DATA SUPPLEMENT

Phosphodiesterase 4B in the cardiac L-type Ca^{2+} channel complex regulates Ca^{2+} current and protects against ventricular arrhythmias in mice

Jérôme Leroy,[1,2*] Wito Richter,[3*] Delphine Mika,[1,2] Liliana R. V. Castro,[1,2] Aniella Abi-Gerges,[1,2] Moses Xie,[3] Colleen Scheitrum,[3] Florence Lefebvre,[1,2] Julia Schittl,[1,2] Philippe Mateo[1,2], Ruth Westenbroek,[4] William A. Catterall,[4] Flavien Charpentier,[5,6] Marco Conti,[3] Rodolphe Fischmeister,[1,2] and Grégoire Vandecasteele[1,2]

[1]INSERM UMR-S 769, Châtenay-Malabry, F-92296, France. [2]University Paris-Sud, IFR141, Châtenay-Malabry, F-92296, France. [3]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, CA 94143, USA. [4]University of Washington School of Medicine, Department of Pharmacology, Seattle, WA 98195-7280, USA. [5]INSERM UMR-S915, CNRS ERL3147, l'institut du thorax, Nantes, F-44007, France. [6]University of Nantes, Nantes, F-44000, France.

*These authors equally contributed to this study

The authors have declared that no conflict of interest exists

Running title: PDE4B regulates L-type Ca^{2+} channels

Address correspondence to:
Rodolphe Fischmeister (e-mail: rodolphe.fischmeister@inserm.fr)
or Grégoire Vandecasteele (e-mail: gregoire.vandecasteele@u-psud.fr)
INSERM UMR-S 769
Université Paris-Sud
Faculté de Pharmacie
5, Rue J.-B. Clément
F-92296 Châtenay-Malabry Cedex
France
Phone: +33-1-46.83.57.57
Fax 33-1-46.83.54.75

Supplemental methods

Reagents. Isoprenaline (Iso) was purchased from Sigma-Aldrich and Ro 20-1724 was from Calbiochem. PAN-selective antibodies against PDE4A (AC55), PDE4B (K118) and PDE4D (M3S1) used for IPs of PDE4 subtypes were described previously (1). Rabbit polyclonal anti-$Ca_V1.2$ (CNC1) was also described previously (2). Rabbit antibody 113-4 raised against the C-terminus of PDE4B was used to detect PDE4B. Antibody against total phospholamban (PLB) was from Affinity Bioreagents. Antibody against Ser-16 phosphorylated PLB was from Upstate. Antibodies against total and Ser-2808 phosphorylated RyR2 were a kind gift from Drs. Steven Reiken and Andrew Marks (Columbia University, New York, USA). Mouse monoclonal anti-α-actinin antibody was from Sigma-Aldrich.

Electrophysiological experiments. Voltage-clamp protocols were generated by a challenger/09-VM programmable function generator (Kinetic Software). The cells were voltage-clamped using a patch-clamp amplifier (model RK-400; Bio-Logic). Currents were filtered at 3 KHz and digitally sampled at 10 KHz using a 12-bit analogue-to-digital converter (DT2827; Data translation) connected to an IBM compatible PC (386/33 Systempro; Compaq Computer Corp.). The maximal amplitude of $I_{Ca,L}$ was measured as the difference between the peak inward current and the current at the end of the 400 ms duration pulse. Currents were not compensated for capacitance and leak currents. The current density-voltage (*I*-*V*) relationships were fitted with a modified Boltzmann equation as follows: $I = G_{max} \times (V-V_{rev})/(1 + \exp(-(V-V_{1/2,act})/k))$, where I is the current density (in pA/pF), G_{max} is the maximum conductance (in nS/pF), V_{rev} is the reversal potential (in mV), $V_{1/2,\ act}$ is the midpoint voltage for current activation (in mV), and k is the slope factor (in

mV). Steady-state inactivation curves were obtained by normalizing the peak current at each test potential to the maximal current, and fitted with a Boltzmann equation: $I/I_{max} = (1-A)/\{1+\exp[(V-V_{½,inac})/k]\} + A$; where $V_{½,\ inact}$ is the potential of half-maximal inactivation, k is the slope factor, A is the amplitude of the non-inactivating $I_{Ca,L}$ current. Activation curves were derived from current–voltage relations and described by: $G/G_{max} = 1/\{1+\exp[(V_{½,act}-V)/k]\}$.

Immunocytochemistry. Ventricular myocytes were plated on laminin-coated glass coverslips and incubated (5% CO_2, 37°C) for 2 h in a minimal essential medium (MEM: M 21475; Gibco®, Invitrogen) containing 1.2 mM $CaCl_2$, 1% penicillin-streptomycin, insulin (1 µg/ml), transferrin (0.55 µg/ml), and selenium (0.5 ng/ml) (ITS Medium Supplement, Sigma) and 10 mM HEPES. Cardiomyocytes were fixed with paraformaldehyde (PFA, 4%, 45 min). Then, PFA was neutralized with NH_4Cl (0.5 M, 5 min). Both antibodies raised against $Ca_V1.2$ and PDE4B were produced in rabbit excluding the possibility to co-localize the two proteins in the same cell. Thus, co-immunostaining of $Ca_V1.2$ and PDE4B with α-actinin was performed in different cardiomyocytes using a mouse monoclonal antibody against α-actinin. Cells were rinsed three times with Phosphate-Buffer Saline (PBS) and blocked sequentially with streptavidin (2%, 15 min) and biotin (2%, 15 min). For double-labelling, myocytes were incubated overnight at 4°C with the two primary antibodies diluted in PBS containing 10% goat serum and 0,25% Triton X-100: rabbit polyclonal anti-$Ca_V1.2$ (CNC1) and mouse monoclonal anti-α-actinin, or rabbit antibody 113-4 raised against the C-terminus of PDE4B and mouse monoclonal anti-α-actinin. Myocytes were then rinsed three times with 1% BSA in PBS (5 min) and then incubated with a biotinylated antibody raised against rabbit

IgG for 1h at room temperature. After three washes with PBS/BSA, cells were incubated with streptavidin AlexaFluor® 488 conjugate to reveal $Ca_v1.2$ or PDE4B and AlexaFluor® 633 to reveal α-actinin. After intensive washes with PBS/BSA, coverslips were mounted on slides using 20 µl of Mowiol medium and examined with a Carl Zeiss (Oberkochen) LSM 510 confocal scanning laser microscope. Optical sections series were obtained with a Plan Apochromat 63x objective (NA 1.4, oil immersion). The fluorescence was observed with a BP 505-550 nm and a LP 649 nm emission filters under 488-nm and 633 nm laser illuminations.

ECG recording and intracardiac recording and pacing. The criteria used to measure RR, PR, QRS and QT intervals on recorded ECG have been described elsewhere (3). The QT interval was corrected for heart rate with Bazett formula adapted to mouse sinus rate, *i.e.* $QTc = QT/(RR/100)^{1/2}$ with QT and RR, expressed in ms (4).

After ECG recording, anesthesia was prolonged by an additional intraperitoneal injection of etomidate. The extremity of a 2F octapolar catheter (Biosense Webster) was placed in the right ventricle through the right internal jugular vein, using intracardiac electrograms as a guide for catheter positioning. Surface ECG (lead I) and intracardiac electrograms were recorded on a computer through an analog-digital converter (IOX 1.585, Emka Technologies) for monitoring and later analysis (ECG-Auto 2.1.4.15, Emka Technologies). Intracardiac electrograms were filtered between 0.5 and 500 Hz. Pacing was performed with a digital stimulator (DS8000, World Precision Instruments). Standard pacing protocols were used to determine the ventricular effective refractory periods (VERP) and to induce ventricular arrhythmias. The stimulus amplitude and duration were set at 1.5 times

the excitation threshold and 2 ms, respectively. VERP were assessed at baseline by using the extrastimulus method. Extrastimuli were delivered following trains of 8 paced beats at a cycle length of 75 ms. The extrastimulus coupling interval was initially set at 70 ms and then reduced by 2 ms at each cycle until ventricular refractoriness was reached. The inducibility of ventricular arrhythmias was assessed in baseline condition and after intraperitoneal infusion of isoprenaline 0.02 mg/kg and 0.2 mg/kg by using the programmed electrical stimulation (PES) method with 1 to 3 extrastimuli (performed twice), and burst pacing. Burst pacing consisted of trains of 30 paced beats at cycle lengths of 70, 60, 50 and 40 ms. For each cycle length, the trains were performed 5 times at 8-s intervals if they induced a 1:1 conduction. If not, the protocol was stopped and an additional train at a cycle length of 5 ms above the last cycle length used was performed.

References

1. Richter W, Jin SL, Conti M. Splice variants of the cyclic nucleotide phosphodiesterase PDE4D are differentially expressed and regulated in rat tissue. *Biochem J*. 2005;388(Pt 3):803-811.
2. Hulme JT, Westenbroek RE, Scheuer T, Catterall WA. Phosphorylation of serine 1928 in the distal C-terminal domain of cardiac $Ca_v1.2$ channels during ß1-adrenergic regulation. *Proc Natl Acad Sci USA*. 2006;103(44):16574-9.
3. Royer A, et al. Mouse model of SCN5A-linked hereditary Lenegre's disease: age-related conduction slowing and myocardial fibrosis. *Circulation*. 2005;111(14):1738-46.
4. Mitchell GF, Jeron A, Koren G. Measurement of heart rate and Q-T interval in the conscious mouse. *Am J Physiol*. 1998;274(3 Pt 2):H747-51.

Supplemental Figure Legends

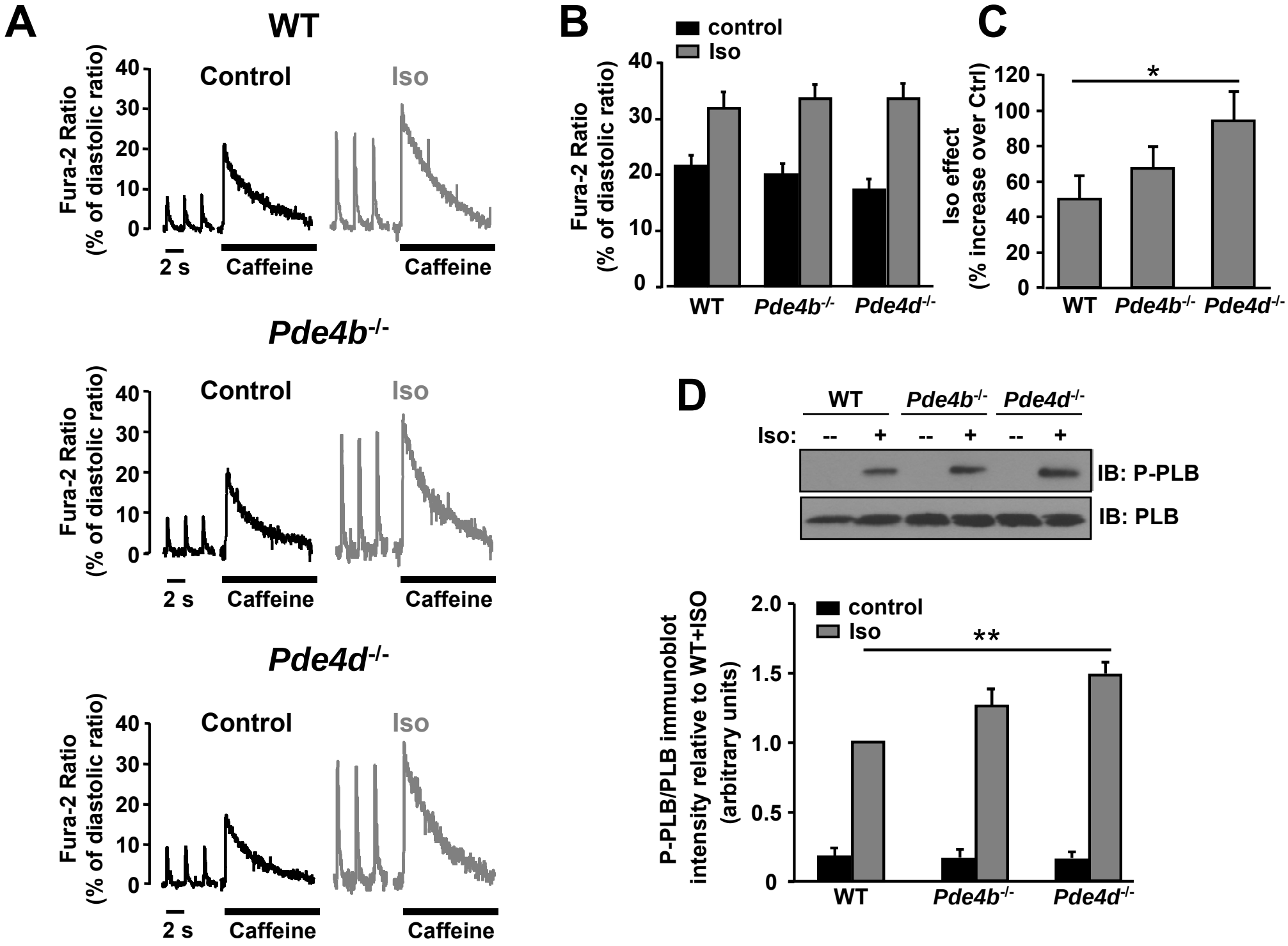
Supplemental Figure 1

SR Ca^{2+} load and PKA phosphorylation level of phospholamban (PLB) in WT, *Pde4b*$^{-/-}$, and *Pde4d*$^{-/-}$ AMVMs. **(A)** Representative examples of rapid caffeine (10 mM) application to AMVMs from the three genotypes after pacing at 0.5 Hz in control external Ringer solution (Ctrl, black traces) and following isoprenaline pulse stimulation (Iso, 100 nM, 15 s, grey traces). The amplitude of the caffeine transient was used as a measure of SR Ca^{2+} load. **(B)** Comparison of average amplitude of caffeine transients in WT, *Pde4b*$^{-/-}$, and *Pde4d*$^{-/-}$ AMVMs in control (black bars) and at the maximum of Iso pulse stimulation (grey bars). n=15 to 18 cells per group. **(C)** Average increase in SR Ca^{2+} load induced by Iso in WT (n=16), *Pde4b*$^{-/-}$ (n=18), and *Pde4d*$^{-/-}$ (n=18) AMVMs. Statistical significance is indicated as *,p<0.05 **(D)** PKA phosphorylation level of PLB in WT, *Pde4b*$^{-/-}$, and *Pde4d*$^{-/-}$ AMVMs in control external Ringer (black bars) and 90 s after Iso pulse (100 nM, 15 s) stimulation (grey bars). The data represent the average ± SEM of seven samples. Statistical significance is indicated as **,p<0.01.

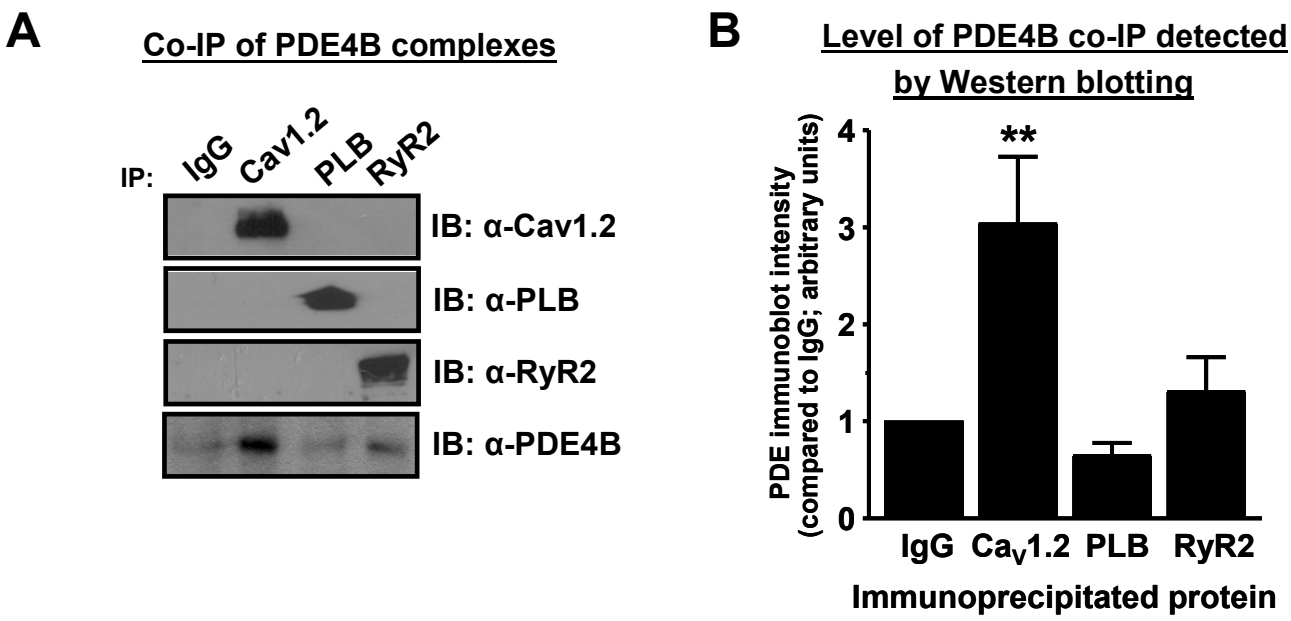
Supplemental Figure 2

Role of PDE4B in ECC. Detergent extracts prepared from adult mouse hearts were subjected to IP with antibodies against $Ca_V1.2$, PLB or RyR2. The amount of the immunoprecipitated signaling proteins as well as the amount of PDE4B recovered in IP pellets was detected by immunoblotting. **(A)** Representative western blot. **(B)** The graph depicts the average ±SEM of three experiments. **,p<0.001 PDE4B is significantly enriched only in IP pellets of $Ca_V1.2$ suggesting that this is the major

signaling complex involving PDE4B. **(C,D)** Measurement of PLB phosphorylation level at Ser-16 in WT and *Pde4b*$^{-/-}$ hearts (n=3). (E,F) Measurement of RyR2 phosphorylation level at Ser-2808 in WT and *Pde4b*$^{-/-}$ hearts (n=3).



Supplemental Figure 1



Supplemental Figure 2