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Single inhibition of either PDE3 or PDE4 unmasks β_2-adrenoceptor-mediated inotropic and lusitropic effects in the left but not right ventricular myocardium of rat

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ABSTRACT

Cyclic nucleotide phosphodiesterase (PDE)3 and PDE4 provide the major PDE activity in cardiac myocytes and shape β_1-adrenoceptor-dependent cardiac cAMP signaling but their role in regulating β_2-adrenoceptor-mediated responses is less well known. We investigated potential differences in PDE3 and PDE4 activities between right (RV) and left (LV) ventricular myocardium, and their role in regulating β_2-adrenoceptor effects. PDE3 activity in the microsomal fraction was lower in RV than in LV but was the same in the cytosolic fraction. However, no significant difference between RV and LV was found when the PDE4 activity was studied. β_2-adrenoceptor activation increased inotropism and lusitropism in LV when measured in the presence of either the PDE3 inhibitor cilostamide, the PDE4 inhibitor rolipram or a non-selective PDE inhibitor IBMX. However, the joint inhibition of both PDE3 and PDE4 was necessary in RV to uncover β_2-adrenoceptor-induced inotropic and lusitropic effects. Our results indicate different regulation of β_2-adrenoceptor-mediated contractility by PDE3 and PDE4 in RV and LV of the rat heart. In the case of PDE3 due to a different contribution of the enzyme in the microsomal fraction whereas in the case of PDE4 it can be attributed to differences in the intracellular distribution and coupling to β_2-adrenoceptors.

1. Introduction

The heart can be considered as two independent pumps, one delivering blood to the lungs and the other to the rest of the body. Because the contractile work performed by each side of the heart is different, the anatomy as well as the mechanical and biochemical signaling of right ventricle (RV) and left ventricle (LV), the most important functional parts of the heart, are also different (Cadete et al., 2012; Pandit et al., 2011).

The sympathetic nervous system plays a pivotal role in regulating cardiac contractility, mainly through the activation of β-adrenoceptors, of which β_1- and β_2-adrenoceptors are the predominant subtypes expressed in the heart in many mammalian species, including human (Woo and Xiao, 2012). The effect of β_1-adrenoceptor activation on cardiac contractility has been extensively studied and it is known to produce a cAMP-dependent contractile effect by activating the stimulatory G protein/adenylyl cyclase/cAMP pathway (Brodde et al., 2006). The inotropic response to cardiac muscle β_2-adrenoceptor activation is less straightforward and, indeed, it was initially thought that this receptor, although present in the myocardium, was not involved in the contractile response (Freyss-Beguin et al., 1983; Juberg et al., 1985). However, more recent evidence indicates that β_2-adrenoceptor can also induce an inotropic effect although it is negated by the activity of cyclic nucleotide phosphodiesterase (PDE) enzymes, which break down cAMP into 5′-AMP (Perez-Schindler et al., 2013). Indeed, non-selective PDE inhibition by IBMX unmasks a positive inotropic effect of salbutamol mediated by β_2-adrenoceptor in the RV of the rat heart (Gonzalez-Muñoz et al., 2009).

PDEs are grouped into different families of which PDE3 and PDE4 account for most of the PDE activity in cardiomyocytes (Verde et al., 1999; Rochais et al., 2006). PDE3 exhibits a very low Michaelis constant (K_m), with a value of between 0.1 and 0.8 μM for both cAMP and cGMP, and a relatively low maximal velocity (V_{max}), which is higher for cAMP than for cGMP (Degerman et al.,

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1997). On the other hand, PDE4 is a cAMP-specific PDE with a K_m in the range of 1 to 10 μM and V_{max} values considerably lower than those of PDE3 (Bender and Beavo, 2006). PDE activity is not evenly distributed throughout the heart (Demirel-Yilmaz et al., 2012) and differences between RV and LV have been reported (Shan and Margulies, 2011; Soler et al., 2015). Both, PDE3 and PDE4 regulate β_1-adrenoceptor mediated contractility in rodent myocardium (Juan-Fita et al., 2003; Rochais et al., 2006). However, the interventricular regulation of β_2-adrenoceptor-mediated effects by PDEs is less well known. In the present work we have investigated the contribution of PDE3 and PDE4 in rat ventricle and whether or not they differentially regulate β_2-adrenoceptor-mediated responses. For this purpose, we isolated microsomal and cytosolic fractions of RV and LV for the biochemical characterization of PDE3 and PDE4 activities at a high exogenous cAMP concentration. Moreover, using a myocardiac preparation with endogenous cAMP, we compared the influence of either the PDE3 inhibitor cilostamide or the PDE4 inhibitor rolipram on contractile effects elicited by β_2-adrenoceptor activation.

2. Materials and methods

2.1. 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. Male Sprague-Dawley rats (250–350 g) were rendered unconscious instantaneously by cerebral concussion and euthanized by rapid exsanguination, after which the chest was opened and the heart rapidly removed and placed in Tyrode solution of the following composition: 136.9 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$ and 5 mM dextrose.

2.2. Cytosolic and microsomal fractions

Subcellular fractionation of the cardiac tissue was achieved by mechanical homogenization and differential sedimentation (Smith et al., 1997; Soler et al., 2015), using free wall from right or left rat ventricle muscle as the starting material. The cytosolic fraction of LV or RV was the supernatant of the 60 min centrifugation at 51,000 × *g* and the microsomal fraction of LV or RV was the corresponding pellet once resuspended and resedimented at 100,000 × *g* for 40 min. Cytosolic extracts were freed from phosphate and nucleotides by gel filtration through chromatographic minicolumns (Penefsky, 1977). Isolated fractions were aliquoted and stored at −80 °C for further use.

2.3. Protein quantitation

The total concentration in the isolated fractions was measured using a kit based on the bicinchoninic acid method and bovine serum albumin as standard protein.

2.4. PDE activity

A colorimetric procedure adapted to a microtiter plate was used. Essentially, the PDE activity product 5′-AMP was further cleaved under non-rate limiting conditions by excess 5′-nucleotidase, and the phosphate (P_i) released was quantified by the malachite green method (Lanzetta et al., 1979). cAMP hydrolysis was measured at 37 °C in a 100 μl reaction medium containing 10 mM Tris–HCl, pH 7.4, 25 mM NaCl, 0.2 mM $MgCl_2$, 100 μM cAMP, 5′-nucleotidase at 50 U/μl and the corresponding aliquot of the cytosolic or microsomal extract. The reaction was stopped at different times by 200 μl malachite green reagent and samples were processed as previously described (Soler et al., 2015). The initial rate of cAMP hydrolysis was determined using a plot of absorbance at 660 nm *vs.* time and the standard curve of 5′-AMP *vs.* P_i. The enzyme activity of PDE3 or PDE4 was evaluated by subtracting the PDE activity measured in the presence of a selective inhibitor from the activity measured in its absence. In these experiments, the cAMP concentration was 100 μM. Since the inhibitory effect may be dependent on substrate concentration (Yung-Chi and Prusoff, 1973), pilot assays were conducted to determine the effective concentration range for each inhibitor (10 μM cilostamide, 30 μM rolipam). In the presence of 100 μM cAMP, the inhibition of PDE3 by 10 μM cilostamide and PDE4 by 30 μM rolipam was additive and total PDE activity was inhibitable by IBMX. Activity data are expressed as nmol P_i/min/mg protein that are equivalent to nmol 5′-AMP/min/mg protein, while the contribution of specific PDE families are expressed as a percentage with respect to the total PDE activity.

2.5. Western blot of PDE3A

The separation and detection of PDE3A were performed by standard procedures. Briefly, aliquots of 100 μg protein were subjected to 7% sodium-dodecyl-sulfate-polyacrylamide minigel electrophoresis in Laemmli buffer and then electroblotted by semi-dry transfer. After blocking, the blotted membrane was successively exposed to rabbit PDE3A antibody (1:200) and peroxidase-conjugated anti-rabbit IgG (1:5000). The protein loading control was performed after stripping by reexposing the blotted membrane to rabbit extracellular signal-regulated kinase (ERK) antibody (1:7500) followed by incubation with anti-rabbit IgG conjugated to peroxidase (1:5000). Immunoreactive bands were detected with the Amersham™ ECL prime western blotting detection reagent from GE Healthcare (Madrid, Spain) and ChemiDoc XRS+ molecular imager from Bio-Rad Laboratories (Madrid, Spain). Densitometric quantitation was carried out with Gel-Pro Analyzer 3.1 software from Sigma.

2.6. Paced rat ventricular tissues

Right ventricular strips (10 mm long, 1 mm wide 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% O_2 and 5% CO_2. The preparations were electrically stimulated for 1 ms with a Grass SD-9 stimulator (Quincy, MA, USA) at a frequency of 1 Hz and supramaximal (threshold+25%) voltage. A length-force curve was obtained and the tissues were left at the length associated with the maximal developed force (Gonzalez-Muñoz et al., 2008). Contractions were measured using a Grass FT-03 force–displacement transducer (Quincy, MA, USA) and displayed on a computer screen using a Stemtech amplifier (Houston, TX, USA) and ACODAS software from Dataq Instruments (Akron, OH, USA). Tissues were allowed to equilibrate for 45–60 min before drug challenge.

To investigate the β_2-adrenoceptor-mediated inotropic effect, concentration-response curves for the β_2-adrenoceptor agonist salbutamol were obtained in the presence of the β_1-adrenoceptor antagonist CGP-20712A. Salbutamol concentrations were increased stepwise by 0.5 log unit, as soon as the response to the previous concentration had stabilised. Cumulative concentration-response curves for the β_2-adrenoceptor-mediated effect of salbutamol, in the presence of 300 nM CGP-20712A, were determined in the absence or after 15 min in the presence of the non-selective PDE inhibitor IBMX (30 μM). Alternatively, selective PDE inhibitors cilostamide (0.1 μM) or rolipram (3 μM) which