated for 24 h with PRE084 (20 μ M) or SKF10047 (20 μ M) ($n = 5$). **(D)** Cells transfected with $K_v1.5$ +S1R were incubated with BD1047 (10 μ M) for 2 or 24 h ($n = 4$). In B and C panels, relative $K_v1.5$ (left) and S1R protein expression (right) by western blot was probed with an anti-GFP or anti-S1R antibody and normalized by vinculin expression. *: $P < 0.05$, **: $P < 0.01$ vs. control. In panels B, data was analysed by a two-tailed unpaired student t-test. In C and D, by a 1-way ANOVA followed by a post-hoc Dunnett test.

3.4. The expression of $K_v1.5$ and S1R is decreased in PAH and AF

Next, we aimed to determine whether the expression of S1R was impaired in cardiovascular diseases associated with altered $K_v1.5$ channel expression such as PAH and AF. Regarding PAH, first we performed a transcriptomic analysis which revealed that, both *KCNA5* and *SIGMA1R* mRNA levels were clearly downregulated in samples from PAH patients (Fig. 5A). Moreover, the expression of both genes showed a positive correlation, with a $P = 0.038$ (Fig. 5B). When the data was stratified according to PAH types, a similar downregulation was observed in all types of PAH studied, although some data did not reach statistical significance, probably due to the low number of samples (Fig. 5C). In line with these data, we found that $K_v1.5$ and S1R protein expressions were also downregulated in lung samples from PAH patients

compared to controls (Fig. 5D). While both proteins exhibited a subtle positive correlation trend this did not reach statistical significance (Fig. 5E).

Western-blot analyses also revealed that $K_v1.5$ and S1R were downregulated in AF samples compared with the control ones (Fig. 6A) and both proteins exhibited a positive correlation with a P-value of 0.0008 (Fig. 6B). Additionally, in all types of AF studied: paroxysmal, permanent, and perioperative, S1R was downregulated, but in the case of $K_v1.5$, it was downregulated in permanent and perioperative AF but not in paroxysmal (Fig. 6C).

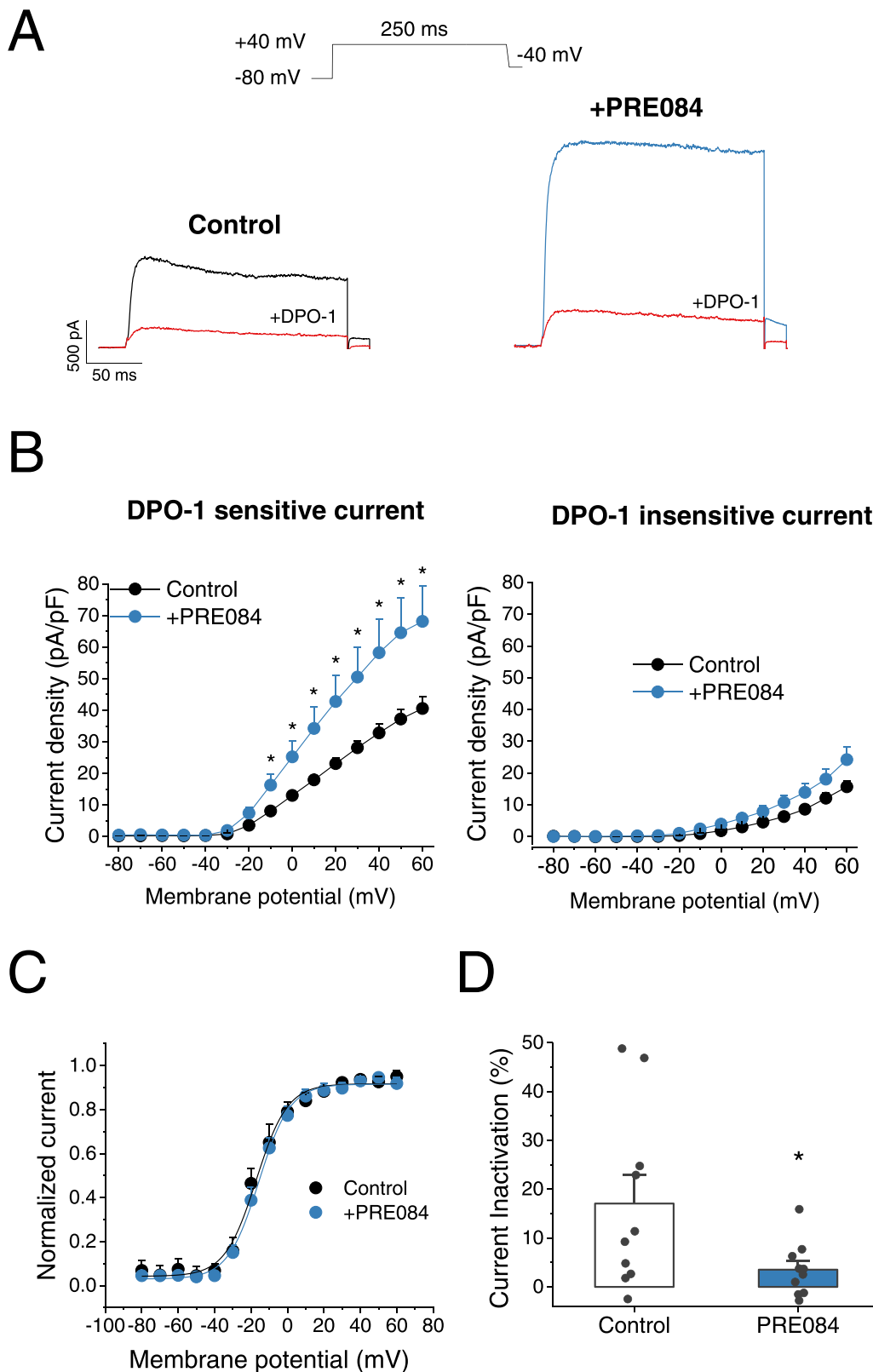


Fig. 3. Effect of PRE084 on K^+ currents in PASM. **(A)** Representative current traces of K^+ current at +40 mV in control condition (left, black) and incubated with PRE084 (20 μ M) for 24 h (right, blue). The current was registered in the absence and in the presence of DPO-1 (red). **(B)** I-V relationships obtained after plotting the maximum current amplitude, normalized by cell capacitance, at each membrane potential ($n = 8-9$ cells from $N = 6$ rats). The DPO-1 sensitive current is shown on the left and the DPO-1 insensitive current on the right. **(C)** Normalized activation curves in control condition and after incubation with PRE084 (20 μ M) ($n = 9$ from $N = 6$ rats per group). **(D)** Degree of inactivation of the current in control condition and after incubation with PRE084 (20 μ M) ($n = 10$ from $N = 6$ rats per group). * : $P < 0.05$ vs. control. Data were analysed by a two-tailed unpaired student t-test.

3.5. The S1R agonist PRE084 preserves $K_v1.5$ channel activity in PASM under hypoxic conditions

Finally, we studied the potential beneficial effects of PRE084 on $K_v1.5$ channel activity in PA after sustained exposure to hypoxia as an in vitro PAH model. Following 48 h exposure to hypoxia K_v currents

present in PASM, which mainly reflect $K_v1.5$ channels as demonstrated by DPO-1 sensitivity, were markedly reduced (Fig. 7A). Notably, treatment with PRE084 was able to markedly increase the attenuated K_v currents after hypoxic exposure (Fig. 7A right). The current-voltage relationships of the total K_v current and the DPO-1-sensitive (i.e. $K_v1.5$) current are shown in Fig. 7B and C, respectively. Under hypoxic

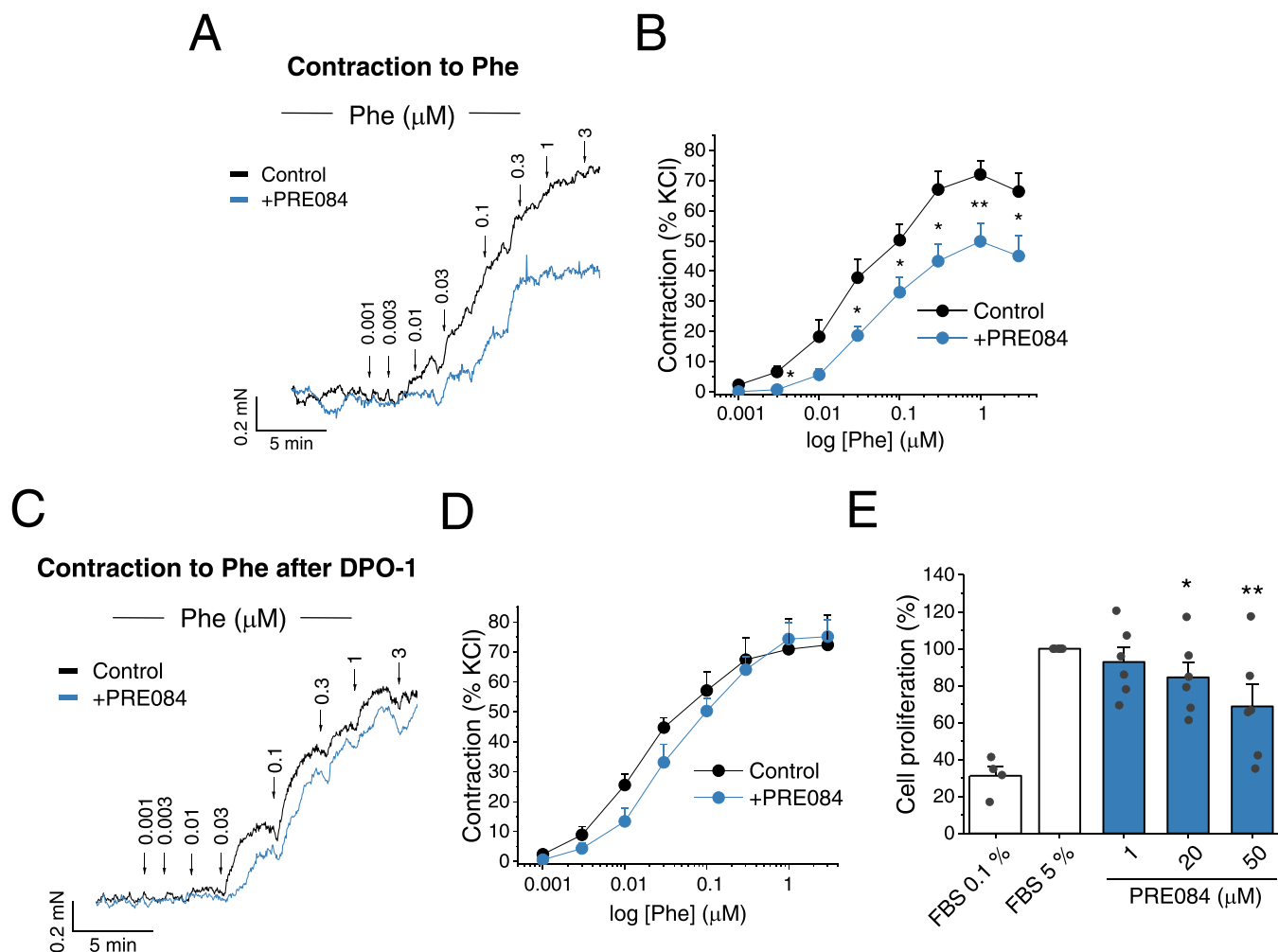


Fig. 4. PRE084 decreases vasoconstriction and proliferation in PA. (A) Representative recording and (B) average data of the concentration-response curves to phenylephrine (Phe, 0.001–3 μM) in rat PA incubated for 24 h in the absence (control) or in the presence of PRE084 (20 μM) ($n = 12$ PA from $N = 8$ rats per group). (C) Representative recording and (D) average data of the concentration-response curves to Phe after 15 min treatment with DPO-1 in PA incubated for 24 h in the absence (control) or in the presence of PRE084 (20 μM) ($n = 9$ PA from $N = 6$ rats per group). (E) Concentration-dependent inhibition of human PASMC proliferation induced by PRE084 (1, 20 and 50 μM) ($n = 6$ from $N = 5$ patients). Data were normalized to the control condition (FBS 5 %). FBS 0.1 % was used as a negative control of proliferation ($n = 4$). *: $P < 0.05$, **: $P < 0.01$ vs. control. Data were analysed by a two-tailed unpaired student t-test (panel B) or 1-way ANOVA followed by a post-hoc Dunnett test (panel E).

conditions the $K_{\text{V}}1.5$ channel component recorded at membrane potentials more positive than -30 mV were significantly increased by PRE084 treatment (Fig. 7C), reaching values similar to those recorded after incubation under normoxic conditions. Finally, pulmonary arteries were maintained in hypoxia for 48 h with and without PRE084 prior to functional experiments in the myograph. The vasoconstrictor response to KCl was unaffected by incubation with 20 μM PRE084 (2.41 mN/mm^2 , $n = 7$ vs. 1.86 mN/mm^2 , $n = 6$). On the other hand, the vasoconstriction induced by DPO-1 after hypoxic exposure was greater in PRE084-treated PA than in untreated ones (Fig. 7D). Moreover, PRE084 attenuated the exacerbated contraction induced by serotonin under hypoxic conditions, with no effect on the potency (see Supplementary material online, Table S3). Finally, we determined $K_{\text{V}}1.5$ channel and S1R protein levels to ascertain whether the effects of PRE084 were associated with changes in protein expression. We found that $K_{\text{V}}1.5$ channel expression was not significantly altered by hypoxic exposure, while the addition of PRE084 led to a substantial augmentation of $K_{\text{V}}1.5$ channel expression (see Supplementary material online, Fig. S7). In contrast, S1R protein levels were comparable in the three groups.

4. Discussion

In the present study, we have characterized the modulation of $K_{\text{V}}1.5$ channels by S1R. We demonstrate a physical interaction between both proteins. Interestingly, co-transfection with S1R exerts a bimodal regulation of $K_{\text{V}}1.5$ channel expression and activity consisting of up-regulation and down-regulation as S1R levels rise. S1R ligands regulate $K_{\text{V}}1.5$ channel expression and activity in a S1R-dependent manner. Thus, the S1R agonist PRE084 increases $K_{\text{V}}1.5$ current amplitude in PASMC and attenuates pulmonary vasoconstriction and proliferation. Furthermore, $K_{\text{V}}1.5$ and S1R protein expressions are reduced in human heart and lung samples from AF and PAH patients, respectively. Finally, the ability of PRE084 to increase $K_{\text{V}}1.5$ activity is preserved under hypoxic conditions. Our data identifies S1R as a novel regulator of $K_{\text{V}}1.5$ channels and a potential pharmacological target to explore for cardiovascular diseases associated with $K_{\text{V}}1.5$ channel dysregulation.

$K_{\text{V}}1.5$ channels are found throughout the cardiovascular system carrying out essential functions. Thus, $K_{\text{V}}1.5$ channels are key regulators of pulmonary and systemic vascular tone and atrial repolarization [4,5,7]. Importantly, $K_{\text{V}}1.5$ expression and activity are altered in different cardiovascular diseases. Thus, downregulation of K^+ channels,

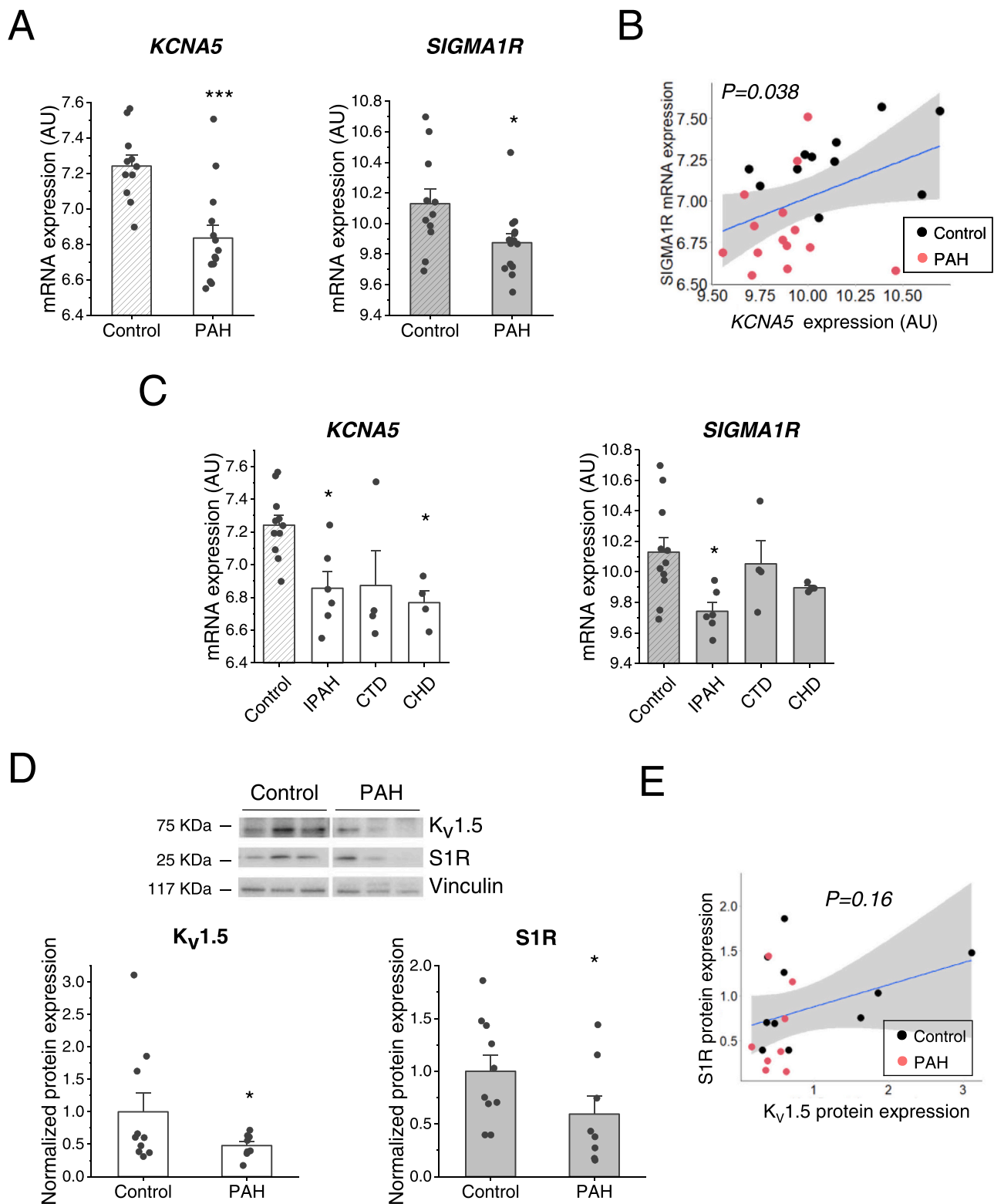


Fig. 5. $K_v1.5$ and S1R mRNA and protein expression in PAH human samples. (A) mRNA expression levels of *KCNA5* (left) and *SIGMA1R* (right) genes in control ($N = 11$) and PAH ($N = 14$) patients after a transcriptomic analysis. (B) Correlation between *SIGMA1R* and *KCNA5* mRNA levels ($N = 25$). The regression line is in blue, and the 95 % confidence interval is highlighted in grey. $P = 0.038$. (C) *KCNA5* (left) and *SIGMA1R* (right) mRNA expression dividing the PAH samples between: IPAHA ($N = 6$), CTD ($N = 4$) and CHD ($N = 4$). (D) Total $K_v1.5$ (left) and S1R (right) protein expression were quantified in human samples from control ($N = 10$) or PAH patients ($N = 8$). Protein expression levels were probed by western blot with an anti- $K_v1.5$ or anti-S1R antibody and normalized by vinculin expression. (E) Correlation between S1R and $K_v1.5$ protein expression ($N = 18$). The regression line is shown in blue, and the 95 % confidence interval, in grey. $P = 0.16$. * : $P < 0.05$, * * : $P < 0.001$ vs. control. Data were analysed by a two-tailed (panel A) or one-tailed unpaired student t-test (panel D) or 1-way ANOVA followed by a post-hoc Dunnett test (panel C).

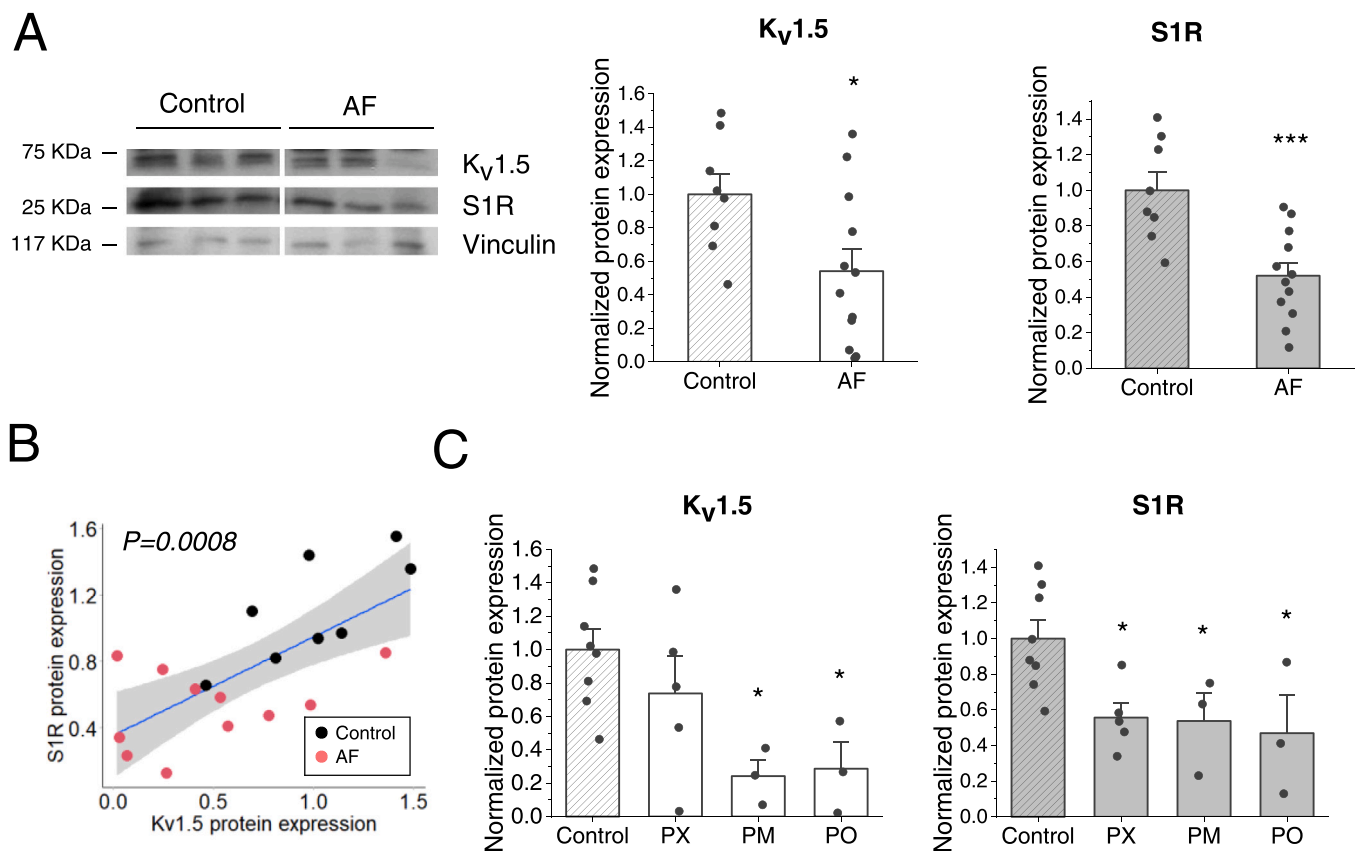


Fig. 6. K_V1.5 and S1R protein expression in AF human samples. (A) Total K_V1.5 (left) and S1R (right) protein expression was quantified in human samples from control (SR) ($N = 8$) or AF patients ($N = 12$). Protein expression levels were probed by western blot with an anti-K_V1.5 or anti-S1R antibody and normalized by vinculin expression. (B) Correlation between S1R and K_V1.5 protein expression. The regression line is in blue and in grey is indicated the 95 % confidence interval. $P = 0.0008$ ($N = 19$). (C) Normalized K_V1.5 (left) and S1R (right) protein expression dividing the AF samples between: PX: paroxysmal ($N = 5$), PM: permanent ($N = 3$), and PO: perioperative ($N = 3$). Protein expression levels were normalized to their control. *: $P < 0.05$, ***: $P < 0.001$ vs. control. Data were analysed by a two-tailed unpaired student t-test (panel A) or 1-way ANOVA followed by a post-hoc Dunnett test (panel C).

particularly K_V1.5 and TASK-1, is considered an early contributor to the pathophysiology of PAH [4,6,30–32]. Similarly, AF is associated with downregulation of most K⁺ channels, including K_V1.5 [33,34]. Thus, the search for strategies to enhance the activity of K_V1.5 channel in these pathological entities has attracted much attention.

S1R regulates the function of a variety of proteins including ion channels, which can occur through direct protein-protein interactions or indirectly through G proteins [9,35]. Moreover, the physical interaction of S1R with an ion channel may affect its gating, trafficking, maturation or expression [12,25,26]. Our data reveal that S1R and K_V1.5 channels physically interact and that K_V1.5 channel activity and expression are closely linked to S1R levels. Thus, S1R causes a bimodal modulation of K_V1.5 channels in both cell types, characterised by an increase and a decrease in activity and expression as S1R levels increase. Aydar E et al. [10] found a similar depression of K_V1.4 currents in *Xenopus oocytes* coexpressed with a 1:1 S1R:Kv1.4 ratio or higher but these authors did not performed experiments overexpressing lower amounts of S1R. Apart from current amplitude, S1R did not significantly influenced voltage-dependent activation and inactivation parameters of K_V1.5 channels hence strongly suggesting that S1R mainly affects their expression rather than their gating. Unlike K_V1.5, K_V2.1 or K_{ir}2.1 currents were not affected by S1R overexpression. Our data on K_V2.1 are consistent with the negligible effect of S1R knockdown previously reported [36] while the lack of effect on K_{ir}2.1 is not in agreement with the proposed S1R-K_{ir}2.1 functional coupling [37]. All these data demonstrate that S1R could represent a new member of the K_V1.5 channelosome [38].

We next explored the effects of S1R ligands on K_V1.5 channels. Two

previous studies have evaluated the effect of S1R agonists on K_V1.5 currents in heterologous systems [10,26]. Aydar et al. reported that in *Xenopus oocytes* transfected with K_V1.5 and S1R, the S1R agonist SKF10047 (100 μM) acutely perfused (2 min) inhibited K_V1.5 current in a S1R-dependent manner [10]. A more recent study found a similar inhibitory effect on K_V1.5 by PRE084 and SKF10047 in HEK293 cells [26]. Herein we also observed that acute administration of these S1R ligands led to a substantial inhibition of the K_V1.5 current. In line with previous studies on K_V1.2 [26], K_V1.3 [13], and K_V2.1 [36] we found that the inhibitory effect of S1R ligands was unaffected when S1R was co-expressed. Moreover, we observed a similar inhibitory effect when testing the S1R antagonist BD1047. These data challenges whether the inhibitory effects observed after acute exposure to S1R ligands are indeed mediated via S1R.

Since our data suggested that K_V1.5 regulation by S1R involves changes in channel expression we examined the chronic effects of S1R ligands on K_V1.5 current and expression. We found that 24 h exposure to SKF10047 and PRE084 led to a substantial increase in K_V1.5 current which was associated with an increased expression of the channel. Importantly, these effects were observed when HEK293 cells were co-expressed with K_V1.5 and S1R but not when cells were only transfected with the channel, indicating that K_V1.5 upregulation by the ligands is S1R dependent. Although studies analysing the chronic effect of S1R ligands on K_V1.5 channels are lacking, there is evidence that other K_V channels can be upregulated in an S1R-dependent manner [12]. Conversely, K_V1.5 channel expression was decreased by the S1R antagonist BD1047. In addition, S1R protein levels in HEK293 cells and isolated PA were not altered by S1R ligands, which suggest that these

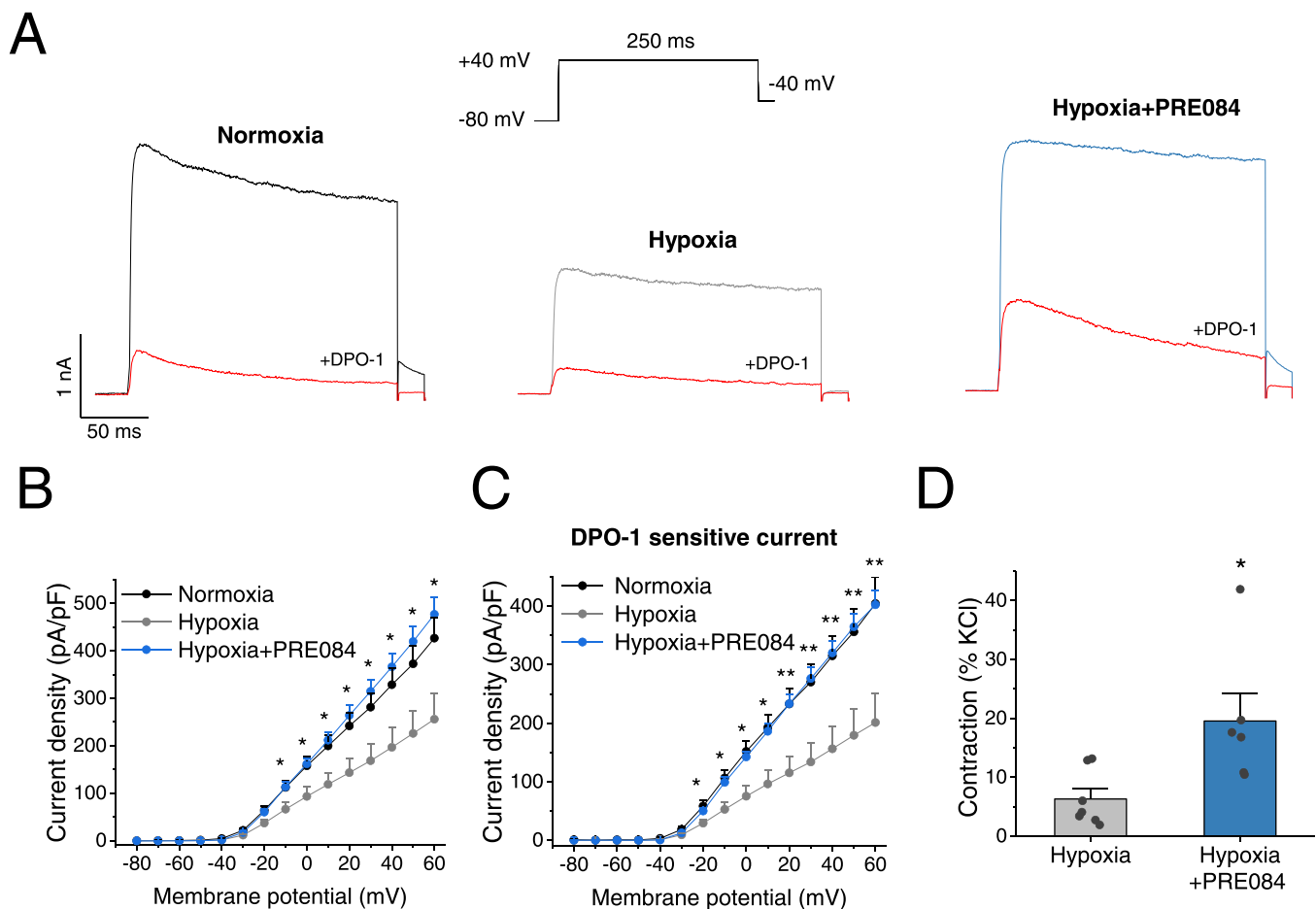


Fig. 7. PRE084 increases $K_v1.5$ currents in PASMCM following hypoxic exposure. (A) Representative current traces of K^+ currents at +40 mV in rat PASMCM after 48 h incubation under normoxic (left, black), hypoxic (grey) or hypoxic plus PRE084 (20 μ M) (right, blue) conditions. The current was recorded in the absence and in the presence of DPO-1 (red). (B) Current-voltage relationships obtained after plotting the maximum current amplitude, normalized by cell capacitance, at each membrane potential (Control $n = 9$, hypoxia $n = 9$, hypoxia+PRE084 $n = 6$ from $N = 6-7$ rats per group) and (C) the DPO-1 sensitive current (Control $n = 8$, hypoxia $n = 8$, hypoxia+PRE084 $n = 6$ from $N = 6-7$ rats per group). * and ** indicate $P < 0.05$ and $P < 0.01$ hypoxia+PRE084 vs hypoxia, respectively. (D) Average data of the vasoconstriction response to DPO-1 in hypoxic-incubated PA untreated or treated with PRE084 (20 μ M). ($n = 6-7$, from $N = 6-7$ rats per group) *: $P < 0.05$ hypoxia vs hypoxia+PRE084. Data were analysed by a 1-way ANOVA followed by a post-hoc Dunnett test (panels B and C) or two-tailed unpaired student t-test (panel D). Data are expressed as mean \pm SEM.

ligands affect the oligomerization state of S1R rather than its expression. Further research is needed to elucidate the precise functional consequences of S1R oligomerization, but it seems to be key for the interaction with target proteins [14].

Overall, our data showed that $K_v1.5$ channel activity and expression may be fine-tuned by S1R ligands and encouraged us to move to a more physiological system. Thus, we evaluated the effects of PRE084 in pulmonary arterial myocytes characterised by high $K_v1.5$ channel activity [30]. We found that 24 h incubation with PRE084 produced a strong increase in the $K_v1.5$ current present in PASMCM. In the vascular system, an increase in the $K_v1.5$ current limits membrane depolarization and thus reduces the open probability of the voltage-gated calcium channels, hence decreasing Ca^{2+} entry and attenuating vasoconstriction [4,5,30]. In this sense, our vascular reactivity experiments showed that PRE084-exposed PA had an attenuated contraction to Phe in a concentration-dependent manner. Importantly, this effect was prevented in the presence of the $K_v1.5$ channel inhibitor DPO-1, which strongly suggests the increase in $K_v1.5$ channel activity as the underlying mechanism for the attenuated vasoconstriction.

In addition to vascular tone regulation, $K_v1.5$ channel activity controls cellular processes such as apoptosis and proliferation, being an increase in the $K_v1.5$ current associated with a decrease in early and late apoptosis in PASMCM [2]. In this regard, K^+ is the dominant cation in the

cytoplasm and thus plays an important role in the maintaining of the cell volume. In early apoptosis, decreased K_v activity inhibits apoptotic cell shrinkage, and in later stages of apoptosis, decreases caspase activation and DNA fragmentation [2,39]. Furthermore, S1R plays an important role in counteracting ER stress and decreasing apoptosis [8,20]. In fact, PRE084 has also been shown to reduce cell apoptosis [40]. Here, we demonstrate that PRE084 decreases PASMCM proliferation in a concentration-dependent manner. Nevertheless, our findings do not support an effect of PRE084 on apoptosis in PASMCM.

Considering the ability of S1R agonists to modulate $K_v1.5$ channels and affect pulmonary vascular function and given the role of $K_v1.5$ channel dysfunction in PAH, we also examined the S1R expression in human PAH samples. A transcriptomic analysis revealed decreased $K_v1.5$ mRNA in lung samples from PAH samples which was concurrent with diminished S1R. In line with these results, protein levels of both $K_v1.5$ and S1R were also decreased in PAH samples. Likewise, a similar protein downregulation of $K_v1.5$ and S1R was observed in cardiac human samples from AF, in which $K_v1.5$ channel dysfunction is also considered a key contributor. Consistent with these data, several experimental studies have also found a reduced S1R expression in the atrium of a rat model of AF [22] and in the right ventricle of PAH rats [41]. In our study mRNA and protein expression of $K_v1.5$ and S1R showed a positive correlation in both pathologies. Nevertheless, further

research is needed to elucidate whether the S1R downregulation has any causal relationship with lower $K_V1.5$ expression in AF and/or PAH and could contribute to the pathophysiology of these diseases. Moreover, studies in animal models of these diseases would be necessary to determine whether the reduced expression of S1R may limit the therapeutic potential of S1R agonists in these pathological conditions.

We finally evaluated the potential beneficial effects of PRE084 on $K_V1.5$ channel dysfunction following sustained hypoxic exposure, which represents a standard in vitro model of PH due to its role as an underlying trigger for different forms of pulmonary hypertension including PAH (group I PH) and especially that due to respiratory diseases (group III PH). Interestingly, we found that PRE084 was able to rescue hypoxic downregulation of $K_V1.5$ channel activity and to attenuate the exacerbated 5-HT contraction observed after hypoxic exposure, pointing to a potential benefit effect in PH associated with hypoxia. Of note, the reduced $K_V1.5$ channel activity in PA after 48 h hypoxic exposure was not associated with changes in $K_V1.5$ channel or S1R protein expression. Previous studies have shown that in PASMC, mRNA levels of $K_V1.5$ are reduced after 48 h incubation with hypoxia [42], while the reduction in the amount of $K_V1.5$ channel protein seems to require longer incubations (≥ 60 h) [42–44]. This suggests that the attenuated $K_V1.5$ activity observed after ≤ 48 h of hypoxia observed herein and previously reported [31,45,46] does not appear to be strictly related to a decrease in the total amount of $K_V1.5$ channel protein but may involve, among others, alterations in gating, trafficking, or surface expression of the channel [4,5].

The ability of PRE084 to increase $K_V1.5$ current under both normoxic and hypoxic conditions may be of great relevance. Since $K_V1.5$ channels are considered a key mechanism for hypoxic pulmonary vasoconstriction [28,43,47–49], their preservation by PRE084 under hypoxic conditions is expected to maintain this crucial protective mechanism that divert blood flow away from hypoxic lung areas to the best oxygenated alveoli [49]. Although more research is needed to fully address these questions, there are several examples of protective effects of S1R agonists such as PRE084 in the cardiovascular system, while the antagonists produce the opposite effects [40,50,51]. Moreover, Sun et al. has recently shown that activation of S1R improves right ventricular dysfunction secondary to pulmonary hypertension [41]. Intriguingly, several therapeutic drugs with S1R agonistic properties such as donepezil, the serotonin reuptake inhibitors fluvoxamine and fluoxetine, or dehydroepiandrosterone have shown beneficial effects in experimental PH [41,52–54]. Moreover, fluoxetine-induced protection was linked to $K_V1.5$ channels upregulation [53,55]. Whether S1R activation contributes to these effects requires further studies.

In conclusion, our data reveal that S1R functionally interacts with the $K_V1.5$ channel and acts as a critical regulator of its expression and activity. In the arterial pulmonary vessels, PRE084 is able to increase $K_V1.5$ currents and reduce contraction and proliferation of PASMC. Of note S1R expression is downregulated in human AF and PAH. Altogether, these data identify S1R as a potential novel pharmacological target in diseases associated with an impaired $K_V1.5$ channel function.

5. Conclusions

Pulmonary arterial hypertension and atrial fibrillation are severe cardiovascular diseases associated with electrical remodelling including $K_V1.5$ channel dysfunction. Thus, novel pharmacological strategies to improve the attenuated $K_V1.5$ channel function are highly demanded. Here, we show that the chaperone S1R finetunes $K_V1.5$ channel expression and function and S1R modulators may effectively enhance $K_V1.5$ channel function. Our study identifies S1R as a novel pharmacological target to rescue the attenuated $K_V1.5$ channel function in cardiovascular diseases associated with channel malfunction.

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CRedit authorship contribution statement

A.C., T.G., N.D. and A.V.-Z., conceived and designed the research. A.V.-Z., M.B.-N., M.V.-E., S.R., B.B., G.T., A.B.-B., F.P.-V. and B.C., performed the experiments and analysed the experimental data. J.A.B. provided the cardiac human samples. B.C., F.P.-V., C.V., N.D, T.G. and A. C. interpreted the data. A.V.-Z and A.C. drafted the manuscript. All authors edited and revised the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2023.106684.

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