

SUPPLEMENTAL MATERIAL

PDE8 governs cAMP/PKA-dependent reduction of L-type calcium current in human atrial fibrillation

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SUPPLEMENTARY METHODS

RNA isolation, cDNA synthesis, real-time qPCR and quantification. Frozen tissue samples from 48 patients (16 SR, 8 pAF, 8 cAF, 8Ctl and 8 HF patients) were weighed and sited in pre-cooled tubes containing TRIzol® reagent (Invitrogen, Life Technologies, France) and rapidly subjected to automated grinding in a Bertin Precellys 24 (Bertin Technologies, France). Total RNA extraction was carried out using standard procedure according to the manufacturer's instructions. RNA concentration and purity were evaluated by optical density (Biophotometer, Eppendorf, BioServ, France). RNA integrity was analysed on a Bioanalyzer 2100 with the RNA6000 Nano Labchip Kit (Agilent Technologies, Santa Clara, CA, USA). First strand cDNA synthesis was performed from 1 µg of total RNA with random primers and MultiScribe™ Reverse Transcriptase according to the provided protocol (Applied Biosystems, Life Technologies, France). RNA integrity number (RIN) values were ranged between 5.6 and 8.5, with a mean of 6.9 ± 0.2 for SR, 6.4 ± 0.3 for pAF, 6.6 ± 0.2 for cAF, 7.0 ± 1.1 for Ctl and 7.4 ± 0.9 for HF. There were no differences between groups in RNA content. Real-time PCR assays were performed as previously described¹⁸ to study the expression of 2 target genes (*PDE8A*, *PDE8B*) related to specifically chosen²⁸ reference genes (*POLR2A*, *YWHAZ*, *GAPDH*, *IPO8*, *PPIA*). Experiments were performed in triplicates for each sample. An average Ct value was calculated for each group of patients. Relative gene expression ratio was determined using the $\Delta\Delta C_t$ method and normalized by the geometric mean of the set of stable reference genes. Normalization was performed using multiple reference genes instead of one in order to measure expression levels of targeted genes accurately. *POLR2A*, *YWHAZ*, *GAPDH*, *IPO8*, *PPIA* were chosen and validated as reference genes for human heart samples a previously described²⁶. The normalization factor was calculated using the geometric mean of these five reference genes instead of

the arithmetic mean in order to control better possible outliers and abundance differences between the different genes.

Western Blot analysis. 54 snap-frozen atrial samples were used to perform western blot. From those, 12 samples were first 5 min incubated with control buffer (CON), 30 nM PF-04957325 (PF), or 100 nM β -adrenergic agonist isoprenaline (ISO), and then snap-frozen. The samples were then homogenized in lysis buffer (Tris 0.03M, EDTA 0.005 M, NaF 0.03 M, 3% SDS, 10% Glycerol, Protease- and Phosphatase-Inhibitor Cocktail, Roche) using a homogenizer (MICCRA D-1). After three to five homogenization steps for 10 seconds each, always followed by cooling of the samples in liquid nitrogen, samples were incubated on ice for 1 hour. After centrifugation (900 g, 15 min, RT) supernatants were stored at -80°C until usage. Protein quantification was performed using BCA Protein Assay (Pierce BCA Protein Assay Kit, Thermo Scientific, #23227), samples were denatured at 95°C 5 min and 50 μ g of total protein were loaded on 8% SDS gels for SDS polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane (Amersham, #106.000.02) using a tank blot system. For immunoblot analysis, the following antibodies were used: PDE8A (Santa Cruz Biotechnology sc-30059, rabbit polyclonal antibody, 1:500 in 3% BSA, sample cooking 70°C 10 min), PDE8B (Santa Cruz Biotechnology sc-17234, goat polyclonal antibody, 1:200 in 3% BSA, 70°C 10 min), Cav1.2 α 1C (Alomone ACC-022, rabbit polyclonal antibody, 1:160 in 5% milk, 70°C 10 min), phosphospecific antibody anti-CH3P against Cav1.2-phosphoSer1928 (kindly provided by William A. Catterall, rabbit polyclonal antibody, 1:1000 in 5% milk, 70°C 10 min) and GAPDH (HyTest #5G4.6C5, mouse monoclonal antibody, 1:20,000 in 5% milk). For quantification, band densitometry analysis was done using ImageJ software.

Co-Immunoprecipitation. 15 snap-frozen samples were homogenized in immunoprecipitation buffer (10 mM HEPES, 5 mM EDTA, 500 mM Sucrose, 0.1% SDS,

0.5% Triton, 20 mM NaF, 1 mM Na_3VO_4 , Protease- and Phosphatase-Inhibitor Cocktail). 500 μg of homogenate were incubated with 2 μg Cav1.2 antibody (Alomone; ACC-003) and 150 μl Protein G Sepharose beads (GE Healthcare) overnight. Subsequently, samples were washed 4x using PBS and centrifuged after each wash step (800 g, 2 min, 4°C). Samples were denatured at 95°C 5 min in 2x loading buffer. After centrifugation (800 g, 2 min, 4°C), 30 μl homogenate was loaded on 8-12% SDS gels. Detection of co-immunoprecipitated proteins were performed using specific antibodies against PDE8A (Santa Cruz Biotechnology sc-30059, rabbit antibody, 1:500), PDE8B (Santa Cruz Biotechnology sc-17234, goat antibody, 1:200), PKAII α reg (Santa Cruz Biotechnology sc-908, rabbit antibody, 1:200) and PKAc (BD Biosciences 610980, mouse antibody, 1:200). For quantification, band densitometry analysis was done using ImageJ software.

Isolation and culture of HAMs. After surgical excision, tissue samples were placed into Custodiol® solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) for the transport to the laboratory. Cell isolations from 47 patients were carried out as previously described⁷. Tissue was cut into small cubes and digested in Enzyme solution I [Ca^{2+} free solution containing 0.5 mg ml⁻¹ collagenase (Worthington type 1, 240 U mg⁻¹; Lakewood, New Jersey, USA), 0.5 mg ml⁻¹ proteinase (Sigma type XXIV, 11 U mg⁻¹; St. Louis, Missouri, USA) and 2% bovine serum albumin (BSA; Sigma, St. Louis, Missouri, USA)] at 200 rpm and 37°C 30 min. Next, tissue cubes were transferred to Stop Ca^{2+} free solution [in mM: 88 Sucrose, 88 NaCl, 5.4 KCl, 4 NaHCO_3 , 0.3 NaH_2PO_4 , 1.1 MgCl_2 , 10 HEPES, 20 Taurine, 10 Glucose, 5 Na^+ pyruvate. 7.4 pH at RT; 5% BSA, and 2 μM blebbistatin (Sigma, St. Louis, Missouri, USA)] and moved up and down in a Pasteur pipette. The remaining tissue was digested for five more rounds maximum with Enzyme solution II (Ca^{2+} -free solution containing 0.4 mg ml⁻¹ collagenase and 2% BSA) at 200 rpm and 37°C 15 min. HAMs were harvested by centrifugation (500 rpm, 5 min,

RT). For patch-clamp experiments, HAMs were resuspended in Stop Ca^{2+} free solution and Ca^{2+} was re-introduced by a gentle increase to 1 mmol/L. For Förster-resonance energy transfer (FRET) experiments, HAMs were resuspended in plating medium M1 [MEM (M4780; Sigma, St Louis, Missouri, USA) containing 2 mmol/L Ca^{2+} , 2.5% fetal bovine serum (FBS, Invitrogen, Cergy-Pontoise, France), 1% penicillin-streptomycin and 2 μM blebbistatin]. HAMs were then plated on Laminin-coated dishes (Cellvis Glasbottom dish, 29 mm with 10 mm bottom well). After 2-hours at 37°C and 5% CO_2 , medium was changed to medium M2 (FBS-free M1) containing adenovirus and HAMs were kept in culture at 37°C and 5% CO_2 for 48-hours. The multiplicity of infection (MOI) was 200 PFU/cell. Only striated, rod-shaped HAMs were used.

Perforated patch-clamp in freshly-isolated and cultured HAMs. Whole-cell perforated patch-clamp configuration was used to record $I_{\text{Ca,L}}$ in 16 HAMs from 10 patients as previously described^{4,11}. Pipettes had a resistance between 1.5–3 $\text{M}\Omega$ and were filled with internal solution containing (in mM): aspartic acid 109, CsCl 47, MgCl_2 1, Mg_2ATP 3, Na_2 -phosphocreatine 5, Li_2GTP 0.42, HEPES 10, adjusted to pH 7.2 with CsOH. Amphotericin (250 $\mu\text{g/mL}$) was added to the pipette solution before each experiment. Extracellular solution contained (in mM): CaCl_2 2, MgCl_2 1.8, NaCl 127.1, NaHCO_3 4, NaH_2PO_4 0.33, D-glucose 10, pyruvic acid 5, HEPES 10, MgCl_2 1.8, adjusted to pH 7.4 with NaOH. Amphotericin-B (250-mg/mL) was added to the tip of the pipette solution. For $I_{\text{Ca,L}}$ measurement, cells were depolarized from -80 to -50 and to 0 mV during 200 ms at 0.5 Hz, and the current-voltage (I-V) dependence was determined by depolarizing the myocytes to potentials between -40 and +50 mV (in increments of 10 mV). A 50 ms pre-pulse of -50 mV was used to inactivate voltage-dependent Na^+ -currents. K^+ -currents were blocked by replacing all K^+ -ions with external and internal Cs^+ . Myocytes were voltage-clamped at room temperature using an EPC-10 patch-clamp

amplifier (HEKA Elektronik, Germany). The maximal amplitude of whole-cell $I_{Ca,L}$ was measured at 0 mV as the difference between the peak inward current and the current at the end of the depolarization step. $I_{Ca,L}$ amplitudes were corrected for membrane capacitance. Membrane capacitance was calculated as the time integral of current responses to 1-mV hyperpolarizing step changes in membrane potential. Experiments were performed under stable access resistance only when it had decreased to a value only 5-fold higher than intrinsic pipette resistance. No series resistance compensation was employed.

FRET-based live-cell imaging of sarcolemmal cAMP in HAMs. FRET measurements were performed on 38 living HAMs from 18 patients, transduced with adenovirus encoding pm-Epac1-camps to measure cAMP at the membrane²⁹. Cells were maintained in a K⁺-Ringer solution containing (in mM): 144 NaCl, 5.4 KCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes, adjusted to 7.4 pH with NaOH at RT and images were taken every 5 seconds. The FRET system used consist of an LED light source (pE-100, CoolLED), a beam splitter (DV2, Photometrix) and a camera (CMOS camera optiMOS, QImaging), together with a standard inverted microscope (Nikon eclipse Ti-4) and a 60x/1.5 oil immersion objective. To excite the donor fluorophore (CFP) a single-wavelength light-emitting diode (LED, 440 nm) was used. The LED was controlled by an Arduino digital-to-analogue input-output board (Arduino). A Dual View beam splitter (Cube 05-EM, 505 dcxr, D480/30m, D535/40) was used to split the emission light into donor (CFP) and acceptor (YFP) channels. Single channels intensities were recorded with a CMOS camera (QImaging). The software Micro Manager 1.4.5 was used to perform time-laps image acquisition. Microsoft Excel was used for data analysis. The corrected FRET ratio was calculated as follows: FRET ratio cAMP measurements= $YFP - (B * CFP) / CFP$. Bleed through (B) corrected FRET traces were normalized to baseline and FRET response was quantified.

Immunocytochemistry and confocal imaging. HAMs were fixed with paraformaldehyde (PFA; 2%) for 15 min. After 15 min incubation at RT, PFA was removed and neutralized with Glycine 0.1 M for 10 min. Myocytes were then 3x washed 5 min with phosphate-buffered saline (PBS) and permeabilized with Triton X-100 (0.5%) for 15 min. HAMs were rinsed again three times with PBS and blocked with 1% BSA in PBS for 40 min. Myocytes were then labeled with a primary rabbit polyclonal PDE8A antibody (Santa Cruz Biotechnology sc-30059, 1:200) or a goat polyclonal PDE8B antibody (Santa Cruz Biotechnology sc-17234, 1:200), diluted in PBS containing 10% goat serum and 0.25% Triton X-100. After an overnight incubation at 4°C, HAMs were washed three times using 1% BSA and then incubated for 2 h with AlexaFluor® 488 conjugated anti-mouse IgG to reveal the PDE8 staining. Images of 74 myocytes from 7 SR patients, 50 myocytes from 5 pAF patients, and 60 myocytes from 7 cAF patients were acquired using a Carl Zeiss (Oberkochen, Germany) LSM 510 confocal laser scanning microscope. Optical section series were obtained with a Plan Apochromat 63x objective (NA 1.4, oil immersion). The fluorescence was observed with a BP 505-550 nm emission filter under 488-nm laser illumination, respectively.

Sharp-electrode AP-recordings. APs were recorded with standard intracellular microelectrodes in atrial trabeculae from 5 SR, 5 pAF and 7 cAF patients³⁰. Trabeculae were then mounted on the organ bath and perfused with 50-mL of recirculating (flow rate 7 mL/min), oxygenized Tyrode's solution containing (in mM): NaCl 127, KCl 4.5, MgCl₂ 1.5, CaCl₂ 1.8, glucose 10, NaHCO₃ 22, NaH₂PO₄ 0.42, equilibrated with 5 % CO₂ in 95 % O₂ at 36 °C, pH 7.4. Microelectrodes filled with 2.5 M KCl. Pipette resistances were between 20 and 80 MΩ. Preparations were stimulated at 1-Hz for at least 1-h before data acquisition.

Chemicals. 3-isobutyl-1-methylxanthine (IBMX) was from AppliChem (Darmstadt, Germany). PF-04957325 (PF, MCE, Sweden), Isoprenaline (ISO), laminin, and all other chemicals were from Sigma-Aldrich (Deisenhofen, Germany).