

## PDE2 activity differs in right and left rat ventricular myocardium and differentially regulates $\beta_2$ adrenoceptor-mediated effects

Fernando Soler<sup>1</sup>, Francisco Fernández-Belda<sup>1</sup>, Joaquín Pérez-Schindler<sup>2</sup>, Christoph Handschin<sup>2</sup>, Teodomiro Fuente<sup>3</sup> and Jesús Hernandez-Cascales<sup>4</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology A, University of Murcia, 30100 Murcia, Spain; <sup>2</sup>Biozentrum, University of Basel, Klingelbergstrasse 50/70, 4056 Basel, Switzerland; <sup>3</sup>Unit of Radiopharmacy, University Hospital Virgen de la Arrixaca, 30120 Murcia, Spain; <sup>4</sup>Department of Pharmacology, Faculty of Medicine, University of Murcia, 30100 Murcia, Spain  
Corresponding author: Jesús Hernandez-Cascales. Email: jehernca@um.es

### Abstract

The important regulator of cardiac function, cAMP, is hydrolyzed by different cyclic nucleotide phosphodiesterases (PDEs), whose expression and activity are not uniform throughout the heart. Of these enzymes, PDE2 shapes  $\beta_1$  adrenoceptor-dependent cardiac cAMP signaling, both in the right and left ventricular myocardium, but its role in regulating  $\beta_2$  adrenoceptor-mediated responses is less well known. Our aim was to investigate possible differences in PDE2 transcription and activity between right (RV) and left (LV) rat ventricular myocardium, as well as its role in regulating  $\beta_2$  adrenoceptor effects. The free walls of the RV and the LV were obtained from Sprague–Dawley rat hearts. Relative mRNA for PDE2 (quantified by qPCR) and PDE2 activity (evaluated by a colorimetric procedure and using the PDE2 inhibitor EHNA) were determined in RV and LV. Also,  $\beta_2$  adrenoceptor-mediated effects ( $\beta_2$ -adrenoceptor agonist salbutamol +  $\beta_1$  adrenoceptor antagonist CGP-20712A) on contractility and cAMP concentrations, in the absence or presence of EHNA, were studied in the RV and LV. PDE2 transcript levels were less abundant in RV than in LV and the contribution of PDE2 to the total PDE activity was around 25% lower in the microsomal fraction of the RV compared with the LV.  $\beta_2$  adrenoceptor activation increased inotropy and cAMP levels in the LV when measured in the presence of EHNA, but no such effects were observed in the RV, either in the presence or absence of EHNA. These results indicate interventricular differences in PDE2 transcript and activity levels, which may distinctly regulate  $\beta_2$  adrenoceptor-mediated contractility and cAMP concentrations in the RV and in the LV of the rat heart.

**Keywords:** PDE2 activity, interventricular differences,  $\beta_2$  adrenoceptor, inotropy, cAMP levels

**Experimental Biology and Medicine 2015; 240: 1205–1213. DOI: 10.1177/1535370214560969**

### Introduction

The heart can be considered as two independent pumps, one delivering blood to the lungs and other to the rest of the body. Because the contractile work performed by each pump is different, the anatomy as well as the mechanical and biochemical properties of the right and left ventricle (RV and LV, respectively) is also different.<sup>1,2</sup>

The second messenger cAMP is an important regulator of cardiac contractility and its cellular levels are determined by a balance between synthesis and degradation. Activation of  $\beta$  adrenoceptors by the sympathetic nervous system or circulating catecholamines is one of the major neurohormonal mechanisms controlling myocardial cAMP production. Stimulation of both  $\beta_1$  and  $\beta_2$  adrenoceptors can induce the activation of the stimulatory G protein (*G $\alpha$ s*)/adenylyl-cyclase (AC)/cAMP/cAMP-dependent protein kinase A (PKA) signaling pathway that leads to the phosphorylation

of several target proteins regulating excitation–contraction coupling within the cardiac myocyte.<sup>3</sup> The  $\beta_1$  adrenoceptor is the most abundant subtype present in the mammalian heart, where it is expressed in a  $\beta_1$ : $\beta_2$  ratio of approximately 75:25%<sup>4,5</sup> and it was initially thought to be the only one responsible for the inotropic response to catecholamines.<sup>6,7</sup> However, recent studies indicate that  $\beta_2$  adrenoceptors can also provide inotropic support and are involved in the regulation of cardiac metabolism as well as in the pathogenesis of a number of diseases, including pathological hypertrophy, arrhythmia, and heart failure.<sup>8</sup>

Degradation of cAMP is regulated by the activity of different cyclic nucleotide phosphodiesterases (PDEs) that break down cAMP into 5'-AMP.<sup>9</sup> On the basis of the structure, kinetics, and substrate specificity, PDEs can be grouped into different families,<sup>9</sup> of which at least four (PDE1–4) are present in the heart of a variety of animal

species, including humans.<sup>9</sup> Among these families, PDE3 and PDE4 provide the major PDE activity in the heart, and their role in regulating cardiac cAMP signaling has been extensively studied.<sup>10–12</sup> There is evidence that PDE2 may also play a significant role in modulating cardiac function. Indeed, a PDE2-dependent decrease of cAMP has been reported to be responsible for attenuating  $\beta$ -adrenergic signaling in rabbit atrioventricular nodal cells.<sup>13</sup> In human cardiac myocytes, PDE2 regulates L-type calcium current, which is involved both in heart rate and contractility.<sup>14</sup> Accordingly,  $\beta_1$  adrenoceptor activation, along with PDE2 inhibition, results in a greater production of cAMP in left ventricular myocytes<sup>15</sup> as well as in the right ventricular free wall of rat heart.<sup>16</sup> These data imply that PDE2 attenuates cAMP production induced by  $\beta_1$  adrenoceptor agonists both in the right and left ventricular myocardium, but the regulation of cardiac  $\beta_2$  adrenoceptor-mediated responses by PDE2 is less well known. Although PDE2 is expressed in a wide variety of tissues, including ventricular myocardium,<sup>9</sup> it is unclear whether PDE2 activity and expression are equally distributed in right and left ventricular myocardium. Therefore, the aim of the present study was to determine the differences in PDE2 transcription and activity in RV and LV, in addition to determining its role in regulating  $\beta_2$  adrenoceptor effects on cardiac function.

## Materials and methods

### Animals

The study was performed in accordance with the European Union Council Directive of 22 September 2010 (2010/63/EU) and reviewed and approved by the Ethical Committee of the University of Murcia.

Sprague–Dawley rats (250–350 g) were kept under standardized conditions: 12 h-light/dark cycle, 22°C, and 70% humidity. Food and water were available *ad libitum*. Animals were stunned by a blow to the head and exsanguinated. The chest was opened and the heart rapidly removed and placed in Tyrode solution of the following composition (mmol/L): NaCl 136.9, KCl 5.0, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1.5, NaH<sub>2</sub>PO<sub>4</sub> 0.4, NaHCO<sub>3</sub> 11.9, and dextrose 5.0.

### RNA isolation and quantitative PCR (qPCR)

To determine PDE2A transcripts, which is the only PDE2 variant expressed in rat and human cardiac tissues,<sup>17</sup> RV and LV were powdered on dry ice and RNA was isolated using TRI Reagent<sup>®</sup> according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). One microgram of RNA was treated with DNase I (Invitrogen<sup>™</sup>, Thermo Fisher Scientific) and then reversed transcribed using hexanucleotide mix (Roche Diagnostics, Hoffmann-La Roche, Basel, Switzerland) and SuperScript<sup>™</sup> II reverse transcriptase (Invitrogen<sup>™</sup>, Thermo Fisher Scientific). Relative mRNA was quantified by qPCR on a StepOnePlus system (Applied Biosystems<sup>®</sup>, Thermo Fisher Scientific) using Power SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems<sup>®</sup>, Thermo Fisher Scientific). Analysis was

performed by the  $\Delta\Delta C_T$  method using TATA binding protein (TBP) as endogenous control. TBP transcript levels did not differ between RV and LV. Primer sequences were as follows:

PDE2A forward CGATCCAACACTGCTTCCACTAC,  
PDE2A reverse CTTCTGGAAGGCCAAGGTACTG,  
TBP forward ATGAGAATAAGAGAGCCACGAACA  
TBP reverse TGCACACCATTTCCAGAA.

### Subcellular fractionation and protein content

Right or left ventricle free walls (~3 g wet weight) were trimmed of connective tissue, pooled and quick-frozen in liquid nitrogen. Cytosolic and microsomal fractions were obtained as previously described.<sup>18</sup> Briefly, samples were homogenized on ice in a sucrose-containing buffer by three 10-s bursts using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA). Next, samples were centrifuged at 5900 g for 10 min at 4°C and then the supernatant was collected and subsequently centrifuged at 51,000 g for 60 min. The supernatant was saved as the cytosolic fraction, i.e. right ventricle cytosol (RVC) or left ventricle cytosol (LVC), and the pellet was resuspended in a medium containing 0.6 mol/L KCl and resedimented at 100,000 g for 40 min to obtain the microsomal fraction, i.e., right ventricle microsomes (RVM) or left ventricle microsomes (LVM). To remove nucleotides and phosphate from the cytosolic extracts, samples were centrifuged at 800 g for 1 min using chromatography minicolumns (38 × 100 mm) filled with BioGel P-6DG (BioRad, Hercules, CA, USA) and pre-equilibrated with 10 mmol/L Tris-HCl buffer (pH 7.4).<sup>19</sup> Isolated samples were aliquotted and stored at –80°C until use.

Protein concentration was measured by the bicinchoninic acid method<sup>20</sup> using the BCA Protein Assay Kit from Pierce<sup>®</sup> (Thermo Fisher Scientific). When the protein content was referred to as g of wet tissue, the yield of protein was 9.1 ± 1.0 mg/g in RVM and 8.5 ± 0.8 mg/g in LVM. Similarly, 24.9 ± 1.3 mg/g and 23.3 ± 0.5 mg/g were the protein yields in RVC and LVC, respectively.

### PDE activity

A two-step colorimetric procedure adapted to a microplate format was used.<sup>21</sup> In this assay, the 5'-AMP (Enzo Life Sciences, Farmingdale, NY, USA) released after cleavage of the substrate cAMP was further hydrolyzed into adenosine and inorganic phosphate (P<sub>i</sub>) by the presence of excess 5'-nucleotidase (Enzo Life Sciences). The time-dependent P<sub>i</sub> accumulation was quantified by the malachite green reagent.<sup>22</sup> The linear dependence between standard 5'-AMP and P<sub>i</sub> was first determined. The reaction was carried out in a 96-well plate containing 10 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaCl, 0.2 mmol/L MgCl<sub>2</sub>, 0–50  $\mu$ mol/L 5'-AMP, and 5'-nucleotidase from *Crotalus atrox* venom at 50 U/ $\mu$ L in final volumes of 100  $\mu$ L. The reaction was allowed to proceed at 37°C for 30 min and stopped by adding 200  $\mu$ L malachite green reagent. After 1 min at

room temperature, 10  $\mu\text{L}$  of 34% sodium citrate was added and the plate was shaken at room temperature for another 30 min for colour development. The 5'-AMP standard curve was constructed by plotting nmol of 5'-AMP versus absorbance at 660 nm. The PDE activity was also measured in the same microplate format. The final composition in each well was 10 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaCl, 0.2 mmol/L  $\text{MgCl}_2$ , 50 U/ $\mu\text{L}$  5'-nucleotidase and the corresponding aliquot of microsomal or cytosolic extract. After equilibration at 37°C, the reaction was started by adding 100  $\mu\text{mol/L}$  cAMP. The enzymatic reaction was stopped at different times by adding 200  $\mu\text{L}$  malachite green reagent and the samples were processed as described above. The time course of cAMP hydrolysis was evaluated by plotting absorbance at 660 nm *versus* time. The blank was performed by adding malachite green reagent before cAMP. PDE2 activity in the subcellular fractions was deduced by subtraction when the PDE activity was measured in the absence or presence of 30  $\mu\text{mol/L}$  erythro-9-[2-hydroxy-3-nonyl] adenine (EHNA). Specific activities are expressed as nmol  $\text{P}_i$ /min/mg protein, which is equivalent to nmol 5'-AMP/min/mg protein.

### Paced rat ventricular tissues

Right ventricular strips (1 mm wide, 10 mm long and 0.5 mm thick) and left ventricular papillary muscles were mounted longitudinally between two platinum electrodes, in Tyrode solution maintained at 37°C, pH 7.4, and gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ . The preparations were electrically stimulated (Grass SD-9 stimulator, Quincy, MA, USA) at a frequency of 1 Hz, 1 ms of duration and supramaximal voltage (threshold + 25%). A length-force curve was obtained and the tissues left at the length associated with the maximal developed force.<sup>23</sup> Contractions were measured using a force-displacement transducer (Grass FT-03, Quincy, MA, USA) and displayed on a computer screen using a Stemtech amplifier (Stemtech Inc., Houston, TX, USA) and ACODAS software (Dataq Instruments, Inc., Akron, OH, USA). Tissues were allowed to equilibrate for 45–60 min before drug challenge.

To investigate  $\beta_2$  adrenoceptor-mediated inotropic effects, concentration-response curves for salbutamol were performed in the presence of the  $\beta_1$  adrenoceptor antagonist ( $\pm$ )-2-hydroxy-5-(2-((2-hydroxy-3-(4-(1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl)phenoxy)propyl)amino)ethoxy)-benzamide methanesulfonate (CGP-20712A). Salbutamol concentrations were increased stepwise by 0.5 log unit as soon as the response to the previous concentration had stabilized. Cumulative concentration-response curves for  $\beta_2$  adrenoceptor-mediated effects of salbutamol in the presence of 300 nmol/L CGP-20712A were determined in the absence or 15 min after the addition of 10  $\mu\text{mol/L}$  EHNA, which effectively inhibits PDE2 activity in rat myocardium.<sup>10</sup> EHNA is also an inhibitor of adenosine deaminase, which catalyzes the metabolism of adenosine.<sup>9</sup> To investigate the possible involvement of adenosine in the results obtained with EHNA, we also obtained concentration-response curves for salbutamol and CGP-20712A in the presence of 10  $\mu\text{mol/L}$  2'-deoxycoformycin (DCF), which

inhibits adenosine deaminase but does not affect PDE2.<sup>24</sup> Only one concentration-response curve for salbutamol and CGP-20712A in the absence or presence of the above mentioned drugs was determined in the same ventricular tissue. Drugs were added to a 30 mL organ bath in a volume smaller than or equal to 0.1 mL. Inotropic responses were expressed as percentages of the basal force. The relaxation phase of the isometric twitch was also assessed by measuring the time-to-half relaxation ( $t_{1/2}$ ), a standard index of isometric relaxation in mammalian myocardium.<sup>25</sup>

### Measurement of cAMP

The levels of cAMP were measured by radioimmunoassay [<sup>125</sup>I] TME-S-cAMP (Diagnostic Pasteur, France) according to the manufacturer's instruction, using strips of RV or LV (50–70 mg). CGP-20712A at 300 nmol/L was present in the media to produce an effective blockade of  $\beta_1$  adrenoceptors, and cAMP was measured in the absence or 15 min after exposure to 10  $\mu\text{mol/L}$  EHNA. The incubation time was similar to that previously used for studying the effect of this PDE inhibitor on intracellular cAMP levels.<sup>10,16</sup> To investigate the effects of  $\beta_2$  adrenoceptor activation on cAMP levels, salbutamol was added and the effect was evaluated after 3 min in the absence or presence of EHNA. The effect of salbutamol in the presence of DCF on cAMP levels was also determined. After incubation with the drugs, the tissues were immediately frozen. The preparation was weighed and homogenized in 1.5 mL of cold perchloric acid (0.3 mol/L) using a Polytron homogenizer and was then centrifuged at 10,000 g at 4°C for 15 min. The supernatants were treated with potassium phosphate until pH of 6.2 was reached. The sensitivity of the assay was 2 pmol/mL. Intra- and inter-assay coefficients of variation were 7.7% and 8.2%, respectively. The antibody cross-reacted 100% with 3',5'-cAMP and less than 0.3% with other nucleotides.

### Chemicals

Chemicals used for this study, unless otherwise noted, were purchased from Sigma-Aldrich (St Louis, MO, USA). DCF was a gift from Pfizer S.A. Madrid, Spain.

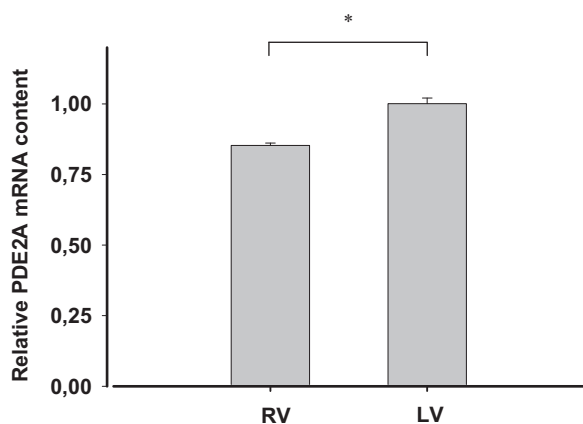
### Statistical analysis

Results are expressed as mean  $\pm$  S.E.M. Student's *t*-test or one-way analysis of variance followed by the Fisher LSD *post hoc* test were used for multiple comparisons. The criterion for significance was  $P < 0.05$ .

## Results

### PDE2A mRNA levels and activity

Quantitative PCR analysis performed on rat ventricular tissue indicated that transcript levels of PDE2A were less abundant in RV than in LV (Figure 1). Moreover, when the right or left ventricle free walls were subjected to subcellular fractionation, total PDE activity was lower in the RVM fraction ( $0.73 \pm 0.07$  nmol  $\text{P}_i$ /min/mg protein) than in the LVM fraction ( $1.09 \pm 0.09$  nmol  $\text{P}_i$ /min/mg protein)



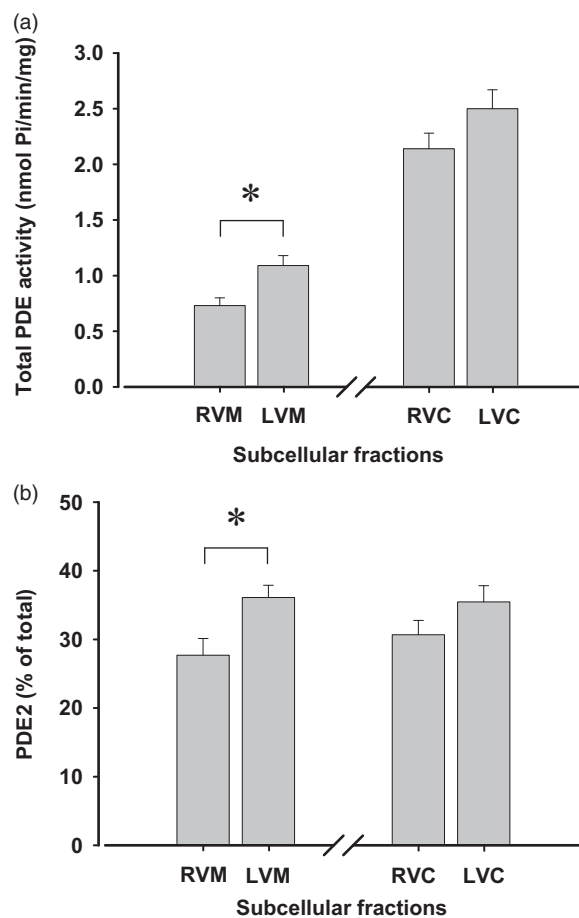
**Figure 1** PDE2A (the only PDE2 variant expressed in both rat and human myocardium) is differentially transcribed in the right and left ventricles. Relative quantification of PDE2A mRNA levels in RV or LV, respectively ( $n = 6$  per group). Values are expressed as mean  $\pm$  SEM., \* $P < 0.05$

( $n = 4-6$  experiments,  $P < 0.05$ ) (Figure 2a). However, total PDE activity was  $2.14 \pm 0.14$  nmol  $P_i$ /min/mg protein in RVC and  $2.50 \pm 0.17$  nmol  $P_i$ /min/mg protein in LVC ( $n = 5-9$  experiments). In this case, the difference between the two values did not reach statistical significance.

The next aim was to evaluate the inhibitory effect of EHNA and thus estimate the contribution of PDE2 to the total PDE activity. PDE2 activity was also lower in RVM ( $0.20 \pm 0.02$  nmol  $P_i$ /min/mg protein) than in LVM ( $0.39 \pm 0.02$  nmol  $P_i$ /min/mg protein) ( $n = 4-9$  experiments,  $P < 0.05$ ). Similar experiments revealed that PDE2 activity was  $0.66 \pm 0.04$  nmol  $P_i$ /min/mg protein in RVC and  $0.89 \pm 0.06$  nmol  $P_i$ /min/mg protein in LVC ( $n = 4-9$  experiments), but no significant difference between the two values was found. When expressed as percentage of total PDE activity in the corresponding fraction, PDE2 activity was 27.4% in RVM, 35.8% in LVM, 30.8% in RVC, and 35.6% in LVC (Figure 2b). Therefore, the contribution of PDE2 was around 25% lower in RVM than in LVM, whereas the difference between RVC and LVC was not statistically significant.

### Contractility

When used alone, the PDE2 inhibitor EHNA had no significant effect in our preparations of RV ( $3.3 \pm 0.5$  mN versus  $3.5 \pm 0.3$  mN in the absence or presence of  $10 \mu\text{mol/L}$  EHNA, respectively;  $n = 4$ ) and LV ( $3.9 \pm 0.3$  mN versus  $4.1 \pm 0.4$  mN in the absence or presence of  $10 \mu\text{mol/L}$  EHNA, respectively;  $n = 5$ ). Moreover,  $\beta_2$  adrenoceptor stimulation (salbutamol +  $300 \text{ nmol/mL}$  CGP-20712A) in LV papillary muscle was devoid of inotropic effect, but in the presence of  $10 \mu\text{mol/L}$  EHNA it produced a concentration-dependent increase in force generation ( $E_{\text{max}}$ :  $35.2 \pm 5.7\%$ ;  $-\log EC_{50}$ :  $6.2 \pm 0.03$ ,  $n = 5$ ) (Figure 3). In contrast, salbutamol plus CGP-20712A did not increase contractility in the absence or presence of EHNA when assayed in RV samples (Figure 3). We also studied whether EHNA affects the  $\beta_2$  adrenoceptor-mediated effects of

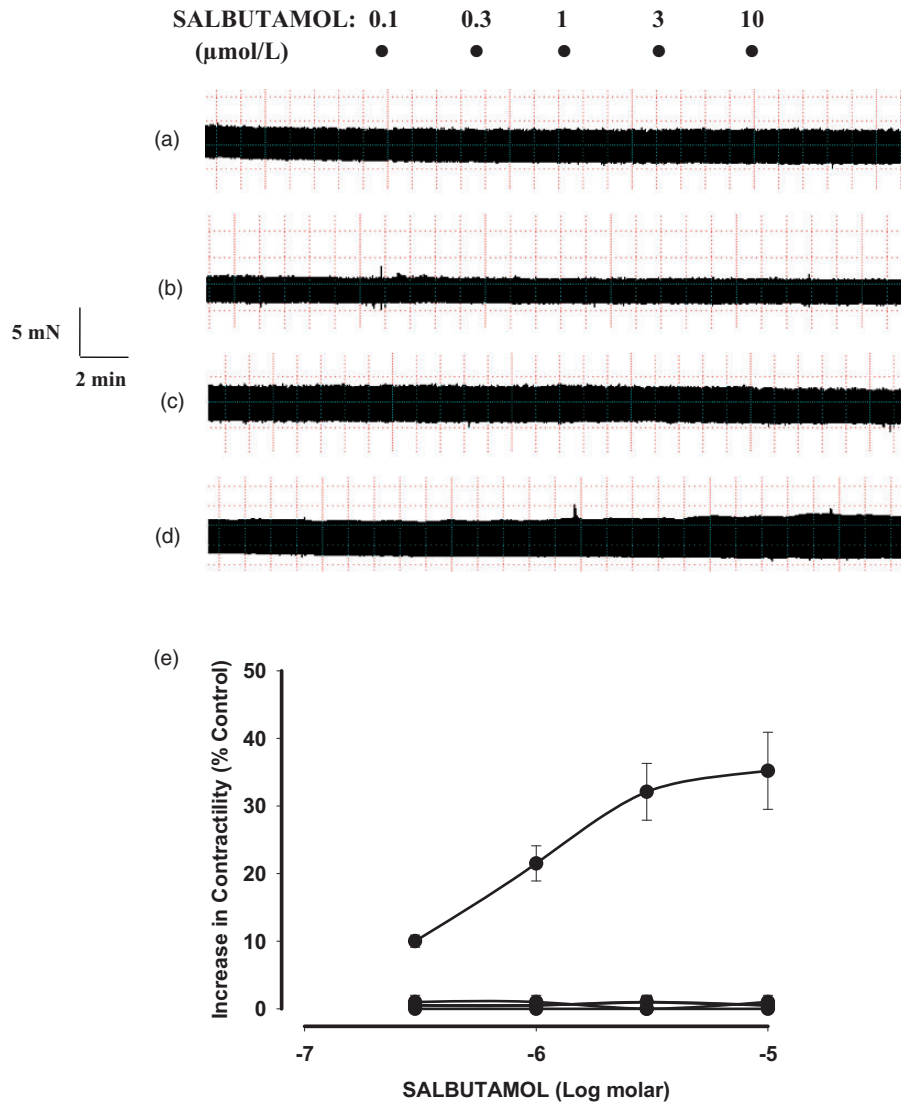


**Figure 2** Total PDE activity and percentage of PDE2 activity in subcellular fractions of right and left ventricle of rat heart. (a) Total PDE activity expressed as nmol  $P_i$ /min/mg protein was measured in RVM, LVM, RVC, and LVC using  $100 \mu\text{mol/L}$  cAMP as a substrate. Values are the mean  $\pm$  S.E.M., \* $P < 0.05$ . (b) PDE2 activity was expressed as percentage with respect to the corresponding total PDE activity displayed in panel (a). PDE2 activity in nmol  $P_i$ /min/mg protein was  $0.20 \pm 0.02$  in RVM,  $0.39 \pm 0.02$  in LVM,  $0.66 \pm 0.04$  in RVC, and  $0.89 \pm 0.06$  in LVC. Values are the mean  $\pm$  S.E.M., \* $P < 0.05$

salbutamol on relaxation time. Salbutamol and CGP-20712A in RV did not affect the relaxation time in the absence or presence of EHNA (Figure 4a). In contrast,  $3 \mu\text{mol/L}$  salbutamol, in the presence of CGP-20712A, which produced a maximal inotropic response in LV, also reduced the relaxation time from  $50 \pm 1.9$  ms to  $40 \pm 1.7$  ms ( $n = 6$ ,  $P < 0.05$ ) when applied in the presence of  $10 \mu\text{mol/L}$  EHNA in this tissue (Figure 4b). The combination of salbutamol and CGP-20712A failed to produce any effect on contractility or relaxation, either in LV or RV, when studied in the presence of DCF (data not shown).

### Effects of salbutamol, in the presence of the $\beta_1$ adrenoceptor antagonist CGP-20712A, and EHNA on cAMP levels

To investigate whether inotropy correlated with cAMP levels, we studied the effect of  $1 \mu\text{mol/L}$  salbutamol, a concentration similar to its  $EC_{50}$  for the  $\beta_2$  adrenoceptor-mediated inotropic effect (see above), and  $300 \text{ nmol/L}$



**Figure 3**  $\beta_2$  adrenoceptor-mediated effects of salbutamol on the basal force of contraction in rat ventricular myocardium.  $\beta_1$  adrenoceptors were blocked in all cases by the presence of 300 nmol/L CGP-20712A. Representative traces, in electrically driven strips, taken from the free wall of right ventricle (a and b) or left ventricle (c and d) showing that in the right ventricular myocardium, salbutamol was devoid of contractile effect both in the absence (a) or in the presence of 10  $\mu$ mol/L EHNA (b). However, in the left ventricular myocardium, salbutamol did not increase contractility alone (c), but produced a concentration-dependent inotropic effect in the presence of EHNA (d). (e) Cumulative concentration–response curves for the effect of salbutamol alone in the right ( $\blacklozenge$ ) or left ( $\blacktriangle$ ) ventricular myocardium and also in the right ( $\blacksquare$ ) or left ( $\bullet$ ) ventricular myocardium when 10  $\mu$ mol/L EHNA was present. Each point represents the mean  $\pm$  S.E.M. of 5–6 experiments

CGP-20712A on cAMP concentrations when measured in the absence or presence of EHNA in rat RV and LV.

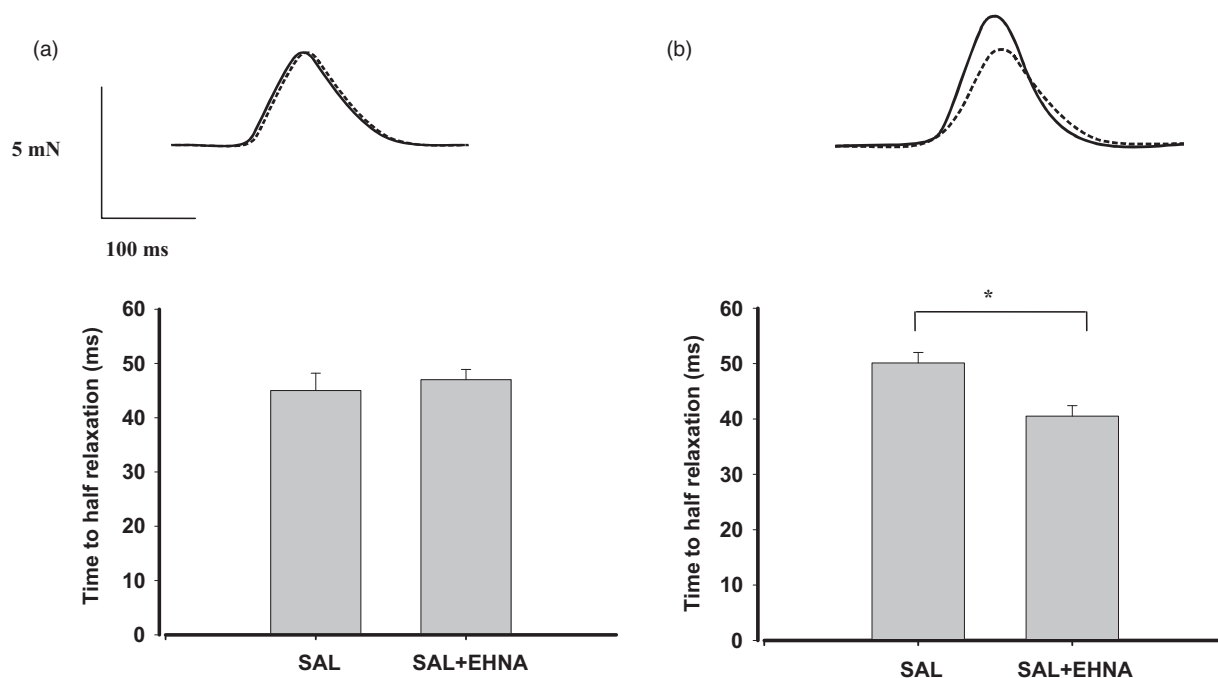
In RV, 10  $\mu$ mol/L EHNA tended to increase cAMP levels, although this effect was not statistically significant when compared to the control (Figure 5a). In the same tissue, salbutamol failed to increase cAMP concentrations, alone or combined with EHNA (Figure 5a). However, EHNA and salbutamol in LV were devoid of any effect when used separately, but their combination produced an increase in cAMP levels (Figure 5b). DCF did not alter the basal cAMP content in RV ( $462 \pm 53$  pmol/g, control,  $n=6$  versus  $457 \pm 37$  pmol/g, in the presence of DCF,  $n=4$ ,  $P > 0.05$ ) or LV ( $463 \pm 43$  pmol/g, control,  $n=6$  versus  $473 \pm 34$  pmol/g, in the presence of DCF,  $n=4$ ,  $P > 0.05$ ). Neither did it modify the effect of salbutamol in RV

( $428 \pm 41$  pmol/g, control,  $n=6$  versus  $443 \pm 38$  pmol/g, in the presence of DCF,  $n=4$ ,  $P > 0.05$ ) or in LV ( $506 \pm 37$  pmol/g, control,  $n=6$  versus  $492 \pm 47$  pmol/g, in the presence of DCF,  $n=4$ ,  $P > 0.05$ ).

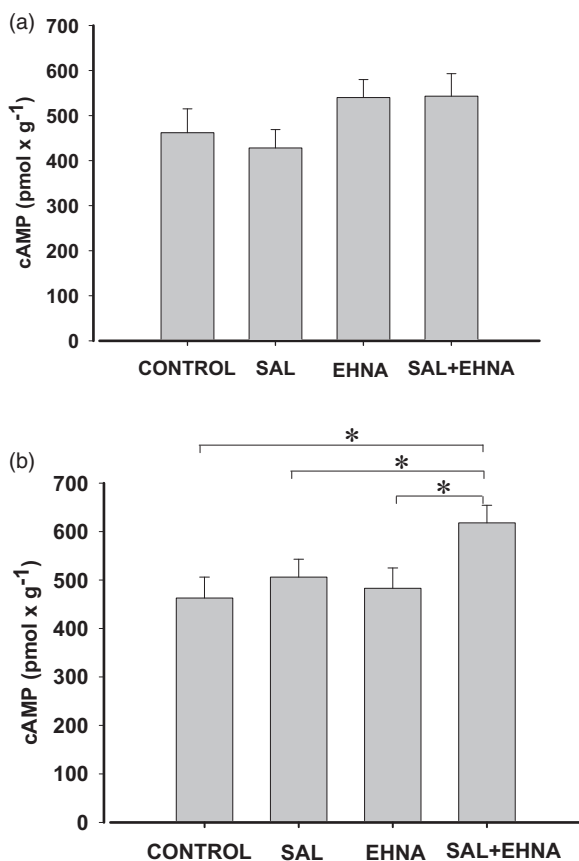
## Discussion

In the present study, we report for the first time, different expression and activity levels of PDE2 in RV and LV. Importantly, we also found that PDE2 differentially regulates  $\beta_2$  adrenoceptor-mediated cAMP production and contractility in the right and left ventricular myocardium.

Transcripts of the PDE2A gene have previously been identified in rat and human myocardium<sup>17</sup> and now we show that PDE2A mRNA levels are significantly lower in



**Figure 4**  $\beta_2$  adrenoceptor-mediated lusitropic responses to salbutamol (SAL) in right and left ventricular myocardium. Strips of rat right ventricle (a) or left ventricular papillary muscle (b) were exposed to 3  $\mu\text{mol/L}$  salbutamol plus 300 nmol/L CGP-20712A to block  $\beta_1$  adrenoceptors either in the absence (dotted lines) or in the presence (solid lines) of the PDE2 inhibitor EHNA (10  $\mu\text{mol/L}$ ). Top: original registration. Bottom: time required to half relaxation time. Data are expressed as means  $\pm$  S.E.M. of six experiments. \* $P < 0.05$



**Figure 5**  $\beta_2$  adrenoceptor-mediated effects of salbutamol, in the presence of the  $\beta_1$  adrenoceptor antagonist CGP-20712A, on cAMP production, in rat right (a) or left (b) ventricular myocardium. cAMP production was induced by 1  $\mu\text{mol/L}$  salbutamol (SAL) plus 300 nmol/L CGP-20712A in the absence or in the presence of 10  $\mu\text{mol/L}$  EHNA. Values are  $\pm$  S.E.M. of six experiments. \* $P < 0.05$

RV compared to LV. The microsomal fraction of heart muscle is enriched in sarcoplasmic reticulum membrane and contains some key proteins involved in cardiac contractility, including PDE2.<sup>9</sup> Our data on enzyme activity indicate that PDE2 activity and the contribution of PDE2 to the total PDE activity were significantly lower in the microsomal fraction of the RV with respect to the LV. It should be noted that PDE2 in the microsomal and cytosolic fractions was approximately one-third of the corresponding total PDE activity and differ from previous results in rat myocardium indicating that the PDE2 contribution was minimal<sup>15,26</sup> or null.<sup>17</sup> This discrepancy may be due to different assay conditions in which the catalytic activity was estimated. Assessment of catalytic activity depends on substrate concentration and the enzyme Michaelis constant ( $K_m$ ). Previous studies on PDE2 activity were performed in the presence of 0.5  $\mu\text{mol/L}$  cAMP<sup>17</sup> or 1  $\mu\text{mol/L}$  cAMP.<sup>26,27</sup> Given that  $K_m$  for PDE2 is 30  $\mu\text{mol/L}$ ,<sup>28</sup> a low substrate concentration will not reveal the maximal enzymatic activity, so we used 100  $\mu\text{mol/L}$  cAMP for the evaluation of the PDE2 activity.

We also studied the interventricular regulation of  $\beta_2$  adrenoceptor-mediated contractile responses by PDE2.  $\beta_2$  adrenoceptors are present in rat ventricular myocardium, but their inotropic effects are blunted by PDE activity. In fact, a global PDE inhibition unmasks a  $\beta_2$  adrenoceptor-mediated inotropic effect in this tissue.<sup>8,29</sup> In the present study,  $\beta_2$  adrenoceptor stimulation was devoid of inotropic effect both in RV and LV, which is consistent with previous results showing a lack of  $\beta_2$  adrenoceptor agonist-mediated inotropic effects in rodent myocardium.<sup>6,7</sup> However, in the presence of EHNA, stimulation of the  $\beta_2$  adrenoceptor with salbutamol increased contractility in LV. The inhibitory

effect of EHNA on adenosine deaminase<sup>9</sup> does not seem to play any role in the response because no  $\beta_2$  adrenoceptor-mediated inotropic effect was observed in the presence of DCF, which also inhibits adenosine deaminase but is devoid of PDE2 inhibitory activity.<sup>24</sup>  $\beta_2$  adrenoceptor activation also hastened relaxation in the presence of EHNA, in LV. This agrees with previous results in adult rat ventricular myocytes showing that the inhibition of PDE2 with EHNA slightly, but significantly enhanced the effect of  $\beta_2$  adrenoceptor stimulation on relaxation kinetics.<sup>30</sup> In contrast, we observed that  $\beta_2$  adrenoceptor stimulation in RV failed to produce any inotropic or lusitropic effect in the absence or presence of EHNA.

To test whether inotropic or lusitropic responses correlate with cAMP levels, we measured the interventricular cAMP production induced by activation of  $\beta_2$  adrenoceptors in the absence or presence of EHNA.  $\beta_2$  adrenoceptor activation did not increase cAMP concentration, which is consistent with previous results whereby  $\beta_2$  adrenoceptor agonists failed to enhance intracellular cAMP levels in rat ventricular myocardium.<sup>29,31,32</sup> In contrast,  $\beta_1$  adrenoceptor activation has been shown to raise cAMP levels in rat ventricular myocardium.<sup>16,32,33</sup> It is known that  $\beta_2$  adrenoceptor-mediated signaling is locally confined, unlike the generalized cAMP production generated by  $\beta_1$  adrenoceptor activation.<sup>34</sup> This is likely due to specific localization of  $\beta_2$  adrenoceptors inside the caveolae, whereas  $\beta_1$  adrenoceptors are also present in other cell fractions.<sup>35</sup> Inside the caveolae,  $\beta_2$  adrenoceptors are colocalized with G protein, AC isoforms, PKA and PDEs, the last limiting the diffusion of cAMP produced by  $\beta_2$  adrenoceptor stimulation.<sup>31</sup> In fact, the inhibition of PDE activity unmasks a  $\beta_2$  adrenoceptor-mediated increase in cAMP production in rat ventricular myocardium.<sup>29</sup> The results of the present study showing that salbutamol + CGP-20712A in the presence of EHNA produces an increase in cAMP concentration in the left ventricular myocardium is consistent with this and indicates that PDE2 limits  $\beta_2$  adrenoceptor-mediated cAMP production in this tissue. In RV, however, we failed to detect any effect of salbutamol + CGP-20712A on cAMP levels, in the absence or presence of EHNA.

Taken together, our results demonstrate a differential interventricular PDE2 mRNA transcription and activity, being higher in LV. They also show that PDE2 inhibition unmasks a  $\beta_2$  adrenoceptor-mediated increase in cAMP concentrations in LV. It is known that the enhancement of cAMP levels activates PKA leading to the phosphorylation of different PKA substrates such as phospholamban, L-type calcium channel or ryanodine receptor which result in increased contractility and relaxation.<sup>3,25</sup> This is consistent with the  $\beta_2$  adrenoceptor-mediated inotropic and lusitropic effects that we observed in LV when PDE2 was inhibited. A disparity in  $\beta_2$  adrenoceptor density does not seem to be responsible for this difference since it is virtually the same both in RV and LV of rat heart.<sup>36-38</sup> A likely explanation is that different interventricular PDE2 activity distinctly modulates  $\beta_2$  adrenoceptor-mediated effects in the RV and LV. However, the possibility that interventricular variations in PDE2 distribution in intracellular microdomains also contribute to these effects cannot be excluded.

Right and left ventricles have different functions and consequently perform different contractile work, meaning that there are important anatomical and electrophysiological disparities between both ventricles.<sup>2,39</sup> Importantly, proteomic analysis of both RV and LV has revealed that sarcomeric proteins such as myosin light chain 2,  $\alpha$ -actin and myosin heavy chain, or metabolic proteins such as peroxiredoxin 3 which elicit an antioxidant effect at mitochondrial level, are differentially expressed.<sup>1</sup> Some evidence also indicates that PDE activity may be different in right and left ventricular myocardium. Indeed, variations of PDE activities in different regions of rodent heart<sup>40,41</sup> and higher PDE5 activity in RV than in LV of feline heart<sup>42</sup> have been reported. Our results, showing a higher PDE2 mRNA transcripts and activity in left than in right rat ventricular myocardium, further support this view and suggest a different interventricular shaping of cAMP signals. This is likely to be of physiological significance and may contribute to better protecting the function of the LV (the most important heart chamber). Indeed,  $\beta_2$  adrenoceptors enhance cAMP tissue levels by activating the  $G_{\alpha s}$ /AC pathway, which increases cardiac contractility but may also induce some deleterious cardiac effects such as arrhythmias, apoptosis, or pathological hypertrophy.<sup>8</sup>  $\beta_2$ -adrenoceptor also regulates an alternative signaling pathway through activation of the inhibitory G protein ( $G_{\alpha i}$ ) and the heterodimer formed by the  $\beta$  and  $\gamma$  subunits of the G protein ( $G_{\beta\gamma}$ ).<sup>8</sup> Besides the inhibition of AC, the main signaling pathway regulated by  $\beta_2$ -adrenoceptors through  $G_{\alpha i}$ / $G_{\beta\gamma}$  appears to be the phosphatidylinositol 3-kinase (PI3K) signaling cascade which improves energy metabolism and protects against apoptosis in cardiac myocytes. Hydrolysis of cAMP by PDE2 may improve the benefit/risk profile of  $\beta_2$ -adrenoceptors by reducing their cAMP-mediated deleterious effects without affecting their PI3K-mediated beneficial effects. This may be particularly true in the case of an increase in cAMP production, whether compartmentalized or throughout the cell.

It is known that the  $K_m$  of PDEs is a key factor in shaping cAMP cell signaling,<sup>43</sup> therefore PDE2 with a  $K_m$  of  $30 \mu\text{mol/L}$ <sup>28</sup> is practically inactive at  $\sim 1.2 \mu\text{mol/L}$  of cAMP, which is the basal level detected in adult ventricular myocytes.<sup>44</sup> Other PDEs with lower  $K_m$ , such as PDE3 and PDE4, are already acting at or close to saturation at the basal cAMP concentration. However, when transient increases of cAMP are elicited, as occurs after adrenergic stimulation, PDE2 may come into play and take on a relevant role in limiting cAMP enhancement. Interestingly, a marked PDE2 upregulation has recently been reported in failing rat and human hearts. This has been considered to be an important defence mechanism during cardiac stress, and the pharmacological activation of PDE2 has been proposed as a novel therapeutic strategy in heart failure.<sup>45</sup>

In conclusion, to the best of our knowledge, this study is the first to show differential interventricular expression and activity levels of PDE2. Our results also indicate that PDE2 differentially regulates  $\beta_2$  adrenoceptor-mediated cAMP production, inotropy, and lusitropy in RV and LV. These results suggest a different interventricular shaping of the cAMP signals, while the higher PDE2 activity in LV may

contribute to better protecting this heart chamber from  $\beta_2$  adrenoceptor/cAMP-mediated cardiac toxicity. Further understanding of the detailed molecular mechanism of the chamber-specific regulation of PDE2 expression and its functional consequences will help us to understand the pathophysiological significance of these findings.

**Authors' contributions:** JHC initiated the study and performed the experiments assessing cardiac contractility. FS and FFB carried out subcellular fractionation and PDE activity. JPS and CH determined PDE2A transcripts and TF measured cAMP tissue levels. All authors have contributed to writing this manuscript.

#### ACKNOWLEDGEMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

#### REFERENCES

- Cadete VJ, Lin HB, Sawicka J, Wozniak M, Sawicki G. Proteomic analysis of right and left cardiac ventricles under aerobic conditions and after ischemia/reperfusion. *Proteomics* 2012;**12**:2366–77
- Pandit SV, Kaur K, Zlochiver S, Noujaim SF, Furspan P, Mironov S, Shibayama J, Anumonwo J, Jalife J. Left-to-right ventricular differences in  $I_{KATP}$  underlie epicardial repolarization gradient during global ischemia. *Heart Rhythm* 2011;**8**:1732–9
- Woo AY, Xiao RP. Beta-Adrenergic receptor subtype signaling in heart: from bench to bedside. *Acta Pharmacol Sin* 2012;**33**:335–41
- Steinberg SF. The molecular basis for distinct beta-adrenergic receptor subtype actions in cardiomyocytes. *Circ Res* 1999;**85**:1101–11
- Brodde OE, Bruck H, Leineweber K. Cardiac adrenoceptors: physiological and pathophysiological relevance. *J Pharmacol Sci* 2006;**100**:323–37
- Freyss-Beguim M, Griffaton G, Lechat P, Picken D, Quennedey MC, Rouot B, Schwartz J. Comparison of the chronotropic effect and the cyclic AMP accumulation induced by beta 2-agonists in rat heart cell culture. *Br J Pharmacol* 1983;**78**:717–23
- Juberg EN, Minneman KP, Abel PW. Beta 1- and beta 2-adrenoceptor binding and functional response in right and left atria of rat heart. *Naunyn Schmiedeberg's Arch Pharmacol* 1985;**330**:193–202
- Pérez-Schindler J, Philp A, Hernandez-Cascales J. Pathophysiological relevance of cardiac  $\beta_2$ -adrenergic receptor and its potential as therapeutic target to improve cardiac function. *Eur J Pharmacol* 2013;**698**:39–47
- Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacol Rev* 2006;**58**:488–520
- Verde I, Vandecasteele G, Lezoualc'h F, Fischmeister R. Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type  $Ca^{2+}$  current in rat ventricular myocytes. *Br J Pharmacol* 1999;**127**:65–74
- Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M. Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. *Circ Res* 2004;**95**:67–75
- Vandecasteele G, Rochais F, Abi-Gerges A, Fischmeister R. Functional localization of cAMP signalling in cardiac myocytes. *Biochem Soc Transac* 2006;**34**:484–8
- Han X, Kobzik L, Balligand JL, Kelly RA, Smith TW. Nitric oxide synthase (NOS3)-mediated cholinergic modulation of  $Ca^{2+}$  current in adult rabbit atrioventricular nodal cells. *Circ Res* 1996;**78**: 998–1008
- Fischmeister R, Castro L, Abi-Gerges A, Rochais F, Vandecasteele G. Species- and tissue-dependent effects of NO and cyclic GMP on cardiac ion channels. *Comp Biochem Phys A* 2005;**142**:136–43
- Mongillo M, Tocchetti CG, Terrin A, Lissandron V, Cheung YF, Dostmann WR, Pozzan T, Kass DA, Paolocci N, Houslay MD, Zaccolo M. Compartmentalized phosphodiesterase-2 activity blunts beta-adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. *Circ Res* 2006;**98**:226–34
- Juan-Fita MJ, Vargas ML, Hernandez J. Comparative actions of diazepam and other phosphodiesterase inhibitors on the effects of nor-adrenaline in rat myocardium. *Pharmacol Toxicol* 2003;**93**:23–8
- Johnson WB, Katugampola S, Able S, Napier C, Harding SE. Profiling of cAMP and cGMP phosphodiesterases in isolated ventricular cardiomyocytes from human hearts: comparison with rat and guinea pig. *Life Sci* 2012;**90**:328–36
- Smith CJ, Huang R, Sun D, Ricketts S, Hoegler C, Ding JZ, Moggio RA, Hintze TH. Development of decompensated dilated cardiomyopathy is associated with decreased gene expression and activity of the milrinone-sensitive cAMP phosphodiesterase PDE3A. *Circulation* 1997;**96**:3116–23
- Penefsky HS. Reversible binding of  $P_i$  by beef heart mitochondrial adenosine triphosphatase. *J Biol Chem* 1977;**252**:2891–9
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;**150**:76–85
- Harder KW, Owen P, Wong LKH, Aebersold R, Clark-Lewis I, Jirik FR. Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase  $\beta$  (HPTP $\beta$ ) using synthetic phosphopeptides. *Biochem J* 1994;**298**:395–401
- Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem* 1979;**100**:95–7
- Gonzalez-Muñoz C, Nieto-Ceron S, Cabezas-Herrera J, Hernández-Cascales J. Glucagon increases contractility in ventricle but not in atrium of the rat heart. *Eur J Pharmacol* 2008;**587**:243–7
- Rivet-Bastide M, Vandecasteele G, Hatem S, Verde I, Bénardeau A, Mercadier JJ, Fischmeister R. cGMP-stimulated cyclic nucleotide phosphodiesterase regulates the basal calcium current in human atrial myocytes. *J Clin Invest* 1997;**99**:2710–8
- Brutsaert DL, Sys SU. Relaxation and diastole of the heart. *Physiol Rev* 1989;**69**:1228–315
- Rochais F, Abi-Gerges A, Horner K, Lefebvre F, Cooper DM, Conti M, Fischmeister R, Vandecasteele G. A specific pattern of phosphodiesterases controls the cAMP signals generated by different Gs-coupled receptors in adult rat ventricular myocytes. *Circ Res* 2006;**98**:1081–8
- Richter W, Xie M, Scheitrum C, Krall J, Movsesian MA, Conti M. Conserved expression and functions of PDE4 in rodent and human heart. *Basic Res Cardiol* 2011;**106**:249–62
- Martins TJ, Mumby MC, Beavo JA. Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J Biol Chem* 1982;**257**:1973–9
- Gonzalez-Muñoz C, Fuente T, Hernandez-Cascales J. Phosphodiesterases inhibition unmask a positive inotropic effect mediated by beta2-adrenoceptors in rat ventricular myocardium. *Eur J Pharmacol* 2009;**607**:151–5
- Macdougall DA, Agarwal SR, Stopford EA, Chu H, Collins JA, Longster AL, Colyer J, Harvey RD, Calaghan S. Caveolae compartmentalise  $\beta_2$ -adrenoceptor signals by curtailing cAMP production and maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular myocyte. *J Mol Cell Cardiol* 2012;**52**:388–400
- Steinberg SF. Beta(2)-Adrenergic receptor signaling complexes in cardiomyocyte caveolae/lipid rafts. *J Mol Cell Cardiol* 2004;**37**:407–15
- Rybin VO, Pak E, Alcott S, Steinberg SF. Developmental changes in beta2-adrenergic receptor signaling in ventricular myocytes: the role of Gi proteins and caveolae microdomains. *Mol Pharmacol* 2003;**63**:1338–48
- Juan-Fita MJ, Vargas ML, Hernandez J. The phosphodiesterase 3 inhibitor cilostamide enhances inotropic responses to glucagon but not to dobutamine in rat ventricular myocardium. *Eur J Pharmacol* 2005;**512**:207–13



34. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. Beta<sub>2</sub>-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science* 2010;**327**:1653–7
35. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of beta – adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* 2000;**275**:41447–57
36. Horinouchi T, Morishima S, Tanaka T, Suzuki F, Tanaka Y, Koike K, Miwa S, Muramatsu I. Pharmacological evaluation of plasma membrane beta-adrenoceptors in rat hearts using the tissue segment binding method. *Life Sci* 2006;**79**:941–8
37. Myslivecek J, Nováková M, Palkovits M, Krizanová O, Kvetnanský R. Distribution of mRNA and binding sites of adrenoceptors and muscarinic receptors in the rat heart. *Life Sci* 2006;**79**:112–20
38. Hardouin S, Bourgeois F, Toraasson M, Oubenaissa A, Elalouf JM, Fellman D, Dakhli T, Swynghedauw B, Moalic JM. β-Adrenergic and muscarinic receptor mRNA accumulation in the sinoatrial node area of adult and senescent rat hearts. *Mech Age Dev* 1998;**100**:277–97
39. Brunet S, Aimond F, Li H, Guo W, Eldstrom J, Fedida D, Yamada KA, Nerbonne JM. Heterogeneous expression of repolarizing, voltage-gated K<sup>+</sup> currents in adult mouse ventricles. *J Physiol* 2004;**559**:103–20
40. Demirel-Yilmaz E, Cenik B, Ozcan G, Deric M.K. Various phosphodiesterase activities in different regions of the heart alter the cardiac effects of nitric oxide. *J Cardiovasc Pharmacol* 2012;**60**:283–92
41. Hua R, Adamczyk A, Robbins C, Ray G, Rose RA. Distinct patterns of constitutive phosphodiesterase activity in mouse sinoatrial node and atrial myocardium. *PLoS ONE* 2012;**7**:e47652
42. Shan X, Margulies KB. Differential regulation of PDE5 expression in left and right ventricles of feline hypertrophy models. *PLoS ONE* 2011;**6**:e19922
43. Matthiesen K, Nielsen J. Cyclic AMP control measured in two compartments in HEK293 cells: phosphodiesterase K<sub>M</sub> is more important than phosphodiesterase localization. *PLoS ONE* 2011;**6**:e24392
44. Iancu RV, Ramamurthy G, Warriar S, Nikolaev VO, Lohse MJ, Jones SW, Harvey RD. Cytoplasmic cAMP concentrations in intact cardiac myocytes. *Am J Physiol Cell Physiol* 2008;**295**:C414–22
45. Mehel H, Emons J, Vettel C, Wittköpper K, Seppelt D, Dewenter M, Lutz S, Sossalla S, Maier LS, Lechene P, Leroy J, Lefevre F, Varin A, Eschenhagen T, Nattel S, Dobrev D, Zimmermann WH, Nikolaev VO, Vandecasteele G, Fischmeister R, El-Armouche A. Phosphodiesterase-2 is upregulated in human failing hearts and blunts β-adrenergic responses in cardiomyocytes. *J Am Coll Cardiol* 2013;**62**:1596–606

(Received June 10, 2014, Accepted September 30, 2014)