

## RESEARCH PAPER

# Functional antagonism of $\beta$ -adrenoceptor subtypes in the catecholamine-induced automatism in rat myocardium

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### Keywords

$\alpha$ -adrenoceptor;  $\beta_1$ -adrenoceptor;  $\beta_2$ -adrenoceptor; spontaneous activity; sarcoplasmic reticulum; calcium; arrhythmia; catecholamines; phosphodiesterases

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## BACKGROUND AND PURPOSE

Myocardial automatism and arrhythmias may ensue during strong sympathetic stimulation. We sought to investigate the relevant types of adrenoceptor, as well as the role of phosphodiesterase (PDE) activity, in the production of catecholaminergic automatism in atrial and ventricular rat myocardium.

## EXPERIMENTAL APPROACH

The effects of adrenoceptor agonists on the rate of spontaneous contractions (automatic response) and the amplitude of electrically evoked contractions (inotropic response) were determined in left atria and ventricular myocytes isolated from Wistar rats.

## KEY RESULTS

Catecholaminergic automatism was  $\text{Ca}^{2+}$ -dependent, as it required a functional sarcoplasmic reticulum to be exhibited. Although both  $\alpha$ - and  $\beta$ -adrenoceptor activation caused inotropic stimulation, only  $\beta_1$ -adrenoceptors seemed to mediate the induction of spontaneous activity. Catecholaminergic automatism was enhanced and suppressed by  $\beta_2$ -adrenoceptor blockade and stimulation respectively. Inhibition of either PDE3 or PDE4 (by milrinone and rolipram, respectively) potentiated the automatic response of myocytes to catecholamines. However, only rolipram abolished the attenuation of automatism produced by  $\beta_2$ -adrenoceptor stimulation.

## CONCLUSIONS AND IMPLICATIONS

$\alpha$ - and  $\beta_2$ -adrenoceptors do not seem to be involved in the mediation of catecholaminergic stimulation of spontaneous activity in atrial and ventricular myocardium. However, a functional antagonism of  $\beta_1$ - and  $\beta_2$ -adrenoceptor activation was identified, the former mediating catecholaminergic myocardial automatism and the latter attenuating this effect. Results suggest that hydrolysis of cAMP by PDE4 is involved in the protective effect mediated by  $\beta_2$ -adrenoceptor stimulation.

## Abbreviations

LA, left atria; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PLB, phospholamban; RL, resting cell length; Rmax, maximum response; SC, spontaneous contractions; SERCA, sarco-endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; SR, sarcoplasmic reticulum; VM, ventricular myocytes

## Introduction

Catecholaminergic signalling in the heart is one of the most physiologically relevant mechanisms for increase of cardiac output, which is achieved by chronotropic and inotropic stimulation. Both effects are mediated predominantly by  $\beta$ -adrenoceptors, whereas the contribution of post-junctional  $\alpha$ -adrenoceptors to the positive inotropic effect is relatively smaller and variable among species (Brodde and Michel, 1999; Bers, 2001).

$\beta$ -adrenoceptors are coupled to the  $G_{\text{os}}$  subunit of GTP-binding proteins, which mediates stimulation of adenylate cyclase catalytic activity, leading to accumulation of cAMP and activation of the cAMP-dependent protein kinase (PKA). The latter phosphorylates several substrates in cardiac myocytes, including the sarcolemmal L-type  $\text{Ca}^{2+}$  channels, and phospholamban (PLB), a negative regulator of the sarcoendoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA). The overall effect of PKA activation is enhancement of  $\text{Ca}^{2+}$  mobilization due to increase in both transmembrane  $\text{Ca}^{2+}$  influx and sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  uptake. The latter contributes not only to accelerate relaxation, but also to increase SR  $\text{Ca}^{2+}$  content (Bers, 2001; ter Keurs and Boyden, 2007). Because the SR is the source of most of the  $\text{Ca}^{2+}$  that activates contraction in mammalian myocardium, and the fraction of the SR  $\text{Ca}^{2+}$  content released at systole is largely dependent on the amplitude of the L-type  $\text{Ca}^{2+}$  current (i.e. the trigger for release) and the SR  $\text{Ca}^{2+}$  content (Bassani *et al.*, 1995), the systolic increase in cytosolic  $[\text{Ca}^{2+}]$  is greatly amplified by  $\beta$ -adrenoceptor stimulation (Ginsburg and Bers, 2004), thus increasing contraction amplitude. However, augmented SR  $\text{Ca}^{2+}$  load may also result in enhanced diastolic  $\text{Ca}^{2+}$  release (Bassani *et al.*, 1997; Shannon *et al.*, 2002), which, by activation of sarcolemmal  $\text{Ca}^{2+}$ -dependent inward currents, may give rise to delayed afterdepolarizations and spontaneous electrical activity in cardiac cells. While diastolic SR  $\text{Ca}^{2+}$  release has been implicated in the generation of pacemaker activity in nodal and subsidiary pacemaker cells from the right atrium, in the  $\text{Ca}^{2+}$ -overloaded myocardium it may favour development of arrhythmia (Bassani *et al.*, 1997; Bogdanov *et al.*, 2001; Nam *et al.*, 2005; ter Keurs and Boyden, 2007; Fujiwara *et al.*, 2008; Venetucci *et al.*, 2008).

Whereas  $\alpha$ -adrenoceptor stimulation has been shown to favour  $\text{Ca}^{2+}$  influx/efflux balance due to action potential prolongation, and to produce modest increase in  $\text{Ca}^{2+}$  transient amplitude (Brodde and Michel, 1999; Bers, 2001), little is known on the role of this adrenoceptor type in the generation of  $\text{Ca}^{2+}$ -dependent automatism. On the other hand, although both  $\beta_1$ - and  $\beta_2$ -adrenoceptor subtypes may exert positive inotropic action by coupling to the  $G_{\text{os}}$ -adenylate cyclase-PKA signalling cascade, evidence shows that the latter subtype may be also coupled to other pathways, such as  $G_{\text{oi}}$ -containing G proteins, whose activation may antagonize some of the effects typically related to the cAMP cascade, and thus limit enhancement of cell  $\text{Ca}^{2+}$  cycling (Kuschel *et al.*, 1999; Xiao *et al.*, 2006; Siedlecka *et al.*, 2008). Regarding  $\beta_2$ -adrenoceptor stimulation, pro-arrhythmic effects have been described in the myocardium from humans (Kaumann and Sanders, 1993), rabbits (DeSantiago *et al.*, 2008) and dogs (Billman *et al.*, 1997; Altschuld and Billman, 2000), whereas Sosunov *et al.* (2000) reported

arrhythmia facilitation by  $\beta_2$ -adrenoceptor blockade in canine ventricle. Thus, it is not clear yet whether this subtype is involved in the stimulation of myocardial automatism by catecholamines.

The main goal of the present study was the identification of the adrenoceptor types involved in the mediation of catecholamine-induced spontaneous activity in left atrial and ventricular myocardium. In addition, because of recent evidence suggesting a link between cAMP hydrolysis by phosphodiesterases (PDEs) and arrhythmogenesis (Lehnart *et al.*, 2005; Galindo-Tovar and Kaumann, 2008; Penna and Bassani, 2010), we also investigated whether catecholaminergic automatism was affected by PDE activity.

## Methods

### Myocardial preparations

Animal care and experimental protocols were in compliance with international and institutional guidelines, and were approved by the Committee on Ethics in Animal Research of the University of Campinas (CEEA/IB/UNICAMP, protocols No. P636-1 and P776-1). Left atria (LA) and left ventricular myocytes (VM) were isolated from adult (4–6-month-old) male Wistar rats (128 animals). Rats were maintained at  $23 \pm 2^\circ\text{C}$ , under a 12 h : 12 h light–dark cycle, and had free access to food and water. Animals were killed by exsanguination following cerebral concussion.

Left atria were mounted in an organ bath containing Krebs–Henseleit solution at  $36.5^\circ\text{C}$  gassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . The preparation was attached to a force transducer (F-60, Narco Biosystems, Houston, TX, USA), and electrically stimulated with voltage pulses (1.2 $\times$  threshold amplitude, 3 ms duration, 0.5 Hz) through a pair of piercing platinum electrodes. After application of 5 mN preload, the muscle was allowed to equilibrate for 45 min.

Ventricular myocytes were enzymatically isolated via retrograde perfusion at  $37^\circ\text{C}$  (Penna and Bassani, 2010). Briefly, after aorta cannulation, the heart was perfused with  $\text{Ca}^{2+}$ -free Krebs–Henseleit solution for 5 min, and then with the same solution containing collagenase type I (0.5 mg·mL<sup>-1</sup>, Worthington Biochem, Lakewood, NJ, USA) for 15–20 min. After perfusion with enzyme-free solution for 5 min, the left ventricle was removed, and myocytes were mechanically dissociated, washed and stored at  $4^\circ\text{C}$  in glutamate solution. Cells were plated on a collagen-coated perfusion chamber placed on the stage of a videomicroscopy system, and perfused with modified Tyrode's solution at 23 or  $36^\circ\text{C}$ . Field electric stimulation (same parameters as for LA, except for the rate at  $36^\circ\text{C}$ , which was 1 Hz) was delivered through a pair of platinum electrodes. Cell shortening was measured with a video-edge detector (Centro de Engenharia Biomédica, UNICAMP, pat# MPI-300.834-7) and converted to percentage of the resting cell length (RL).

### Concentration–effect curves

In this study, the automatic response to adrenoceptor agonists was considered as the development of spontaneous contractions (SC) in the absence of electrical stimulation (Boer and Bassani, 2004; Carvalho *et al.*, 2006; Penna and Bassani,

2010). For determination of the concentration–effect relationship in LA, we used an experimental protocol developed for multicellular myocardial preparations (Boer and Bassani, 2004), in which the high variability of response among preparations precludes determination of individual curves. Briefly, the number of SCs was computed during three successive 1 min rest periods intercalated by stimulation at 5 Hz for 30 s. This stimulation/rest protocol was applied in the absence of drugs, as well as after 2 min exposure to the agonist. The average SC rate at each agonist concentration was used for determination of the parameters of the concentration–effect curve. For VM experiments, a similar protocol was used, except that the stimulation rate was not increased before the rest periods. In this case, it was possible to obtain concentration–effect curves for individual cells. The SC rate was computed by visual inspection and from force/cell length traces recorded during the rest periods. We have previously reported that, with catecholamine exposure, the SC rate in the absence of electric stimulation is strongly correlated to the rate of extrasystolic contractions during electric stimulation (Penna and Bassani, 2010).

All experiments with LA were performed in the presence of 0.1  $\mu\text{M}$  atropine to avoid interference from endogenously released acetylcholine. Preparations were incubated (LA) or perfused (VM) with receptor antagonists for 50 or 30 min, respectively, before agonist addition and throughout the experiment. Twitch amplitude, measured immediately before application of the stimulation/rest protocol, was used for assessment of the agonist inotropic effect. Preliminary experiments showed that sensitivity, but not the maximum inotropic response to agonists, was affected by the stimulation/rest protocol in LA. Thus, only the maximum response ( $R_{\text{max}}$ ) was used for comparisons.

To investigate the role of the SR in the generation of spontaneous activity, VM were treated with 5  $\mu\text{M}$  thapsigargin for 5 min, which inhibits SERCA completely and irreversibly. The effectiveness of the treatment was confirmed by abolition of SR reloading upon electrical stimulation, tested by challenge with 10 mM caffeine (Bassani *et al.*, 1995). In LA, SERCA was inhibited by incubation with 50  $\mu\text{M}$  2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (Nakamura *et al.*, 1992) for at least 20 min.

### Solutions

The composition of the Krebs–Henseleit solution was (mM): 115 NaCl, 4.5 KCl, 25  $\text{NaHCO}_3$ , 2.5  $\text{CaCl}_2$ , 1.2  $\text{KH}_2\text{PO}_4$ , 1.2  $\text{MgSO}_4$ , 11 glucose; pH 7.4. For experiments with catecholamines, 1 mM ascorbic acid was added. The composition of the Tyrode's solution was (mM): 140 NaCl, 6 KCl, 1  $\text{CaCl}_2$ , 1 MgCl, 5 HEPES, 11 glucose; pH adjusted to 7.4 with NaOH. The composition of the glutamate solution was (mM): 30 KCl, 70 glutamic acid, 1  $\text{MgCl}_2$ , 10  $\text{KH}_2\text{PO}_4$ , 20 taurine, 10 HEPES, 11 glucose; pH adjusted to 7.4 with KOH.

All solutions were prepared with salts of analytical grade and type I water. Stock solutions of the drugs were prepared with water (except for milrinone, rolipram and thapsigargin, which were dissolved in dimethylsulphoxide), stored at  $-20^\circ\text{C}$ , and diluted immediately before use. Except for thapsigargin (Calbiochem, La Jolla, CA, USA), all drugs were from Sigma Chem Co (St Louis, MO, USA).

### Data analysis

A sigmoidal function was adjusted to the concentration–effect relationship for estimation of the  $R_{\text{max}}$  and the negative logarithm of the agonist molar concentration that evoked half-maximal response ( $\text{pD}_2$ ). For LA, the standard error (SE) of each parameter was obtained from the curve fitting to the average points. For VM, mean and SE were calculated from values determined in individual cells. These values were similar to the mean parameters of the average curve determined in the same way as for LA, which indicates that both approaches produce equivalent results. For concentration–effect curves determined in VM,  $n$  refers to the number of cells studied, which were isolated from at least four hearts for a given experimental protocol.

Data were compared by either Student's *t*-test for unpaired or paired samples, or by one- or two-way analysis of variance followed by Bonferroni *t*-test for multiple comparisons, when appropriate.  $P < 0.05$  was considered to show statistical significance.

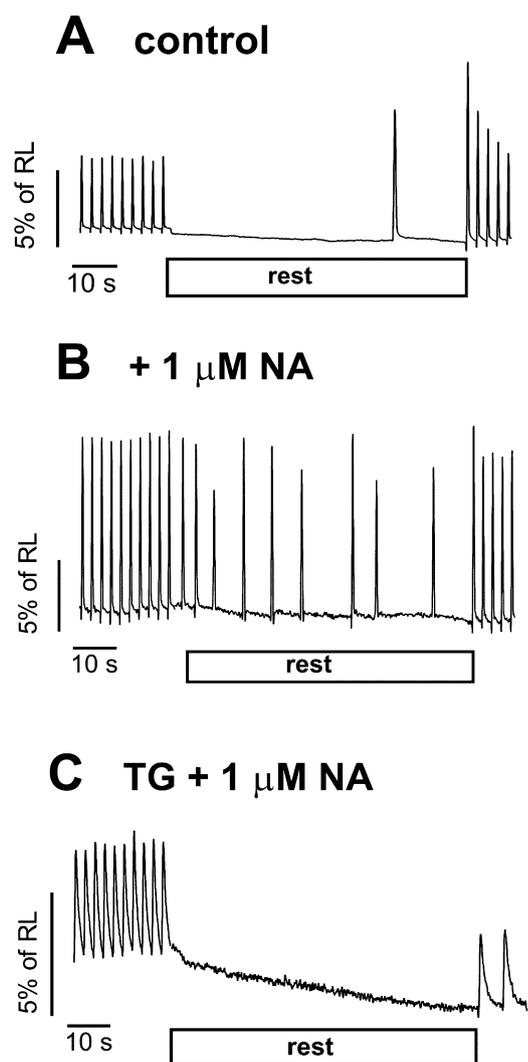
### Results

Under basal conditions, contraction amplitude was  $2.98 \pm 0.37$  mN ( $n = 81$ ) in LA, and  $9.31 \pm 0.24\%$  and  $6.47 \pm 0.41$  of RL in VM at 23 ( $n = 185$ ) and  $36^\circ\text{C}$  ( $n = 59$ ) respectively. In both preparations, the SC rate during stimulatory rest in the absence of agonists was low, especially in LA, in which it was not statistically different from zero ( $0.2 \pm 0.1$  SC $\cdot\text{min}^{-1}$  in LA;  $0.6 \pm 0.1$  and  $1.1 \pm 0.2$  SC $\cdot\text{min}^{-1}$  in VM at 23 and  $36^\circ\text{C}$  respectively). Such low rates indicate that the stimulation/rest protocol *per se* is not effective at promoting automatism. None of the adrenoceptor antagonists alone changed significantly the contraction amplitude or SC rate in either LA or VM.

Spontaneous activity was greatly enhanced by exposure to catecholamines (Figure 1A,B). In LA, SCs commonly developed at a quite regular rate ( $\sim 3$ – $5$  Hz), as described by Boer and Bassani (2004). In VM, spontaneous activity was manifested mainly as propagated contractile waves, or less often as contractions nearly as rapid as electrically evoked twitches. However, after treatment with thapsigargin, not only did twitch amplitude markedly decrease, but also spontaneous activity was totally abolished, even in the presence of high agonist concentrations (Figure 1C) in all of the six studied cells. In LA, di-(*tert*-butyl)-1,4-benzohydroquinone decreased the developed force by  $\sim 60\%$  both in the absence and presence of 10  $\mu\text{M}$  noradrenaline, whereas the increase in SC rate in response to the agonist was reduced by 90% (not shown). These results confirm that spontaneous activity in both preparations depends strongly, if not completely, on the SR function.

### Adrenoceptor types involved in the mediation of the automatic effect of noradrenaline

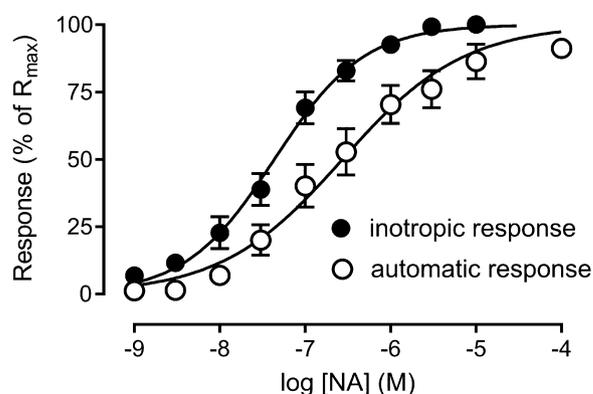
The potency of catecholamines was greater for the production of positive inotropic response than for automatism stimulation during rest (Figure 2), as previously observed (Boer and Bassani, 2004; Carvalho *et al.*, 2006; Penna and Bassani, 2010). Extrasystolic contractions, on the other hand,



**Figure 1**

Records of ventricular myocyte shortening showing development of spontaneous contractions during interruption of electric stimulation (rest), in the absence (A) and presence (B and C) of noradrenaline (NA). Disabling sarcoplasmic reticulum function by thapsigargin (TG) treatment abolished spontaneous activity, even in the presence of noradrenaline (C). RL, resting cell length.

developed in some cells, at agonist concentrations that evoked inotropic responses close to the maximum (not shown). Figure 3 shows the automatic response to noradrenaline, which stimulated spontaneous activity in both LA and VM, although with greater potency in the latter (Table 1). This figure also shows concentration–effect curves to noradrenaline obtained in the presence of the competitive  $\alpha$ - and  $\beta$ -adrenoceptor antagonists phentolamine (1  $\mu$ M) and propranolol (1  $\mu$ M), respectively, which are not selective for receptor subtypes. At these concentrations, phentolamine and propranolol did not depress LA ( $3.26 \pm 0.30$  and  $3.35 \pm 0.29$  mN before and after phentolamine, respectively;  $3.25 \pm 0.58$  and  $2.88 \pm 0.50$  mN before and after propranolol, respectively;  $P > 0.65$ , paired *t*-test) or VM basal contraction amplitude ( $8.82 \pm 1.20$  and  $8.61 \pm 0.48\%$  of RL before and



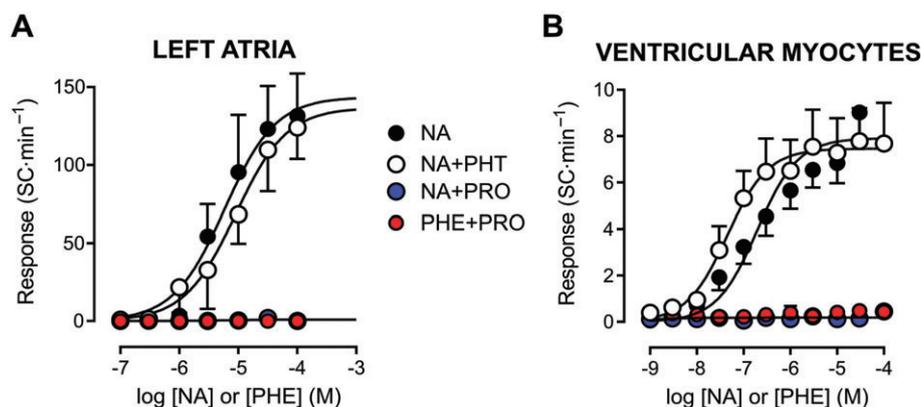
**Figure 2**

Inotropic (increase in systolic cell shortening) and automatic (rate of spontaneous contractions in the absence of electric stimulation) responses to noradrenaline (NA) determined in the same set of ventricular myocytes ( $n = 18$ ). The responses were normalized to the respective maximum responses ( $R_{\max}$ ). The mean  $pD_2$  value was significantly greater for the inotropic ( $7.36 \pm 0.14$ ) than for the automatic response ( $6.76 \pm 0.16$ ;  $P < 0.05$ ).

after phentolamine, respectively;  $8.22 \pm 1.53$  and  $7.95 \pm 1.38\%$  of RL before and after propranolol, respectively;  $P > 0.46$ ). These phentolamine and propranolol concentrations were previously shown to largely suppress the development of contraction and relaxation in response to 1  $\mu$ M noradrenaline and isoprenaline, respectively, in endothelium-free rat aorta (Lai and Bassani, unpublished results).

In both preparations, the automatic  $R_{\max}$  to noradrenaline was significantly influenced by the antagonist used ( $P < 0.001$ , one-way analysis of variance). Whereas  $\alpha$ -adrenoceptor blockade with phentolamine did not significantly change  $R_{\max}$  or  $pD_2$  values ( $P > 0.11$ ),  $\beta$ -adrenoceptor antagonism by propranolol abolished noradrenaline-induced spontaneous activity (non significant regression,  $R^2 < 0.17$ ; Table 1 and Figure 3). Additionally,  $\alpha_1$ -adrenoceptor blockade by prazosin (0.3–1  $\mu$ M) in VM produced results similar to those with phentolamine (not shown). Selective  $\alpha$ -adrenoceptor stimulation with phenylephrine in the presence or absence of propranolol also failed to evoke automatism in both LA and VM (Table 1 and Figure 3).

Adrenoceptor type-dependent differences were observed also in the inotropic  $R_{\max}$  to noradrenaline ( $P < 0.001$ ). In LA,  $R_{\max}$  was comparable in the absence and presence of phentolamine ( $2.92 \pm 0.34$  and  $2.76 \pm 0.59$  mN respectively).  $\beta$ -Adrenoceptor blockade by propranolol did not abolish inotropic responsiveness to noradrenaline, but decreased the response at 100  $\mu$ M noradrenaline by  $\sim 45\%$  ( $1.66 \pm 0.14$  mN,  $P < 0.01$ ). phenylephrine at this concentration evoked a response similar to that to noradrenaline in the presence of propranolol ( $1.78 \pm 0.12$  mN). Likewise, the  $R_{\max}$  to noradrenaline in VM was not significantly affected by phentolamine ( $11.2 \pm 1.1$  and  $10.8 \pm 1.2\%$  of RL in the absence and presence of phentolamine, respectively), but the response to 100  $\mu$ M noradrenaline was reduced to  $4.4 \pm 0.4\%$  of RL by propranolol a value comparable to the inotropic response to phenylephrine in the presence of propranolol ( $4.0 \pm 0.4\%$  of RL).



**Figure 3**

Concentration–effect curves for adrenoceptor agonists in isolated rat left atria (A) and ventricular myocytes (B). The automatic response is expressed as the rate of spontaneous contractions (SC) during rest. Curves to noradrenaline (NA) were obtained in the absence of antagonists, as well as in the presence of the  $\alpha$ - and  $\beta$ -adrenoceptor antagonists phentolamine (PHT, 1  $\mu$ M) and propranolol (PRO, 1  $\mu$ M) respectively. The response to the  $\alpha$ -adrenoceptor agonist phenylephrine (PHE) was determined in the presence of PRO. Atropine (0.1  $\mu$ M) was present throughout in all experiments with left atria. Values shown are means and SE. Curve parameters are shown in Table 1.

**Table 1**

Automatic response to adrenoceptor agonists, in the presence and absence of other compounds. Data are expressed as mean  $\pm$  SE. Experiments with left atria were performed in the presence of 0.1  $\mu$ M atropine

Agonist	Left atria			Ventricular myocytes		
	Basal (SC·s <sup>-1</sup> )	R <sub>max</sub> (SC·s <sup>-1</sup> )	pD <sub>2</sub>	Basal (SC·s <sup>-1</sup> )	R <sub>max</sub> (SC·s <sup>-1</sup> )	pD <sub>2</sub>
NA	0.6 $\pm$ 0.4 (n = 13)	161 $\pm$ 12	5.34 $\pm$ 0.15	1.3 $\pm$ 0.6 (n = 18)	8.6 $\pm$ 1.0	6.76 $\pm$ 0.16
NA + PHT	0.4 $\pm$ 0.2 (n = 10)	152 $\pm$ 13	4.99 $\pm$ 0.14	2.5 $\pm$ 1.3 (n = 10)	7.5 $\pm$ 0.9	7.13 $\pm$ 0.11
NA + PRO	0.2 $\pm$ 0.2 (n = 6)	0.6 $\pm$ 0.5 <sup>a,b</sup>	–	1.3 $\pm$ 0.7 (n = 11)	0.5 $\pm$ 0.2 <sup>a,b</sup>	–
PHE + PRO	0.2 $\pm$ 0.1 (n = 10)	0.2 $\pm$ 0.1 <sup>a,b</sup>	–	0.4 $\pm$ 0.2 (n = 13)	0.5 $\pm$ 0.2 <sup>a,b</sup>	–
ISO	0.5 $\pm$ 0.4 (n = 12)	83 $\pm$ 5 <sup>b,d</sup>	7.12 $\pm$ 0.18 <sup>d</sup>	0.5 $\pm$ 0.4 (n = 13)	4.4 $\pm$ 0.4 <sup>b,d</sup>	7.64 $\pm$ 0.09 <sup>b,d</sup>
ISO + BUT	0.9 $\pm$ 0.5 (n = 6)	122 $\pm$ 15 <sup>c</sup>	6.99 $\pm$ 0.23	0.1 $\pm$ 0.1 (n = 11)	9.5 $\pm$ 1.3 <sup>c</sup>	7.16 $\pm$ 0.07 <sup>c</sup>
ISO + ICI	–	–	–	0.4 $\pm$ 0.2 (n = 15)	11.9 $\pm$ 1.1 <sup>c</sup>	7.43 $\pm$ 0.10
ISO + SAL	0.4 $\pm$ 0.2 (n = 6)	38 $\pm$ 4 <sup>d</sup>	6.85 $\pm$ 0.23	–	–	–

<sup>a</sup>SC rate recorded at the agonist concentration of 100  $\mu$ M.

<sup>b</sup>P < 0.01 versus NA.

<sup>c</sup>P < 0.01 versus ISO.

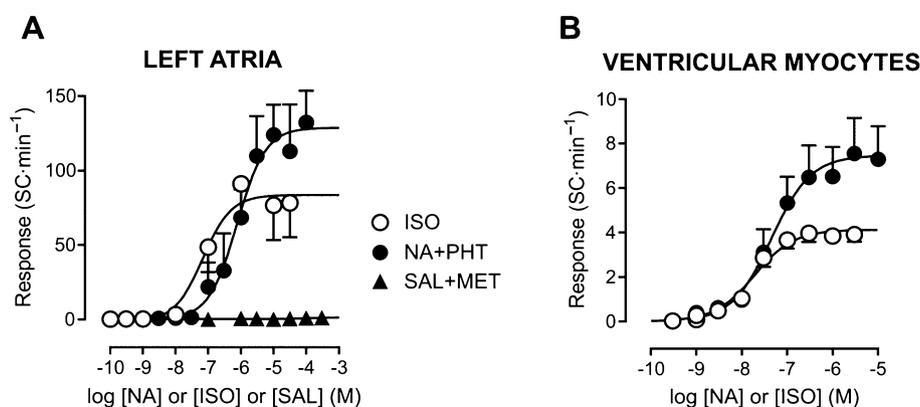
<sup>d</sup>P < 0.01 versus NA + PHT.

BUT, butoxamine (0.3  $\mu$ M); ICI, ICI118551 (0.1  $\mu$ M); ISO, isoprenaline; NA, noradrenaline; PHE, phenylephrine; PHT, phentolamine (1  $\mu$ M); PRO, propranolol (1  $\mu$ M); R<sub>max</sub>, maximum responses; SAL, salbutamol (10  $\mu$ M); SC, spontaneous contractions; SE, standard error.

### Functional antagonism of $\beta_1$ - and $\beta_2$ -adrenoceptors in the mediation of automatic effects of catecholamines

As the results above indicate that stimulation of atrial and ventricular spontaneous activity by noradrenaline is mediated by  $\beta$ -adrenoceptors, it might be expected that isoprenaline, a full  $\beta$ -adrenoceptor agonist, would exert a comparable effect. However, the R<sub>max</sub> to isoprenaline was only 50% of that to noradrenaline under  $\alpha$ -adrenoceptor blockade, although isoprenaline potency was considerably higher (P < 0.01; Table 1, Figure 4). It is interesting to notice that the absolute increase in SC rate in VM was similar for noradrenaline and

isoprenaline up to the concentration of 30 nM, above which noradrenaline was more effective in stimulating spontaneous activity (Figure 4B). The inotropic R<sub>max</sub> values to isoprenaline and to (noradrenaline + phentolamine) were similar (LA: 2.73  $\pm$  0.73 and 2.76  $\pm$  0.59 mN, respectively; VM: 10.2  $\pm$  1.4 and 10.8  $\pm$  1.2% of RL, respectively; P > 0.75). Thus, the discrepant automatic responses to noradrenaline and isoprenaline were not paralleled by differences in the inotropic responses recorded in the same preparations. The apparent efficacy of isoprenaline in LA could be increased by manipulations aimed at enhancing diastolic SR Ca<sup>2+</sup> release, such as addition of 0.5 mM caffeine (R<sub>max</sub> = 121  $\pm$  13 SC·min<sup>-1</sup>, n = 11, P < 0.05) or diastolic membrane depolarization by raising extracellular



**Figure 4**

Concentration–effect curves for  $\beta$ -adrenoceptor agonists in isolated rat left atria (A) and ventricular myocytes (B). Curves are shown as in Figure 2. In both preparations, the maximum automatic response was greater for preferential activation of  $\beta_1$ -adrenoceptors by noradrenaline (NA) under  $\alpha$ -adrenoceptor blockade by phentolamine (PHT, 1  $\mu$ M) than by non-selective  $\beta$ -adrenoceptor stimulation by isoprenaline (ISO).  $\beta_2$ -adrenoceptor stimulation by salbutamol (SAL) in the presence of the  $\beta_1$ -adrenoceptor antagonist metoprolol (MET, 0.5  $\mu$ M) did not evoke automatism. All experiments with left atria were performed in the presence of 0.1  $\mu$ M atropine. Values shown are means and SE. Curve parameters are shown in Table 1.

K<sup>+</sup> concentration from 5.7 to 8.2 mM ( $R_{\max} = 142 \pm 16$  SC·min<sup>-1</sup>,  $n = 5$ ,  $P < 0.05$ ).

The major difference in the mode of action of isoprenaline and noradrenaline is that only the former acts on  $\beta_2$ -adrenoceptors (Lands *et al.*, 1967). Figure 4A shows that the  $\beta_2$ -adrenoceptor agonist salbutamol was unable to induce spontaneous activity in LA exposed to the  $\beta_1$ -adrenoceptor antagonist metoprolol (0.5  $\mu$ M, 100-fold greater than the  $K_B$  value in rat atrial tissue, Bassani and De Moraes, 1988). The SC rate was  $0.2 \pm 0.2$  and  $1.5 \pm 1.2$  SC·min<sup>-1</sup> in the absence and presence of 300  $\mu$ M salbutamol ( $n = 4$ ;  $P > 0.32$ ). Metoprolol-treated LA also failed to develop spontaneous activity when isoprenaline was used as the agonist (not shown).

Nevertheless, as shown in Figure 5, the automatic effect of isoprenaline was affected by the competitive  $\beta_2$ -adrenoceptor antagonist butoxamine (0.3  $\mu$ M, 100-fold greater than the  $K_B$  value in rat atria, Bassani and De Moraes, 1988). In both LA (Figure 5A) and VM (Figure 5B), butoxamine significantly enhanced the automatic  $R_{\max}$  to isoprenaline ( $P < 0.01$ ; Table 1). The  $pD_2$  value was not significantly changed by butoxamine in LA ( $P > 0.67$ ), but was decreased in VM ( $P < 0.01$ ; Table 1). However, it should be noted that, in the latter preparation, the absolute automatic response up to 30 nM isoprenaline was essentially the same in both conditions, whereas above this concentration isoprenaline produced higher SC rates in butoxamine-treated myocytes (Figure 5B), as observed for noradrenaline. The highly selective  $\beta_2$ -adrenoceptor antagonist ICI118551 (0.1  $\mu$ M; Brodde and Michel, 1999) also increased the automatic  $R_{\max}$  to isoprenaline in VM ( $P < 0.01$ ), although isoprenaline  $pD_2$  value was unaffected (Table 1; Figure 5B). It is important to notice that, in the presence of butoxamine or ICI118551, the automatic  $R_{\max}$  to isoprenaline was not significantly different from that to noradrenaline in either LA ( $P > 0.07$ ) or VM ( $P > 0.58$ ). These results were reproduced in experiments with VM performed at 36°C (Table 2, Figure 5C), that is, potentiation of

**Table 2**

Automatic response to noradrenaline (NA) and isoprenaline (ISO) determined in rat ventricular myocytes at 36°C. The latter agonist was tested in the absence and presence of the  $\beta_2$ -adrenoceptor antagonist ICI118551 (ICI, 0.1  $\mu$ M). Data are presented as mean  $\pm$  SE

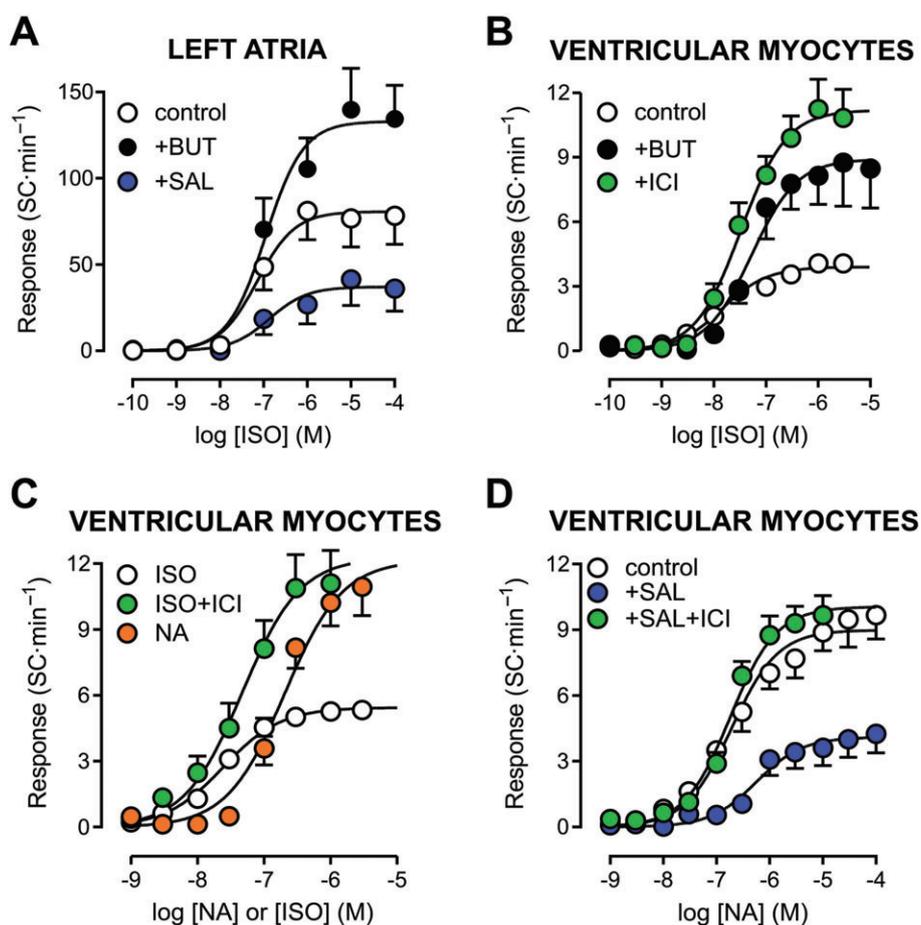
Agonist	Basal (SC·s <sup>-1</sup> )	$R_{\max}$ (SC·s <sup>-1</sup> )	$pD_2$
ISO ( $n = 20$ )	1.1 $\pm$ 0.3	5.7 $\pm$ 0.4	7.61 $\pm$ 0.08
ISO + ICI ( $n = 20$ )	0.9 $\pm$ 0.2	11.7 $\pm$ 1.4 <sup>a</sup>	7.47 $\pm$ 0.11
NA ( $n = 19$ )	1.0 $\pm$ 0.3	12.4 $\pm$ 1.4 <sup>a</sup>	6.70 $\pm$ 0.05

<sup>a</sup> $P < 0.01$  versus ISO in the absence of ICI; one-way analysis of variance:  $P > 0.77$  for basal values, and  $P < 0.001$  for  $R_{\max}$ ;  $t$ -test for ISO versus ISO + ICI:  $P > 0.31$ .

$R_{\max}$ , maximum responses; SC, spontaneous contractions; SE, standard error.

the automatic response to isoprenaline by ICI118551, resulting in a  $R_{\max}$  value similar to that to NA. At a supramaximal agonist concentration (3  $\mu$ M), the percentage of cells that developed extrasystoles at 36°C was greater in the presence of noradrenaline (71%) than that of isoprenaline (30%). However, in the presence of ICI118551, 75% of the cells showed extrasystolic contractions in response to isoprenaline. Thus, in terms of apparent efficacy, the automatic response to isoprenaline under  $\beta_2$ -adrenoceptor blockade resembles that to noradrenaline under  $\alpha$ -adrenoceptor blockade; in both cases, the response is expected to be mediated preferentially by  $\beta_1$ -adrenoceptors.

In LA and VM, the inotropic  $R_{\max}$  to isoprenaline was 30–40% lower in the presence of butoxamine (1.81  $\pm$  0.33 mN and 7.8  $\pm$  1.8% of RL, respectively), but this decrease did not reach statistical significance ( $P > 0.14$ ).



**Figure 5**

Concentration–effect curves for isoprenaline (ISO) and noradrenaline (NA) in isolated rat left atria (A) and ventricular myocytes (B–D). Curves were also determined during  $\beta_2$ -adrenoceptor blockade with butoxamine (BUT, 0.3  $\mu\text{M}$ ) or ICI118551 (ICI, 0.1  $\mu\text{M}$ ), and in the presence of the  $\beta_2$ -adrenoceptor agonist salbutamol (SAL, 10  $\mu\text{M}$ ). Experiments with left atria were performed in the presence of 0.1  $\mu\text{M}$  atropine. Panels A and C show results obtained at 36°C, whereas the temperature was 23°C for experiments in panels B and D. Curve parameters are shown in Tables 1–3.

ICI118551 did not affect significantly the inotropic  $R_{\text{max}}$  to isoprenaline in VM ( $8.8 \pm 2.2\%$  of RL,  $P > 0.60$ ). At 36°C, the inotropic  $R_{\text{max}}$  in VM was comparable for noradrenaline and isoprenaline ( $12.6 \pm 1.3$  and  $10.3 \pm 1.0\%$  of RL), and the latter was not significantly affected by ICI118551 ( $9.5 \pm 1.1\%$  of RL,  $P > 0.61$ ).

The ability of  $\beta_2$ -adrenoceptor antagonists to enhance isoprenaline-induced automatism suggests that some mechanism coupled to  $\beta_2$ -adrenoceptors might limit the automatism stimulation mediated by  $\beta_1$ -adrenoceptors. If so, one would expect that sustained  $\beta_2$ -adrenoceptor activation could attenuate the development of spontaneous activity during  $\beta_1$ -adrenoceptor stimulation. To test this possibility, the automatic response to catecholamines was determined in the presence of 10  $\mu\text{M}$  salbutamol, which was applied 5 min before and during agonist exposure. Salbutamol alone did not produce significant increase in basal twitch force in LA (from  $2.64 \pm 0.27$  to  $2.94 \pm 0.35$  mN) or cell shortening in VM (from  $7.8 \pm 1.2$  to  $9.3 \pm 1.3\%$  of RL;  $P > 0.20$ , paired  $t$ -test). Continuous exposure to salbutamol did not significantly change the inotropic  $R_{\text{max}}$  to isoprenaline ( $3.65 \pm 0.63$  mN) or

noradrenaline ( $9.2 \pm 1.6\%$  of RL) in LA and VM respectively ( $P > 0.27$ ).

The basal SC rate was not significantly affected by salbutamol in LA (from  $0.5 \pm 0.3$  to  $0.4 \pm 0.2$   $\text{SC}\cdot\text{min}^{-1}$ ) or in VM (from  $1.7 \pm 0.7$  to  $1.1 \pm 0.5$   $\text{SC}\cdot\text{min}^{-1}$ ;  $P > 0.14$ , paired  $t$ -test). As shown in Figure 5A and Table 1, salbutamol decreased the automatic  $R_{\text{max}}$  to isoprenaline by more than 50% in LA ( $P < 0.001$ ), without affecting significantly isoprenaline  $\text{pD}_2$  ( $P > 0.38$ ). In VM, salbutamol also suppressed the automatic  $R_{\text{max}}$  to noradrenaline ( $P < 0.01$ ; Table 3, Figure 5D), in addition to decreasing the sensitivity to the agonist ( $P < 0.05$ ). The effects of salbutamol on the noradrenaline-stimulated automatism of VM were completely abolished by ICI118551 (Table 3; Figure 5D).

#### *Catecholaminergic automatism: effects of PDE inhibition*

Because PDE activity seems able to prevent PKA phosphorylation of SR proteins in response to  $\beta_2$ -adrenoceptor activation (Soto *et al.*, 2009), we hypothesized that the anti-automatic effect of  $\beta_2$ -adrenoceptor stimulation might

involve PDEs. To test this hypothesis, the automatic response of VM to noradrenaline, both in the presence and absence of salbutamol, was determined under inhibition of PDE3 and PDE4 by milrinone and rolipram respectively (3  $\mu$ M; 15 min pre-exposure and throughout the experiment).

Incubation with milrinone did not significantly affect contraction amplitude ( $9.9 \pm 0.7$  to  $9.5 \pm 0.7\%$  of RL;  $n = 26$ ;  $P > 0.17$ , paired *t*-test) or SC rate ( $1.0 \pm 0.3$  to  $0.6 \pm 0.2$  SC $\cdot$ min $^{-1}$ ;  $P > 0.10$ ). The inotropic  $R_{\max}$  to noradrenaline was decreased by 25% by milrinone treatment (from  $11.2 \pm$

$1.1$  to  $8.6 \pm 1.0\%$  of RL), while the automatic  $R_{\max}$  was increased by 20% (Table 3), but these changes did not attain statistical significance ( $P > 0.05$ ). However, the noradrenaline pD<sub>2</sub> was significantly greater in the presence of milrinone ( $P < 0.001$ ; Table 3).

In milrinone-treated cells, salbutamol addition increased both contraction amplitude (from  $10.1 \pm 1.1$  to  $11.9 \pm 1.1\%$  of RL;  $n = 7$ ;  $P < 0.02$ ; paired *t*-test) and SC rate (from  $0.4 \pm 0.4$  to  $3.6 \pm 0.8$  SC $\cdot$ min $^{-1}$ ;  $P < 0.02$ ). In contrast to the effects of salbutamol in the absence of milrinone, a two-way analysis of variance to investigate the combination of salbutamol and milrinone effects revealed that these compounds exerted significant ( $P < 0.01$ ), but independent effects ( $P > 0.43$  for milrinone–salbutamol interaction) on the automatic  $R_{\max}$  to noradrenaline, the former decreasing and the latter increasing it (Figure 6A). The *post hoc* Bonferroni test showed that milrinone attenuated the depression of the automatic  $R_{\max}$  by salbutamol ( $P < 0.01$ ). A similar result was observed for noradrenaline potency: salbutamol decreased, whereas milrinone increased the pD<sub>2</sub> value for noradrenaline ( $P < 0.02$ ), and, again, their effects were independent ( $P > 0.88$  for interaction). Treatment with milrinone plus salbutamol, but not with salbutamol alone, depressed the inotropic  $R_{\max}$  to noradrenaline ( $3.3 \pm 0.7\%$  of RL;  $P < 0.01$ ).

As observed with milrinone, rolipram alone did not significantly affect baseline contraction amplitude (from  $9.3 \pm 0.6$  to  $8.8 \pm 0.6\%$  of RL;  $n = 32$ ;  $P > 0.22$ , paired *t*-test), although the SC rate was decreased (from  $0.8 \pm 0.3$  to  $0.4 \pm 0.2$  SC $\cdot$ min $^{-1}$ ;  $P < 0.04$ ). Rolipram by itself increased the pD<sub>2</sub>, but did not affect the automatic (Table 3) or the inotropic ( $10.4 \pm 1.6\%$  of RL)  $R_{\max}$  to noradrenaline. In the presence of rolipram, salbutamol markedly enhanced basal contraction amplitude (from  $9.2 \pm 0.7$  to  $14.3 \pm 1.4\%$  of RL), and also increased and SC rate (from  $0.7 \pm 0.4$  to  $1.9 \pm 0.6$  SC $\cdot$ min $^{-1}$ ;  $n = 16$ ;  $P < 0.04$ ; paired *t*-test). Nevertheless, in contrast with what happened with milrinone, a significant interaction ( $P < 0.03$ ) was detected between the effects of rolipram and salbutamol on the automatic responsiveness to noradrenaline. In the presence of rolipram, salbutamol failed to produce the significant decrease in both pD<sub>2</sub> and  $R_{\max}$  to noradrenaline

**Table 3**

Automatic response to noradrenaline (NA) determined in rat ventricular myocytes in the presence of the compounds indicated in the left column. Data are presented as mean  $\pm$  SE. The parameters of the response to NA in the absence of other drugs, shown in Table 1, are presented again in this table for clarity

Pretreatment	Basal (SC $\cdot$ s $^{-1}$ )	$R_{\max}$ (SC $\cdot$ s $^{-1}$ )	pD <sub>2</sub>
None ( $n = 18$ )	$1.3 \pm 0.6$	$8.6 \pm 1.0$	$6.76 \pm 0.16$
SAL ( $n = 17$ )	$1.1 \pm 0.5$	$4.3 \pm 1.0^a$	$6.30 \pm 0.17^a$
ICI + SAL ( $n = 19$ )	$1.6 \pm 0.3$	$10.7 \pm 0.7^b$	$6.80 \pm 0.06^b$
MLR ( $n = 19$ )	$0.4 \pm 0.2$	$10.4 \pm 0.5$	$7.44 \pm 0.10^a$
MLR + SAL ( $n = 7$ )	$3.6 \pm 0.8^{a,c}$	$7.6 \pm 0.5$	$7.03 \pm 0.20^d$
ROL ( $n = 16$ )	$0.1 \pm 0.1^a$	$9.5 \pm 0.5$	$7.36 \pm 0.09^a$
ROL + SAL ( $n = 16$ )	$1.9 \pm 0.6^c$	$9.1 \pm 0.6$	$7.21 \pm 0.1^{a,d}$

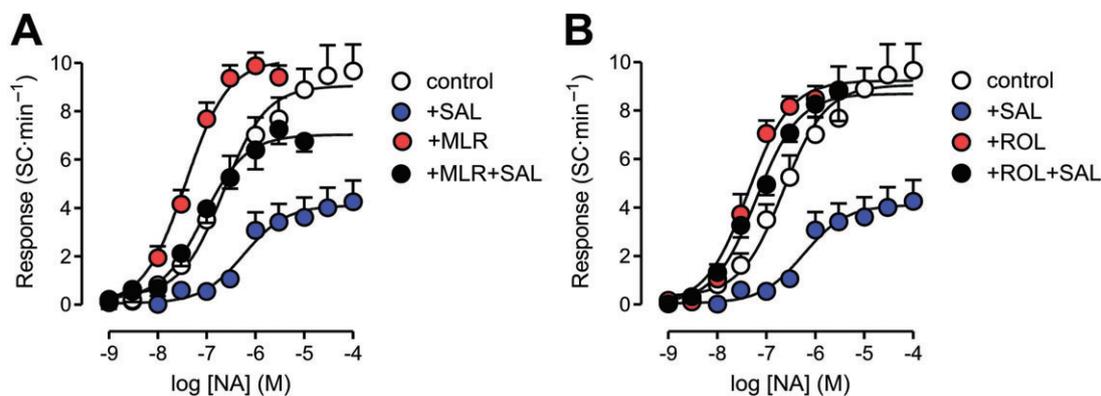
<sup>a</sup> $P < 0.05$  versus NA in the absence of other drugs.

<sup>b</sup> $P < 0.05$  versus SAL alone.

<sup>c</sup> $P < 0.05$  versus the same phosphodiesterase inhibitor in the absence of SAL.

<sup>d</sup> $P < 0.05$  versus in the absence of the phosphodiesterase inhibitor.

ICI, ICI118551 (0.1  $\mu$ M); MLR, milrinone (3  $\mu$ M);  $R_{\max}$ , maximum responses; ROL, rolipram (3  $\mu$ M); SAL, salbutamol (10  $\mu$ M); SC, spontaneous contractions; SE, standard error.



**Figure 6**

Concentration–effect curves for noradrenaline (NA) in isolated rat ventricular myocytes, determined in the presence and absence of the phosphodiesterase inhibitors milrinone (MLR, 3  $\mu$ M, panel A) or rolipram (ROL, 3  $\mu$ M, panel B). Some curves were determined during perfusion with the  $\beta_2$ -adrenoceptor agonist salbutamol (SAL, 10  $\mu$ M). Curve parameters are shown in Table 2.

that was observed in absence of rolipram (Table 3 and Figure 6B). In other words, exposure to rolipram abolished the effects of salbutamol on the automatic responsiveness to noradrenaline. The inotropic  $R_{max}$  to noradrenaline was depressed after (rolipram + salbutamol) treatment (to  $3.9 \pm 0.7\%$  of RL;  $P < 0.01$ ), probably because before noradrenaline challenge, cells treated with salbutamol presented greater basal contraction amplitude. Accordingly, systolic shortening at maximal noradrenaline concentrations under rolipram exposure was not significantly different in the absence and presence of salbutamol ( $18.3 \pm 1.5$  and  $17.6 \pm 1.5\%$  of RL, respectively;  $P > 0.75$ ).

## Discussion

The general aim of this study was to investigate the mechanisms involved in the production of spontaneous activity in rat myocardium by catecholamines. The present results indicate that: (i) the SR is crucially involved in the production of this effect; (ii) enhancement of spontaneous activity is mediated by  $\beta_1$ -adrenoceptors, whereas  $\alpha$ - or  $\beta_2$ -adrenoceptor stimulation is unable to evoke automatism in either atrial or ventricular myocardium; (iii)  $\beta_2$ -adrenoceptor stimulation is able to antagonize the  $\beta_1$ -mediated enhancement of spontaneous activity by catecholamines; and (iv) although both PDE3 and PDE4 seem to affect catecholaminergic automatism, only inhibition of PDE4 abolished the effect of  $\beta_2$ -adrenoceptor stimulation.

The occurrence of spontaneous activity in myocardial cells exposed to high catecholamine concentrations is generally attributed to development of  $Ca^{2+}$  overload (Nam *et al.*, 2005; ter Keurs and Boyden, 2007; DeSantiago *et al.*, 2008; Venetucci *et al.*, 2008). The primordial role of the SR in the generation of automatism by catecholamines in rat myocardium was confirmed by the marked suppression of SCs by inhibition of SR function, even at maximal agonist concentrations.

$Ca^{2+}$ -dependent triggered activity has been implicated in atrial fibrillation of sympathetic origin (Chen and Tan, 2007). Our findings in the isolated LA model of catecholaminergic automatism are in agreement with this main mechanism. For instance, spontaneous activity in this preparation seems to arise from focal electrical activity (Zafalon *et al.*, 2009), requires previous rapid pacing (Boer and Bassani, 2004) and is sensitive to SERCA inhibition (present results). In addition, we observed that isoprenaline-induced automatism could be enhanced by maneuvers that increase diastolic SR  $Ca^{2+}$  release directly, such as exposure to low-caffeine concentration (Ferraz *et al.*, 2001), or indirectly, such as increase in extracellular  $K^+$  concentration (which is expected to induce SR  $Ca^{2+}$  overload by impairment of  $Ca^{2+}$  extrusion via the  $Na^+/Ca^{2+}$  exchanger due to sarcolemmal depolarization).

### *$\beta_1$ -adrenoceptors mediate catecholaminergic automatism in rat myocardium*

While  $\alpha$ -adrenoceptor blockade did not significantly affect the inotropic and automatic responses to noradrenaline,  $\beta$ -adrenoceptor blockade with propranolol reduced by 50% the inotropic response to  $100 \mu M$  noradrenaline and phe-

nylephrine, and totally abolished noradrenaline-induced automatism. These results indicate that  $\alpha$ -adrenoceptor signalling, although mediating a modest inotropic stimulation, is not required for the production of catecholaminergic automatism in rat myocardium.

On the other hand,  $\beta_2$ -adrenoceptor stimulation by either salbutamol or isoprenaline completely failed to induce spontaneous activity in LA treated with the  $\beta_1$ -adrenoceptor antagonist metoprolol. Both  $\beta_1$ - and  $\beta_2$ -adrenoceptors are coupled to the  $G_s$ -PKA pathway. Nevertheless, selective  $\beta_2$ -adrenoceptor stimulation typically produces smaller increase in  $Ca^{2+}$  transient and contraction amplitude than that of  $\beta_1$ -adrenoceptors in the myocardium of several species (e.g. Xiao *et al.*, 1994; 2006; Cui *et al.*, 1996; Kuschel *et al.*, 1999; DeSantiago *et al.*, 2008), and may even depress contractility (Siedlecka *et al.*, 2008). Although  $\beta_2$ -adrenoceptors have been implicated in the generation of  $Ca^{2+}$ -dependent arrhythmias, this was observed mostly under pathophysiological conditions, such as myocardial ischaemia or heart failure (Billman *et al.*, 1997; Altschuld and Billman, 2000; DeSantiago *et al.*, 2008), conditions in which  $\beta_1$ -adrenoceptors are typically down-regulated, and  $\beta_2$ -adrenoceptor involvement in  $Ca^{2+}$  cycling is abnormally enhanced (Brodde and Michel, 1999; Altschuld and Billman, 2000; DeSantiago *et al.*, 2008). On the other hand, Steinberg *et al.* (2002) attributed to the  $\beta_1$ -adrenoceptor subtype the induction of catecholaminergic spontaneous activity in canine ventricle, although  $\beta_2$ -adrenoceptor activation was able to produce substantial inotropic stimulation. Accordingly, it has been shown that  $\beta_1$ -, but not  $\beta_2$ -adrenoceptor blockade, can suppress catecholaminergic automatism in ventricular myocardium (Galindo-Tovar and Kaumann, 2008; Penna and Bassani, 2010). These reports are in line with our present findings that  $\beta_1$ -adrenoceptors play a major role in the mediation of myocardial automatism induction by catecholamines. On the other hand, Kaumann and Sanders, (1993) reported induction of  $\beta_2$ -adrenoceptor-mediated automatism in right atrial tissue from non-failing human hearts. It remains to be determined whether this is also the case in human ventricular myocardium.

### *$\beta_2$ -adrenoceptors attenuate $\beta_1$ -adrenoceptor mediated catecholaminergic automatism in rat myocardium: a possible link with PDE4*

Compared to  $\beta_1$ -adrenoceptors,  $\beta_2$ -adrenoceptors are less effective in raising cAMP levels, as well as inducing positive inotropic and lusitropic effects, a difference largely attributed to the spatial restriction of cAMP accumulation (Nikolaev *et al.*, 2006; Xiao *et al.*, 2006). Compartmentalization of the  $\beta_2$ -adrenoceptor signalling, associated with briefer PKA activation (Soto *et al.*, 2009), may be the cause of the lack of significant PKA phosphorylation of internal proteins such as PLB, which is typically phosphorylated during  $\beta_1$ -adrenoceptor activation (Xiao *et al.*, 1994; 2006; Kuschel *et al.*, 1999; DeSantiago *et al.*, 2008; Soto *et al.*, 2009). PLB phosphorylation by PKA is considered a requisite for increase in SR  $Ca^{2+}$  content and release during  $\beta$ -adrenergic stimulation (Li *et al.*, 2002), and, as shown by us and others (Fujiwara *et al.*, 2008), SR function is crucial to the generation of  $Ca^{2+}$ -dependent spontaneous activity and/or arrhythmias. Here we found evidence of  $\beta_2$ -adrenoceptor involvement in

catecholaminergic automatism, but by antagonizing the  $\beta_1$ -adrenoceptor-mediated enhancement in spontaneous activity, rather than contributing to it. This evidence is based on: (i) greater apparent efficacy of noradrenaline (preferential  $\beta_1$ -adrenoceptor stimulation) over isoprenaline (similar potency on both subtypes) at inducing automatism; (ii) potentiation of isoprenaline-stimulated automatism by  $\beta_2$ -adrenoceptor competitive antagonists; and (iii) attenuation of isoprenaline- and noradrenaline-evoked spontaneous activity by background  $\beta_2$ -adrenoceptor stimulation with salbutamol, which was completely reversed by ICI118551.

Opposing influences of  $\beta_1$ - and  $\beta_2$ -adrenoceptor stimulation have been previously described. The former is associated with deleterious effects, such as worsening of doxorubicin and catecholamine cardiotoxicity and apoptosis, and the latter was shown to exert cardioprotective effects (see Communal *et al.*, 1999; Fajardo *et al.*, 2006; Xiao *et al.*, 2006). Protection against arrhythmia by  $\beta_2$ -adrenoceptors was previously reported by Sosunov *et al.*, (2000) in a canine model of inherited arrhythmia, as well as in normal dogs. In the present study, we demonstrate that this functional antagonism of  $\beta_1$ - and  $\beta_2$ -adrenoceptor-mediated effects also extends to the potentially arrhythmogenic induction of myocardial automatism in apparently healthy, normal, wild type rodents. It remains to be determined if this type of  $\beta_2$ -adrenoceptor-mediated cardioprotection is also observed in other species, including humans.

Increasing cAMP accumulation, independently of  $\beta$ -adrenoceptor stimulation, is sufficient to induce automatism in VM (Penna and Bassani, 2010). The PDE-catalysed degradation of cAMP limits the effects of activation of the  $\beta$ -adrenoceptor pathway, especially at high stimulation levels. Among the PDE families, only PDE3 and PDE4 are involved in the regulation of myocardial contractility (Osadchii, 2007). PDE families appear to be compartmentalized (Mongillo *et al.*, 2004) and this would allow the creation of cAMP concentration gradients and/or microdomains that may favour or attenuate PKA activation in different regions of the cell. In this study, selective inhibition of PDE3 or PDE4 by non-saturating concentrations of milrinone and rolipram, respectively, did not produce significant inotropic or automatic effects, but markedly potentiated the automatic response to NA, increasing the  $pD_2$  to a value comparable to that observed for the inotropic response of VM in the absence of the inhibitors (Penna and Bassani, 2010). This result indicates that both isoenzymes seem to attenuate the production of spontaneous activity in rat ventricle, and is in agreement with the observation by Galindo-Tovar and Kaumann (2008) that rolipram can enhance the  $\beta_1$ -adrenoceptor-mediated induction of spontaneous activity by catecholamines in mouse ventricle.

Several reports indicate that inhibition of PDE4 is more effective than that of PDE3 at potentiating catecholamine effects in rodent myocardium, particularly those mediated by  $\beta_2$ -adrenoceptors (Mongillo *et al.*, 2004; Rochais *et al.*, 2006; Vargas *et al.*, 2006), including PLB phosphorylation (Soto *et al.*, 2009). Although the  $\beta_2$ -adrenoceptor agonist salbutamol does not affect contraction amplitude in rat VM (Siedlecka *et al.*, 2008; present results), we observed that inhibition of either PDE3 or PDE4 unmasked significant inotropic stimulation by this agonist. However, this effect was more

prominent after PDE4 than PDE3 antagonism. Moreover, the attenuation of noradrenaline-induced automatism by salbutamol was abolished by rolipram, but not by milrinone. These observations indicate that cAMP degradation by PDEs seems to play a role in blunting the automatic response to  $\beta_2$ -adrenoceptor stimulation in the rat ventricle. Additionally, they are suggestive that PDE4, but not PDE3, is closely involved in the functional antagonism between  $\beta_1$ - and  $\beta_2$ -adrenoceptor signalling pathways regarding the generation of catecholaminergic automatism.

The involvement of  $\beta_2$ -adrenoceptors and PDE4 in the attenuation of catecholaminergic,  $Ca^{2+}$ -dependent automatism in the myocardium may have pathophysiological implications. Deficiency of PDE4D results in cardiomyopathy and arrhythmias, and the amount of this isoform associated with the SR is decreased in human heart failure (Lehnart *et al.*, 2005). Interestingly, in failing, but not in control hearts,  $\beta_2$ -adrenoceptor stimulation is able to promote PLB phosphorylation by PKA, marked positive inotropic effect, SR  $Ca^{2+}$  overload and arrhythmias (DeSantiago *et al.*, 2008). Decrease in ventricular PDE4 activity was observed in rats with compensated ventricular hypertrophy due to aortic banding (Abi-Gerges *et al.*, 2009), a condition in which catecholaminergic automatism is enhanced (Carvalho *et al.*, 2006). Finally, stress, recognized as a risk factor for arrhythmia in humans (see Lane *et al.*, 2005), decreased cardiac PDE activity associated with the SR (Okruhlicová *et al.*, 2004). We recently observed depressed inotropic responsiveness and greater apparent efficacy of catecholamines at increasing extrasystolic activity in VM from stressed rats, which was consistent with impaired cAMP degradation and loss of  $\beta_2$ -adrenoceptor-mediated protection (Penna and Bassani, 2010). Although further investigation is required, the current information on the mechanisms involved in catecholaminergic arrhythmogenesis points out that  $\beta_2$ -adrenoceptors and PDEs might be promising targets for the pharmacological treatment of arrhythmias.

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## Conflicts of interest

None.

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