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Phospholipase Cβ_4 isozyme is expressed in human, rat, and murine heart left ventricles and in HL-1 cardiomyocytes

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Abstract Phospholipase C-β (PLCβ) isozymes (PLCβ_1 and PLCβ_3) have been extensively characterized in cardiac tissue, but no data are available for the PLCβ_4 isozyme. In this study, PLC$\beta_{(1–4)}$ isozymes mRNA relative expression was studied by real-time PCR (RT-PCR) in human, rat, and murine left ventricle and the presence of PLCβ_4 isozyme at the protein level was confirmed by Western blotting in all species studied. Confocal microscopy experiments carried out in HL-1 cardiomyocytes revealed a sarcoplasmic subcellular distribution of PLCβ_4. Although there were unexpected significant interspecies differences in the PLC$\beta_{(1–4)}$ mRNA expression, PLCβ_4 mRNA was the main transcript expressed in all left ventricles studied. Thus, whereas in human and rat left ventricles PLCβ_4 > PLCβ_3 > PLCβ_2 > PLCβ_1 mRNA pattern of expression was found, in murine left ventricle the pattern of expression was different, i.e., PLCβ_4 > PLCβ_1 > PLCβ_3 > PLCβ_2. However, results obtained in mouse HL-1 cardiomyocytes showed PLCβ_3 ≈ PLCβ_4 > PLCβ_1 > PLCβ_2 pattern of mRNA expression indicating a probable cell type specific expression of the different PLCβ isozymes in cardiomyocytes. Finally, RT-PCR experiments showed a trend, even though not significant ($P = 0.067$), to increase PLCβ_4 mRNA levels in HL-1 cardiomyocytes after angiotensin II treatment. These results demonstrate the presence of PLCβ_4 in the heart and in HL-1 cardiomyocytes showing a different species-dependent pattern of expression of the PLC$\beta_{(1–4)}$ transcripts. We discuss the relevance of these findings in relation to the development of cardiac hypertrophy.

Keywords Phospholipase Cβ_4 · Human · Rat · Mouse · Left ventricle · RT-PCR · Angiotensin II

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Introduction

Myocardial hypertrophy can be initiated by different stressors such as physiological stress of physical exercise or pathological stress, like volume and/or pressure overload [1, 2]. In cellular models, hypertrophy can be induced by activation of certain receptors coupled to the heterotrimeric proteins belonging to the $G_{q/11}$ subfamily (angiotensin II AT_1 receptor, endothelin-1 ET_A receptor, and α_1-adrenergic receptor) [3]. Even if other signaling effectors of $G\alpha_{q/11}$ have recently been revealed [4], phospholipase Cβ is the best characterized effector of the $G\alpha_{q/11}$ mediated signaling [5]. In this sense, a recent study has shown that α_1-adrenergic receptor-mediated hypertrophic response is mediated by the PLCβ_{1b} isozyme in neonatal rat cardiomyocytes [6]. Thus, pharmacological interventions that interfere with PLCβ signaling in myocytes have been proposed as a new strategy to influence the hypertrophic process of the heart [6]. PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) producing two second messengers, inositol

1,4,5-trisphosphate (IP_3), and diacylglicerol (DAG). Both second messengers are involved in signaling pathways that can contribute to the development of cardiac hypertrophy [7, 8]. Four members of the PLC$\beta_{(1-4)}$ subfamily and multiple splice variants have been described and two of them (PLCβ_1 and PLCβ_3) have been extensively studied in cardiac tissue [9]. However, neither PLCβ_2 and PLCβ_4 isozymes nor the splice variants have been studied to the same extent. Furthermore, only one report has described PLCβ_2 expression in rat heart membranes [10], and no data is available to our knowledge on the presence of PLCβ_4 in myocardial tissue. However, a recent study using expressed sequence tags (EST) from the human Unigene database has proposed the presence of PLCβ_4 (Hs 472101) in human heart [11]. Taking into account the different regulatory properties of the PLCβ isozymes [12] and the different coupling of PLC isozymes to different receptor systems [13], we have considered of interest to study the pattern of expression of the different PLCβ isozymes in human, rat, and murine left ventricles. The aim of this study was to confirm the presence of PLCβ_4 in cardiac tissue and to study the pattern of expression of the different isozymes in different species. Furthermore, we have assessed whether key substances in the cardiac hypertrophy development as angiotensin II can influence the mRNA expression levels of this PLCβ isozyme.

Materials and methods

Human left ventricle and blood samples

Human left ventricle biopsy was obtained after informed consent. The patient (50 years old) was diagnosed of eccentric left ventricular hypertrophy secondary to severe aortic valve regurgitation and the myocardial sample was obtained at operation time. Blood sample was obtained from healthy control subject (33 years old) after informed consent. This study has been approved by the institutional review committee and follows the recommendations from the Helsinki Declaration.

Preparation of crude heart membranes

Animals used in this study were used following the US National Institutes of Health guiding principles in the care and use of animals. Adult male Wistar-Kyoto rats (20–22 weeks old) (CRL: Wi (Han), Charles River, Barcelona, Spain) and adult male BALB/c mice (20–22 weeks old) (BALB/cByJ, Charles River, Barcelona, Spain) fed ad libitum. Animals were killed by decapitation after handling, and heart left ventricles (20–150 mg) were dissected in ice-cold 20 mM Tris–HCl buffer at pH 7.0, containing 1 mM EGTA (Tris-EGTA buffer) prior to homogenization and then homogenized in 20 volumes of the same hypotonic buffer using a polytron homogenizer. A crude membrane preparation was isolated by repeated centrifugations and rehomogenizations in hypotonic buffer as described previously [14, 15]. Briefly, the homogenate was centrifuged for 15 min at 40,000 × g. The pellet obtained was then resuspended in Tris/EGTA buffer, rehomogenized, and centrifuged again. Protein concentration was determined by the Bradford's method [16]. The samples were aliquoted in microcentrifuge eppendorf tubes and the pellets were kept at −80°C.

Western blot of PLCβ_4

Adult human, rat, and murine crude heart membrane proteins were solubilized in 2× sample buffer (final concentration: 10% glycerol, 5% 2-mercaptoethanol, 2% sodium dodecylsulphate (SDS) and 62.5 mM Tris–HCl, pH 6.8), and equal protein amounts (20 μg of membrane proteins) were resolved by electrophoresis in 8% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Spain). Blots were blocked in 5% non-fat dry milk/phosphate-buffered saline overnight at 4°C and incubated for 2 h at room temperature with the rabbit polyclonal anti-PLCβ_4 antibody (1:400 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Blots were washed and incubated with the secondary antibody goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co. St. Louis, MO, USA) diluted 1:1000 in blocking buffer for 1 h at room temperature. Immunoreactive bands were visualized with the enhanced chemiluminescence reagents (ECL-Plus, Amersham Pharmacia, Spain) and analyzed by imaging analyzer (Chemi-Doc XRS, Bio-Rad, Philadelphia, PA, USA). Blotting with the primary antibody preincubated with blocking peptide specific for the anti-PLCβ_4 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as negative control and mouse cerebellum as positive control. Each gel contained a prestained broad-range protein ladder (Kaleidoscope™, BIO-RAD, CA, USA) to calculate the molecular masses of the individual bands.

Cell culture

HL-1 murine cardiomyocytes (a gift from Dr. W. C. Claycomb, Louisiana State University Medical Center, USA) (passages 61–65) were cultured on fibronectin-covered flasks with Claycomb media™ supplemented with 0.1 mM norepinephrine (Sigma–Aldrich), 2 mM L-glutamine (Life technologies), 100 U/ml/100 μg/ml Penicillin/Streptomycin (Life technologies), and 10% FBS (JRH Biosciences) (maintenance medium). For real-time PCR (RT-PCR) experiments HL-1 cells (10^6 per treatment) were

treated for 24 h with 1 μmol/l angiotensin II without norepinephrine and FBS, and a control group was carried out in parallel. Cell area was quantified from manually outlined cells digitized microscopic images (recorded by a Nikon phase contrast microscope; Nikon corporation, Tokyo, Japan) of randomly chosen cell fields using NIS-Elements AR v3.0 software (Nikon Instruments Europe, Badhoevedorp, Netherlands). 40 cells were measured for each independent experiment, and the experiments were repeated 4 times. For confocal microscopy experiments HL-1 cells were plated on 24-well plates (4×10^4 cells per well) with glass slides previously coated with fibronectin.

Confocal microscopy

HL-1 cardiomyocytes grown on fibronectin-coated slides were stained using DAPI for nuclear visualization and the localization of PLCβ_4 was examined by immunofluorescence using a rabbit polyclonal antibody from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and an anti-rabbit Alexa Fluor®-594 conjugated secondary antibody from Invitrogen. Confocal images were captured using a Zeiss Meta510 LSM with ×60 oil-immersion objectives (Carl Zeiss, Oberkochen, Germany).

Real-time PCR

Adult rat and murine left ventricles samples ($n = 6$) were processed to isolate the RNA using RNeasy® Fibrous Tissue Minikit (Qiagen, Germantown, MD, USA). HL-1 cardiomyocytes ($n = 4$) were processed to isolate the RNA using RNeasy® Mini Kit (Qiagen, Germantown, MD, USA). Total RNA was quantified using Nano Drop® ND-100 UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Pool of human PolyA RBA, mRNA, human heart, left ventricle from 15 control subjects was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The cDNA was obtained using random primers and the Taqman Reverse Transcription Reagents (Ref N808-0234, applied Biosystems, Foster City, CA, USA) following the manufacturers instructions.

Rat and murine left ventricle and HL-1 cardiomyocyte PLC$\beta_{(1-4)}$ genes were amplified using predesigned SYBR probes from Qiagen (Germantown, MD, USA) (rat assay IDs: QT01685537, QT00188062, QT01620563, and QT00 175266, respectively; mouse assay and murine HL-1 cardiomyocytes IDs: QT00173817, QT01052324, QT00155 274, and QT00117621, respectively). A rat, mouse, and murine HL-1 cardiomyocyte endogenous control of GAPDH gene was used (assay IDs: QT00199633 and QT00309099). Human PLCβ_{1-4} genes were amplified using predesigned TaqMan probes from Applied Biosystems (Foster City, CA, USA) (assay IDs: Hs01080542_m1, Hs00248563_m1, Hs00184504_m1, and Hs00168656_m1, respectively). An endogenous control of GAPDH gene was used (assay ID: Hs99999905_m1). Reactions were carried out in an ABI 7300, following manufacturer's instructions working with 50 ng of cDNA in each well. Each sample was analyzed in triplicate in each plate, and each plate was processed 3 times to test intra- and inter-plate variability, respectively. The relative quantity of the gene target was determined by the $2^{-\Delta\Delta Ct}$ method. All RQ values are expressed as mean ± SEM.

Statistical methods

All Western blot experiments were performed processing simultaneously the human, rat, and murine left ventricle samples within one experiment. Significance of mean difference in PLCβ_4 immunodetection and RT-PCR experiments were analyzed using Student's *t*-test. *P* value <0.05 was considered as being statistically significant.

Results

Real-time PCR experiments in human, rat, and mouse ventricle

Overall, the mRNA expression pattern analysis of the left ventricle PLCβ isozymes shows PLCβ_4 as the main transcript expressed in all the studied species (Fig. 1). However, the relative mRNA expression of the different PLC$\beta_{(1-4)}$ isozymes in the human left ventricle differed from those found in rat and murine left ventricles. Thus, in the human left ventricle the PLCβ_4 mRNA relative expression was 2.2-fold higher than the PLCβ_2 transcript (Fig. 1a). In the rat and murine left ventricles PLCβ_4 mRNA relative expression was 115- and 152-fold greater, respectively, than the mouse PLCβ_2 transcript (Fig. 1b). It should be noted here that the mouse PLCβ_2 transcript was chosen as reference value for the mouse and rat data analysis. Human and murine blood samples were used as a positive control for PLCβ_2 mRNA expression and as a control tissue where this PLCβ isozyme expression is well-defined (Fig. 1). PLCβ_2 mRNA expression was confirmed in all the studied left ventricles but some differences were shown among the species studied being the rat left ventricle where PLCβ_2 mRNA expression was lower. Concerning the PLC$\beta_{(1,3)}$ mRNA relative expression there were striking differences among species and the results showed that PLCβ_1 is more abundant than PLCβ_3 in murine left ventricle whereas the converse (PLCβ_3 > PLCβ_1) was found in human and rat left ventricles. The relative expression values for PLCβ_1 and PLCβ_3 transcripts were 48- and 24-fold over the PLCβ_2 transcript in murine left ventricle,

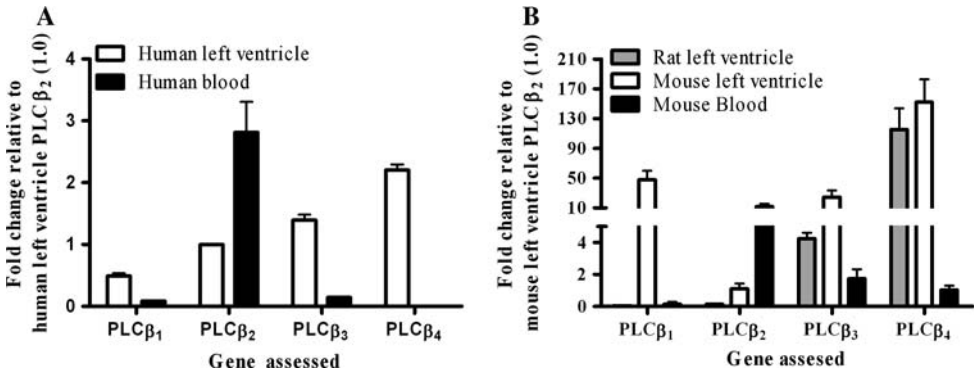

Fig. 1 Real-time PCR analysis of PLC$\beta_{(1-4)}$ gene expression. **a** Human left ventricle and blood were used and gene expression was normalized to GAPDH mRNA and expressed as fold change relative to PLCβ_2 transcript from human left ventricle. **b** Rat and mouse left ventricle and mouse blood and gene expression was normalized to GAPDH mRNA and expressed as fold change relative to PLCβ_2 gene from mouse left ventricle normalized. mRNA was prepared and quantified by RT-PCR as described in the "Material and methods" section. The results represent the mean ± SEM of three independent experiments carried out in triplicate

respectively, whereas 0.036/4.25 and 0.49/1.39-fold values were obtained for rat and human left ventricles, respectively (Fig. 1).

Effect of angiotensin II treatment in PLC$\beta_{(1-4)}$ mRNA expression in HL-1 cardiomyocytes

The mRNA expression pattern of the PLC$\beta_{(1-4)}$ isozymes in untreated HL-1 cardiomyocytes differed from results obtained from ventricles, showing similar pattern of mRNA expression for PLCβ_3 and PLCβ_4 (Fig. 2) and in a lower expression levels for PLCβ_1 and PLCβ_2. Cell area was significantly increased in HL-1 cardiomyocyte treated with 1 μmol/l of angiotensin II ($P < 0.05$, data not shown). Treatment with angiotensin II increased, even though not significantly, the mRNA expression levels for all the isozymes studied. The data obtained for PLCβ_4 was borderline with the statistical significance ($P = 0.067$) (Fig. 2).

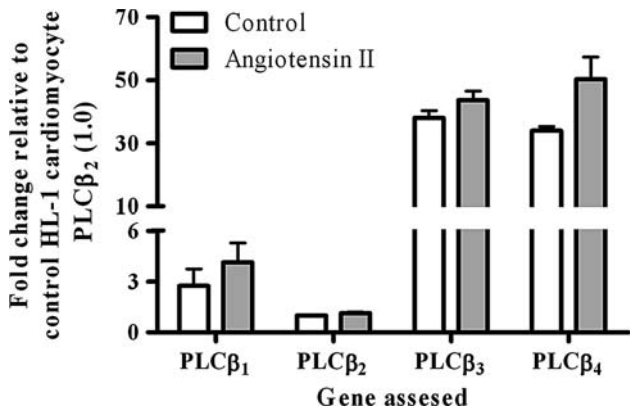

Fig. 2 Real-time PCR analysis of PLC$\beta_{(1-4)}$ gene expression in HL-1 cardiomyocytes after angiotensin treatment. HL-1 cardiomyocytes were treated for 24 h with 1 μmol/l angiotensin II and control cells were carried out in parallel. Gene expression was normalized to GAPDH mRNA and expressed as fold change relative to PLCβ_2 transcript from control HL-1 cardiomyocyte. mRNA was prepared and quantified by RT-PCR as described in the "Material and methods" section. The results represent the mean ± SEM of four independent experiments carried out in triplicate

Protein analysis for PLCβ_4

Western blot experiments performed in human, rat, and murine left ventricles showed the presence of PLCβ_4 at the protein level in all the studied samples (Fig. 3). Bands migrating as expected molecular masses corresponding to the two different splice variants [26] (130 and 116 KDa) are shown in all left ventricles studied. Furthermore, the signal obtained with the primary antibody alone disappeared when the sample was preincubated with the specific blocking peptide for the used anti-PLCβ_4 antibody. However, the results obtained showed differences at molecular masses of the bands in the different species. Thus, the bands detected for rat and mouse ventricles migrated at lower KDa than the band detected in human left ventricle. Optical density and statistical analysis of the results did not show any significant difference in the PLCβ_4 expression among human, rat, and murine left ventricles.

Subcellular distribution of the PLCβ_4 isozyme

Confocal microscopy experiments of immunostained HL-1 cardiomyocytes were performed in order to assess the subcellular localization of PLCβ_4. Confocal image analysis showed a sarcoplasmic distribution of PLCβ_4 in HL-1 cardiomyocytes ruling out a sarcolemmal or nuclear localization (Fig. 4).

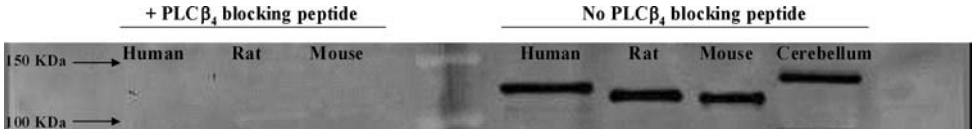

Fig. 3 Representative Western blot from three independent experiments of PLCβ_4 isozyme from adult heart tissues. Membranes prepared as described in "Material and methods" section were resolved by SDS-PAGE on 8% gels. Each *lane* represented 20 μg of membrane proteins. *Lanes* were as follows: human, rat, and mouse left ventricle with and without blocking peptide preincubation and as positive control we used mouse cerebellum

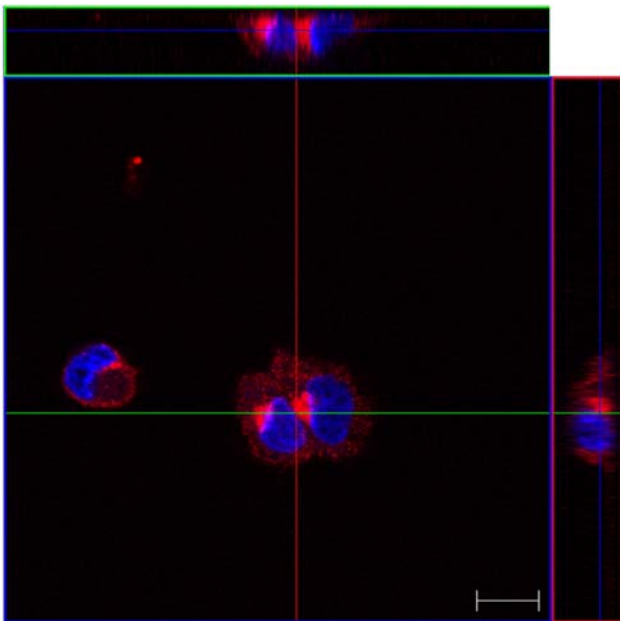

Fig. 4 Confocal image analysis of immunofluorescent staining of HL-1 cardiomyocytes with PLCβ_4 primary antibody and Alexa 594 anti-rabbit secondary antibody (*red channel*), and nuclear staining (DAPI). PLCβ_4 shows a sarcoplasmic distribution and is not present in the nuclei or in sarcolemma. At least 100 cells were examined. *Scale bar* = 10 μm

Discussion

Although PLCβ_1 and PLCβ_3 isozymes have been extensively studied in cardiac tissue and several studies have linked the cardiac hypertrophy to PLC$\beta_{(1,3)}$ activation [6, 17–19], no studies have been carried out in order to study the presence of PLCβ_4 in mammalian heart and the role that PLCβ_4 could play in the development of cardiac hypertrophy. The inhibition induced by ribonucleotides on PLCβ_4 activity and the fact that PLCβ_4 was discovered after the characterization of PLCβ isozymes in cardiac tissue done by Rhee and coworkers in 1993 [20] could be the reason for the unavailable data characterizing PLCβ_4 in cardiac tissue [21, 22]. However, in the absence of ribonucleotides the specific PLCβ_4 induced PIP_2 hydrolysis is 4–5 times the average specific activity of PLCβ_1 and PLCβ_3 [22] and data obtained in vivo showed a $G_{q/11}$ -dependent activation of the PLCβ_4 [23]. Although the PLCβ_4 expression has been circumscribed to retina and cerebellum [12, 24] and visual and motor functions are related to this isozyme by data obtained in studies using knockout mice [25], a recent report using the EST from human Unigene database has suggested the presence of PLCβ_4 in human heart [11]. The data obtained in our study demonstrate that PLCβ_4 is expressed in human, rat, and murine left ventricles and in HL-1 cardiomyocytes. Furthermore, RT-PCR results obtained showed that PLCβ_4 is the main transcript expressed in the left ventricles of all the studied species and one of the most expressed in HL-1 cardiomyocytes. In addition, results obtained by Western blot in human, rat, and murine left ventricles and by confocal microscopy in HL-1 cardiomyocytes confirm the protein expression of PLCβ_4. However, the results obtained by Western blot experiments showed differences in the molecular mass of PLCβ_4 detected in the different species. The results obtained are in agreement with the presence of two different splice variants, PLCβ_{4a} expression in human ventricle whereas PLCβ_{4b} would be the splice variant expressed in rat and murine ventricles. Differences between rat and mouse PLCβ_4 mRNA length could explain the differences observed. However, post-transcriptional modifications or peptide splicing processes can not be excluded. Confocal imaging of murine HL-1 cardiomyocytes showed a sarcoplasmic distribution of PLCβ_4 which is in agreement with the well-documented cytoplasmic distribution of PLCβ_{4b} [26]. The eventual differential splice variant expression of PLCβ_4 in human ventricle could be functionally relevant since whereas PLCβ_{4a} is activated by $G_{q/11}$, PLCβ_{4b} is insensitive to Gα_q and G$\beta\gamma$ activation [26].

Treatment of HL-1 cardiomyocytes with angiotensin II increased, even though not significantly, all the PLCβ isozymes mRNA expression but this increase was more pronounced for PLCβ_4 mRNA. Further studies will be required in order to dissect whether the PLCβ_4 expression is altered by hypertrophic agents in other cardiomyocyte models as neonatal rat cardiomyocytes. In relation to this, recently it has been reported that α_1-adrenergic receptor-mediated hypertrophy is mediated by PLCβ_{1b} in neonatal rat cardiomyocytes [6]. However, this study has not analyzed whether PLCβ_4 is involved in this effect or whether other hypertrophic agents as angiotensin II mediate their

effect through PLCβ_{1b}. In this respect, differences among receptor coupling to the different PLCβ isozymes have been described [13] and therefore, different receptor-PLCβ isozyme coupling could be possible depending on which PLCβ isozymes are present downstream to these receptors. In this sense, the special regulatory properties of the PLCβ_4 led us to speculate that the interconversion from ATP or GTP, which inhibit PLCβ_4, into cyclic guanine nucleotides (cGMP and cAMP), which exert no inhibitory effect on PLCβ_4, induced by increased norepinephrine levels and NOS1 activity found in cardiac hypertrophy and heart failure, could relieve the inhibition of PLCβ_4 activity and therefore increase the activity of downstream signaling pathways related to cardiac hypertrophy and heart failure development in human ventricle [27–29]. Therefore, further studies will be necessary, in order to dissect the role of the PLCβ_4 and its splice variants in the etiopathology of cardiac hypertrophy and heart failure.

The data obtained in the present study showed PLCβ_4 as the main transcript expressed in all the studied left ventricles and one of the most expressed in HL-1 cardiomyoctes. Moreover, we found remarkable interspecies differences when considering the relative expression of the better studied PLCβ_1 and PLCβ_3 transcripts. Expression of PLCβ_1 is greater than PLCβ_3 in murine left ventricle, whereas the opposite was found in human and rat left ventricles. No studies are available in the literature where the PLCβ_1 and PLCβ_3 mRNA expression pattern have been compared across human, rat and murine left ventricles to contrast our findings. However, there are several reports where PLCβ activity was determined in mouse and rat cardiomyocytes showing interspecies differences in response to selective $G_{q/11}$-coupled receptor agonists that are reminiscent of our results [30, 31]. The differences found in those studies and the data obtained in the present study highlight the use of caution when comparing the results obtained in different animal models. In this regard, the present results suggest a more similar pattern of expression between rat and human left ventricles. However, the differences found at the PLCβ_1 and PLCβ_2 mRNA level of expression and the PLCβ_{4a} and PLCβ_{4b} splice variants between human and rat left ventricles raise the necessity to study in greater depth the differences existing among the different species in the PLCβ isozyme expression profile and more importantly the functional significance of these differences in the role of PLCβ in the development of cardiac hypertrophy.

In conclusion, our findings obtained by RT-PCR show PLCβ_4 as the main PLCβ transcript in human, rat, and murine left ventricles and one of the most expressed in HL-1 cardiomyocytes. On the other hand, PLCβ_4 protein expression was confirmed by Western blotting in human, rat, and murine left ventricles and by confocal microscopy in HL-1 cardiomyocytes. Even though angiotensin II increase in PLCβ expression did not reach statistical significance, the results obtained for PLCβ_4 mRNA expression levels raise the necessity to study in greater depth the role of PLCβ_4 in the cardiac hypertrophic development. Further studies will be required to understand the pathophysiological relevance of PLCβ_4 in the origin of cardiac hypertrophy and heart failure.

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