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RESEARCH PAPER

Phosphodiesterase-4 blunts inotropism and arrhythmias but not sinoatrial tachycardia of (−)-adrenaline mediated through mouse cardiac β_1-adrenoceptors

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Background and purpose: β_1 and β_2-adrenoceptors coexist in murine heart but β_2-adrenoceptor-mediated effects have not been detected in atrial and ventricular tissues, possibly due to marked phosphodiesterase (PDE) activity. We investigated the influence of the PDE3 inhibitor cilostamide and PDE4 inhibitor rolipram on the effects of (−)-adrenaline in three regions of murine heart.
Experimental approach: (−)-Adrenaline-evoked cardiostimulation was compared on sinoatrial beating rate, left atrial and right ventricular contractile force in isolated tissues from 129SvxC57B1/6 cross mice. Ventricular arrhythmic contractions were also assessed.
Key results: Both rolipram (1 μM) and cilostamide (300 nM) caused transient sinoatrial tachycardia but neither enhanced the chronotropic potency of (−)-adrenaline. Rolipram potentiated 19-fold (left atrium) and 7-fold (right ventricle) the inotropic effects of (−)-adrenaline. (−)-Adrenaline elicited concentration-dependent ventricular arrhythmias that were potentiated by rolipram. All effects of (−)-adrenaline were antagonized by the β_1-adrenoceptor-selective antagonist CGP20712A (300 nM). Cilostamide (300 nM) did not increase the chronotropic and inotropic potencies of (−)-adrenaline, but administered jointly with rolipram in the presence of CGP20712A, uncovered left atrial inotropic effects of (−)-adrenaline that were prevented by the β_2-adrenoceptor-selective antagonist ICI118551.
Conclusions and implications: PDE4 blunts the β_1-adrenoceptor-mediated effects of (−)-adrenaline in left atrium and right ventricle but not in sinoatrial node. Both PDE3 and PDE4 reduce basal sinoatrial rate in a compartment distinct from the β_1-adrenoceptor compartment. PDE3 and PDE4, acting in concert, prevent left atrial β_2-adrenoceptor-mediated inotropy. PDE4 partially protects the right ventricle against (−)-adrenaline-evoked arrhythmias.
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Keywords: phosphodiesterase-4; murine heart; arrhythmias; β_1- and β_2-adrenoceptors; (−)-adrenaline

Abbreviations: CGP20712A, (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide); ICI118551, (1-[2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxy-3-[(1-methylethyl)amino]-2-butanol); RyR2, ryanodine receptor 2

Introduction

The mouse heart has been used as a model for human β_1-and β_2-adrenoceptors. The murine ventricular β-adrenoceptor population consists of 70% β_1-adrenoceptor and 30% β_2-adrenoceptor (Heubach *et al*., 1999), similar to the non-failing human ventricle (Molenaar *et al*., 2000) and atrium (Molenaar *et al*., 1997). It has been proposed that murine cardiac β_2-adrenoceptors couple concurrently to G_s and G_i proteins in murine hearts and that G_s protein-mediated cardiostimulant effects only become apparent after inactivating G_i protein with *Pertussis* toxin (PTX) in ventricular cardiomyocytes (Xiao *et al*., 1999). However, the work of Oostendorp and Kaumann (2000) in murine left atria and Heubach *et al*. (2002) in murine ventricle and sinoatrial node has failed to detect cardiostimulant effects of adrenaline through β_2-adrenoceptors, even after treatment with PTX. Furthermore, these findings agree with recent work on murine ventricular myocytes, demonstrating that PTX failed to affect the β_2-adrenoceptor-mediated increase in cAMP (Nikolaev *et al*., 2006). A plausible reason for the lack of

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detectable function of β_2-adrenoceptors in the murine heart could be avid phosphodiesterase-catalysed hydrolysis of the cAMP produced through agonist-evoked receptor activation.

The ryanodine receptor 2 (RyR2) is the main cardiac intracellular channel for Ca^{2+} release from the sarcoplasmic reticulum of myocytes from the sinoatrial node (Rigg *et al.*, 2000; Vinogradova *et al.*, 2005a), left atrium (Vest *et al.*, 2005) and ventricle (Li *et al.*, 2002; Wehrens *et al.*, 2005). The cardiomyocyte RyR2 is crucial in mediating excitation–contraction coupling, which in turn is strongly modulated by the sympathetic nervous system. Upon catecholamine-evoked stimulation through β-adrenoceptors, cAMP activates the cAMP-dependent PKA, which phosphorylates several proteins including phospholamban and RyR2. Phosphorylated phospholamban dis-inhibits the sarcoplasmic reticulum (SR) calcium pump allowing refilling of the SR calcium stores and making calcium available for release through the RyR2 channel. Murine RyR2 can be phosphorylated by PKA at Serine^{2008} (Wehrens *et al.*, 2006) and Serine^{2030} (Xiao *et al.*, 2006). Murine RyR2 phosphorylation, for example, at Serine^{2008} (Wehrens *et al.*, 2006), appears to reduce binding of the channel-stabilizing subunit calstabin 2 (formerly FKBP12.6) thereby facilitating calcium leak and arrhythmias (Vest *et al.*, 2005). Rolipram-sensitive phosphodiesterase-4D3 (PDE4D3) forms a complex with RyR2. It has been reported that reduction of PDE4D3 levels in heart failure contributes to PKA-induced hyperphosphorylation, resulting in leaky RyR2 channels that facilitate cardiac arrhythmias (Lehnart *et al.*, 2005).

To elucidate whether the activity of PDE3 and/or PDE4 prevent the manifestation of cardiostimulation through murine β_2-adrenoceptor, we investigated the effects of the PDE3 inhibitor cilostamide and PDE4 inhibitor rolipram (Vargas *et al.*, 2006) on the responses to (−)-adrenaline under conditions of β_1-adrenoceptor blockade in murine cardiac tissues. To investigate whether there are regional differences in the roles of PDE3 and PDE4, we first studied the effects of the PDE inhibitors on the responses to (−)-adrenaline, mediated through β_1-adrenoceptor in three cardiac regions: sinoatrial node, left atrium and right ventricle. Right ventricular walls tend to become arrhythmic with high catecholamine concentrations (Heubach *et al.*, 2004). To inquire whether inhibition of PDE4 increases catecholamine-evoked arrhythmias, we investigated the influence of rolipram on concentration-dependent arrhythmias elicited by (−)-adrenaline on murine right ventricular wall.

Methods

Mice

All mice were bred and used in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. We used genetically heterogenous, outbred 129Sv × C57Bl/6 cross mice of either sex. The mice were studied at 6 months of age. All animals were maintained at 21 °C on a 12 h light/dark cycle and allowed free access to standard rodent chow and water.

Isolated cardiac tissues

Mice of either sex were killed by dislocation of the neck and the hearts were dissected and placed in oxygenated, modified Tyrode's solution at room temperature containing (in mM): NaCl 136.9, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.5, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, EDTA 0.04, ascorbic acid 0.2, pyruvate 5 and glucose 5.0. The pH of the solution was maintained at pH 7.4 by bubbling a mixture of 5% CO_2 and 95% O_2. Spontaneously beating right atria, left atria and the free wall of the right ventricle were rapidly dissected, mounted in pairs and attached to Swema 4-45 strain gauge transducers in an apparatus containing modified Tyrode's solution at 37 °C. Left atria and right ventricular walls were paced at 2 Hz and stretched as described (Oostendorp and Kaumann, 2000; Heubach *et al.*, 2002). Contractile force was recorded through PowerLab amplifiers on a Chart for Windows, version 5.0 recording programme (ADInstruments, Castle Hill, NSW, Australia).

All tissues were exposed to phenoxybenzamine (5 μM) for 90 min followed by washout, to irreversibly block α-adrenoceptors and tissue uptake of (−)-adrenaline (Gille *et al.*, 1985; Heubach *et al.*, 2002). Some experiments were carried out in the presence of 2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]propyl]amino] ethoxy]-benzamide (CGP20712A) (300 nM) to selectively block β_1-adrenoceptor (Oostendorp and Kaumann, 2000) and conceivably uncover CGP20712A-resistant effects, mediated through β_2-adrenoceptor (Heubach *et al.*, 2002, 2003). To corroborate that CGP20712A-resistant effects were mediated through β_2-adrenoceptor, the β_2-adrenoceptor-selective antagonist 1-[2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxy-3-[(1-methylethyl)amino]-2-butanol (ICI118551) (50 nM; Oostendorp and Kaumann, 2000) was used in the presence of CGP20712A.

Cumulative concentration–effect curves for (−)-adrenaline were carried out in the absence and presence of the PDE3 inhibitor cilostamide (300 nM) or PDE4 inhibitor rolipram (1 μM) (Vargas *et al.*, 2006), followed by the administration of a saturating concentration of (−)-isoprenaline (200 μM). For inotropic studies, the experiments were terminated by elevating the $CaCl_2$ concentration to 9 mM as shown in representative experiments for left atrium (Figure 1) and right ventricular wall (Figure 2).

Paced right ventricular walls tend to become arrhythmic with high catecholamine concentrations (Heubach *et al.*, 2004). Arrhythmic contractions consisted of extrasystoles and ventricular tachycardia (Figure 3). The incidence of arrhythmic contractions was assessed from fast-speed tracings as a function of (−)-adrenaline concentration as shown in the representative experiments of Figure 3. The percentage incidence of these arrhythmic events, regardless of whether they were extrasystoles or tachycardia or both, was computed for each (−)-adrenaline concentration across all used right ventricles. The number of preparations with arrhythmic contraction was divided by the total number of preparations, and a standard error calculated. Positive inotropic effects of (−)-adrenaline were measured only from non-arrhythmic ventricles or during periods of stable non-arrhythmic contractions.

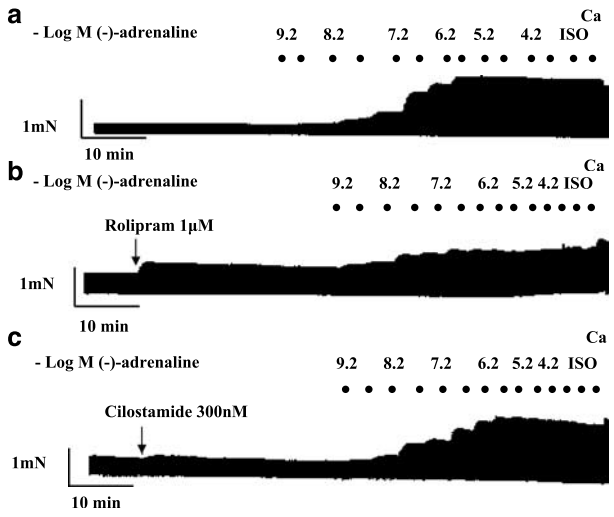

Figure 1 Potentiation of the effects of (−)-adrenaline by rolipram but not by cilostamide on left atrium. Representative experiments, depicting cumulative concentration–effect curves for (−)-adrenaline in the absence of PDE inhibitors (**a**), in the presence of rolipram (**b**) and in the presence of cilostamide (**c**). Black spots indicate −log(−)-adrenaline concentrations, achieved by cumulative administration. Ca, $CaCl_2$ (9 mM); Iso, (−)-isoprenaline (200 μM).

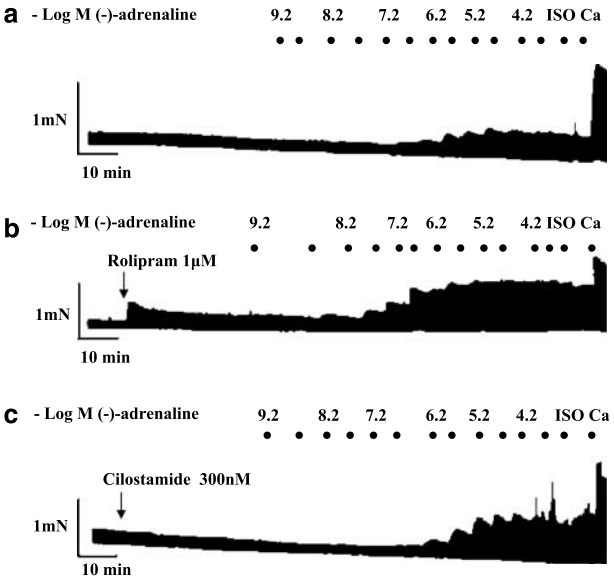

Figure 2 Positive inotropic and arrhythmic effects of (−)-adrenaline on right ventricular walls; influence of PDE inhibitors. Representative experiments, depicting cumulative concentration–effect curves for (−)-adrenaline in the absence of PDE inhibitors (**a**), presence of rolipram (**b**) and presence of cilostamide (**c**). Black spots indicate −log(−)-adrenaline concentrations, achieved by cumulative administration. Ca, $CaCl_2$ (9 mM); Iso, (−)-isoprenaline (200 μM).

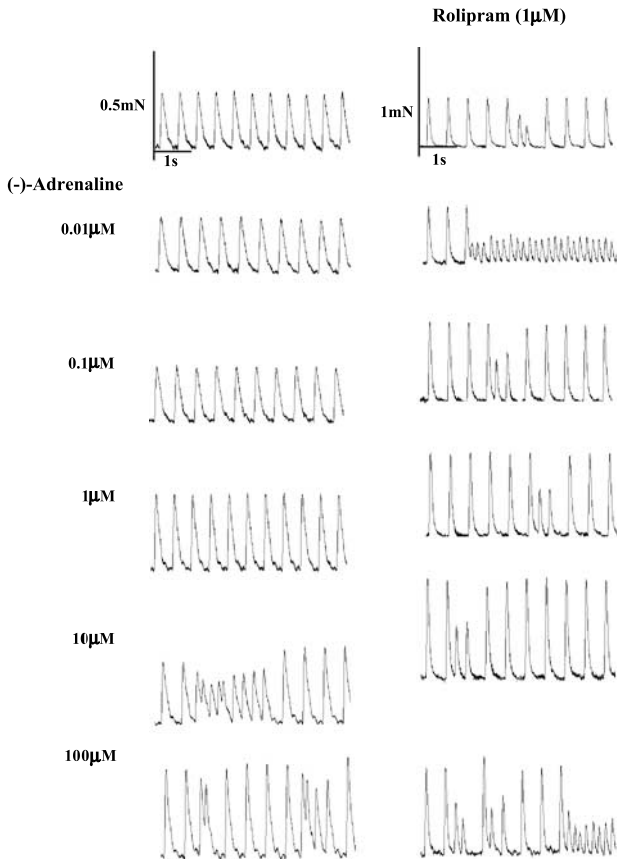

Figure 3 Rolipram potentiates the positive inotropic and arrhythmic effects of (−)-adrenaline on murine right ventricular wall. Comparison of the effects of increasing (−)-adrenaline concentrations on a right ventricular wall in the absence (left hand panels) and another right ventricular wall in the presence of rolipram (right hand panels). Please note extrasystoles at 10 and 100 μM (−)-adrenaline in the absence of rolipram and with rolipram and all (−)-adrenaline concentrations in the presence of rolipram. (−)-Adrenaline 10 μM in the absence of rolipram and (−)-adrenaline 0.01 and 100 μM in the presence of rolipram caused episodes of tachycardia.

Statistics

$-\log EC_{50} M$ values of (−)-adrenaline were estimated from fitting a Hill function with variable slopes to concentration–effect curves from individual experiments. When appropriate, we used an equation for two receptor populations, taken from GraphPad Prism 4 for Windows. The data are expressed as mean ± s.e.mean of n = number of mice. Significance of differences between means was assessed with paired and unpaired Student's t-test using GraphPad 4 Software Inc. (San Diego, CA, USA). The distribution of the arrhythmia data is a Bernoulli (0, 1) distribution. The statistical data are the sum of Bernoulli distributions that yields a binomial distribution (Feller, 1968). Since the sample size was sufficiently large, the binomial distribution was approximated to a normal distribution (Feller, 1968). ANOVA with repeated measurements was applied, using the SPSS programme (Chicago, IL, USA). $P<0.05$ was considered significant.

Drugs

CGP20712A was from Novartis (Basel, Switzerland). ICI118551 was from Tocris (Bristol, UK); (−)-adrenaline, (−)-isoprenaline, rolipram, phenoxybenzamine and cilostamide were from Sigma (Poole, Dorset, UK).

Results

Basal cardiac force and rate. Effects of (−)-isoprenaline and high calcium

Basal left atrial and right ventricular force, as well as sinoatrial rate, are shown in Table 1. The high Ca^{2+} concentration (9 mM) did not increase further the maximum (−)-isoprenaline response in left atrium (Figure 1 and Table 1). However, 9 mM Ca^{2+} caused a considerably greater increase of ventricular force than 200 μM (−)-isoprenaline (Figure 2 and Table 1). CGP20712A (300 nM) did not significantly modify atrial force but reduced ventricular force. However, (−)-isoprenaline and 9 mM Ca^{2+} elevated contractile force to values similar to those in the absence of CGP20712A (Table 1). The combination of CGP20712A (300 nM) and ICI118551 (50 nM) did not affect basal force and the responses to (−)-isoprenaline and 9 mM Ca^{2+} in left atrium and right ventricle. CGP20712A and CGP20712A plus ICI118551 affected neither basal sinoatrial rate nor the response to (−)-isoprenaline (200 μM).

Rolipram potentiates the effects of (−)-adrenaline on left atria but not on the sinoatrial node

Rolipram (1 μM) increased left atrial contractile force (Figures 1 and 4b). Rolipram tended to transiently increase sinoatrial rate maximally from 288 ± 22 to 313 ± 21 beats min^{-1} ($n = 5$, $P = 0.08$, paired Student's *t*-test). Rolipram potentiated 19-fold the positive inotropic effects of (−)-adrenaline on left atrium (Figure 4b and Table 2) but did not potentiate the positive chronotropic effects of (−)-adrenaline on sinoatrial node (Figure 4a and Table 2).

As observed previously (Heubach *et al*., 2002), CGP20712A tended to cause bradycardia (Figures 4a and 6a and Table 1) but the effect did not reach statistical significance (Table 2). CGP20712A caused a 3 log unit rightward and surmountable shift of the concentration–effect curve of (−)-adrenaline for sinoatrial tachycardia (Figure 4a and Table 2) and left atrial contractile force (Figure 4b and Table 2). Rolipram, in the presence of CGP20712A, increased sinoatrial rate from 297 ± 7 to 329 ± 11 beats min^{-1} ($n = 8$, $P < 0.005$). Rolipram, in the presence of CGP20712A, potentiated sixfold the positive inotropic effects of (−)-adrenaline on left atria (Figure 4b and Table 2) but did not affect the positive chronotropic effects of (−)-adrenaline on sinoatrial node (Figure 4a and Table 2).

Table 2 Cardiostimulant potencies ($-\log EC_{50}$ *M*) of (−)-adrenaline

	$-log\ EC_{50}$ M			
	Control		*CGP20712A (300 nM)*	
	n		n	
Right atrium (sinus rate)				
Control	4	7.24 ± 0.19	6	4.83 ± 0.07
Rolipram	3	7.26 ± 0.24	8	4.87 ± 0.06
Cilostamide	4	7.35 ± 0.11	4	4.71 ± 0.11
Rolipram + cilostamide	5	8.11 ± 0.11*	6	4.95 ± 0.05
Left atrium (contractile force)				
Control	5	7.19 ± 0.12	8	4.22 ± 0.19
Rolipram	4	8.47 ± 0.11***	8	4.97 ± 0.16**
Cilostamide	4	7.66 ± 0.20	4	4.55 ± 0.14
Rolipram + cilostamide			13	5.53[a] ± 0.16***[#]
Rolipram + cilostamide			13	7.93[b] ± 0.30
Ventricle (contractile force)				
Control	7	6.54 ± 0.12		
Rolipram	9	7.40 ± 0.14**		
Cilostamide	4	6.37 ± 0.41		
Rolipram + cilostamide	8	7.42 ± 0.30**	6	4.32 ± 0.05

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control.
[#]$P < 0.01$, compared to rolipram.
[a]β_1-adrenoceptor component.
[b]β_2-adrenoceptor component.

Table 1 Contractile force of the left atria and right ventricles as well as sinoatrial rate of right atria

		Force (mN)		
	n	*Basal*	*(−)-Isoprenaline (200 μM)*	*Ca^{2+} (9 mM)*
Left atrium				
No β-antagonist	57	0.94 ± 0.11	2.10 ± 0.20	2.19 ± 0.20
CGP20712A	26	0.80 ± 0.18	2.14 ± 0.29	2.27 ± 0.28
CGP20712A + ici 118551	5	1.01 ± 0.24	2.40 ± 0.49	3.49 ± 0.31
Right ventricle				
No β-antagonist	49	0.53 ± 0.07	1.08 ± 0.12	2.11 ± 0.23*
CGP20712A	17	0.23 ± 0.04[#]	1.14 ± 0.28	2.32 ± 0.47
CGP20712A + ici 118551	4	0.54 ± 0.11	1.50 ± 0.52	2.67 ± 0.95
		Sinoatrial rate (beats min^{-1})		
No β-antagonist	42	294 ± 8	508 ± 10	
CGP20712A	24	282 ± 9**	499 ± 12	
CGP20712A + ici 118551	5	319 ± 36	521 ± 41	

[#]$P < 0.05$ with respect to the absence of β-antagonist.
*$P < 0.01$ with respect to (−)-isoprenaline.
**$P = 0.06$ with respect to the absence of β-antagonist.

Rolipram potentiates ventricular effects of (−)-adrenaline

Rolipram tended to increase basal force (Figures 2 and 4c) to a variable extent by an average of 4.5 ± 1.7% of the response to 9 mM $CaCl_2$ but the effect did not reach significance ($P = 0.056$). Rolipram potentiated sevenfold the positive inotropic effects of (−)-adrenaline (Table 2, Figure 4c) and increased the (−)-adrenaline-evoked maximum contraction (E_{max} as % of the response to 9 mM $CaCl_2$, $P < 0.03$; Figure 4c). CGP20712A caused insurmountable blockade of the inotropic effects of (−)-adrenaline on ventricle in the absence of rolipram or partially surmountable blockade in the presence of rolipram (Figure 4c).

High (−)-adrenaline concentrations tended to produce arrhythmic contractions on the free wall of the right ventricle (Figures 3 and 5a). The arrhythmic contractions consisted of extrasystoles and/or episodes of ventricular tachycardia. A contraction due to an extrasystole appeared prematurely with reduced force and also reduced the force of the following paced contraction (Figure 3). Ventricular tachycardia appeared as a sequence of non-paced contractions at a faster rhythm than the paced contractions and with decreased contractile force. Some ventricular preparations on occasion produced spontaneous arrhythmic contractions in the absence or presence of rolipram (Figures 5a and b). The incidence of spontaneous arrhythmic contractions was not significantly enhanced by rolipram in the absence of CGP20712A ($P = 0.164$, $n = 16$, paired Student's test; $P = 0.258$, $n = 24$ controls vs $n = 16$ rolipram-treated, unpaired Student's test; Figure 5a) or presence of CGP20712A ($P = 0.172$, $n = 7$, paired Student's test; Figure 5b). The increase of (−)-adrenaline-evoked arrhythmic contractions was linearly related to −log concentration but the slope was steeper in the absence of rolipram (slope 0.126, r^2 0.715) than in the presence of rolipram (slope 0.096, r^2 0.831) (Figure 5a). CGP20712A prevented the incidence of the (−)-adrenaline-evoked arrhythmias, both in the absence and presence of rolipram (Figure 5b).

Cilostamide does not potentiate the positive chronotropic and inotropic effects of (−)-adrenaline

Cilostamide (300 nM) did not significantly increase left atrial contractility (Figure 1c); force was 1.13 ± 0.52 and 1.19 ± 0.53 mN in the absence and presence of cilostamide. Cilostamide did not affect ventricular contractility (Figure 2d). Cilostamide transiently increased sinoatrial rate maximally from a basal rate of 294 ± 23 to 351 ± 28 beats min^{-1} ($P < 0.01$, $n = 4$, paired Student's test). In the presence of CGP20712A, cilostamide increased sinoatrial rate from 241 ± 10 to 283 ± 6 beats min^{-1} ($P < 0.005$, $n = 4$). Cilostamide did not significantly affect the potency of (−)-adrenaline on sinoatrial node (Figure 4a) and left atrium (Figure 4b) in the absence or presence of CGP20712A (Figures 4a and b and Table 2). Cilostamide did

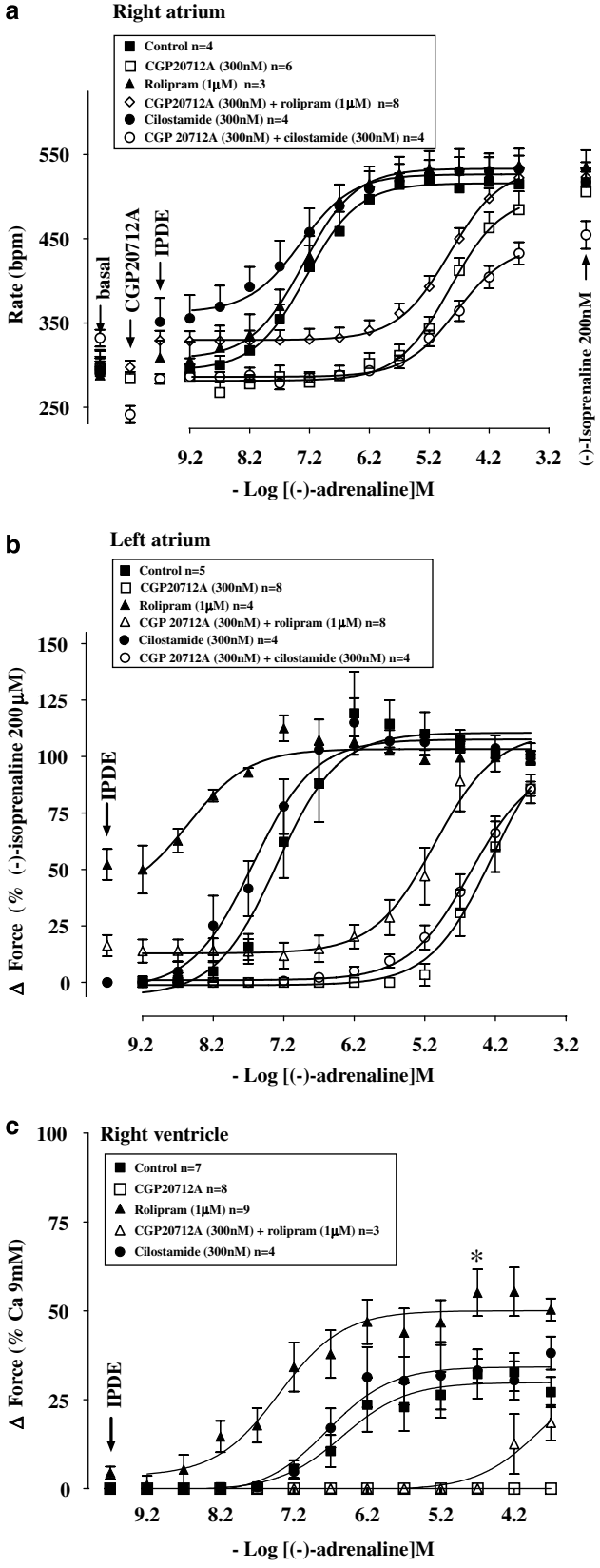

Figure 4 Potentiation of the effects of (−)-adrenaline by rolipram, mediated through β_1-adrenoceptors, on left atria (**b**) and right ventricular walls (**c**), but not on sinoatrial pacemaker (**a**) in the absence and presence of CGP20712A. Lack of effect of cilostamide on (−)-adrenaline potency. A single concentration–effect curve for (−)-adrenaline was determined in the absence or presence of CGP20712A. IPDE, PDE inhibitor. *Increase of the E_{max} of (−)-adrenaline by rolipram (*$P < 0.03$).

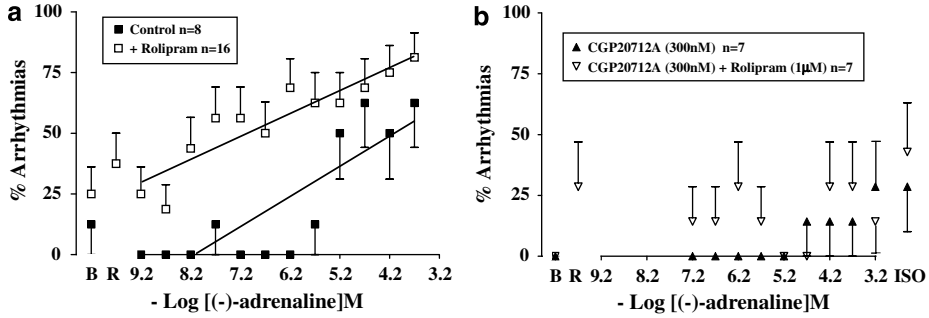

Figure 5 Increased incidence of (−)-adrenaline-evoked arrhythmias by rolipram (**a**) and blockade by CGP20712A (**b**). %Arrythmias is the % of ventricles that showed extrasystoles and/or ventricular tachycardia at each (−)-adrenaline concentration. Lines depict the dependence of arrhythmias on −log(−)-adrenaline concentration. The slopes of the lines were 0.126 and 0.096 in the absence and presence of rolipram, respectively. B, basal; ISO, (−)-isoprenaline 200 μM; R, rolipram.

not affect the potency of (−)-adrenaline in right ventricle (Figure 4c and Table 2).

Concurrent cilostamide and rolipram uncover functional β_2-adrenoceptors in left atrium but not in sinoatrial node and right ventricle

Cilostamide and rolipram, administered together, caused marked increases in sinoatrial rate as well as of left atrial and right ventricular force (Figures 6a–c). Concurrent cilostamide and rolipram potentiated the sinoatrial and ventricular effects of (−)-adrenaline (compare Figures 6a and c with Figures 4a and c; Table 2). However, the ventricular potency of (−)-adrenaline in the presence of both cilostamide and rolipram was not different from the potency of (−)-adrenaline in the presence of rolipram alone (Table 2). The maximum increase in contractility by combined cilostamide and rolipram prevented further effects of (−)-adrenaline in left atrium (Figure 6b). In the presence of CGP20712A, the increases in left atrial and right ventricular force and sinoatrial rate caused by the combination of cilostamide and rolipram were smaller ($P<0.001$, $P<0.01$ and $P=0.02$, respectively) than in the absence of CGP20712A and the concentration–effect curves of (−)-adrenaline were shifted to the right by 3 log units (Figure 6 and Table 2). CGP20712A-resistant effects of (−)-adrenaline were detected on left atrium (Figure 6b) but not on sinoatrial node and right ventricle (Figures 6a and c). The concentration–effect curve for (−)-adrenaline on left atrium in the presence of CGP20712A was biphasic (Figure 6b) with a high-potency and low-potency component (Table 2). The CGP20712A-resistant effects of (−)-adrenaline were prevented by the β_2-adrenoceptor-selective antagonist ICI118551 (50 nM) (Figure 6b), consistent with mediation through β_2-adrenoceptors. The fractions of left atrial β_1- and β_2-adrenoceptor-mediated effects of (−)-adrenaline in the presence of cilostamide, rolipram and CGP20712A amounted to 0.74 ± 0.07 vs 0.26 ± 0.07, respectively (Table 2).

Discussion

Our results point to regional differences in the role of PDE4 in murine heart. First, (−)-adrenaline-evoked sinoatrial tachycardia was not potentiated by either cilostamide or rolipram, inconsistent with modulation by either PDE3 or PDE4 alone. However, both isoenzymes appear to control basal sinoatrial beating rate. Second, in contrast, the positive inotropic effects of (−)-adrenaline, mediated through left atrial and right ventricular β_1-adrenoceptors, were potentiated by rolipram, but not by cilostamide, suggesting hydrolysis of inotropically relevant cAMP by PDE4 but not by PDE3. Third, the effects of (−)-adrenaline in the three cardiac regions were antagonized by CGP20712A, consistent with mediation through β_1-adrenoceptors. Next, CGP20712A-resistant effects, mediated through β_2-adrenoceptor, were only observed on left atrium in the presence of both cilostamide and rolipram, suggesting that PDE3 and PDE4 act in concert to prevent manifestation of β_2-adrenoceptor function. Finally, rolipram potentiated (−)-adrenaline-evoked right ventricular arrhythmias.

PDE3 and PDE4 modulate basal sinoatrial beating but not β_1-adrenoceptor-mediated tachycardia of (−)-adrenaline

Sinoatrial cells exhibit a considerably higher basal cAMP content and basal PKA-mediated phosphorylation of phospholamban than atrial or ventricular cells (Vinogradova *et al.*, 2006). Submaximal PKA inhibition slows the spontaneous firing rate of sinoatrial action potentials and it has been postulated that basal PKA activity is obligatory for rhythmical Ca^{2+} release from RyR2 channels involved in generating spontaneous sinoatrial beating (Maltsev *et al.*, 2006; Vinogradova *et al.*, 2006). Interestingly, basal PDE activity also appears to be elevated in sinoatrial cells (Vinogradova *et al.*, 2005b) and our data, showing that cilostamide and rolipram increase sinoatrial rate, suggest that both PDE3 and PDE4 reduce tonically sinoatrial beating rate by hydrolysing cAMP. The tachycardia elicited by either cilostamide or rolipram suggests mediation through an increase of cAMP in sinoatrial cells. Blockade of β_1-adrenoceptors with CGP20712A did not prevent the tachycardia of cilostamide or rolipram, ruling out an interaction of endogenously released noradrenaline with β_1-adrenoceptors. The tachycardia of cilostamide or rolipram is likely to result from the inhibition of either PDE3 or PDE4 respectively, followed by elevation of sinoatrial cAMP and increase of PKA-dependent beating rate. In addition, the increased

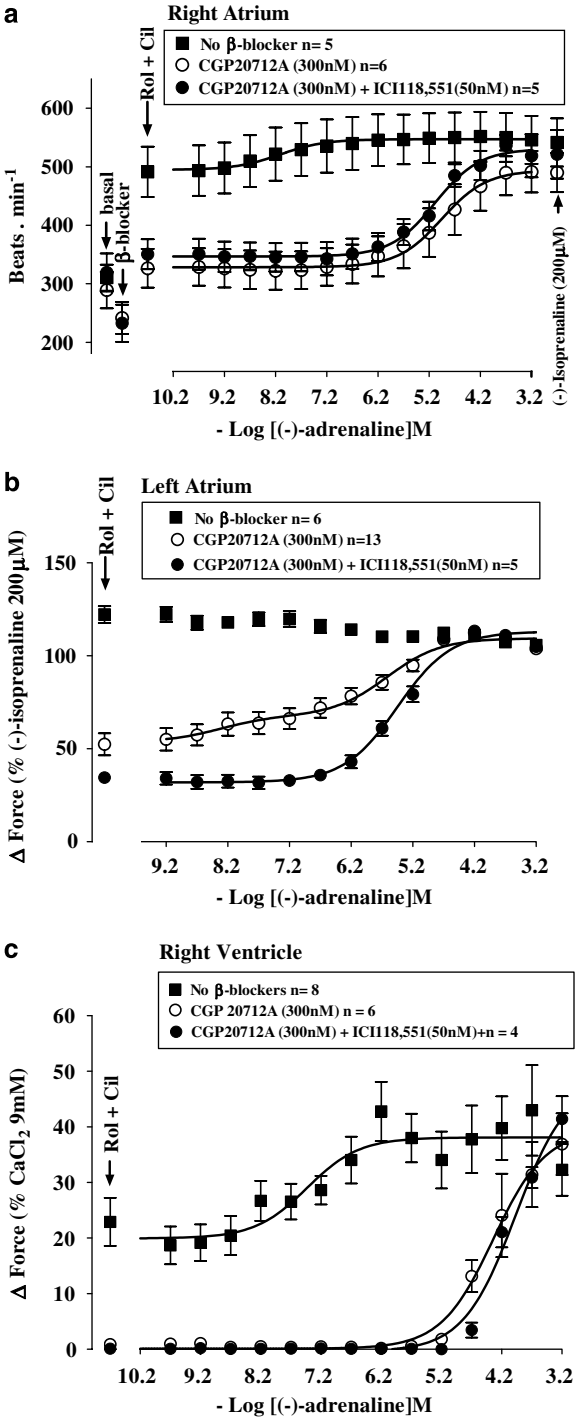

Figure 6 Cilostamide (Cil) and rolipram (Rol), administered together, potentiate the chronotropic effects of (−)-adrenaline (**a**) and uncover β_2AR-mediated inotropic effects in the presence of CGP20712A, sensitive to blockade by ICI118551, in left atrium (**b**) but not in right ventricle (**c**). Data from left atria in the presence of CGP20712A were fitted for two β-adrenoceptor populations with $-\log EC_{50}$ *M* of 7.93 ± 0.30 (β_2-adrenoceptor) and 5.53 ± 0.16 (β_1-adrenoceptor) and fractional E_{max} of 0.74 ± 0.07 (β_1-adrenoceptor) and 0.26 ± 0.07 (β_2-adrenoceptor). The left atrial $-\log EC_{50}$ *M* of the curve for (−)-adrenaline in the presence of both CGP20712A and ICI118551 was 5.44 ± 0.08.

sinoatrial cAMP in the presence of cilostamide or rolipram may also directly bind to, and open the channels responsible for the current activated by hyperpolarization, I_f (DiFrancesco and Tortora, 1991). Our evidence is consistent with the tachycardia produced by several other PDE3-selective inhibitors in a variety of species (Brunkhorst *et al*., 1989; Sato *et al*., 1999; Herring and Paterson, 2001).

The modulation of sinoatrial beating rate by PDE3 is in marked contrast to the lack of influence of cilostamide on the β_1-adrenoceptor-mediated increase in sinoatrial rate elicited by (−)-adrenaline. This discrepancy suggests that the PDE3-sensitive pool of cAMP that modulates sinoatrial beating frequency is separated from a PDE3-insensitive pool of cAMP through which (−)-adrenaline increases sinoatrial rate. The rolipram-evoked tachycardia is also at variance with the lack of potentiation of the positive chronotropic effects of (−)-adrenaline by rolipram, suggesting the existence of a PDE4-sensitive cAMP compartment for basal heart rate but not for sinoatrial β_1-adrenoceptor stimulation by (−)-adrenaline.

Does inhibition of both PDE3 and PDE4 potentiate (−)-adrenaline-evoked tachycardia?

Neither inhibition of PDE3 nor PDE4 alone affected the chronotropic potency of (−)-adrenaline. In contrast, the combined administration of rolipram (1 μM) and cilostamide (300 nM) caused tachycardia and appeared to potentiate the positive chronotropic effects of (−)-adrenaline (Figure 6a and Table 2). However, in the presence of CGP20712A, which shifted the concentration–effect curve of (−)-adrenaline by 3 log units to the right, the combination of rolipram and cilostamide failed to potentiate the positive chronotropic effects of (−)-adrenaline (Figure 6a). The tachycardia caused by the combination of rolipram and cilostamide was less marked in the presence than in the absence of CGP20712A (Figure 6a), and could be plausibly related to an inverse agonist effect of CGP20712A and perhaps to blockade of β_1-adrenoceptors activated by traces of endogenously released noradrenaline. Since the chronotropic potency of (−)-adrenaline was not significantly increased by the combination of rolipram and cilostamide in the presence of CGP20712A, the apparent potentiation of the effects of (−)-adrenaline in the absence of CGP20712A appears mainly due to the additivity of the effects of rolipram, cilostamide and (−)-adrenaline. However, since both PDE3 and PDE4 appear to reduce the cAMP required for basal sinoatrial rate, it cannot be excluded that some cAMP from this compartment may leak into the β_1-adrenoceptor compartment of cAMP when both isoenzymes are inhibited. Alternatively, both PDE3 and PDE4 may actually hydrolyse cAMP in the β_1-adrenoceptor compartment but, when one enzyme is inhibited, the resulting increase of cAMP induced PKA-catalysed phosphorylation of the other enzyme, thereby facilitating cAMP hydrolysis and reducing both increases of cAMP and sinoatrial rate by (−)-adrenaline. The hydrolytic activity of both PDE3 (Gettys *et al*., 1987; Smith *et al*., 1991) and PDE4 (MacKenzie *et al*., 2002) are enhanced by PKA-dependent phosphorylation. Only when both PDE3 and PDE4 were inhibited in the sinoatrial β_1-adrenoceptor

compartment was cAMP enhanced and some potentiation of (−)-adrenaline-evoked tachycardia followed. Further evidence is needed to support or reject these interpretations.

PDE4 limits the β_1-adrenoceptor inotropic function and PDE3 and PDE4 jointly prevent β_2-adrenoceptor function in left atrium

Rolipram caused marked potentiation of the effects of (−)-adrenaline on left atrium in the absence and presence of CGP20712A but cilostamide failed to affect the inotropic potency. These effects are consistent with an exclusive role of PDE4 in controlling the inotropically relevant cAMP generated through left atrial β_1-adrenoceptor stimulation.

Inhibition of both PDE3 and PDE4 uncovered functional β_2-adrenoceptors in left atrium. The CGP20712A-resistant effects of (−)-adrenaline in the presence of both cilostamide and rolipram were prevented by ICI118551, consistent with mediation through β_2AR. Our results indicate that PDE3 and PDE4, acting in concert, prevent the manifestation of β_2AR-mediated effects of (−)-adrenaline in murine left atrium. The potentiation by rolipram of the effects of (−)-adrenaline in the presence of CGP20712A (Figure 4b) is similar to that observed in the absence of CGP20712A. Importantly, the leftward shift of the concentration–effect curve was parallel and expected from the high affinity of CGP20712A for β_1-adrenoceptors without evidence for CGP20712A-resistant effects. Therefore inhibition of PDE4 alone does not appear to uncover β_2-adrenoceptor-mediated effects of (−)-adrenaline. Previous work failed to detect β_2-adrenoceptor-mediated effects in murine left atrium, even after inactivation of G_i protein with PTX (Oostendorp and Kaumann, 2000; Heubach *et al*., 2002). On the other hand, β_2-adrenoceptor stimulation increases cAMP but this was not affected by PTX (Nikolaev *et al*., 2006). Taken together, this evidence is inconsistent with the concept that activation of G_i protein through β_2-adrenoceptor blunts G_s protein-mediated effects in murine heart (Xiao *et al*., 1999). β_2-Adrenoceptor-mediated increases of cAMP are spatially confined and do not propagate and, further, β_2-adrenoceptor-mediated effects are mainly blunted by both PDE3 and PDE4 in murine myocytes (Nikolaev *et al*., 2006). Our present results with β_2-adrenoceptor-mediated inotropic effects on left atrium are in line with the conclusions of Nikolaev *et al*. (2006).

Under our conditions, the β_2-adrenoceptor function was uncovered only with the concurrent use of cilostamide and rolipram under β_1-adrenoceptor blockade with CGP20712A in left atrium but not in sinoatrial node and right ventricle. Genetic deletion of the β_2-adrenoceptor does not modify the chronotropic response to (−)-isoprenaline, which is entirely mediated through β_1-adrenoceptors (Chruscinski *et al*., 1999). It is therefore unknown whether the murine sinoatrial node possesses functional β_2-adrenoceptors. However, 30% of murine ventricular β-adrenoceptors are β_2-adrenoceptors (Heubach *et al*., 1999). A possible reason for the lack of β_2-adrenoceptor-mediated responses in right ventricle could be an involvement of PDE2, an option which requires further research.

The $-\log EC_{50} M$ of the left atrial β_1AR-mediated component of the inotropic effects of (−)-adrenaline in the presence of rolipram, cilostamide and CGP20712A was 3.6-fold (that is 0.56 log units) larger than the $-\log EC_{50} M$ in the presence of rolipram and CGP20712 (Table 2). These results suggest that when PDE4 is inhibited, PDE3 may become activated, by an excess of cAMP, possibly through PKA-catalysed phosphorylation, and contribute to hydrolyse inotropically relevant cAMP.

The effects of (−)-adrenaline, mediated through β_1-adrenoceptors, are blunted by PDE4 but not by PDE3 in right ventricle

The effects of (−)-adrenaline were antagonized by CGP20712A, consistent with mediation through β_1-adrenoceptors and the 3 log surmountable shift of the concentration–effect curves did not reveal CGP20712A-resistant effects of (−)-adrenaline in right ventricular wall, inconsistent with the participation of β_2-adrenoceptors. The addition of cilostamide to rolipram did not cause additional potentiation (Table 2), pointing towards an exclusive function of PDE4, and no role of PDE3, in murine ventricle. The potentiation of the positive inotropic effects of (−)-adrenaline by rolipram, but not by cilostamide, and the lack of additional potentiation by cilostamide in the presence of rolipram, are consistent with hydrolysis of inotropically relevant cAMP by PDE4 but not by PDE3 in right ventricle. Our results are consistent with the conclusion of recent work by Nikolaev *et al.* (2006) demonstrating that β_1-adrenoceptor-mediated cAMP signals are entirely controlled by PDE4 in murine ventricular myocytes.

Our results are at variance with data of Xiang *et al*. (2005), showing that PDE4 blunted the effects of isoprenaline mediated through β_2-adrenoceptor in spontaneously beating ventricular myocytes from new-born mice. These authors demonstrated that the fade of (−)-isoprenaline-induced increase in myocyte beating rate was prevented by a PDE4-selective inhibitor and that fade did not occur in PDE4D3-KO mice, clearly proving involvement of this PDE4 isoenzyme. However, in contrast to our demonstration that the effects of (−)-adrenaline are mediated through β_1-adrenoceptors and potentiated by the PDE4 inhibitor rolipram, in their work, the effects of (−)-isoprenaline, mediated through β_1-adrenoceptors, were not affected by PDE4 inhibition (Xiang *et al*., 2005). Xiang *et al*. (2005) also found that cilostamide did not affect the responses to (−)-isoprenaline, mediated through β_2-adrenoceptors in their experimental model, ruling out the role of PDE3. Comparison of the results of the experiments of Xiang *et al*. (2005) in ventricular myocardium from new-born mice and our results from myocardium of adult mice suggests that the β_2AR inotropic function is reduced (left atrium) or lost (right ventricle) in adult mice. Consistent with this suggestion are the results of Heubach *et al*. (2002), as well as our present results, demonstrating a lack of functional β_2-adrenoceptors in adult murine ventricular myocardium, even after inactivation of G_i protein with PTX (Heubach *et al*., 2002). Furthermore, the results of Kuznetsov *et al*. (1995) in rat myocardium is also in agreement with work on murine hearts, because activation of β_2-adrenoceptor, at low agonist concentrations that cause positive inotropic and lusitropic effects in neonatal cardiomyocytes, is lost in adult myocytes.

In the adult rat, the ventricular inotropic responses to (−)-noradrenaline, mediated through β_1-adrenoceptors, are potentiated by rolipram but not by cilostamide (Vargas *et al*., 2006), findings compatible with our present work using (−)-adrenaline in adult murine right ventricle and left atrium.

Unlike murine and rat heart, human atrial and ventricular myocardium respond to catecholamines with positive inotropic effects, mediated through β-adrenoceptors, which are potentiated by cilostamide but not by rolipram, that is, modulated by PDE3 but not by PDE4 (Kaumann *et al*., 2007; Christ *et al*., 2006a, b). Moreover, in human isolated myocardium, cilostamide potentiates the effects of (−)-adrenaline, mediated through β_2-adrenoceptors, more than the effects of (−)-noradrenaline, mediated through β_1-adrenoceptors, perhaps suggesting a more marked phosphorylation of PDE3 by PKA via β_2-adrenoceptors than via β_1-adrenoceptors (Christ *et al*., 2006a, b). In contrast to murine and rat myocardium, in human myocardium, rolipram does not affect the positive inotropic effects of physiological catecholamines, mediated through either β_1AR (Christ *et al*., 2006a; Kaumann *et al*., 2007) or β_2AR (Christ *et al*., 2006a, b). Thus, murine and rat cardiac myocardial models do not mimic the control by specific PDE isoenzymes of the positive inotropic responses to physiological catecholamines, mediated through β_1- and β_2-adrenoceptors in human myocardium.

Murine right ventricle, a model for catecholaminergic polymorphic ventricular tachycardia

(−)-Adrenaline caused concentration-dependent arrhythmias in the right ventricular wall. The extrasystolic contractions showed reduced force. The contractions of ventricular tachycardia, initiated by an extrasystole, also exhibited markedly reduced force. Larger mammals including man with non-failing hearts, increase cardiac contractile force when heart rate is increased, the Bowditch staircase. In rodents, however, contractile force of cardiac tissues and myocytes is decreased when heart rate is increased and this negative staircase has also been demonstrated in mice (Wussling *et al.,* 1987; Ceylan-Isik *et al.,* 2006). The reduced contractile force of ventricular extrasystoles and tachycardia is probably a manifestation of negative staircase.

The arrhythmias were greatly attenuated by CGP20712A and are therefore mediated through β_1-adrenoceptors. In the presence of rolipram, (−)-adrenaline elicited arrhythmic contractions at lower concentrations than in the absence of rolipram (Figure 5a), consistent with potentiation. However, the concentration–effect curve for (−)-adrenaline was flatter in the presence than in the absence of rolipram, so that an additive effect of rolipram cannot be ruled out, despite our finding that rolipram-evoked arrhythmias did not reach statistical significance. Taken together, these results are consistent with a protective role of PDE4 through hydrolysis of cAMP, thereby preventing both PKA-catalysed phosphorylation of the RyR2 channels (Wehrens *et al*., 2006; Xiao *et al*., 2006) and Ca^{2+} leak that would lead to ventricular arrhythmias. Our results with ventricular arrhythmias are consistent with results in isolated cardiomyocytes from adult mice in which the PDE4D3 isoform was found to participate in a macromolecular complex including RyR2 and PKA (Lehnart *et al*., 2005). PDE4D3 ablation in mice hastened the appearance of heart failure after myocardial infarction and of arrhythmias, associated with increased cAMP-dependent signals at RyR2 sites after low catecholamine concentrations, compared to wild-type mice (Lehnart *et al*., 2005).

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a cardiac arrhythmia that occurs under conditions of adrenergic stimulation during exercise or under emotional stress. Affected individuals present syncope and/or sudden cardiac death in childhood and adolescence (Coumel *et al*., 1978; Leenhardt *et al*., 1995). The mortality rate is approximately one-third of CPVT patients by age of 35 years (Lehnart *et al*., 2004). The disease was linked to chromosome 1q42–q43 (Rampazzo *et al*., 1995) and subsequently both Priori *et al*. (2001) and Laitinen *et al*. (2001) showed that individuals with the autosomal dominant form of CPVT had mutations in the human cardiac RyR2. Over 30 mutations have been identified, which cluster within three regions of the RyR2 receptor, the first 450 amino acids at N terminus, a central region (amino acids 2240–2510) and the C terminus (amino acids 3378–5000) (Jiang *et al*., 2005).

Some clinically relevant RyR2 mutations of patients with CPVT have been reproduced in mice. These include $RyR2^{R4496C}$ mice that exhibit proarrhythmic delayed afterdepolarizations (Liu *et al*., 2006) and $RyR2^{R176Q}$ mice that exhibit a high incidence of ventricular tachycardia and cardiocyte Ca^{2+} oscillations (Kannankeril *et al*., 2006). Ventricular arrhythmias, including tachycardia and fibrillation, which occur in patients with RyR2 mutations have been attributed to enhanced store overload-induced Ca^{2+} release from the RyR2 (Jiang *et al*., 2004, 2005).

We attribute the right ventricular arrhythmias, observed as a function of (−)-adrenaline concentration, to pro-arrhythmic Ca^{2+} leaking out from the RyR2 channels, due to cAMP and resultant PKA-catalysed RyR2 phosphorylation (Lehnart *et al*., 2005; Wehrens *et al*., 2006; Xiao *et al*., 2006). The potentiation of the (−)-adrenaline-evoked arrhythmias we observed with rolipram is consistent with the work of Lehnart *et al*. (2005). We suggest that the murine right ventricular wall would provide an experimental model for CPVT. Mice generated to carry human CPVT mutations of RyR2 channels should exhibit a greater sensitivity to (−)-adrenaline-evoked arrhythmias, mediated through β_1-adrenoceptors.

Conclusions

Rolipram revealed regional differences in the role of PDE4 in murine heart. (−)-Adrenaline-evoked cardiostimulation was blunted considerably by PDE4, but not by PDE3, in murine left atrium and in right ventricle. Although both PDE3 and PDE4 modulated basal sinoatrial beating rate, inhibition of either of these phosphodiesterases did not potentiate the β_1-adrenoceptor-mediated tachycardia elicited by (−)-adrenaline. Concurrent inhibition of both PDE3 and PDE4 uncovered cardiostimulant effects of (−)-adrenaline

mediated through β_2-adrenoceptors of left atrium but not of sinoatrial node or right ventricle. Rolipram potentiated (−)-adrenaline-evoked arrhythmias, mediated through β_1-adrenoceptors in right ventricular wall. The murine right ventricle may serve as a model for (−)-adrenaline-evoked arrhythmias in mice carrying RyR2 mutations corresponding to human CPVT.

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Conflict of interest

The authors state no conflict of interest.

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