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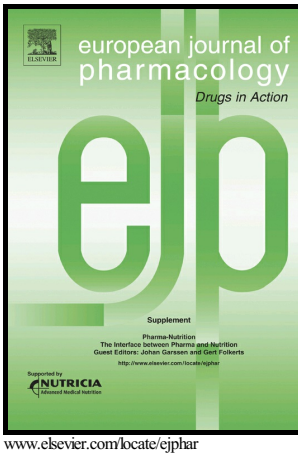

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Single inhibition of either PDE3 or PDE4 unmasks β_2-adrenoceptor-mediated inotropic and lusitropic effects in the left but not right ventricular myocardium of rat

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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.

Keywords:

PDE3-4 activities

β_2-adrenoceptor activation

Inotropy

Lusitropy

Interventricular difference

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., 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., 2014). 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., 2014), 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 ^{o}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 ^{o}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., 2014). 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 ºC, 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 effectively inhibit PDE3 (Verde et al., 1999) or PDE4 (Monguillo et al., 2004) activity, respectively were used. Only one concentration-response curve for salbutamol + CGP-20712A in the absence or presence of the above indicated drugs was determined in the same ventricular tissue.

For comparison, we also investigate the regulation of β_1-adrenoceptor-mediated contractility by PDE3 or PDE4 in left ventricular papillary muscle since it has already been studied in electrically-driven right ventricular strips (Juan-Fita et al., 2003). For this purpose, we used noradrenaline and preparations were pretreated with desmethylimipramine (2 μM), phentolamine (1 μM) and ICI-118551 (50 nM) to inhibit noradrenaline neuronal uptake and α- and β_2-adrenoceptors, respectively. In these conditions, cumulative concentration-response curves for noradrenaline were obtained in the absence or presence of either cilostamide (0.1 μM) or rolipram (3 μM). To confirm whether PDE inhibition only affected cAMP-mediated inotropy, concentration-response curves were obtained for ouabain, as a prototype of non-cAMP-dependent inotropic drug, in the absence or presence of the non-selective PDE inhibitor IBMX.

Drugs were added to a 30 ml organ bath in a volume smaller than or equal to 0.1 ml. Experiments were terminated by raising the Ca^{2+} concentration to 9 mM, which produced a maximal inotropic response, and the results are expressed as percentages of this effect and also in mN. The relaxation phase of the isometric twitch was also studied

by measuring the time-to-half relaxation ($t_{1/2}$), which is a good index of isometric relaxation in mammalian myocardium (Brutsaert and Sys, 1989)

2.7. Drugs and other products

Salbutamol, (±)-2-hydroxy-5-(2-((2-hydroxy-3-(4-(1-methyl-4-(trifluoromethyl)-1H-imidazol-2-l)phenoxy)propyl)amino)ethoxy)-benzamide methanesulfonate (CGP-20712A), (±)-1-(2,3-(dihidro-7-methyl-1H-inden-4-yl)oxy)-3-(1-methylethyl)amino)-2-butanol hydrochloride (ICI-118551), 3-isobutylmethylxantine (IBMX), cilostamide, rolipram, noradrenaline and ouabain were obtained from Tocris Bioscience (Bristol, UK) whereas desmethylimipramine and phentolamine were from Sigma-Aldrich (Madrid, Spain).

cAMP, 5'-AMP and 5'-nucleotidase from *Crotalus atrox* venom were products from Enzo Life Sciences (Farmingdale, NY, USA). Pierce® BCA protein assay kit was from Thermo Fisher Scientific (Rockford, IL, USA) and BioGel P-6DG for gel filtration was from Bio-Rad (Hercules, CA, USA). Polyvinylidene difluoride Amersham Hybond P 0.45 membrane for electroblotting was from GE Healthcare (Madrid, Spain). Rabbit antibodies anti-human PDE3A (sc-20792) and anti-rat ERK (sc-154) were from Santa Cruz Biotechnology (Heidelberg, Germany) and secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (W4011) was from Promega Biotech Ibérica (Madrid, Spain). All other reagents for the biochemical procedures were analytical grade and provided by Sigma-Aldrich (Madrid, Spain).

2.8. Statistics

Enzyme activity data are mean values of a number of determinations (n) ± S.E.M. Observed differences were analyzed by the Student t-test using SigmaPlot 11.0

from Systat software (San Jose, CA, USA). Functional results are also expressed as mean ± 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. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. PDE3 and PDE4 in subcellular fractions

The *in vitro* enzyme activity was measured in the presence of selective inhibitors to evaluate the contribution of specific PDE families. Experiments performed at saturating conditions of substrate both in the absence or presence of 10 μM cilostamide revealed that the contribution of microsomal PDE3 to total PDE activity was lower in RV (28.5 ± 4.2%) than in LV (40.3 ± 1.1%) ($n = 4$, $P < 0.05$) (Fig. 1A). That is, PDE3 activity in the microsomal fraction was around 29% lower in RV than in LV. When the cytosolic fraction was analyzed, PDE3 activity was 43.0 ± 2.9% in RV and 48.9 ± 5.1% in LV ($n = 4$, $P > 0.05$). In this case, the difference was not statistically significant.

A similar set of experiments performed in the absence or presence of 30 μM rolipram indicated that the contribution of PDE4 to the total activity was relatively low in both microsomal and cytosolic fractions, displaying values of 9.4 ± 1.6% in microsomes of RV ($n = 4$), 10.6 ± 5.0% in microsomes of LV ($n = 4$), 26.5 ± 2.5% in cytosol of RV ($n = 4$) and 26.8 ± 4.5% in cytosol of LV ($n = 4$) (Fig. 1B). The difference between microsomes of RV and LV or cytosol of RV and LV did not reach the significance level.

PDE3A and PDE3B subfamilies are products of homologous genes that exhibit different patterns of expression. Since PDE3A is more abundantly expressed in murine heart than PDE3B (Chung et al., 2015), the observed differences in PDE3 activity were also analyzed by Western blot. As can be seen (Fig. 1C), the relative expression of PDE3A was higher in LV than in RV and the difference was more evident in the microsomal than in the cytosolic fraction.

3.2. Salbutamol + IBMX

To ascertain whether inhibition of PDE activity unmasks β_2-adrenoceptor-mediated inotropic and lusitropic effects in LV, as it did in RV (Gonzalez-Muñoz et al., 2009), we tested the effect of salbutamol + CGP-20712A, in the absence or presence of the non-selective PDE inhibitor IBMX, in left ventricular papillary muscle. Salbutamol alone at 0.1 to 10 μM had no inotropic effect in LV but produced a concentration-dependent contractile effect in the presence of 30 μM IBMX. This effect was prevented by the β_2-adrenoceptor antagonist ICI-118551 (50 nM), indicating that it was dependent on β_2-adrenoceptor activation (Fig. 2). As expected, IBMX (30 μM) failed to increase the contractile effect of the non-cAMP-dependent inotropic agent ouabain both in right and left ventricular myocardium. Indeed, neither the –log EC_{50} (5.5 + 0.9, n = 4 in the absence and 6.0 + 0.7, n = 4 in the presence of IBMX, P > 0.05), nor the E_{max} (48.3 + 6.4, n = 4 in the absence and 40.6 + 2.7, n = 4 in the presence of IBMX, P > 0.05) of ouabain were modified by IBMX in RV. Similarly, IBMX in LV did not alter the –log EC_{50} (5.8 + 0.4, n = 4 in the absence and 5.5 + 0.6, n = 4 in the presence of IBMX, P > 0.05) or the E_{max} (30.3 + 5.2, n = 4 in the absence and 37.5 + 4.2, n = 4 in the presence of IBMX, P > 0.05) of ouabain. Moreover, IBMX had no inotropic effect in the presence of the β_1-adrenoceptor antagonist CGP-20712A (when investigating the interaction IBMX-β_2-adrenoceptor activation). However, in the absence of CGP-20712A (when studying the IBMX-ouabain interaction), IBMX increased inotropy by 46.8 + 7.3% (n = 4) in RV and 37.8 + 6.7% (n = 4) in LV, compared to basal contractility. This indicates that PDE inhibition enhances inotropic effect of basal cAMP levels, mainly generated by endogenous catecholamines through β_1-adrenoceptor activation.

Salbutamol at 3 μM had no lusitropic effect in LV when added after preincubation with CGP-20712A, the $t_{1/2}$ relaxation time being 48.5 + 1.9 ms (n = 5) in the absence and 47.2 + 1.5 ms (n = 5, P > 0.05) in the presence of salbutamol + CGP-20712A. However, the application of 3 μM salbutamol when papillary muscles were preincubated with CGP-20712A and IBMX, which produced a maximal inotropic effect (see Fig. 2), produced a lusitropic effect that was again prevented by preincubation with 50 nM ICI-118551 (Fig. 3).

3.3. Contractile effects regulation by PDE3 and PDE4

Selective inhibition of PDE3 by cilostamide (0.1 μM) or PDE4 by rolipram (3 μM) in cardiac muscle preparations failed to produce an inotropic effect by themselves or to promote β_2-adrenoceptor- mediated contractile effect induced by salbutamol in RV. Nevertheless, the joint inhibition of PDE3 and PDE4 by cilostamide + rolipram unmasked a concentration–dependent positive inotropic effect of salbutamol + CGP-20712A in this tissue (Fig. 4). However, the single inhibition of either PDE3 or PDE4 in LV, when samples were already preincubated with CGP-20712A was devoid of contractile effect but had a positive inotropic effect when salbutamol was added. This inotropic effect was further increased when both PDE3 and PDE4 were simultaneously inhibited (Fig. 5).

For comparison, we studied the effect of PDE3 and PDE4 inhibition on β_1-adrenoceptor-mediated inotropy in LV following its previous evaluation in RV by our group (Juan-Fita et al., 2003). For this purpose, we tested the effect of noradrenaline in the absence or presence of either 0.1 μM cilostamide or 3 μM rolipram. Each inhibitor produced a leftward shift of the concentration-response curve for noradrenaline (Fig. 6) and significantly changed the –log EC_{50} value from 6.75 + 0.06 (n = 4) when added

alone to 7.1 + 0.10 in the presence of 0.1 μM cilostamide (n = 4, $P < 0.05$) or 7.2 + 0.15 in the presence of 3 μM rolipram (n = 4, $P < 0.05$).

Whether cilostamide and rolipram affected the β_2-adrenoceptor-mediated lusitropic effect of salbutamol in ventricular myocardium was also studied. Neither cilostamide nor rolipram on their own affected the $t_{1/2}$ relaxation time in RV and only the joint inhibition of PDE3 plus PDE4 reduced this time in this tissue (Fig 7A). However, salbutamol + CGP-20712A reduced the $t_{1/2}$ relaxation time after the single inhibition of either PDE3 or PDE4 in LV, and the joint inhibition of PDE3 and PDE4 further increased the lusitropic effect of salbutamol + CGP-20712A (Fig. 7B).

4. Discussion

PDE3 and PDE4 are crucial in regulating cAMP-mediated effects (Verde et al., 1999; Rochais et al., 2006) since they account for the largest part of cAMP degradation under physiological conditions in myocardium. However, whether the catalytic activity and contribution of PDE3 and PDE4 differs in RV and LV is unknown and has been evaluated in the present study.

Current data on the contribution of PDE families in heart muscle are difficult to compare due to different experimental conditions used for evaluation, namely sample preparation and concentration of substrate (Rochais et al., 2006; Richter et al., 2011; Johnson et al., 2012). It is known that cAMP is compartmentalized and subjected to time-dependent changes, however if we assume the average basal value of ~1.2 μM (Iancu et al., 2008), PDE3 activity is working at ~73% saturation whereas PDE4 only reaches ~18%. Under non-saturating conditions, the contribution of PDE families is dependent on cAMP concentration. Our biochemical assays were performed in the presence of saturating substrate concentration in order to evaluate the activity of all the PDE families, including the high K_m (low affinity) PDE2 (Soler et al., 2014). In this way, the contribution of specific PDEs is independent of cAMP concentration. In fact, the contribution of PDE3 or PDE4 to the total activity is dependent on kinetic constants, cAMP concentration and protein content. This means that for any cAMP concentration, differences in activity between RV and LV are only dependent on protein content. It should be noted that our aim was to identify potential differences in PDEs regulation. In this respect, we analyzed the contribution of PDE3 and PDE4 in microsomal and cytosolic fractions. The microsomal fraction is the most directly associated with cardiac contractility regulation owing to the presence of sarcoplasmic reticulum membrane that

is known to contain PDE3A as component of a multiprotein signaling complex (Beca et al., 2013) and members of the PDE4 family (Houslay et al., 2007).

The functional results of the present work show that, as in RV (Gonzalez-Muñoz et al., 2009), PDE activity also blunts the inotropic and lusitropic effects elicited by β_2-adrenoceptor in the LV of rat heart. However, β_2-adrenoceptor-mediated inotropic and lusitropic responses are differentially regulated by PDE3 and PDE4 in right and left ventricular myocardium. Indeed, the sole inhibition of either PDE3 or PDE4 unmasks β_2-adrenoceptor-mediated inotropic and lusitropic effects in LV but not in RV. In the latter case, the combined inhibition of both isoenzymes was necessary to produce such effects. The single inhibition of either PDE3 by cilostamide, or PDE4 by rolipram had no effect on basal contractility in our preparation, which is consistent with the notion that in rat myocardium neither a selective inhibitor of PDE3 nor of PDE4 increases force of contraction when given alone, but they do increase contractility when administered in combination. Thus, inhibition of a sufficient proportion of PDEs to rise cAMP generated mainly through β_1-adrenoceptor activated by endogenous catecholamines is necessary (see Nicholson et al., 1991 for review). This agrees with our results in which the joint inhibition of PDE3 and PDE4 by IBMX increased ventricular contractility when the β_1-adrenoceptor was not inhibited by CGP-20712A (when assessing ouabain-IBMX interactions). However, in the presence of the β_1-adrenoceptor antagonist CGP-20712A, the combined inhibition of PDE3 and PDE4 by IBMX or rolipram+cilostamide failed to enhance contractility.

Activation of β-adrenoceptors by catecholamines plays a pivotal role in regulating cardiac contractility, and β_1- and β_2-adrenoceptors are predominantly expressed in the heart of various species, including human (Brodde et al., 2006). PDEs play an essential role in tailoring β-adrenoceptors responses (Bender and Beavo, 2006)

but their activities do not seem to be evenly distributed throughout the myocardium (Shan and Margulies, 2011; Demirel-Yilmaz et al., 2012; Soler et al., 2014). Acute stimulation of β_1-adrenoceptors produces an inotropic effect, which is regulated by PDE3 and PDE4 in both right (Juan-Fita et al 2003) and left (present work) ventricular myocardium of rat heart**.** However, the mechanism(s) underlying the inotropic effect of β_2-adrenoceptor activation is less straightforward. Indeed, β_2-adrenoceptor agonists failed to enhance cardiac contractility in rodent, unlike β_1-adrenoceptor agonists (Freyss-Beguin et al., 1983; Juberg et al., 1985). This seems to be due to lower cAMP production resulting from β_2-adrenoceptor activation, compared to β_1-adrenoceptor activation, as has been demonstrated in rat ventricular myocardium (Juan-Fita et al., 2003; Gonzalez-Muñoz et al., 2009) as well as in isolated rat ventricular myocytes (Rochais et al., 2006). In addition, the lower cAMP that is produced seems to be avidly hydrolysed by PDEs (Perez-Schindler et al., 2013) and so the overall inhibition of PDEs unmasked β_2-adrenoceptor-mediated inotropic and lusitropic effects in the RV of rat myocardium (Gonzalez-Muñoz et al., 2009). Our results show that IBMX promoted β_2-adrenoceptor-mediated inotropic effect and lusitropic effects in left ventricular papillary muscle thus indicating that β_2-adrenoceptor-mediated contractile effects are negated by PDE activity in both RV (Gonzalez-Muñoz et al., 2009) and LV (present results).To explore β_2-adrenoceptor-mediated effects we have used salbutamol which is an effective β_2-adrenoceptor agonist widely used in clinic for treating asthma, particularly during acute exacerbations (Bel, 2013; Neame et al., 2015). Our results also indicate that the single inhibition of PDE3 or PDE4 in LV unmasks β_2-adrenoceptor-mediated inotropic and lusitropic responses and this effect is further enhanced by the combined inhibition of both PDE families. This agrees with previous results in adult rat ventricular myocytes showing that the inhibition of either of these two PDE families enhances the effect of

β_2-adrenoceptors on the L-type Ca^{2+} current, which plays a determinant role in triggering myocardial contractility (Rochais et al., 2006). However, the joint inhibition of both PDE3 and PDE4 by IBMX (Gonzalez-Muñoz et al., 2009) or cilostamide + rolipram (present results) was necessary to uncover β_2-adrenoceptor-induced inotropic and lusitropic effects in RV. These results indicate that both PDEs are necessary to circumvent the inotropy elicited by β_2-adrenoceptor in RV, whereas either PDE3 or PDE4 individually is sufficient to prevent β_2-adrenoceptor-induced contractility in LV. Disparities in β_2-adrenoceptor density do not seem to be responsible for this difference since β_2-adrenoceptors expression is virtually the same in both RV and LV of the rat heart (Horinouchi et al., 2006; Myslivecek et al., 2006). The lower contribution of microsomal PDE3 activity in RV with respect to LV, accompanied by lower protein expression, could be expected to provide smaller cAMP signals when inhibited. However, interventricular differences in PDE4 activity cannot explain the differential regulation of β_2-adrenoceptor-mediated contractile effects in RV and LV. In this case, it can be attributed to the different intracellular distribution and coupling to β_2-adrenoceptors in right and left ventricular myocardium (Bethke et al., 1993; Smith et al., 1993; Molina et al., 2014).

RV and LV differ in structure and function and they work under very different mechanical conditions. RV ejects blood at low pressure to the lungs in an almost continuous manner, in contrast to the relatively high pressure pulsatile flow generated by LV. Thus, the existence of anatomical, functional and biochemical differences between right and left ventricle, such as wall thickness, maximal sarcomere shortening, Ca^{2+} dynamics, α-myosin heavy chain isoenzymes expression and ionic currents density (Brunet et al., 2004; Walker and Buttrick, 2009) is not surprising. Some evidence also points to differential contribution of PDEs in right and left ventricular myocardium.

Indeed, there are reports of higher PDE5 activity in right than in left ventricle of feline heart (Shan and Margulies, 2011) and also variations of PDE activities in different regions of the rodent heart (Demirel-Yilmaz et al., 2012; Hua et al., 2012; Soler et al., 2014). The results of the present work showing that PDE3 and PDE4 differently regulate β_2-adrenoceptor-mediated inotropic and lusitropic effects in right and left ventricular myocardium further support this view. This could be of interest, particularly in the setting of the right and left ventricular failure which may require drugs with selective ventricular inotropic effect in order to avoid interference with the function of the counterpart ventricle (Haddad et al., 2010). However, the clinical relevance of this finding remains to be determined.

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Figure Legends

Fig. 1. PDE activity and protein expression in subcellular fractions of rat ventricle. (A) The contribution of PDE3 activity was evaluated in microsomal and cytosolic fractions isolated from RV (white bars) or LV (grey bars) and deduced by subtraction from the corresponding total activity (n = 4). Significant differences are shown (*). (B) Contribution of PDE4 activity in microsomal and cytosolic fractions from RV (white bars) or LV (grey bars) were deduced by subtraction from the corresponding total activity (n = 4). Differences between groups were not significant. (C) Blot images of PDE3A and ERK in microsomes and cytosol from RV and LV. Protein levels of PDE3A in RV (white bars) and LV (grey bars) were normalized against the corresponding content of ERK2 and expressed on a relative scale. Weak detection of ERK1 was observed.

Fig. 2. β_2-adrenoceptor- mediated effect of salbutamol on the contraction basal force of left ventricular myocardium. In all cases, β_1-adrenoceptors were blocked by 300 nM CGP-20712A. Representative traces in three electrically-driven papillary muscles showing that salbutamol is devoid of any contractile effect (A) but preincubation with the non-selective PDE inhibitor IBMX (30 μM) unmasked a concentration-dependent positive inotropic effect of salbutamol (B). The effect was eliminated when the β_2-

adrenoceptor antagonist ICI-118551 (50 nM) was added in preincubation (C). (D) Cumulative concentration-response curves for the effect of salbutamol (●), salbutamol in the presence of IBMX (■) or salbutamol in the presence of IBMX + ICI-118551 (▲). Inotropic responses are expressed as percentage of the effect caused by 9 mM Ca^{2+}. Each point represents the mean value + S.E.M. (vertical bars) of 5 experiments.

Fig. 3. β_2-adrenoceptor-mediated lusitropic effect of salbutamol on left ventricular myocardium. Papillary muscles of rat heart were preincubated with 300 nM of the β_1-adrenoceptors blocker CGP-20712A and 30 μM of the non-selective PDE inhibitor IBMX (Control, C). In these conditions, tissues were exposed to 3 μM salbutamol (SAL). (A) original recordings. (B) $t_{1/2}$ relaxation times. The lusitropic effect of salbutamol in the presence of CGP-20712A plus IBMX was prevented by the β_2-adrenoceptor antagonist ICI 118551 (50 nM). Data are expressed as means + S.E.M. of 5 experiments. * $P < 0.05$.

Fig. 4. Effect of PDE3 and PDE4 on β_2-adrenoceptor-mediated inotropic effect in RV. Representative traces showing the effect of 3 μM rolipram (A), 0.1 μM cilostamide (B), or the combination of both (C) on salbutamol-evoked contractile response in electrically-driven strips of right ventricular myocardium. β_1-adrenoceptors were blocked by 300 nM CGP-20712A. (D) Cumulative concentration-response curves for salbutamol in the presence of the indicated concentrations of either rolipram (○), cilostamide (●) or rolipram + cilostamide (▼). Further details as in the legend of figure 2. Values are the means + S.E.M. of 4-5 experiments.

Fig. 5. Effect of PDE3 and PDE4 on β_2-adrenoceptor-mediated inotropic effect in LV. Representative traces showing the effect of 3 μM rolipram (A), 0.1 μM cilostamide (B) or the combination of both (C) on salbutamol-induced contractile response in electrically-driven papillary muscles. β_1-adrenoceptors were blocked by 300 nM CGP-20712A. (D) Cumulative concentration-response curves for β_2-adrenoceptor-mediated inotropic effect of salbutamol when measured in the presence of the indicated concentrations of either cilostamide (●), rolipram (○) or the combination of both (▼). Further details as in the legend of figure 2. Each point represents the mean value + S.E.M. of 4-5 experiments.

Fig 6. Effect of PDE3 or PDE4 on β_1-adrenoceptor- mediated inotropy in LV. Cumulative concentration-response curves in electrically-driven ventricular papillary muscle for noradrenaline alone (●) and in the presence of either 0.1 μM cilostamide (▼) or 3 μM rolipram (○). Preparations were pretreated with desmethylimipramine (2 μM), phentolamine (1 μM) and ICI-118551 (50 nM) to inhibit noradrenaline uptake and α- and β_2-adrenoceptors, respectively. Further details as in the legend to figure 2. Values are means + S.E.M. of 4 experiments.

Fig 7. Effect of PDE3 and PDE4 on β_2-adrenoceptor-mediated lusitropic response ($t_{1/2}$ relaxation time) in rat ventricular myocardium. Preparations were preincubated with 300 μM of β_1-adrenoceptor blocker CGP-20712A. In these conditions, electrically-driven right ventricular strips (A) or left ventricular papillary muscle (B) were exposed to 3 μM salbutamol (SAL) in the absence (C) or presence of either 0.1 μM cilostamide

(CIL), 3 μM rolipram (ROL) or both (CIL + ROL). Values are means + S.E.M. of 4-5 experiments.

Figure

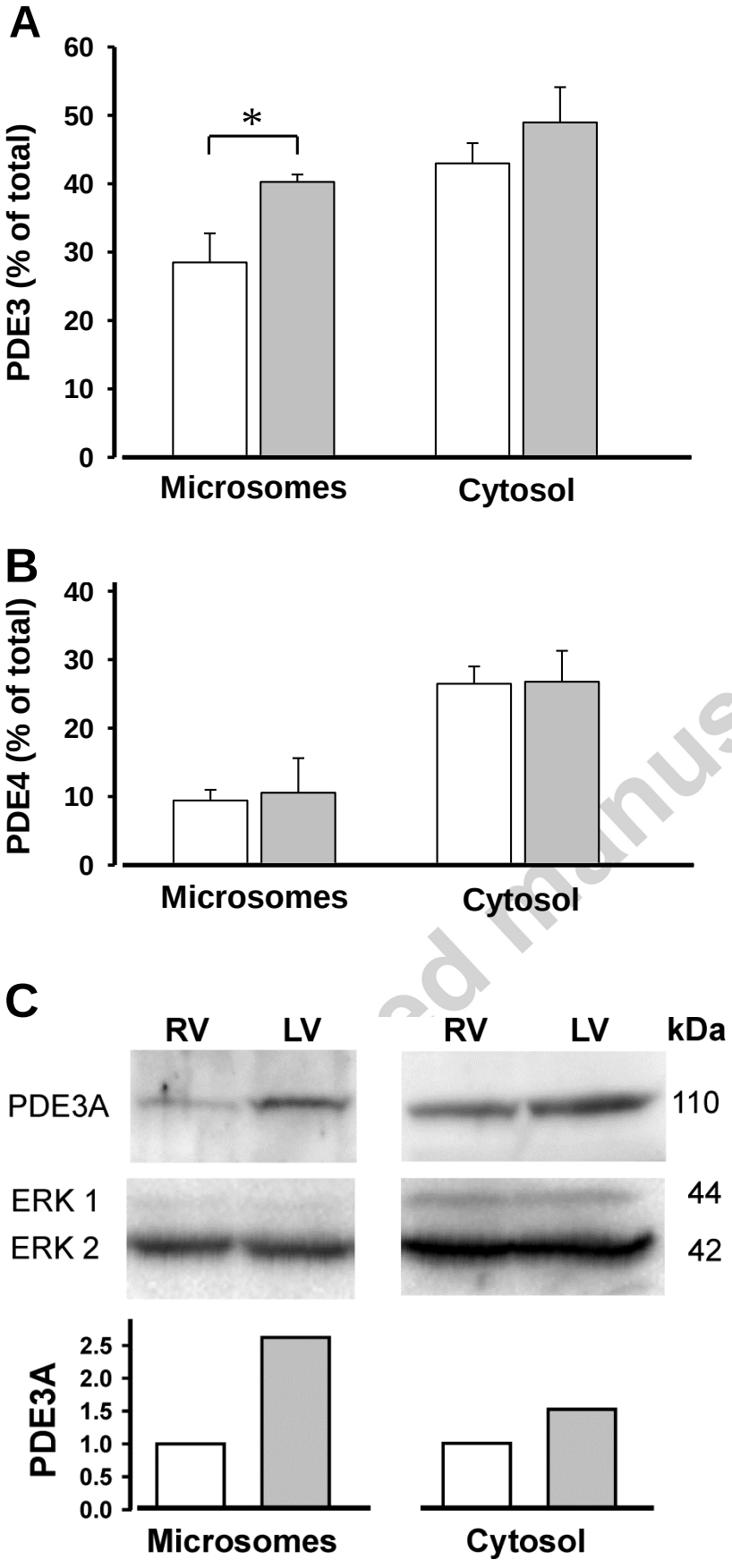

Figure 1

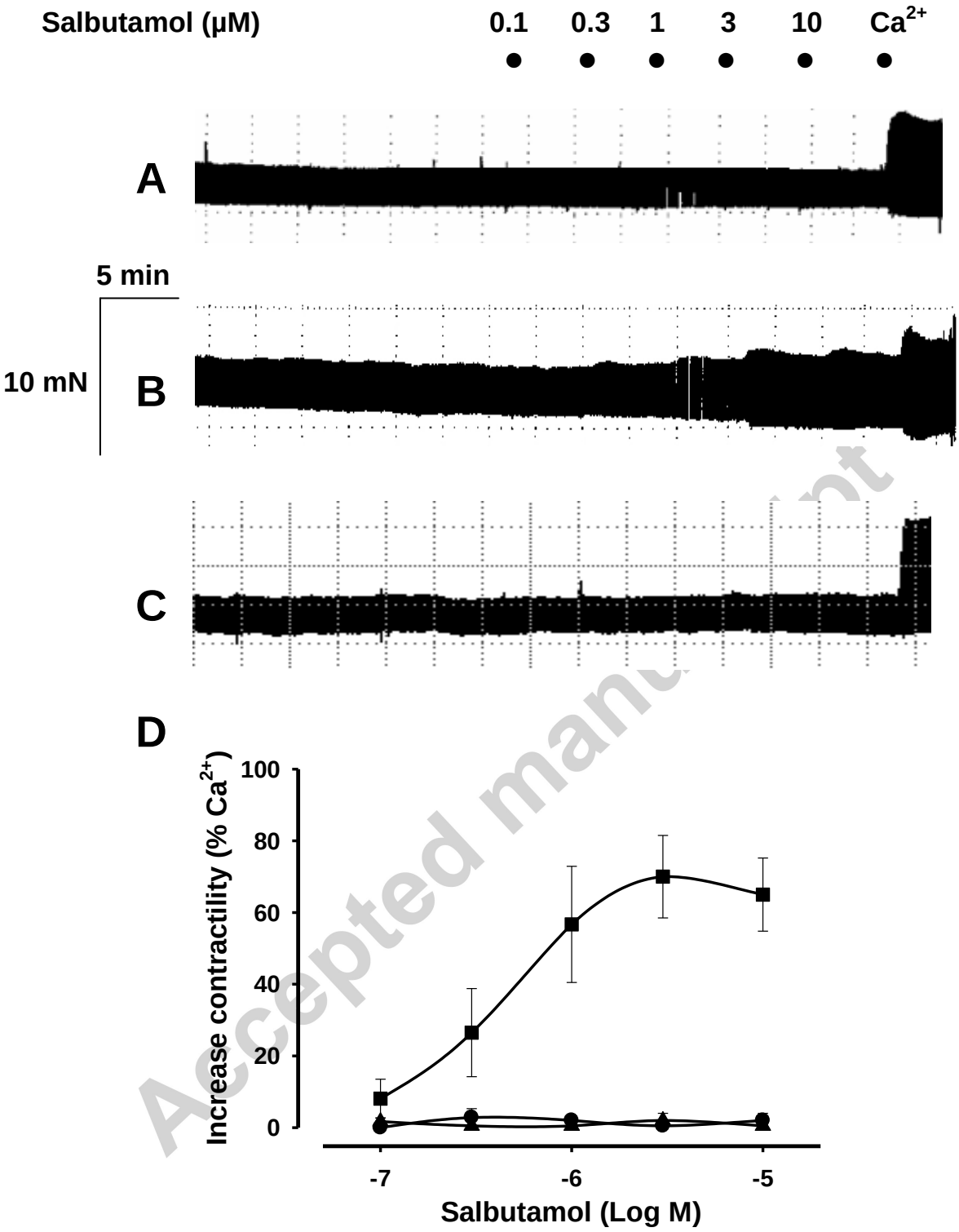

Figure 2

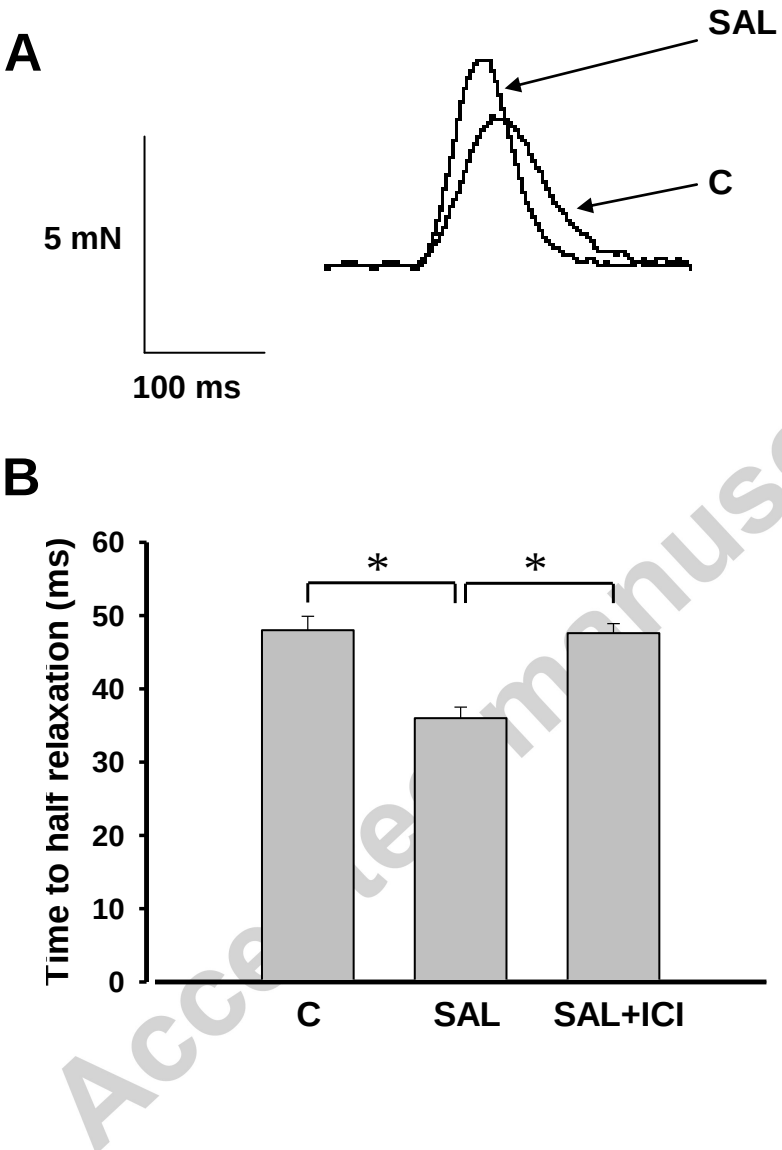

Figure 3

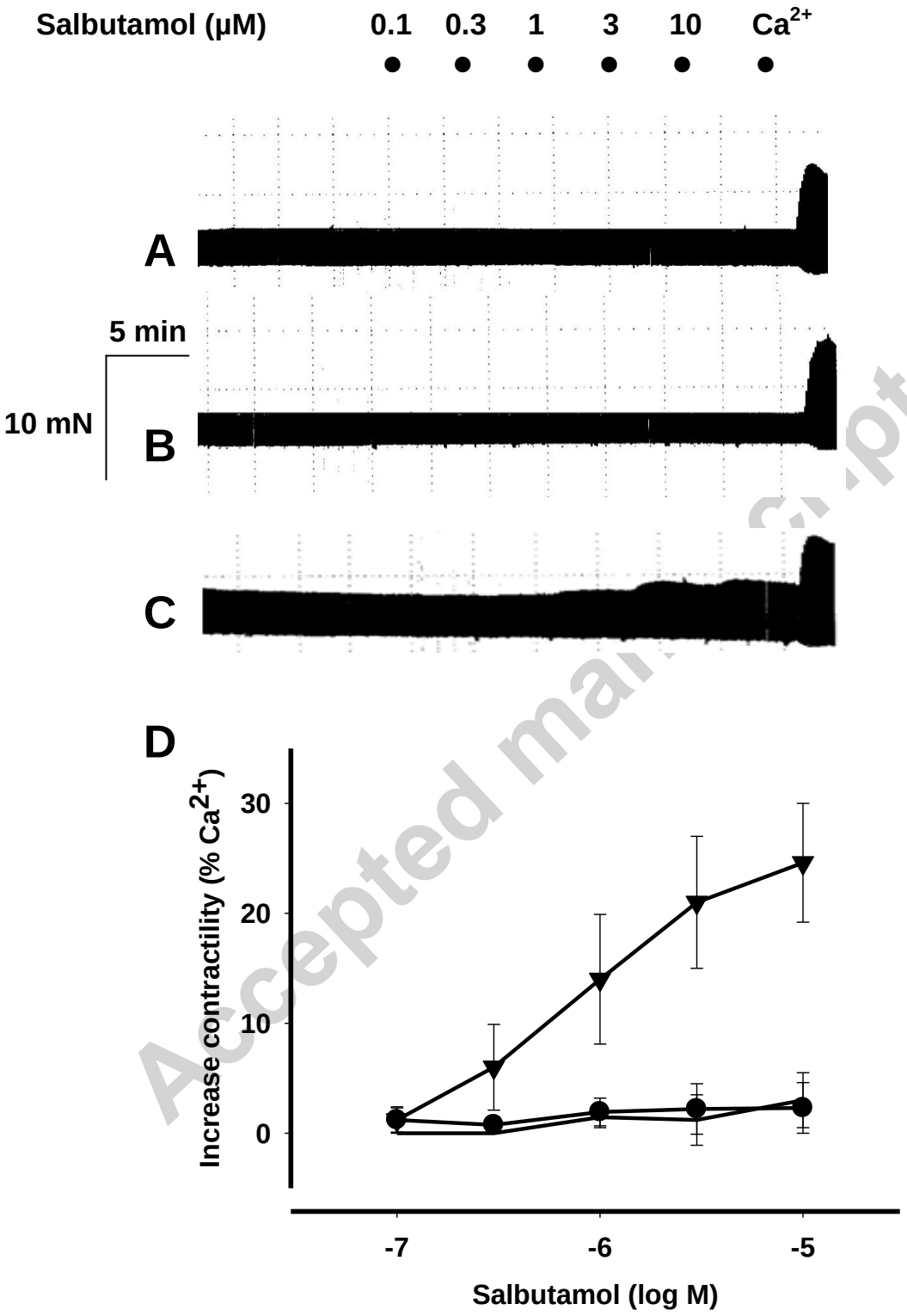

Figure 4

Figure 5

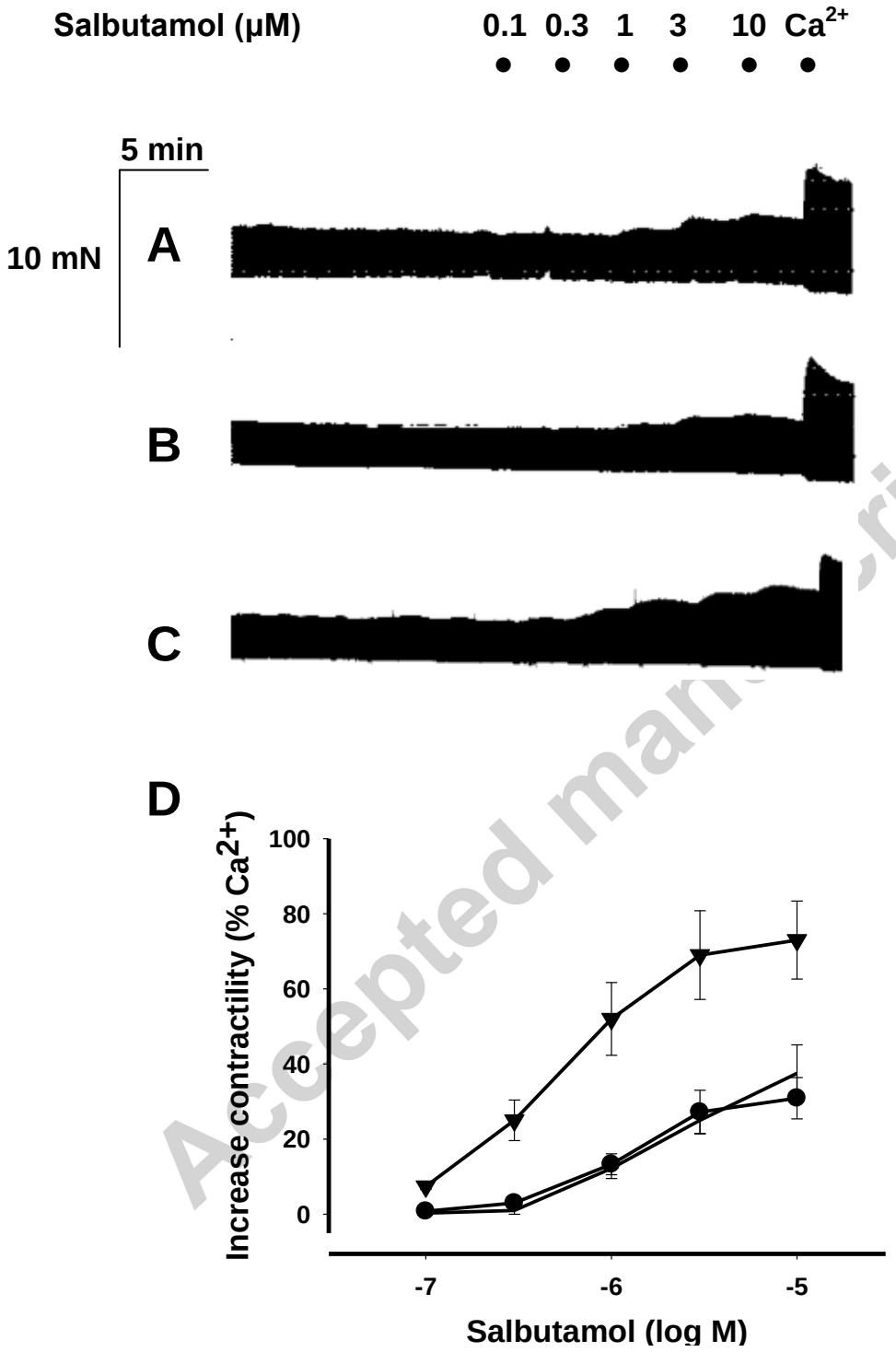

Figure 5

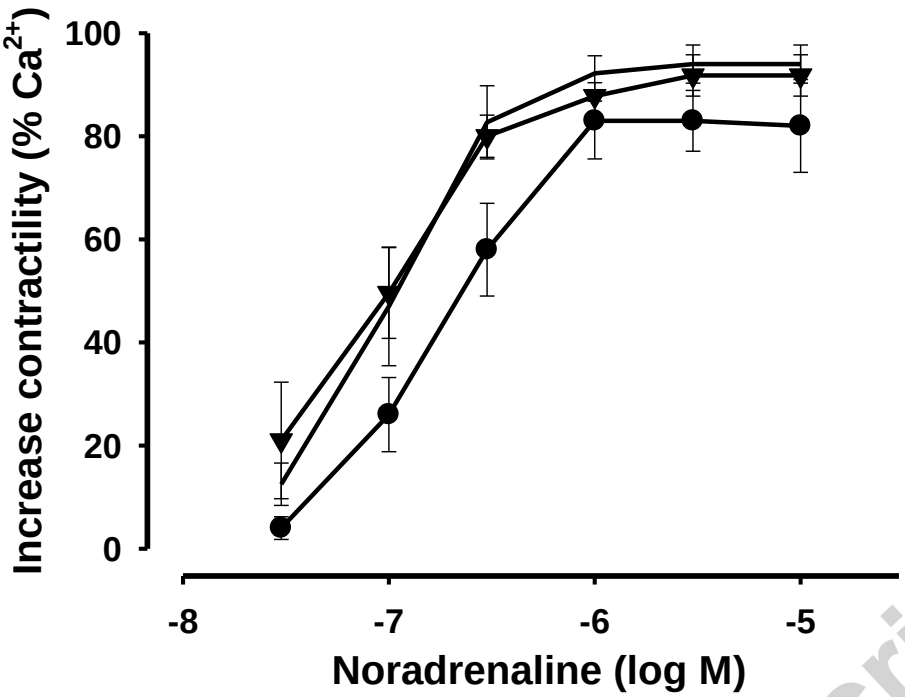

Figure 6

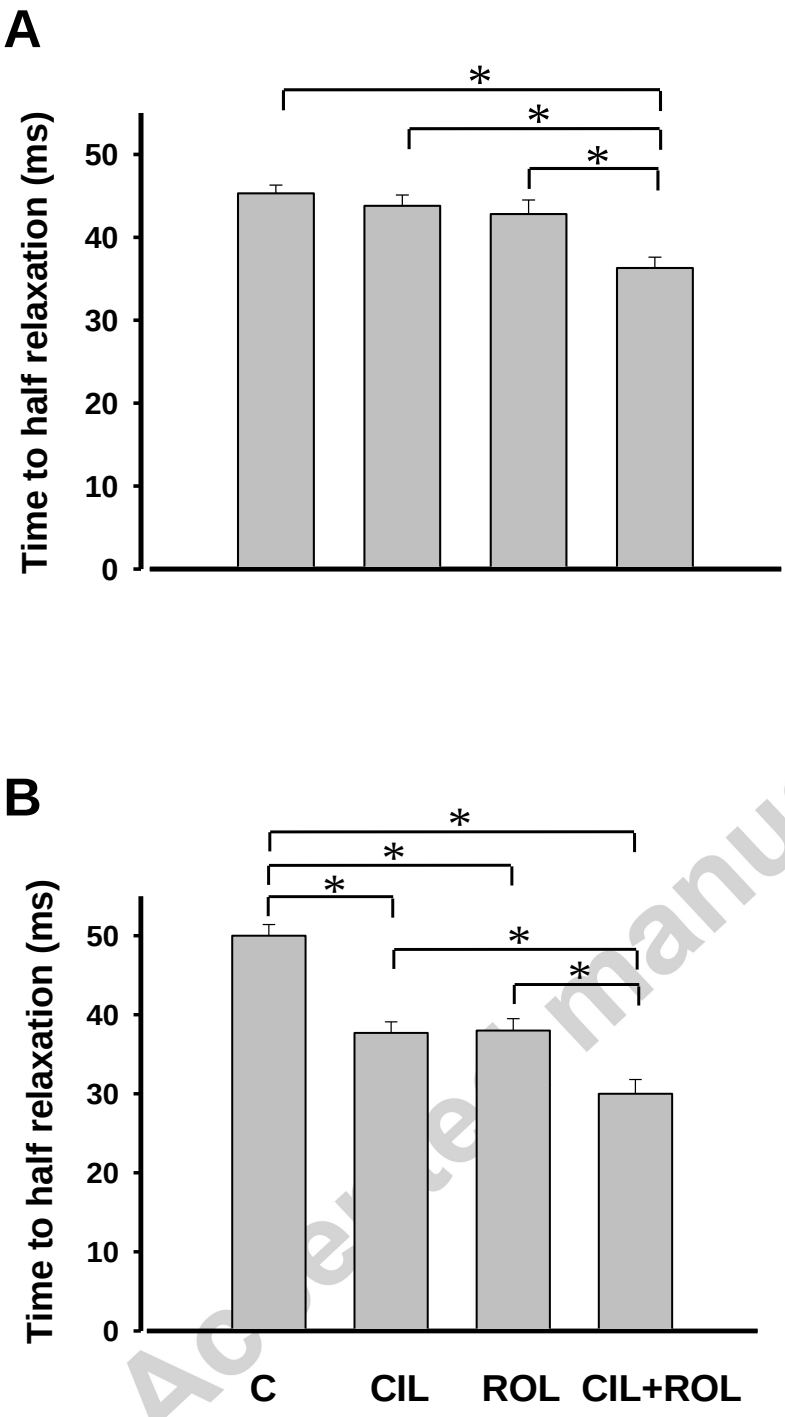

Figure 7