Universidad de Murcia
Facultad de Medicina
Departamento de Farmacología

Control por fosfodiesterasas de la función cardíaca activada por los receptores acoplados a proteína G

Alejandro Galindo Tovar

Memoria presentada para optar al grado de doctor con "Mención de Doctorado Europeo"

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La tesis doctoral titulada *"**Control por fosfodiesterasas de la función cardiaca activada por receptores acoplados a proteína G**"* es un compendio de trabajos previamente publicados.

Los artículos que componen el cuerpo de esta tesis son los siguientes:

- Galindo-Tovar A, Kaumann AJ (2008) **Phosphodiesterase-4 blunts inotropism and arrhythmias but not sinoatrial tachycardia of (-)-adrenaline mediated through mouse cardiac β_1-adrenoceptors.** *British Journal of Pharmacology 153: 710-720.*

- Christ T, Galindo-Tovar A, Thoms M, Ravens U, Kaumann AJ (2009) **Inotropy and L-type Ca^{2+} current, activated by β_1- and β_2-adrenoceptors, are differently controlled by phosphodiesterases 3 and 4 in rat heart.** *British Journal of Pharmacology 156: 62-83.*

- Galindo-Tovar A, Vargas ML, Escudero E, Kaumann AJ (2009) **Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT_4 receptors.** *British Journal of Pharmacology 156:237-249.*

- Galindo-Tovar A, Vargas ML, Kaumann AJ (2009) **Phosphodiesterases PDE3 and PDE4 jointly control the inotropic effects but not chronotropic effects of (-)-CGP12177 despite PDE4-evoked sinoatrial bradycardia in rat atrium.** *Naunyn-Schmiedeberg's Archives of Pharmacology 379:379-384.*

Alejandro Galindo Tovar

Dr. Alberto Julio Kaumann, del Departamento de Fisiología, Desarrollo y Neurociencia de la Universidad de Cambridge, Reino Unido y Profesor Investigador Visitante del Departamento de Farmacología de la Facultad de Medicina de la Universidad de Murcia y **Dra. María Luisa Vargas Álvarez-Castellanos,** Profesora Titular del Departamento de Farmacología de la Facultad de Medicina de la Universidad de Murcia,

CERTIFICAN:

Que la presente Tesis Doctoral titulada ***"Control por Fosfodiesterasas de la función cardiaca activada por receptores acoplados a Proteína G"***, constituye el trabajo de investigación realizado bajo nuestra dirección, en el Grupo Farmacología Celular y Molecular de la Facultad de Medicina, por Don Alejandro Galindo Tovar desde el año 2004. Este trabajo pertenece al ámbito de la Farmacología y Fisiología Cardiaca. Mediante diferentes técnicas experimentales se profundiza en el conocimiento de la función de las fosfodiesterasas en la regulación de los efectos mediados a través de distintos receptores acoplados a Proteína G.

Esta Tesis Doctoral reúne la calidad y el rigor científico necesarios para ser defendida en la Universidad de Murcia como requisito para que Don Alejandro Galindo Tovar aspire al Grado de Doctor y a la ***"Mención de Doctorado Europeo"***.

Murcia, 29 de junio de 2009

Alberto Julio Kaumann

María Luisa Vargas Álvarez-Castellanos

UNIVERSIDAD DE MURCIA
Facultad de Medicina
Departamento de Farmacología

Carlos Mario Cárceles Rodríguez Profesor Titular y Director del Departamento de Farmacología,

HACE CONSTAR:

Que la Tesis Doctoral como Compendio de Publicaciones y con Mención de Doctorado Europeo titulada **"Control por Fosfodiesterasas de la función cardiaca activada por receptores acoplados a Proteína G"** que presenta Don Alejandro Galindo Tovar, ha sido realizada bajo la dirección de Alberto Julio Kaumann y María Luisa Vargas Álvarez-Castellanos y que el Departamento de Farmacología ha dado su conformidad para que se presente ante la Comisión de Doctorado.

En Murcia, a 1 de julio de 2009

Carlos Mario Cárceles Rodríguez

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Vista la solicitud presentada el día 20 de enero de 2009 por D. Alejandro Galindo Tovar, con DNI número 48501225, sobre autorización para presentación de tesis doctoral como compendio de publicaciones con carácter previo a la tramitación de la misma en la Universidad de Murcia, le comunico que la Comisión de General de Doctorado, vistos:

- El informe previo del Departamento de Farmacología, responsable de la autorización de la tesis doctoral en fase de elaboración, de esta Universidad, y
- El visto bueno de la Comisión de Ramas de Conocimiento de Ciencias de la Salud,

resolvió, en su sesión de 13 de marzo de 2009, **ACCEDER** a lo solicitado por el interesado pudiendo, por lo tanto, presentar su tesis doctoral en la modalidad de compendio de publicaciones.

Lo que en cumplimiento del artículo 58 de la vigente Ley 30/1992, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común, de 26 de noviembre, se **notifica a** D. Alejandro Galindo Tovar, significándole que contra esta resolución, que pone fin a la vía administrativa, se podrá interponer potestativamente ante el mismo órgano que la ha dictado, recurso de reposición, en el plazo de un mes a contar desde el día siguiente a su notificación, de acuerdo con lo dispuesto en el art. 116 de la citada Ley.

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Murcia, 16 de marzo de 2009
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UNIVERSIDAD DE MURCIA Vicerrectorado de Estudios

Durante el bienio 2004-2006 se realizaron los Cursos de Doctorado en el Programa "Integración y Modulación de señales Biomedicina" de la Universidad de Murcia, con la obtención del Diploma de Estudios Avanzados (DEA).

Durante la realización de esta tesis doctoral se han realizados dos colaboraciones en el extranjero. La primera de un mes en el año 2006 en el laboratorio del Dr. Alberto Julio Kaumann, Departamento de Fisiología, Desarrollo y Neurociencia, Universidad de Cambridge, Cambridge, Reino Unido. La segunda durante tres meses en los años 2006-2007 en el laboratorio del Dr. Torsten Christ, Departamento de Farmacología y Toxicología, Facultad de Medicina, Universidad Tecnológica de Dresde, Dresde, Alemania.

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La estancia del Dr. Alberto Julio Kaumann ha sido parcialmente financiada por la Fundación Seneca.

En primer lugar quiero expresar mi agradecimiento a mis directores de tesis, los doctores Alberto Kaumann y Mª Luisa Vargas, sin ellos esta tesis doctoral no habría sido posible.

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"Si buscas resultados distintos, no hagas siempre lo mismo"

Albert Einstein

A mis padres

Isabel y Basilio

Glosario de abreviaturas

5-HT: 5-hidroxitriptamina. Serotonina

5-HT_4: Receptor de serotonina 4

AC: Adenilil ciclasa

Ach: Acetilcolina

ADN: Acido desoxirribonucleico

AKAP: Proteína de anclaje de PKA

AKAPm: Proteina de anclaje de PKA muscular

AMPc: Adenosín monofosfato cíclico

ATP: Adenosín trifosfato

BB: Haz de Hiss

Ca^{2+}: Calcio

CaMKII: Proteína quinasa dependiente de calmodulina

CGP12177: Clorhidrato de 4-[3-[(1,1-Dimetiletil)amino]-2-hidroxipropoxi]-1, 3-dihidro-2H-benzimidazol-2-ona clorhidrato

CGP20712A: Metanosulfonato de (±)-2-Hidroxi-5-[2-[[2-hidroxi-3-[4-[1-metil-4-(trifluorometil)-1H-imidazol-2-il]fenoxi]propil]amino]etoxi]-benzamida

CICR: Liberación de calcio inducida por calcio

CNG: Canal activado por nucleótidos cíclicos

Cs^{+}: Cesio

DHPR: Receptor de dihidropiridinas

DMSO: Dimetil sulfóxido.

EC_{50}: Concentración necesaria de agonista para alcanzar la mitad del efecto máximo

EDTA: Ácido Etilendiamintetracético

EGTA: Ácido Etilenglicoltetraacético

EHNA: Clorhidrato de *eritro*-9-(2-Hidroxi-3-nonil)adenina

EPAC: Proteína de intercambio de nucleótidos de guanina activada por AMPc

ERK: Señal extracelular regulada por quinasas

FP: Fibras de Purkinje

FRET: Resonancia de transferencia de energía fluorescente

GC: Guanilil ciclasa

G_i: Proteína G inhibitoria

GMPc: Guanosín monofosfato cíclico

GPCR: Receptor acoplado a proteína G

GRK: Proteína quinasa dependiente del receptor acoplado a proteína G

G_s: Proteína G estimulante

HCN: Canal activado por hiperporalización y nucleótidos cíclicos

HEPES: Ácido N-2-Hidroxietilpiperacina-N'-2'-Etanesulfónico

IBMX: 3-isobutil-1-metil-xantina

IC_{50}: Concentración necesaria de inhibidor para alcanzar la mitad de la inhibición máxima

I_{CaL}: Corriente de calcio tipo L

I_{CaT}: Corriente de calcio tipo T

ICI118551: Clorhidrato de (±)-1-[2,3-(Dihidro-7-metil-1H-inden-4-il)oxi]-3-[(1-metiletil)amino]-2-butanol

ICV: Vena cava inferior

I_f: Corriente "funny". Corriente activada por hiperpolarizacion

I_K: Corriente de potasio

I_{KR}: Componente rápida de la corriente de potasio

I_{KS}: Componente lenta de la corriente de potasio

I_{NCX}: Corriente del intercambiador sodio/calcio

iPDE: Inhibidor de fosfodiesterasa

ISO: Isoproterenol

K^+: Potasio

K_M: Concentracion de sustrato a la que se da la mitad de la velocidad máxima de reacción de la enzima.

LA: Aurícula izquierda

LCICR: Liberación local de calcio inducida por calcio

LV: Ventrículo izquierdo

MV: Válvula mitral

NAV: Nódulo auriculoventricular

NCX: Intercambiador sodio/calcio

NS: Nódulo sinusal

PA: Potencial de acción

PDE: Fosfodiesterasa

PKA: Proteína quinasa dependiente de AMPc

PKC: Proteína quinasa C

PKI: Inhibidor proteico de la Proteína quinasa A

PLB: Fosfolambano

PTX: Toxina pertussis

RA: Aurícula derecha

RS: Retículo sarcoplasmático

RT-PCR: Reacción en cadena de la polimerasa en tiempo real

RV: Ventrículo derecho

RyR: Receptor de rianodina

RyR2: Receptor de rianodina tipo 2

SERCA: Bomba de calcio dependiente de ATP del retículo sarcoplasmático

Tm: Tropomiosina

TnC: Troponina C

TnI: Troponina I

VC: Fijación del voltaje

V_{Max}: Velocidad máxima de catálisis de una enzima

VT: Válvula tricúspide

β_1AR: Receptor beta1-adrenérgico

β_{1H}AR: Sitio de alta afinidad del receptor beta1-adrenérgico

β_{1L}AR: Sitio de baja afinidad del receptor beta1-adrenérgico

β_2AR: Receptor beta2-adrenérgico

β_3AR: Receptor beta3-adrenérgico

βAR: Receptor beta-adrenérgico

ÍNDICE

1. Compendio de publicaciones

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

British Journal of Pharmacology (2008) 153, 710–720
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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

A Galindo-Tovar and AJ Kaumann

Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK

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.

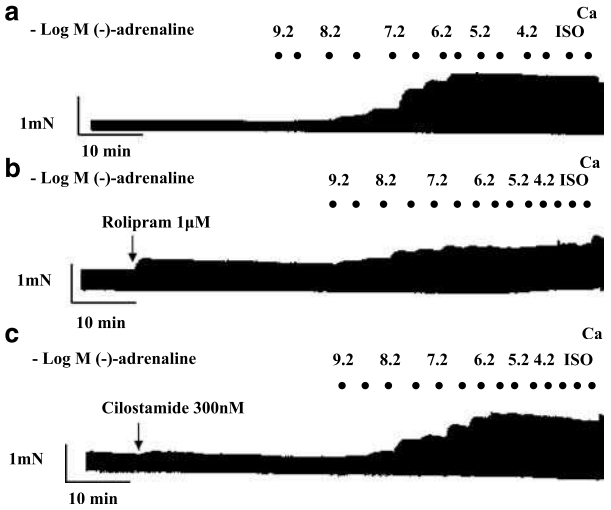

Figure 1 Potentiation of the effects of (−)-adrenaline by rolipram but not by cilostamide on left atrium. Representative experiments, depicting cumulative concentration–effect curves for (−)-adrenaline in the absence of PDE inhibitors (**a**), in the presence of rolipram (**b**) and in the presence of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

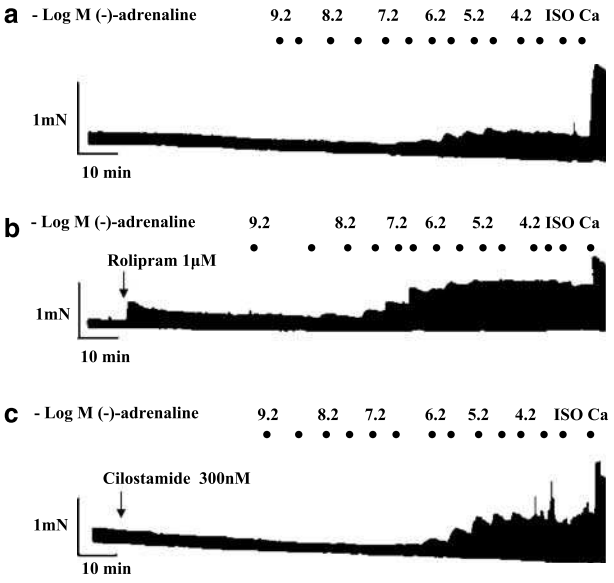

Figure 2 Positive inotropic and arrhythmic effects of (−)-adrenaline on right ventricular walls; influence of PDE inhibitors. Representative experiments, depicting cumulative concentration–effect curves for (−)-adrenaline in the absence of PDE inhibitors (**a**), presence of rolipram (**b**) and presence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

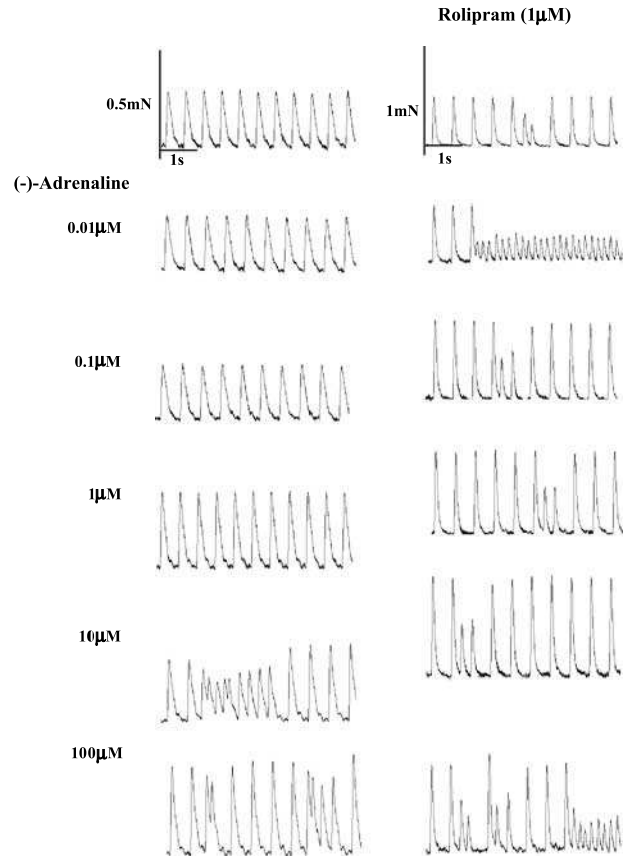

Figure 3 Rolipram potentiates the positive inotropic and arrhythmic effects of (−)-adrenaline on murine right ventricular wall. Comparison of the effects of increasing (−)-adrenaline concentrations on a right ventricular wall in the absence (left hand panels) and another right ventricular wall in the 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

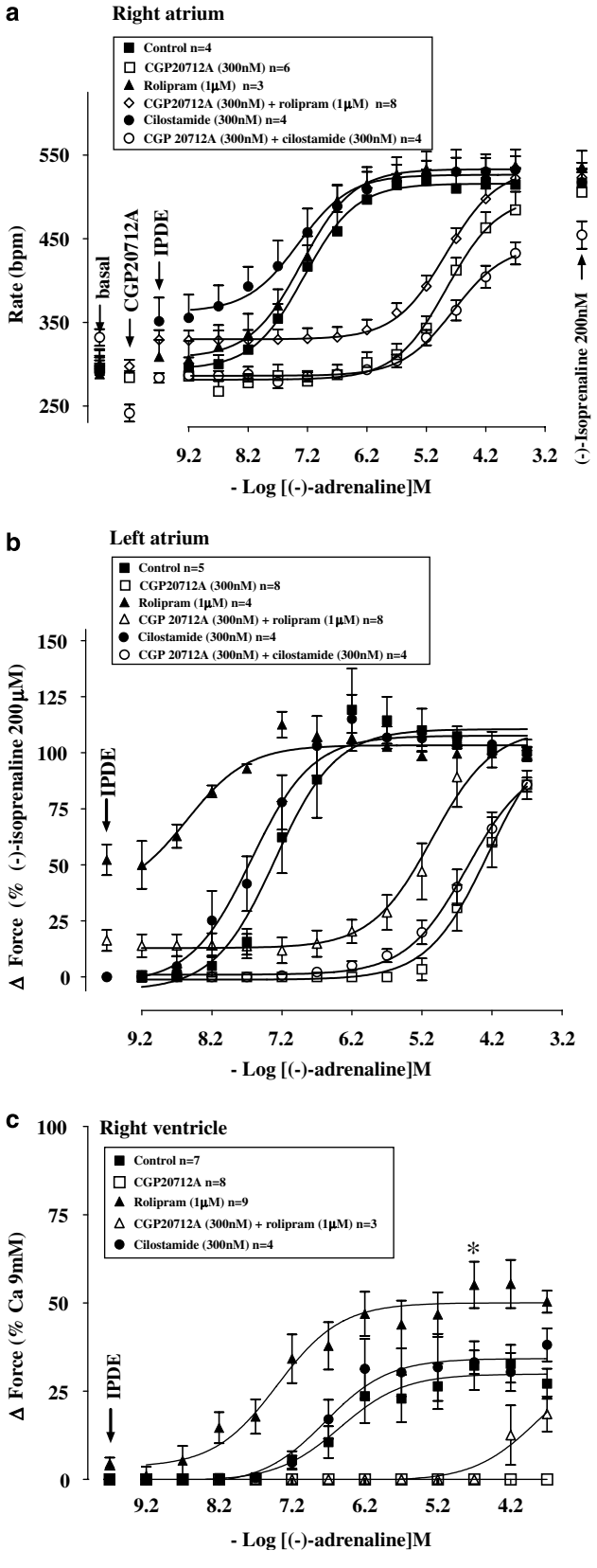

Figure 4 Potentiation of the effects of (−)-adrenaline by rolipram, mediated through β_1-adrenoceptors, on left atria (**b**) and right ventricular walls (**c**), but not on sinoatrial pacemaker (**a**) in the absence and presence of CGP20712A. Lack of effect of cilostamide on (−)-adrenaline potency. 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 (*$P < 0.03$).

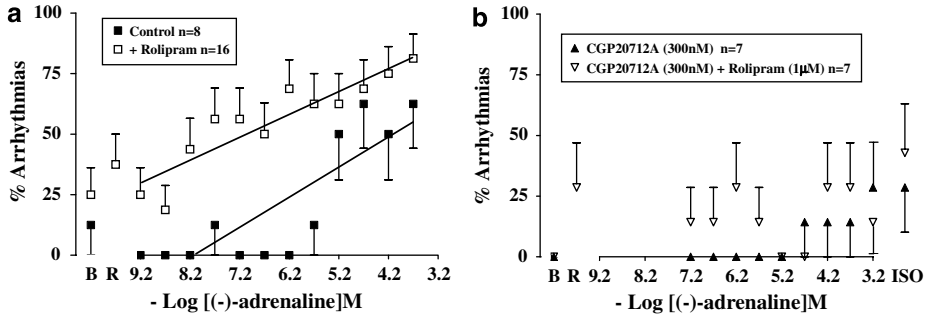

Figure 5 Increased incidence of (−)-adrenaline-evoked arrhythmias by rolipram (**a**) and blockade by CGP20712A (**b**). %Arrythmias is the % of ventricles that showed extrasystoles and/or ventricular tachycardia at each (−)-adrenaline concentration. Lines depict the dependence of arrhythmias on −log(−)-adrenaline 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

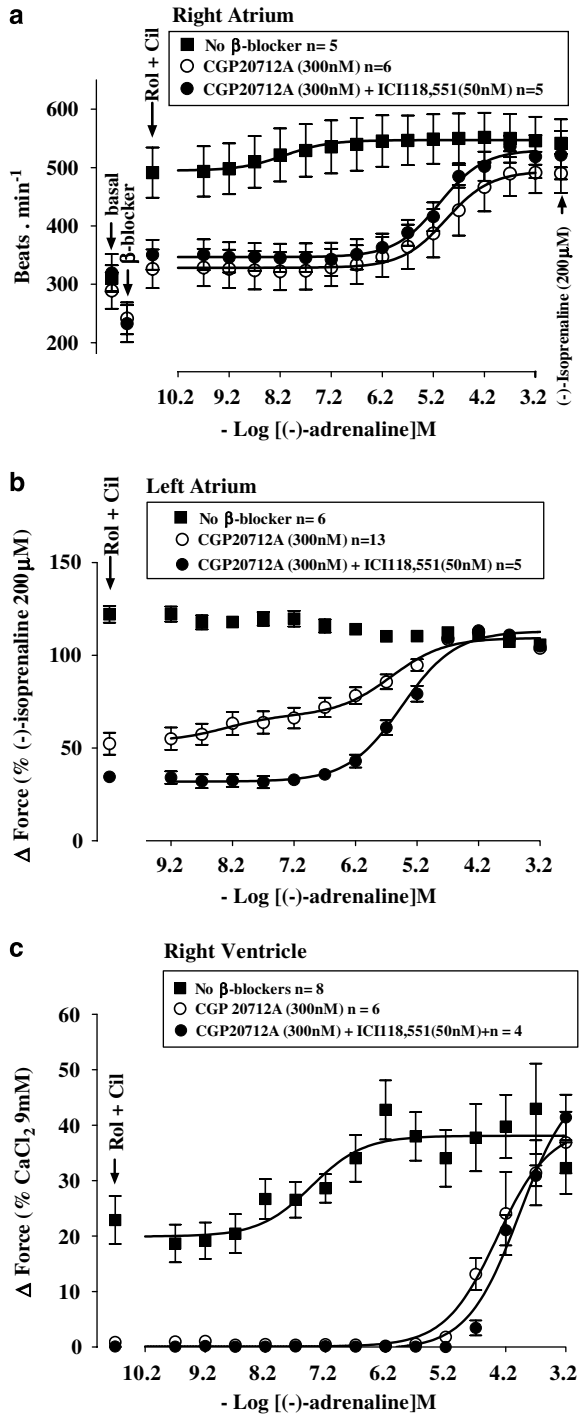

Figure 6 Cilostamide (Cil) and rolipram (Rol), administered together, potentiate the chronotropic effects of (−)-adrenaline (**a**) and uncover β_2AR-mediated inotropic effects in the presence of CGP20712A, sensitive to blockade by ICI118551, in left atrium (**b**) but not in right ventricle (**c**). 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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1.2 Inotropy and L-type Ca2+ current, activated by β_1- and β_2-adrenoceptors, are differently controlled by phosphodiesterases 3 and 4 in rat heart.

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Inotropy and L-type Ca^{2+} current, activated by β_1- and β_2-adrenoceptors, are differently controlled by phosphodiesterases 3 and 4 in rat heart

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Background and purpose: β_1- and β_2-adrenoceptors coexist in rat heart but β_2-adrenoceptor-mediated inotropic effects are hardly detectable, possibly due to phosphodiesterase (PDE) activity. We investigated the influence of the PDE3 inhibitor cilostamide (300 nmol·L^{-1}) and the PDE4 inhibitor rolipram (1 μmol·L^{-1}) on the effects of (–)-catecholamines.
Experimental approach: Cardiostimulation evoked by (–)-noradrenaline (ICI118551 present) and (–)-adrenaline (CGP20712A present) through β_1- and β_2-adrenoceptors, respectively, was compared on sinoatrial beating rate, left atrial and ventricular contractile force in isolated tissues from Wistar rats. L-type Ca^{2+}-current ($I_{Ca\text{-}L}$) was assessed with whole-cell patch clamp.
Key results: Rolipram caused sinoatrial tachycardia. Cilostamide and rolipram did not enhance chronotropic potencies of (–)-noradrenaline and (–)-adrenaline. Rolipram but not cilostamide potentiated atrial and ventricular inotropic effects of (–)-noradrenaline. Cilostamide potentiated the ventricular effects of (–)-adrenaline but not of (–)-noradrenaline. Concurrent cilostamide + rolipram uncovered left atrial effects of (–)-adrenaline. Both rolipram and cilostamide augmented the (–)-noradrenaline (1 μmol·L^{-1}) evoked increase in $I_{Ca\text{-}L}$. (–)-Adrenaline (10 μmol·L^{-1}) increased $I_{Ca\text{-}L}$ only in the presence of cilostamide but not rolipram.
Conclusions and implications: PDE4 blunts the β_1-adrenoceptor-mediated inotropic effects. PDE4 reduces basal sinoatrial rate in a compartment distinct from compartments controlled by β_1- and β_2-adrenoceptors. PDE3 and PDE4 jointly prevent left atrial β_2-adrenoceptor-mediated inotropy. Both PDE3 and PDE4 reduce $I_{Ca\text{-}L}$ responses through β_1-adrenoceptors but the PDE3 component is unrelated to inotropy. PDE3 blunts both ventricular inotropic and $I_{Ca\text{-}L}$ responses through β_2-adrenoceptors.
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Keywords: phosphodiesterases; rat atrium; sinoatrial node; ventricle; β_1- and β_2-adrenoceptors; (–)-adrenaline; (–)-noradrenaline; calcium current

Abbreviations: $I_{Ca\text{-}L}$, (L-type calcium current); CGP20712A, (2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide); EHNA, Erythro-9-[2-Hydroxy-3-nonyl]adenine; IBMX, 3-isobutyl-1-methylxanthine; ICI118551, (1-[2,3-dihydro-7-methyl-1H-inden-4-yl)oxy-3-[(1-methylethyl)amino]-2-butanol); PDE, phosphodiesterase

Introduction

Activation of the sympathetic nervous system causes cardiostimulation through release of catecholamines. (–)-Noradrenaline and (–)-adrenaline increase rate and force of the mammalian heart through coexisting β_1- and β_2-adrenoceptors (receptor nomenclature follows Alexander *et al.*, 2008) coupled to cAMP-dependent pathways. However, access of cAMP to effectors is different for cardiac β_1-adrenoceptors and β_2-adrenoceptors (Xiao *et al.*, 1995; Kuschel *et al.*, 1999) possibly due in part to involvement of different phosphodiesterases (PDEs). Hydrolysis of cAMP by PDEs protects the heart against overstimulation by sympathetic nerves but there are differences between β-adrenoceptor subtypes. Several PDE isoenzymes modulate catecholamine-evoked cardiostimulation (Fischmeister *et al.*, 2006; Nikolaev *et al.*, 2006). In the rat ventricle, PDE activity is mostly due to PDE3 and PDE4 (Mongillo *et al.*, 2004; Rochais *et al.*, 2004; Rochais *et al.*, 2006). Rochais *et al.* (2006)

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investigated the effects of PDE inhibitors on the relationship between (–)-isoprenaline-evoked increases of subsarcolemmal cAMP (monitored from cyclic nucleotide-gated channels used as biosensors) and L-type Ca^{2+} current, $I_{Ca\text{-}L}$, mediated through β_1- and β_2-adrenoceptors of rat ventricular myocytes. (–)-Isoprenaline increased myocytic cAMP through both β_1- and β_2-adrenoceptors and these effects were markedly potentiated by the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX). However, (–)-isoprenaline increased subsarcolemmal cAMP only through β_1- but not β_2-adrenoceptors. Inhibition of PDE3 or PDE4 caused robust enhancement of the β_1AR-mediated subsarcolemmal cAMP increase. Although inhibition of either PDE3 or PDE4 uncovers transient subsarcolemmal cAMP increases through β_2-adrenoceptors, only the concomitant inhibition of PDE3 and PDE4 caused stable increases of cAMP through these receptors. Comparable results were reported with $I_{Ca\text{-}L}$ measurements. (–)-Isoprenaline-evoked increases in $I_{Ca\text{-}L}$ through β_1- or β_2-adrenoceptors are enhanced by inhibition of PDE3 or PDE4. Taken together, the work of Rochais *et al.* (2006) illustrates differences and similarities of PDE-evoked modulation of the function of β_1- and β_2-adrenoceptors in a microdomain of rat ventricular cell membranes. How do these β_1- and β_2-adrenoceptor-mediated events in the membrane microdomain translate into increased ventricular contractility? How do PDEs modulate β_1- and β_2 adrenoceptor activity in non-ventricular cardiac regions of the rat?

Although β_1- and β_2-adrenoceptors coexist in the sinoatrial node (Saito *et al.*, 1989), left atrium (Juberg *et al.*, 1985) and ventricles (Minneman *et al.*, 1979) of the rat heart, the cardiostimulant function of β_1- but not β_2-adrenoceptors is well accepted. There is no evidence for an inotropic function of β_2-adrenoceptors in left atrium (Juberg *et al.*, 1985) and (–)-adrenaline only causes modest tachycardia through rat sinoatrial β_2-adrenoceptors, compared with β_1-adrenoceptor-mediated tachycardia (Kaumann, 1986). The situation in ventricle is particularly complex, apparently because coupling of rat β_2-adrenoceptors to *Pertussis* toxin (PTX)-sensitive G_i protein was reported to prevent G_s protein-mediated increases in Ca^{2+} transients and myocyte contractions and relaxations through these receptors (Xiao *et al.*, 1995). However, others failed to detect β_2-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ and Ca^{2+} transients after PTX pre-treatment in rat ventricular myocytes (LaFlamme and Becker, 1998). Another possible reason for the subdued functional expression of rat cardiac β_2-adrenoceptor activity could be a nearly complete hydrolysis of otherwise cardiostimulant cAMP by PDEs, particularly PDE3 and PDE4.

We therefore sought to investigate the β_2-adrenoceptor-mediated effects of (–)-adrenaline on sinoatrial node, left atrium and ventricle under conditions of inhibition of PDE3 and PDE4 with cilostamide and rolipram, respectively, and compare the influence of the PDE inhibitors on the effects of (–)-noradrenaline, mediated through β_1-adrenoceptors (Vargas *et al.*, 2006). To better understand the link between PDE-controlled cAMP in the $I_{Ca\text{-}L}$ domain and contractility events, the influence of the effects of cilostamide and rolipram on β_1-adrenoceptors- and β_2-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ was compared in ventricular and atrial myocytes.

Methods

Isolated tissue experiments

Rats were killed following protocols approved by the Regierungspräsident Dresden, (permit number: 24D-9168.24-1/2007-17), in accordance with the guidelines of the European Community. Male Wistar rats (10–12 weeks old, ~200 g weight) were anaesthetized with O_2 30%/CO_2 70% and killed with pure CO_2. The hearts were dissected and placed in oxygenated, modified Tyrode's solution at room temperature containing ($mmol \cdot L^{-1}$): NaCl 126.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 22, NaH_2PO_4 0.45, 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 as well as left ventricular papillary muscles were rapidly dissected, mounted in pairs and attached to Swema 4–45 strain gauge transducers in an apparatus containing the modified Tyrode's solution at 37°C. Left atria, right ventricular walls and left ventricular papillary muscles were paced at 1 Hz and stretched as described (Kaumann and Molenaar, 1996; Oostendorp and Kaumann, 2000; Vargas *et al.*, 2006). 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 ($5\ \mu mol \cdot L^{-1}$) for 90 min followed by washout, to irreversibly block α-adrenoceptors and tissue uptake of the catecholamines (Gille *et al.*, 1985; Heubach *et al.*, 2002). Experiments with (–)-noradrenaline were carried out in the presence of ICI118551 ($50\ nmol \cdot L^{-1}$) to block β_2-adrenoceptors. Experiments with (–)-adrenaline were carried out in the presence of CGP20712A ($300\ nmol \cdot L^{-1}$) to selectively block β_1-adrenoceptors and conceivably uncover CGP20712A-resistant effects, mediated through β_2-adrenoceptors (Kaumann, 1986; Oostendorp and Kaumann, 2000; Heubach *et al.*, 2002). To corroborate that CGP20712A-resistant effects of (–)-adrenaline were mediated through β_2-adrenoceptors, the β_2-adrenoceptor-selective antagonist ICI118551 ($50\ nmol \cdot L^{-1}$) (Kaumann, 1986; Oostendorp and Kaumann, 2000) was used in the presence of CGP20712A.

Cumulative concentration-effect curves for the catecholamines were carried out in the absence and presence of the PDE3 inhibitor cilostamide ($300\ nmol \cdot L^{-1}$) or PDE4 inhibitor rolipram ($1\ \mu mol \cdot L^{-1}$) (Vargas *et al.*, 2006), followed by the administration of a saturating concentration of (–)-isoprenaline ($200\ \mu mol \cdot L^{-1}$). With $300\ nmol \cdot L^{-1}$ cilostamide approximately 86% of PDE3 and <0.4% of PDE4 would be inhibited; with $1\ \mu mol \cdot L^{-1}$ rolipram approximately 50% of PDE4 and 0.4% of PDE3 would be inhibited (see Vargas *et al.*, 2006). For inotropic studies, the experiments were terminated by elevating the $CaCl_2$ concentration to $8\ mmol \cdot L^{-1}$. The (–)-catecholamines caused, on occasion, ventricular arrhythmias. Positive inotropic effects of (–)-catecholamines were only measured from non-arrhythmic ventricular preparations or during periods of stable non-arrhythmic contractions. Times to peak force and to half-maximal relaxation ($t_{1/2}$) were obtained fromfast speed tracings using ChartPro for Windows version 5.51 analysis programme (ADInstruments, Castle Hill, NSW, Australia).

Measurements of $I_{Ca\text{-}L}$

Ventricular and atrial myocytes of male rats were enzymatically dissociated as described earlier (Christ *et al.*, 2001). Myocytes were stored at room temperature until use in a solution containing (mmol·L^{-1}): NaCl 100, KCl 10, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 5, taurine 50, MOPS 5 and glucose 50, pH 7.4. The single electrode patch clamp technique was used to measure $I_{Ca\text{-}L}$ at 37°C (Christ *et al.*, 2006a). Holding potential was –80 mV. K^+ currents were blocked by replacing K^+ with Cs^+. The external perfusing solution contained (mmol·L^{-1}): tetraethylammonium 120, CsCl 10, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 20 with pH adjusted with CsOH. The pipette solution contained (mmol·L^{-1}): Cs methanesulphonate 90, CsCl 20, HEPES 10, Mg-ATP 4, Tris-GTP 0.4, EGTA 10 and $CaCl_2$ 3 with a calculated free Ca^{2+} concentration of 60 nmol·L^{-1} (EQCAL, Biosoft, Cambridge, UK) and pH 7.2, adjusted with CsOH. Current amplitude was determined as the difference between peak inward current and current at the end of the 200 ms depolarizing step to +10 mV. The effects of catecholamines on $I_{Ca\text{-}L}$ were expressed in per cent of control (Christ *et al.*, 2006a). To minimize the effects of desensitization, myocytes were exposed only to one concentration of catecholamine.

Statistics

–Log EC_{50}M values of the catecholamines were estimated from fitting a Hill function with variable slopes to concentration-effect curves of catecholamines from individual experiments. To decide whether a model of one or two receptor populations could be used to fit concentration-effect curves, we used the extra sum-off squares *F* test with $P < 0.05$ to reject the hypothesis of one receptor population. Data from tissue and myocyte experiments were expressed as mean ± SEM of *n* = number of mice or number of myocytes (from ≥3 rats) respectively. Significance of differences between means was assessed with paired and unpaired Student's *t*-test using GraphPad 5 Software Inc. (San Diego, CA).

Drugs

CGP20712A was from Novartis (Basel, Switzerland). ICI118551 was from Tocris (Bristol, UK); (–)-adrenaline, (–)-isoprenaline, rolipram, phenoxybenzamine, isobutylmethylxanthine (IBMX), Erythro-9-[2-Hydroxy-3-nonyl] adenine (EHNA), cilostamide and PTX were from Sigma (Poole Dorset, UK).

Results

Rolipram but not cilostamide increases sinoatrial rate

Mean beating rate was 234 ± 5 beats min^{-1} ($n = 52$) and 314 ± 9 beats min^{-1} ($n = 45$) in the presence of CGP20712A and ICI118551 respectively. CGP20712A caused bradycardia (Fig. 1C) but ICI118551 did not significantly change sinoatrial rate (Fig. 1A,B). An average decrease of 12 ± 5 beats min^{-1} by ICI118551 ($n = 45$ pooled data) was not significantly different from spontaneous rate decrease in time-matched controls (16 ± 3 beats min^{-1}, $n = 8$). The CGP20712A-evoked bradycardia (Fig. 1A) was also reported in mouse heart (Heubach *et al.*, 2002; Galindo-Tovar and Kaumann, 2008) and could be related to inverse agonism or blockade of β-adrenoceptors activated by traces of endogenously released noradrenaline. Cilostamide did not significantly modify sinoatrial beating rate in the presence of ICI118551 ($P = 0.26$, $n = 8$) or CGP20712A ($P = 0.29$, $n = 6$) (Fig. 1A,C). Rolipram increased sinoatrial rate by 37.3 ± 6.0% of the effect of 200 µmol·L^{-1} (–)-isoprenaline ($P < 0.01$, $n = 5$) and 24.4 ± 7.5% ($P = 0.035$, $n = 6$) in the presence of ICI118551 (Fig. 1A,B) or CGP20712A (Fig. 1C) respectively. The combination of cilostamide + rolipram increased beating rate by 59.8 ± 7.4% ($P < 0.002$, $n = 10$) and 43.9 ± 3.7% ($P < 0.001$, $n = 6$) in the presence of ICI118551 (Fig. 1A) and CGP20712A (Fig. 1C) respectively. The increase of sinoatrial rate by the combination of cilostamide + rolipram was significantly greater from that by rolipram alone in the presence of ICI118551 ($P < 0.04$) or CGP20712A ($P < 0.05$). IBMX (100 µmol·L^{-1}) in the presence of CGP20712A increased sinoatrial rate by 94 ± 2% of (–)-isoprenaline ($n = 4$, not shown), precluding analysis of experiments with (–)-adrenaline under these conditions.

Cilostamide and rolipram fail to affect the chronotropic potency of catecholamines at sinoatrial β_1- and β_2-adrenoceptors

Neither cilostamide nor rolipram, administered separately or in combination, or IBMX (10 µmol·L^{-1}) significantly altered the chronotropic potency of (–)-noradrenaline in the presence of ICI118551 (Fig. 1A, Table 1). (–)-Adrenaline in the presence of ICI118551 increased sinoatrial rate with $-\log EC_{50}$ = 7.11 ± 0.07 ($n = 6$) through β_1-adrenoceptors (Fig. 1B). Cilostamide did not affect the potency of (–)-adrenaline in the presence of ICI118551 ($-\log EC_{50}$ = 7.10 ± 0.10, Fig. 1B).

As described before (Kaumann, 1986), the concentration-effect curves of (–)-adrenaline were biphasic in the presence of CGP20712A with a high-potency, CGP20712A-resistant component and low-potency, CGP20712A-sensitive component. Low (–)-adrenaline concentrations produced a very small increase of sinoatrial rate, mediated through β_2-adrenoceptors, while high concentrations partially surmounted the blockade of β_1-adrenoceptors caused by CGP20712A (Fig. 1B,C, Table 1). The PDE inhibitors neither alone or in combination did not affect the potency of (–)-adrenaline for β_2-adrenoceptor-mediated chronotropic effects (Table 1). However, the fraction of the chronotropic effect mediated via β_2-adrenoceptors (f_2) was markedly enhanced by cilostamide, cilostamide + rolipram and IBMX (Fig. 1C, Table 1). The CGP20712A-resistant component f_2 was completely antagonized by ICI118551, as demonstrated for the combination of cilostamide and rolipram (Figs 1C and 2a), consistent with mediation through β_2-adrenoceptors.

Effects of PDE inhibitors on left atrial contractile force

Mean contractile force was 2.8 ± 0.3 mN ($n = 58$) and 5.9 ± 0.3 mN ($n = 44$) in the presence of CGP20712A and ICI118551 respectively. Cilostamide did not significantly enhance contractile force. Rolipram and IBMX (10 µmol·L^{-1}) increased contractile force in the presence of ICI118551 by 56.4 ± 7.9% ($P = 0.001$, $n = 7$) and 80.5 ± 3.5% ($P < 0.001$,

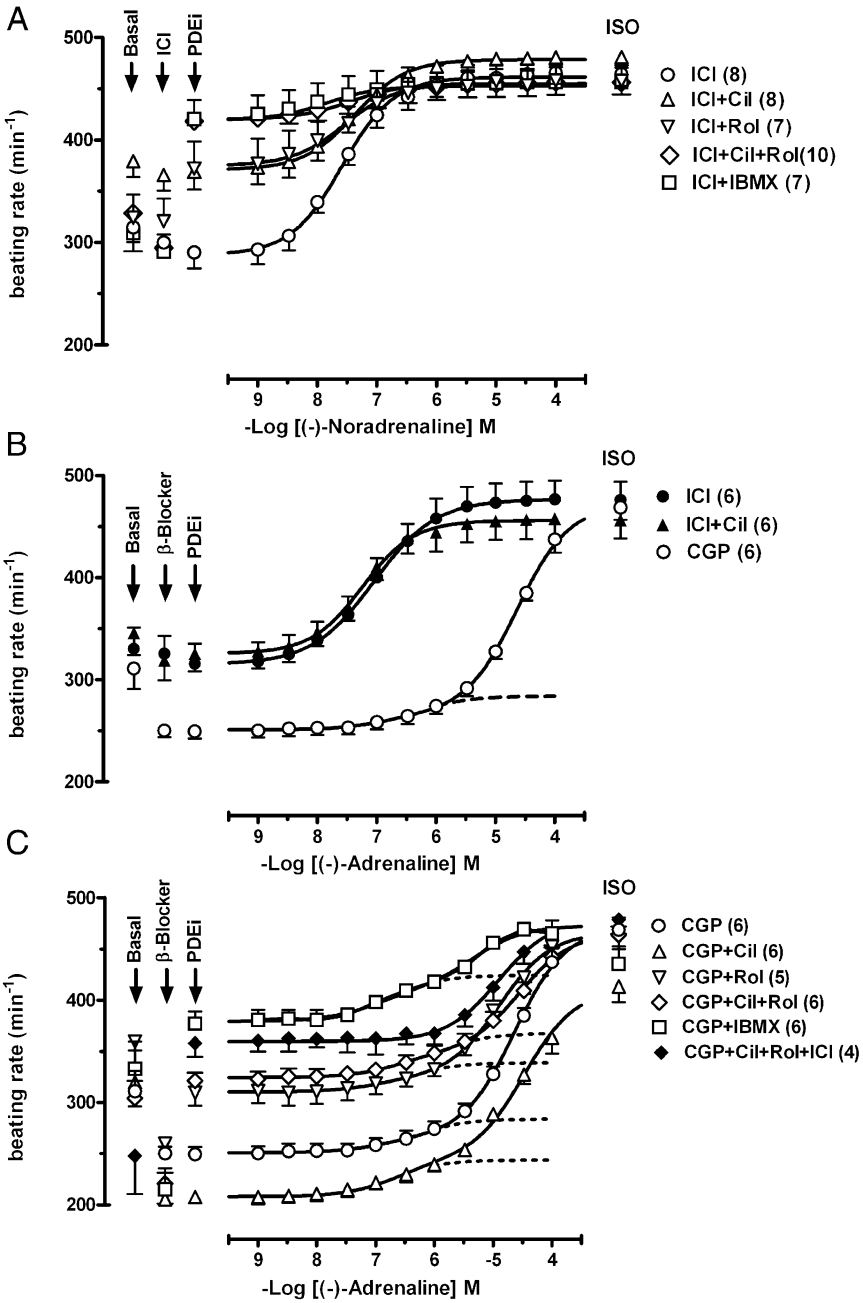

Figure 1 The influence of cilostamide (300 nmol·L^{-1}, Cil), rolipram (1 μmol·L^{-1}, Rol) and IBMX (10 μmol·L^{-1}) on the sinoatrial tachycardia elicited by (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors. (A) Lack of potentiation of the positive chronotropic effects of (–)-noradrenaline by PDE inhibitors in the presence of ICI118551 (50 nmol·L^{-1}, ICI). (B) Effects of (–)-adrenaline mediated through β_1-adrenoceptors in the presence of ICI118551 and through both β_1- and β_2-adrenoceptors in the presence of CGP20712A (300 nmol·L^{-1}, CGP). Lack of potentiation of the effects of (–)-adrenaline by cilostamide in the presence of ICI118551. (C) Lack of potentiation of the effects of (–)-adrenaline by cilostamide, rolipram and IBMX through β_2-adrenoceptors in the presence of CGP20712A. Blockade by ICI118551 of the β_2-adrenoceptor-mediated tachycardia of (–)-adrenaline in the presence of both cilostamide and rolipram (see also Fig. 2A). Fits of some biphasic curves were constrained by using the effect of (–)-isoprenaline as maximum. Broken lines in (B) and (C) depict a small β_2-adrenoceptor-mediated chronotropic component. Means ± SEM, numbers of right atria are given in parenthesis. ISO, (–)-isoprenaline (200 μmol·L^{-1}); PDEi, PDE inhibitor.

$n = 6$) of (–)-isoprenaline respectively (Fig. 3A). In the presence of CGP20712A rolipram, IBMX 10, 30 and 100 μmol·L^{-1} increased contractile force by 20.6 ± 6.0% ($P < 0.01$, $n = 8$), 23.6 ± 3.1% ($P < 0.02$, $n = 6$), 65.9 ± 5.5% ($P < 0.001$, $n = 8$) (Fig. 3C) and 108 ± 2% ($P < 0.001$, $n = 4$, not shown) respectively. The increases in contractile force by rolipram and IBMX (10 μmol·L^{-1}) in the presence of CGP20712A were significantly smaller ($P = 0.05$ and $P < 0.001$ respectively) than in the presence of ICI118551. The combination of cilostamide + rolipram increased contractile force significantly more ($P = 0.03$) in the presence of ICI118551 (105 ± 1% of (–)-isoprenaline, $P < 0.001$, $n = 8$, Fig. 3A) than in the presence of CGP20712A (61.2 ± 5.2%, $P < 0.001$, $n = 8$, Fig. 3C).

Table 1 Cardiostimulant potencies (–logEC$_{50}$)

(–)-Noradrenaline (ICI118551 50 nmol·L^{-1})		β_1		
	n		*–LogEC$_{50}$*	
Right atrium (sinus rate)				
Control	8		7.64 ± 0.07	
Rolipram	7		7.47 ± 0.13	
Cilostamide	8		7.43 ± 0.09	
Rolipram + cilostamide	10		7.51 ± 0.09	
IBMX (10 μmol·L^{-1})	7		7.73 ± 0.13	
Left atrium (contractile force)				
Control	9		7.69 ± 0.06	
Rolipram	7		8.75 ± 0.06**	
Cilostamide	8		7.89 ± 0.09	
Rolipram + cilostamide	8		not determined	
IBMX (10 μmol·L^{-1})	6		8.41 ± 0.12**	
Right ventricle (contractile force)				
Control	6		6.88 ± 0.02	
Rolipram	5		7.18 ± 0.10*	
Cilostamide	7		7.06 ± 0.13	
Rolipram + cilostamide	5		7.91 ± 0.04**	
IBMX (10 μmol·L^{-1})	8		7.81 ± 0.08**	
Left ventricular papillary muscle (contractile force)				
Control	6		6.79 ± 0.03	
Rolipram	6		7.06 ± 0.10*	
Cilostamide	10		6.91 ± 0.05	
Rolipram + cilostamide	6		7.32 ± 0.13**	
IBMX (10 μmol·L^{-1})	7		7.61 ± 0.10*	
(–)-Adrenaline (CGP20712A 300 nmol·L^{-1})	n	β_1 *–LogEC$_{50}$*	β_2 *–LogEC$_{50}$*	f_2
Right atrium (sinus rate)				
Control	6		6.68 ± 0.17	0.08 ± 0.02
Rolipram	5		6.67 ± 0.26	0.07 ± 0.03
Cilostamide	6		6.89 ± 0.14	0.16 ± 0.03*
Rolipram + cilostamide	6		7.01 ± 0.25	0.23 ± 0.07*
IBMX (10 μmol·L^{-1})	6		6.90 ± 0.13	0.30 ± 0.07*
Left atrium (contractile force)				
Control	8	4.27 ± 0.04		
Rolipram	8	5.10 ± 0.09**		
Cilostamide	6	4.62 ± 0.08**		
Rolipram + cilostamide	10	5.96 ± 0.11**	8.17 ± 0.08	0.26 ± 0.02
IBMX (10 μmol·L^{-1})	7	5.23 ± 0.09**		
IBMX (30 μmol·L^{-1})	5	5.53 ± 0.16**	7.02 ± 0.08	0.25 ± 0.09
Right ventricle (contractile force)				
Control	7		6.09 ± 0.08	0.09 ± 0.07
Rolipram	6		6.15 ± 0.18	0.07 ± 0.03
Cilostamide	6		6.48 ± 0.08*	0.15 ± 0.02*
Rolipram + cilostamide	8		6.83 ± 0.12*	0.92 ± 0.03**
IBMX (10 μmol·L^{-1})	16		6.62 ± 0.09*	0.84 ± 0.09**
IBMX (100 μmol·L^{-1})	4		6.81 ± 0.16	0.99 ± 0.01**
Left ventricular papillary muscle (contractile force)				
Control	4		6.05 ± 0.2	0.06 ± 0.03
Rolipram	4		6.06 ± 0.10	0.08 ± 0.04
Cilostamide	10		6.44 ± 0.11*	0.17 ± 0.05
Rolipram + cilostamide	5		6.84 ± 0.08**	0.98 ± 0.03**
IBMX (10 μmol·L^{-1})	7		6.54 ± 0.10*	0.82 ± 0.04**
IBMX (100 μmol·L^{-1})	4		6.91 ± 0.12*	1.01 ± 0.04**

P values compared with control.
*$P < 0.05$, **$P < 0.001$.

Rolipram and IBMX but not cilostamide potentiate the effects of (–)-noradrenaline through β_1-adrenoceptors on left atrium

Rolipram and IBMX potentiated the effects of (–)-noradrenaline in the presence of ICI118551 11.5-fold and fivefold respectively (Fig. 3A, Table 1). Cilostamide caused a small leftward shift of the concentration-effect curve for (–)-noradrenaline (Fig. 3A) but the difference in potency (Table 1) did not reach significance ($P = 0.078$). The maximum increase in contractility by combined cilostamide and rolipram prevented further effects of (–)-noradrenaline (Fig. 3A).

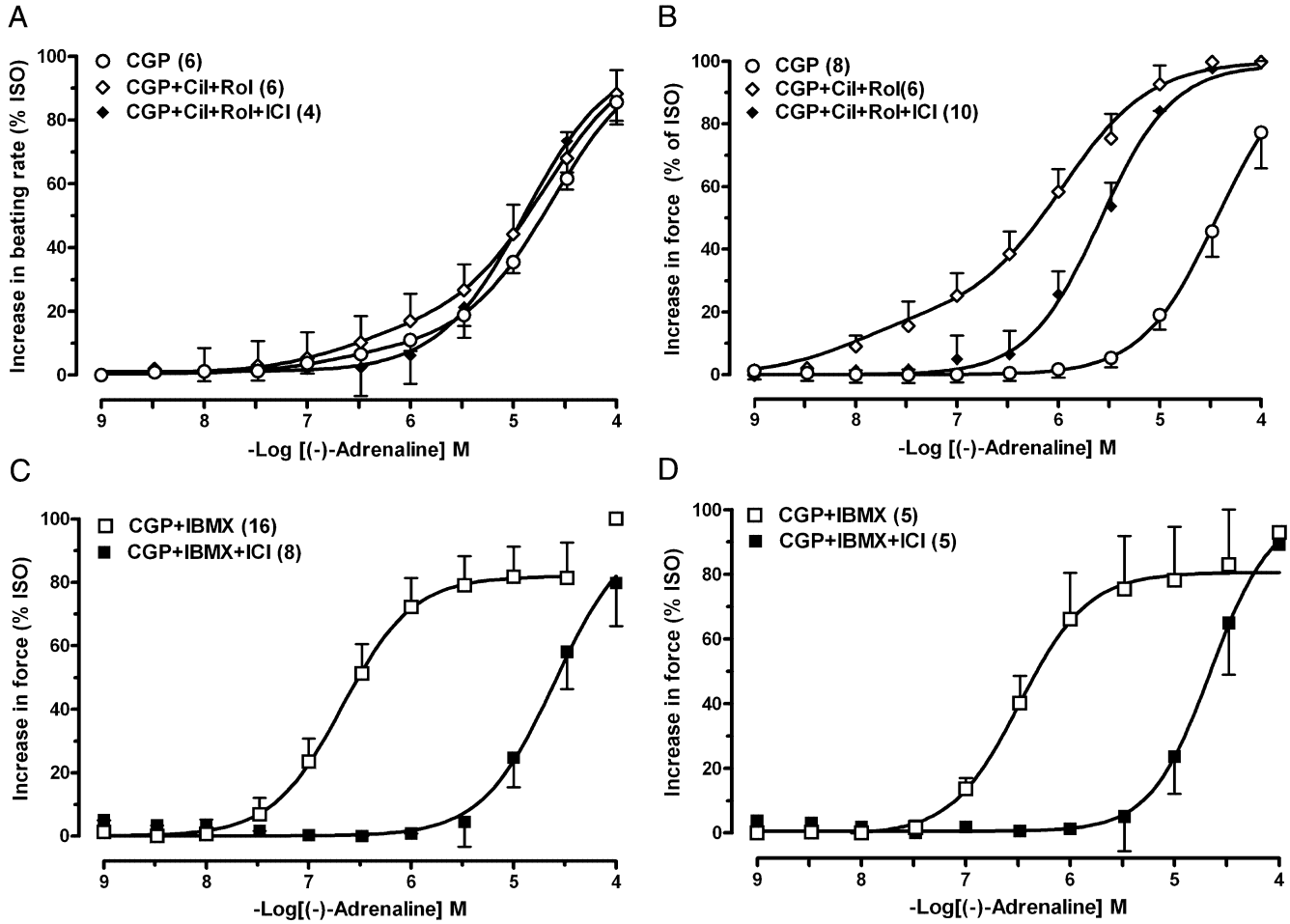

Figure 2 Comparison of the β_2-adrenoceptor-mediated effects of (–)-adrenaline in the presence of CGP20712A (CGP) and the inhibition of both PDE3 and PDE4 in four cardiac regions. Antagonism by ICI118551 (ICI). (A) Sinoatrial tachycardia. (B) Positive inotropic effects on left atrium. (C) Positive inotropic effects on free right ventricular wall. (D) Positive inotropic effects on left ventricular papillary muscle. For further details see Figure 1. Cil, cilostamide; Rol, rolipram.

Concurrent cilostamide + rolipram uncovers functional β_2-adrenoceptors in left atrium

CGP20712A caused a 2.8 log unit nearly surmountable rightward shift of the concentration-effect curve of (–)-adrenaline (Fig. 3B, Table 1), compared with the curve of (–)-adrenaline in the presence of ICI118551 ($-\log EC_{50} = 7.06 \pm 0.06$, $n = 4$) (Fig. 3B), consistent with mediation through β_1-adrenoceptors. CGP20712A-resistant components of the effects of (–)-adrenaline were not observed (Fig. 3B), suggesting absence of β_2-adrenoceptor-mediated effects.

Cilostamide and rolipram potentiated the effects of (–)-adrenaline at high concentrations in the presence of CGP20712A twofold and sevenfold respectively (Fig. 3C, Table 1). IBMX (10 μmol·L^{-1}) potentiated the effects of (–)-adrenaline ninefold (Fig. 3C, Table 1). Concurrent cilostamide + rolipram uncovered consistent biphasic concentration-effect curves for (–)-adrenaline with a high-sensitivity component H ($-\log EC_{50} = 8.2$, $f_2 = 0.26$) and low-sensitivity component L ($-\log EC_{50} = 6.0$, $f_1 = 0.74$) (Fig. 3C, Table 1). ICI118551, in the presence of CGP20712A, prevented the appearance of the high-affinity component of (–)-adrenaline (Figs 2B and 3C), consistent with mediation through β_2-adrenoceptors.

IBMX (10 μmol·L^{-1}) increased contractile force less than the combination of cilostamide and rolipram and failed to reveal a biphasic curve for (–)-adrenaline (data in Table 1). However, 30 μmol·L^{-1} IBMX markedly increased basal force and unconcealed biphasic curves for (–)-adrenaline (Fig. 3C, Table 1).

Rolipram but not cilostamide potentiates the right ventricular effects of (–)-noradrenaline through β_1-adrenoceptors

Mean contractile force was 1.9 ± 0.1 mN ($n = 35$) and 2.1 ± 0.1 mN ($n = 46$) in the presence of CGP20712A and ICI118551 respectively. Cilostamide, rolipram and IBMX (10 μmol·L^{-1}) failed to increase right ventricular contractile force in the presence of ICI118551 (Fig. 4A) or CGP20712A (Fig. 4C). The combination of cilostamide + rolipram caused a small increase of contractile force (14 ± 10%, $P < 0.05$) in the presence of ICI118551 (Fig. 4A) but not in the presence of CGP20712A (Fig. 4C). IBMX (100 μmol·L^{-1}) in the presence of CGP20712A increased ventricular force by 47 ± 14% ($n = 4$, not shown).

Cilostamide did not significantly change the inotropic potency of (–)-noradrenaline (Fig. 4A, Table 1). Rolipram

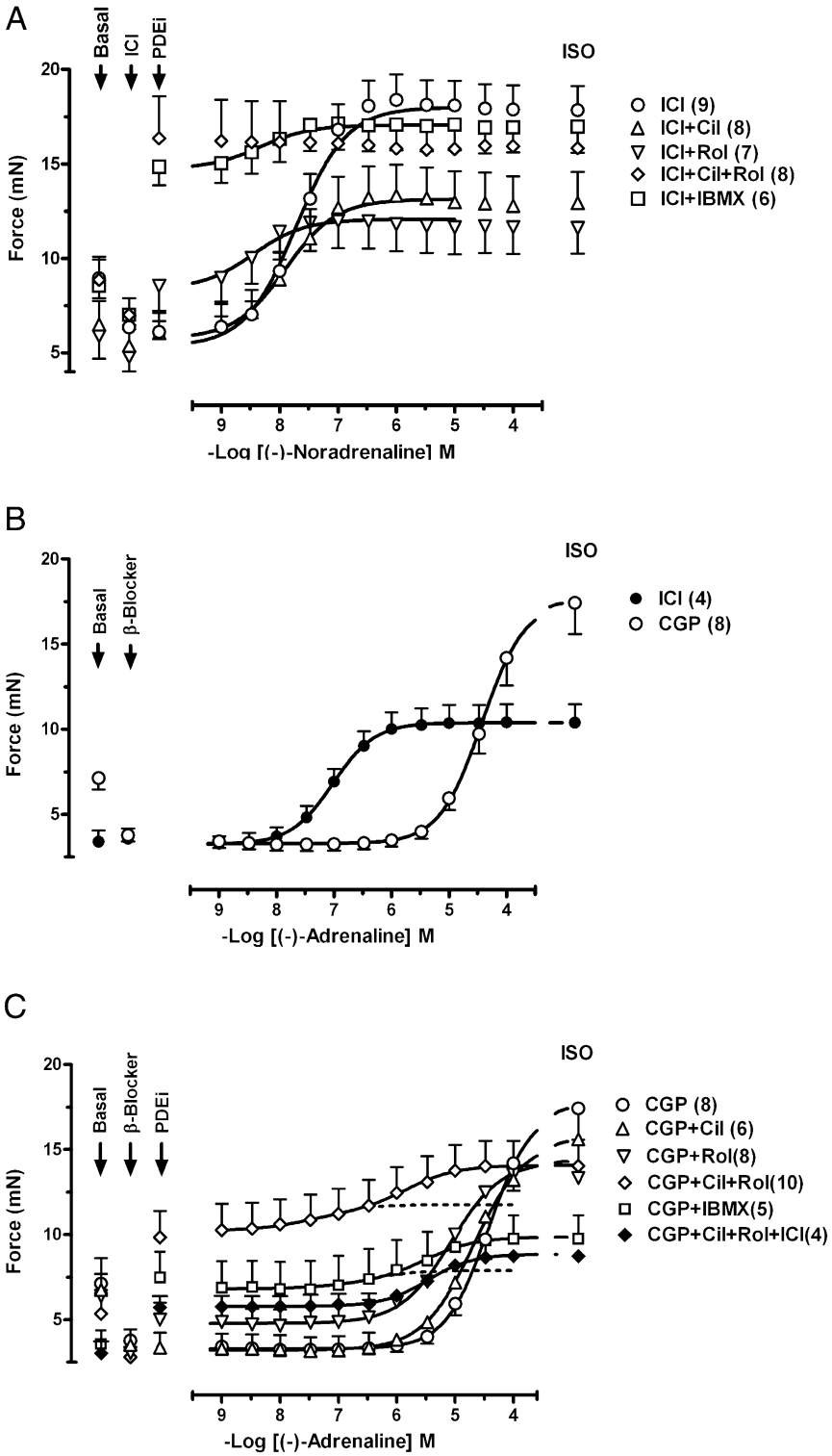

Figure 3 The influence of PDE inhibitors on the positive inotropic effects of (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors on left atrium. (A) Potentiation of the effects of (–)-noradrenaline by rolipram (Rol), concurrent rolipram + cilostamide and IBMX (10 $\mu mol \cdot L^{-1}$) but not by cilostamide (Cil) alone. (B) Effects of (–)-adrenaline mediated through β_1-adrenoceptors in the presence of ICI118551 (ICI) and antagonism by CGP20712A (CGP). (C) Unmasking of functional β_2-adrenoceptors by concurrent cilostamide + rolipram or IBMX (30 $\mu mol \cdot L^{-1}$) and antagonism by ICI118551. Fits of some biphasic curves were constrained by using the effect of (–)-isoprenaline as maximum. For further details see legend to Figure 1. Error bars are omitted in the range of curve overlap.

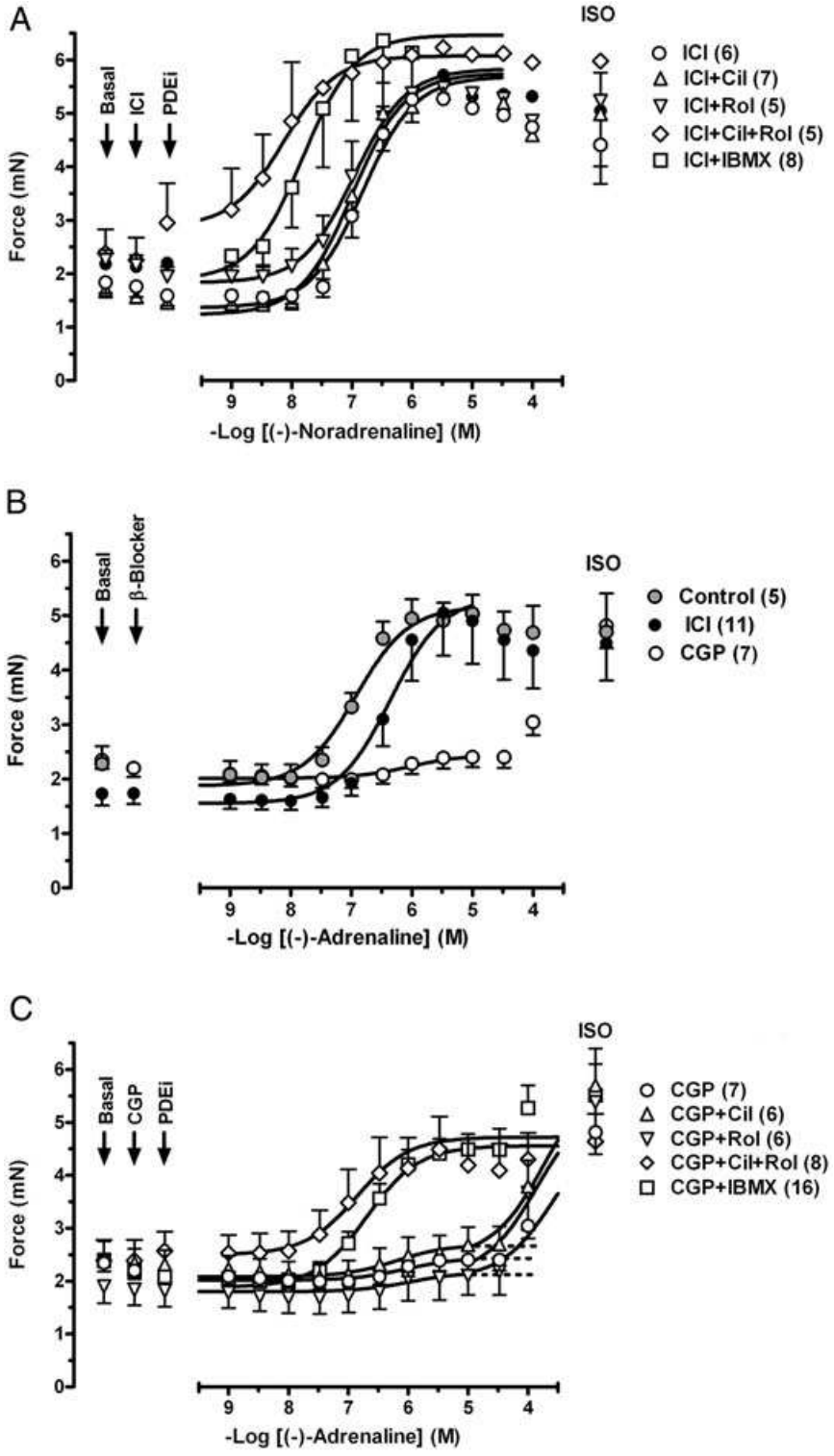

Figure 4 The influence of PDE inhibitors on the positive inotropic effects of (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors of the free right ventricular wall. (A) Potentiation of the positive inotropic effects of (–)-noradrenaline by rolipram (Rol), concurrent cilostamide + rolipram and IBMX but not by cilostamide (Cil) alone. (B) Effects of (–)-adrenaline mediated through both β_1-adrenoceptors and β_2-adrenoceptors in the absence of antagonists (controls), through β_1-adrenoceptors in the presence of ICI118551 (ICI) and through β_2-adrenoceptors in the presence of CGP20712A (CGP). (C) Potentiation of the effects of (–)-adrenaline through β_2-adrenoceptors by cilostamide, concurrent cilostamide + rolipram and IBMX but not by rolipram. Fits of some biphasic curves were constrained by using the effect of (–)-isoprenaline as maximum. For further details see Figure 1.

potentiated twofold the right ventricular effects of (–)-noradrenaline in the presence of ICI118551 (Fig. 4A, Table 1). The effects of (–)-noradrenaline were potentiated 8.5-fold by IBMX and 11-fold by concurrent cilostamide + rolipram (Fig. 4A). The potentiation of the effects of (–)-noradrenaline by IBMX and cilostamide + rolipram were both significantly greater than the potentiation by rolipram alone ($P < 0.001$ for both conditions).

Concurrent cilostamide and rolipram potentiate the right ventricular effects of (–)-adrenaline through β_2-adrenoceptors

(–)-Adrenaline increased right ventricular contractile force with $-\log EC_{50} = 6.93 \pm 0.05$, ($n = 5$) (Fig. 4B). ICI118551 caused a threefold rightward shift ($P < 0.001$) of the concentration-effect curve for (–)-adrenaline, suggesting involvement of a minor β_2-adrenoceptor-mediated component. In the presence of ICI118551, the $-\log EC_{50}$ was 6.40 ± 0.06 ($n = 11$) for the effects of (–)-adrenaline mediated through β_1-adrenoceptors (Fig. 4B). CGP20712A caused a nearly 3 log unit rightward and partially surmountable shift of the curve for (–)-adrenaline, compared with the curve in the presence of ICI118551, and revealed a small CGP20712A-resistant component with $f_2 = 0.09 \pm 0.02$ compared with (–)-isoprenaline (200 μmol·L^{-1}) (Figs 2C and 4B,C, Table 1). Cilostamide in the presence of CGP20712A significantly increased the potency and size (f_2) of the CGP20712A-resistant component (Fig. 4C, Table 1). Rolipram in the presence of CGP20712A did not significantly affect the effects of (–)-adrenaline compared with CGP20712A alone (Fig. 4C and Table 1). Rolipram combined with cilostamide caused a fivefold potentiation and markedly increased f_2 to 0.92 ± 0.03 ($n = 8$) of the CGP20712A-resistant component, precluding assessment of the CGP20712A-sensitive component (Fig. 4C, Table 1). IBMX (10 μmol·L^{-1}) in the presence of CGP20712A potentiated threefold the effects of (–)-adrenaline and markedly increased f_2 to 0.84 ± 0.09 ($n = 16$) of the CGP20712A-resistant component of the effects of (–)-adrenaline (Fig. 4C and Table 1). A total of IBMX (100) μmol·L^{-1} did not cause additional potentiation of the effects of (–)-adrenaline compared with 10 μmol·L^{-1} IBMX (Table 1). The effects of (–)-adrenaline in the presence of CGP20712A and IBMX were prevented by ICI118551 (Fig. 2C), consistent with mediation through β_2-adrenoceptors.

Rolipram but not cilostamide potentiates β_1-adrenoceptor-mediated effects of (–)-noradrenaline on left ventricular papillary muscle

Mean contractile force was 1.00 ± 0.06 mN ($n = 34$) and 1.13 ± 0.10 mN ($n = 31$) in the presence of CGP20712A and ICI118551, respectively, in left ventricular papillary muscles. In the presence of ICI118551 cilostamide and rolipram did not significantly change contractile force (Fig. 5A). Concurrent rolipram + cilostamide and IBMX (10 μmol·L^{-1}) tended to increase but these effects did not reach statistical significance (Fig. 5A). Cilostamide did not modify the potency of (–)-noradrenaline (Fig. 5A, Table 1). Rolipram, concurrent cilostamide + rolipram and IBMX (10 μmol·L^{-1}) potentiated twofold, sevenfold and tenfold the effects of (–)-noradrenaline, respectively, in the presence of ICI118551 (Fig. 5A, Table 1).

Cilostamide and concurrent cilostamide and rolipram, but not rolipram, potentiate β_2-adrenoceptor-mediated effects of (–)-adrenaline on left ventricular papillary muscles

In the presence of CGP20712A cilostamide, rolipram, IBMX (10 μmol·L^{-1}) and rolipram + cilostamide did not increase force (Fig. 5B). IBMX (100 μmol·L^{-1}) increased contractile force by $43 \pm 7\%$ of (–)-isoprenaline ($n = 4$, $P < 0.05$). (–)-Adrenaline in the presence of CGP20712A caused very small increases in contractility ($-\log EC_{50} = 6.0$), with $f_2 = 0.06 \pm 0.03$ ($n = 4$) compared with (–)-isoprenaline (Fig. 5B, Table 1). Cilostamide but not rolipram increased the CGP20712A-resistant component (Fig. 5B, Table 1). In the presence of IBMX and concurrent presence of cilostamide + rolipram the effects of (–)-adrenaline were potentiated three- and sixfold with f_2 values of 0.82 ± 0.04 ($n = 7$) and 0.98 ± 0.03 ($n = 5$), respectively, compared with (–)-isoprenaline (Fig. 5B, Table 1). A total of IBMX (100) μmol·L^{-1} did not cause additional potentiation of the effects of (–)-adrenaline compared with 10 μmol·L^{-1} IBMX (Table 1). The effects of (–)-adrenaline in the presence of CGP20712A and IBMX (10 μmol·L^{-1}) were prevented by ICI118551 (Fig. 2D), consistent with mediation through β_2-adrenoceptors.

Lusitropic effects of catecholamines. Cilostamide and rolipram cause (–)-adrenaline to hasten relaxation through β_2-adrenoceptors in left ventricular papillary muscles

To compare lusitropic effects of the catecholamines mediated through β_1- and β_2-adrenoceptors, we assessed the relaxation accompanying the approximately matching effects produced by (–)-noradrenaline (0.1 μmol·L^{-1}) and (–)-adrenaline (1 μmol·L^{-1}) in the presence of IBMX (10 μmol·L^{-1}) from the experiments of Figure 5. The positive lusitropic effects of these catecholamine concentrations in the absence and presence of cilostamide and rolipram were also assessed. Basal times to peak force and relaxation $t_{1/2}$ times were 51.5 ± 0.5 ms ($n = 71$) and 30.8 ± 0.4 ms ($n = 71$) respectively. (–)-Noradrenaline shortened both the times to peak force and relaxation $t_{1/2}$ (Figs 6 and 7). Cilostamide, rolipram and concurrent cilostamide + rolipram tended to enhance the relaxant effects of (–)-noradrenaline but none of the effects of the PDE inhibitors reached statistical significance. IBMX itself reduced both times to peak force and relaxation $t_{1/2}$ (Figs 6 and 7). (–)-Noradrenaline in the presence of IBMX caused significantly greater reductions of time to peak force and relaxation $t_{1/2}$ than in the absence of IBMX (Figs 6 and 7).

(–)-Adrenaline did not hasten relaxation (Figs 6 and 7). However, in the presence of cilostamide, rolipram and IBMX, (–)-adrenaline significantly reduced both times to peak force and relaxation $t_{1/2}$ (Figs 6 and 7).

(–)-Noradrenaline increases ventricular $I_{Ca\text{-}L}$ exclusively through β_1-adrenoceptors

Basal current density of $I_{Ca\text{-}L}$ in rat ventricular myocytes was 10.2 ± 0.2 pA pF^{-1} ($n = 495$). Representative experiments of

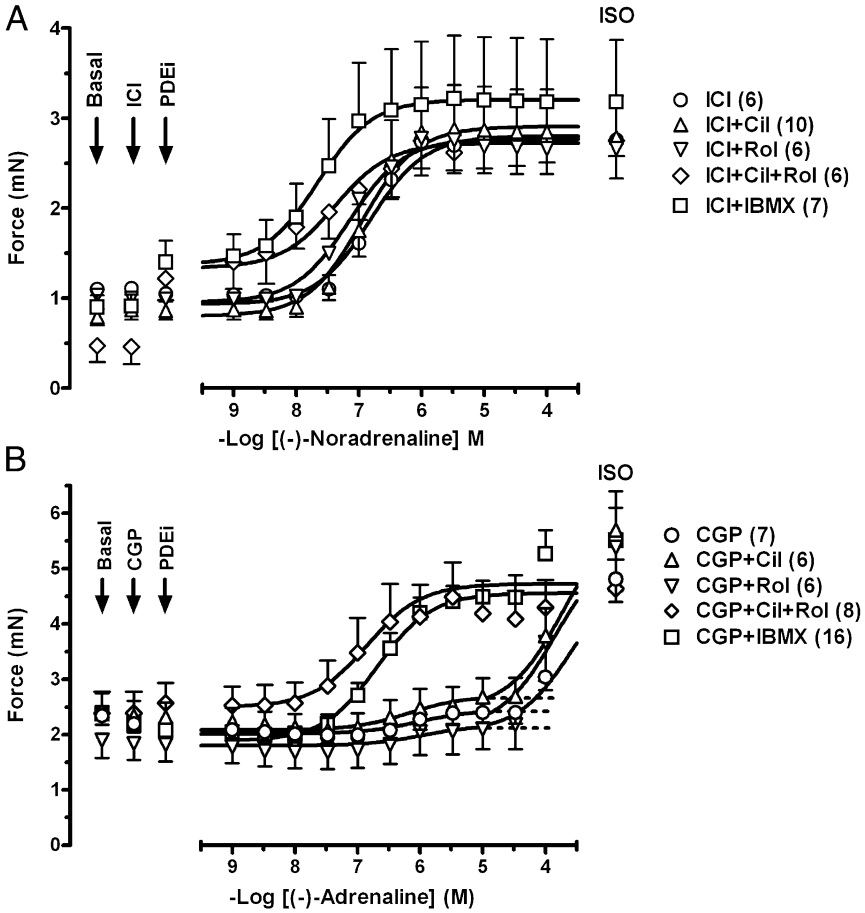

Figure 5 The influence of PDE inhibitors on the positive inotropic effects of (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors of left ventricular papillary muscles. (A) Potentiation of the positive inotropic effects of (–)-noradrenaline by rolipram (RoI), concurrent rolipram + cilostamide and IBMX but not by cilostamide (CiI) alone. (B) Potentiation of the effects of (–)-adrenaline by cilostamide, cilostamide + rolipram and IBMX but not rolipram in the presence of CGP20712A (CGP). Fits of some biphasic curves were constrained by using the effect of (–)-isoprenaline as maximum. For further details see Figure 1.

(–)-noradrenaline-evoked increases in $I_{Ca\text{-}L}$ and the effects of rolipram, cilostamide and IBMX are shown in Figure 8 (left hand panels). (–)-Noradrenaline increased $I_{Ca\text{-}L}$ in a concentration-dependent manner with $-\log EC_{50} = 5.95 \pm 0.15$ (Fig. 9A). The effects of (–)-noradrenaline were resistant to blockade by the β_2-adrenoceptor-selective antagonist ICI118551 but antagonized by the β_1-adrenoceptor-selective antagonist CGP20712A (Fig. 9A), consistent with exclusive mediation through β_1-adrenoceptors. Prazosin failed to alter the concentration-effect curve for (–)-noradrenaline, ruling out participation of α_1-adrenoceptors (Fig. 9A). The effects of (–)-isoprenaline on $I_{Ca\text{-}L}$ are shown for comparison in Fig. 9C. As found with (–)-noradrenaline, the effects of (–)-isoprenaline were antagonized by CGP20712A but not by ICI118551, consistent with mediation through β_1- but not through β_2-adrenoceptors. (–)-Isoprenaline ($-\log EC_{50} = 7.70 \pm 0.20$, Fig. 9C) was approximately 50-fold more potent than (–)-noradrenaline.

Both cilostamide and rolipram augment (–)-noradrenaline-evoked $I_{Ca\text{-}L}$ increases through ventricular β_1-adrenoceptors

The PDE inhibitors did not significantly modify basal $I_{Ca\text{-}L}$ in the presence of ICI118551 (Fig. 10C) or CGP20712A (Fig. 10D). (–)-Noradrenaline (1 μmol·L^{-1}) (ICI118551 present) caused an approximately half maximal increase of $I_{Ca\text{-}L}$ (Figs 9A,B and 10C). The responses to (–)-noradrenaline (1 μmol·L^{-1}) were significantly increased by cilostamide ($P < 0.05$), rolipram ($P < 0.01$) and the combination of cilostamide + rolipram ($P < 0.01$), consistent with a role of both PDE3 and PDE4 (Figs 8,9 and 10C). IBMX (10 μmol·L^{-1}) enhanced the (–)-noradrenaline response more than cilostamide ($P < 0.01$) but not significantly more than the responses to rolipram or concurrent cilostamide + rolipram (Figs 9B and 10C). The effects of 100 nmol·L^{-1} (–)-noradrenaline were only increased by IBMX and the combination of cilostamide + rolipram but not by cilostamide or rolipram alone (Fig. 9B).

Cilostamide and concurrent cilostamide + rolipram, but not rolipram alone, cause (–)-adrenaline-evoked increases of $I_{Ca\text{-}L}$ through ventricular β_2-adrenoceptors

(–)-Adrenaline (10 μmol·L^{-1}) (CGP20712A present) caused a marginal increase of $I_{Ca\text{-}L}$ which did not reach statistical significance (Figs 8 and 10D). In the presence of cilostamide, but not rolipram, (–)-adrenaline significantly ($P < 0.05$) increased $I_{Ca\text{-}L}$ (Figs 8 and 10D). In the presence of concurrent cilostamide + rolipram, (–)-adrenaline enhanced $I_{Ca\text{-}L}$ signifi-

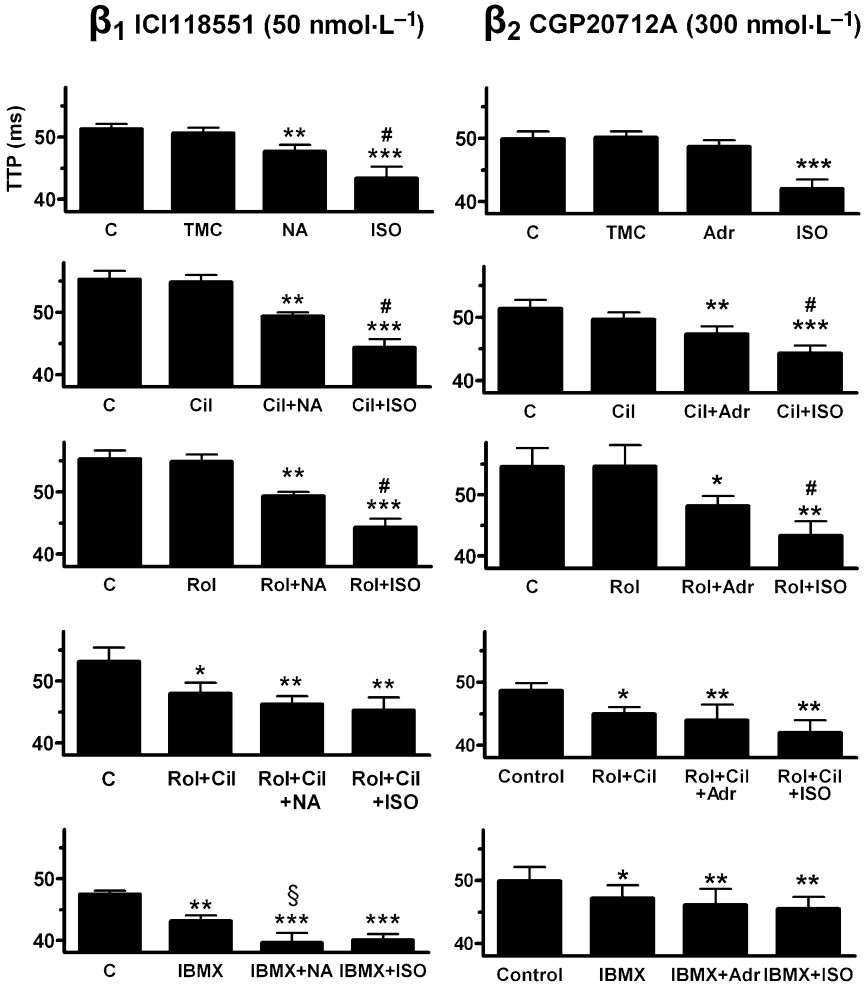

Figure 6 Influence of PDE inhibitors on shortening of time to peak force (TTP) by (–)-noradrenaline (100 nmol·L^{-1}, NA) through β_1-adrenoceptors (left hand panels) and (–)-adrenaline (1 μmol·L^{-1}, Adr) through β_2-adrenoceptors (right hand panels). Comparison with the responses to (–)-isoprenaline (100 μmol·L^{-1}, ISO). C, control; TMC, time-matched control; Cil, cilostamide (300 nmol·L^{-1}); Rol, rolipram (1 μmol·L^{-1}) and IBMX (10 μmol·L^{-1}). Data from the experiments on left ventricular papillary muscles (see Fig. 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control; #$P < 0.05$ vs. catecholamine + PDE inhibitor; §$P < 0.05$ vs. PDE inhibitor alone.

cantly more ($P < 0.01$) than in the presence of cilostamide alone (Figs 8 and 10D). ICI118551 (50 nmol·L^{-1}) nearly abolished the (–)-adrenaline-evoked increase in the presence of both CGP20712A and IBMX (10 μmol·L^{-1}) (Fig. 10D). The (–)-adrenaline-evoked increases of $I_{Ca\text{-}L}$ in the presence of IBMX and CGP20712A were 42 ± 16% ($n = 16$) and 6.5 ± 1% ($n = 8$) in the absence and presence of ICI118551, respectively ($P < 0.01$), consistent with exclusive mediation through β_2-adrenoceptors.

To investigate whether G_i inactivation facilitated β_2-adrenoceptor responses, myocytes were incubated at least 3 h with PTX 1.5 μg mL^{-1} at 37°C under constant agitation as described (Gong *et al.*, 2000). However, even after PTX treatment of ventricular myocytes 10 μmol·L^{-1} (–)-adrenaline in the presence 300 nmol·L^{-1} CGP20712A failed to increase $I_{Ca\text{-}L}$ ($n = 10$ cells, Fig. 8). In contrast, the PTX treatment nearly abolished the blunting effect of carbachol (10 μmol·L^{-1}) on the (–)-isoprenaline (1 μmol·L^{-1}) evoked increase in $I_{Ca\text{-}L}$ ($n = 7$ cells, not shown).

(–)-Noradrenaline increases atrial $I_{Ca\text{-}L}$ through β_1-adrenoceptors in the presence of IBMX

Basal current density of $I_{Ca\text{-}L}$ in rat atrial myocytes was 6.5 ± 0.3 pA pF^{-1} ($n = 95$). The PDE inhibitors did not significantly modify basal $I_{Ca\text{-}L}$ in the presence of ICI118551 (Fig. 10A). The response to (–)-noradrenaline (1 μmol·L^{-1}), a concentration 50-fold greater than the concentration causing half maximal inotropic effects (Fig. 3, Table 1), was studied in the absence and presence of PDE inhibitors (Fig. 10A). (–)-Noradrenaline produced a moderate increase in $I_{Ca\text{-}L}$ that was not significantly enhanced by EHNA, cilostamide, rolipram or concurrent rolipram + cilostamide, but markedly increased by both IBMX (10 μmol·L^{-1}) and the combination of cilostamide, rolipram and EHNA (Fig. 10A). The responses to 1 μmol·L^{-1} (–)-noradrenaline in the presence of IBMX or the combination of EHNA, cilostamide and rolipram (Fig. 10A) are not significantly different from the response to 10 μmol·L^{-1} (–)-noradrenaline which increased $I_{Ca\text{-}L}$ by 80 ± 11% ($n = 6$, not shown).

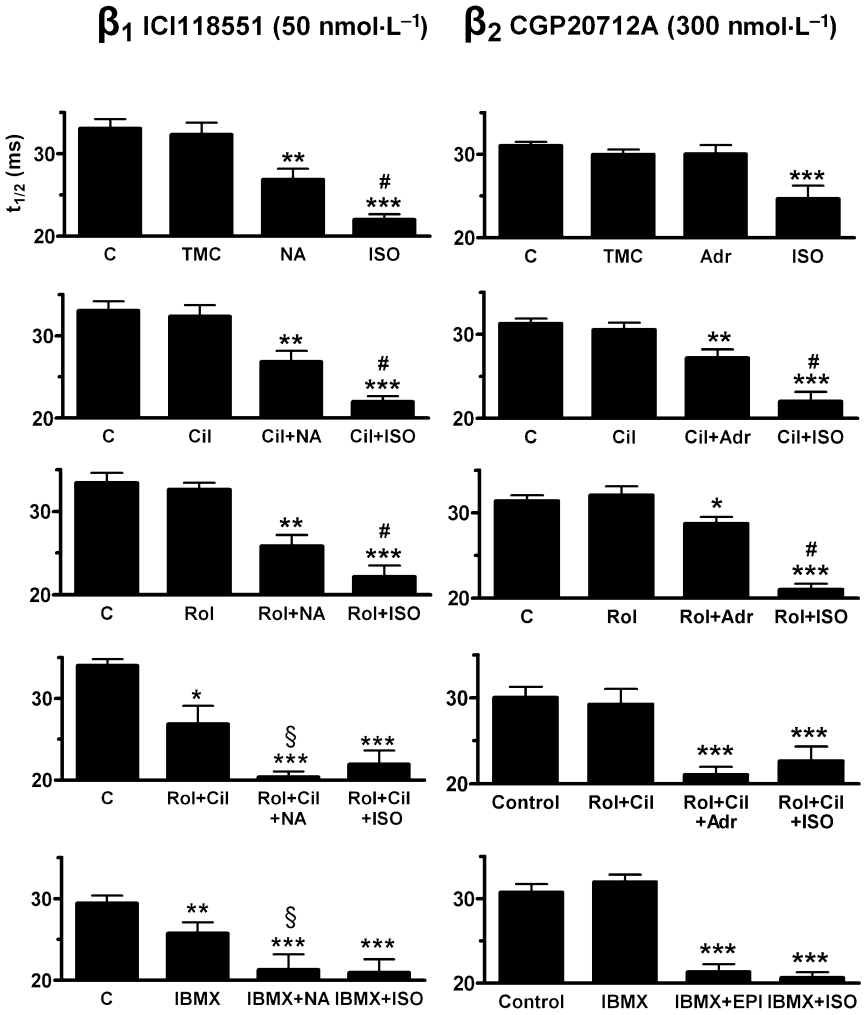

Figure 7 Influence of PDE inhibitors on the shortening of relaxation time ($t_{1/2}$) by (–)-noradrenaline (100 nmol·L^{-1}, NA) through β_1-adrenoceptors (left hand panels) and (–)-adrenaline (1 μmol·L^{-1}, Adr) through β_2-adrenoceptors (right hand panels). Comparison with the responses to (–)-isoprenaline (ISO). C, control; TMC, time-matched control; Cil, cilostamide (300 nmol·L^{-1}); Rol, rolipram (1 μmol·L^{-1}) and IBMX (10 μmol·L^{-1}). Data from the experiments on left ventricular papillary muscles (see Fig. 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control; #$P < 0.05$ vs. catecholamine + PDE inhibitor; §$P < 0.05$ vs. PDE inhibitor alone.

(–)-Adrenaline (10 μmol·L^{-1} in the presence of CGP20712A 300 nmol·L^{-1}) only produced marginal increases of $I_{Ca\text{-}L}$ which were not significantly affected by concurrent rolipram + cilostamide or IBMX (Fig. 10B).

Discussion

Our results point to region-specific and isoenzyme-dependent differences of the roles of PDE3 and PDE4 in reducing β_1- and β_2-adrenoceptor function in the rat heart. These conclusions are based on a range of findings. The sinoatrial tachycardia caused by (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors was not potentiated by cilostamide or rolipram, inconsistent with modulation by either PDE3 or PDE4. However, rolipram caused tachycardia, suggesting that PDE4 reduces basal sinoatrial beating rate by decreasing the PKA-controlled pacemaker rhythm, independently of β-adrenoceptor activation. In contrast, the positive inotropic effects to (–)-noradrenaline, mediated through atrial and ventricular β_1-adrenoceptors, were potentiated by rolipram, but not by cilostamide, suggesting hydrolysis of inotropically relevant cAMP by PDE4 but not by PDE3. Further, cilostamide but not rolipram potentiated the minor inotropic ventricular effects of (–)-adrenaline mediated through β_2-adrenoceptors, suggesting a blunting effect of PDE3. The combination of cilostamide + rolipram uncovered functional cardiostimulation mediated through β_2-adrenoceptors in left atrium and markedly potentiated the effects of (–)-adrenaline in right and left ventricular myocardium through β_2-adrenoceptors, suggesting that PDE3 and PDE4 act in concert to prevent manifestation of an inotropic function through β_2-adrenoceptors. Increases of ventricular $I_{Ca\text{-}L}$ through β_1-adrenoceptor activation were augmented by either cilostamide or rolipram, consistent with modulation by both PDE3 and PDE4, but inconsistent with the selective blunting by PDE4, but not PDE3, of the positive

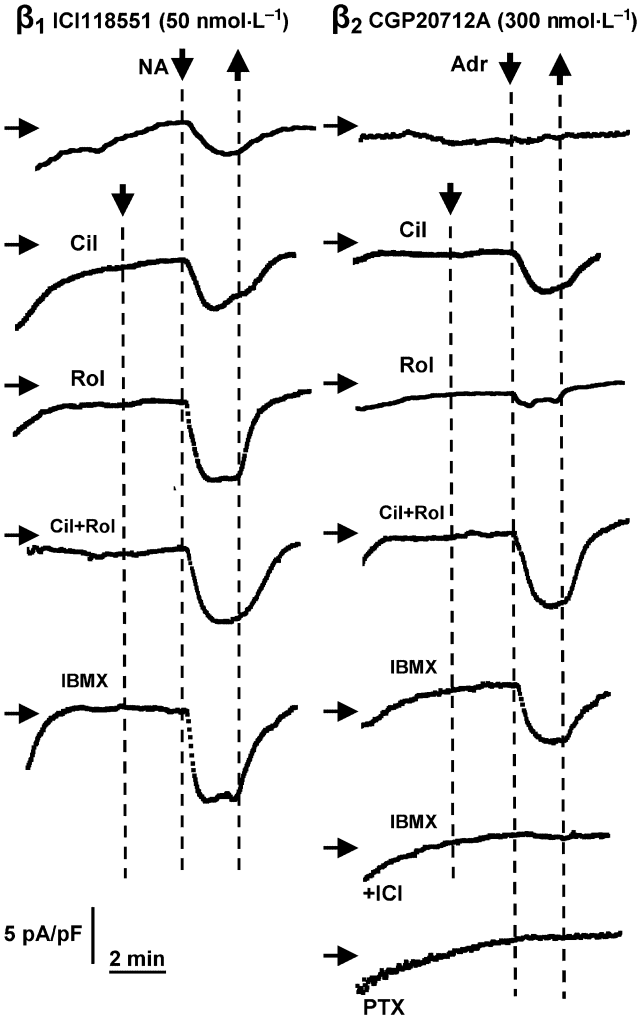

Figure 8 Augmentation by PDE inhibitors of increases in $I_{Ca\text{-}L}$ evoked by (–)-noradrenaline (NA) through β_1-adrenoceptors (ICI118551 present, left hand panels) and unmasking of (–)-adrenaline (Adr) responses mediated through β_2-adrenoceptors (CGP20712A present, right hand panels). Representative experiments of ventricular myocytes. Displayed are time courses of $I_{Ca\text{-}L}$ amplitudes measured in individual cells. Scaling is given in the left lower corner (same scaling for all graphs). Cilostamide (300 nmol·L^{-1}, Cil), alone or combined with rolipram (1 μmol·L^{-1}, Rol) and IBMX (10 μmol·L^{-1}) uncovered (–)-adrenaline-evoked increases in $I_{Ca\text{-}L}$ through β_2AR. Prevention of effects of (–)-adrenaline in the presence of IBMX by ICI118551 (50 nmol·L^{-1}, ICI). *Pertussis* toxin (PTX) did not uncover effects of (–)-adrenaline. Horizontal arrows indicate the level of 10 pA pF^{-1}; downward-pointing arrows and upward-pointing arrows indicate addition and washout of PDE inhibitors and catecholamines respectively.

inotropic effects of (–)-noradrenaline. Cilostamide but not rolipram facilitated ventricular $I_{Ca\text{-}L}$ activation through β_2-adrenoceptors, suggesting selective modulation by PDE3, consistent with the blunting effects of PDE3 of the positive inotropic effects of (–)-adrenaline. Our ventricular findings with β_2-adrenoceptor stimulation are consistent with an important control by PDE3 of cAMP generated in the subsarcolemmal space ($I_{Ca\text{-}L}$), but a more strict control of contractility by both PDE3 and PDE4 near the contractile machinery.

PDE4 modulates basal sinoatrial beating but not the tachycardia mediated with (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors

The basal beating rate of sinoatrial cells is tightly controlled by cAMP-dependent protein kinase (PK)-A phosphorylation of phospholamban and probably other PKA substrates (Vinogradova *et al*., 2006). Basal PDE activity is high in sinoatrial cells compared with ventricular myocytes (Vinogradova *et al*, 2005; 2007; 2008). The PDE3-selective inhibitor milrinone markedly increases beating rate and phospholamban phosphorylation in rabbit sinoatrial node cells, consistent with a control of basal heart rate by PDE3 (Vinogradova *et al*., 2007; 2008). Vinogradova *et al.* (2008) provided evidence that milrinone increased Ca^{2+} loading of the sarcoplasmic reticulum (SR), attributed to enhanced Ca^{2+} ATPase pumping expected from the phospholamban phosphorylation, leading to enhanced Ca^{2+} release from subsarcolemmal ryanodine RyR2 channels which in turn activated the Na^+/Ca^{2+} exchanger inward current that accelerated diastolic depolarization and sinoatrial beating rate.

In contrast to the results in rabbit (Vinogradova *et al*., 2008), in the rat, the PDE4 inhibitor rolipram but not the PDE3-selective cilostamide caused tachycardia. The rolipram-evoked tachycardia in the rat sinoatrial node persisted during β_1-adrenoceptor blockade with CGP20712A, ruling out sensitization by rolipram of β_1-adrenoceptor-mediated effects by traces of endogenously released noradrenaline. Our results with rolipram-evoked tachycardia and the failure of cilostamide to change sinoatrial rate are consistent with a direct reduction of the basal sinoatrial rate by PDE4 but not by PDE3 in the rat. The tachycardia caused by rolipram is likely to result from the inhibition of PDE4, followed by elevation of sinoatrial cAMP, increased PKA-catalysed phosphorylation of proteins leading to accelerated Ca^{2+}-cycling and enhanced beating rate. In addition, the increased sinoatrial cAMP in the presence of rolipram may directly open cyclic nucleotide-gated HCN channels responsible for the current activated by hyperpolarisation, I_f (DiFrancesco and Tortora, 1991), thereby contributing to tachycardia. However, the milrinone-evoked sinoatrial tachycardia, described by Vinogradova *et al.* (2008), was hardly reduced by I_f inhibition with Cs^+, so that a role for I_f appears unlikely.

Recent work shows that, in contrast to the selective reduction of sinoatrial beating rate by PDE3 in the rabbit proposed by Vinogradova *et al.* (2008) and PDE4 in the rat (this work), both PDE3 and PDE4 reduce murine basal sinoatrial rate (Galindo-Tovar and Kaumann, 2008). Unlike mouse and rat, in the dog the PDE4 inhibitor rolipram does not change sinoatrial rate but PDE3 inhibitors cause tachycardia (Heaslip *et al*., 1999). Thus, there are species differences as to which PDE isoforms reduce basal sinoatrial rate.

The IBMX (100 μmol·L^{-1}) caused greater tachycardia than IBMX (10 μmol·L^{-1}). Additional PDEs (PDE2?) may control basal sinoatrial rate, when PDE3 and PDE4 are inhibited. Alternatively, IBMX (100 μmol·L^{-1}) may have caused a more complete blockade of PDE4 than rolipram (1 μmol·L^{-1}).

Vinogradova *et al.* (2008) suggested that the PDE-dependent control of local Ca^{2+} release and basal spontaneous sinoatrial beating rate used the same mechanism as that used by β-adrenoceptor stimulation to accelerate sinoatrial rate

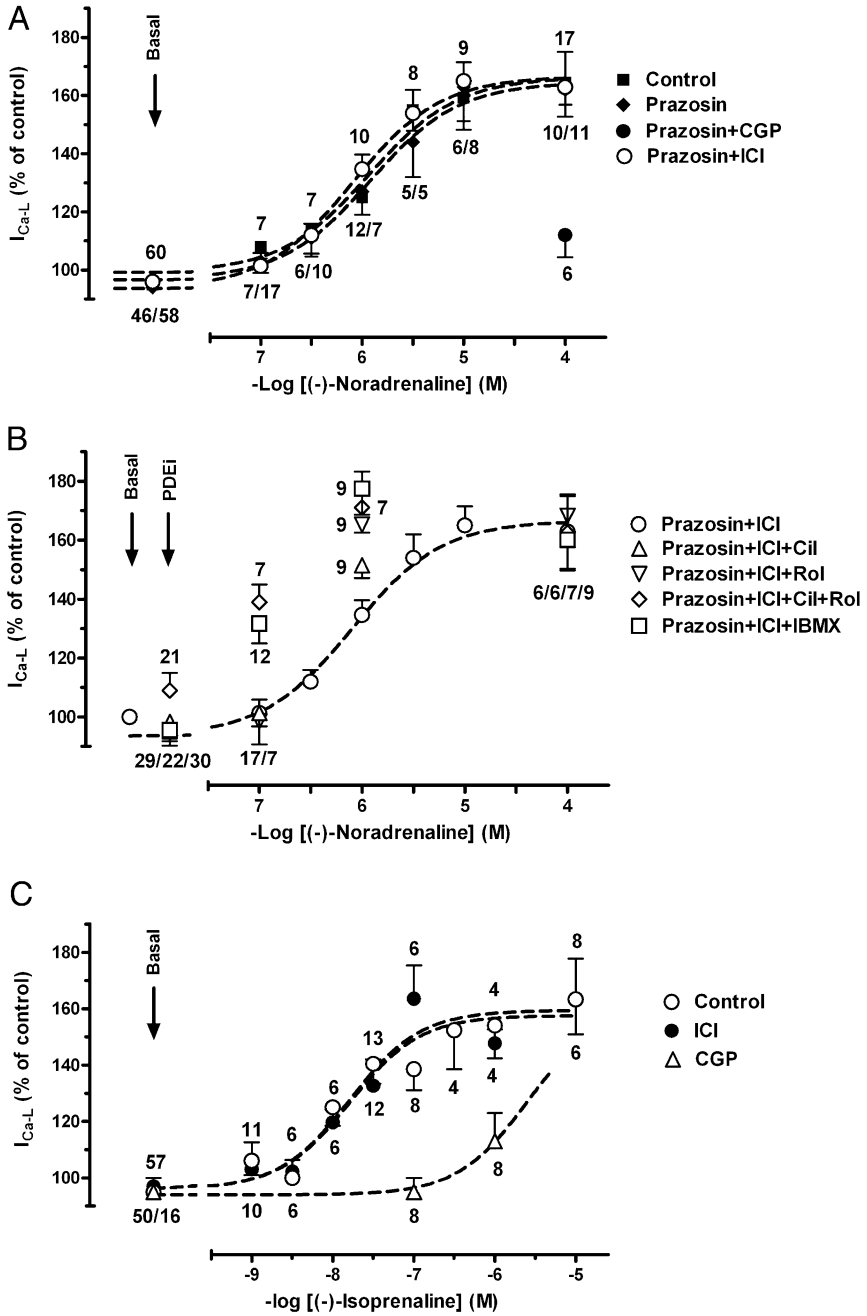

Figure 9 Concentration-dependent increases of I_{Ca-L} by (–)-noradrenaline and (–)-isoprenaline. (A) (–)-Noradrenaline-induced increases in I_{Ca-L} is not affected by prazosin (1 μmol·L^{-1}) and ICI118551 (50 nmol·L^{-1}, ICI) but suppressed by CGP20712A (300 nmol·L^{-1}, CGP). (B) Potentiation of (–)-noradrenaline-induced increases of I_{Ca-L} by cilostamide (300 nmol·L^{-1} Cil), rolipram (1 μmol·L^{-1}, Rol), concurrent cilostamide + rolipram and IBMX (10 μmol·L^{-1}). (C) Effects of (–)-isoprenaline are exclusively mediated through β_1-adrenoceptors. Numbers indicate number of myocytes.

(Vinogradova *et al.*, 2002). However, our results disclosed an unexpected difference. The positive chronotropic effects of (–)-noradrenaline through β_1-adrenoceptors and (–)-adrenaline through β_2-adrenoceptors in the rat were not potentiated by the separate or joint presence of cilostamide and rolipram. This is in contrast to the β-adrenoceptor-independent tachycardia elicited by rolipram, suggesting that the sinoatrial cAMP pools, presumably enhanced through activation of β_1- and β_2-adrenoceptors, are distinct from the rolipram-sensitive cAMP pool controlled by PDE4 that modulates basal sinoatrial rate. A similar discrepancy was recently reported in murine sinoatrial node, in which both PDE3 and PDE4 reduce basal sinoatrial beating rate but neither controls the β_1-adrenoceptor-mediated tachycardia of (–)-noradrenaline (Galindo-Tovar and Kaumann, 2008).

Could the catecholamine-evoked tachycardia occur through β-adrenoceptors via a cAMP-independent pathway? Yatani *et al.* (1987) suggested that effects of isoprenaline on L-type Ca^{2+} channels occur through direct coupling to G_s protein and without diffusible cAMP in guinea-pig myocytes.

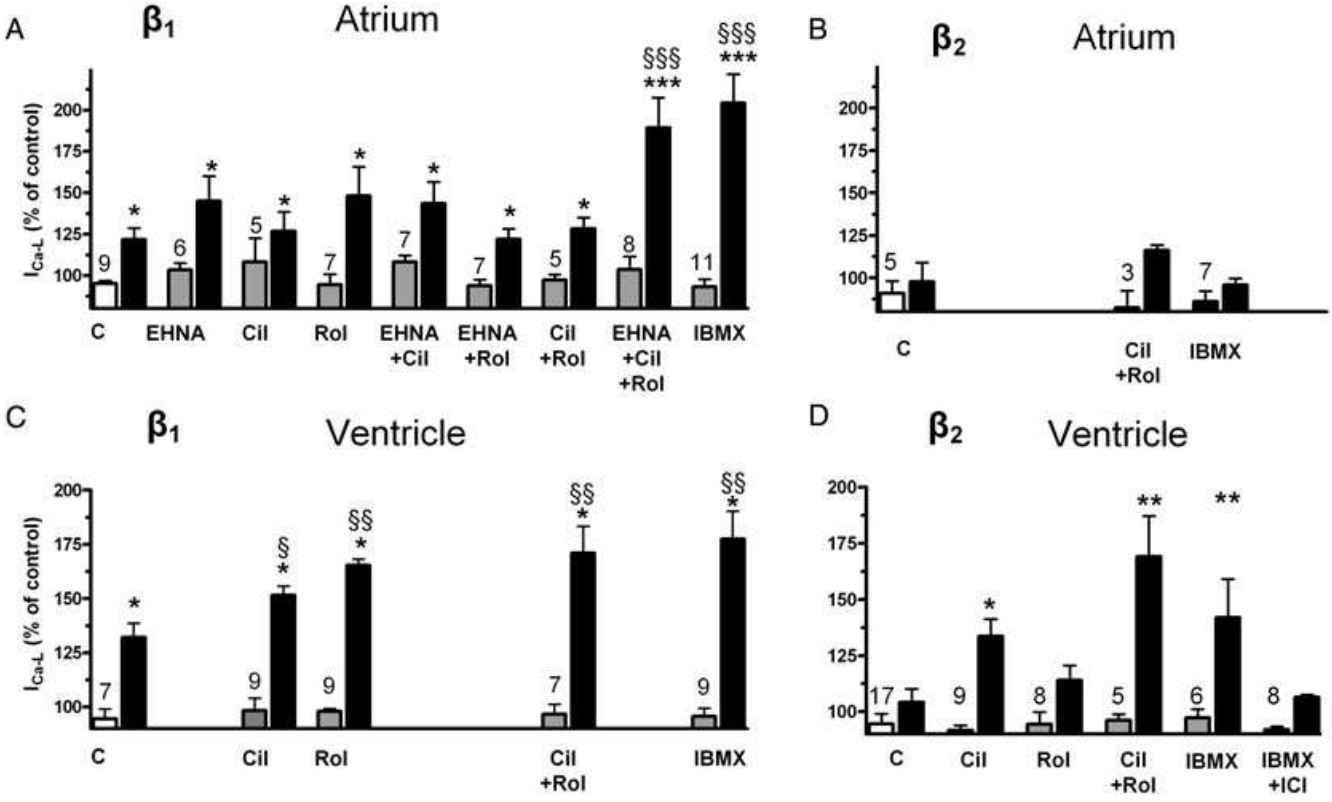

Figure 10 Effects of PDE inhibitors on $I_{Ca\text{-}L}$ responses to (–)-noradrenaline (1 μmol·L^{-1}) through β_1-adrenoceptors (ICI118551 50 nmol·L^{-1} present; A, C) and (–)adrenaline (10 μmol·L^{-1}) through β_2-adrenoceptors (CGP20712A 300 nmol·L^{-1} present; B, D) in atrial (A, B) and ventricular (C, D) myocytes. Responses to EHNA (10 μmol·L^{-1}), cilostamide (300 nmol·L^{-1}, Cil), rolipram (1 μmol·L^{-1}, Rol) and IBMX (10 μmol·L^{-1}) are depicted in grey columns, responses to agonists in black columns. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the corresponding basal; §$P < 0.05$, §§$P < 0.01$, §§§$P < 0.001$ compared with the effect in the absence of PDE inhibitor. Numbers on grey columns indicate number of myocytes.

However, the comprehensive evidence obtained directly from sinoatrial cells by Vinogradova *et al*. (2006; 2008), as well as classical work, points to an obligatory involvement of a cAMP-dependent pathway. Taniguchi *et al*. (1977) reported that noradrenaline caused a maximum elevation of cAMP and increase of beating rate by 1 min, but the cAMP response faded to control levels while the tachycardia remained stable by the 5th minutes in rabbit sinoatrial pacemaker cells. The non-selective PDE inhibitor, theophylline, prevented the fade of the noradrenaline-evoked cAMP response, consistent with a role for PDEs. Taniguchi *et al*. (1977) suggested that the adenylyl cyclase was involved. However, the work of Taniguchi *et al*. (1977) showing that the noradrenaline-evoked tachycardia persists despite the PDE-induced fade of the cAMP signal, is consistent with the failure of PDE inhibitors to influence the chronotropic potency of (–)-noradrenaline in the rat (this work) and murine sinoatrial node (Galindo-Tovar and Kaumann, 2008), but not necessarily consistent with an involvement of cAMP. On the other hand, Tanaka *et al*. (1996) provided conclusive evidence for an obligatory involvement of cAMP and PKA in catecholamine-evoked sinoatrial tachycardia through β-adrenoceptors in rabbit sinoatrial cells. Positive chronotropic responses and increases in $I_{Ca\text{-}L}$ to flash photolysis of caged isoprenaline and cAMP were abolished by the PKA inhibitor Rp-cAMP in rabbit sinoatrial cells.

PDE4 limits the inotropic function of left atrial β_1-adrenoceptors

Rolipram caused marked potentiation (11-fold) of the positive inotropic effects of (–)-noradrenaline on left atrium 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. IBMX also potentiated the effects of (–)-noradrenaline but not more than rolipram (Fig. 3A, Table 1), making it unlikely that, apart from PDE4, other PDE isoforms modulate β_1-adrenoceptor-mediated effects of (–)-noradrenaline in left atrium. The selective control by PDE4 of left atrial inotropic β_1-adrenoceptor function observed in the rat is quite similar in the mouse (Galindo-Tovar and Kaumann, 2008).

Rolipram and IBMX (10 μmol·L^{-1}) potentiated not only the left atrial effects of low (–)-noradrenaline concentrations (11-fold and fivefold) observed in the presence of ICI118551 (Fig. 3A, Table 1) but also similarly the effects of high (–)-adrenaline concentrations (sevenfold and ninefold) in the presence of CGP20712A (Fig. 3C, Table 1). These latter findings can be interpreted as surmountability by high (–)-adrenaline concentrations of the β_1-adrenoceptor blockade by CGP20712A. Under this condition of reactivation of β_1-adrenoceptors, PDE4 appears to limit the inotropically relevant cAMP generated by (–)-adrenaline to a similar extent as that generated by receptor interaction with (–)-noradrenaline

in the absence of CGP20712A. In contrast to the failure of cilostamide to significantly increase the potency of (–)-noradrenaline, cilostamide produced a small fold potentiation of the effects of high (–)-adrenaline concentrations in the presence of CGP20712A, suggesting a small role of PDE3 at β_1-adrenoceptors, activated by (–)-adrenaline. However, after cilostamide, the f_2 fraction was also increased, so that participation of β_2-adrenoceptors cannot be excluded. The ninefold potentiation of the effects of (–)-adrenaline by IBMX (10 $\mu mol \cdot L^{-1}$) in the presence of CGP20712A appears to be mainly due to PDE4 inhibition at β_1-adrenoceptors because it is close to the sevenfold potentiation caused by rolipram. The twofold greater potentiation with 30 $\mu mol \cdot L^{-1}$ IBMX, compared with 10 $\mu mol \cdot L^{-1}$ IBMX, could be caused by an additional small involvement of PDE2 or alternatively, be due merely to an additive cardiostimulant component produced by IBMX.

PDE3 and PDE4 jointly prevent β_2-adrenoceptor function in left atrium

The concentration-effect curves of (–)-adrenaline were monophasic without a CGP20712A-resistant component, even in the presence of cilostamide or rolipram, consistent with an interaction with a single receptor population (i.e. β_1-adrenoceptors). Therefore, inhibition of PDE3 or PDE4 alone does not appear to reveal β_2-adrenoceptor-mediated effects of (–)-adrenaline. However, the concurrent inhibition of PDE3 and PDE4 with cilostamide and rolipram in the presence of CGP20712A uncovered functional β_2-adrenoceptors in the left atrium. The CGP20712A-resistant effects of low (–)-adrenaline concentrations in the presence of both cilostamide and rolipram were prevented by ICI118551, consistent with mediation through β_2-adrenoceptors (Figs 2B and 3C). Therefore, PDE3 and PDE4, acting in concert, appear to prevent the manifestation of β_2-adrenoceptor-mediated effects of (–)-adrenaline in rat left atrium. It is not clear why consistent β_2-adrenoceptor-mediated effects of (–)-adrenaline were not observed in the presence of IBMX (10 $\mu mol \cdot L^{-1}$), perhaps because inhibition of PDE3 and PDE4 was less complete than with the combination of cilostamide + rolipram. Indeed, a higher IBMX concentration of 30 $\mu mol \cdot L^{-1}$ also revealed a CGP20712A-resistant component of the (–)-adrenaline effects (Fig. 3C, Table 1) with $f_2 = 0.25$, probably mediated through β_2-adrenoceptors. However, even under pronounced non-selective PDE inhibition with IBMX, the E_{max} of the β_2-adrenoceptor-mediated effects was small compared with the E_{max} of β_1-adrenoceptor-mediated effects. The inotropic properties of rat and murine (Galindo-Tovar and Kaumann, 2008) left atrial β_2-adrenoceptors are remarkably similar: β_1-adrenoceptor-mediated effects of the catecholamines are blunted by PDE4 but the modest β_2-adrenoceptor-mediated effects of (–)-adrenaline are completely suppressed by concurrent action of PDE3 and PDE4.

The ventricular inotropic effects of (–)-noradrenaline, mediated through β_1-adrenoceptors, are selectively blunted by PDE4

Rolipram but not cilostamide potentiated twofold the positive inotropic effects of (–)-noradrenaline on both right ventricular strips and left ventricular papillary muscles (Figs 4A and 5A, Table 1), consistent with selective hydrolysis of inotropically relevant cAMP by PDE4 but not PDE3 reported previously for β_1-adrenoceptors activated by (–)-noradrenaline (Vargas *et al.*, 2006) or (–)-isoprenaline (Katano and Endoh, 1992). Although cilostamide did not significantly modify the potency of (–)-noradrenaline, concurrent cilostamide + rolipram caused an eightfold potentiation of the effects of (–)-noradrenaline in right ventricular preparations (Fig. 4A, Table 1). The synergistic potentiation of the effects of (–)-noradrenaline by concurrent cilostamide + rolipram, that is, when both PDE3 and PDE4 are inhibited, confirms similar observations in rat right ventricle and are accompanied by marked increases in cAMP levels (Vargas *et al.*, 2006). The accumulation of inotropically relevant cAMP during inhibition of PDE4 by rolipram may leak into the compartment of PDE3 and produce PKA-catalysed phosphorylation (Gettys *et al.*, 1987; Smith *et al.*, 1991) thereby activating PDE3 to further hydrolyze inotropically relevant cAMP.

Interestingly, in the presence of concurrent cilostamide + rolipram (or IBMX 10 $\mu mol \cdot L^{-1}$) approximately 10-fold lower (–)-noradrenaline concentrations sufficed to produce approximately half maximal increases in $I_{Ca\text{-}L}$ compared with the absence of the PDE inhibitors (Fig. 9B). The inotropic potency of (–)-noradrenaline was nearly 10-fold higher than the potency to increase $I_{Ca\text{-}L}$. However, both effects were potentiated approximately 10-fold by concurrent cilostamide + rolipram (compare Figs 4A and 9B). The similarity of these potentiations is consistent with the elimination of local cAMP gradients at the Ca^{2+} channel domain and SR and a homogenous distribution of a high cAMP level across the sarcoplasm when both PDE3 and PDE4 are inhibited (see also Fig. 11D).

PDE3 but not PDE4 moderately blunts (–)-adrenaline responses, but acting in concert with PDE4, markedly reduces β_2-adrenoceptor-mediated effects of (–)-adrenaline in ventricle

Low (–)-adrenaline concentrations caused a small increase in contractility in the presence of CGP20712A with an f_2 of 0.09 and 0.06 in right and left ventricular preparations, respectively, presumably mediated through β_2-adrenoceptors (Figs 4B,C and 5B). Very high (–)-adrenaline concentrations (30–100 $\mu mol \cdot L^{-1}$) partially surmounted the β_1-adrenoceptor blockade by (–)-adrenaline (Figs 4B,C and 5B). Rolipram failed to affect the (–)-adrenaline responses, ruling out hydrolysis by PDE4 of inotropically relevant cAMP through β_2-adrenoceptors. Cilostamide, instead, caused twofold potentiation of the effects of (–)-adrenaline with small increases in f_2 to 0.15–0.17 in both right and left ventricle (Figs 4C and 5B, Table 1), consistent with a small role of PDE3 in blunting β_2-adrenoceptor-mediated responses. However, concurrent cilostamide + rolipram caused a marked increase of f_2 to 0.92 and 0.99, and fivefold and sixfold potentiation of the effects of (–)-adrenaline in right and left ventricle (Figs 4C and 5B, Table 1).

The IBMX (10 $\mu mol \cdot L^{-1}$) caused a similar increase in f_2 to 0.82–0.84 and threefold potentiation of the right and left ventricular effects of (–)-adrenaline; these effects were prevented by ICI118551 (Fig. 2C,D), consistent with mediation

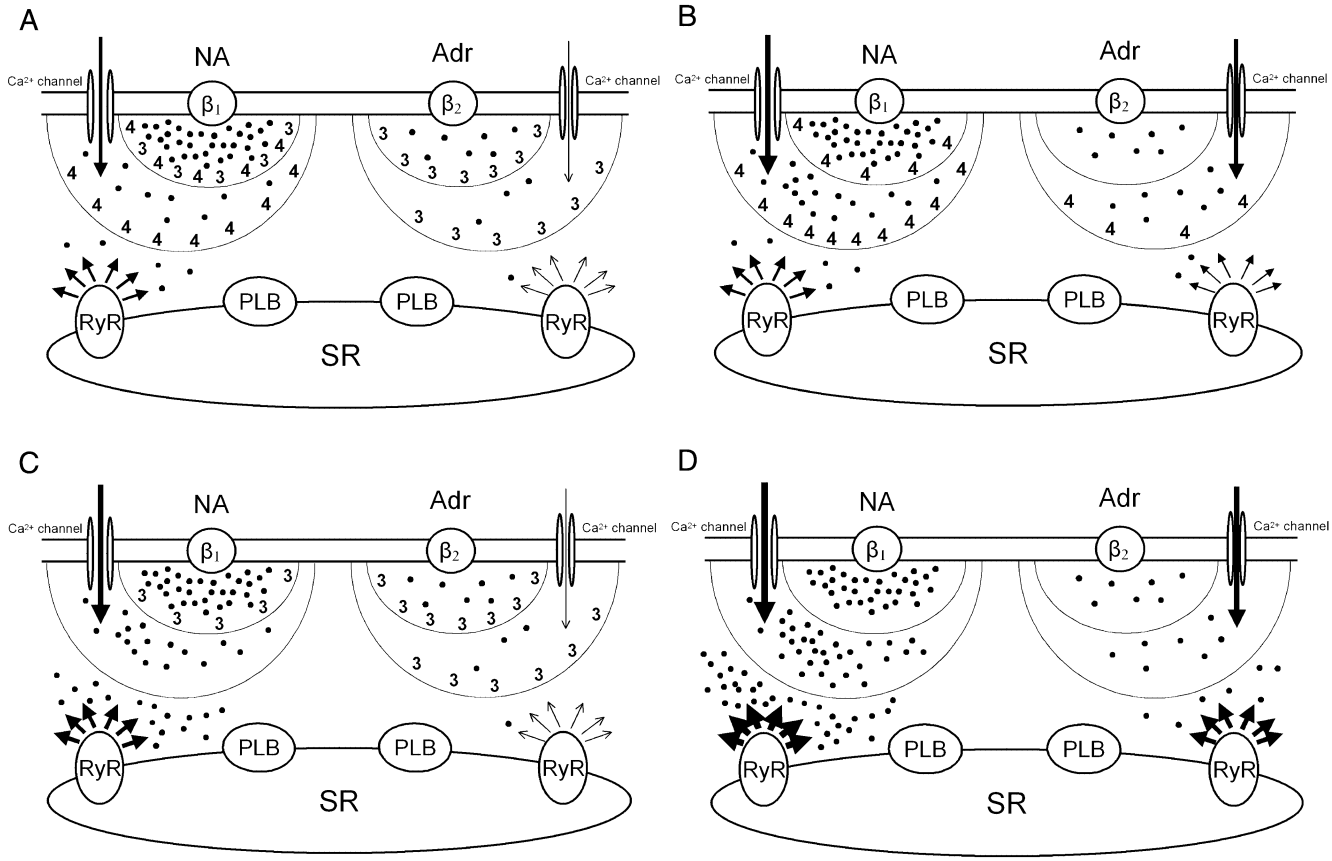

Figure 11 Hypothetical regulation of cAMP levels by activated PDE3 (3) and PDE4 (4) in compartments at different distances from activated β_1- and β_2-adrenoceptors in rat ventricle. (A) Physiological situation. (B) Inhibition of PDE3. (C) Inhibition of PDE4. (D) Inhibition of both PDE3 and PDE4. Black dots represent steady state concentrations of cAMP in each domain. (–)-Noradrenaline (NA) and (–)-adrenaline (Adr) stimulate β_1- and β_2-adrenoceptors, respectively, thereby increasing the production of cAMP and activating PDE3 and PDE4. Two sequential barriers, formed by activated PDEs, break down cAMP. The composition of PDEs in both barriers is different for β_1- and β_2-adrenoceptors. L-type Ca^{2+} channel function is enhanced by cAMP and only reduced by PDE activity of the first barrier (smaller half-circle), whereas inotropically relevant cAMP has to pass through the more distal second barrier (larger half-circle) to reach the SR. The first barrier corresponds to PDE3 and PDE4 bound to the sarcolemma. The second barrier is produced by PDE3 and PDE in the cytoplasm and at the SR. Arrows represent Ca^{2+} current through L-type channels and Ca^{2+} release from RyR2 channels (RyR). Arrow thickness is proportional to $I_{Ca\text{-}L}$ and Ca^{2+} release. The inotropic responses are assumed to be proportional to Ca^{2+} released from the SR through RyR2 channels. The cAMP-dependent activation of PKA and subsequent PKA-catalysed phosphorylation of Ca^{2+} channels, phospholamban (PLB), PDE4 and RyR2 is not represented to avoid overcrowding. Both PDE3 and PDE4 blunt the $I_{Ca\text{-}L}$ response to (–)-noradrenaline (NA) mediated through β_1-adrenoceptors but only PDE3 blunts the $I_{Ca\text{-}L}$ response to (–)-adrenaline mediated through β_2-adrenoceptors. Positive inotropic responses to both (–)-noradrenaline (β_1-adrenoceptors) and (–)-adrenaline (β_2-adrenoceptors) are assumed to occur at least in part through PKA-catalysed phosphorylation of Ca^{2+} channels, RyR2 channels and phospholamban (PLB). The positive inotropic effects of catecholamines are controlled by PDE4 through β_1-adrenoceptors but by PDE3 through β_2-adrenoceptors.

through β_2-adrenoceptors. IBMX (100 μmol·L^{-1}) did not cause greater potentiation than IBMX (10 μmol·L^{-1}) or concurrent rolipram + cilostamide. The similar potentiation of the effects of (–)-adrenaline by IBMX compared with concurrent cilostamide + rolipram suggests that mostly PDE3 and PDE4 control inotropically relevant cAMP and make unlikely a contribution of other PDE isoenzymes (e.g. PDE2).

To account for the synergistic potentiation of the effects of (–)-adrenaline by concurrent cilostamide + rolipram, the accumulation of inotropically relevant cAMP during inhibition of PDE3 by cilostamide may leak into the compartment of PDE4 and produce PKA-catalysed phophorylation (MacKenzie *et al.*, 2002) thereby activating PDE4 to further hydrolyze cAMP that has become inotropically relevant.

PDE4 blunts β_1-adrenoceptor-mediated increases of both $I_{Ca\text{-}L}$ and contractile force but PDE3 reduces only $I_{Ca\text{-}L}$

In line with previous work with (–)-isoprenaline on rat ventricular myocytes (Verde *et al.*, 1999; Rochais *et al.*, 2006), our results with (–)-noradrenaline show that both cilostamide and rolipram enhance the β_1-adrenoceptor-mediated $I_{Ca\text{-}L}$ responses. These results suggest that both PDE3 and PDE4 hydrolyze subsarcolemmal cAMP generated through β_1-adrenoceptor stimulation in the vicinity of the $I_{Ca\text{-}L}$ channels as reported previously (Rochais *et al.*, 2006) and are consistent with the immunocytochemical localisation of both PDE3 and PDE4 in the sarcolemma (Okruhlicova *et al.*, 1996). In contrast and, as reported before (Katano and Endoh, 1992; Vargas *et al.*, 2006), only inhibition of PDE4 but not of PDE3

potentiates the positive inotropic effects of catecholamines, suggesting that the PDE3-catalysed hydrolysis of subsarcolemmal cAMP reduces β_1-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ without affecting contractile events.

The dissociation between PDE3-evoked reduction of β_1-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ but not of contractile responses suggests that β_1-adrenoceptor/PDE3/L-type Ca^{2+} channel complexes are located at a separate compartment from the domains of phospholamban and RyR2 channels that contribute to enhanced contractility through PKA-catalysed phosphorylation, via greater Ca^{2+} uptake and release from and to the SR respectively. On the other hand, PDE4 reduces both the inotropically relevant cAMP (Vargas *et al.*, 2006) and the cAMP in the $I_{Ca\text{-}L}$ domain (Rochais *et al.*, 2006) through β_1-adrenoceptor stimulation. Rat ventricular PDE4 activity was initially only detected in the cytosol (Weishaar *et al.*, 1987). Interestingly, inhibition of PDE4 causes a more marked increase in cAMP in the cytosol than in the subsarcolemmal compartment in rat cardiomyocytes (Leroy *et al.*, 2008), suggesting that the control of cytosolic cAMP by PDE4 contributes to the blunting of the β_1-adrenoceptor-mediated inotropic responses.

The β_1-adrenoceptor-evoked increases of cAMP lead to PKA-dependent phosphorylation and activation of PDE4 (Leroy *et al.*, 2008). PDE4-catalysed hydrolysis of cAMP reduces the free diffusion of cAMP and causes gradients of both cAMP and PKA (Saucerman *et al.*, 2006). During inhibition of PDE4, more cAMP accumulates at the domains of L-type Ca^{2+} channels, phospholamban as well as RyR2 channels, as schematically depicted in Figure 11. This interpretation has to be restricted to rat ventricle because, for example, in human ventricular and atrial myocardium β_1-adrenoceptor-mediated increases in contractility are greatly reduced by PDE3 activity, but not by PDE4 activity (Christ *et al.*, 2006b; Kaumann *et al.*, 2007), presumably because PDE3, rather than PDE4, is bound to the SR (Lugnier *et al.*, 1993). Unfortunately, a comparison of the expression and activity of rat and human myocardial PDE3 and PDE4 (see Osadchii, 2007) does not yet appear to explain species-dependent control of β_1-adrenoceptors and their function by these PDE isoenzymes.

β_2-Adrenoceptor-mediated increases of ventricular $I_{Ca\text{-}L}$ and contractility are blunted by PDE3 but not PDE4

In contrast to the increase in $I_{Ca\text{-}L}$ obtained with (–)-noradrenaline, mediated through β_1-adrenoceptors, the effect of (–)-adrenaline through β_2-adrenoceptors was reduced by PDE3 only. This apparently contradicts the results of Rochais *et al.* (2006). They demonstrated on rat ventricular myocytes cultured for 24 h that cilostamide and to a greater extent the PDE4 inhibitor Ro 20-1724 enhanced the increases of $I_{Ca\text{-}L}$ induced by isoprenaline in the presence of CGP20712A, attributed to mediation through β_2-adrenoceptors. In contrast, using freshly isolated myocytes, we found that in the presence of CGP20712A only cilostamide, but not rolipram, enhanced the (–)-adrenaline-evoked increase in $I_{Ca\text{-}L}$. The discrepancy between our results and those of Rochais *et al.* (2006) is puzzling but could be due to different experimental conditions. We worked at 37°C, used 300 nmol·L^{-1} CGP20712A as well as 10 μmol·L^{-1} (–)-adrenaline and demonstrated that the effects of (–)-adrenaline on $I_{Ca\text{-}L}$ were mediated through β_2-adrenoceptors because they were prevented by 50 nmol·L^{-1} ICI118551 (at least in the presence of IBMX). On the other hand, Rochais *et al.* worked at 21–27°C, used 1 μmol·L^{-1} CGP20712A as well as 5 μmol·L^{-1} (–)-isoprenaline but did not verify whether the effects of (–)-isoprenaline in the presence of (–)-CGP20712A were antagonized by ICI118551. It is possible that (–)-isoprenaline in the presence of CGP20712A did partially surmount the blockade of β_1-adrenoceptors in the experiments of Rochais *et al.* (2006). CGP20712A 300 nmol·L^{-1} produced an approximate 2 log incomplete shift of the concentration-effect curve of (–)-isoprenaline for increases in $I_{Ca\text{-}L}$ density (Fig. 9C). The three-fold greater concentration of CGP20712A (1 μmol·L^{-1}) used by Rochais *et al.* (2006) would be expected to cause a 2.5 log unit shift of the curve for (–)-isoprenaline. Assuming that the $-\log EC_{50}$ for the effects of (–)-isoprenaline on $I_{Ca\text{-}L}$ is 7.7 (Fig. 9C), its $-\log EC_{50}$ at β_1-adrenoceptors will be decreased by 2.5 log units in the presence of 1 μmol·L^{-1} CGP20712A to 5.2, that is, to 6 μmol·L^{-1} which is close to the 5 μmol·L^{-1} (–)-isoprenaline used by Rochais *et al.* (2006). Thus, under the conditions of Rochais *et al.* (2006) it is conceivable that (–)-isoprenaline actually interacted mainly with β_1-adrenoceptors and only to a lesser extent with β_2-adrenoceptors.

We also found that the selective cilostamide-sensitive PDE3 blunting of the $I_{Ca\text{-}L}$ response to (–)-adrenaline correlated with the small but significant potentiation by cilostamide of the β_2-adrenoceptor-mediated inotropic effects of (–)-adrenaline (Figs 4, 5, Table 1) on both right as well as left ventricle and were accompanied by hastened relaxation in left ventricle (Figs 6 and 7). If the small potentiation were solely due to enhanced Ca^{2+}-induced Ca^{2+} release from RyR2 channels, relaxation should not have been hastened. However, not only cilostamide, but even rolipram induced (–)-adrenaline to hasten relaxation (Figs 6 and 7), so that PKA-catalysed phosphorylation of phospholamban seems to have occurred when either PDE3 or PDE4 were inhibited. Rolipram only induced (–)-adrenaline to hasten relaxation but not to enhance ventricular $I_{Ca\text{-}L}$ or potentiate the inotropic effects through β_2-adrenoceptors of left ventricular papillary muscles, suggesting that the relevant PDE4 is hydrolyzing cAMP near phospholamban but perhaps not near the Ca^{2+} channels or RyR2 channels .

The predominant control of β_2-adrenoceptor-mediated inotropic effects of (–)-adrenaline by PDE3 in rat ventricle is similar to the exclusive control by PDE3, but not PDE4, encountered in human atrium (Christ *et al.*, 2006b).

Concurrent cilostamide and rolipram caused marked potentiation of the ventricular inotropic effects of (–)-adrenaline through β_2-adrenoceptors. Cyclic AMP, accumulating after cilostamide-evoked PDE3 inhibition during β_2-adrenoceptor stimulation, may have overflowed its normal limits and reached compartments, including Ca^{2+} channels and RyR2 channels in which PDE4 could be phosphorylated and activated by PKA, thus hydrolyzing cAMP (MacKenzie *et al.*, 2002; Leroy *et al.*, 2008) and reducing its levels at both $I_{Ca\text{-}L}$ channels and proteins involved in enhancing contractile force (Fig. 11D). Concurrent inhibition of PDE3 and PDE4 would therefore preserve large amounts of cAMP which would diffuse to produce PKA-catalysed phosphorylation of $I_{Ca\text{-}L}$ and

Table 2 Densities of β_1- and β_2-adrenoceptors (as % total) in rat cardiac regions

Tissue	*β_1-Adrenoceptor*	*β_2-Adrenoceptor*	*Reference*
Sinoatrial node	93.3	6.8	Matthews *et al.* (1996)
Atrium	74.3	25.7	Mysliveček *et al.* (2003)
Left ventricle	81.5	18.5	Witte *et al.* (2000)
Right ventricular papillary muscle	80.4	19.7	Matthews *et al.* (1996)
Ventricles	79.0	21.0	Mysliveček *et al.* (2003)
Ventricles	73.0	27.0	Kitagawa *et al.* (1995)
Ventricular myocytes	90.0	10.0	Kitagawa *et al.* (1995)
Ventricles	70.0	30.0	Mauz and Pelzer (1990)
Ventricular myocytes	100	0	Mauz and Pelzer (1990)

of proteins, including RyR2 Ca^{2+} release channels and phospholamban, involved in the positive inotropic effects of (–)-adrenaline through β_2-adrenoceptors.

(–)-Noradrenaline-evoked increases of atrial $I_{Ca\text{-}L}$ through β_1-adrenoceptors are only increased by the concurrent inhibition of PDE1, PDE2 and PDE3. Discrepancies with inotropic effects

The small increase in $I_{Ca\text{-}L}$ by (–)-noradrenaline, mediated through β_1-adrenoceptors, was unaffected by EHNA, cilostamide, rolipram or concurrent cilostamide + rolipram, inconsistent with control by PDE2, PDE3 or PDE4. However, both IBMX and concurrent EHNA, cilostamide and rolipram markedly increased the (–)-noradrenaline response, suggesting that at the L-type Ca^{2+} channel domain the three PDE isoenzymes act in concert to blunt the response to (–)-noradrenaline.

The function of PDE isoenzymes controlling atrial $I_{Ca\text{-}L}$ responses to (–)-noradrenaline is fundamentally at variance with the atrial inotropic responses to (–)-noradrenaline, which are selectively controlled by PDE4. Inhibition of PDE2 is mandatory to permit effects of concomitant inhibition of PDE3 and PDE4 on the level of $I_{Ca\text{-}L}$. In contrast, it is also unlikely that PDE2 is modulating atrial positive inotropic responses to (–)-noradrenaline and (–)-adrenaline through β_1-adrenoceptors and β_2-adrenoceptors, respectively, because responses and potencies in the presence of IBMX were not significantly greater than in the presence of concurrent cilostamide + rolipram (Fig. 3, Table 1). The discrepancy could be related to structural peculiarities of the atrial myocardium. Unlike ventricular myocardium, atrial myocardium virtually lacks t-tubules which in ventricle couple to the junctional SR and form the SR network around the myofibrils (Hüser *et al.*, 1996; Lipp *et al.*, 1996; Yamasaki *et al.*, 1997). In atrial myocardium, slender sarcotubular connections are connected to Z-tubules (Yamasaki *et al.*, 1997). Due to inhomogenous Ca^{2+} increases upon depolarization, Ca^{2+}-induced Ca^{2+} release largely fails in deeper layers of the atrial myocytes (Mackenzie *et al.*, 2001). We speculate that the selective control by PDE4 of the positive inotropic effects of (–)-noradrenaline is related to the localisation of this isoenzyme at or near the SR, as also proposed for ventricular myocardium.

Are regional differences in cardiac β_2-adrenoceptor function related to both PDE activity and receptor density?

In the absence of PDE inhibition, (–)-adrenaline elicited weak cardiostimulation through β_2-adrenoceptors of sinoatrial node and both right and left ventricles but not at all in the left atrium (Figs 1–5). Concurrent cilostamide and rolipram did not potentiate the positive chronotropic effects of (–)-adrenaline and only caused an increase in f_2 to 0.23 from 0.08 in the absence of the PDE inhibitors (Table 1). On the left atrium, in contrast, combined cilostamide and rolipram uncovered potent positive inotropic effects of (–)-adrenaline with $f_2 = 0.26$, mediated through β_2-adrenoceptors. On the ventricles, the effect of IBMX (10 $\mu mol \cdot L^{-1}$) or the combination of cilostamide and rolipram potentiated the effects of (–)-adrenaline and greatly enhanced f_2 to 0.82–0.84 and 0.92–0.98 respectively (Table 1). PDE3 and PDE4, acting in concert, appeared to hydrolyze inotropically relevant cAMP effects elicited by (–)-adrenaline through β_2-adrenoceptors in ventricle and left atrium but hardly affected chronotropically relevant cAMP in sinoatrial cells. Can the differences between cardiac regions in the magnitude of the β_2-adrenoceptor fraction f_2 after combined inhibition of PDE3 and PDE4 be explained in part by differences in regional β_2-adrenoceptor densities? The literature on β_2-adrenoceptor density, compared with β_1-adrenoceptor density, in the rat heart, summarized in Table 2, reveals that the sinoatrial node has the lowest density of β_2-adrenoceptors amounting only to about 25–30% of atrial or ventricular β_2-adrenoceptors. Furthermore, a large fraction of rat right atrial β_2-adrenoceptor population is not located in myocytes but in ganglion cells (Mysliveček *et al.*, 2006). Yet, (–)-adrenaline enhanced sinoatrial beating through β_2-adrenoceptors (This report; Kaumann, 1986) even in the absence of PDE inhibitors while left atrial β_2-adrenoceptors only reveal inotropic function when both PDE3 and PDE4 are inhibited, despite the fourfold greater β_2-adrenoceptor density, relative to β_1-adrenoceptors. Clearly the minor β_2-adrenoceptor-mediated tachycardia is conspicuous, despite the very low receptor density, because it is unaffected by PDEs. Ventricular β_2-adrenoceptor function after concomitant cilostamide and rolipram is even more marked than that of left atrial β_2-adrenoceptors under this condition, despite the similar β_2/β_1-adrenoceptor proportion in both regions. Physiological and anatomical differences between left atrium and ventricle may account for the difference in β_2-adrenoceptor function, even after combined inhibition of PDE3 and PDE4.

Enzymatic disaggregation of rat ventricular myocytes markedly reduced (Kitagawa *et al*, 1995) or even made undetectable β_2-adrenoceptors (Mauz and Pelzer, 1990) compared with intact ventricle (Table 2). The reduced ventricular β_2-adrenoceptor density, compared with β_1-adrenoceptor

density, in rat ventricular myocytes, may explain the high (–)-adrenaline concentration used (10 μmol·L^{-1}) to unambiguously demonstrate increases in $I_{Ca\text{-}L}$ through β_2-adrenoceptors. Our failure to detect effects on $I_{Ca\text{-}L}$ by (–)-adrenaline through β_2-adrenoceptors of atrial myocytes could also be due to further reduction of the low receptor density by enzymatic disaggregation.

Conclusions

The PDE4 reduced basal sinoatrial beating rate but neither PDE3 nor PDE4 affected the chronotropic potencies of (–)-noradrenaline and (–)-adrenaline through β_1- and β_2-adrenoceptors, respectively, suggesting that the cAMP hydrolysis by PDE4 controlled beating rate in a compartment distinct from the region at which β-adrenoceptor activation modified ionic channel activity and Ca^{2+} cycling leading to tachycardia. PDE4 but not PDE3 reduced the atrial and ventricular inotropic effects of (–)-noradrenaline through β_1-adrenoceptors. The β_1-adrenoceptor-mediated increases of ventricular $I_{Ca\text{-}L}$ were blunted by both PDE3 and PDE4 at the sarcolemmal domain but increases in contractile force are blunted only by PDE4, suggesting that cAMP in the β_1-adrenoceptor/PDE3/Ca^{2+}channel compartment does not reach proteins that produce increases in contractility such as RyR2 channels and phospholamban (Fig. 11). PDE3, but not PDE4 alone, reduced both increases in $I_{Ca\text{-}L}$ and ventricular positive inotropic effects of (–)-adrenaline through β_2-adrenoceptors. However, not only PDE3 but also PDE4 appeared to prevent hastening of relaxation by (–)-adrenaline though β_2-adrenoceptors. Concurrent inhibition of PDE3 and PDE4 uncovered left atrial inotropic effects of (–)-adrenaline through β_2-adrenoceptors and markedly potentiated ventricular inotropic effects of (–)-adrenaline mediated through β_2-adrenoceptors. PDE3 and PDE4 protected the rat heart against ventricular and left atrial overstimulation but not against tachycardia through β_1- and β_2-adrenoceptors.

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Conflicts of interest

The authors state no conflict of interest.

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1.3 Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT4 receptors.

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RESEARCH PAPER

Ontogenic changes of the control by phosphodiesterase-3 and -4 of 5-HT responses in porcine heart and relevance to human atrial 5-HT$_4$ receptors

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Background and purpose: Atrial inotropic responses to 5-HT mediated through 5-HT$_4$ receptors fade, presumably through phosphodiesterase (PDE) activity. We investigated the influence of a selective inhibitor of PDE3 (cilostamide) or of PDE4 (rolipram) on the fade of 5-HT responses in atrial muscle.
Experimental approach: 5-HT responses were compared, *ex vivo*, on sinoatrial beating rate of newborn piglets, porcine atrial and ventricular force, and human atrial force. cAMP levels were assessed in piglet atrium.
Key results: 5-HT-evoked sinoatrial tachycardia did not fade and was not potentiated by cilostamide (300 nmol·L^{-1}) or rolipram (1 μmol·L^{-1}). Inotropic responses to 5-HT faded in atria from piglets, adolescent pigs and humans. Cilostamide reduced atrial fade of 5-HT responses in adolescent pigs and humans but not in newborn piglets. Cilostamide disclosed 5-HT ventricular responses in newborn, but not adolescent pigs. Rolipram reduced fade of atrial 5-HT responses in newborn and adolescent pigs but not in humans. Concurrent cilostamide + rolipram abolished fade of 5-HT responses in porcine left atria and facilitated ventricular 5-HT responses, but did not reduce residual fade in human atrium in the presence of cilostamide. 5-HT-evoked increases in cAMP faded; fade was abolished by concurrent cilostamide + rolipram.
Conclusions and implications: PDE3-induced control of porcine 5-HT responses differed in atrium and ventricle and changed with age. PDE3 and PDE4 jointly prevented fade of inotropic and cAMP responses to 5-HT in porcine atrium. Unlike porcine atria, only PDE3 induced fade of 5-HT responses in human atria.
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Keywords: Phosphodiesterases-3 and -4; porcine and human atrium; sinoatrial node; ventricle; 5-HT$_4$ receptors; cAMP

Abbreviations: IBMX, isobutyl-methylxanthine; PDE3, phosphodiesterase-3; PDE4, phosphodiesterase-4

Introduction

Human (Kaumann *et al.*, 1990; Sanders and Kaumann, 1992; Brattelid *et al.*, 2004) and porcine (Kaumann, 1990; Parker *et al.*, 1995) hearts express functional 5-HT$_4$ receptors that mediate cardiostimulation including arrhythmias (Kaumann, 1994; Kaumann and Sanders, 1994; Rahme *et al.*, 1999; Pau *et al.*, 2003; Leftheriotis *et al.*, 2005) through cAMP-dependent pathways (see Kaumann and Levy, 2006a). Cyclic AMP is hydrolysed by phosphodiesterases (PDEs) and recent evidence has disclosed that the role of these enzymes is so important that they virtually prevent the functional manifestation of effects of 5-HT through human and porcine ventricular 5-HT$_4$ receptors. Only when PDE activity is inhibited with the non-selective PDE inhibitor, isobutyl-methylxanthine (IBMX), can positive inotropic and lusitropic effects of 5-HT, as well as stimulation of cAMP-dependent protein kinase (PKA) and even arrhythmias mediated by ventricular 5-HT$_4$ receptors, become apparent (Brattelid *et al.*, 2004), but which PDE isoenzymes are responsible is unknown.

The inotropic responses to 5-HT and 5-HT$_4$ receptor partial agonists tend to fade in human (Kaumann *et al.*, 1991; Sanders and Kaumann, 1992) and porcine (Parker *et al.*, 1995) atria. De Maeyer *et al.* (2006) recently reported that IBMX prevents the fade of 5-HT responses in porcine atria but which PDE isoenzymes are mainly involved is still an open question (Kaumann and Levy, 2006b).

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At least four PDE isoenzymes, PDEs 1–4, are expressed in the human heart and PDEs 2–4 have also been found in porcine heart (Zimmermann *et al.*, 1994). PDE3 and PDE4 are the most important isoenzymes responsible for the hydrolysis of inotropically relevant cAMP, generated through receptor activation, but there are important species differences. The inotropic effects of noradrenaline, mediated through β_1-adrenoceptors, are controlled mainly by PDE4 in murine (Galindo-Tovar and Kaumann, 2008) and rat (Katano and Endoh, 1992; Vargas *et al.*, 2006) hearts, but controlled by PDE3 in human heart (Christ *et al.*, 2006; Kaumann *et al.*, 2007).

As the PDE isoenzymes that blunt 5-HT$_4$-receptor-mediated cardiostimulation were unknown (Kaumann and Levy, 2006b), we sought to investigate the role of PDE3 and PDE4 in three cardiac regions of the heart of newborn piglets, a model for human cardiac 5-HT$_4$ receptors. Cilostamide and rolipram were used to selectively inhibit PDE3 and PDE4 respectively (Vargas *et al.*, 2006; Galindo-Tovar and Kaumann, 2008). We investigated the effects of the PDE inhibitors on 5-HT-evoked sinoatrial tachycardia, as well as left atrial inotropic and cAMP responses to 5-HT in isolated atria from newborn piglets. To investigate how relevant data from newborn piglets were to older pigs and humans, we also assessed the effects of the PDE inhibitors on the fade of 5-HT responses in atrial trabeculae from adolescent pigs and adult humans. Finally, we investigated whether selective inhibition of PDE3 and/or PDE4 could uncover effects of 5-HT on ventricular trabeculae of newborn and adolescent pigs.

Methods

The drug/molecular target nomenclature conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Patients

This study was approved by the Ethical Committees of the University of Murcia and the University Hospital. The patients provided written informed consent. Right atrial appendages were obtained from 10 patients (mean age 59 ± 4 years, 8 men, 2 women) with stable sinus rhythm and not in heart failure (ejection fraction 68 ± 5%), undergoing cardiac surgery for aortic valve replacement (7) and coronary artery bypass (2) or both (1) at the University Hospital. Two coronary patients and two aortic valve patients were chronically treated with the β-blockers atenolol and bisoprolol respectively. Two aortic valve patients took salbutamol. Other chronically administered drugs included an angiotensin-converting enzyme inhibitor (1), H^+ pump antagonists (6), aspirin (5), lipid lowering drugs (simvastatin/atorvastatin, 7), diuretics (7), hypoglycemic agents (3), calcium antagonist (1), benzodiazepines (3) and nitrates (4).

Human right atrial trabeculae

After excision, the atrial appendages were immediately placed into oxygenated, modified cold Tyrode's solution and transported to the laboratory in less than 5 min. Up to eight atrial trabeculae were dissected in oxygenated, modified Tyrode's solution at room temperature containing (mmol·L^{-1}): 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 solution was maintained at pH 7.4 by bubbling a mixture of 5% CO_2 and 95% O_2. The atrial trabeculae were mounted in pairs, attached to Swema 4-45 strain gauge transducers in an apparatus containing the above solution at 37°C, paced at 1 Hz, and stretched as described previously (Kaumann *et al.*, 1990). Force was recorded on a 12-channel Watanabe polygraph.

Isolated tissues from pigs

All animal care and procedures complied with the guidelines of the European Communities Council Directive of 23 March 1998 (1999/575/CE) and were approved by the Ethical Committee of the University of Murcia. Newborn piglets (1–2 days, either sex, 0.9 ± 0.05 kg) and adolescent pigs (12–14 weeks old, either sex, 22 ± 2 kg) were obtained from the animal farm of the School of Veterinary Sciences, University of Murcia. Newborn piglets and pigs were anaesthetized with sodium pentobarbital, 100 mg·kg^{-1} i.p. and 50 mg·kg^{-1} i.v. respectively. The hearts were removed and dissected in oxygenated, modified Tyrode's solution, as detailed above, at room temperature. Spontaneously beating right atria (Kaumann, 1990), left atrial strips (Parker *et al.*, 1995) and right ventricular trabeculae (width <1 mm, Brattelid *et al.*, 2004) from newborn piglets, as well as left atrial trabeculae (width <1 mm) (De Maeyer *et al.*, 2006) and ventricular trabeculae from adolescent pigs were rapidly dissected and mounted to contract in modified Tyrode's solution at 37°C, often in pairs. Two to four atrial strips (newborn piglets) or trabeculae (adolescent pigs) were obtained from a single left atrium. Left atrial preparations and right ventricular trabeculae were paced at 1 Hz and stretched as described earlier (Parker *et al.*, 1995; Brattelid *et al.*, 2004). Contractile force of left atrial trabeculae from adolescent pigs and human right atrium was recorded on a 12-channel Watanabe polygraph. Force and time to peak force of left atrial strips from newborn piglets were recorded through PowerLab amplifiers on a Chart for Windows, Version 5.5.6 recording programme (ADInstruments, Castle Hill, NSW, Australia).

Protocols

All experiments were carried out in the presence of (-)-propranolol (200 nmol·L^{-1}) to avoid indirect effects due to 5-HT-evoked release of noradrenaline and interaction with β-adrenoceptors.

Cumulative concentration–effect curves for 5-HT were carried out on spontaneously beating right atria and paced left atrial strips of newborn piglets in the absence and presence of the PDE3 inhibitor cilostamide (300 nmol·L^{-1}) or PDE4 inhibitor rolipram (1 μmol·L^{-1}) (Vargas *et al.*, 2006; Galindo-Tovar and Kaumann, 2008), followed by the administration of a saturating concentration of (-)-isoprenaline (200 μmol·L^{-1}). For inotropic studies, the experiments were terminated by elevating the $CaCl_2$ concentration to 9 mmol·L^{-1}.

Measurement of cyclic AMP

In order to investigate whether contractile responses correlated with cyclic AMP levels in the same left atrial strip, basal force and agonist-induced force were measured and the tissues frozen in liquid nitrogen immediately 2 or 20 min after the addition of a maximum inotropically effective concentration of 5-HT (10 μmol·L^{-1}). The effects of a maximum inotropically effective concentration of (-)-isoprenaline (200 μmol·L^{-1}), incubated for 2 min, were studied for comparison. Two to four strips from one left atrium of a newborn piglet were set up into separate organ baths and exposed to two to four different experimental conditions (for a representative experiment, see Figure 1).

Levels of cyclic AMP were measured by radioimmunoassay [^{125}I]TME-S-cAMP (Diagnostic Pasteur, France), according to the manufacture's instructions. Incubation time with the PDE inhibitors was 30 min, instead of 15 min used by other authors (Katano and Endoh 1992; Verde *et al.*, 1999). After freezing, the tissue was weighed and homogenized in cold perchloric acid (0.3 mol·L^{-1}; 1:30, weight : volume) with a Polytron homogenizer (setting 8 for 15 s) and centrifuged (10 000× *g*, 4°C, 15 min). The supernatants were treated with potassium hydroxide until pH 6.7 was reached. The sensitivity of the assay was 2 pmol·mL^{-1}. The pellet was re-disolved in KOH 2 mol·L^{-1} for protein determination. Intra- and inter-assay coefficients of variation were 7.7% and 8.2% respectively. The antibody cross-reacted 100% with 3′, 5′-cyclic AMP and less than 0.3% with other nucleotides. Cyclic AMP concentrations were expressed as pmol·mg^{-1} of tissue.

Statistics

The data are expressed as mean ± SEM of *n* = number of right atria, left atrial strips or trabeculae and ventricular trabeculae. –LogEC$_{50}$M values of 5-HT were estimated from fitting a Hill function with variable slopes to concentration–effect curves from individual experiments. Significance of differences between means was assessed with either paired and unpaired Student's *t*-test using GraphPad 4 Software Inc. (San Diego, CA) and/or ANOVA (Tukey's multiple comparison test). $P < 0.05$ was considered significant.

Drugs

5-Hydroxytryptamine HCl, (-)-propranolol, rolipram and cilostamide were purchased from Sigma Chemicals (Madrid, Spain). Rolipram and cilostamide were dissolved in dimeth-

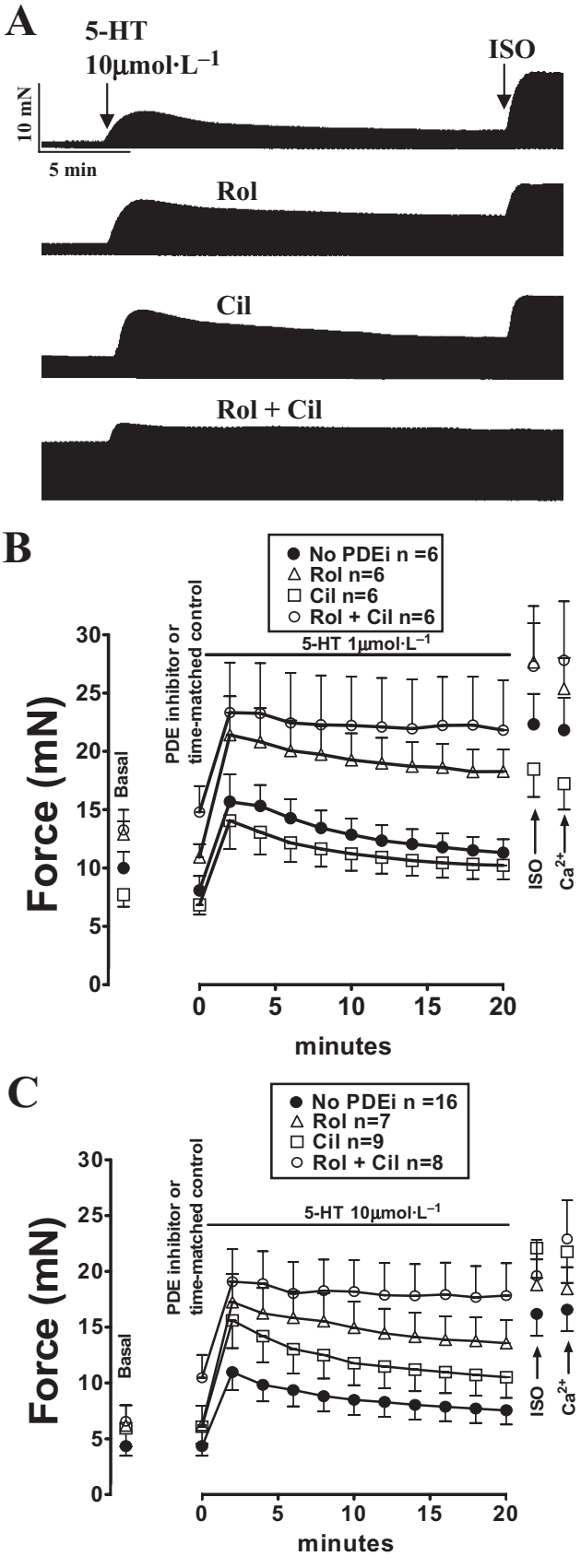

Figure 1 Rolipram (Rol) reduces fade and concurrent rolipram + cilostamide (Rol + Cil) abolished fade of the inotropic response to 5-HT on left atria from newborn piglets. (A) Represtative experiment showing the response to 10 μmol·L^{-1} 5-HT in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide on four strips obtained from the same atrium. After the 20th min of 5-HT administration, (-)-isoprenaline (200 μmol·L^{-1}, ISO) was added. (B) Data of experiments with fade of the response to 1 μmol·L^{-1} 5-HT. Experiments were carried out on two strips from each atrium. (C) Data of experiments with fade of the response to 10 μmol·L^{-1} 5-HT. Experiments were carried out on two to four strips from each atrium. *n* refers to number of strips. The experiments were terminated by raising the Ca^{2+} concentration to 9 mmol·L^{-1} (Ca^{2+}). PDEi, PDE inhibitor.

ylsulphoxide and Tyrode's solution (20% dimethylsulphoxide in Tyrode's solution). Drugs were added to the organ bath so that the concentration of dimethylsulphoxide was less than 0.1%, which by itself did not modify contractile force.

Results

Cilostamide and rolipram increased sinoatrial rate but did not potentiate the effects of 5-HT in newborn piglets

Rolipram (1 μmol·L^{-1}) significantly ($P < 0.05$, $n = 15$) increased beating rate by 13 ± 3% (Figure 2A–C). Cilostamide (300 nmol·L^{-1}) tended to increase sinoatrial beating rate by 10 ± 4% of the effect of (-)-isoprenaline (200 μmol·L^{-1}) (Figure 2A–C), but the effect did not reach statistical significance ($P = 0.09$, $n = 17$, paired Student's *t*-test). Concurrent rolipram + cilostamide increased sinoatrial rate by 39 ± 12% ($P < 0.05$, $n = 7$) of the effect of (-)-isoprenaline (Figure 2A–C).

5-HT (10 μmol·L^{-1}) elicited tachycardia that was maximal approximately at 1 min and remained stable until the 20th min of observation in the absence and presence of rolipram and cilostamide (Figure 2A).

5-HT caused concentration-dependent increases of sinoatrial rate in the absence and presence of cilostamide, rolipram and concurrent rolipram + cilostamide (Figure 2C). The PDE inhibitors, given separately or in combination, did not significantly change the chronotropic potency of 5-HT (Figure 2D). Rolipram and concurrent rolipram + cilostamide, but not cilostamide, significantly increased the E_{max} of 5-HT (Figure 2C).

Rolipram reduced fade and concurrent rolipram + cilostamide abolished fade of inotropic responses to 5-HT in left atria of newborn piglets

Rolipram (1 μmol·L^{-1}) and cilostamide (300 nmol·L^{-1}) did not significantly increase contractile force but concurrent rolipram + cilostamide caused variable increases in left atrial force with an average of 30 ± 6% of (-)-isoprenaline ($P < 0.001$, $n = 23$, pooled data of Figs 1 and 3). The responses to 1 and 10 μmol·L^{-1} 5-HT were maximal by approximately the second min and faded to 39 ± 7 and 45 ± 2% by the 20th min of administration respectively (Figure 1). Cilostamide did not significantly affect the residual responses at the 20th min to 1 μmol·L^{-1} (54 ± 7%) and 10 μmol·L^{-1} (42 ± 6%) 5-HT, but rolipram significantly enhanced the residual responses to 77 ± 7% ($P < 0.01$) and 67 ± 3% ($P < 0.001$) respectively (Figure 1). Concurrent rolipram + cilostamide nearly abolished fade of the

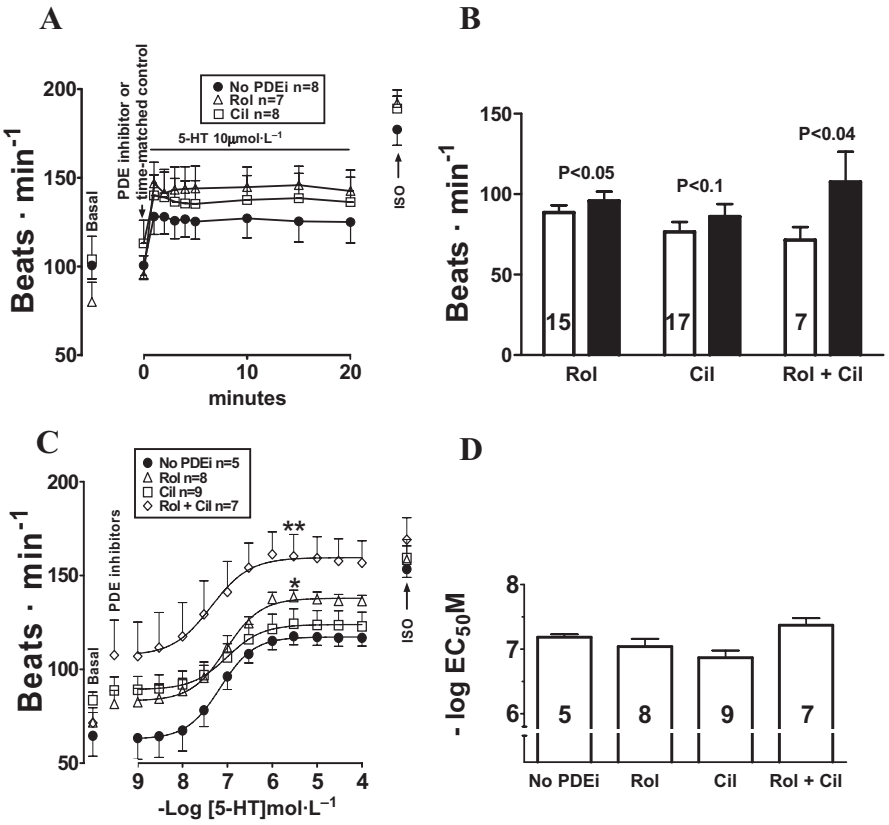

Figure 2 (A) 5-HT induced stable sinoatrial tachycardia on right atria from newborn piglets. (B) Rolipram (Rol) and concurrent rolipram + cilostamide (Rol + Cil) cause sinoatrial tachycardia. Open and black columns represent sinoatrial rate in the absence and presence of PDE inhibitors (PDEi). Data were from A, B or a pool of both. (C) The effects of 5-HT were not potentiated by rolipram, cilostamide or concurrent rolipram + cilostamide. (D) –LogEC$_{50}$M data from the experiments in (C). *$P < 0.05$ E_{max} of 5-HT with rolipram, **$P < 0.01$ E_{max} of 5-HT with concurrent rolipram + cilostamide, compared with the E_{max} of 5-HT in the absence of PDE inhibitors. Numbers in open columns refer to number of right atria.

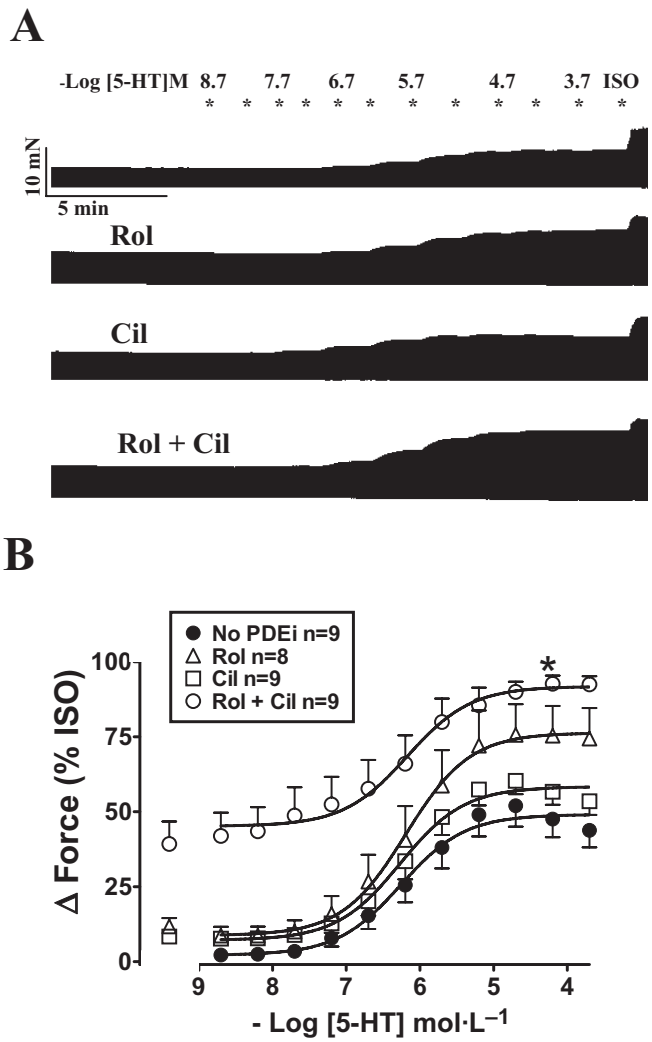

Figure 3 Rolipram and cilostamide, administered separately or in combination, did not potentiate the effects of 5-HT on left atria from newborn piglets. (A) Representative experiment of cumulative concentration–effect curves of 5-HT in the absence and presence of rolipram (Rol), cilostamide (Cil) and concurrent rolipram + cilostamide (Rol + Cil) on four strips from the same left atrium. The experiment was terminated with 200 μmol·L^{-1} (-)-isoprenaline (ISO). (B) Data from n strips of nine piglets. Basal force was 7.0 ± 0.8 mN and force in the presence of (-)-isoprenaline was 14.2 ± 1.5 mN (n = 35 atrial strips).

responses to both 1 μmol·L^{-1} and 10 μmol·L^{-1} 5-HT at the 20th min (91 ± 6 and 87 ± 8%, respectively, of the maximum response) (Figure 1).

Cilostamide and rolipram failed to potentiate the inotropic effects 5-HT in left atria from newborn piglets

Cumulative concentration–effect curves to 5-HT were not significantly shifted to the left by rolipram, cilostamide and concurrent rolipram + cilostamide. The potency of 5-HT was not significantly changed (Figure 3B). –LogEC$_{50}$ values for 5-HT were 6.04 ± 0.21 (n = 9), 6.04 ± 0.22 (n = 8), 6.20 ± 0.14 (n = 9) and 6.44 ± 0.24 (n = 9) in the absence of PDE inhibitors and presence of rolipram, cilostamide and concurrent rolipram + cilostamide respectively. Concurrent rolipram + cilostamide increased the E_{max} of 5-HT with respect to the effect of (-)-isoprenaline (Figure 3B).

Concurrent rolipram + cilostamide reduced fade of both the inotropic response and cAMP response to 5-HT in left atria from newborn piglets

Two minutes after administration, 5-HT (10 μmol·L^{-1}) increased contractile force in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide by 3.9 ± 0.6, 9.2 ± 1.0, 9.8 ± 1.2 and 8.5 ± 1.3 mN respectively (Figure 4). The increase in force by the second min of 5-HT administration was significantly greater (ANOVA) in the presence of rolipram (P < 0.05), cilostamide (P < 0.01) and concurrent rolipram + cilostamide (P < 0.05) compared with the absence of PDE inhibitors (Figure 4). Twenty minutes after administration, the increases in force by 5-HT were reduced to (mN) –0.6 ± 0.3, 4.5 ± 0.8 (P < 0.01), 3.5 ± 0.8 (P < 0.05) and 4.7 ± 0.9 (P < 0.01) in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide respectively. The inotropic responses to 5-HT at the 20th min in the separate presence of rolipram and cilostamide, but not in the presence of concurrent rolipram + cilostamide, were significantly smaller (P < 0.05, Figure 4) than the corresponding response to 5-HT at the second min of administration.

Rolipram, cilostamide and concurrent rolipram + cilostamide did not significantly increase left atrial cAMP levels (ANOVA) (Figure 4). At the second min of administration, 5-HT (10 μmol·L^{-1}) significantly increased the cAMP levels in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide by 66%, 46%, 59% and 173% of basal cAMP levels respectively (Figure 4). The cAMP levels in the presence of both 5-HT (second min) and concurrent rolipram + cilostamide were significantly higher (P < 0.05) than in the presence of 5-HT alone (Figure 4A,D). At the 20th min of administration, 5-HT (10 μmol·L^{-1}) increased the cAMP levels in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide by 22%, 13%, 17% and 124% of basal cAMP levels respectively (Figure 4). Only the cAMP level in the presence of both 5-HT (20th min) and concurrent rolipram + cilostamide was significantly higher (P < 0.01) than in the presence of 5-HT alone (Figure 4A,D).

Rolipram, but not cilostamide, markedly increased the left atrial cAMP signal produced by (-)-isoprenaline in newborn piglets

(-)-Isoprenaline, incubated for 2 min, increased the cAMP level approximately twofold in the absence or presence of cilostamide. The effects of (-)-isoprenaline on cAMP levels in the absence and presence of cilostamide were not significantly different to the corresponding effects of 5-HT (10 μmol·L^{-1}) at the second min of administration (Figure 4A,C). In contrast, with rolipram, the cAMP levels in the presence of (-)-isoprenaline were considerably higher (P < 0.02) than in the presence of 5-HT. With concurrent rolipram + cilostamide, the cAMP levels in the presence of (-)-isoprenaline were also markedly higher (P < 0.001) than in

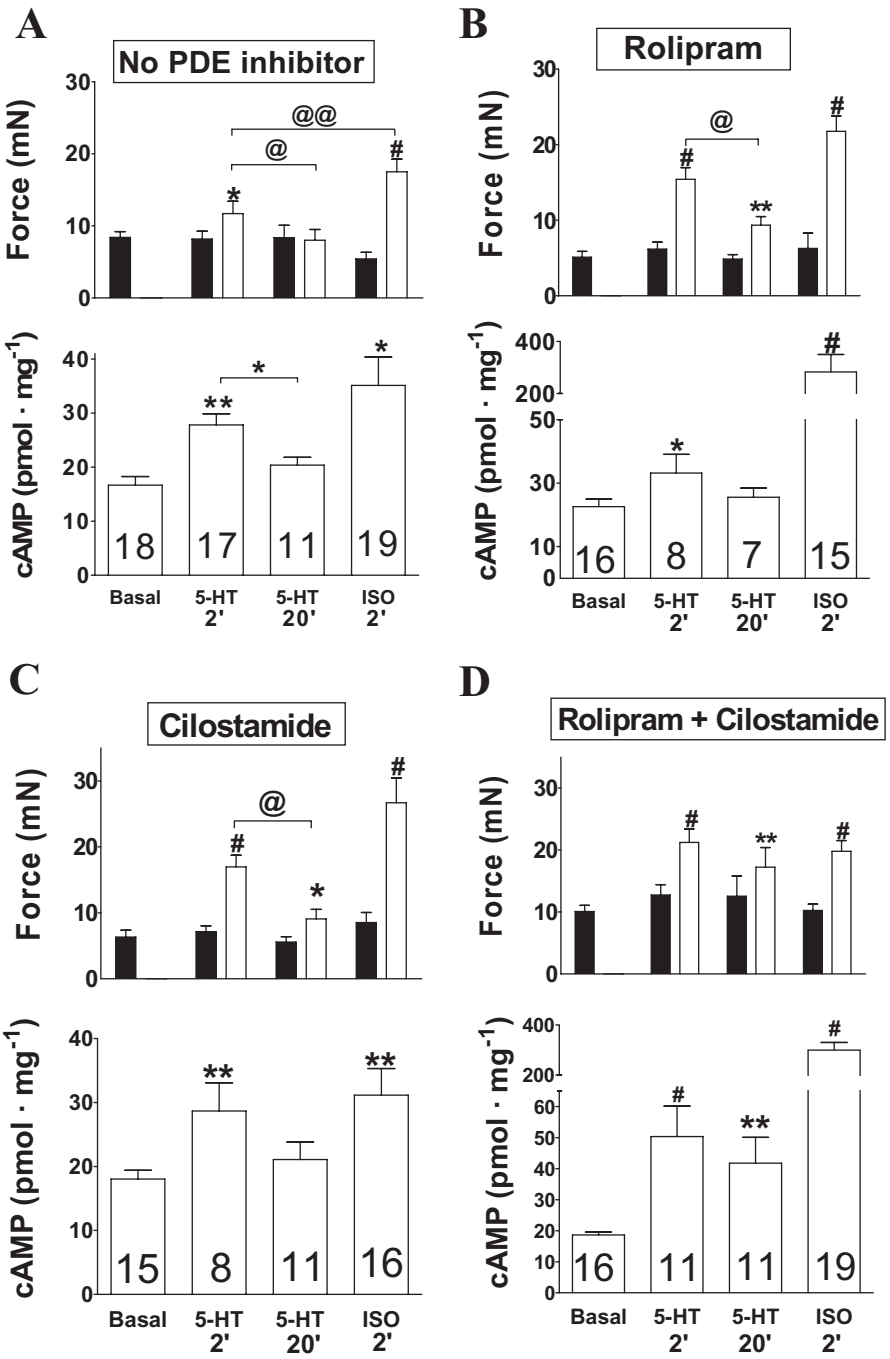

Figure 4 Fade of the inotropic and cAMP responses to 5-HT in left atria from newborn piglets in the absence (A) and presence of rolipram (1 μmol·L^{-1}, B) and cilostamide (300 nmol·L^{-1}, C), but lack of fade with concurrent rolipram + cilostamide (D). Top and bottom columns of each panel represent contractile force data and cAMP data from the same tissues. Black columns: contractile force in the absence of 5-HT. Top open columns of each panel represent the inotropic effects of 5-HT (10 μmol·L^{-1}) at the second min and 20th min, and of (-)-isoprenaline (200 μmol·L^{-1}, ISO). Numbers in each open column refers to number of atrial strips from at least five piglets. *$P < 0.05$, **$P < 0.01$, #$P < 0.001$ compared with the absence of 5-HT or (-)-isoprenaline (paired Student's *t*-test). $^{@}P < 0.05$ between the response to 5-HT at the second min and 20th min of administration (unpaired Student's test). $^{@@}P < 0.02$ between the response to 5-HT at the second min and the response to (-)-isoprenaline. Please note the expansion of the cAMP ordinate for (-)-isoprenaline (200 μmol·L^{-1}, ISO) in (B) and (D).

the presence of 5-HT (Figure 4B,D), but virtually the same as with rolipram alone. The (-)-isoprenaline-evoked increases of contractile force were not significantly different in the absence and presence of rolipram, cilostamide and concurrent rolipram + cilostamide (Figure 4A–D).

5-HT accelerated the onset of relaxation in left atria of newborn piglets

Both 5-HT (10 μmol·L^{-1}) and (-)-isoprenaline (200 μmol·L^{-1}) shortened the time to peak force by the second min of administration, but the effect of 5-HT was significantly smaller

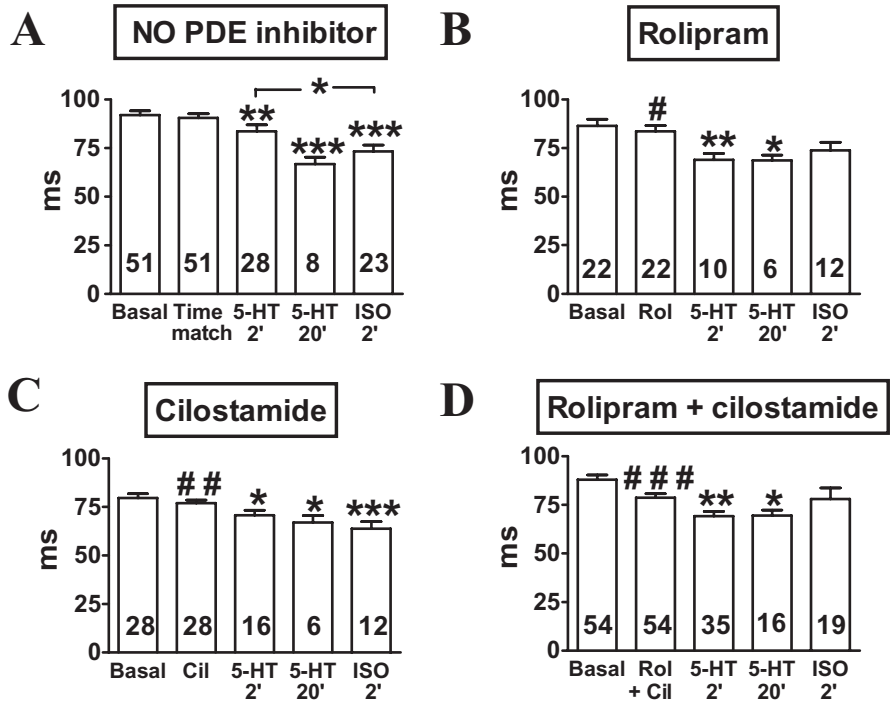

Figure 5 Faster onset of atrial relaxation by 5-HT (10 μmol·L^{-1}) (A) in newborn piglets. Effects of rolipram (1 μmol·L^{-1}, Rol) (B), cilostamide (300 nmol·L^{-1}, Cil) (C) and concurrent rolipram + cilostamide (Rol + Cil) (D), incubated for 30 min, and comparison with (-)-isoprenaline (200 μmol·L^{-1}, ISO). Data shown are means (± SEM) of time to peak force measurements. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with respect to time-matched control or after 30 min incubation with indicated phosphodiesterase inhibitors. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ with respect to basal.

(Figure 5A). Rolipram, cilostamide and concurrent rolipram + cilostamide also shortened time to peak force (Figure 5B–D). In the presence of rolipram, cilostamide and concurrent rolipram + cilostamide 5-HT caused shortenings of time to peak force (Figure 5B–D), which were not significantly different from the effects of (-)-isoprenaline under these conditions (Figure 5B–D). The shortening of the time to peak force caused by 5-HT persisted by the 20th min in the absence and presence of the PDE inhibitors (Figure 5A–D).

Cilostamide but not rolipram disclosed 5-HT responses in ventricular trabeculae from newborn piglets

5-HT did not enhance contractile force of ventricular trabeculae from newborn piglets unless all PDE activity was inhibited with IBMX (Brattelid *et al.*, 2004). Cilostamide and concurrent rolipram + cilostamide, but not rolipram alone, disclosed significant inotropic responses to 5-HT (Figure 6A). However, the response to 5-HT in the presence of concurrent rolipram + cilostamide was not significantly greater than in the presence of cilostamide alone ($P = 0.2$).

Concurrent rolipram + cilostamide, but not rolipram or cilostamide alone, disclosed 5-HT responses in ventricular trabeculae from adolescent pigs

5-HT does not increase contractions of ventricular trabeculae from newborn and adult pigs unless PDEs are inhibited with IBMX (Brattelid *et al.*, 2004). Rolipram and cilostamide separately did not change force, but concurrent rolipram + cilostamide significantly increased force (Figure 6B). In the separate presence of either rolipram or cilostamide, 5-HT failed to increase force (Figure 6B). In contrast, concurrent rolipram + cilostamide allowed 5-HT to significantly increase force (Figure 6B).

Cilostamide reduced fade, and concurrent rolipram + cilostamide abolished fade of the response to 5-HT in atria from adolescent pigs

The data are shown in Figure 7. Rolipram alone did not significantly increase contractile force. Cilostamide tended to increase force but the effect was not significant ($P = 0.11$, Student's paired *t*-test). Concurrent rolipram + cilostamide increased force by 31 ± 12% of the effect of 200 μmol·L^{-1} (-)-isoprenaline ($P < 0.01$). The positive inotropic response to 5-HT faded and disappeared between the 20th min and 30th min of administration. In the separate presence of rolipram and cilostamide, 20.2 ± 8.8% ($P = 0.19$) and 30.6 ± 9.7% ($P < 0.04$) of the initial response were observed, respectively, by the 30th min of administration. Concurrent rolipram + cilostamide completely prevented fade of the 5-HT response ($P < 0.001$).

Cilostamide but not rolipram partially prevented fade of the inotropic response to 5-HT in human atrial trabeculae

Rolipram and cilostamide, administrated separately or together, did not significantly change atrial force. Force (mN) was 3.4 ± 0.5 ($n = 33$ trabeculae), 4.4 ± 1.0 ($n = 10$), 4.3 ± 0.9 ($n = 10$) and 2.7 ± 0.9 ($n = 5$) in the absence and presence of

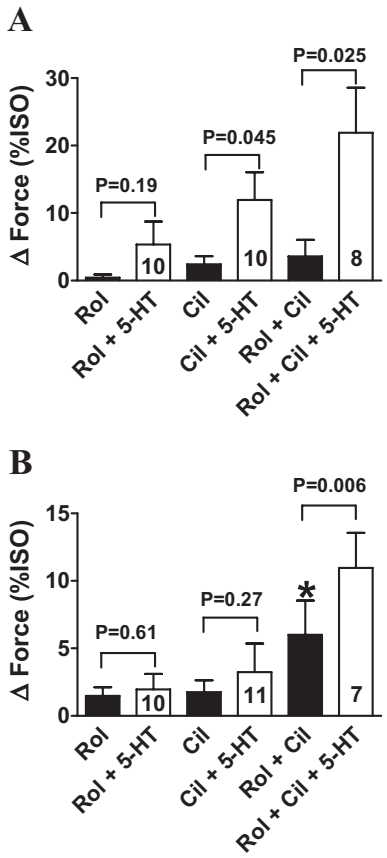

Figure 6 Cilostamide (300 nmol·L^{-1}, Cil) disclosed ventricular 5-HT responses in newborn piglets (A) but not in adolescent pigs (B). 5-HT enhances ventricular force in the presence of concurrent rolipram (1 μmol·L^{-1}, Rol) + cilostamide in both newborn piglets (A) and adolescent pigs (B). Basal force and force in the presence of (-)-isoprenaline (200 μmol·L^{-1}, ISO) were 2.7 ± 0.5 and 10.0 ± 1.7 mN, respectively, in newborn (28 trabeculae from 20 piglets), and 2.5 ± 0.5 and 10.1 ± 1.1 mN in adolescents (28 trabeculae from 10 pigs). *$P < 0.05$ with respect to absence of rolipram. Open and closed columns summarize data in the presence and absence of 5-HT (10 μmol·L^{-1}).

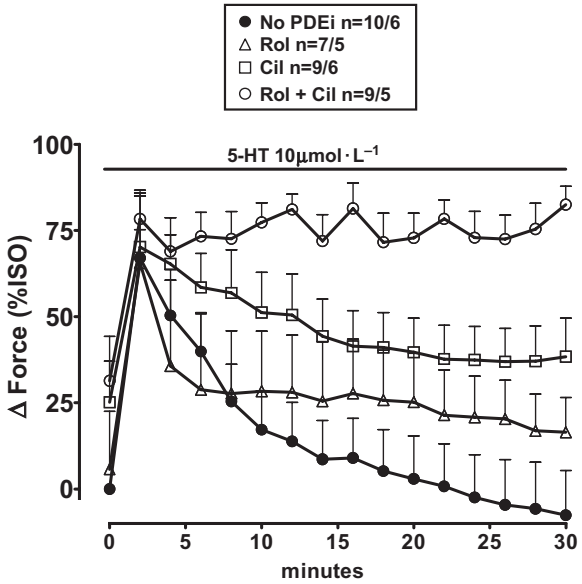

Figure 7 Fade of the inotropic response to 5-HT (10 μmol·L^{-1}) in the absence and presence of phosphodiesterase inhibitors (PDEi) on left atrial trabecule of adolescent pigs. Marginal partial reduction of fade by rolipram (Rol), partial reduction by cilostamide (Cil) and prevention of fade by concurrent rolipram + cilostamide (Rol + Cil). Basal force and force in the presence of (-)-isoprenaline was 2.5 ± 0.3 and 5.4 ± 0.7 respectively (35 trabeculae from 9 pigs).

rolipram, cilostamide and concurrent rolipram + cilostamide respectively. The inotropic response to 5-HT (1 μmol·L^{-1}) was approximately maximal at the third min and faded to 15 ± 6% and 14 ± 5% by the 60th min of administration in the absence and presence of rolipram respectively (Figure 8). Cilostamide but not rolipram partially reduced fade of the 5-HT response. Concurrent rolipram + cilostamide did not reduce fade more than cilostamide. In the presence of cilostamide and concurrent rolipram + cilostamide, the response to 5-HT faded at the 60th min to 54 ± 5% and 61 ± 4% of the maximum response ($P < 0.001$), compared with fade in the absence and presence of rolipram.

Discussion

Our main findings were: (i) PDE3 and PDE4 reduced heart rate but neither isoenzyme affected the stable tachycardia elicited by 5-HT, mediated through sinoatrial 5-HT$_4$ receptors of newborn piglets; (ii) the fade of the porcine atrial inotropic responses and cAMP responses to 5-HT was caused by both PDE3 and PDE4; (iii) the control of atrial 5-HT responses by PDEs switched from a predominant role of PDE4 in newborn piglet to a predominant role of PDE3 in adolescent pigs; (iv) in porcine ventricle, PDE3 prevented 5-HT responses in newborn but, PDE3 and PDE4, acting in concert, prevented 5-HT responses in adolescents; and (v) fade of human atrial responses to 5-HT was due to the action of PDE3, but not that of PDE4.

PDE3 and PDE4 reduced sinoatrial beating rate but did not modulate 5-HT$_4$ receptor-mediated tachycardia

High cAMP levels and activity of cAMP-dependent PKA of sinoatrial cells are obligatory for the maintenance of basal heart beat (Vinogradova *et al.*, 2006). Partial inhibition of PKA (Maltsev *et al.*, 2006; Vinogradova *et al.*, 2006) or reducing sinoatrial cAMP levels through PDE-catalysed hydrolysis (Vinogradova *et al.*, 2008) slows sinoatrial beating rate. The PDE isoenzymes that control sinoatrial rate depend on species, PDE3 in the rabbit (Vinogradova *et al.*, 2008), PDE4 in the rat (Christ *et al.*, 2008) and both PDE3 and PDE4 in the mouse (Galindo-Tovar and Kaumann, 2008). The sinoatrial tachycardia produced by concurrent rolipram + cilostamide in newborn piglets was threefold greater than the tachycardia produced by each PDE inhibitor alone (Figure 2B). Therefore, both PDE3 and PDE4, acting in concert, reduced sinoatrial beating rate in newborn piglets.

In agreement with a similar observation by De Maeyer *et al.* (2006), we found that 5-HT-evoked tachycardia did not fade. Sinoatrial tachycardia elicited by 5-HT in newborn piglets is mediated through 5-HT$_4$ receptors (Kaumann, 1990) and, as

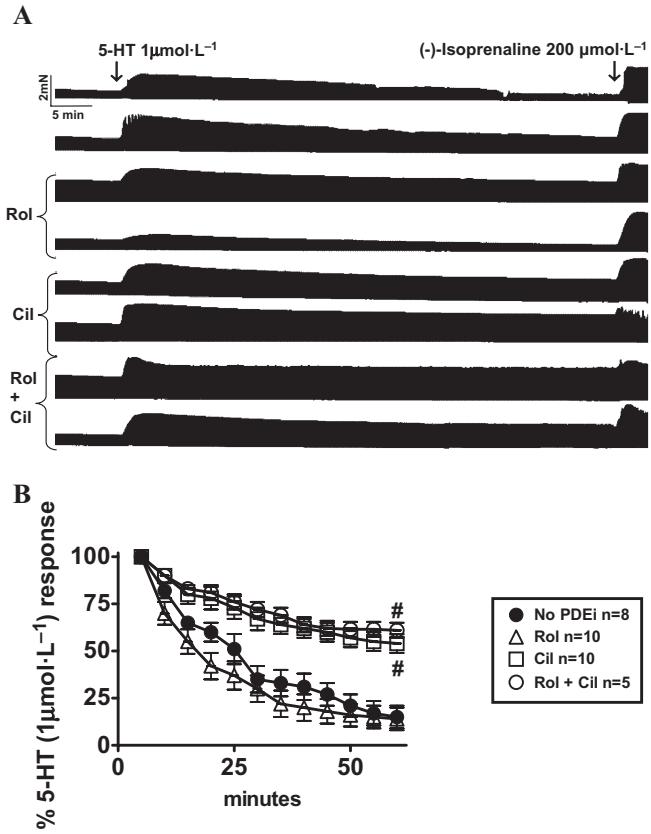

Figure 8 Cilostamide, but not rolipram, partially prevents fade of the inotropic response to 5-HT in human atrial trabeculae. (A) Representative experiment on eight trabeculae from a 73-year-old man with aortic insufficiency without heart failure (ejection fraction 63%). Pairs of trabeculae were set up into the same organ bath, in the absence and presence of rolipram (1 μmol·L^{-1}, Rol), cilostamide (300 nmol·L^{-1}, Cil) and concurrent rolipram + cilostamide. After 1 h incubation with 5-HT (1 μmol·L^{-1}), (-)-isoprenaline (ISO) was administered. (B) Data from the fade of the response to 5-HT in the absence and presence of indicated PDE inhibitors. Basal contractile force was 3.4 ± 0.5 mN, and force in the presence of (-)-isoprenaline 7.2 ± 0.8 mN (n = 33 trabeculae). Number of trabeculae (n) from 10 patients.

these receptors are coupled to G_s proteins, they are assumed to mediate a rise of cAMP levels in the neighbourhood of both the receptors and ion channels critically involved in increasing pacemaker firing rate. Atrial channel currents that increase upon 5-HT$_4$ receptor stimulation include the L-type Ca^{2+} channel with an obligatory participation of PKA (Ouadid *et al.*, 1992) and the pacemaker current I_f, activated by hyperpolarization (Pino *et al.*, 1998). The stable tachycardia induced by 5-HT is probably due to the absence of PDE activity in the sinoatrial cAMP pool involved in the increase of pacemaker rate through 5-HT$_4$ receptors. The lack of potentiation of the chronotropic effects by rolipram, cilostamide and concurrent rolipram + cilostamide (Figure 2) supports the hypothesis that the cAMP pool that governs 5-HT$_4$ receptor-mediated sinoatrial tachycardia is protected from PDE3 and PDE4 and represents a compartment that is distinct from the cAMP compartment in which both PDE3 and PDE4 reduce basal sinoatrial beating. The increases in E_{max} of 5-HT in the presence of rolipram and concurrent rolipram + cilostamide (Figure 2C) could merely be due to additivity of the tachycardia caused by 5-HT and the PDE inhibitors.

The distinct compartmentalization of cAMP between basal sinoatrial rate, controlled by PDEs, and 5-HT$_4$ receptor-mediated tachycardia, unaffected by PDEs in newborn piglets, resembles similar situations for β_1-adrenoceptor-evoked tachycardia. Basal sinoatrial beating rate is reduced by both PDE3 and PDE4 in the mouse (Galindo-Tovar and Kaumann, 2008) and by PDE4 in the rat (Christ *et al.*, 2008), but in contrast, (-)-noradrenaline-evoked tachycardia, mediated through β_1-adrenoceptors, is minimally affected by PDE3 and PDE4 activity in these species.

The fade of the inotropic and cAMP responses to 5-HT in left atria from newborn piglets was caused by PDE3 and PDE4 acting in concert

The positive inotropic response to 5-HT (10 μmol·L^{-1}) by the second min of exposure was accompanied by an increase in the atrial cAMP level (Figure 4A). The fade of the inotropic response to 5-HT by the 20th min was mirrored by the fade of the cAMP response (Figure 4A) and concurrent rolipram and cilostamide prevented both fades (Figure 4D). This evidence indicates that PDE3 and PDE4 jointly reduced cAMP generated through 5-HT$_4$ receptor activation. However, are the measured atrial cAMP signals inotropically relevant?

In two situations there were discrepancies between the cAMP level and inotropic response to 5-HT (10 μmol·L^{-1}). First, cilostamide induced 5-HT to produce a significantly greater inotropic response by the second min of administration, compared with the absence of cilostamide, but the 5-HT-evoked cAMP signal was not increased. Furthermore, by the 20th min, when the cAMP response to 5-HT had completely faded, there was still a residual inotropic response in the presence of cilostamide (Figure 4C). In the second situation, the inotropic response to 5-HT by the second min, but not the cAMP response, was increased by rolipram, compared with the absence of rolipram (Figure 4A,B). Moreover, although rolipram reduced the inotropic fade of the 5-HT response by the 20th min, the corresponding cAMP signal disappeared (Figure 4B).

These discrepancies between inotropic responses and cAMP levels are inconsistent with the assumption that the measured cAMP levels reflect cAMP surges in inotropically relevant compartments. Clearly, hypothetical increases of cAMP that activate PKA in small compartments, such as the 5-HT$_4$ receptor/L-type Ca^{2+} channel domain or a domain in the vicinity of the membrane of the sarcoplasmic reticulum (SR), have escaped detection. PDE3 and PDE4 are mainly associated with the SR and sarcolemma respectively (Lugnier *et al.*, 1993, Lugnier, 2006). PKA-catalysed phosphorylation of phospholamban (PLB) dis-inhibits the SR Ca^{2+}-ATPase, which then pumps Ca^{2+} faster back into the SR, lowering sarcoplasmic Ca^{2+} with consequent faster relaxation of contractile proteins (MacLennan and Kranias, 2003). The reduction of the time to peak force observed with 5-HT on left atria from newborn piglets (Figure 5) is consistent with a faster onset of relaxation due to PKA-catalysed PLB phosphorylation and phosphorylation of other proteins involved in the temporal control of relaxation, such as troponin-I (Garvey *et al.*, 1988). Hypothetical cAMP changes in left

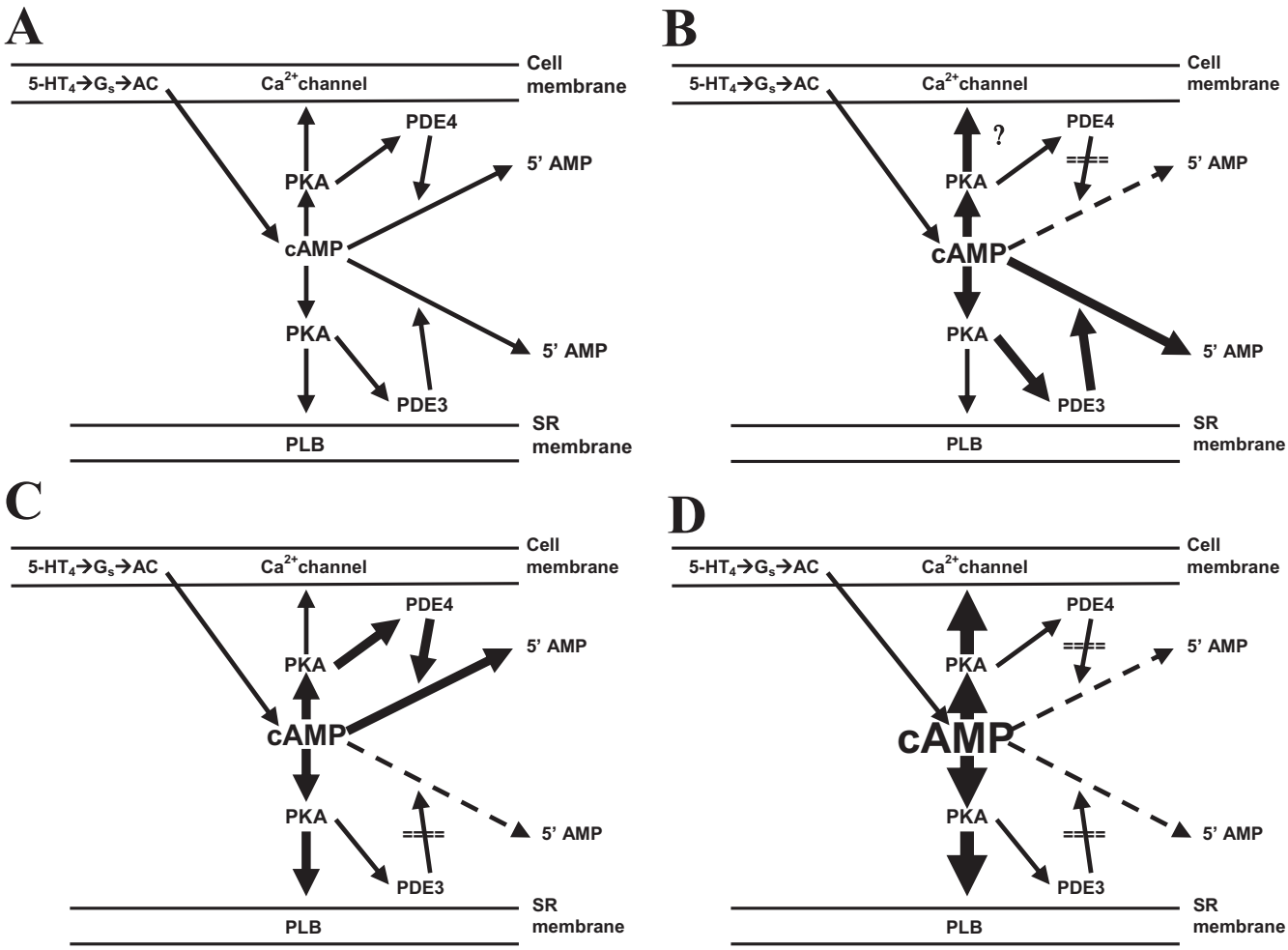

Figure 9 Schematic illustration of the decreases by phosphodiesterases PDE3 and PDE4 of hypothetical cAMP surges caused by 5-HT_4 receptor (5-HT_4) stimulation, subsequent coupling to Gs protein and activation of adenylyl cyclase (AC) in left atrial cells of newborn pigs. (A) PDE3 is assumed to hydrolyse cAMP mainly in the neighbourhood of the sarcoplasmic reticulum (SR), thereby partially reducing activation of protein kinase (PKA) and PKA-catalysed phosphorylation of phospholamban (PLB). PDE4 is assumed to hydrolyse cAMP mainly in the neighbourhood of the L-type Ca^{2+} channel, thereby partially preventing activation of PKA and PKA-dependent phosphorylation of the Ca^{2+} channel. (B) Inhibition of PDE4 would increase cAMP- and PKA-dependent phosphorylation of the Ca^{2+} channels, thereby initially enhancing Ca^{2+}-induced Ca^{2+} release and transiently increase contractility. The increased cAMP would also cause PKA-catalysed phosphorylation and activation of PDE3, thereby reducing cAMP at the PLB domain and blunting the inotropic response to 5-HT. (C) Inhibition of PDE3 would increase cAMP and PKA-dependent phosphorylation of PLB, thereby augmenting contractility and accelerating the onset of relaxation. The increased cAMP would also cause PKA-catalysed phosphorylation and activation of PDE4, thereby reducing cAMP at the Ca^{2+} channel domain, decreasing Ca^{2+}-induced Ca^{2+} release and contractility. (D) Inhibition of both PDE3 and PDE4 maintains stable cAMP concentrations in inotropically relevant compartments, thereby allowing sustained inotropic responses to 5-HT.

atria of newborn piglets,observed during 5-HT_4 receptor activation in the absence and presence of PDE inhibitors are represented in Figure 9.

Inhibition of PDE4 with rolipram would increase cAMP and enhance PKA-dependent phosphorylation of the Ca^{2+} channels (Ouadid *et al.*, 1992), thereby initially stimulating Ca^{2+}-induced Ca^{2+} release (Fabiato, 1985) and transiently increase contractility (Figure 9B) as observed experimentally with 5-HT (second min) in Figure 4B. The increased cAMP could also cause PKA-catalysed phosphorylation and activation of PDE3 (Gettys *et al.*, 1987), thereby reducing cAMP at the PLB domain and blunting the inotropic response to 5-HT.

After inhibition of PDE3 with cilostamide, the contractile response to 10 $\mu mol \cdot L^{-1}$ 5-HT was increased (Figure 4C) at the second min of administration and the onset of relaxation, compared with (-)-isoprenaline, further shortened (Figure 5C), consistent with enhanced PLB phosphorylation (Figure 9C). However, the inotropic response to 5-HT faded by the 20th min even in the presence of cilostamide (Figs 1 and 4c). Furthermore, the inotropic potency of 5-HT in the presence of cilostamide, estimated from cumulative concentration–effect curves to 5-HT, was not enhanced compared with the absence of cilostamide (Figure 3). The persistence of the fade of the inotropic response to 5-HT, despite the inhibition of PDE3 and the early enhanced inotropic response to 10 $\mu mol \cdot L^{-1}$ 5-HT, suggests a time-dependent increase of cAMP hydrolysis. We propose that the persistent fade is due to accumulation of cAMP in a PDE3-inhibited compartment, followed by leak into compartments where

PKA can be activated to phosphorylate and stimulate PDE4 (MacKenzie *et al.*, 2002), thereby again reducing inotropically relevant cAMP. The activation of PDE4 and reduction of inotropically relevant cAMP take time, and are assumed to occur at several increasing 5-HT concentrations during the cumulative concentration–effect curve (Figure 3). The net effect of PDE4 activation in the presence of cilostamide is a lack of potentiation of the effects of 5-HT observed in Figure 3.

The fade of the inotropic cAMP responses to 5-HT was prevented by concurrent rolipram + cilostamide, consistent with the assumption that the combined activities of PDE3 and PDE4 converge to reduce cAMP in cell compartments that contribute to the increase in contractile force through activation of 5-HT$_4$ receptors (Figure 9D). cAMP may have overflowed from inotropically relevant compartments and flooded the sarcoplasm, consistent with the enhanced cAMP signals measured under concurrent rolipram + cilostamide (Figure 4D), compared with the absence of PDE inhibitors or separate presence of rolipram and cilostamide (Figure 4A–C).

In the absence of PDE inhibitors, 5-HT (2 min) accelerated the onset of relaxation, although less than (-)-isoprenaline (Figure 5A), consistent with PKA-dependent phosphorylation of PLB. This acceleration of the onset of relaxation by 5-HT and (-)-isoprenaline were, however, not significantly different in the presence of cilostamide (Figure 5C), rolipram (Figure 5B) or concurrent rolipram + cilostamide (Figure 5D). Interestingly, unlike the inotropic response to 5-HT that faded by the 20th min in the absence and presence of PDE inhibitors, the faster onset of relaxation persisted (Figure 5A–D), probably reflecting slow reversal kinetics due to dephosphorylation of some proteins involved in this lusitropic effect (e.g. troponin-I) (England, 1976; Perry, 1979; Garvey *et al.*, 1988).

The (-)-isoprenaline-evoked cAMP signal was greatly reduced by PDE4 but not by PDE3 in left atria of newborn piglets

The twofold (-)-isoprenaline-evoked increase in cAMP was not affected by cilostamide, but was approximately 10-fold greater in the presence of rolipram or concurrent rolipram + cilostamide (Figure 4). Therefore, unlike the 5-HT$_4$ receptor-mediated increase of cAMP that is hydrolysed jointly by PDE3 and PDE4, the β-adrenoceptor-mediated increase of cAMP appears to be hydrolysed exclusively by PDE4. The β-adrenoceptor subtype that mediates the effects of (-)-isoprenaline is still unknown, but probably is mainly the β_1-adrenoceptor.

The positive inotropic responses to (-)-isoprenaline were not significantly different in the absence and presence of the PDE inhibitors, presumably because at the high concentration used (200 μmol·L^{-1}), the contractile system was saturated at each condition.

Ontogenic regional changes of the function of porcine PDE3 and PDE4

Early reports failed to find evidence for 5-HT-evoked increases of force in porcine (Schoemaker *et al.*, 1992) and human ventricles (Jahnel *et al.*, 1992; Schoemaker *et al.*, 1993). However, mRNA for the 5-HT$_4$ receptor splice variants 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ receptors was detected in human ventricle by Bach *et al.* (2001). More recently, Brattelid *et al.* (2004) provided evidence for functional ventricular 5-HT$_4$ receptors in newborn piglets, adult pigs and humans. They found that 5-HT increased ventricular force in the presence of the non-selective PDE inhibitor IBMX, suggesting that PDEs protect the ventricular myocardium against 5-HT$_4$ receptor-mediated stimulation, but the PDE isoenzymes involved were not identified. Here, we found that in newborn piglets, but not adolescent pigs, cilostamide discloses increases of ventricular contractile force with 5-HT. Concurrent rolipram + cilostamide caused 5-HT to produce significant increases of ventricular force in adolescent pigs, suggesting that the preferential control by PDE3, found in newborn pigs, was lost in the adolescent hearts and that PDE3 and PDE4, acting in concert, suppressed the responses to 5-HT.

Curiously, in left atria there was also a change of the role of PDE3 and PDE4 with age, but somewhat opposite to the change observed in porcine ventricle. Rolipram partially prevented the fade of the inotropic response to 5-HT in atria from newborn, but not in atria from adolescent pigs. Conversely, cilostamide did not prevent fade of the inotropic response to 5-HT in newborn but reduced fade in adolescents. The mechanism of these age-dependent changes in the relative role of PDE3 and PDE4 in reducing inotropically relevant cAMP pools is unknown. Cardiac PDE activities have been reported to be markedly decreased in the left ventricle of 150-day-old pigs compared with newborn piglets (Mersmann *et al.*, 1977). However, we found that the fade of the atrial inotropic 5-HT response was greater in adolescent pigs (≈100 days of age) than in newborn piglets, actually suggesting greater activities of both PDE3 and PDE4 in the former than in the latter.

Concurrent rolipram + cilostamide prevented the fade of both the inotropic and cAMP responses to 5-HT in newborn piglets, as well as inotropic fade in adolescent pigs. These results suggest that fade after 5-HT$_4$ receptor stimulation is mostly produced by the activities of PDE3 and PDE4, but not by other PDE isoenzymes. Activation of 5-HT$_4$ receptors produces rapid desensitization in several systems, as observed with the adenylyl cyclase responses to 5-HT in neurones of murine colliculi (Ansanay *et al.*, 1995), rat oesophagus (Ronde *et al.*, 1995) and recombinant receptors (Barthet *et al.*, 2005; Ponimaskin *et al.*, 2005). However, when both PDE3 and PDE4 were inhibited, responses to 1 and 10 μmol·L^{-1} 5-HT were sustained during 20–30 min agonist exposure, inconsistent with 5-HT$_4$ receptor desensitization.

PDE3, but not PDE4, induced fade of the inotropic response to 5-HT in human atrium

The inotropic response to 5-HT partially faded in human atrial trabeculae. Cilostamide but not rolipram reduced the fade, consistent with the hypothesis that PDE3 activity, but not PDE4, hydrolyses inotropically relevant cAMP, thereby contributing to tachyphylaxis. Concurrent rolipram + cilostamide did not reduce fade more than cilostamide alone, but a residual fade persisted under these two conditions (Figure 8). The residual fade could be produced by additional PDEs. However, the non-selective PDE inhibitor

IBMX (10 μmol·L^{-1}) does not potentiate more than cilostamide the positive inotropic effects of 5-HT on human atrial trabeculae (A. Kaumann, unpubl. experiments), consistent with an involvement of PDE3 but not of additional PDEs. By exclusion, the small cilostamide-resistant fade of the human atrial 5-HT responses could be due to 5-HT$_4$ receptor desensitization.

PDE3, but not PDE4, is also responsible for the fade of inotropic responses to (-)-noradrenaline and (-)-CGP12177, mediated through β_1-adrenoceptors in human atrium (Kaumann *et al*., 2007). Furthermore, the positive inotropic responses to (-)-adrenaline, mediated through human atrial β_2-adrenoceptors, are only potentiated by cilostamide but not by rolipram (Christ *et al*., 2006). Thus, unlike effects mediated through porcine 5-HT$_4$ receptors, which are blunted by both PDE3 and PDE4, and porcine β-adrenoceptors (β_1?) controlled by PDE4, effects mediated through human Gs protein-coupled receptors appear to be controlled only by PDE3. This conclusion is reinforced by the observation that in human atria, concurrent rolipram + cilostamide does not reduce more than cilostamide alone, the fade of the inotropic responses to 5-HT through 5-HT$_4$ receptors (this work) or to (-)-noradrenaline and (-)-CGP12177 through β_1-adrenoceptors (Kaumann *et al*., 2007).

5-HT$_4$ receptors of porcine cardiac tissues have been used as a model for human cardiac 5-HT$_4$ receptors (Kaumann, 1990; Kaumann and Levy, 2006b). However, there are some important differences of 5-HT$_4$ receptor function in the two species. The density of 5-HT$_4$ receptors is 10-fold lower in porcine atrium (Kaumann *et al*., 1995) than human atrium (Kaumann *et al*., 1996) and there are fundamental differences between the 5-HT$_4$ receptor splice variants between the two species (De Maeyer *et al*., 2008). Our present work, showing that both PDE3 and PDE4 control porcine cardiac 5-HT responses but only PDE3 controls human atrial 5-HT responses, adds another functional difference between porcine and human 5-HT$_4$ receptors. Therefore, the use of porcine cardiac 5-HT$_4$ receptors as a model of human cardiac 5-HT$_4$ receptors has to be treated with caution.

Conclusions

5-HT caused sustained sinoatrial tachycardia. PDE3 and PDE4 reduced sinoatrial beating rate but did not potentiate the 5-HT-evoked sinoatrial tachycardia, suggesting that these PDEs reduce the cAMP responsible for basal heart rate but do not have access to a cAMP compartment in the vicinity of the 5-HT$_4$ receptors. PDE3 and PDE4 jointly prevent the fade of the inotropic and cAMP responses to 5-HT in left atria of both newborn and adolescent pigs, making unlikely any involvement of additional PDEs. However, while left atrial PDE4 was more active in the newborn, PDE3 was more active in adolescents. For still unknown reasons, there is a selective suppression of the ventricular 5-HT responses by PDE3 in newborn pigs that changes into a joint control by both PDE3 and PDE4 in older pigs. The inotropic response to 5-HT in human atrium partially fades but, unlike porcine myocardium, fade was caused only by PDE3 and not by PDE4.

Acknowledgements

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Conflicts of interest

The authors state no conflicts of interest.

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1.4 Phosphodiesterases PDE3 and PDE4 jointly control the inotropic effects but not chronotropic effects of (-)-CGP12177 despite PDE4-evoked sinoatrial bradycardia in rat atrium.

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SHORT COMMUNICATION

Phosphodiesterases PDE3 and PDE4 jointly control the inotropic effects but not chronotropic effects of (−)-CGP12177 despite PDE4-evoked sinoatrial bradycardia in rat atrium

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Abstract Acting through a low-affinity site of the β_1-adrenoceptor (β_{1L}AR), CGP12177 causes sinoatrial tachycardia and positive inotropic effects in left atrium but not in the ventricle of the rat. However, inhibition of either PDE3 or PDE4 also uncovers positive inotropic effects of CGP12177 in ventricle, but whether these phosphodiesterases also control the atrial agonist effects of CGP12177 was unknown. We, therefore, investigated the effects of the PDE3-selective inhibitor cilostamide (300 nM) and PDE4 inhibitor rolipram (1 μM) on the (−)-CGP12177-evoked increases of sinoatrial beating rate and force of paced left atria of the rat. Rolipram (n=8) increased basal sinoatrial rate by 27±5 bpm but cilostamide (n=8) had no effect. The chronotropic potency of (−)-CGP12177 (−logEC_{50}M=7.5) was not changed by rolipram and cilostamide or their combination. (-)-CGP12177 increased left atrial force with intrinsic activity 0.25 compared to (-)-isoprenaline. Rolipram (n=8) and cilostamide (n=8) did not change basal force of left atria but concurrent rolipram+cilostamide (n=8) increased force by 52±9% of the effect of 200 μM (−)-isoprenaline. Neither rolipram nor cilostamide affected the inotropic potency of (−)-CGP12177 (−logEC_{50}M=7.4) but concurrent rolipram+cilostamide caused potentiation (−logEC_{50}M=8.2) and converted (-)-CGP12177 into a full agonist compared to (-)-isoprenaline. Cyclic AMP appears to maintain sinoatrial rate and PDE4 elicits bradycardia through hydrolysis of cAMP in a compartment distinct from the β_{1L}AR-induced cAMP compartment through which (−)-CGP12177 causes tachycardia. In contrast to the (−)-CGP12177-evoked tachycardia, not controlled by PDE3 and PDE4, these isoenzymes jointly reduce (−)-CGP12177-evoked increases of left atrial contractility through β_{1L}AR.

Keywords Rat atrial β_1-adrenoceptors · (−)-CGP12177 · Sinoatrial tachycardia · Cilostamide and rolipram · Contractile force · Phosphodiesterases PDE3 and PDE4

Introduction

Cardiac (Lowe et al. 2002) and recombinant (Baker et al. 2003; Joseph et al. 2004) β_1-adrenoceptors can be activated through two sites, β_{1H}AR and β_{1L}AR, with high and low affinity for β-adrenoceptor blockers, respectively. β-adrenoceptor blockers that cause cardiostimulation through β_{1L}AR are designated non-conventional partial agonists. CGP12177 is an experimentally used non-conventional partial agonist that has uncovered quantitative differences of β_{1L}AR function between species and even between different heart regions (reviewed by Kaumann and Molenaar 2008). CGP12177 is a non-conventional partial agonist on rat sinoatrial node and left atrium but not on ventricle (Kaumann and Molenaar 1996), unless phosphodiesterases (PDEs) are inhibited by isobutyl-1-methylxanthine (IBMX, Sarsero et al. 1999). Eleven families of PDE are known (Bender and Beavo 2006). Cyclic AMP is mainly hydrolysed

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by PDE3 and PDE4 in rat cardiomyocytes (Rochais et al. 2006). In rat ventricle, both PDE3 and PDE4 hydrolyze inotropically relevant cAMP produced by CGP12177 through β_{1L}AR while the cAMP produced by (−)-noradrenaline through β_{1H}AR is only hydrolyzed by PDE4 (Vargas et al. 2006). However, it is unknown which phosphodiesterases control the atrial effects of CGP12177.

Galindo-Tovar and Kaumann (2008) recently found that both PDE3 and PDE4 reduce murine sinoatrial beating rate but neither isoenzyme reduces the positive chronotropic effects of (−)-adrenaline mediated through β_{1H}AR. Similarly, in the rat, the sinoatrial tachycardia caused by (−)-noradrenaline is not blunted by PDE3 and PDE4 but PDE4 reduces sinoatrial rate (Christ et al. 2009). In contrast, the positive inotropic effects of (−)-adrenaline and (−)-noradrenaline, mediated through β_{1H}AR, are controlled by PDE4 but not PDE3 in both murine and rat left atrium and ventricle (Galindo-Tovar and Kaumann 2008; Christ et al. 2009; Vargas et al. 2006). These regional differences of cardiac PDE3 and PDE4 functions, as well as their different controls of rat ventricular effects through β_{1H}AR and β_{1L}AR (Vargas et al. 2006), prompted us to investigate the effects of the PDE3-selective inhibitor cilostamide and PDE4 inhibitor rolipram on the (−)-CGP12177-evoked sinoatrial tachycardia and increased left atrial contractility, mediated through rat β_{1L}AR.

Materials and methods

Rat atria

All animal care and procedures complied with the guidelines of the European Communities Council Directive of 23 March 1998 (1999/575/CE) and were approved by the Ethical Committee of the University of Murcia. Thirty two Sprague–Dawley rats of either sex (10–12 weeks old, ~200 g weight) were stunned and exsanguinated. The rapidly removed heart was dissected in warm oxygenated Tyrode's solution, containing (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 95% O_2/5% CO_2. Left atria and spontaneously beating right atria were rapidly mounted in pairs and attached to Swema 4–45 strain gauge transducers in an apparatus containing the above solution at 37°C. Left atria were paced at 1 Hz with just over threshold voltage and stretched as described (Kaumann and Molenaar 1996). Spontaneously beating rate of right atria and left atrial contractile force were recorded through PowerLab amplifiers on a Chart for Windows, Version 5.0 recording program (ADInstruments, Castle Hill, NSW, Australia).

All experiments were carried out in the presence of (−)-propranolol (200 nM) to selectively block β_{1H}AR (Joseph et al. 2004). Blockade of the effects of traces of endogenously-released noradrenaline probably causes the cardiodepressant effects of low (−)-CGP12177 concentrations. The slight cardiodepressant effects of (−)-CGP12177 are prevented by (−)-propranolol through β_{1H}AR, thereby, enhancing its cardiostimulant effects through β_{1L}AR (Kaumann 1996; Kaumann and Molenaar 2008).

Concentration–effects curves for (−)-CGP12177 were determined by cumulative addition of half-log increments in concentration. Only a single curve was determined on each tissue in the absence or presence of the PDE3-selective 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). As discussed by Vargas et al (2006), 300 nM cilostamide inhibits approximately 86% of PDE3 but less than 0.4% of PDE4; 1 μM rolipram inhibits approximately 50% of PDE4 but less than 0.4% of PDE3. The PDE inhibitors were preincubated for 30 min before a concentration–effect curve for (−)-CGP12177 was begun.

Drugs used

(−)-CGP12177 ((−)-4-(3-tertiarybutylamino-2-hydroxypropoxy) benzimidazol-2-one) was a gift from GlaxoSmithKline (Epsom, UK). (−)-Propranolol, cilostamide, and rolipram were purchased from Sigma Chemicals (Madrid, Spain). Cilostamide and rolipram were dissolved in 20% dimethylsulfoxide in Tyrode's solution. Stock solutions (3 mM) were diluted into prewarmed and preoxygenated bathing solution at the desired concentration.

Statistical analysis

-LogEC_{50}M values of (-)-CGP12177 were estimated from fitting a Hill function with variable slopes to concentration-effect curves from individual experiments. Results are expressed as mean±s.e.m. values. Student's paired *t*-test and one-way analysis of variance with the Newman–Keuls multiple comparison test were used. $P<0.05$ was considered significant. The number of experiments refers to number of rats.

Results

Rolipram but not cilostamide caused sinoatrial tachycardia as shown in the representative experiments of Fig. 1. Rolipram enhanced sinoatrial beating rate from 241±10 bpm to 268±11 bpm ($n=8$, $P<0.002$). Sinoatrial rate was 266±10 bpm and 267±10 bpm ($n=8$) in the absence

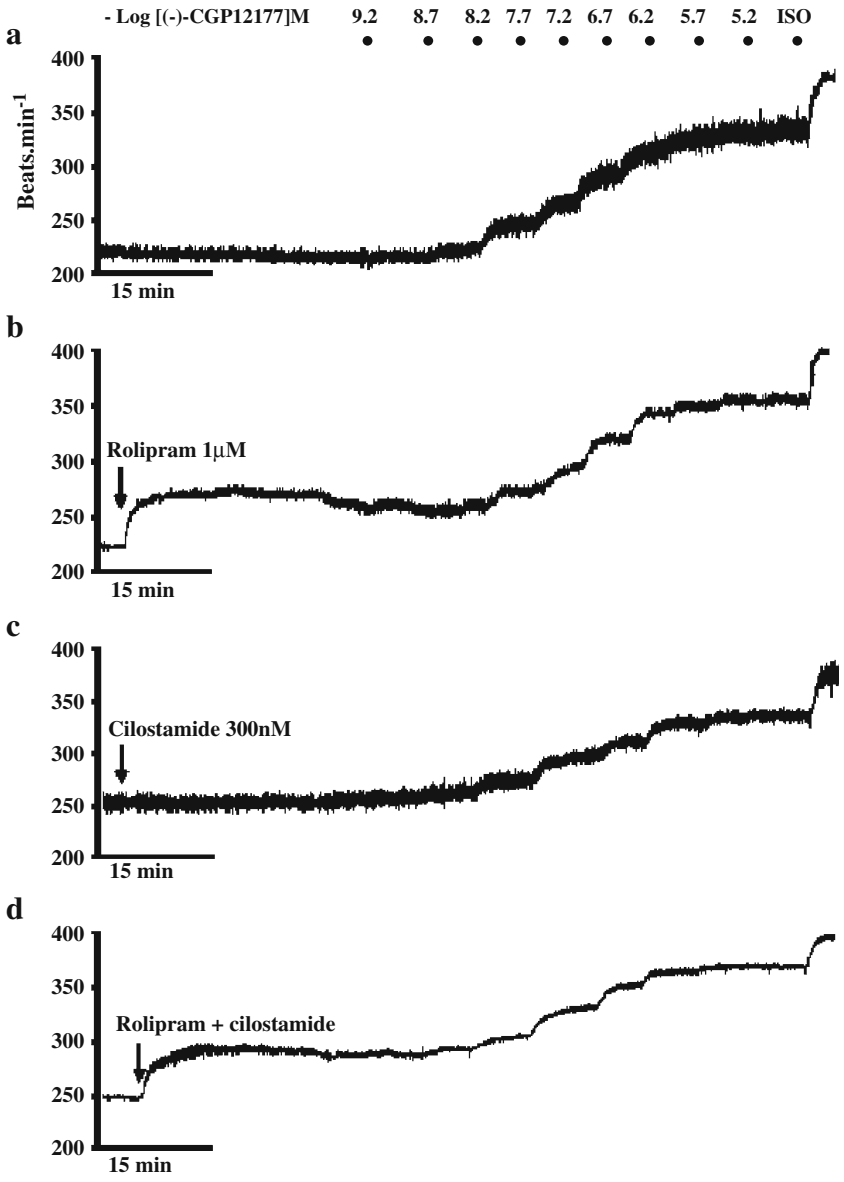

Fig. 1 Representative experiments depicting the tachycardia caused by rolipram and the lack of potentiation of the positive chronotropic effects of (−)-CGP12177 by PDE inhibitors. Concentration–effect curves for (−)-CGP12177 are shown in the absence (**a**) and presence of rolipram (1 μM, **b**), cilostamide (300 nM, **c**) and concurrent rolipram+cilostamide (**d**). *ISO* (−)-isoprenaline (200 μM)

and presence of cilostamide, respectively. Concurrent rolipram+cilostamide increased sinoatrial rate from 236± 12 bpm to 279±9 bpm ($P<0.001$). The increase in sinoatrial rate by 43±4 bpm caused by concurrent rolipram+ cilostamide was greater ($P<0.02$) than the increase in sinoatrial rate of 27±5 bpm caused by rolipram alone.

Rolipram and cilostamide, administered separately or together, did not increase the chronotropic potency and intrinsic activity of (−)-CGP12177 (Figs. 1 and 2a).−LogEC$_{50}$M values were not significantly different in the absence or presence of the PDE inhibitors (Fig. 2a).

Rolipram and cilostamide did not significantly change basal contractile force of left atria but concurrent rolipram+ cilostamide increased force by 52.9±9% of the effect of (−)-isoprenaline (Fig. 2b). The intrinsic activity of (-)-CGP12177 with respect to (-)-isoprenaline was 0.25 in the absence of the PDE inhibitors. Neither rolipram nor cilostamide caused increases in potency and intrinsic activity of (−)-CGP12177 (Fig. 2b). However, concurrent rolipram+ cilostamide converted (−)-CGP12177 into a full agonist with respect to (−)-isoprenaline and potentiated its effects by increasing the−logEC$_{50}$M by 0.78 log units compared to the absence of the PDE inhibitors (Fig. 2b).

Discussion

Our results demonstrate that: (1) cilostamide and rolipram, administered separately or together fail to potentiate the (−)-CGP12177-evoked tachycardia despite the tachycardia

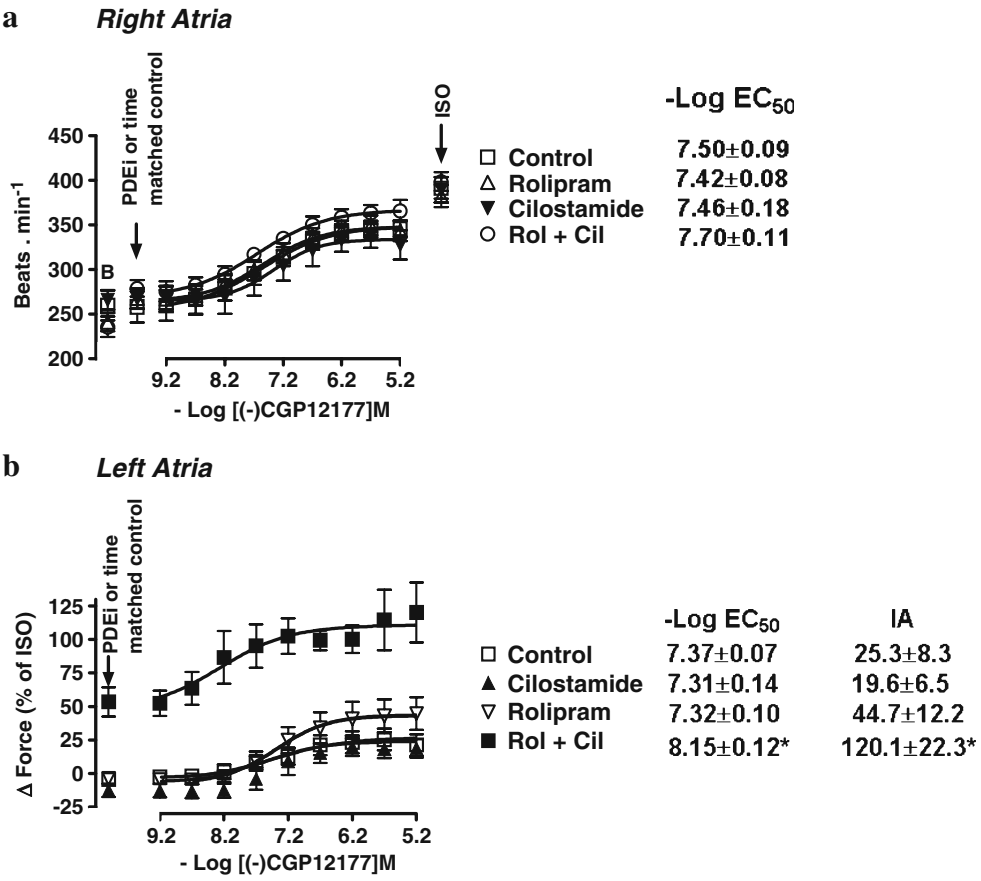

Fig. 2 Cilostamide (300 nM, *Cil*) and rolipram (1 μM, *Rol*) do not potentiate the positive chronotropic effects of (−)-CGP12177 on right atrium (**a**). Concurrent rolipram+cilostamide potentiate the positive inotropic effects and increase the intrinsic activity of (−)-CGP12177 in left atrium (**b**). *$P<0.05$ compared to control. *B* Basal sinoatrial rate before the addition of PDE inhibitors (*PDEi*). Basal and (−)-isoprenaline-evoked contractile force (mN) was 2.1±0.4/6.5±1.2, 2.4±0.4/8.2±1.5, 2.6±0.4/7.5±0.8 and 2.4± 0.4/7.3±1.3 in the absence and presence of rolipram, cilostamide, and concurrent rolipram+cilostamide respectively. *ISO* (−)-Isoprenaline (200 μM). *IA* intrinsic activity

produced by rolipram and, in contrast, (2) concurrent rolipram+cilostamide potentiate the positive inotropic effects of (−)-CGP12177 in left atrium. These results are consistent with a joint control by PDE3 and PDE4 of the agonist effects of (−)-CGP12177 through β_{1L}AR in left atrium but not in sinoatrial cells.

Basal cAMP and cAMP-dependent protein kinase (PKA) activity appear obligatory for spontaneous sinoatrial action potentials because inhibition of adenylyl cyclases or PKA greatly reduces spontaneous firing (Vinogradova et al. 2006; Younes et al. 2008). Both cAMP levels and phosphodiesterase activity are higher in sinoatrial cells than ventricular myocytes and the PDE3-selective inhibitor milrinone markedly increases beating rate and phospholamban phosphorylation in rabbit sinoatrial node cells, consistent with a reduction of basal heart rate by PDE3 (Vinogradova et al. 2008). Milrinone increases Ca^{2+} loading of the sarcoplasmic reticulum (SR), attributed to enhanced Ca^{2+} ATPase pumping expected from the phosphorylation of phospholamban, leading to enhanced Ca^{2+} release from subsarcolemmal RyR2 channels which in turn activates the Na^{+}/Ca^{2+} exchanger inward current that accelerated diastolic depolarization and sinoatrial beating rate (Vinogradova et al. 2008). The increased sinoatrial cAMP in the presence of milrinone may directly open cyclic nucleotide-gated HCN channels responsible for the current activated by hyperpolarization, I_f (DiFrancesco and Tortora 1991; Barbuti and DiFrancesco 2008), thereby contributing to tachycardia. However, inhibition of I_f with CsCl only causes minor bradycardia compared to the bradycardia produced by the inhibition of adenylyl cyclases or PKA (Younes et al. 2008) and hardly affects the milrinone-evoked sinoatrial tachycardia (Vinogradova et al. (2008).

In contrast to the tachycardia caused by the PDE3-selective inhibitor milrinone in the rabbit (Vinogradova et al. 2008), in Sprague–Dawley rats (this work) and Wistar rats (Christ et al. 2009), the PDE4 inhibitor rolipram, but not the PDE3-selective cilostamide, causes tachycardia. Since the rolipram-evoked tachycardia in the rat sinoatrial node was observed during blockade of β_1AR and β_2AR with (−)-propranolol, sensitization by rolipram of β_1AR-mediated effects by traces of endogenously released noradrenaline can be ruled out. Our results with the rolipram-evoked tachycardia and the failure of cilostamide to change sinoatrial rate are consistent with a direct reduction of the basal sinoatrial rate by PDE4 but not PDE3. The tachycardia caused by rolipram probably results from the inhibition of PDE4, followed by elevation of sinoatrial cAMP, increased PKA-catalyzed

phosphorylation of proteins leading to accelerated Ca^{2+}-cycling and enhanced beating rate. This is consistent with previous work with rat right atrium, showing that IBMX causes tachycardia, presumably at least in part through inhibition of PDE4, together with an increase in PKA activity (Kaumann and Lynham 1997).

Concurrent rolipram+cilostamide caused greater tachycardia than rolipram alone. The accumulation of chronotropically relevant cAMP during inhibition of PDE4 may activate PKA in the neighborhood of PDE3 and produce its phophorylation (Smith et al. 1991), thereby activating PDE3 to further hydrolyse cAMP which in turn would partially blunt the rolipram-evoked tachycardia. If in addition to PDE4 inhibition PDE3 is also inhibited with cilostamide, more cAMP would accumulate and the tachycardia would be greater than with inhibition of PDE4 alone. However, neither the sinoatrial tachycardia of (−)-CGP12177, mediated through β_{1L}AR (this work), nor the tachycardia of (−)-adrenaline and (−)-noradrenaline, mediated through β_{1H}AR (mouse, Galindo-Tovar and Kaumann 2008; Wistar rat, Christ et al. 2009) are potentiated by inhibition of PDE3 and PDE4. Furthermore, the tachycardia caused by (−)-adrenaline through rat sinoatrial β_2AR is not potentiated by inhibition of PDE3 or PDE4 (Christ et al. 2009). Inhibition of PDE3 and PDE4 also produces sinoatrial tachycardia in right atria from new-born piglets but does not potentiate the positive chronotropic effects of 5-hydroxytryptamine through sinoatrial 5-HT_4 receptors (Galindo-Tovar et al. 2009). Thus, depending on species, PDE3 and/or PDE4 appear to hydrolyze the cAMP that maintains basal sinoatrial rate but do not seem to have access to the cAMP pool produced by activation of the sinoatrial Gs protein-coupled receptors β_{1H}AR, β_{1L}AR, β_2AR, and 5-HT_4.

Could other PDE isoenzymes, PDE1 and/or PDE2, control the tachycardia caused by the Gs-protein-coupled receptors? This seems unlikely for β_{1H}AR and β_2AR because the non-selective PDE inhibitor IBMX (10 μM), albeit causing marked tachycardia, does not potentiate the positive chronotropic effects of (−)-noradrenaline and (−)-adrenaline, respectively (Christ et al. 2009). However, IBMX appears to enhance the positive chronotropic potency of (−)-CGP12177 in Wistar rats, although the additive tachycardia caused by IBMX and (−)-CGP12177 might have led to an overestimation of potentiation (Kaumann and Lynham 1997). Nevertheless, it can not be excluded that the β_{1L}AR-mediated tachycardia is controlled by some phosphodiesterase, but not by PDE3 or PDE4.

Concurrent rolipram+cilostamide increased left atrial force, suggesting that basal force is under control of the cAMP hydrolyzed by PDE3 and PDE4 (Fig. 2b). This finding is in line with an increase of both left atrial force and PKA activity by IBMX (10 μM; Kaumann and Lynham 1997). As previously observed with (±)-CGP12177 in rat ventricle (Vargas et al. 2006), concurrent rolipram+cilostamide also potentiate the positive inotropic effects of (−)-CGP12177, mediated through left atrial β_{1L}AR, consistent with a joint control by PDE3 and PDE4. However, the positive inotropic effects of (−)-noradrenaline, mediated through β_{1H}AR of rat ventricle and left atrium are only potentiated by rolipram but not cilostamide (Vargas et al. 2006; Christ et al. 2009), consistent with control by PDE4 but not PDE3. In contrast to rat myocardium, the positive inotropic effects of both (−)-noradrenaline and (−)-CGP12177 on human atrial myocardium, mediated through β_{1H}AR and β_{1L}AR, respectively, are both controlled by PDE3 but not by PDE4 (Christ et al. 2006; Kaumann et al. 2007).

We conclude that in the Sprague–Dawley rat, the β_{1L}AR-mediated sinoatrial tachycardia is not controlled by PDE3 and PDE4, despite the bradycardia caused by PDE4. In contrast, as previously observed on rat ventricle, both PDE3 and PDE4 reduce the β_{1L}AR-mediated increases in left atrial force.

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2. Introducción

2.1 Fisiología de la Función Cardíaca

El funcionamiento normal del corazón depende de la frecuencia cardíaca y de la contracción apropiada y secuencial, de sus distintas cavidades. Cada zona del corazón presenta unas propiedades estructurales y eléctricas específicas (Schram et al., 2002). Las células implicadas en la actividad contráctil se caracterizan, fisiológicamente, por la incapacidad para iniciar de forma espontánea su ciclo de trabajo, y confían su activación, a un impulso externo que se origina en una región altamente específica, el nódulo sinusal (NS).

2.1.1 Regulación del Calcio Intracelular

Es bien conocido el papel esencial que desempeña el calcio en el proceso de regulación de las fases de contracción y relajación del ciclo cardiaco. Durante cada ciclo cardiaco, la cantidad de iones de calcio (Ca^{2+}) que realmente entran en la célula y la abandonan, es relativamente reducida, mientras que en el retículo sarcoplasmático (RS) hay un movimiento de Ca^{2+} mucho mayor (Bers y Perez-Reyes, 1999). La liberación de calcio inducida por calcio (CICR) (Fabiato, 1983) se fundamenta en la liberación por parte del RS de cantidades relativamente grandes de Ca^{2+} al citosol, en respuesta a la cantidad mucho menor que penetra en el cardiomiocito con cada onda de despolarización (Bouchard et al,. 1995). Este proceso da como resultado la elevación unas 10 veces de la concentración de Ca^{2+} en el citosol resultando una mayor interacción de los iones Ca^{2+} con la maquinaria contráctil. Esta teoría está apoyada por los siguientes hallazgos: la caracterización molecular del receptor rianodínico (RyR) del RS que libera calcio cuando es activado por Ca^{2+} (Fan y Palade, 1999), los datos electrofisiológicos que relacionan estrechamente la duración del potencial de acción (PA) con la magnitud de la liberación del Ca^{2+} (Bouchard et al., 1995), la proximidad anatómica de los RyR con los canales de calcio tipo L del sarcolema y la membrana citoplasmática de las fibras musculares (Bers y Perez-Reyes, 1999).

2.1.2 Mecanismos de aumento de Ca^{2+}

Ciertas características especiales del ritmo cardiaco se relacionan con las corrientes de Ca^{2+}. En el corazón existen dos tipos diferentes de canales de Ca^{2+}: los canales de estructuras intracelulares y los canales de Ca^{2+} de la membrana plasmática regulados por voltaje.

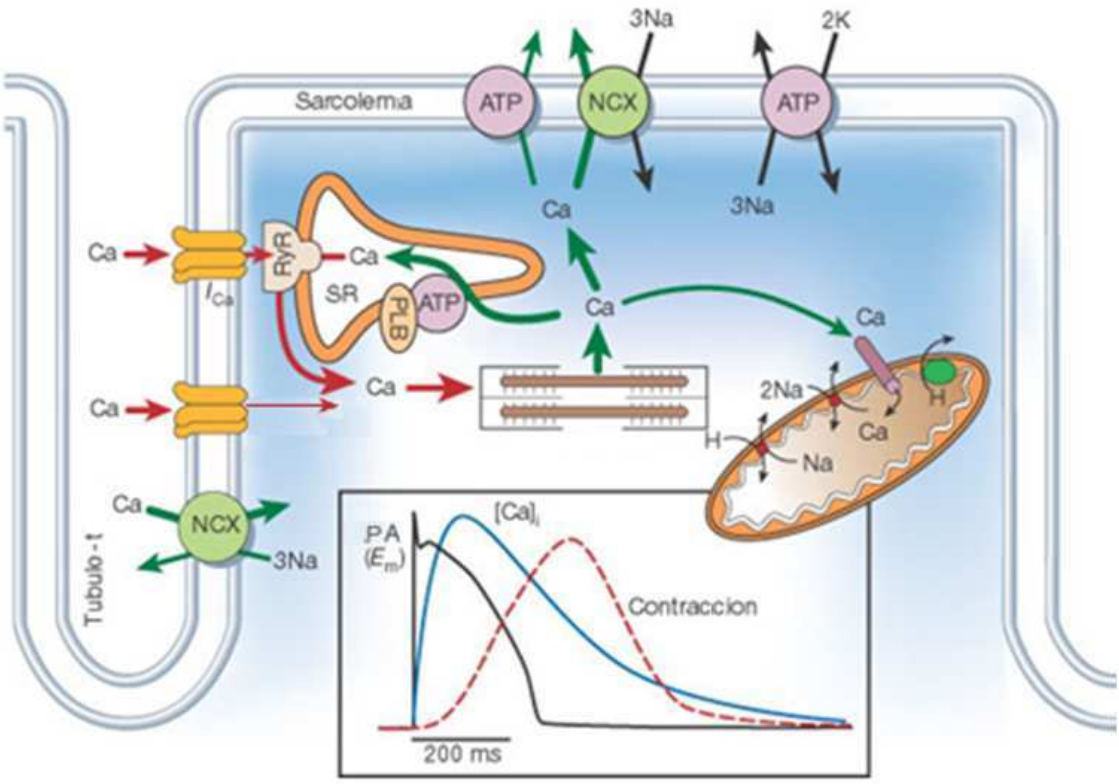

Figura 1: Transporte de Ca^{2+} en miocitos ventriculares. El cuadro interior muestra el transcurso temporal de un potencial de acción, el transiente de Ca^{2+}, y la contracción medidas en un miocito ventricular. NCX, intercambiador Na^{+}/Ca^{2+}; ATP, ATP-asa; PLB, fosfolambano; SR, retículo sarcoplasmático; RyR, receptor de rianodina; PA, potencial de acción (Modificada de Bers, 2002).

Los principales mecanismos de control de la concentración de Ca^{2+} se resumen en la figura 1. Hay dos clases de canales de Ca^{2+} dependiente de voltaje, los canales tipo L y T (Bers, 2002). La vía de entrada más importante son los canales de calcio tipo L, de cinética relativamente lenta, conocidos como receptores de dihidropiridina (DHPRs), que se localizan en los túbulos T, invaginaciones del sarcolema perpendiculares a la longitud de la fibra cardíaca que forman un conjunto de

sarcómero – túbulo t – sarcómero denominado tríada. Los canales Tipo T tienen un papel menor en la contractilidad, aunque si parecen resultar importantes en el crecimiento y proliferación celular (Katz, 1996). Con cada PA, se produce un aumento del flujo de Ca^{2+} por los canales tipo L (I_{CaL}) y produce un aumento inmediato de la concentración de Ca^{2+}. La parte más importante para la activación de la maquinaria contráctil es la liberación secundaria de Ca^{2+} por parte del RS, activada por aumentos de I_{CaL} causando pequeños incrementos de Ca^{2+} localizados próximos a los canales de calcio citoplasmáticos subyacentes a los túbulos T de la membrana. El aumento local de la concentración de Ca^{2+}, actúa sobre los grandes canales de calcio en los RyR, que se abren para liberar grandes cantidades de Ca^{2+} desde el interior de las cisternas hacia el citoplasma. El Ca^{2+} liberado desde el retículo sarcoplasmático también contribuye a la inactivación dependiente de Ca^{2+} de los canales L (Sipido et al., 1998).

El Ca^{2+} que fluye a través de los canales de Ca^{2+} tipo L activa los RyR2, para mediar la CICR (Fabiato y Fabiato, 1977) que es la principal fuente de intracelular de Ca^{2+} para la contracción cardíaca (Wang et al., 2001). La fracción de Ca^{2+} responsable de la contracción que procede del sarcolema varía entre las diferentes especies. Así, en roedores es aproximadamente un 10 %, frente a un 90 % por parte del RS, y en otras especies de mamíferos, como el perro, el conejo, el gato y en el ventrículo humano, el balance es de un 70 % del RS frente a un 25-28 % del sarcolema (Bers, 2002).

2.1.3 Receptor Rianodínico

Cada uno de los canales de calcio de la membrana tipo L controla un grupo de cuatro a diez RyR del RS (Bers y Perez-Reyes, 1999; Wier et al., 1994), gracias a la proximidad anatómica entre los canales de calcio de los túbulos T y los canales liberadores de calcio ubicados en el RS. Los RyR son unas estructuras proteicas que forman complejos tetraméricos; cada uno de estos complejos actúa como un canal de Ca^{2+}. Parte del RyR se extiende desde la membrana del RS hasta los túbulos T formando una estructura basal denominada complejo del canal que establece la conexión entre el RS y los túbulos T. Existen tres isoformas del receptor de rianodina: 1) RyR1 en células de músculo esquelético; 2) RyR2 en células cardíacas y 3) RyR3 en

otro tipo de células no musculares, como las células neuronales. (Franzinia-Armstrong 2004).

Los eventos espacios-temporales de la concentración de Ca^{2+} que siguen al estimulo se denominan transientes de Ca^{2+} del inglés "*transients".* Los "*sparks"* de Ca^{2+}, son eventos localizados de liberación de Ca^{2+} del RS. Su descripción está basada en la observación de chispazos o briznas de Ca^{2+}, transientes espontáneos de Ca^{2+}. Éstos reflejan el sincronismo de activación de un racimo de 6 a 20 receptores de RyR2, que es muy importante para el acoplamiento excitación-contracción (Cheng et al., 1993; Bridge et al., 1999). Los chispazos de calcio son la unidad fundamental de liberación de Ca^{2+} del RS. Durante el acoplamiento excitación-contracción, miles de chispazos de Ca^{2+} en cada célula, son sincronizados en el tiempo por el PA, cada uno de los aumentos en la concentración de Ca^{2+} están completamente solapados en el espacio y en el tiempo, dando lugar a que el transiente de Ca^{2+} parezca totalmente uniforme. Los sucesos locales de liberación de Ca^{2+} pueden ser incluso visualizados bloqueando el 90 % de I_{CaL} (Cheng et al., 1995; Lopez-Lopez et al., 1995).

2.1.4 Mecanismos de disminución de Ca^{2+} citoplasmático.

El aumento de la concentración citosólica de Ca^{2+} que desencadena la contracción, concluye cuando pasa la onda de excitación, deja de entrar Ca^{2+} y se interrumpe la liberación del mismo por el RS. Esto podría deberse a varias razones: 1) que la concentración citosólica de Ca^{2+} se hubiera elevado lo suficiente para inhibir el proceso de CICR (Bers y Perez-Reyes, 1999); 2) que la liberación de Ca^{2+} a partir del RS estuviera ligada estrechamente a la apertura de los canales L de Ca^{2+}, de modo que cuando estos se cierran, cesa la liberación (Balke et al., 1994); 3) que la elevada concentración de Ca^{2+} citosólica activara la bomba de captación de Ca^{2+} localizada en el RS (Lytton et al., 1992) y 4) que el RS liberara Ca^{2+} solamente durante el tiempo que dura el PA (Bouchard et al,. 1995). El resultado global de estos procesos es la reducción de la concentración de iones Ca^{2+} en el citosol, que da inicio a la diástole.

Para contrarrestar la entrada de Ca^{2+} extracelular durante la despolarización, se reduce el Ca^{2+} celular principalmente por medio del intercambiador Na^{+}/Ca^{2+} (NCX) (figura 1) y de menor importancia por la bomba de Ca^{2+} del sarcolema (figura 1). Al

final de la diástole, para restaurar de nuevo el potencial de membrana de reposo, los iones de sodio importados son eliminados hacia fuera, en intercambio con los iones de potasio, por medio de la bomba sodio-potasio. Los cambios en la concentración intracelular de calcio tienen efectos importantes sobre la acción de las proteínas contráctiles en el miocardio (figura 1).

2.1.5 Transporte de Ca^{2+} por ATP-asa cálcica del RS

El RS capta Ca^{2+} gracias a la actividad de la bomba de Ca^{2+} llamada SERCA (ATP-asa de Ca^{2+} del RS) que constituye la mayoría del componente proteico de la membrana del RS (Lompré et al., 1991*)*.

El fosfolambano (PLB) es el regulador principal de esta bomba de calcio. La actividad del PLB está regulada por su estado de fosforilación, proceso que altera la configuración molecular de SERCA. El PLB es un inhibidor endógeno de SERCA. La fosforilación de PLB por la proteína quinasa dependiente de AMPc (PKA) o la proteína quinasa dependiente de calmodulina (CaMKII), deshace esta inhibición, aumentando la afinidad de Ca^{2+} por SERCA, lo que conlleva una relajación más rápida por disminución de la concentración citosólica de Ca^{2+} (Brittsan y Kranias, 2000). El Ca^{2+} captado por el RS se almacena en el mismo antes de ser liberado. Existe una proteína almacenadora fuertemente cargada llamada calsecuestrina, localizada en la porción del RS cercana a los túbulos T. Se cree que el calcio almacenado con la calsecuestrina se libera cuando esta proteína lo descarga hacia la cara interna del RyR.

2.1.6 Proteínas contráctiles y contractilidad cardíaca:

La estructura y las proteínas que forman la maquinaria contráctil se encuentran representadas en la figura 2.

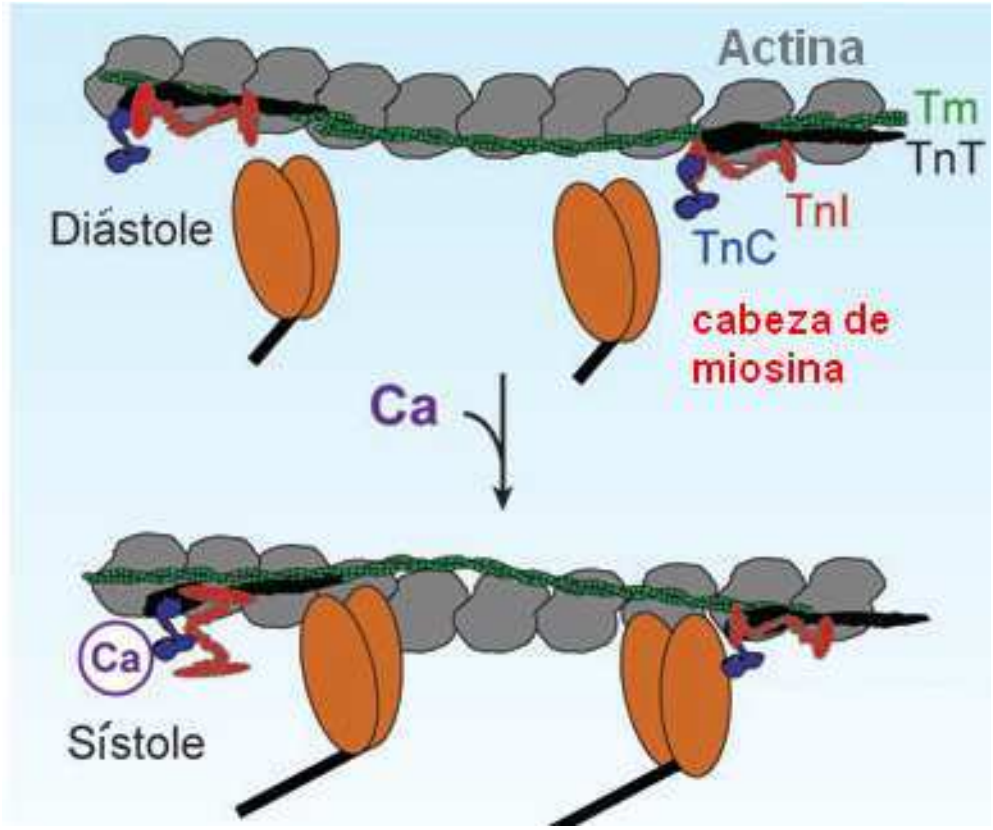

Figura 2: Activación de los miofilamentos por calcio. TnC, troponina-C; TnI, troponina-I; TnT, troponina-T; Tm, tropomiosina. (Modificada de Bers, 2008.)

La maquinaria contráctil de la célula miocárdica se basa en la interacción entre los filamentos de actina y miosina. El complejo troponina esta formado por troponina C (TnC), troponina I (TnI), troponina T y tropomiosina. La TnC posee zonas de unión para tres o cuatro iones Ca^{2+}. Cuando aumenta la concentración de Ca^{2+}, éste se une a la TnC y la conformación del complejo de troponina cambia y se produce el desplazamiento de la tropomiosina. Este hecho deja al descubierto los sitios de unión de la miosina en los filamentos de actina, lo que da como resultado la unión entre los puentes cruzados de miosina con actina, iniciándose el proceso contráctil. Estos cambios se producen cuando la concentración de Ca^{2+} intracelular, excede 10^{-7}M, activándose el sistema completamente con 10^{-6}M. Hay dos maneras principales de

cambiar la fuerza de la contracción cardíaca: 1) alterando la duración de los transientes de Ca^{2+} y 2) alterando la sensibilidad de los miofilamentos al Ca^{2+}. La sensibilidad de los miofilamentos al Ca^{2+} se incrementa dinámicamente al estirar los miofilamentos (cuando el corazón se llena de sangre), resultando una mayor contracción. Esto es debido, en parte, a la compresión de los filamentos transversos que incrementan la interacción actina-miosina. Esta compresión lateral es un importante mecanismo de autorregulación con el que el corazón se ajusta a diástoles alteradas, según la Ley de Frank-Starling (Shiels y White, 2008).

Cuando la concentración de Ca^{2+}es baja, se produce la relajación porque la molécula de tropomiosina impide la unión de miosina con las moléculas de actina.

2.2 Fisiología del nódulo sinusal

El nódulo sinusal (NS), presenta unas características fisiológicas distintas al resto del corazón por lo que lo trataremos separadamente.

La existencia de una distribución eléctrica regional especializada en el tejido cardiaco fue evidenciada por primera vez a mediados del siglo XIX por Stannius (1852). En 1907, Keith y Flack fueron los primeros en describir el NS como una estructura anatómica.

Las células del NS poseen actividad espontánea y generan potenciales de acción repetitivos a una frecuencia controlada variable, lo que determina la frecuencia cardíaca. Son, por lo tanto, origen de la actividad del marcapasos fisiológico del corazón.

En el corazón humano adulto, el NS se encuentra situado en la unión entre la vena cava superior y la pared de la aurícula derecha, dentro del surco terminal. Tiene una forma de luna creciente y una longitud media de 1,3 cm. Al microscopio óptico, las células nodales aparecen de menor tamaño y más pálidas que los miocitos auriculares con función contráctil. Estas células nodales se disponen sobre una matriz de tejido conectivo denso formando cordones que se entrelazan entre sí (Guerra y Cinca, 2007). En la periferia del NS, los miocitos nodales se mezclan con los miocitos contráctiles e incluso forman digitaciones que penetran en el miocardio normal, sin que se evidencie una capsula fibrosa que separe ambas estructuras (Verheijck et al., 1998; Sanchez-Quintana et al., 2005).

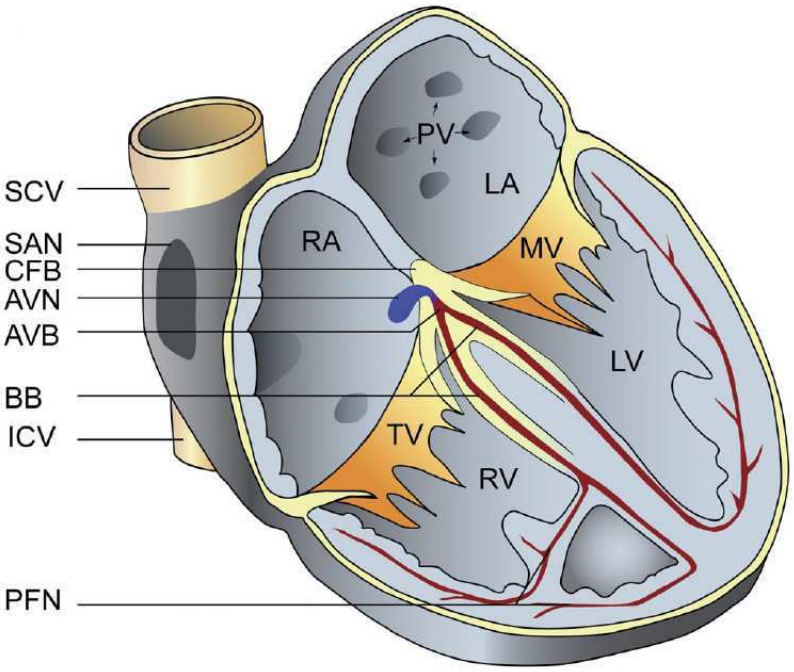

Figura 3: El corazón de mamíferos. El nódulo sinusal (SAN) está localizado en la entrada de la vena cava superior (SCV) en la aurícula derecha (RA). LA, aurícula izquierda; RV, ventrículo derecho; LV, ventrículo izquierdo; PV, venas pulmonares; TV, válvula tricúspide; MV, válvula mitral; ICV, vena cava inferior; PFN, fibras de Purkinje; AVN, nódulo auriculoventricular; CFB, cuerpo fibroso central; BB, haz de Hiss (Mangoni y Nargeot, 2008).

En el corazón de mamíferos, hay tres estructuras principales involucradas en el automatismo que son capaces de regular el latido cardiaco: el NS, que constituye el marcapasos fisiológico, el nódulo auriculoventricular (NAV), y las fibras de Purkinje (FP). El ritmo intrínseco del NS es normalmente más rápido que el sistema de conducción cardiaco y suprime la actividad marcapasos en el AVN y en las FP. El automatismo del AVN puede ser dominante en caso de bloqueo o fallo del NS (James, 2003). Las FP puede generar también un ritmo cardiaco viable en condiciones de bloqueo auriculoventricular. Por estas razones, la región del NS está descrita como el marcapasos principal, mientras el NAV y las FP están descritos como marcapasos secundarios o accesorios.

El NS es estructuralmente heterogéneo. Se han propuesto dos puntos de vista sobre la organización del NS. El modelo del "gradiente" del NS (Boyett et al., 2000),

propone que hay una transición progresiva en el tamaño y las propiedades eléctricas de las células marcapasos entre las células localizadas en el centro del NS y las de la periferia. Las células del centro son pequeñas y tienen un ritmo y velocidad de repolarización lentos. Las células de la periferia tienen un mayor ritmo y velocidad de repolarización que las células auriculares. Contrario al modelo de "gradiente", no habría una distribución preferencial de las células con propiedades de marcapasos en el centro del NS (Verheicjk et al., 1998). Estas células con diferentes grados de automaticidad y diferentes configuraciones de PA, estarían supuestamente distribuidas uniformemente en toda la región del NS. Verheijck et al. (1998) han indicado que las células auriculares están presentes en el NS, aunque su densidad es máxima en la periferia y mínima en el centro del NS. Esta visión de la estructura del NS ha sido llamada modelo "mosaico". El modelo "gradiente" parece explicar mejor el comportamiento electrofisiológico del NS (Mangoni y Nargeot, 2008).

Las células centrales del NS pueden liderar la actividad marcapasos a pesar de su ritmo de latido más lento, porque las células de la periferia están electrónicamente inhibidas por la aurícula derecha. Las diferencias en el ritmo intrínseco de disparo de las células centrales y de la periferia del NS son debidas a la expresión heterogénea de canales iónicos (Kodama et al., 1997; Boyett et al., 1998) y de proteínas involucradas en la homeostasis del Ca^{2+} (Musa et al., 2002; Lancaster et al., 2004).

Los mecanismos que parecen ser más importantes para la generación de la actividad marcapasos son la corriente *"funny"* (I_f) y la regulación del ciclo de Ca^{2+}. Existen otros mecanismos involucrados en la génesis de la actividad marcapasos, que se han discutido extensamente en una revisión reciente (Mangoni y Nargeot, 2008). La relevancia de la I_f en la génesis de la actividad marcapasos es apoyada por diferentes líneas de evidencia electrofisiológica, farmacológica y genética (Barbuti y DiFrancesco, 2008). La importancia de la liberación espontánea de Ca^{2+} en la generación de la actividad marcapasos se ha demostrado en experimentos realizados en células de NS de conejo tras la inhibición farmacológica de los RyR2 (Bogdanov et al., 2001).

Desde un punto de vista teórico, la actividad marcapasos puede ser considerada como un oscilador generado por una corriente de salida variable en el

tiempo y una corriente de entrada dependiente de voltaje activada durante la fase de repolarización. Diferentes canales iónicos con distintas propiedades biofísicas y farmacológicas están potencialmente involucrados en la fase de despolarización diastólica (Mangoni y Nargeot 2008). El PA de las células del NS muestras dos características importantes:

- Ausencia de fase de reposo.
- Baja velocidad de despolarización del nuevo PA.

Esta forma de PA explica el automatismo de las células del marcapasos sinusal. No es necesaria la llegada de un estímulo para provocar el cambio de la permeabilidad de la membrana a los iones, sino que dicha permeabilidad al sodio primero y al potasio se instaura espontánea y cíclicamente.

2.2.1 Potencial de Acción de las Células con actividad Marcapasos.

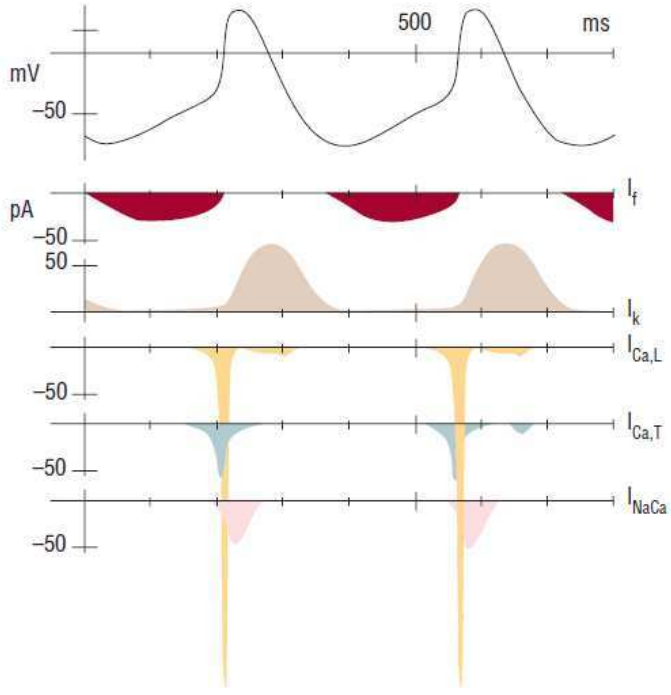

Figura 4: Simulación por ordenador de las corrientes iónicas implicadas en la generación del potencial de acción de una célula marcapasos del nódulo sinusal (Modificado de Vaquero et al., 2007).

En el NS hay 3 iones particularmente importantes en la generación del potencial de acción del marcapasos:

- El PA del NS y las principales corrientes responsables de su forma están resumidas en la figura 4. Al final de la repolarización, cuando el potencial de membrana es muy negativo (-60mV), se abren los canales iónicos que producen una corriente de entrada lenta despolarizante de Na^+ y K^+ (I_f). La activación de esta corriente causa que el potencial de membrana comience espontáneamente a despolarizarse, iniciándose así el prepotencial. Cuando el potencial de membrana llega a -50mv, se abren otro tipo de canales, los canales tipo T. El Ca^{2+} que entra a la célula a través de estos canales sigue disminuyendo el potencial de la membrana, que sigue despolarizando más la célula, hasta que el potencial de membrana llega a -40mV activándose los canales de Ca^{2+} tipo L, prosiguiendo la entrada de Ca^{2+} a la célula que continua su despolarización hasta llegar al umbral (entre -40 y -30mV). La activación de la corriente generada por el NCX (I_{NCX}) causa el aumento exponencial de la última parte del prepotencial (Maltsev y Lakatta, 2009).
- La fase de despolarización del PA es primariamente causada por el incremento de la conductancia al Ca^{2+} a través de I_{CaL}. Durante esta fase la I_f y la I_{CaT} disminuyen hasta que se cierran sus canales respectivos. Debido a que el movimiento de Ca^{2+} no es muy rápido, la curva de despolarización no es tan rápida como en otras células cardíacas.
- La repolarización ocurre por la apertura de los canales de K^+, incrementando la corriente de salida hiperpolarizante de K^+ (I_{KR}, I_{KS}, componentes rápida y lenta, respectivamente). Al mismo tiempo la I_{CaL} se inactiva y se cierra el canal. Al final de la repolarización comienza de nuevo el prepotencial con el inicio espontáneo de activación de I_f.

2.2.2 Papel de la corriente I_f

La relevancia fisiológica de la corriente I_f en la actividad marcapasos ha sido materia de debate en los últimos años. Abundantes evidencias demuestran que I_f juega un papel clave en la generación de la actividad marcapasos en el NS. Como características principales se destacan: 1) presentar una permeabilidad combinada

para el Na^+ y el K^+, 2) ser activada por hiperpolarización y 3) presentar una cinética de activación muy lenta (Yanagihara e Irisawa, 1980). Posteriormente se llegó a la conclusión de que la despolarización diastólica refleja la activación lenta de la I_f, la cual tiene lugar al final de un PA cuando el potencial de la membrana es más negativo, alcanzando así el rango de activación de la I_f (DiFrancesco, 1985).

La I_f inicia el prepotencial, hasta alcanzar el umbral de activación de las corrientes de Ca^{2+}, I_{CaL} principalmente, y finalmente de la corriente de NCX iniciándose luego un nuevo PA.

En el NS, la corriente I_f es la única corriente voltaje dependiente activada por hiperporalización de la membrana. Los canales f se abren durante la fase tardía de repolarización, cercana al potencial de membrana máximo diastólico. La I_f iniciaría la primera parte de la despolarización diastólica hasta que el umbral de activación de los canales tipo L y T se alcanzase (DiFrancesco, 2006). Se ha cuestionado, sin embargo, si el tamaño y cinéticas de la I_f a voltajes diastólicos puede ser compatible con la cantidad de corriente de entrada necesaria para iniciar la fase de despolarización en presencia de una corriente de salida (Denyer y Brown, 1990a).

Actualmente se esta discutiendo el papel de I_f en la actividad marcapasos y su aceleración por la estimulación β-adrenérgica (DiFrancesco 2006; Maltsev et al., 2006).

Los canales f son bloqueados por cesio (Cs^+). Este catión ha sido utilizado para cuantificar su participación en la actividad marcapasos del NS (Noma et al., 1983; Denyer y Brown, 1990b) incluso humano (Verkerk et al., 2007). Cuando se probó en células latiendo espontáneamente, el Cs^+ (2-5mM) enlenteció significativamente el ritmo de latido e indujo cronotropismo negativo sin afectar a ninguna de las otras corrientes (Denyer y Brown, 1990b). El hecho de que el Cs^+ enlentezca, pero no pare, la actividad marcapasos ha sido interpretado como una indicación de que I_f contribuye a la actividad marcapasos sin que sea un requisito absoluto para el automatismo (Noma et al., 1983; Denyer y Brown, 1990b). Sin embargo un antagonista de I_f como ivabradina enlentece hasta un 20 % la actividad marcapasos *in vitro* de células aisladas de NS de conejo, demostrando un papel funcional importante (Thollon et al., 1997).

La cinética y el rango de activación de la I_f están modulados por diferentes mecanismos entre los que destacan: 1) la presencia de unidades auxiliares (Qu et al., 2004); 2) fenómenos de fosforilación (Accili et al., 1997); 3) interacción con proteínas estructurales de membrana (Barbuti et al., 2004); 4) modulación por el sistema nervioso autónomo a través del AMPc. El AMPc es el segundo mensajero que media la activación por el sistema nervioso simpático de I_f. La activación de los canales f por el AMPc se produce tras la unión directa de esta molécula al canal, en vez de una fosforilación mediada por PKA (DiFrancesco y Tortora, 1991).

El mayor avance del conocimiento de los canales f se produjo con la clonación de los canales activados por hiperporalización y nucleótidos cíclicos (HCN) (Zagotta et al., 2003). En los mamíferos se han clonado 4 isoformas (HCN 1-4). Los canales HCN forman parte de la superfamilia de los canales de K^+ modulados por voltaje y los canales activados por nucleótidos cíclicos (CNG). Los canales HCN están formados por un tetrámero (Zagotta et al., 2003) y se caracterizan por presentar: 6 dominios transmembrana con un sensor de voltaje situado en el cuarto dominio, una secuencia del poro típica de los canales de K^+ y un dominio de unión a nucleótidos cíclicos, idéntico al de los canales CNG, localizado en el extremo C-terminal. En el NS se expresa fundamentalmente HCN4 (80 %) aunque se han descrito también ciertos grados de expresión de HCN1 y HCN2, dependiendo de la especie (Moroni et al., 2001)

2.2.3 Liberación de Ca^{2+} desde el retículo sarcoplasmático y automatismo.

Dos estudios, de Rubenstein y Lipsius (1989) y de Li y colaboradores (1997) suministraron evidencia inicial de que la liberación de Ca^{2+} del RS y el NCX estaban involucrados en la generación de la actividad marcapasos. Rubenstein y Lipsius (1989) observaron que la rianodina enlentecía el automatismo en células marcapasos auriculares reduciendo la pendiente de despolarización diastólica tardía (la fase exponencial), mientras el Cs^+ (presumiblemente mediante el bloqueo de I_f) disminuia la fracción lineal del prepotencial. Estos autores concluyeron que había numerosos mecanismos involucrados en la automaticidad de las células y que la liberación de Ca^{2+} desde el RS era importante en la generación de la fase tardía del prepotencial. El

enlentecimiento del automatismo por rianodina también fue descrito en preparaciones de cobaya (Rigg y Terrar, 1996) y conejo (Li et al., 1997). Rigg y Terrar (1996) hallaron una reducción de los transientes de Ca^{2+} intracelulares. Li et al. (1997) demostraron la abolición de I_{NCX} por rianodina y un quelante de Ca^{2+}. Estos estudios indicaron que el Ca^{2+} intracelular tenía un papel importante en el mantenimiento del automatismo, pero todavía no se había establecido una unión entre la señal del Ca^{2+} y la actividad marcapasos. En el corazón de sapo sin embargo han sido descritos distintos tipos de señales de Ca^{2+}: un transiente citosólico, ligado a la duración del PA, una segunda señal de Ca^{2+} retrasada vinculada a un cambio en el contenido de Ca^{2+} nuclear, y una tercera señal posiblemente debida a la liberación local de Ca^{2+} inducida por Ca^{2+} subsarcolémico (LCICR) (Ju y Allen, 2000).

Durante los últimos años el grupo liderado por el Edward Lakatta ha investigado el mecanismo celular que genera LCICR y ha enfatizado su relevancia en determinar la frecuencia sinusal y ha descrito una señal de Ca^{2+} que precede al PA debidas a los LCICR (Bogdanov et al., 2001). Estas señales son debidas a la liberación de Ca^{2+} desde el RS, y son abolidas por rianodina.

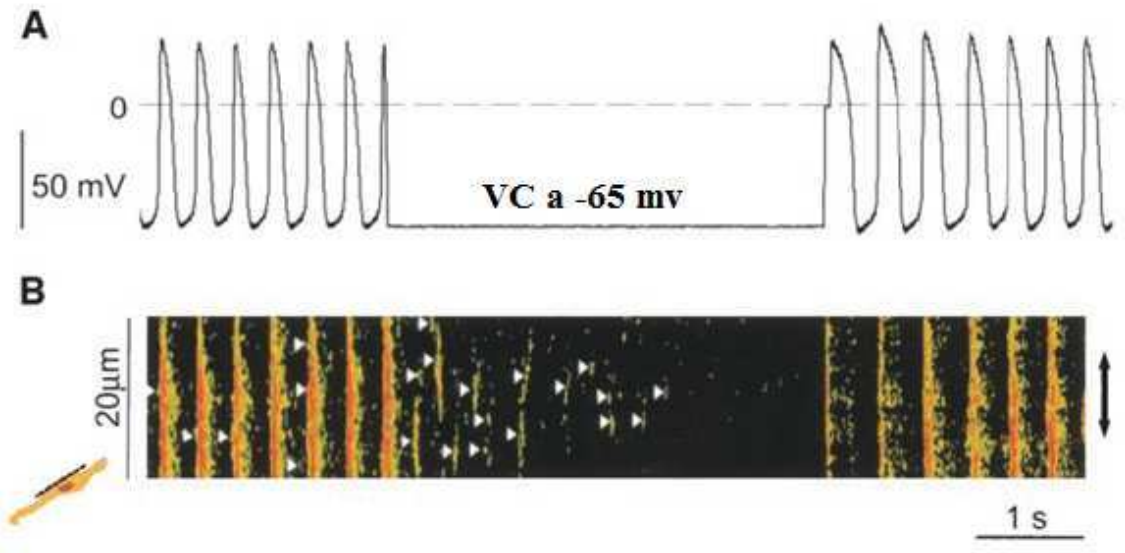

Figura 5: Los LCICR no desaparecen inmediatamente a pesar de la fijación de voltaje de la membrana. A. Registros de potencial de acción, B, LCICR (Microscopia confocal linescan sobre la célula, ver ilustración al margen inferior izquierdo); VC, fijación de voltaje. (Adaptada de Vinogradova et al., 2004).

Vinogradova y colaboradores (2004) han publicado evidencias que indican que en células de NS de conejo se puede generar LCICR sin que se produzca un cambio en el voltaje de la membrana (figura 5). El tamaño de los LCICR depende de la concentración extracelular de Ca^{2+}. Además, si en células latiendo espontáneamente se fija el voltaje en el potencial diastólico máximo o en voltajes positivos *(voltage clamp)*, los LCICR no desaparecen inmediatamente, si no que persisten por algunos segundos en ausencia de PA.

Con potencial de membrana fijo, los LCICR muestran un comportamiento al azar aproximadamente periódico (Vinogradova et al., 2004) y mantienen una periodicidad similar durante la actividad espontánea. Este resultado apoya la interpretación de que los LCICR no están directamente unidos a la actividad de los canales iónicos de la membrana, pero reflejan la existencia de un "reloj" intracelular de Ca^{2+} independiente mediado por la liberación espontánea de Ca^{2+} del RS. La razón de la desaparición de LCICR se produce posiblemente tras el vaciado del RS (figura 6).

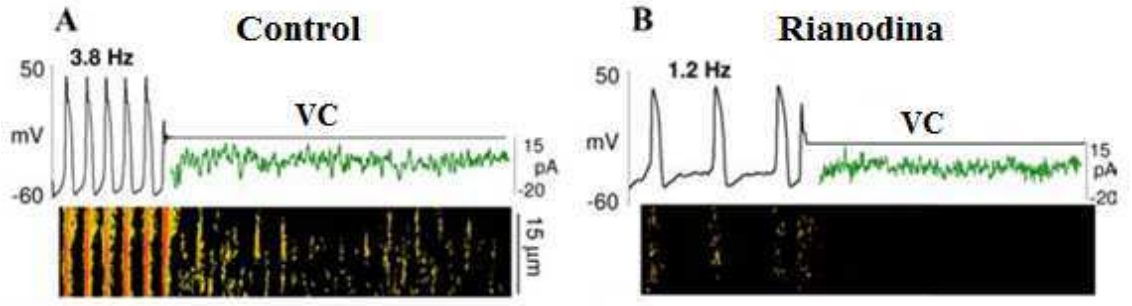

Figura 6: Grabaciones simultaneas de potencial de membrana (arriba) y microscopia confocal (abajo) en células de NS de conejo latiendo espontáneamente y durante la fijación de voltaje a -10mV en células control (A) y en presencia de rianodina (3µM) (B). Rianodina enlentece el ritmo de latido espontáneo y elimina los LCICR, VC; fijación de voltaje (Adaptada de Lakatta et al., 2008).

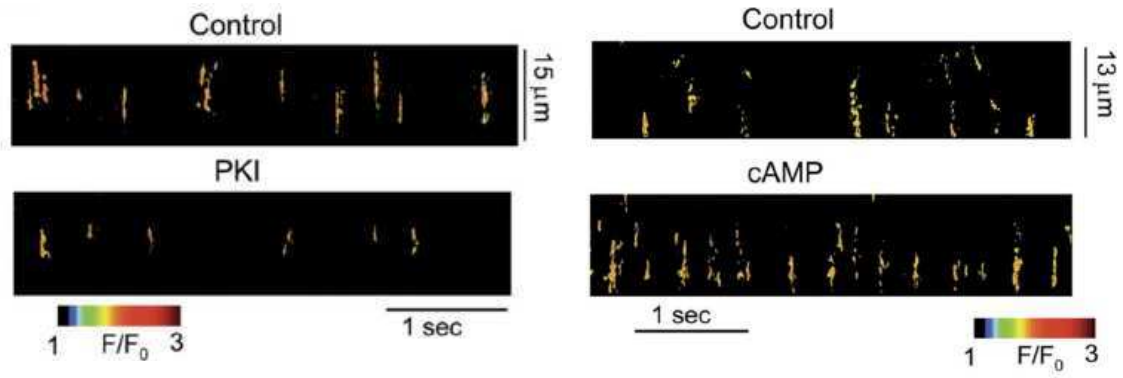

Figura 7: Control de LCICRs por PKA. A la izquierda disminuye en presencia PKI (inhibidor protéico de PKA) y a la derecha se estimula en presencia de AMPc. (Vinogradova et al., 2006).

Los mecanismos celulares subyacentes a este "reloj" de Ca^{2+} no están completamente aclarados en la actualidad, pero recientemente se ha demostrado que los LCICRs se estimulan por AMPc y se suprimen por la inhibición de la actividad PKA (figura 7) (Vinogradova et al., 2006). Estos autores también han encontrado que los niveles de AMPc y la actividad basal PKA son casi 10 veces mayores en las células del NS que en los miocitos auriculares y ventriculares (figura 8), y que la alta actividad de PKA parece ser un requisito previo para la actividad marcapasos (figura 7).

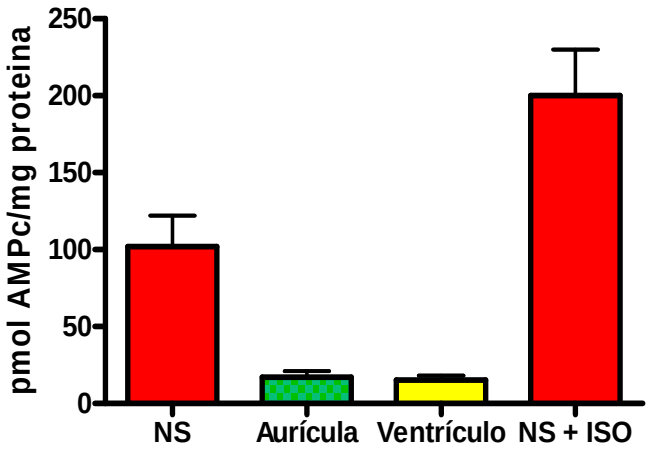

Figura 8: Niveles de AMPc en tres regiones cardíacas.; NS, nódulo sinusal; ISO, isoproterenol. (Modificado Vinogradova et al., 2006).

En conclusión, los LCICR mediados por el RS participan en la generación de la fase exponencial del prepotencial. Los dos elementos clave de este mecanismo, antes del PA, son los LCICR y NCX. El Ca^{2+} liberado por RyR2 activa al NCX que genera una corriente de entrada contribuyendo a la fase exponencial del prepotencial hasta alcanzar el umbral. En un trabajo reciente, Lyashkov et al. (2007) han detectado la co-localización del NCX y de RyR2 en células de NS de conejo y propusieron han propuesto que la proximidad entre NCX y RyR2 permite una rápida conversión de los LCICR en oscilaciones de voltaje de la membrana.

2.3 Vía de señalización de los receptores acoplados a proteínas G.

Los receptores son proteínas mediadoras de los efectos de hormonas, neurotransmisores, proteínas o iones. Los receptores acoplados a proteínas G (GPCRs) son muy numerosos y participan en gran número de rutas de señalización. Según un estudio reciente del genoma humano se estima que hay más de 800 GPCRs que reúnen a una gran variedad de ligandos de muy diversa naturaleza y tamaño (Takeda et al., 2002). Los GPCRs son proteínas transmembrana que tienen en común el dominio intracitoplasmático de unión a la proteína G, mientras que muestran una gran heterogeneidad en la porción extracelular que lleva a cabo la unión con el ligando. La proteínas G son capaces de actuar sobre una gran gama de efectores intracelulares desencadenando una gran variedad de respuestas. Los GPCRs son una familia de receptores de 7 dominios transmembrana. Estos receptores son una de las dianas más importantes de la terapéutica, con una frecuencia aproximada del 30 % de las prescripciones médicas (figura 9) (Hopkins y Groom, 2002). Un ejemplo de GPCRs es el receptor $5HT_4$ y sus *splice variants* que se ilustra en la figura 10.

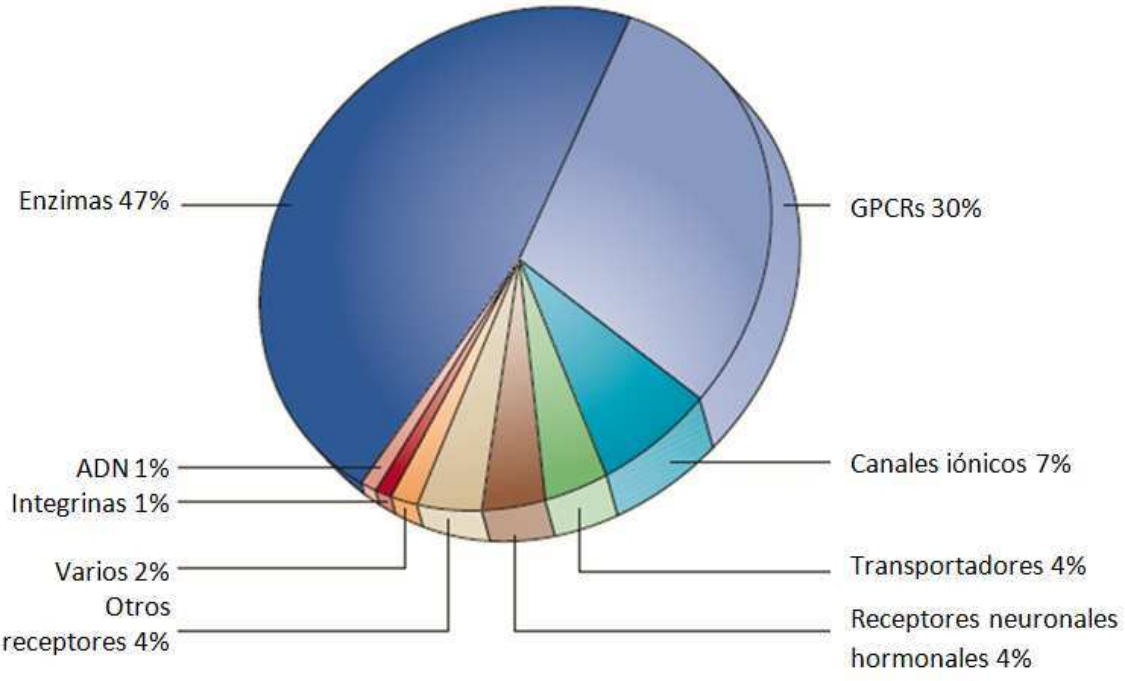

Figura 9: Dianas farmacológicas de las prescripciones médicas (adaptada de Hopkins y Groom, 2002).

Dependiendo del ligando y del tipo de GPCRs, los sitios activos incluyen el fragmento N-terminal, las horquillas *(loops)* extracelulares, los dominios transmembrana y las conexiones de las horquillas transmembranales exteriores (hélices) (Gether y Kobilka, 1998).

Las proteínas G son heterotrímeros compuestos por tres subunidades distintas, α, β y γ. La subunidad α es el principal regulador de la proteína efectora, las subunidades β y γ, actúan como un complejo, como una sola subunidad βγ, que también puede interactuar y modular la actividad de algunas proteínas efectoras. En la proteína G el GDP se encuentra unido herméticamente a la subunidad α, cuando el receptor no está ocupado y el efector se encuentra inactivo. Cuando el ligando y el receptor se unen, el receptor interacciona con el heterotrímero promoviendo un cambio conformacional y la disociación del GDP del sitio de unión en la subunidad α. Inmediatamente este sitio de unión para nucleótidos de guanina es rápidamente ocupado por GTP. La unión de GTP con la subunidad α induce un cambio conformacional con dos consecuencias: 1) la proteína G se disocia del complejo ligando-receptor dejando libre el receptor para otro acoplamiento con otra proteína G vecina y 2) también reduce la afinidad de la subunidad α por βγ, dando lugar a su disociación. La subunidad α posee actividad GTPasa. La hidrólisis catalizada por la subunidad α deja a GDP en el sitio de unión, causando la disociación y desactivación del complejo activo. Esta actividad en esencia es un reloj interno, que controla el cambio de activación/desactivación. La forma de GDP unida a α, tiene alta afinidad por βγ, dando la consecuente reasociación de α GDP con β y γ devolviendo al sistema a su estado inactivo (Hepler y Gilman, 1992). Las diferentes familias de proteínas Gα ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$) difieren en la cascada de señalización que activan y sus consecuentes respuestas fisiológicas. Los nuevos descubrimientos sobre los GPCRs repercuten en el diseño de fármacos más eficaces.

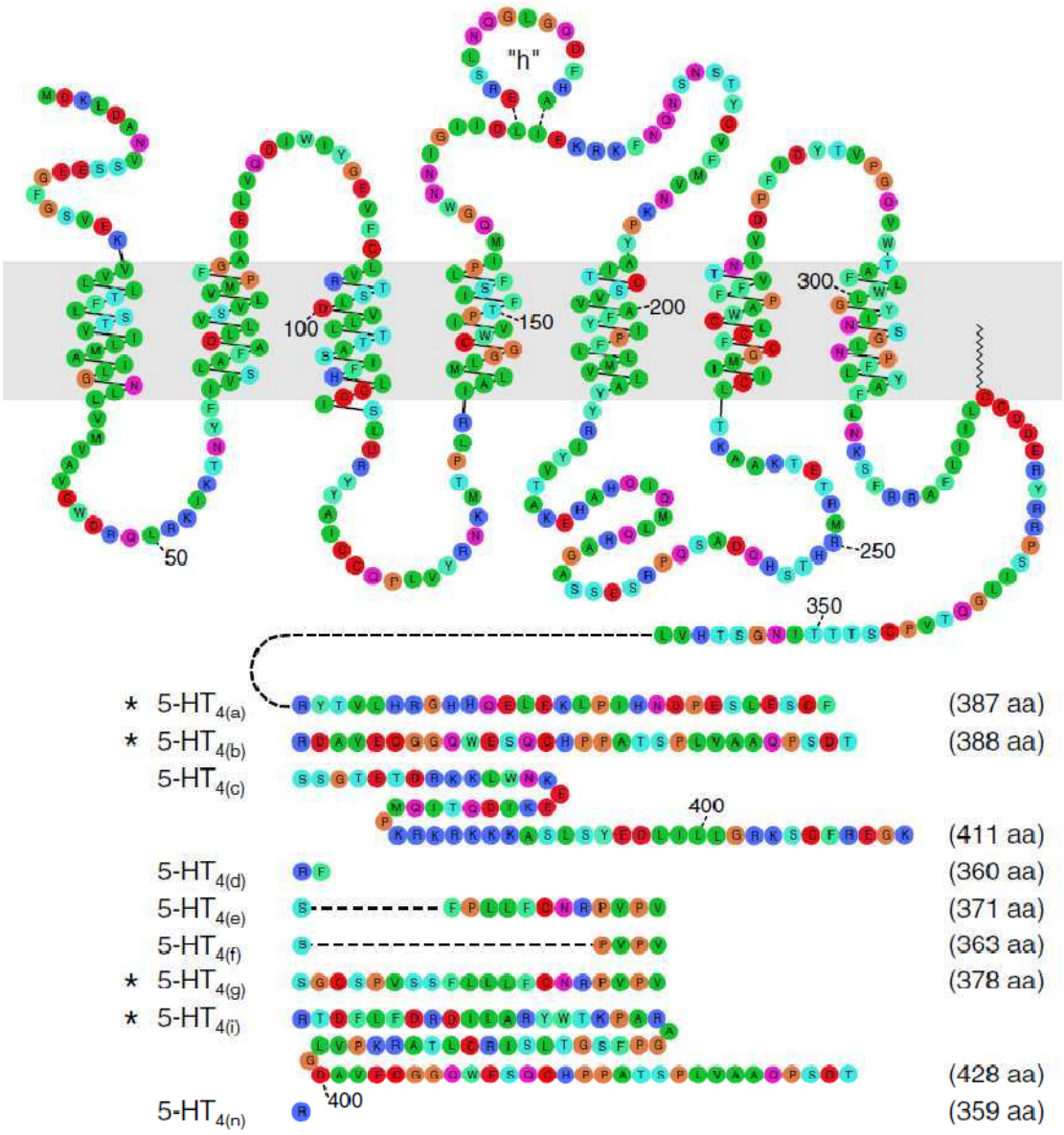

Figura 10: Receptor 5-HT$_4$ con sus diferentes "splice variants". El asterisco señala los que se expresan en corazón humano. "h" muestra unos aminoácidos extra que solo se han encontrado en asociación con la cola tipo b debido a un exón extra del gen (Kaumann y Levy, 2006).

Los ligandos de los receptores unidos a proteínas G son muy diversos, desde iones o moléculas pequeñas hasta grandes proteínas. A pesar de la gran variedad y abundancia de receptores unidos a proteínas G que participan en las distintas rutas de señalización, su estructura tridimensional aún no está totalmente resuelta. Existen dificultades para su cristalización por motivos de producción, purificación, estabilidad de los receptores y homogeneidad. El problema de la estabilidad se debe a que hay receptores que precisan detergentes específicos para su extracción que impiden la formación de cristales. La

homogeneidad también es un problema, ya que en una misma célula hay gran variedad de GPCRs y se necesita aislar un único receptor en cantidad y pureza suficientes para poderlo cristalizar (Kobilka, 2007).

Después de la estimulación por el ligando, la subunidad disociada $G_{\alpha s}$ activa la señalización con la estimulación de adenilil ciclasa (AC). Esta enzima es la responsable principal del incremento intracelular de producción del segundo mensajero 3'5'-adenosin monofosfato ciclico (AMPc) (Sutherland y Robison, 1966). El AMPc modula la contractilidad cardíaca por la activación de la proteína quinasa dependiente de AMPc (PKA).

2.3.1 Adenilil ciclasa

La AC es la enzima responsable de la conversión del trifosfato de adenosina (ATP) a cAMP y pirofosfato. Se han descrito, clonado y secuenciado en mamíferos nueve isoformas (AC1-AC9) (Hanoune y Defer, 2001). Las ACs están ancladas en la membrana citoplasmática. La amplia diversidad de isoformas permite localizar la expresión de AC en diferentes dominios de la membrana, permitiéndole tener diferentes respuestas compartiméntales dentro de una misma célula, así como el control de la síntesis de AMPc a través de la modulación específica de otros procesos de transducción de señales (Sunahara et al., 1996).

Existen diferentes mecanismos de regulación de estos enzimas tales como: fosforilación por parte de PKA o PKC, cambios en niveles intracelulares de Ca^{2+} (Ca^{2+}/Calmodulina), mediante la subunidad α de la proteína G inhibitoria ($G_{i\alpha}$) o bien por el complejo βγ de proteínas G ($G_{\beta\gamma}$) (Hanoune y Defer, 2001). En función del mecanismo de regulación se han clasificado en 4 grupos (tabla 1).

Grupo	Adenilil ciclasa	Regulación
1	AC1	Estimulación por Ca^{2+}/calmodulina
	AC3	Estimulación por forscolina
	AC8	
2	AC2	Estimulación por subunidades βγ
	AC4	Estimulación por fosforilacion por PKC
	AC7	Estimulación por forscolina
3	AC5	Inhibición por $[Ca^{2+}]$ <1µM
	AC6	Inhibición por β1γ2 de la proteína G
		Estimulación por forscolina
4	AC9	Inhibición por calcineurina
		Estimulación por forscolina

Tabla 1: Propiedades reguladoras de las AC de mamíferos. (Modificada de Hanoune y Defer, 2001).

2.3.2 AMPc

El AMPc es un segundo mensajero intracelular. Este concepto comenzó a utilizarse en 1958, cuando se descubrió que la acción sobre el metabolismo hepático de la adrenalina y el glucagón estaba mediada por AMPc (Sutherland y Rall, 1958).

El AMPc está involucrado en el mecanismo de acción y en los procesos de transducción de la señal de múltiples moléculas, como son las hormonas, los neurotransmisores, las citocinas y otros factores. Como consecuencia de la gran variedad de moléculas que median su acción a través AMPc, éste se encuentra implicado en diversos procesos como son la contracción muscular, la agregación plaquetaria, los procesos metabólicos, la neurotransmisión, la síntesis de esteroides o la movilización de glucosa (Iyengar, 1996).

La concentración intracelular de AMPc en las células cardíacas varía de unas regiones a otras (figura 8). La concentración intracelular de AMPc es un factor importante en su mecanismo de señalización y puede variar rápidamente,

aumentando o disminuyendo, en respuesta a estímulos extracelulares. Los dos factores principales para la regulación de la concentración intracelular de AMPc son la modulación de la velocidad de síntesis a través de AC y la velocidad de degradación por parte de las fosfodiesterasas (PDEs). Estas enzimas son las encargadas de hidrolizar los nucleótidos cíclicos.

2.3.3 Proteina quinasa A (PKA)

Es una proteína heterotetramérica formada por dos subunidades reguladoras que contiene los sitios de unión de AMPc, y otras dos subunidades catalíticas. En ausencia de AMPc, el tetrámero está inactivo. La unión de AMPc a las subunidades reguladoras reduce su afinidad por las subunidades catalíticas, que son activas por sí mismas (Scott, 1991). Las subunidades catalíticas liberadas, fosforilan residuos de serina y treonina. En el músculo cardiaco la subunidad catalítica de PKA modula la contractilidad mediante la fosforilación de proteínas del miocito entre las que se incluyen el canal de Ca^{2+} tipo L (Haase et al., 1996), el RyR2 (Marx et al., 2000), PLB (Tada y Toyofuku, 1998), la TnI (Solaro et al., 1976) y la proteína C (Kaumann et al., 1999).

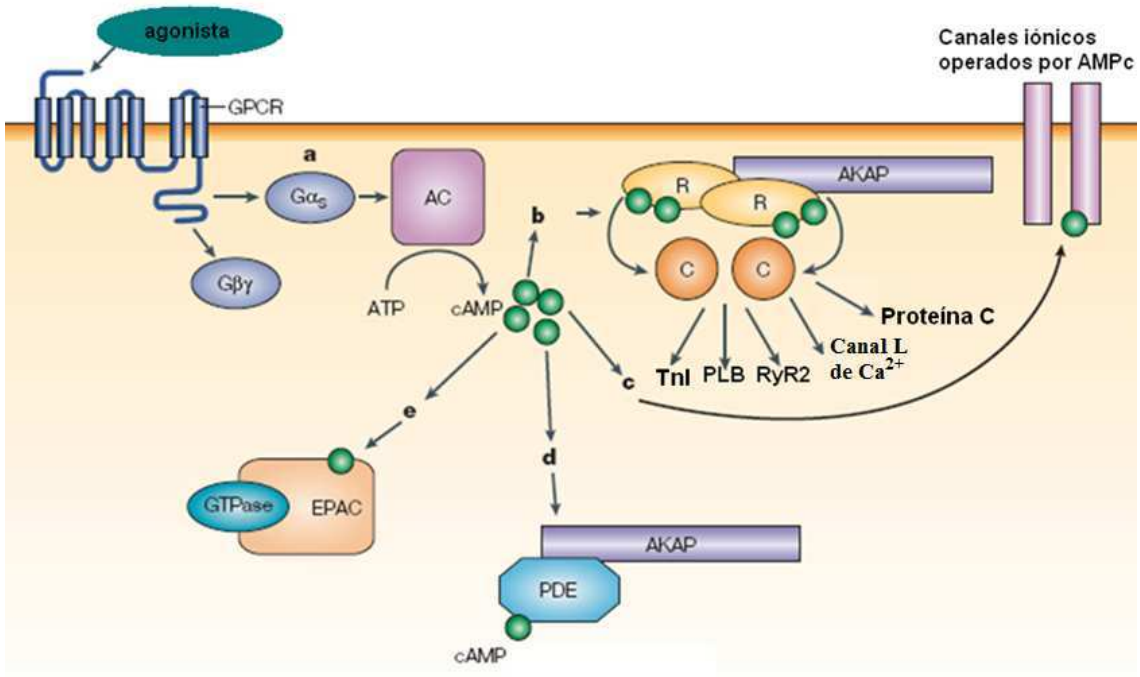

Figura 11. Rutas de señalización del AMPc. Estimulación por el agonista del receptor acoplado a proteína Gαs (GPCR) (a) activando adenilil ciclasa (AC) que cataliza la síntesis de AMPc. El AMPc se une a las subunidades reguladoras (R) de de la proteína quinasa A (PKA) (b) liberando las subunidades catalíticas activas (C). El AMPc también puede activar canales iónicos operados por AMPc (c); fosfodiesterasas (PDEs) (d) y factores de intercambio de nucleótidos de guanina conocidas como proteínas de intercambio activadas por AMPc (EPAC) (e). AKAP, proteína de anclaje de PKA; TnI, troponina I; PLB, fosfolambano; RyR2, canal de rianodina tipo 2 (Modificado Wong y Scott, 2004).

2.3.4 Desensibilización de los receptores.

La actividad de los GPCR representa un balance coordinado entre los mecanismos moleculares responsables de la señalización del receptor, desensibilización y resensibilización.

La exposición prolongada de los GPCRs a los agonistas, con frecuencia, da lugar a una rápida atenuación de la respuesta denominada desensibilización. Este proceso,

en cierto modo, actúa como un mecanismo para proteger a la célula de un exceso de señales, ya sea en cantidad o en duración.

La desensibilización de la señalización de los GPCR es un proceso con diferentes pasos, clásicamente descrito por 4 procesos principales. En el primer paso, el GPCR es desacoplado de la proteína G después de la estimulación prolongada del receptor por el agonista. Esto sucede a concentraciones relativamente bajas de agonista o incluso en ausencia de este, dando como resultado la atenuación de la respuesta primaria, por ejemplo, disminución de la producción del segundo mensajero. Este proceso puede estar mediado por PKA que fosforila al receptor dando como resultado el desacoplamiento de G_s(Bouvier et al., 1988; Hausdorff et al., 1989). En el segundo caso, la desensibilización de los GPCR ocurre por fosforilación del mismo por la quinasa del receptor de proteína G (GRK), de las que se han descrito 7 genes (Fergusson, 2001). Este proceso tiene lugar a concentraciones elevadas de agonista unido al receptor. La fosforilación de los GPCR por GRK permite la unión con la β-arrestina. El desacoplamiento del receptor de la proteína G es habitualmente debido a la unión de la β-arrestina. La β-arrestina, unida al receptor, puede formar un complejo con las clatrinas que será responsable de la internalización y endocitosis del receptor. Sin embargo, puede darse el desacoplamiento sin la fosforilación del receptor (en presencia de GRK) e incluso en ausencia de β-arrestina, como ocurre con el receptor 5-HT_4 (Barthet et al., 2005). Las características moleculares involucradas en la interacción entre el receptor y la β-arrestina determinan las particularidades de la endocitosis, del desplazamiento intracelular, el reciclaje y la resensibilización (tercer paso) o la degradación (cuarto paso, también llamado *"down-regulation"*) (Ferguson, 2001). El tiempo en el que ocurren estos procesos puede ser muy variable, desde segundos en la fosforilación, minutos en la endocitosis, hasta horas en la disminución de RNAm y degradación del receptor (Ferguson, 2001; Kohout y Lefkowitz, 2002). Además de su papel en la desenbilización, las arrestinas han mostrado recientemente tener otras funciones como la de transportar la PDE4 hacia la proximidad del GPCR (Perry et al., 2002) o de activar otras vías de señalización (Lefkowitz y Shenoy, 2005).

Los GPCRs han sido divididos en dos grupos basados en su forma de unirse a la β-arrestina. La clase A, entre ellos los β_2AR, que cuenta con un conjunto de residuos de

serina y treonina desordenados, que tienen un rápido reciclaje de vuelta a la superficie de la célula. La clase B, por ejemplo el $5HT_4$ (Barthet et al., 2005), que cuenta con los residuos de serina y treonina agrupados en racimos, que forman un complejo estable con la β-arrestina y predominantemente, son dianas de lisosomas para su degradación *("down-regulation")* (Oakley et al., 2001).

2.4 Receptores β-adrenérgicos

En el corazón humano coexisten tres receptores β-adrenérgicos (βAR) llamados β_1AR, β_2AR y β_3AR. Cuando son activados, los β_1AR en mamíferos median fuertes incrementos en la fuerza contráctil y aceleración de la relajación, mientras en los β_2AR, estos efectos, dependen de la especie (Kaumann, 1986; Kaumann y Lemoine, 1987; Kaumann y Molenaar, 1996; Kaumann et al., 1996a; Heubach et al., 1999; Kaumann et al., 1999). Los efectos de la estimulación de ambos receptores, β_1AR y β_2AR, en contractilidad son consecuentes del acoplamiento a la vía proteína $G_{s\alpha}$ → AC → cAMP →PKA, con la consiguiente fosforilación de las dianas responsables de la contracción y la aceleración de la relajación por PKA (Kaumann et al., 1996a; Kaumann et al., 1999; Molenaar et al., 2000; Molenaar et al., 2007a) incluyendo canales de Ca^{2+} tipo-L y PLB (Walsh y Van Patten, 1994; Kaumann et al., 1999). La fosforilación de los canales de Ca^{2+} tipo-L promueve la entrada de Ca^{2+} e incrementa la contracción. La fosforilación de PLB está involucrada en el incremento de la relajación diastólica debido al incremento de la recaptación de Ca^{2+} por parte de SERCA (figura 12).

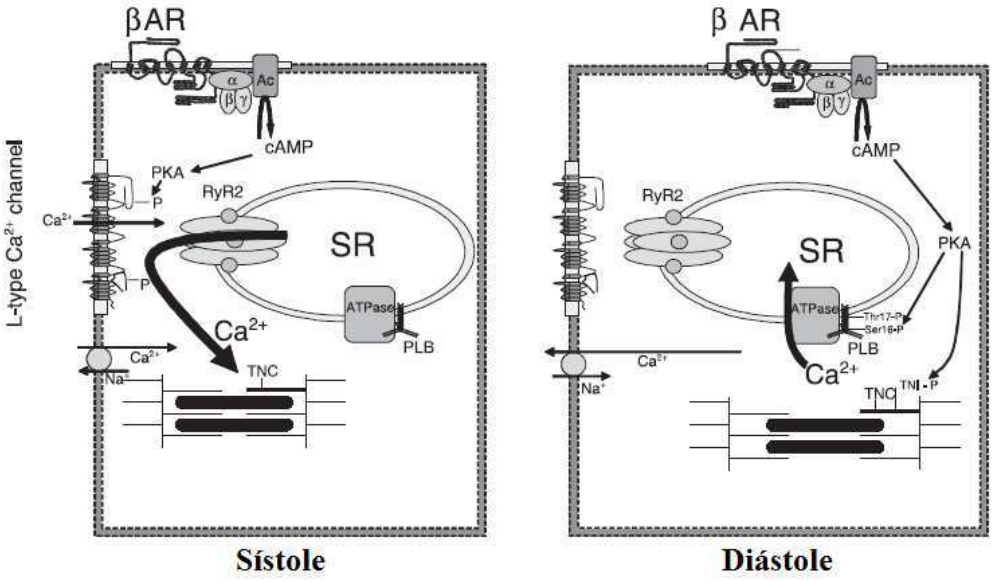

Figura 12: Activación del receptor β*-adrenérgico y dianas de fosforilación fundamentales para el acoplamiento de la excitación-contracción en la célula miocárdica humana.* βAR*, receptor* β*-adrenérgico; PLB, fosfolambano; AC, adenilil ciclasa; TNC, troponina C; TNI-P, troponina I fosforilada; RyR2, receptor de rianodina. (Modificada de Molenaar et al., 2007b).*

Los receptores βAR median las señales excitatorias del sistema nervioso simpático sobre el sistema cardiovascular. En el corazón humano sano el 80 % de los receptores son β_1ARs, están acoplados a proteínas G_s y median incrementos de frecuencia y contractilidad (Rockman et al., 2002). En tejidos cardíacos humanos (Kaumann et al., 1996a; Kaumann et al., 1999; Molenaar et al., 2000) y miocitos aislados (Del Monte et al., 1993) ambos, β_1AR y β_2AR, también aceleran la relajación miocárdica, mejorando la función diastólica. Los efectos de la estimulación de la contractilidad por ambos β_1AR y β_2AR son consistentes con el acoplamiento a la ruta de la proteína $G_{s\alpha}$=>AC=>AMPc=> PKA, con la consiguiente fosforilación de las dianas enzimáticas responsables de la contracción y la relajación miocárdica por PKA (Kaumann et al, 1996a; Kaumann et al, 1999; Molenaar et al. 2000; Molenaar et al. 2007a). Los β_2AR humanos tienen mayor potencia activadora de la proteína G_s/AC que los β_1AR (Kaumann y Lemoine, 1987; Levy et al., 1993). Green y Liggett (1994) demostraron que una región rica en prolinas del β_1AR, que no tiene el β_2AR, causa un impedimento parcial estérico en la proteína G_s.

La señal del los βAR tiene 4 papeles principales en el corazón: incrementar la frecuencia de latido cardiaco (cronotrópica), la contractilidad (inotrópica), la relajación miocárdica (lusitrópica) y modular el metabolismo en función de lo requerido por estos incrementos de demanda energética (metabólica). La frecuencia cardíaca es principalmente controlada por el NS, que contiene las células marcapasos más especializadas. Los neurotransmisores noradrenalina y adrenalina aumentan la frecuencia de latido del marcapasos via I_f (DiFrancesco y Tortora, 1991) y por la liberación de Ca^{2+} del RS (Bogdanov et al., 2001). Las respuestas inotrópicas son principalmente atribuidas a la fosforilación, catalizada por PKA, del canal de Ca^{2+} tipo L, PLB y TnI (figura 11). La señal de los βAR incrementa I_{CaL}, aumentando la entrada del Ca^{2+} a los miocitos (Reuter 1983). La fosforilación de PLB libera su inhibición sobre la bomba de Ca^{2+} del RS, captando más Ca^{2+} dentro del RS que estará disponible para su liberación en contracciones subsecuentes (Kirchberber et al., 1975). La fosforilación de PLB por catecolaminas contribuye a la función lusitrópica, por el incremento de captacion de Ca^{2+} desde el citosol al RS por la SERCA acelerándose la relajación de los miofilamentos. La TnI también es fosforilada por PKA y reduce la afinidad por el Ca^{2+}

de la TnC (Solaro et al., 1976). Tanto la respuesta inotrópica, por el incremento del trabajo de los miofilamentos como el aumento de actividad de SERCA necesitan mayor aporte energético para poder generar mayores contracciones. Para mantener estas demandas, la PKA también fosforila y activa a la quinasa fosforilasa, una enzima metabólica que hidroliza el glucógeno liberando glucosa para la síntesis de ATP (Cohen, 1973). Durante la insuficiencia cardíaca se produce un incremento crónico de noradrenalina que aumenta la demanda metabolica. Ambos β_1AR y β_2AR causan arritmias (Kaumann y Sanders, 1993; Del Monte et al., 1993; Desantiago et al., 2008) y para prevenir estos efectos nocivos se administran los β-bloqueantes (Bristow, 2000).

2.4.1 Sitio β_1AR de baja afinidad (β_{1L})

Algunos β-bloqueantes, como el (-)-CGP12177 y el (-)-pindolol tienen actividad agonista parcial (Kaumann 1989; Kaumann, 1996; Kaumann y Molenaar, 1997; Molenaar et al., 1997a; Sarsero et al., 2003; Joseph et al., 2003; Kaumann y Molenaar, 2008), pero a concentraciones aproximadamente 2 unidades logarítmicas más altas que las requeridas para bloquear el receptor. Una propiedad característica de este receptor activado por (-)-CGP12177 es resistir el bloqueo por propanolol. Su identidad como receptores β_1AR no fue inmediatamente obvia. Experimentos posteriores comparando los efectos inotrópicos positivos de (-)-CGP12177 en aurícula izquierda de ratones *knockout* β_2AR y doble *knockout* β_1/β_2AR revelaron un papel imprescindible de los receptores β_1AR (Kaumann et al., 2001). Los receptores β_1AR pueden ser considerados que existen en dos "estados" farmacológicos diferentes, el receptor β_{1H}AR, un sitio de alta afinidad, que es activado por noradrenalina y bloqueado con alta afinidad por β-bloqueantes; y el receptor β_{1L}AR, un sitio de baja afinidad, que es activado por (-)-CGP12177, (-)-pindolol, y otros β-bloqueantes (Kaumann y Molenaar, 2008), y antagonizados con relativamente poca afinidad por los β-bloqueantes sin acción agonista. Sarsero et al. (2003) marcaron los sitios H y L con ^{3}H-CGP12177 y observaron que ambos están disminuidos en la insuficiencia cardíaca. La mutación del aspartato 138 a glutamato 138 reduce 1.000.000 de veces la potencia estimulante de AC por isoproterenol mediada por H y elimina la propiedad bloqueante del bupranolol. Por el contrario, la mutacion deja casi intacta la estimulación de AC por (-)-CGP12177 y la capacidad bloqueante del bupranolol, mediados ambos efectos por L (Joseph et al., 2004).

2.4.2 β_1AR y β_2AR en tejidos cardíacos humanos

Ambos β_1AR y β_2AR están distribuidos por los tejidos y miocitos auriculares y ventriculares del corazón humano (Gille et al., 1985; Buxton et al., 1987; Kaumann y Lemoine 1987; Del Monte et al., 1993; Molenaar et al., 2006). La estimulación de cada subtipo βAR por noradrenalina o adrenalina modifica la contractilidad basal por el incremento de la fuerza contráctil y la aceleración de la relajación. La investigación de los receptores βAR en corazón humano ha sido facilitada por el uso del apéndice de aurícula derecha obtenido de la cirugía cardíaca.

2.4.2.1 Aurícula derecha humana

El proceso de la contracción y relajación auricular está modulado por la activación de ambos β_1AR y β_2AR. La estimulación independiente de ambos receptores β_1AR, por (-)-noradrenalina en presencia de un antagonista β_2AR ICI 118551 (50 nmol/L) (Lemoine et al., 1985), o β_2AR por (-)-adrenalina en presencia de un bloqueante β_1AR CGP20712A (300 nmol/L) (Kaumann, 1986) produce máximas contracciones (Gille et al., 1985; Kaumann et al 1989b; Molenaar et al., 2006; Molenaar et al., 2007a). La habilidad de los β_2ARs para mediar efectos máximos en aurícula humana ocurre a pesar de que la población de β_2AR es solo un 35 % de la población de ambos receptores (Kaumann y Molenaar, 1997). Esto es debido a que los β_2AR están acoplados con mayor eficacia a la proteína G_s que los β_1AR (Kaumann et al., 1996a; Molenaar et al., 2007a; Gille et al., 1985), confirmados con β_1AR y β_2AR co-transfectados (Levy et al., 1993).

La activación de los β_1AR y β_2AR de aurícula derecha humana causa incrementos en la fuerza contráctil y acelera la relajación (Hall et al., 1990; Kaumann et al., 1996a; Molenaar et al., 2007a). La aceleración de la relajación está asociada con la fosforilación de PLB (Kaumann et al., 1996a; Kaumann y Molenaar et al., 2007a).

En aurícula derecha humana con insuficiencia cardíaca está reducida la potencia inotrópica de catecolaminas (Molenaar et al., 2007a). En pacientes tratados crónicamente con bloqueantes selectivos β_1, las repuestas mediadas por β_2AR están aumentadas mientras las respuestas a través de β_1AR están solo marginalmente

incrementadas (Kaumann et al,. 1989b; Hall et al., 1990; Motomura et al., 1990; Molenaar et al., 1997b). El bloqueo crónico de los βAR favorece la aparición de arritmias por noradrenalina /adrenalina mediante los β_1AR y β_2AR respectivamente (Kaumann y Sanders, 1993).

En pacientes con insuficiencia cardíaca avanzada, la densidad de los receptores en la superficie de la membrana y los niveles intracelulares de RNAm de los β_1AR están reducidos (Ungerer et al., 1993; Bristow et al., 1993). En estos pacientes, la contribución relativa de los β_2AR es similar a la de los β_1AR en el mantenimiento de la función cardíaca (Kaumann et al., 1999).

2.4.2.2 Ventrículo humano

Ambos β_1AR y β_2AR, median poderosos incrementos de fuerza contráctil y aceleración de la relajación. Las propiedades características del acoplamiento a proteína G_s son similares a las de la aurícula humana. La activación de ambos β_1AR y β_2AR, causa fosforilación de PLB, de TnI y de proteína C en ventrículos de corazones con insuficiencia cardíaca (Kaumann et al., 1999) y corazones de infantes sin insuficiencia cardíaca (Molenaar et al., 2000).

Una diferencia fundamental entre la aurícula y el ventrículo es la duración de la contracción, que es considerablemente mayor en el ventrículo (Kaumann et al., 1999; Molenaar et al., 2007a). Esto parece ser debido a una mayor expresión de PLB y a la existencia de menores niveles de SERCA IIa en ventrículo con respecto a la aurícula (Boknik et al., 1999) y posiblemente también a diferencias en las cadenas de miosina (Morano, 1999).

2.4.3 Diferencias en la señalización de β_2AR entre humanos y otras especies.

Una diferencia entre los β_2AR humanos y de otras especies es su reducida habilidad para acoplarse a proteína $G_{i\alpha}$. En roedores, los receptores cardíacos β_2AR se acoplan simultáneamente a ambas, proteína $G_{s\alpha}$ y $G_{i\alpha}$ (Xiao et al., 1995; Xiao et al.,

1999; Zheng et al., 2005). Las vías de señalización β_2AR, a través de $G_{s\alpha}$ y $G_{i\alpha}$, se oponen mutuamente, dando como resultado una contractilidad atenuada y la abolición de la habilidad para acelerar la relajación (Xiao et al., 1995; Zheng et al., 2005). Estos autores se basaron en un estudio realizado por Lemoine y Kaumann (1991) donde encontraron que (-)-noradrenalina, actuando por β_1AR, acelera la relajación del músculo papilar felino pero no (-)-adrenalina por el β_2AR. La inhibición de la proteína $G_{i\alpha}$ con toxina pertussis (PTX) causa un incremento de la potencia de los efectos inotrópicos, amplitud y cinéticas de la contracción y transientes de Ca^{2+} de los agonistas a través de receptores β_2AR pero sin la intervención de los β_1AR (Xiao et al., 1995). La fosforilación de PLB fue observada después de la activación de los receptores β_2AR, pero no β_1AR, dando una explicación para las diferencias en los efectos, por la activación de receptores β_1 y β_2AR, sobre las cinéticas en la contracción y relajación (Xiao et al., 1994). El efecto mediado por proteína $G_{s\alpha}$ se demostró tras la inhibición de $G_{i\alpha}$ mediante el tratamiento con PTX (Xiao et al., 1995; Xiao et al., 1999). Hay gran controversia sobre este tema, en corazón de ratón, Heubach et al. (2002) no han podido detectar efectos cardioestimulantes por el β_2AR incluso tras el tratamiento con PTX. Laflamme y Becker (1998) tampoco encontraron que, tras el tratamiento con PTX, hubiese efectos mediados por el β_2AR sobre el I_{CaL}. En β_2AR humanos sobreexpresados en el corazón ratón (TG4), el acoplamiento de β_2AR a G_i es dependiente de la concentración de agonista. La adrenalina a concentraciones 1000 veces más altas, pero no la noradrenalina, interactúa con el sitio de unión del β_2AR que dará lugar al acoplamiento con proteína G_i y que es diferente del lugar que induce el acoplamiento a G_s (Heubach et al., 2004). Estos hallazgos sugieren dos cosas, una, que solo adrenalina, y no noradrenalina, es capaz de cambiar la configuración del β_2AR humano para acoplarse a G_i y dos, pero solo a concentraciones muy altas no fisiológicas. Estos experimentos están de acuerdo con el acoplamiento a G_i del β_2AR de aurícula humana con una concentración muy elevadas de isoproterenol del orden 10^{-4} M (Kilts et al., 2000).

2.5 Receptores 5-HT

Hay 14 tipos diferentes de receptores de serotonina agrupados en 7 grupos (5-HT_1 a 5-HT_7) basado en su estructura molecular, propiedades de transducción de señal y farmacológicas (Hoyer et al., 2002). En el sistema cardiovascular se han demostrado multitud de efectos de serotonina (5-hidroxitriptamina, 5-HT) (Kaumann et al., 1993; Kaumann y Sanders, 1998).

Receptores 5-HT_4

El receptor 5-HT_4 esta positivamente acoplado a proteína G_s y al sistema AC-AMPc (Langlois y Fischmeister, 2003). El receptor 5-HT_4 humano existe con múltiples *splice variants*, todos son idénticos hasta el aminoácido Leucina-358, menos uno que tiene aminoácidos extra en el segundo loop extracelular (Bender et al., 2000), seguidos de diferentes fragmentos C-terminales (figura 10).

Cada uno de los diferentes *splice variants*, revisado por Kaumann y Levy (2006), es llamado 5-$HT_{4\ (a)}$, $5HT_{4\ (b)}$ … y así hasta 5-$HT_{4\ (i)}$ que ha sido la última incorporación a la lista (Brattelid et al., 2004a). Es my difícil determinar la expresión relativa de cada uno, ya que los anticuerpos específicos no resultan muy eficaces para su determinación. Además en el corazón la expresión de estos receptores es muy baja, menor de 4 fmol/mg de proteína en aurícula derecha humana (Kaumann et al., 1996b). La expresión relativa de las diferentes variantes solo ha sido informada desde el comienzo de los estudios de reacción en cadena de la polimerasa en tiempo real (RT-PCR) cuantitativa.

2.5.1 Receptores Cardíacos 5-HT_4

Los receptores cardioestimulantes 5-HT_4 han sido descritos en aurícula derecha (Kaumann et al., 1990), aurícula izquierda (Sanders y Kaumann, 1992) y en ambos ventrículos (Brattelid et al, 2004a) donde median incrementos de contractilidad. Los receptores cardíacos 5-HT_4 humanos median arritmias (Kaumann, 1994; Kaumann y Sanders, 1994; Pau et al., 2003; Brattelid et al., 2004b).

Los receptores 5-HT_4 están presentes en condiciones fisiológicas en los miocitos auriculares humanos (Sanders et al., 1995; Blondel et al., 1997; Pau et al., 2003) y porcinos, mientras que no se encuentran en el corazón de otros animales de laboratorio pequeños. El cerdo es el único modelo animal, no primate, en el que se puede estudiar la función cardíaca de los receptores 5-HT_4 (Kaumann 1990, Villalón et al., 1990; De Maeyer et al., 2006) en condiciones fisiológicas. Hasta el momento se han descrito 9 variantes del receptor porcino 5-HT_4 que son notablemente distintos al humano, incluido uno con 9 segmentos transmembrana (De Maeyer et al., 2008).

2.5.1.1 Frecuencia cardíaca

La 5-HT administrada intravenosamente puede producir taquicardia (Hollander et al., 1957, Le Mesurier et al., 1959) y/o bradicardia (Harris et al., 1960) en el hombre.

Taquicardia

La 5-HT produce taquicardia dosis dependiente en voluntarios sanos (Le Mesurier et al., 1959), probablemente a través del receptor 5-HT_4 (Kaumann y Sanders, 1998). La naturaleza 5-HT_4 de los receptores 5-HT en el NS humano fue sugerida en un estudio doble ciego aleatorio en el cual se encontró una leve taquicardia producida por cisaprida administrada como procinético (Bateman, 1986). Cisaprida es un agonista parcial de los receptores 5-HT_4 que aumenta la contractilidad de trabéculas aisladas de aurícula humana (Kaumann et al., 1991). La taquicardia provocada por 5-HT y cisaprida fue imitada en lechones recién nacidos y se demostro estar mediada por los receptores 5-HT_4 del NS (Kaumann, 1990). Es posible que la taquicardia producida por 5-HT este mediada a través de AMPc generado por la activación del receptor 5-HT_4 y la consiguiente activación de la corriente de marcapasos I_f. La 5-HT activa los canales f causando un desplazamiento hacia potenciales de acción menos negativos, acelerando así la despolarización diastólica, que deriva en taquicardia. A pesar de que no existen pruebas directas para este mecanismo en células humanas del NS, hay evidencia de estimulación de I_f provocada por 5-HT en miocitos auriculares humanos (Pino et al., 1998; Workman y Rankin,

1998). Sin embargo, no se puede descartar que otros mecanismos activados por PKA contribuyan a la taquicardia producida por 5-HT.

2.5.1.2 Fuerza cardíaca

2.5.1.2.1 Aurícula

Los receptores 5-HT_4 humanos, identificados en 1989 (Kaumann et al., 1989a), median los efectos inotrópicos y lusitrópicos positivos de 5-HT, la elevación de AMPc y la activación de PKA en aurícula derecha (Kaumann et al., 1990) e izquierda (Sanders y Kaumann, 1992). Los agonistas parciales, renzaprida y cisaprida, también producen efectos inotrópicos a través de la vía AMPc/PKA (Kaumann et al., 1991). La activación de PKA a través del receptor humano 5-HT_4 sugirió la fosforilación de los canales de Ca^{2+} tipo L (Kaumann et al., 1990, 1991) que fue verificado por Ouadid et al. (1992) y Jahnel et al. (1992). La aceleración de la relajación, efecto lusitrópico, observada con 5-HT a través del receptor auricular 5-HT_4 fue sugerido estar mediada a través de la fosforilación dependiente de PKA de PLB y TnI (Kaumann et al., 1991) y recientemente verificado por Gergs et al. (2009)

La 5-HT eleva 6 veces la corriente del canal de Ca^{2+} tipo L por estimulación de PKA a través de receptores 5-HT_4 en miocitos auriculares de pacientes en ritmo sinusal (Ouadid et al., 1992), pero los efectos son más pequeños en miocitos de corazones en insuficiencia cardíaca (Ouadid et al., 1995). El incremento de I_{CaL} debe producir en el miocito una sobrecarga de Ca^{2+}, facilitando así la aparición de arritmias (Kaumann, 1994). Kaumann y Sanders, (1994) observaron arritmias experimentales con 5-HT, mediadas por receptores 5-HT_4, en aurículas humanas. La incidencia de arritmias provocadas por 5-HT es inversamente proporcional a la frecuencia de estimulación (Kaumann y Sanders, 1994). La 5-HT también puede producir contracciones arrítmicas (Sanders et al., 1995) y eventos electrofisiológicos proarrítmicos en miocitos humanos auriculares (Pau et al., 2003). Incrementos en I_{CaL} (Pau et al., 2005), asociados con efectos inotrópicos positivos (Krobert et al., 2005), han sido también observados con el agonista parcial del receptor 5-HT_4 prucaloprida, un agente procinético del sistema gastroinstestinal (Prins et al., 2000). De cualquier forma, la prucaloprida nunca produjo contracciones arrítmicas (Krobert et al., 2005) ni cambios electrofiosiológicos (Pau et

al., 2005). Con el agonista parcial ML10302 tambien se han observado pequeños incrementos en I_{CaL}, comparados con 5-HT, en miocitos auriculares humanos (Blondel et al., 1997).

La 5-HT también aumenta la corriente I_f a través de receptores 5-HT_4 en aurícula humana (Pino et al., 1998; Lonardo et al., 2005), por lo que se podría esperar que facilitase la formación de impulsos espontáneos o actividad ectópica.

Los efectos inotrópicos y la elevación de AMPc por 5-HT (Sanders et al., 1995) así como los efectos arrítmicos (Kaumann y Sanders 1994) y electrofisiológicos (Pau et al., 2003) de 5-HT, están aumentados en aurículas humanas y miocitos obtenidos de pacientes que están crónicamente tratados con bloqueantes β-adrenérgicos. Los β_2AR (Kaumann et al., 1989b; Hall et al., 1990) y los receptores de histamina H_2 (Sanders et al., 1996), ambos acoplados a proteína G_s, también median respuestas inotrópicas y arrítmicas aumentadas (Kaumann y Sanders 1993; Sanders et al., 1996) en trabéculas auriculares obtenidas de pacientes tratados crónicamente con bloqueantes β_1 selectivos. Un posible mecanismo de las respuestas aumentadas mediadas por receptores acoplados a G_s, en aurícula humana, podría estar relacionada con su promiscuo acoplamiento a proteína G_i (Kilts et al., 2000). En pacientes con insuficiencia cardíaca se sabe que el bloqueo crónico de receptores β adrenérgicos con metoprolol produce un disminución de la actividad de la proteína $G_{\alpha i/o}$ sensible a PTX (Sigmund et al., 1996), reduciendo así probablemente la inhibición de AC producida por G_i, permitiendo mayores señales de la vía G_s=>AMPc=>PKA.

2.5.1.2.2 Ventrículo

Hasta recientemente se desconocía un papel funcional del receptor 5-HT_4 en el ventrículo humano. Los resultados iníciales mostraban que en contraste con la aurícula humana, la 5-HT no incrementaba la fuerza contráctil en preparaciones de ventrículo humano (Jahnel et al., 1992; Schoemaker et al., 1993). Similar a la situación en el hombre, 5-HT mostraba efectos cronotrópicos e inotrópicos positivos (Kaumann 1990; Parker et al., 1995) a través del receptor 5-HT_4 auricular porcino pero aparentemente no por el ventrículo porcino (Lorrain et al., 1992; Saxena et al., 1992; Schoemaket et al., 1992). Los receptores auriculares humanos 5-HT_4 están expresados con una

densidad algo menor que la de los β_1AR y β_2AR (Kaumann et al., 1996b) y median señales de AMPc menores que la de estos receptores β-adrenérgicos (Kaumann et al 1990, 1991; Sander y Kaumann 1992; Sander et al., 1995). La densidad de los receptores 5-HT_4 auriculares en el lechón recién nacido es aún 10 veces más baja que en aurícula humana adulta (Kaumann et al., 1995). La densidad de los receptores 5-HT_4 en el ventrículo humano podría ser aun menor que en la aurícula humana y que la hidrólisis de AMPc mediada por las PDEs evitara la liberación de unidades catalíticas de PKA necesarias para la fosforilación de proteínas envueltas en la cardioestimulación.

Los receptores funcionales 5-HT_4 fueron descubiertos en ventrículo, humano y porcino, en presencia del inhibidor no selectivo de PDEs IBMX (3-isobutil-1-metil-xantina) (Brattelid et al., 2004b). La 5-HT causa habitualmente un incremento de la fuerza contráctil en trabéculas de ventrículo derecho. La 5-HT, en presencia de IBMX, produce efectos inotrópicos consistentes (46 % respecto de isoproterenol) y también efectos lusitrópicos (25 % de isoproterenol) en trabéculas de ventrículo izquierdo. En algunos pacientes la 5-HT provocó contracciones ventriculares arrítmicas. La 5-HT, aun en ausencia de IBMX produjo ocasionalmente pequeños incrementos de la fuerza contráctil en trabéculas de ventrículo izquierdo. Todos los efectos de 5-HT fueron prevenidos con antagonistas selectivos de 5-HT_4, lo que indica la mediación de este receptor (Brattelid et al., 2004b).

Como se demostró en ventrículo humano, en presencia de IBMX, la 5-HT también causó efectos inotrópicos positivos en trabéculas ventriculares de lechones recién nacidos (16 % respecto de isoproterenol) y en cerdos adultos (32 % respecto de isoproterenol). Los efectos de 5-HT fueron antagonizados por antagonistas selectivos del receptor 5-HT_4 (Brattelid et al., 2004b). La 5-HT, en presencia de IBMX, aumentó la actividad PKA en ventrículo de lechón recién nacido y de cerdo adulto, consistente con el mayor efecto inotrópico en adultos. La señal de PKA provocada por 5-HT fue prevenida por antagonistas selectivos de 5-HT_4, cconsecuente con la mediación de receptores 5-HT_4 (Brattelid et al., 2004b). Estos resultados apoyan el uso del ventrículo porcino como un modelo experimental de receptor ventricular humano 5-HT_4.

2.5.2 Remodelación cardíaca, aparición del receptor 5-HT$_4$

Se ha observado que en pacientes que habían sufrido un infarto y tenían insuficiencia cardíaca, se obtenían respuestas más elevadas a 5-HT (Brattelid et al., 2004b). Por eso se realizó un estudio en rata, que normalmente no expresa el receptor 5-HT$_4$ pero si expresan su RNAm. En este estudio, seis semanas después de un infarto de miocardio inducido, aparecieron receptores funcionales 5-HT$_4$ que mediaban efectos inotrópicos de 5-HT tan marcados como los del isoproterenol. Consecuentemente se propuso el corazón infartado con insuficiencia cardíaca como modelo experimental para la investigación de receptores miocárdicos ventriculares 5-HT$_4$ (Qvigstad et al., 2005).

2.6 Regulación simpática de la actividad marcapasos

La activación de los βAR es la base del efecto cronotrópico positivo inducido por catecolaminas. Las catecolaminas aumentan la actividad de canales iónicos, así como la liberación intracelular de Ca^{2+}. La importancia relativa de los canales iónicos sarcolemicos y de la LCICRs en la regulación β-adrenérgica de la actividad de marcapasos sigue estando debatida.

La activación de las corrientes I_f ha sido propuesta para constituir un mecanismo importante en la aceleración de la frecuencia cardíaca por catecolaminas (Brown et al., 1979; Bucchi et al., 2003; DiFrancesco 1993). La probabilidad de abrir canales f, HCN-4 modulados por voltaje y AMPc, aumenta incluso con un pequeño aumento de AMPc intracelular. Un incremento en los niveles intracelulares AMPc, lleva a voltajes más positivos la curva de activación de $I_{f,}$ con lo que suministra más corriente de entrada durante la parte lineal de la despolarización diastólica (DiFrancesco, 1993). Este investigador ha propuesto que la activación de I_f es el mecanismo predominante para el incremento de la pendiente de la curva de despolarización diastólica en situación de bajo tono adrenérgico. DiFrancesco, (1993) se ha basado en la observación de que a dosis bajas, el isoproterenol incrementa la pendiente del prepotencial y la acetilcolina reduce la pendiente del prepotencial sin afectar a la forma del PA (figura 13).

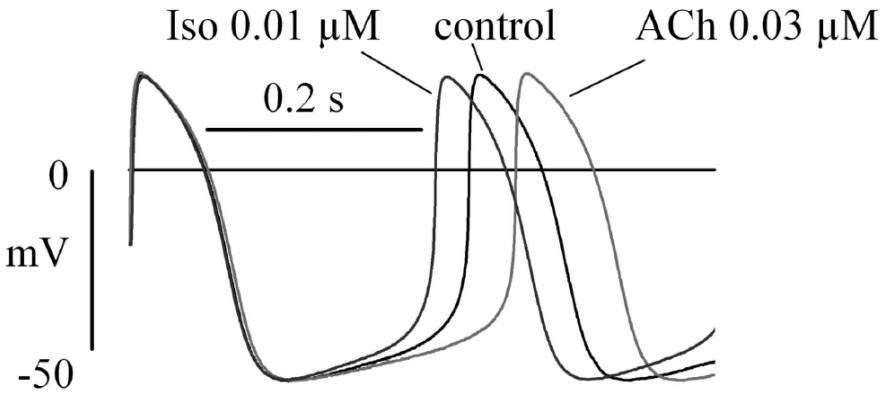

Figura 13: Bajas concentraciones de isoproterenol (Iso) y acetilcolina (Ach) modulan la pendiente del prepotencial en células aisladas de nódulo sinusal de conejo, sin afectar la forma del potencial de acción. (DiFrancesco, 1993).

Otra línea de evidencia experimental sobre el papel de los canales f para estimular la actividad marcapasos es la aceleración de los latidos en células de NS de conejo por análogos de AMPc. Isoproterenol y un análogo de AMPc, que mimetiza sus efectos, incrementan de forma similar la pendiente inicial del prepotencial en células de NS de conejo (Bucchi et al., 2003).

El papel funcional de la I_{CaL} en la regulación adrenérgica de la frecuencia cardíaca no está totalmente aclarado. Las catecolaminas incrementan robustamente la $I_{CaL,}$ en el mismo rango de concentración que la actividad marcapasos (Zaza et al., 1996; Vinogradova et al., 2002; Mangoni et al., 2003). Las corrientes I_f e I_{CaL} muestran una sensibilidad similar a isoproterenol en células de NS de conejo (Zaza et al., 1996). Tras la activación de los βAR aumenta la I_{CaL} que acelera más la despolarización diastólica (Mangoni et al., 2003).

La rianodina suprime el aumento de liberación de Ca^{2+} subsarcolemial y los LCICR en miocitos de NS de conejo (Vinogradova et al., 2002). Este efecto está acompañado por una fuerte reducción de los efectos cronotrópicos positivos inducido por isoproterenol a pesar de que se mantiene el incremento de I_{CaL}, particularmente a dosis relativamente bajas (Vinogradova et al., 2002). Por lo tanto estos autores, han propuesto que la liberación de Ca^{2+} del RS es el principal responsable de los efectos cronotrópicos positivos mediados por los βAR.

La activación de βAR estimula la actividad de la AC, que convierte el ATP en AMPc. La elevación de AMPc promueve una apertura directa de los canales f y la activación de PKA (DiFrancesco y Mangoni, 1994). Las subunidades catalíticas de PKA aumenta la actividad de diferentes canales iónicos en la membrana mediante su fosforilación.

La actividad basal de PKA es mayor en células marcapasos que en células atriales (figura 8) (Vinogradova et al., 2006). La mayor fosforilación de PLB y RyR2 genera LCICRs periódicos causando oscilaciones del voltaje de la membrana por I_{NCX}, que contribuyen al control del estado cronotrópico de la célula (Bogdanov et al., 2006; Vinogradova et al., 2006). La activación de los βAR eleva la actividad de PKA, la

frecuencia de latidos, incrementando el número de LCICR y éstos estimulando I_{NCX} (Vinogradova et al., 2008).

El mecanismo de la actividad marcapasos basado en I_f (DiFrancesco, 2006) y el basado en el "reloj de Ca^{2+}" espontáneo del RS (Maltsev et al., 2006), son modelos probablemente complementarios y tienen en común el principal mensajero que es el AMPc.

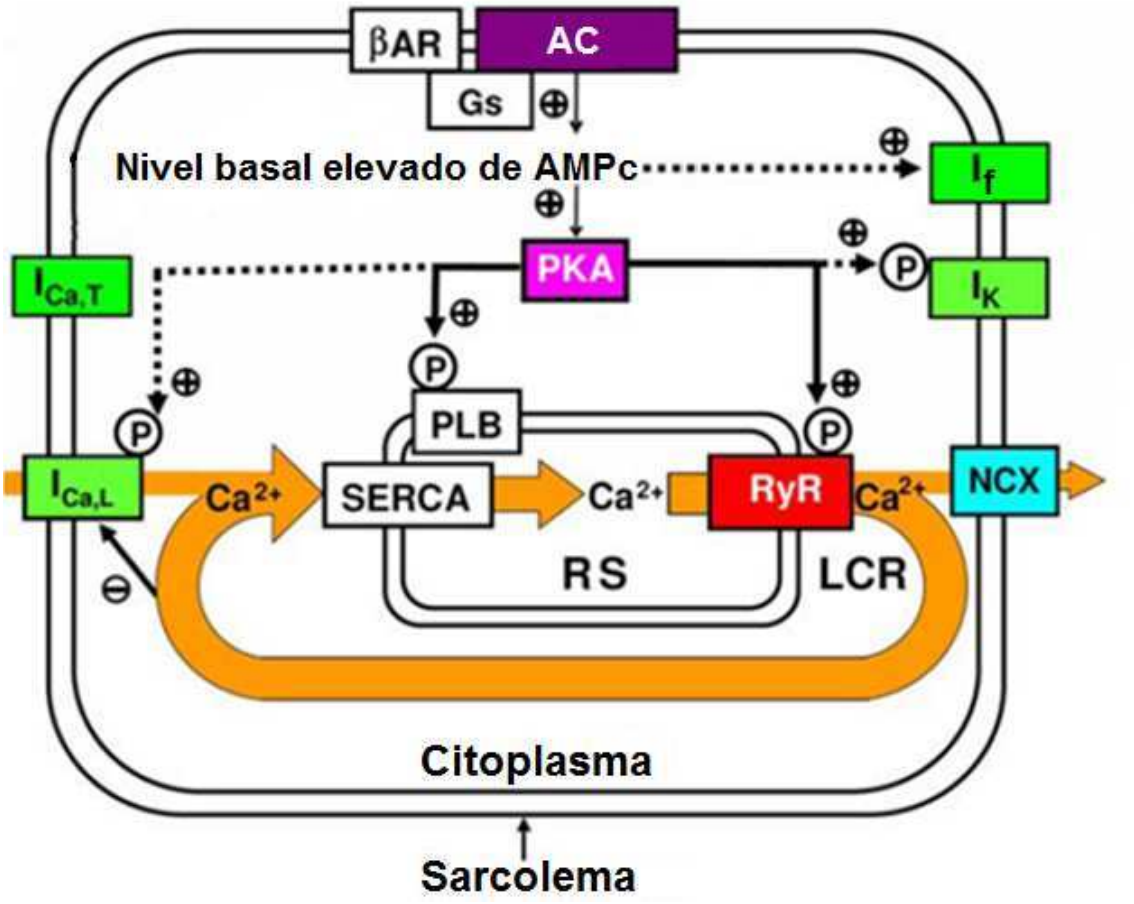

Figura 14: Ilustración esquemática de la integración funcional y regulación del ciclo de Ca^{2+} en la membrana y en el citoplasma. Las flechas gruesas indican el ciclo de Ca^{2+} espontáneo. El verdadero comienzo de la automaticidad del marcapasos cardiaco comienza durante la parte exponencial del prepotencial, generada por la I_{NCX}, cuando el reloj de Ca^{2+} "enciende" la excitación de la superficie de la membrana. Eventos anteriores, como el potencial diastólico máximo y la fase lineal del prepotencial, mediada por I_f, aseguran la reposición del reloj de la membrana. Éste es activado durante el PA anterior y necesita ser reestablecido antes del siguiente PA. El

espontáneo, pero precisamente controlado, reloj rítmico de Ca^{2+} del RS de las células del NS, asegura la estabilidad del ritmo basal y establece la frecuencia basal de disparo, integrando diferentes funciones dependientes de Ca^{2+} y haciendo que interaccionen rítmicamente, sincronizando el reloj de la membrana y el reloj del RS. Además, de la generación del PA, los canales de Ca^{2+} y la I_{NCX}, aseguran una regulación robusta del reloj de Ca^{2+} manteniendo balanceada la concentración intracelular de Ca^{2+}. AC, adenilil ciclasa; βAR, receptor β-adrenérgico; G_s, proteína G estimulante; PKA, proteína quinasa A; PLB, fosfolambano; RyR, receptor de rianodina; LCR, liberación local de Ca^{2+}; RS, retículo sarcoplasmático; NCX, intercambiador Na^+/Ca^{2+}; SERCA, bomba de Ca^{2+} dependiente de ATP del retículo sarcoplasmático; *I_f, corriente "funny"; I_K, corriente de K^+; $I_{Ca,L}$, corriente de Ca^{2+} tipo L; $I_{ca,T}$, corriente de Ca^{2+} tipo T; P, indica fosforilación. (Modificado de Lakatta et al., 2008).*

2.7 Fosfodiesterasas:

Las fosfodiesterasas (PDE) son una familia diversa de enzimas que hidrolizan los nucleótidos cíclicos, juegan un papel muy importante en la regulación de los niveles intracelulares de los segundos mensajeros AMPc y GMPc, y por lo tanto, en la función celular. Las fosfodiesterasas son las únicas enzimas capaces de degradar el AMPc y por eso son responsables de la homeostasis interna de AMPc. El AMPc controla una gran variedad de funciones celulares y regula numerosos procesos biológicos, como son la memoria, aprendizaje, función inmune, secreción de insulina, así como la frecuencia cardíaca y la fuerza de contracción cardíaca. La generación de AMPc está principalmente producida por la activación, mediante un agonista, de un receptor de membrana acoplado a proteína G_s y la consiguiente activación transmembrana de AC en la cara interna de la membrana. El AMPc activa principalmente la PKA, que fosforila diversas dianas citoplasmáticas y nucleares, mediando diversas funciones celulares (Zaccolo, 2006). El AMPc también activa los factores intercambiadores de nucleótidos de guanina (*exchange protein activated by cAMP*, EPAC) y los canales HCN (figura 11) (Bos, 2006).

Las PDE se denominan mediante un número arábigo que indica la familia, seguida de una letra mayúscula que identifica el gen dentro de la familia. Las distintas variantes dentro de dicho gen son identificadas por un número arábigo tras la letra mayúscula.

Actualmente se conocen 11 familias de PDEs codificadas por 21 genes diferentes. Esta clasificación está basada en la distribución tisular, en las propiedades bioquímicas y la sensibilidad a inhibidores químicos. Cada célula o tejido expresa un conjunto diferente de PDEs (Soderling y Beavo; 2000). En miocitos cardíacos se han descrito múltiples familias de PDEs, conocidas como PDE1, PDE2, PDE3, PDE4 Y PDE5. Las PDE1, PDE2 y PDE3 muestran especificidad de sustrato dual en condiciones “in vitro”, es decir pueden hidrolizar selectivamente AMPc y GMPc, mientras PDE4 y PDE5 hidrolizan específicamente AMPc y GMPc respectivamente (Tabla 2). La existencia de diferentes isoformas de PDEs dentro de una misma célula garantiza una distribución espacio-temporal heterogénea de la señalización por nucleótidos cíclicos y

la versatilidad de las funciones mediadas por nucleótidos cíclicos. Las alteraciones en la expresión de PDEs y de su actividad pueden distorsionar el balance y/o localización de los nucleótidos cíclicos y producir eventos patológicos. Por ejemplo, en miocitos cardíacos de ratas recién nacidas, con una estimulación β-adrenérgica, se produce una elevación de AMPc y una activación de PKA en unos compartimentos específicos próximos a los túbulos transversales. La inhibición inespecífica de PDEs distorsiona los gradientes de AMPc, y causa una activación global de PKA, que conduce a la manifestación de efectos no específicos y tóxicos (Zaccolo y Pozzan, 2002).

La contribución relativa de cada familia varía entre las especies, estados de desarrollo, tipos de miocitos (auriculares o ventriculares) y la condición celular (fisiológica o patológica). Cada PDE regula diferentes funciones celulares mediante el acoplamiento selectivo a diferentes conjuntos de AMPc. La expresión y función de las nuevas familias clonadas de PDEs (7-11) en el corazón no ha sido bien documentada, probablemente debido a la ausencia de inhibidores selectivos y al inadecuado funcionamiento de los anticuerpos (Yan et al., 2007).

PDE	*Numero de isoformas*	*Sustrato*	*Km(µM) AMPc*	*Km (µM) GMPc*	*Expresión*	*Inhibidores específicos*
1	8	AMPc/ GMPc	1-30	3	Corazón, cerebro, pulmón, músculo liso	K8-505a
2		AMPc/ GMPc	50	50	Glándula adrenal, corazón, pulmón, hígado, plaquetas	EHNA
3	4	AMPc/ GMPc	0.2	0.3	Corazón, pulmón, hígado, plaquetas, tejido adiposo, células inflamatorias	Cilostamida, enoximona, milrinona
4	20	AMPc	4		Células de sertoli, riñón, cerebro, pulmón, células inflamatorias, corazón	Rolipram, roflumilast, cilomilast
5	3	GMPc	150	1	Pulmón, plaquetas, músculo liso vascular	Sildenafilo, zaprinast
6		GMPc		60	Fotoreceptores	Dipiridamol
7	3	AMPc	0.2		Músculo esquelético, corazón, riñón, cerebro, páncreas, linfocitos-T	BRL-50481
8		AMPc	0.06		Testículos, ojos, hígado, músculo esquelético, corazón, riñón, ovarios, cerebro, linfocitos-T	
9	4	GMPc		0.17	Riñón, hígado, pulmón, cerebro	BAY 73-6691
10	2	AMPc/ GMPc	0.5	3.0	Testículos, cerebro	
11	4	AMPc/ GMPc	0.7	0.6	Músculo esquelético, próstata, riñón, hígado, pituitaria, glándulas salivares, testículos.	

Tabla 2. Características de la superfamilia de las fosfodiesterasas. Modificada de Boswell-Smith et al,(2006).

2.7.1 Compartimentalización del AMPc

Dada la gran diversidad de dianas de PKA en la célula, es esencial que exista una señalización que esté regulada con precisión para que exista una respuesta específica. Para que un estímulo extracelular específico sea correctamente transducido en la respuesta apropiada, deben existir vías de señalización paralelas de AMPc/PKA dentro de la célula (Zaccolo et al,. 2002). Un papel importante en la organización de estas vías de señalización de AMPc/PKA es llevado a cabo por proteínas de anclaje de PKA (AKAP). Estas proteínas anclan PKA a una localización específica cercana a moduladores específicos y a las dianas (Colledge y Scott, 1999; Langeberg y Scott, 2005; Taskén y Aandahl, 2004). Estos grupos anclados de PKA deben de ser selectivamente activados y esto requiere que el AMPc esté disponible en compartimentos discretos (Wong y Scott, 2004) (Figura 11).

En el corazón, la regulación del acoplamiento excitación-contracción cardíaca depende de la regulación que ejerce el AMPc sobre la fosforilación catalizada por PKA, del canal de Ca^{2+} tipo L y el RyR2. Esto lleva a un incremento de iones Ca^{2+} para la contracción del sarcómero durante la sístole (figura 12). La PKA además participa en la captación del Ca^{2+} por el RS durante la diástole, fosforilando PLB. La PKA también fosforila proteínas sarcoméricas, tales como la TnI y la proteína C de unión a la miosina. Ambas proteínas controlan la sensibilidad del sarcómero al Ca^{2+}. Para que toda esta regulación suceda de forma coordinada es necesario que PKA y AMPc estén altamente compartimentalizados en dominios intracelulares, cada uno controlando los efectos de diferentes cascadas de señalización (Zaccolo, 2006).

La hipótesis de la compartimentalización de las vías de señalización dependientes de AMPc en miocitos cardíacos, fue formulada hace mas de 25 años (Hayes et al., 1980). El trabajo de Hayes et al. (1980) está basado en el descubrimiento de que la prostaglandina E1 (PGE1) inducia un aumento en la actividad de AC, pero no de la contractilidad en ventrículo felino, mientras el isoproterenol estimulaba ambos (Kaumann y Birnbaumer, 1974). Como el isoproterenol es un agente inotrópico, mientras PGE1 no, la respuesta inotrópica depende del incremento de AMPc en un determinado compartimento específicamente funcional (Zaccolo, 2006). Este hecho ha

sido fuertemente reforzado en recientes estudios usando la técnica de FRET (Resonancia de transferencia de energía fluorescente) (Zaccolo et al., 2000). Esta es una técnica muy sensible que permite la monitorización de AMPc en células intactas con una alta resolución en tiempo y espacio. Con el uso de esta técnica fue posible visualizar directamente los microdominios de AMPc inducidos por catecolaminas en miocitos cardíacos y demostrar que pequeños pools de AMPc llevan a la activación de pequeños subgrupos de PKA (Zaccolo y Pozzan, 2002). Estos descubrimientos confirman que en miocitos cardíacos, la estimulación de βAR activa respuestas específicas mediante la inducción de aumentos de la concentración intracelular de AMPc que están espacialmente restringidos. Las PDEs juegan un papel muy importante en la determinación de estos microdominios específicos de AMPc (Zaccolo, 2006).

La noción de la compartimentalización del AMPc contrasta con la observación de que el cAMP se comporta en el citoplasma como una molécula que difunde libremente (Nikolaev et al., 2004). Es interesante resaltar que la hidrólisis de AMPc, cuando se mide en células intactas, parece ser mucho más rápida que su síntesis (Nikolaev et al., 2005). Cuando la AC esta totalmente activada, la actividad de las PDEs es suficiente para anular completamente la respuesta de AMPc provocada por catecolaminas (Mongillo et al., 2004). Diversas isoformas de PDEs están asociadas con sitios específicos subcelulares (Houslay, 2001), incluyendo a AKAPs (Dogde et al., 2001; Baillie et al., 2005; Taskén et al., 2001), por diferentes mecanismos (Houslay et al., 1998; Baillie et al., 2002). Como consecuencia, por la rápida degradación del AMPc en los compartimentos seleccionados, las PDEs pueden delimitar la difusión de AMPc, creando gradientes intracelulares del segundo mensajero y modulando grupos definidos de eventos intracelulares mediados por PKA.

La inhibición selectiva de PDEs ha recibido una atención notable durante los últimos años para el tratamiento de diversos problemas cardiovasculares. El objetivo de los tratamientos con inhibidores selectivos de PDE fue aumentar la contractilidad cardíaca en la insuficiencia cardíaca crónica. Desafortunadamente pronto se hizo evidente que el tratamiento crónico de estos enfermos aumentaba la mortalidad (Packer et al., 1991). Las razones para estos efectos negativos a largo plazo están aún sin resolver completamente (Movsesian, 1999) pero es comprensible que se deban a

una elevación de los niveles de AMPc en diferentes compartimentos y la consecuente activación indiscriminada de PKA (Movsesian y Bristow, 2005), que puede dar lugar a una mayor aparición de arritmias ventriculares y muerte súbita. Sin embargo todavía se usan en la insuficiencia cardíaca en situación de emergencia a corto plazo.

Los inhibidores selectivos de fosfodiesterasas (iPDE) han sido y son usados para el tratamiento de problemas cardiovasculares. PDE3 es la más abundante en el corazón humano y esta es la razón por la que se usa inhibidores de PDE3 como agentes inotrópicos. La PDE4 parece que se expresa en pequeño niveles y se sabe muy poco acerca de su función (Zaccolo, 2006).

En el corazón de mamífero se expresan principalmenten la PDE1, 2, 3, 4, y 5.

2.7.2 Fosfodiesterasas que hidrolizan AMPc en el corazón.

Las PDEs estimuladas por Ca^{2+}/Calmodulina (familia PDE1) constituyen una gran familia de enzimas que están codificadas por 3 genes diferentes: PDE1A, PDE1B y PDE1C (Beavo, 1995). Se han identificado múltiples *splice variants* para cada gen. PDE1A y PDE1B tienen mayor afinidad por GMPc que por AMPc, mientras PDE1C hidroliza ambos con alta afinidad, in vitro. *In vivo*, la inhibición de PDE1A ha mostrado una preferencia para elevar GMPc pero no AMPc (Nagel et al., 2006). La inhibición de PDE1C es capaz de elevar los niveles de AMPc en la célula, pero no está claro si PDE1C es capaz de regular GMPc *in vivo* (Han et al., 1999; Rybalkin et al., 2002). La actividad de PDE1, ha sido detectada en la fracción citosólica de miocardio humano aunque en ausencia de Ca^{2+}, la actividad AMPc-PDE en el citosol miocárdico es mayoritariamente ejecutada por PDE3 (Hambleton et al., 2005). En presencia de Ca^{2+}, la actividad AMPc-PDE es mayormente llevada a cabo por PDE1. Esto sugiere que PDE1 y PDE3 contribuyen de forma diferente a la hidrólisis de AMPc en el miocardio bajo condiciones basales o estimuladas por Ca^{2+}.

Las PDE2 y PDE3 son capaces de hidrolizar AMPc y GMPc con alta afinidad. Los miembros de la familia de PDE2 son a veces referidos como PDEs estimuladas por GMPc por la vinculación del GMPc a la región N-terminal de los dominios GAF de PDE2, estimulando ampliamente su actividad catalítica (Soderling y Beavo, 2000). En

contraste, la Vmax de PDE3 para la hidrólisis de AMPc es 10 veces superior que para GMPc. Por esto, GMPc se comporta como un inhibidor competitivo de AMPc debido a la alta afinidad y a la baja velocidad de hidrólisis para GMPc (Leroy et al., 1996). Por esta razón, las isoenzimas de PDE3 son llamadas también enzimas inhibidos por GMPc. La regulación dependiente de GMPc de la actividad de hidrólisis de AMPc de PDE2 y PDE3 representa un importante mecanismo con el que la señal de GMPc regula las repuestas al AMPc en diferentes tipos celulares. En miocitos cardíacos, el papel de PDE2 sobre la función cardíaca y la corriente de Ca^{2+} tipo L, varía entre las diferentes especies y distintas regiones cardíacas. Estas variaciones están relacionadas con la expresión relativa de PDE2 frente a PDE3, la concentración de GMPc y/o la compartimentalizacion de PDEs (Lohmann et al., 1991; Fischmeister et al., 2005). En cardiocitos humanos, pero no de rata, PDE2 controla la actividad basal de I_{CaL}, Ambas PDE2 y PDE3 contribuyen a mantener los niveles de AMPc y GMPc en ausencia de estimulación de AC o de guanilil ciclasa (GC) (Rivet-Bastide et al., 1997).

La PDE2 tiene valores K_m para AMPc y GMPc mayores que la PDE1. La PDE2 es activa en tejido cardiaco de rana, humano y porcino y es selectivamente inhibida por EHNA (eritro-9-[2-Hidroxi-3-nonil]adenina) (Lugnier et al., 1992). PDE2 es la isoenzima más abundante en el miocardio de rana (Lugnier et al., 1992). Por otro lado, la PDE2 solo es responsable del 3 % de la actividad de hidrólisis de AMPc en cardiomiocitos de rata (Mongillo et al., 2006). En ambas especies, la principal actividad de PDE2 en el miocardio se ha detectado en la fracción microsomal, indicando la presencia de PDE2A2, una isoforma PDE2 asociada a la membrana (Mongillo et al., 2006).

La PDE3 es una enzima muy selectiva para la hidrólisis de AMPc y es selectivamente inhibida por una serie de agentes cardiotónicos no glicosídicos como milrinona, enoximona, etc…, que reducen la actividad de la enzima con valores de IC_{50} que son uno o dos órdenes de magnitud inferiores comparados con la concentración con la que inhiben a otras isoenzimas pertenecientes a otras subfamilias (Weishaar et al., 1987a). La familia de PDE3 incluye dos genes PDE3A y PDE3B, pero en tejido miocárdico de rata (Rochais et al., 2006), perro (Smith et al., 1997) y humano (Ding et al., 2005; Hambleton et al., 2005) solo se expresa PDE3A. En cambio, PDE3A y PDE3B se expresan por igual en miocitos ventriculares de ratón (Patrucco et al., 2004). La

PDE3A provee la mayoría de la actividad hidrolizante del AMPc en la fracción microsomal del tejido miocárdico humano y más del 50 % de la actividad total de PDEs en la fracción citosólica en ausencia de Ca^{2+} (Wechsler et al., 2002; Hambleton et al., 2005).

La PDE3 es la isoenzima mas abundante del tejido miocárdico en algunos mamíferos, por ejemplo, en tejido ventricular de perro (Smith et al., 1997; Lugnier et al., 1993) y conejo (Shakur et al., 2002). En el raton y la rata, la actividad PDE es principalmente debida a PDE3 y PDE4 (Mongillo et al., 2004; Rochais et al., 2004; Rochais et al., 2006; Nikolaev et al., 2006). La forma asociada a la membrana de PDE3 esta mayormente localizada en el RS de perro (Lugnier et al., 1993) y rata (Mongillo et al., 2004).

La PDE4 pertenece a una gran familia de enzimas que hidrolizan específicamente el AMPc. Se han identificado 4 genes de PDE4 (PDE4A, PDE4B, PDE4C Y PDE4D), y de ellos resultan múltiples *splice variants* por diferentes promotores. Las isoenzimas de PDE4 se han encontrado en la mayoría de tipos celulares. Cada célula expresa frecuentemente más de un tipo diferente de PDE4.

La expresión y función de las PDE4s en corazón no ha sido estudiada en profundidad anteriormente, porque los inhibidores de PDE4 solo producían efectos inotrópicos mínimos en humanos. Sin embargo, recientemente, se vió que la eliminación del gen PDE4D en ratón produce una cardiomiopatía progresiva en ratones seniles y acelera la aparición de una insuficiencia cardíaca después de un infarto de miocardio (Lehnart et al., 2005). Los efectos deletéreos cardíacos en los ratones knock-out PDE4D se cree que se atribuyen a la perdida de PDE4D3 del complejo macromolecular del RyR2 (Lehnart et al., 2005). La pérdida de PDE4D3 del complejo RyR2 causa una hiperfosforilación por PKA de RyR2, dando lugar a fuga de Ca^{2+} (Lehnart et al., 2005) favoreciendo la aparición de arritmias cardíacas. Además, PDE4D3 se ha encontrado en el macrocomplejo del RyR2 en el hombre, y está disminuida en corazones humanos con insuficiencia cardíaca asociado con un aumento de la fosforilación de RyR2 por PKA (Lehnart et al., 2005).

En cardiomiocitos de ratas recién nacidas, tras la estimulación del β_2AR por isoproterenol, las isoenzimas PDE4D3 y PDE4D5 son transportadas hasta la membrana en la proximidad del β_2AR por la proteína de anclaje β-arrestina. Esto juega un papel muy importante en la regulación de la fosforilación del receptor β_2AR por PKA y el cambio de acoplamiento del receptor de la proteína G_s a G_i (Perry et al., 2002; Baillie et al., 2003). Asociado con la membrana del núcleo del miocito se ha encontrado un complejo macromolecular que está formado por AKAP muscular (AKAPm), PKA, PDE4D3, ERK5 (una quinasa activada por señal extracelular), Rap1 (una pequeña GTPasa) y EPAC (Dogde-Kafka et al., 2005). La PDE4D3 funciona como una proteína adaptadora que combina EPAC, la cual activa Rap1 que a su vez inhibe la activación de ERK5. La activación de ERK5 lleva a la hipertrofia de las células cardíacas a concentraciones bajas de AMPc. Como EPAC solo funciona a concentraciones citoplasmáticas elevadas de AMPc, ERK solo lleva a hipertrofia cardíaca cuando las concentraciones citoplasmático de AMPc son bajas (Dogde-Kafka et al., 2005).

La PDE5 es una isoenzima especifica de GMPc que consta de un único gen PDE5A con tres isoformas PDE5A1, PDE5A2 (aislada en pulmón bovino) y PDE5A3 (tejido cavernoso del pene) (Loughney et al., 1998; Kotera et al., 1999; Lin et al., 2000). En los últimos años diferentes estudios han descrito los potentes efectos cardiovasculares mediados por sildenafilo, un inhibidor selectivo de PDE5A. Recientemente Corbin et al. (2003) mostraron la presencia de PDE5 en miocardio humano.

Mediante tinción han sido evidenciadas partículas difusas de la PDE5 entre las bandas Z en miocitos cardíacos aislados de ratón (Takimoto et al., 2005) y perro (Senzaki et al., 2001). La PDE5A provee un 35-45 % de la actividad de hidrolisis de GMPc en el miocardio de ratón (Takimoto et al., 2005) y alrededor de un 50 % en el humano (Corbin et al., 2003).

No está claro como la inhibición de PDE5A podría producir algún efecto cardiaco directo. El sildenafilo incrementa la concentración de AMPc en tejido auricular humano (Stief et al., 2000) y en músculo papilar de perro (Sugiyama et al., 2002). Estos efectos son presumiblemente mediados por la inhibición de PDE3, debido

a la acumulación de GMPc producida por el sildenafilo, ya que la elevación de AMPc es similar a la producida por milrinona un inhibidor selectivo de PDE3 (Stief et al., 2000). Estos cambios en el metabolismo de los nucleótidos cíclicos parecen no causar efectos funcionales en el corazón. El sildenafilo no afectó a la contractilidad miocárdica en tejidos aislados auriculares y ventriculares de perros y humanos (Cremers et al., 2003; Corbin et al., 2003).

¿Que isoenzima de PDE es la más importante en la regulación de la contractilidad cardíaca?

En miocitos cardíacos de mamíferos, alrededor del 90 % del total de la actividad de hidrólisis de AMPc es llevada a cabo por PDE3 y PDE4 sugiriendo un papel fundamental de estas isoenzimas en la señalización mediada por AMPc (Weishaar et al., 1987a; Weishaar et al., 1987b; Kaasik y Ohisalo, 1996; Shakur et al., 2002; Mongillo et al., 2004). PDE1 y PDE2 también están presentes en el miocardio de rata pero tienen un papel menor (Verde et al., 1999).

Numerosos estudios farmacológicos han demostrado un incremento en la contractilidad ventricular después de la inhibición de PDE3. Las respuestas inotrópicas a inhibidores selectivos de PDE3 han sido demostradas en miocardio auricular y ventricular (Wallis et al., 1999; Malecot et al., 1985; Fujino et al., 1988; Alousi et al., 1983a; Alousi et al., 1983b; Artman et al., 1989; Muller et al., 1990; Rapundalo et al., 1989; Katano y Endoh, 1992) y en miocitos ventriculares aislados (Xiong et al., 2001; Xiong et al., 2004; Wynne et al., 1993) de varias especies mamíferas incluyendo la humana (Bethke et al., 1991; Von der Leyen et al., 1991; Bartel et al., 1996; Christ et al., 2006a). La cilostamida, un inhibidor selectivo de PDE3, potencia mas las respuestas inotrópicas de adrenalina mediadas por β_2AR, que las de noradrenalina mediadas por β_1AR en aurícula humana (Christ et al., 2006a). Los efectos inotrópicos mediados por (-)-CGP12177 mediados por el receptor β_1AR_L en aurícula humana (Kaumann et al., 2007) y en ventrículo de rata (Vargas et al., 2006) también fueron potenciados por cilostamida. En corazones con insuficiencia cardíaca, la reducción de respuestas inotrópicas a agonistas βAR es revertida por la coadministración de inhibidores de PDE3 (Wynne et al., 1993; Gilbert et al., 1995).

En trabajos recientes se ha mostrado que en roedores la PDE4 en lugar de PDE3 juega un papel principal en la regulación de la contracción del miocito cardiaco, en la amplitud y en la duración de la señal del AMPc, tanto en condiciones basales como tras la exposición a un agonista βAR. Esto ha sido demostrado por la mayor efectividad producida en presencia inhibidores de PDE4 comparado con inhibidores de PDE3 (; Mongillo et al., 2004; Nikolaev et al., 2006). Estudios inmunocitoquímicos han mostrado que PDE3A y PDE4D tienen distinta localización subcelular en miocitos cardíacos de ratón, donde PDE3A está localizada uniformemente en el RS y PDE4D esta mayormente anclada a las estructuras del sarcómero (Nikolaev et al., 2006). No está claro si estas diferencias contribuyen a una mayor importancia en la regulación de la contractilidad cardíaca por PDE4. El inhibidor de PDE4 (RO 20-1724) no parece producir efectos cardíacos *in vivo*, salvo una pequeña taquicardia, menor del 10 % (Herzer et al., 1998).

Además, en contraste con los inhibidores de PDE3, rolipram, no produce efectos contráctiles propios en aurícula humana (Christ et al., 2006a), aunque potencia respuestas inotrópicas positivas así como la acumulación intracelular de AMPc inducida por agonistas βAR o forscolina en otras especies (Shahid y Nicholson, 1990; Muller et al., 1990; Katano y Endoh, 1992; Nikolaev et al., 2006; Vargas et al., 2006).

3. Objetivos

Durante la introducción se ha descrito en primer lugar la fisiología de la función inotrópica, cronotrópica y lusitrópica, así como los procesos bioquímicos responsables de las mismas, en las diferentes regiones cardiacas. Después, se ha descrito como son controladas estas funciones, por diferentes receptores que median sus efectos a través del segundo mensajero, el AMPc. Y por último, como es regulada la actividad de este segundo mensajero. El AMPc es hidrolizado por las PDEs, pero aún no se conoce que isoforma de PDE es responsable de la hidrólisis del AMPc generado en cada una de las regiones cardiacas por la activación de cada uno de los receptores que hemos estudiado.

Por ello, los objetivos de esta tesis doctoral se han centrado en 5 aspectos:

1. Elucidar si la actividad de PDE3 y/o PDE4 previene la manifestación de los efectos cardioestimulantes de (-)-adrenalina mediados por activación de los β_2AR en el corazón de roedores, e investigar si existen diferencias regionales en el papel de PDE3 y PDE4 en el control de las respuestas mediadas por β_1AR y β_2AR.

2. Investigar el papel de la PDE3 y la PDE4 en el control de la respuesta producida por la activación del receptor 5-HT_4 en las diferentes regiones cardiacas del corazón de lechón recién nacido, así como su relación con las respuestas producidas en miocardio de cerdos adolescentes y en miocardio humano adulto.

3. Investigar la relación entre los incrementos de I_{CaL} y de contractilidad, mediados por β_1AR y β_2AR, y su reducción por PDEs en miocitos ventriculares de rata.

4. Investigar la influencia de la inhibición de PDE4 en las arritmias mediadas por (-)-adrenalina en la pared del ventrículo derecho de ratón.

5. En el transcurso de los primeros experimentos realizados en ratón, descubrimos un hecho no descrito en la literatura. La inhibición de PDE3 y/o PDE4 produjo un incremento de la frecuencia cardiaca basal sin modificar los efectos mediados por la activación de los β_1ARs. Este hecho nos llevo a pensar en la posibilidad de que existieran dos conjuntos diferentes de AMPc. Uno que regula la frecuencia basal y que es controlado por PDE3 y/o PDE4, y otro que media los efectos cronotrópicos del β_1AR y no es controlado por PDE3 y/o PDE4. Como resultado de esta observación, decidimos estudiar sistemáticamente si se reproducía en diferentes receptores, β_{1H}, $\beta_{1L,}$ $\beta_{2,}$ $5HT_4$ y en distintas especies como rata y lechón.

4. Material y métodos

4.1 Pacientes

Este estudio fue aprobado por el Comité Ético de la Universidad de Murcia y del Hospital Universitario Virgen de la Arrixaca. Los pacientes firmaron el consentimiento informado. Los apéndices de aurícula derecha fueron obtenidos de 10 pacientes con ritmo sinusal estable y sin insuficiencia cardíaca.

4.2 Tejidos aislados

4.2.1 Tejidos aislados humanos

Después del corte, los apéndices auriculares fueron inmediatamente sumergidos en solución Tyrode oxigenada y fría y transportados al laboratorio en menos de 5 minutos. Se disecaron hasta 8 trabéculas en solución Tyrode oxigenada a temperatura ambiente de composición (mM): NaCl 136.9, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.5, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, ácido etilendiamintetracético (EDTA) 0.04, acido ascórbico 0.2, piruvato 5.0 y glucosa 5.0. La solución era mantenida a pH 7.4 burbujeando una mezcla de 5 % CO_2 y 95 % O_2. Las trabéculas fueron montadas en parejas, sujetadas a transductores Swema 4-45, en baños de órganos que contenían solución Tyrode a 37º, estimulados eléctricamente a 1Hz. Los tejidos fueron estirados como se describió anteriormente por Kaumann et al. (1990). La fuerza fue registrada en un polígrafo Watanabe de 12 canales.

4.2.2 Tejidos aislados porcinos

Lechones recién nacidos (1 o 2 días, de ambos sexos) y cerdos adolescentes (22 ± 2 kg, de ambos sexos) fueron obtenidos de la granja veterinaria de la Universidad de Murcia. Ambos fueron anestesiados con pentobarbital sódico 100 $mg.kg^{-1}$ intraperitoneal y 50 $mg.kg^{-1}$ intravenoso respectivamente. Los corazones fueron disecados en solución Tyrode oxigenada como la descrita arriba a temperatura ambiente. Aurículas derechas latiendo espontáneamente, tiras de aurícula izquierda, y trabéculas del ventrículo derecho (diámetro menor de 1mm) de lechones recién nacidos, así como trabéculas de aurícula izquierda (diámetro menor de 1mm) y trabéculas ventriculares de cerdos adolescentes fueron rápidamente disecadas y

montadas para contraerse en solución Tyrode a 37º. De 2 a 4 tiras de aurícula izquierda (lechones recién nacidos) o trabéculas (cerdos adolescentes) fueron obtenidas por cada aurícula izquierda. Las preparaciones de aurícula izquierda y ventriculares fueron estimuladas a 1Hz y estiradas como describieron Parker et al. (1995) y Brattelid et al. (2004b). La fuerza contráctil de las aurículas izquierdas de los cerdos adolescentes fue registrada en un polígrafo Watanabe de 12 canales. La fuerza y el tiempo para la fuerza pico de las tiras de aurícula izquierda de los lechones recién nacidos fueron registrados en un amplificador Power Lab en un Chart para Windows, versión 5.5.6 (ADInstruments, Australia).

4.2.3 Tejidos aislados de roedores

Las roedores fueron sacrificados siguiendo los protocolos aprobados por el Regierungspräsident de Dresde, (permiso numero: 24D-9168.24-1/2007-17), los Procedimientos Científicos de "Home Office" del Reino Unido y el comité ético de la Universidad de Murcia de acuerdo con los normas de la Comunidad Económica Europea. Los corazones fueron disecados y puestos en solución Tyrode oxigenada a temperatura ambiente. La aurícula derecha que latía espontáneamente, aurícula izquierda y la pared libre del ventrículo derecho, así como el músculo papilar izquierdo, fueron rápidamente disecados, montados en parejas, fijados a transductores Swema 4-45 en baños de órganos que contenían la solución Tyrode a 37º. Aurícula izquierda, pared de ventrículo derecho y músculo papilar izquierdo fueron estimulados eléctricamente a 1Hz en rata y a 2Hz en ratones. Los tejidos fueron estirados como fue descrito por Kaumann y Molenaar (1996), Oostendorp y Kaumann (2000) y Vargas et al. (2006). La fuerza contráctil y la frecuencia cardíaca fueron registradas a través de un sistema de registro digital Power Lab en un programa de registro gráfico Chart para Windows Versión 5.5.6.

4.3 Protocolos

4.3.1 Experimentos con 5-HT

Todos los experimentos fueron realizados en presencia de (-)-propanolol (200nM) para evitar efectos indirectos debidos a una liberación endógena de noradrenalina provocada por 5-HT y su interacción con receptores βAR.

Las curvas acumulativas concentración-efecto para la 5-HT fueron realizadas en aurículas derechas latiendo espontáneamente y en tiras de aurícula izquierda de lechón recién nacido estimuladas a 1Hz en ausencia y en presencia del inhibidor de PDE3 cilostamida (300 nM) y del inhibidor de PDE4 rolipram (1 μM), seguidos por una concentración saturante de (-)-isoproterenol (200μM). Para los estudios inotrópicos los experimentos se finalizaron elevando la concentración de $CaCl_2$ a 9 mM.

4.3.2 Medida del AMP cíclico

Para estudiar la correlación entre la fuerza contráctil y el nivel de AMP cíclico, ambos parámetros fueron analizados en el mismo tejido. En tiras de auricula izquierda, se midió la fuerza basal y la fuerza inducida por el agonista y el tejido fue congelado en nitrógeno liquido a los 2 o 20 minutos después de la adición de la concentración inotrópica máxima efectiva de 5-HT (10 μM). Tambien se estudió el efecto de una concentración inotrópica máxima efectiva de (-)-isoproterenol (200 μM), incubada 2 minutos, para comparar con la respuesta obtenida por receptores $5HT_4$ y βAR.

Los niveles de AMPc fueron medidos por radioinmunoensayo [^{125}I]TME-S-$_c$AMP; (Diagnostic Pasteur, France), conforme a las instrucciones del fabricante. La incubación con los inhibidores de PDE fue de 30 minutos. Después de congelado, el tejido fue pesado y homogenizado en acido perclórico frio 0.3 M (1/30 peso/volumen) con un homogenizador Politron (posición 8, 15 segundos) y centrifugado (10,000xg, 4˚C, 15 min). Los sobrenadantes fueron tratados con hidróxido potásico hasta conseguir un pH entre 6 y 7. La sensibilidad del análisis es 2pmol.ml^{-1}. El precipitado fue redisuelto en hidróxido potásico 2 M para la determinación de proteínas por el método del ácido bicinconínico. El anticuerpo reaccionaba al 100 % con 3’ , 5’ –AMP cíclico y menos de

un 0.3 % con otro nucleótidos. Las concentraciones de AMP cíclico fueron expresadas en pmol.mg^{-1} de proteína.

4.3.3 Experimentos con agonistas β-adrenérgicos

Todos los tejidos fueron expuestos a fenoxibenzamina (5 μM), (a excepción de los realizados con CGP12177 que no fueron expuestos) durante 90 minutos, seguidos de un lavado, para bloquear de manera irreversible la recaptación de catecolaminas por parte de los tejidos, así como los receptores α-adrenérgicos (Gille et al., 1985; Heubach et al., 2002). Los experimentos con (-)-noradrenalina fueron realizados en presencia de ICI118551 (50 nM) para bloquear los receptores β_2AR. Los experimentos con (-)-adrenalina fueron realizados en presencia de CGP20712A (300 nM) para bloquear selectivamente los receptores β_1AR y descubrir efectos ocultos resistentes al CGP20712A, mediados por receptores β_2AR (Kaumann, 1986; Oostendorp y Kaumann, 2000; Heubach et al., 2002). Los experimentos con CGP12177 fueron realizados en presencia de propanolol (Vargas et al., 2006) para impedir los efectos cardiodepresivos del CGP12177 debidos al bloqueo de los efectos tónicos de noradrenalina endógena a través del β_{1H}AR (Kaumann, 1996; Kaumann y Molenaar, 2008). Para corroborar que los efectos resistentes al CGP20712A de (-)-adrenalina eran mediados a través del receptor β_2AR, el antagonista selectivo de receptores β_2AR ICI118551 (Kaumann, 1986; Oostendorp and Kaumann, 2000) fue usado en presencia de CGP20712A.

Curvas acumulativas concentración-efecto para las catecolaminas fueron llevadas a cabo en la ausencia y presencia de diferentes inhibidores de PDEs; inhibidor de PDE3 cilostamida; el inhibidor de PDE4 rolipram; el inhibidor de PDEs no selectivo IBMX. La curva fue seguida de la administración de una concentración de (-)-isoproterenol (200 μM) que satura los βAR. Con 300 nM de cilostamida aproximadamente el 86 % de PDE3 y menos de 0.4 % de PDE4 están inhibidas. Con 1 μM de rolipram, aproximadamente, el 50 % de PDE4 esta inhibida y menos de 0.4 % de PDE3 (Vargas et al., 2006). Para los estudios inotrópicos, los experimentos fueron finalizados elevando la concentración de $CaCl_2$ a 8 mM. Las catecolaminas a veces causaron arritmias ventriculares. Los efectos inotrópicos positivos causados por catecolaminas sólo fueron medidos en los tejidos no arrítmicos o durante periodos

estables no arrítmicos. Los tiempos para la fuerza pico y para la mitad de la relajación ($t_{1/2}$) fueron obtenidos de los trazados rápidos usando Chart Pro para Windows, versión 5.51 (AD instruments, Australia).

4.3.4 Medida de la corriente de calcio tipo L. I_{Ca-L}

Los miocitos ventriculares y auriculares fueron disociados enzimáticamente (Christ et al., 2001) y conservados a temperatura ambiente hasta su uso en una solución que contenía (mM): NaCl 100, KCl 10, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgSO_4$ 5, taurina 50, ácido morfolino propano sulfónico (MOPS) 5.0 y glucosa 50, pH 7.4. La técnica patch clamp de electrodo simple fue usada para medir I_{Ca-L} a 37º (Christ et al., 2006b). El potencial de inicio era -80mV. Las corrientes de potasio fueron bloqueadas intercambiando K^+ por Cs^+. La solución externa de perfusión contenía (mM): tetraetilamonio 120, CsCl 10, Ácido N-2-Hidroxietilpiperacina-N'-2'-Etanesulfónico (HEPES) 10, $CaCl_2$ 2, $MgCl_2$ 1 y glucosa 20 con pH ajustado con CsOH. La solución de la pipeta contenía (mM): sulfonato de metano 90, CsCl 20, HEPES 10, Mg-ATP 4, Tris-GTP 0.4, ácido etilenglicoltetraacético (EGTA) 10 y $CaCl_2$ 3 con una concentración de Ca^{2+} libre de 60 nM (EQCAL, Biosoft, Cambridge, UK) y un pH 7.2, ajustado con CsOH. La amplitud de la corriente fue determinada como la diferencia entre el pico inferior y la corriente a 200ms después del escalón de despolarización a +10mV. Los efectos de las catecolaminas en la I_{Ca-L} fueron expresados en porcentaje del control (Christ et al., 2006b). Los miocitos tratados con PTX fueron incubados (1,5 µg/ml) durante 3 horas a 37º bajo agitación constante (Heubach et al., 2001) Para minimizar los posibles efectos debidos a la desensibilización, los miocitos solo fueron expuestos a una concentración de catecolaminas.

4.3.5 Medida de las arritmias

Las arritmias en las paredes de ventrículo derecho fueron medidas durante los trazados rápidos de las contracciones. Para cada concentración de catecolaminas se contabilizó el número de tejidos que presentaron arritmia frente a los que no.

4.4 Estadísticas

Los datos están expresados como media ± error estándar del número de aurículas derechas, tiras o trabéculas de aurícula izquierda y trabéculas ventriculares. Los valores de –Log EC_{50}M de agonista fueron estimados ajustando a una función de Hill con pendiente variable las curvas individuales de concentración efectos. Para los tejidos donde podían actuar dos poblaciones de receptores las curvas fueron analizadas conforme a una ecuación para dos poblaciones de receptores. La significación de las diferencias fue analizada con el test de la t de Student para muestras apareadas o no apareadas usando el software Graph Pad Inc (San Diego, California, USA) y/o ANOVA (Test múltiple de Tukey). La distribución de los datos de las arritmias es una distribución de Bernoulli (0, 1). Los datos estadísticos son la suma de las distribuciones de Bernoulli que produce una distribución binomial (Feller, 1968). Como la muestra era suficientemente grande, la distribución binomial fue aproximada a una distribución normal (Feller, 1968). El análisis de varianza (ANOVA) de medidas repetidas se aplicó a esta distribución con el software SPSS (Chicago, IL, USA).Un valor ode P inferior a 0.05 fue considerado significativa.

4.5 Fármacos

CGP20712A fue obtenido de Novartis (Basel, Switzerland). ICI118551 obtenido de Tocris. (-)-CGP12177 fue gentilmente donado por GlaxoSmithKline. Serotonina HCL, (-)-propanolol, rolipram, cilostamida, EHNA, PTX, (-)-isoproterenol, (-)-noradrenalina, (-)-adrenalina y fenoxibenzamina, fueron obtenidos de Sigma. Rolipram, cilostamida, EHNA e IBMX, fueron disueltos en dimetilsulfoxido y solución Tyrode (20 % DMSO). Los fármacos fueron añadidos a los baños de órganos de manera que la concentración de DMSO fuera menor de 0.1 % que por sí solo no es capaz de modificar la fuerza contráctil.

5. Resultados

5.1 La fosfodiesterasa 4 reduce el inotropismo y las arritmias pero no la taquicardia sinusal de (-)-adrenalina mediados a través del receptor adrenérgico β_1 cardiaco de ratón.

Ambos rolipram (1 μM) y cilostamida (300 nM) causaron una taquicardia sinusal transitoria pero ninguno de ellos incrementó la potencia cronotrópica de (-)-adrenalina. Rolipram potenció 19 veces (aurícula izquierda) y 7 veces (ventrículo derecho) los efectos inotrópicos de (-)-adrenalina. (-)-Adrenalina produjo arritmias ventriculares dependientes de la concentración que fueron potenciadas por rolipram. Todos los efectos de (-)-adrenalina fueron antagonizados por el antagonista β_1 selectivo CGP20712A (300 nM). Cilostamida no incrementó las potencias cronotrópicas e inotrópicas de (-)-adrenalina, pero administrado conjuntamente con rolipram en presencia de CGP20712A, descubrieron efectos inotrópicos en la aurícula izquierda mediados por (-)-adrenalina que fueron prevenidos por el antagonista selectivo β_2 ICI118551.

El inhibidor de PDE4 rolipram muestra diferencias regionales en el papel de PDE4 en el corazón de ratón. La cardioestimulación provocada por (-)-adrenalina, mediada por el β_1AR, fue controlada por PDE4 y no por PDE3 en aurícula izquierda y ventrículo derecho de corazón de ratón. Ambas, PDE3 y PDE4, reducen el ritmo basal sinusal y la inhibición de ambas, no potencia los efectos cronotrópicos de (-)-adrenalina. La inhibición conjunta de PDE3 y PDE4 descubre efectos cardioestimulantes de (-)-adrenalina mediados por β_2AR en la aurícula izquierda pero no en el nódulo sinusal y en el ventrículo derecho. El rolipram potenció las arritmias provocadas por (-)-adrenalina, a través del β_1AR en la pared de ventrículo derecho. La pared de ventrículo derecho de ratón puede servir como modelo para arritmias provocadas por (-)-adrenalina en ratones transfectados que llevan la mutación en el RyR2 y desarrollen taquicardia ventricular por polimorfismo genético.

5.2 El inotropismo y la corriente de Ca^{2+} tipo-L, activados mediante los receptores β_1 y β_2-adrenérgicos, son diferentemente controlados por las fosfodiesterasas 3 y 4 en el corazón de rata.

Rolipram produjo taquicardia en el nódulo sinusal. Cilostamida y rolipram no incrementaron la potencia cronotrópica de (-)-noradrenalina y (-)-adrenalina. Rolipram, pero no cilostamida, potenció los efectos inotrópicos auriculares y ventriculares de (-)-noradrenalina. Cilostamida potenció los efectos inotrópicos de (-)-adrenalina pero no de (-)-noradrenalina. Administrándolos conjuntamente, rolipram y cilostamida, descubrieron efectos inotrópicos en aurícula izquierda producidos por (-)-adrenalina y mediados por el β_2AR. Ambos, rolipram y cilostamida, aumentaron el incremento en $I_{Ca\text{-}L}$ producido por (-)-noradrenalina (1 μM). (-)-Adrenalina (10 μM) aumentó I_{CaL} solo en presencia de cilostamida pero no en presencia de rolipram.

La PDE4 reduce el ritmo basal sinusal pero la inhibición de la PDE3 o la PDE4 no afecta a las potencias cronotrópicas de (-)-noradrenalina o (-)-adrenalina a través de los receptores β_1AR o β_2AR respectivamente. Esto sugiere que el AMPc hidrolizado por PDE4 controla el ritmo de latidos basal en un compartimento distinto a la región donde la activación de los βAR modifica la actividad de los canales iónicos y del ciclo de Ca^{2+} dando lugar a taquicardia. La PDE4 pero no la PDE3 reduce los efectos inotrópicos auriculares y ventriculares de (-)-noradrenalina a través del receptor β_1AR. Los incrementos de $I_{Ca\text{-}L}$ ventriculares mediados por receptores β_1AR son reducidos por ambos PDE3 y PDE4 en el dominio del sarcolema pero los incrementos en fuerza contráctil son solo bloqueados por PDE4. Esto sugiere que el AMPc en el compartimento β_1AR/PDE3/Canal de Ca^{2+} no emite señales relevantes para la contractilidad. La PDE3 sola, pero no la PDE4, reduce ambos incrementos ventriculares en $I_{Ca\text{-}L}$ y efectos inotrópicos positivos de (-)-adrenalina a través de receptores β_2AR. La inhibición conjunta de la PDE3 y la PDE4 desenmascara efectos inotrópicos positivos de (-)-adrenalina mediados a través de receptores β_2AR en aurícula izquierda y potencia marcadamente los efectos inotrópicos ventriculares de (-)-adrenalina a través de receptores β_2AR. La PDE3 y la PDE4 protegen al corazón de rata de la

sobreestimulación por catecolaminas en aurícula izquierda y ventrículo pero no de la taquicardia provocada a través de receptores β_1AR y β_2AR.

5.3 Cambios ontogénicos en el control por fosfodiesterasa 3 y 4 de las respuestas de 5-HT en corazón porcino y la relevancia a los receptores auriculares 5-HT$_4$ humanos.

La taquicardia sinusal producida por 5-HT no desapareció y no fue potenciada por cilostamida (300nM) o rolipram (1μM). Las respuestas inotrópicas a 5-HT desaparecieron en la aurícula de lechones recién nacidos, de cerdos adolescentes y de humanos. La cilostamida redujo la desaparición del efecto de 5-HT en la aurícula de cerdos adolescentes y en humanos pero no en lechones recién nacidos. La cilostamida desenmascaro respuestas ventriculares a 5-HT en ventrículo de lechones recién nacidos, pero no en cerdos adolescentes. El rolipram redujo la desaparición de los efectos auriculares a 5-HT en lechones recién nacidos y cerdos adolescentes, pero no en humanos. Administrados conjuntamente, rolipram y cilostamida, previnieron la desaparición de las respuestas a 5-HT en aurícula izquierda y facilitaron las respuestas ventriculares a 5-HT en tejidos porcinos, pero no redujeron la desaparición residual de los efectos auriculares a 5-HT en presencia de cilostamida. Los incrementos de AMPc producidos por 5-HT desaparecieron, la desaparición de los efectos fue abolida por la administración conjunta de rolipram y cilostamida.

La 5-HT produce taquicardia sinusal estable. La PDE3 y PDE4 reducen el ritmo sinusal pero su inhibición no potencia la taquicardia provocada por 5-HT, sugiriendo que estas fosfodiesterasas regulan el AMPc encargado del ritmo sinusal basal, pero no tienen acceso al AMPc en el compartimento del dominio del receptor 5-HT$_4$. Ambas, PDE3 y PDE4 conjuntamente, previenen la disminución del efecto inotrópico en la aurícula izquierda lechones recién nacidos y cerdos adolescentes y la respuesta de AMPc a 5-HT en la aurícula izquierda de lechones recién nacidos, sugiriendo que no parece existir actividad por parte de otras fosfodiesterasas. De cualquier forma, mientras en la aurícula izquierda la PDE4 es más activa en lechones recién nacidos, la

PDE3 es más activa en cerdos adolescentes. Por razones aún desconocidas, hay una supresión de la actividad de 5-HT en el ventrículo de lechón recién nacido mediada principalmente por PDE3 que cambia a un control conjunto por ambas PDE3 y PDE4 en cerdos adolescentes. A diferencia del miocardio porcino, la disminución de la respuesta inotrópica a 5-HT en aurícula humana está causada parcialmente por la PDE3 pero no por la PDE4.

5.4 Las fosfodiesterasas PDE3 y PDE4 conjuntamente controlan los efectos inotrópicos pero no cronotrópicos del (-)-CGP12177 a pesar de la bradicardia sinusal causada por PDE4 en la aurícula de rata.

El rolipram (n=8) incrementó la frecuencia de latido sinusal basal en 27±5 latidos por minuto pero cilostamida (n=8) no tuvo efecto. La potencia cronotrópica del (-)-CGP12177 (-log EC_{50}M=7.5) no fue modificada por rolipram, cilostamida ni por su combinación. El (-)-CGP12177 incrementó la fuerza de la aurícula izquierda con una actividad intrínseca de un 25 % respecto al (-)-isoproterenol (200µM). El rolipram (n=8) y la cilostamida (n=8) no modificaron la fuerza basal de la aurícula izquierda pero su combinación (n=8) incrementó la fuerza hasta un 52±9 % respecto a (-)-isoproterenol. Ni el rolipram ni la cilostamida afectaron la potencia inotrópica de (-)-CGP12177 (-log EC_{50}M=7.4) pero conjuntamente, rolipram y cilostamida, causaron potenciación (-log EC_{50}M=8.2) y convirtió al (-)-CGP12177 en un agonista total comparado con (-)-isoproterenol.

La taquicardia sinusal mediada por β_{1L}AR no es controlada por la PDE3 ni por la PDE4, a pesar de la taquicardia provocada por la inhibición de la PDE4. Como vimos anteriormente, la PDE4 reduce el ritmo basal del nódulo sinusal. En cambio, como se había observado anteriormente, en el ventrículo de rata, ambas, la PDE3 y la PDE4 reducen los incrementos de fuerza en la aurícula izquierda mediados por el β_{1L}AR.

6. Conclusiones

En el transcurso de las investigaciones realizadas hemos obtenido las siguientes conclusiones:

Como conclusión general, las PDEs se pueden comportan de manera diferente en las distintas regiones cardiacas y compartimentos celulares.

La frecuencia basal del nódulo sinusal está controlada por PDEs. Difiere en cada especie la PDE o las PDEs que la controlan, pero en ninguna especie, de las que hemos estudiado (ratón, rata y lechón), las PDEs ejercen control sobre la taquicardia provocada por diferentes receptores, 5-HT_4, β_{1H}AR, β_{1L}AR y β_2AR.

El miocardio porcino es la única especie no primate que en condiciones fisiológicas expresa receptores 5-HT_4. En el miocardio humano y porcino, los efectos inotrópicos agudos producidos por 5-HT desaparecen con el tiempo. En esta desaparición están involucradas las PDEs pero son diferentes las que actúan en la aurícula humana y en lechón recién nacido. Hemos observado, que con la edad, en el miocardio porcino, se produce un cambio en el papel relativo de PDE4 y PDE3, ya que en los cerdos adolescentes predomina más la PDE3 acercándose más a la situación del humano donde solo actúa la PDE3.

En el ventrículo de rata, los incrementos de I_{caL} mediados por los receptores β_1AR están controlados por la PDE3 y la PDE4, pero los incrementos de la fuerza contráctil solo son controlados por PDE4. Esto sugiere que existen diferencias en los distintos dominios dentro del miocito. En cambio la PDE3, actuando solitariamente, si que controla los efectos ventriculares mediados por el β_2AR en I_{CaL} y la fuerza contráctil.

En aurículas izquierdas de roedores se observan pequeños (pero significativos) efectos mediados por el β_2AR tras la inhibición conjunta de la PDE3 y PDE4. Esto nos sugiere que ambas PDEs previenen conjuntamente la manifestación de los efectos mediados por el β_2AR en la aurícula izquierda. Además, la inhibición conjunta de la PDE3 y PDE4, potencia marcadamente los efectos de los β_2ARs ventriculares.

Las PDEs protegen al corazón de los posibles efectos inotrópicos adversos de una sobreestimulación por catecolaminas. La inhibición de PDEs acompañada de un

agonista β-adrenérgico podría llevar a la aparición de arritmias nocivas mediadas por ambos β_1AR y β_2AR.

La extrapolación a humanos de experimentos con PDEs y GPCRs en modelos animales es muchas veces inadecuada.

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9. Anexo I

English report

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Regulation by phophodiesterases of cardiac function activated by G protein-coupled receptors

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2009

English report

Myocardial β-adrenoceptors (βAR) and 5-HT_4 receptors mediate their signals through the receptor→G_s→AC→cAMP pathway. The phosphodiesterases (PDEs) are a large enzyme family that degrade the cAMP and limit the effects mediated through these receptors. It was unknown which PDE isoenzymes are responsible for the hydrolysis of the cAMP in different cardiac regions. The aim of this doctoral thesis is to investigate which isoenzymes have a role in the human, porcine and rodent myocardium. We have investigated the function of the PDEs in the sinoatrial node, left atrium and ventricle. We performed chronotropic, inotropic studies in isolated tissues, as well as biochemical studies quantifying cAMP. To elucidate the function of PDEs at the L type Ca^{2+} channel and its relation to inotropy, we also used the patch clamp technique in isolated myocytes. The key results are: 1. PDEs have different roles in different cardiac regions and cellular compartments. 2. The basal beating rate of the sinoatrial node is controlled by PDE3 and / or PDE4, but these PDEs do not limit the tachycardia mediated through the stimulation of β_1AR, β_2AR and 5-HT_4 receptors. The evidence is consistent with the existence of two cAMP pools, one controlled by PDE3 and/or PDE4 that modulates basal sinoatrial beating, the other not controlled by PDEs, and responsible for G-protein-coupled receptor (GPCR)-mediated tachycardia. 3. In the rat ventricular myocardium increases of I_{CaL} through activation of β_1AR are reduced by both PDE3 and PDE4 but the increases in contractile force are only controlled by PDE4 suggesting distinct intracellular compartimentalization of PDEs. Both I_{CaL} and inotropic responses through activation of β_2AR are limited by PDE3. 4. The concomitant inhibition of PDE3 and PDE4 uncovers inotropic effects in rodents mediated through β_2ARs. 5. PDE3 is responsible, at least in part, for the fade of the inotropic responses to 5HT mediated through human atrial 5-HT_4 receptors. 6. The control of porcine cardiac 5-HT_4 function undergoes regional and age-dependent changes. The atrial myocardium of adolescent pigs resembles more adult human atrium, in which the fade of 5-HT responses is partially due to PDE3. 7. The PDEs protect the heart from the harmful effects, for example arrhythmia, caused by the overstimulation of the GPCR. 8. Given the diverse roles of PDE3 and PDE4 and their dependence on species, extrapolation to humans should be done cautiously because these animal models usually do not reflect the situation of human myocardium.

1. Introduction

Cardiac β-adrenoceptors (βAR) and 5-HT_4 receptors mediate their signals through the receptor→G_s→AC→cAMP pathway. The phosphodiesterases (PDE) are a wide enzyme family that degrade the cAMP and limit the effects mediated by these receptors. It was unknown which isoenzyme of PDEs are responsible for the hydrolysis of the cAMP in the different cardiacs regions.

The mouse heart has been used as a model for human β_1-and β_2-adrenoceptors (β_1AR and β_2AR). The murine ventricular βAR population consists of 70% β_1AR and 30% β_2AR (Heubach et al., 1999), similar to the nonfailing human ventricle (Molenaar et al., 2000) and atrium (Molenaar et al., 1997). It has been proposed that murine cardiac β_2AR 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_2AR, even after treatment with PTX. Furthermore, these findings agree with recent work on murine ventricular myocytes, demonstrating that PTX failed to affect the β_2AR-mediated increase in cAMP (Nikolaev et al., 2006). A plausible reason for the lack of detectable function of β_2AR in the murine heart could be avid phosphodiesterase-catalysed hydrolysis of the cAMP produced through agonist-evoked receptor activation.

Activation of the sympathetic nervous system causes cardiostimulation through release of catecholamines. (-)-Noradrenaline and (-)-adrenaline increase rate and force of the mammalian heart through coexisting β_1AR and β_2AR coupled to cAMP-dependent pathways. However, access of cAMP to effectors differs for activation of cardiac β_1-ARs and β_2ARs (Xiao et al., 1995; Kuschel et al., 1999) possibly due in part to involvement of different PDEs. Hydrolysis of cAMP by PDEs protects the heart against overstimulation by sympathetic nerves but there are differences between βAR subtypes. Several PDE isoenzymes modulate catecholamine-evoked cardiostimulation (Fischmeister et al., 2006; Nikolaev et al., 2006). In the rat ventricle, PDE activity is

mostly due to PDE3 and PDE4 (Mongillo et al., 2004; Rochais et al., 2004; Rochais et al., 2006).

Human (Kaumann et al., 1990; Sanders and Kaumann, 1992; Brattelid et al., 2004) and porcine (Kaumann, 1990; Parker et al., 1995) hearts express functional 5-HT_4 receptors that mediate cardiostimulation including arrhythmias (Kaumann, 1994; Kaumann and Sanders, 1994; Rahme et al., 1999; Pau et al., 2003; Leftheriotis et al., 2005) through cAMP-dependent pathways (see Kaumann and Levy, 2006a). Cyclic AMP is hydrolysed by PDEs and recent evidence has disclosed that the role of these enzymes is so important that they virtually prevent the functional manifestation of effects of 5-HT through human and porcine ventricular 5-HT_4 receptors. Only when PDE activity is inhibited with the non-selective PDE inhibitor, isobutylmethylxanthine (IBMX), can positive inotropic and lusitropic effects of 5-HT, as well as stimulation of cAMP-dependent protein kinase (PKA) and even arrhythmias mediated by ventricular 5-HT_4 receptors, become apparent (Brattelid et al., 2004), but which PDE isoenzymes are responsible is unknown. The inotropic responses to 5-HT and 5-HT_4 receptor partial agonists tend to fade in human (Kaumann et al., 1991; Sanders and Kaumann, 1992) and porcine (Parker et al., 1995) atria. De Maeyer et al., (2006) reported that IBMX prevents the fade of 5-HT responses in porcine atria but which PDE isoenzymes are mainly involved is still an open question (Kaumann and Levy, 2006b).

Cardiac (Lowe et al., 2002) and recombinant (Baker et al., 2003; Joseph et al., 2004) β_1AR can be activated through two sites, β_{1H}AR and β_{1L}AR, with high and low affinity for βAR blockers, respectively. βAR blockers that cause cardiostimulation through β_{1L}AR at concentrations considerably higher than those that cause receptor blockade, are designated non-conventional partial agonists. CGP12177 is an experimentally used non-conventional partial agonist that has uncovered quantitative differences of β_{1L}AR function between species and even between different heart regions (reviewed by Kaumann and Molenaar, 2008). CGP12177 is a non-conventional partial agonist on rat sinoatrial node and left atrium but not on ventricle (Kaumann and Molenaar, 1996), unless PDEs are inhibited by IBMX (Sarsero et al., 1999) but the PDE isoenzyme are unknown.

2. Aims

1. To elucidate whether the activity of PDE3 and / or PDE4 prevents cardiostimulants effects of activation of β_2AR by (-)-adrenaline in the heart of rodents, and to investigate whether there are regional differences in the role of PDE3 and PDE4 in the control of responses mediated through β_1AR and β_2AR.

2. To investigate the influence of inhibition of PDE4 in arrhythmias mediated by (-)-adrenaline in the wall of the right ventricle of the mouse.

3. To investigate the relationship between increases in I_{CaL} and contractility, mediated by β_1AR and β_2AR, and its reduction by PDEs in rat ventricular myocytes.

4. To investigate the role of PDE3 and PDE4 in the control of the responses produced by activation of 5-HT_4 receptor in different heart regions of newborn piglets and their relation to the responses produced in the myocardium of adolescents pigs and adult human atrium.

5. During the initial experiments carried out in mice, we uncovered an original situation not reported in the literature. Inhibition of PDE3 and / or PDE4 produced an increase in basal heart rate without modifying the effects mediated through activation of β_1ARs. This finding led us to consider the possible existence of two different pools of cAMP, one that regulates the sinoatrial basal frequency and controlled by PDE3 and / or PDE4, the other causing the chronotropic effects mediated though β_1AR but not controlled by PDE3 and / or PDE4. As a result of this observation, we decided to investigate whether the tachycardia mediated through β_{1H}AR, β_{1L}AR, β_2AR and 5HT_4 receptors is also resistant to modulation by PDE3 and / or PDE4.

3. Methods

(-)-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.

Cardiostimulation evoked by (-)-noradrenaline (ICI118551 present) and (-)-adrenaline (CGP20712A present) through β_1AR and β_2AR, respectively, was compared on sinoatrial beating rate, left atrial and ventricular contractile force in isolated tissues from Wistar rats. I_{CaL} was assessed with whole-cell patch clamp.

5-HT responses mediated through 5-HT_4 receptors were compared, *ex vivo*, on sinoatrial beating rate of newborn piglets, porcine atrial and ventricular force, and human atrial force. cAMP levels were assessed in piglet left atrium.

We investigated the effects of the PDE3-selective inhibitor cilostamide (300 nM) and PDE4 inhibitor rolipram (1 µM) on the (−)-CGP12177-evoked increases of sinoatrial beating rate and force of paced left atria of the Sprague-Dowley rat.

4. Results

In murine tissues 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_1AR-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_2AR-selective antagonist ICI118551.

In the rat rolipram caused sinoatrial tachycardia. Cilostamide and rolipram did not enhance chronotropic potencies of (-)-noradrenaline and (-)-adrenaline, mediated through β_1AR and β_2AR respectively. Rolipram but not cilostamide potentiated atrial and ventricular inotropic effects of (-)-noradrenaline, mediated through β_1AR. Cilostamide potentiated the ventricular effects of (-)-adrenaline mediated through β_2AR but not the effects of (-)-noradrenaline mediated through β_1AR. Concurrent cilostamide and rolipram uncovered left atrial effects of (-)-adrenaline mediated through β_2AR. Both rolipram and cilostamide augmented the (-)-noradrenaline-evoked increase in I_{CaL}. (-)-Adrenaline increased I_{CaL} only in the presence of cilostamide but not rolipram. Concurrent cilostamide and rolipram caused marked potentiation of the inotropic effects of (-)-adrenaline, mediated through β_2AR.

In new born piglets 5-HT-evoked sinoatrial tachycardia did not fade and was not potentiated by cilostamide (300 nM) or rolipram (1 μM). Inotropic responses to 5-HT faded in atria from piglets, adolescent pigs and humans. Cilostamide reduced the atrial fade of 5-HT responses in adolescent pigs and humans but not in newborn piglets. Cilostamide disclosed ventricular responses to 5-HT in newborn, but not adolescent pigs. Rolipram reduced fade of atrial 5-HT responses in newborn and adolescent pigs but not in human atria. Concurrent cilostamide and rolipram abolished fade of 5-HT

responses in porcine left atria and facilitated ventricular 5-HT responses, but did not reduce residual fade observed in human atrium in the presence of cilostamide. 5-HT-evoked increases in left atrial cAMP faded; fade was abolished by concurrent cilostamide and rolipram.

In tissues from Sprague-Dawley rats rolipram (n=8) increased basal sinoatrial rate by 27±5 bpm but cilostamide (n=8) had no effect. The chronotropic potency of (−)-CGP12177 (−logEC_{50}M=7.5) was not changed by rolipram and cilostamide or their combination (-)-CGP12177 increased left atrial force with intrinsic activity 0.25 compared to (-)-isoprenaline. Rolipram (n=8) and cilostamide (n=8) did not change basal force of left atria but concurrent rolipram and cilostamide (n=8) increased force by 52±9% of the effect of 200 μM (−)-isoprenaline. Neither rolipram nor cilostamide affected the inotropic potency of (−)-CGP12177 (−logEC_{50}M=7.4) but concurrent rolipram+cilostamide caused potentiation (−logEC_{50}M=8.2) and converted (-)-CGP12177 into a full agonist compared to (-)-isoprenaline.

5. Conclusions

Rolipram revealed regional differences in the role of PDE4 in murine heart. (-)-Adrenaline-evoked cardiostimulation through β_1AR 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_1AR-mediated tachycardia elicited by (-)-adrenaline. Concurrent inhibition of both PDE3 and PDE4 uncovered cardiostimulant effects of (-)-adrenaline mediated through β_2AR of left atrium but not of sinoatrial node or right ventricle. Rolipram potentiated (-)-adrenaline-evoked arrhythmias, mediated through β_1AR 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 Catecholaminergic polymorphic ventricular tachycardia.

In the Wistar rat heart, PDE4 reduced basal sinoatrial beating rate but neither PDE3 nor PDE4 affected the chronotropic potencies of (-)-noradrenaline and (-)-adrenaline through β_1AR and β_2AR respectively, suggesting that the cAMP hydrolysis by PDE4 controlled beating rate in a compartment distinct from the region at which βAR activation modified ionic channel activity and Ca^{2+} cycling leading to tachycardia. PDE4 but not PDE3 reduced the atrial and ventricular inotropic effects of (-)-noradrenaline through β_1AR. The β_1AR-mediated increases of ventricular I_{CaL} were blunted by both PDE3 and PDE4 at the sarcolemmal domain but increases in contractile force are blunted only by PDE4, suggesting that cAMP in the β_1AR/PDE3/Ca^{2+} channel compartment does not reach proteins that produce increases in contractility such as RyR2 channels and phospholamban. PDE3, but not PDE4 alone, reduced both increases in I_{CaL} and ventricular positive inotropic effects of (-)-adrenaline through β_2AR. Concurrent inhibition of PDE3 and PDE4 uncovered left atrial inotropic effects of (-)-adrenaline through β_2AR and markedly potentiated ventricular inotropic effects of (-)-adrenaline mediated through β_2ARs. PDE3 and PDE4 protected the rat heart against ventricular and left atrial overstimulation but not against tachycardia through β_1AR and β_2AR.

In new-born piglets 5-HT caused sustained sinoatrial tachycardia. PDE3 and PDE4 reduced sinoatrial beating rate but did not potentiate the 5-HT-evoked sinoatrial tachycardia, suggesting that these PDEs reduce the cAMP responsible for basal heart rate but do not have access to a cAMP compartment in the vicinity of the 5-HT_4 receptors. PDE3 and PDE4 jointly prevent the fade of the inotropic and cAMP responses to 5-HT in left atria of both newborn and adolescent pigs, making unlikely any involvement of additional PDEs. However, while left atrial PDE4 was more active in newborn, PDE3 was more active in adolescents. For still unknown reasons, there is a selective suppression of the ventricular 5-HT responses by PDE3 in newborn pigs that changes into a joint control by both PDE3 and PDE4 in older pigs. The inotropic response to 5-HT in human atrium partially fades but, unlike porcine myocardium, fade was caused only by PDE3 but not by PDE4.

In the Sprague–Dawley rat, the β_{1L}AR mediated sinoatrial tachycardia is not controlled by PDE3 and PDE4, despite the bradycardia caused by PDE4. In contrast, and as previously observed on rat ventricle, both PDE3 and PDE4 reduce the β_{1L}AR-mediated increases in left atrial force.

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10. Anexo II

Otras comunicaciones

P014 Contractile responses through G_s-coupled receptors are reduced by phosphodiesterase3 activity in human isolated myocardium

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The cAMP hydrolysing phosphodiesterases PDE3 and PDE4 coexist in mammalian cardiomyocytes but their role in modulating contractile force increases through G_s-coupled receptors in human myocardium is unknown. We investigated the influence of the PDE3 inhibitor cilostamide (100-300 nM) and PDE4 inhibitor rolipram (1 μM) on the positive inotropic responses to noradrenaline (β_2-selective antagonist ICI118551, 50 μM present) and adrenaline (β_1-selective antagonist CGP20712, 300 μM present), mediated through β_1- and β_2-adrenoceptors respectively. In ventricular trabeculae from 5 patients with terminal heart failure, cilostamide increased the $-\log EC_{50}M$ of noradrenaline from 5.66±0.20 to 5.96±0.15 ($P<0.05$) and for adrenaline from 5.09±0.47 to 6.32±0.53 ($P=0.08$). In atrial trabeculae from 8 patients without heart failure, cilostamide increased the $-\log EC_{50}M$ of adrenaline and noradrenaline from 7.54±0.13 and 7.08±0.19 to 8.27±0.12 ($P<0.005$) and 7.30±0.30 ($P=0.05$) respectively. 5-hydroxytryptamine (5-HT, 1 μM) increased contractility of atrial trabeculae from 6 non-failing hearts; the responses faded by 93±4% 60 min after administration. Cilostamide reduced the fade to 50.5±8% ($P<0.003$). Rolipram failed to affect the responses to adrenaline, noradrenaline and 5-HT. PDE3, but not PDE4, protects the human heart against overstimulation through β_1-adrenoceptors, β_2-adrenoceptors and 5-HT_4 receptors.

Contractile responses through G_s-coupled receptors are reduced by phosphodiesterase3 activity in human isolated myocardium

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Background

Activation of the G_s-coupled β_1- and β_2-adrenopceptors, as well as 5-HT_4 receptors, increases contracile force through a cAMP pathway in the human heart. The cAMP hydrolysing phosphodiesterases PDE3 and PDE4 coexist in human heart and their activity could limit the contractile effects mediated through G_s-coupled receptors, but their role is unknown. Fade of contractile responses to 5-HT in human atrium (Kaumann & Sanders 1992 *Arch Pharmacol* **345**, 382-386) could be due to avid PDE hydrolysis. We investigated the influence of the PDE3 inhibitor cilostamide (100-300 nM) and PDE4 inhibitor rolipram (1 μM) on the positive inotropic effects of noradrenaline, adrenaline and 5-hydroxytryptamine (5-HT), mediated through human cardiac β_1-adrenoceptors, β_2-adrenoceptors and 5-HT_4 receptors respectively.

.

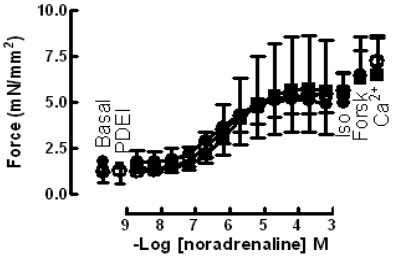
Methods

Isolated ventricular and atrial trabeculae, obtained from human hearts, were set up to contract as described (Molenaar et al., 2006 *Cardiovasc. Res*. **69**, 128-139). β_1-and β_2-adrenoceptor-mediated effects were studied in the presence of the β_1- and β_2-selective antagonists ICI118551 (50 nM) and CGP20712 (300nM) respectively. 5-HT_4 receptor-mediated effects were investigated in the presence of (-)-propranolol (200 nM).

Results and Conclusions

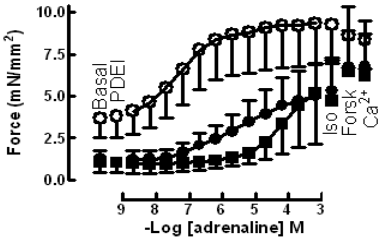
- The effects of the cardiostimulant effects of (-)-noradrenaline, mediated through β_1-adrenoceptors, and of (-)-adrenaline, mediated through β_2-adrenoceptors, were potentiated by cilostamide but not by rolipram.
- Atrial responses to 5-HT faded. The fade was reduced by cilostamide but not by rolipram.
- PDE3, but not PDE4, protects the heart against potentially harmful stimulation through β_1-adrenoceptors, β_2-adrenoceptors and 5-HT_4 receptors.

Noradrenaline +/- PDE inhibitors
Right Ventricle
Heart Failure



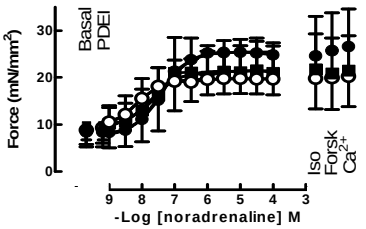
Noradrenaline pEC_{50} 5.74 ± 0.19 (17/6)
+ cilostamide pEC_{50} 6.12 ± 0.20 (13/6)
+ rolipram pEC_{50} 5.81 ± 0.23 (6/4)

Adrenaline +/- PDE inhibitors
Right Ventricle
Heart Failure



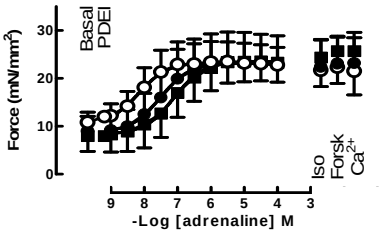
Adrenaline pEC_{50} 5.25 ± 0.41 (17/6)
+ cilostimide pEC_{50} 6.43 ± 0.44 (12/6)
+ rolipram pEC_{50} 5.42 ± 0.65 (7/4)

Noradrenaline +/- PDE inhibitors
Right Atrium
non-failing Hearts



Noradrenaline pEC_{50} 7.08 ± 0.19 (7/7)
+ cilostamide pEC_{50} 7.48 ± 0.30 (4/4)
+ rolipram pEC_{50} 7.01 ± 0.20 (6/6)

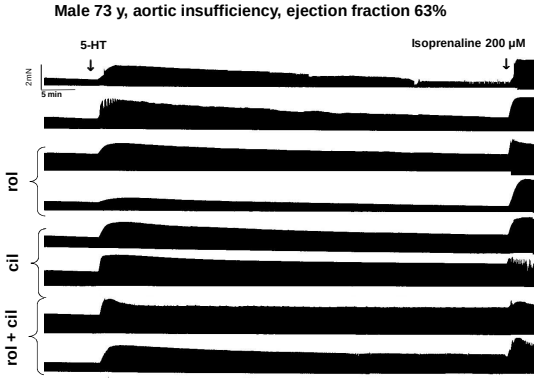
Adrenaline +/- PDE inhibitors
Right Atrium
non-failing Hearts



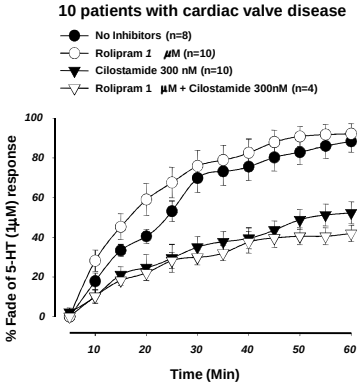
Adrenaline pEC_{50} 7.54 ± 0.13 (9/9)
+ cilostamide pEC_{50} 8.27 ± 0.12 (8/7)
+ rolipram pEC_{50} 7.42 ± 0.19 (6/6)

Kinetics of 5-HT 1μM in the absence and presence of rolipram (rol) and cilostamide (cil)

Male 73 y, aortic insufficiency, ejection fraction 63%



10 patients with cardiac valve disease



REGIONAL DIFFERENCES OF THE INFLUENCE OF ROLIPRAM ON THE EFFECTS OF (-)-ADRENALINE *VIA* β_1-ADRENOCEPTORS IN MURINE HEART

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Catecholamines cause cardiostimulation through murine β_1-adrenoceptors (β_1-AR) but *not* through β_2-AR (Oostendorp & Kaumann, 2000; Heubach et al., 2002), conceivably due to avid hydrolysis of cAMP by phosphodiesterases (PDE). The isoenzymes PDE3 and PDE4 catalyse more than 90% of total cAMP in rat cardiomyocytes (Mongillo et al., 2004). We investigated the effects of PDE3 inhibition with cilostamide (300 nM) and PDE4 inhibition with rolipram (1 μM) on the (-)-adrenaline-evoked cardiostimulation mediated through β_1-AR and β_2-AR. Experiments were carried out on cardiac tissues from mixed 129Sv-C57Bl/6 mice of either sex. The effects of the PDE inhibitors were investigated on sinoatrial beating rate, and force of paced left atrium and right ventricular wall. The tissues were pretreated 60 min with 5 μM phenoxybenzamine, followed by washout. Cilostamide and rolipram increased sinoatrial rate by 34±8 bpm and 9±9 bpm in the absence of the β_1-AR antagonist CGP20712 and by 42±9 bpm and 25±4 bpm in its presence respectively. Rolipram but not cilostamide increased left atrial force by 0.9±0.4 mN and 0.2±0.1 mN in the absence and presence of CGP20712 respectively. Cilostamide and rolipram did not change ventricular force. Rolipram potentiated the left atrial effects of (-)-adrenaline both in the absence and presence of CGP20712, and enhanced the maximum ventricular response to (-)-adrenaline ($P<0.03$). Rolipram did not potentiate the sinoatrial effects of (-)-adrenaline. Cilostamide did not modify the effects of (-)-adrenaline in the 3 cardiac regions. CGP20712 shifted the concentration-effect curves to (-)-adrenaline to the left without revealing CGP20712-resistant effects of (-)-adrenaline.

Table 1. Potencies (-logEC_{50}M) of (-)-adrenaline. Data are mean ± s.e.m.

		n	Control	n	CGP 20712 (300 nM)
Right Atrium	Basal	4	7.2 ± 0.2	6	4.8 ± 0.1
(beats.min^{-1})	Rolipram	3	7.3 ± 0.2	8	4.9 ± 0.1
	Cilostamide	4	7.4 ± 0.1	4	4.7 ± 0.1
Left Atrium	Basal	5	7.2 ± 0.1	8	4.2 ± 0.2
(contractile	Rolipram	4	8.5 ± 0.1 P<0.001	8	5.0 ± 0.2 P<0.01
force)	Cilostamide	4	7.7 ± 0.2	4	4.6 ± 0.1
Ventricle	Basal	4	6.1 ± 0.3	8	Unsurmountable blockade
(Contractile	Rolipram	3	6.3 ± 0.3	8	Unsurmountable blockade
force)	Cilostamide	4	6.4 ± 0.4	Not determined	

P values with respect to the corresponding basals of the control and CGP20712 groups.

PDE4 limits the cardiostimulant effects of (-)-adrenaline, mediated through β_1-AR, in left atrium and to a smaller extent in ventricle but not in sinoatrial node. Inhibition of PDE4 failed to reveal β_2-AR-mediated effects of (-)-adrenaline.

Heubach, J. et al. (2002). *Br. J. Pharmacol*., **136**, 217-229.
Mongillo, M. et al. (2004). *Circ. Res*., **95**, 67-75.
Oostendorp, J. & Kaumann, A.J. (2000). *Arch. Pharmacol*., **361**, 134-145.

REGIONAL DIFFERENCES OF THE INFLUENCE OF ROLIPRAM ON THE EFFECTS OF (-)-ADRENALINE *VIA* β_1-ADRENOCEPTORS IN MURINE HEART

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Introduction:

Catecholamines cause cardiostimulation through murine β_1-adrenoceptors (β_1-AR) but *not* through β_2-AR (Oostendorp & Kaumann, 2000; Heubach et al., 2002), conceivably due to avid hydrolysis of cAMP by phosphodiesterases (PDE). The isoenzymes PDE3 and PDE4 catalyse more than 90% of total cAMP in rat cardiomyocytes (Mongillo et al., 2004). We investigated the effects of PDE3 inhibition with cilostamide (300 nM) and PDE4 inhibition with rolipram (1 μM) on the (-)-adrenaline-evoked cardiostimulation mediated through β_1-AR and possibly β_2-AR.

Material and methods:

Experiments were carried out on cardiac tissues from mixed 129Sv-C57Bl/6 mice of either sex. The effects of the PDE inhibitors were investigated on sinoatrial beating rate, and force of paced left atrium (Oostendorp & Kaumann, 2000) and right ventricular wall (Heubach et al, 2002). To inhibit neuronal and extraneuronal uptake of the (-)-adrenaline, as well as to block α.adrenoreceptors the tissues were pretreated 60 min with 5 μM phenoxybenzamine, followed by washout. The PDE inhibitors were incubated for 30 min before a cumulative concentration-effect curve for (-)-adrenaline was started.

Results:

Cilostamide and rolipram transciusly increased sinoatrial rate by 57±8 bpm and 21±9 bpm in the absence of the β_1-AR antagonist CGP20712 and by 42±5 bpm and 32±8 bpm in its presence respectively. Rolipram, but not cilostamide, increased left atrial force by 0.9±0.4 mN from 0.9±0.25mN and 0.2±0.1 mN from 0.5±0.1mN, in the absence and presence of CGP20712 respectively. Cilostamide and rolipram did not change ventricular force (figure 3ª). Rolipram potentiated the left atrial effects of (-)-adrenaline both in the absence and presence of CGP20712 (Fig 2, table 1), and enhanced the maximum ventricular response to (-)-adrenaline (P<0.03) (Fig 3b). Rolipram did not potentiate the sinoatrial effects of (-)-adrenaline (fig. 1). Cilostamide did not modify the potency of (-)-adrenaline in the 3 cardiac regions (table 1). CGP20712 shifted the concentration-effect curves for (-)-adrenaline to the left without revealing CGP20712-resistant effects of (-)-adrenaline. (Fig. 1-3).

Table 1. Potencies (-logEC$_{50}$M) of (-)-adrenaline. Data are mean ± s.e.m.

		n	Control	n	CGP 20712 (300 nM)
Right Atrium	Basal	4	7.2 ± 0.2	6	4.8 ± 0.1
	Rolipram	3	7.3 ± 0.2	8	4.9 ± 0.1
	Cilostamide	4	7.4 ± 0.1	4	4.7 ± 0.1
Left Atrium	Basal	5	7.2 ± 0.1	8	4.2 ± 0.2
	Rolipram	4	8.5 ± 0.1 P<0.001	8	5.0 ± 0.2 P<0.01
	Cilostamide	4	7.7 ± 0.2	4	4.6 ± 0.1
Ventricle	Basal	4	6.1 ± 0.3	8	Unsurmountable blockade
	Rolipram	3	6.3 ± 0.3	8	Unsurmountable blockade
	Cilostamide	4	6.4 ± 0.4		Not determined

P values with respect to the corresponding basals of the control and CGP20712 groups.

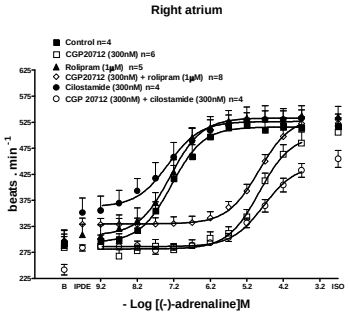


Figure 1. Effects of (-)-adrenaline, mediated through β_1AR on sinoatrial pacemaker in the absence and presence of CGP20712. Lack of effects cilostamide and rolipram on (-)-adrenaline potency. B = basal sinoatrial rate. IPDE = PDE inhibitor or time-matched control. n = number of mice.

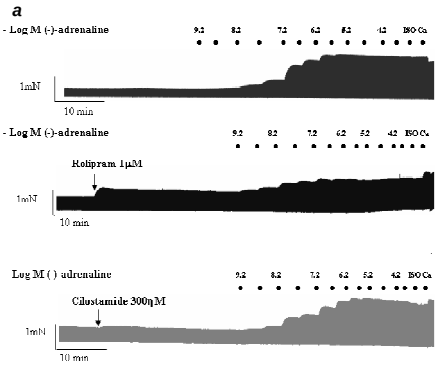
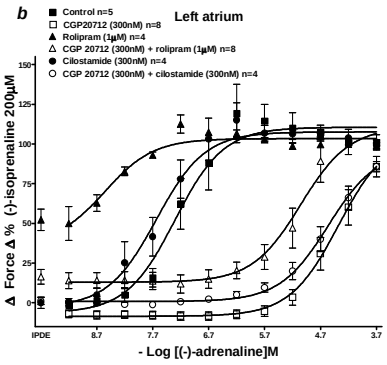


Figure 2. Potentiation of the effects of (-)-adrenaline on murine left atrium by rolipram but not cilostamide. a) Representative experiments of the effects of adrenaline in the absence of PDE inhibitors or presence of rolipram or cilostamide. ISO = (-)-isoprenaline 200 μM. Ca = $CaCl_2$ 9mM. b) Potentiation of the effects of (-)-adrenaline, mediated through β_1AR, by rolipram on left atria in the absence and presence of CGP 20712. IPDE = PDE inhibitor or time-matched control. n = number of mice.



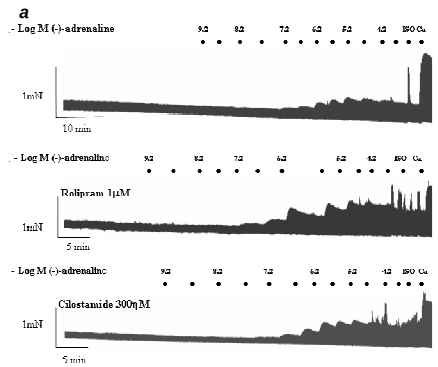
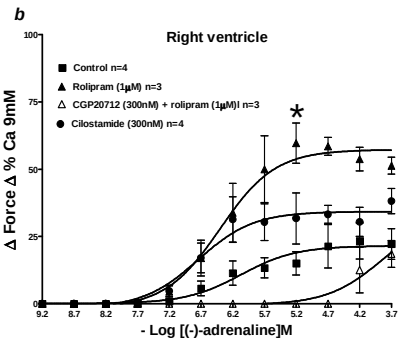


Figure 3. Effects of (-)-adrenaline on murine right ventricular wall. a) Representative experiments of the effects of (-)-adrenaline in the absence of PDE inhibitors or presence of rolipram or cilostamide. ISO = (-)-isoprenaline 200 μM. Ca = CaCl2 9mM. b) Increase of the E_{max} of (-)-adrenaline by rolipram in right ventricular walls (* P<0.03) and lack of effect of cilostamide on (-)-adrenaline potency. n = number of mice.



Conclusions:

PDE4 limits the cardiostimulant effects of (-)-adrenaline, mediated through β_1-AR, in left atrium and to a smaller extent in ventricle but not in sinoatrial node. Inhibition of PDE4 failed to reveal β_2-AR-mediated effects of (-)-adrenaline.

References:

Heubach, J. et al. (2002). *Br. J. Pharmacol.*, **136**, 217-229.
Mongillo, M. et al. (2004). *Circ. Res.*, **95**, 67-75.
Oostendorp, J. & Kaumann, A.J. (2000). *Arch. Pharmacol.*, **361**, 134-145.

Supported by the Seneca Foundation of Spain

FADE OF INOTROPIC RESPONSES BUT NOT OF SINOATRIAL TACHYCARDIA ELICITED BY 5-HYDROXYTRYPTAMINE IN ATRIA FROM NEW-BORN PIGLETS. ROLE OF PHOSPHODIESTERASES 3 AND 4

[1]María Luisa Vargas, [1]Alejandro Galindo-Tovar and [2]Alberto J Kaumann. [1]Department of Pharmacology and H.U.V.Arrixaca, Murcia, Spain and [2]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK.

5-Hydroxytryptamine (5-HT) increases porcine atrial contractility through 5-HT_4 receptors but the inotropic responses fade. Fade is prevented by the non-selective phosphodiesterase (PDE) inhibitor isobutyl-methyl-xanthine (De Maeyer et al., 2006) but the role of PDE isoenzymes is unknown (Kaumann & Levy, 2006). We investigated the influence of the PDE3-selective blocker cilostamide (Cil, 300 nM) and PDE4-selective blocker rolipram (Rol, 1 μM) on the kinetics of 5-HT responses in spontaneously beating right atria and paced left atria from new-born piglets euthanised with pentobarbital 100mg.kg^{-1}. Experiments were carried out in the presence of (-)-propranolol (200 nM). Cil and Rol increased sinoatrial rate from 76.5±6.2 to 86.0±7.8 bpm (n=14, P<0.02, paired Student´s test) and from 88.6±4.4 to 95.8±5.8 bpm (n=11, P<0.03) respectively. 5-HT (1 μM and 10 μM) caused sinoatrial tachycardia that did not fade (n=8) and was not affected by Cil and Rol or their combination and $-\log EC_{50}M$ values for 5-HT, estimated from concentration-effect curves (n=5-8 per group), were not different. 5-HT (10 μM), incubated for 2 min, increased left atrial force from 4.2±1.5 to 8.4±2.3 mN (n=8, P<0.003) by the 20th min the response faded to 5.4±1.4 mN. In the presence of Cil or Rol 5-HT increased force from 5.5±0.8 to 14.0±2.1 (n=12, P<0.0001) and 4.8±0.6 to 12.9±1.3 mN (n=8, P<0.0001) respectively at the 2nd min; these responses partially faded to 9.0±1.5 and 9.3±1.2 mN respectively by the 20th min. The inotropic responses to (-)-isoprenaline (200 μM) were not different in the absence (n=14) or presence of Cil (n=16) or Rol (n=18). Left atrial cAMP was determined by radioimmunoassay in freeze-clamped tissues (Table 1). 5-HT only increased cAMP by the 20th min. Both Rol and Cil caused increases of cAMP that faded. The (-)-isoprenaline-evoked cAMP response was markedly increased by Rol but not significantly by Cil.

Table 1. Effects of PDE inhibitors, 5-HT (10 μM) and (-)-isoprenaline (200 μM) (ISO), on left atrial cAMP levels (pmol.mg^{-1} protein, mean ± s.e.m.)

	Control		5-HT				ISO	
	n		n	2 min	n	20 min	n	
Basal	21	17.8±1.6	13	19.6±2.7	8	27.0±4.9[a]	21	27.3±2.1[d]
Rol	16	22.6±2.4	8	33.2±5.9[b]	7	25.6±2.	15	298.7±86.5[c]
Cil	20	20.4±2.7	8	28.7±4.4[c]	11	21.1±2.7	20	31.2±4.2[a]
Rol+Cil	19	18.6±0.9	8	32.3±3.9[d]	8	26.6±4.0[d]	18	275.9±31.3[d]

[a] P<0.05, [b]P=0.058, [c]P<0.005, [d]P<0.001; P values refer to comparisons with controls

Conclusions: 1. Both PDE3 and PDE4 appear to modulate basal sinoatrial rate but not 5-HT_4 receptor-mediated tachycardia. 2. Both PDE3 and PDE4 partially contribute to the fade of left atrial inotropic responses to 5-HT. 3. Some inconsistencies between cAMP data and inotropy of 5-HT reflect still unknown compartmentalization. 4. 5-HT_4 receptors signal to both PDE3 and PDE4 while β-adrenoceptors signal mainly to PDE4.

De Maeyer, J.H. *et al.* (2006) *Br. J. Pharmacol*., **147**, 140-157.
Kaumann, A.J. & Levy, F.O. (2006). *Br. J. Pharmacol*., **147**, 128-130

FADE OF INOTROPIC RESPONSES BUT NOT OF SINOATRIAL TACHYCARDIAELICITED BY 5-HYDROXYTRYPTAMINE IN ATRIA FROM NEW-BORN PIGLETS. ROLE OF PHOSPHODIESTERASES 3 AND 4.

[1]María Luisa Vargas, [1]Alejandro Galindo-Tovar & [2]Alberto J Kaumann.

[1]Department of Pharmacology, University of Murcia, Reasearch Unit of Universtary Virgen Arrixaca Hospital, Murcia, Spain and [2]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK.

Introduction:

5-Hydroxytryptamine (5-HT) increases porcine atrial contractility through 5-HT4 receptors but the inotropic responses fade. Fade is prevented by the non-selective phosphodiesterase (PDE) inhibitor isobutyl-methyl-xanthine (De Maeyer et al., 2006) but the role of PDE isoenzymes is unknown (Kaumann & Levy, 2006). We investigated the influence of the PDE3-selective blocker cilostamide (Cil, 300 nM) and PDE4-selective blocker rolipram (Rol, 1 µM) on the kinetics of 5-HT responses in spontaneously beating right atria and paced left atria from new-born piglets.

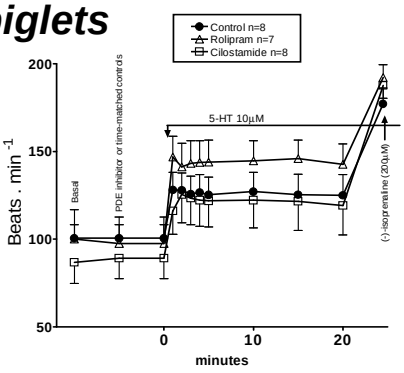
Methods:

Experiments were carried out in the presence of (-)-propranolol (200 nM) , the PDE inhibitors were incubated during 30 minutes. Left atrial cAMP was determined by radioimmunoassay in freeze-clamped tissues .

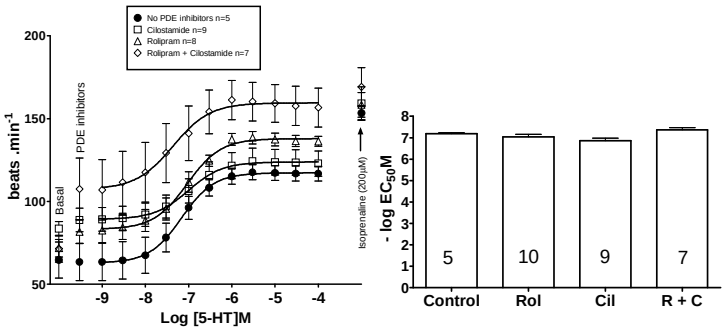
Results:

Spontaneously beating right atria of new-born piglets

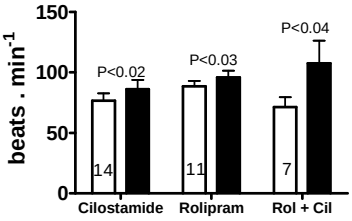
Kinetics of the positive cronotropic effects of 5-HT in the absence or presence of PDE inhibitors.



PDE inhibitors do not affect the chronotropic potency of 5-HT



Both, cilostamide and rolipram cause transient sinotratrial tachycardia.



Conclusions:

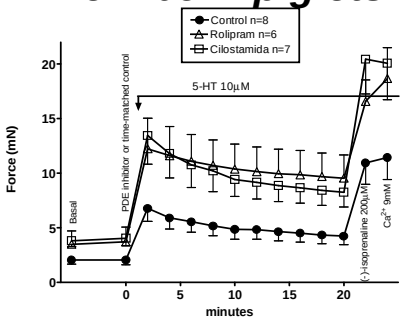
1. *Both PDE3 and PDE4 appear to modulate basal sinoatrial rate but not 5-HT4 receptor-mediated tachycardia.*
2. *Both PDE3 and PDE4 partially contribute to the fade of left atrial inotropic responses to 5-HT.*
3. *Some inconsistencies between cAMP data and inotropy of 5-HT reflect still unknown compartmentalization.*
4. *5-HT4 receptors signal to both PDE3 and PDE4 while β-adrenoceptors signal mainly to PDE4.*

References:

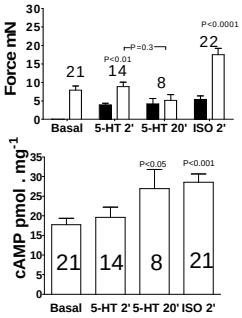
De Maeyer et al., Br J Pharmacol. 2006 Jan;147(2):140-57.
Kaumann &Levy, Pharmacol Ther. 2006 Sep;111(3):674-706.

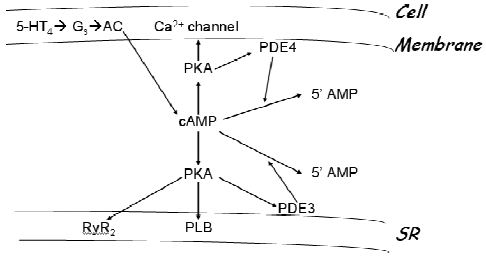
Left atria from new-born piglets

Kinetics of the positive inotropic effects of 5-HT in the absence or presence of PDE inhibitors.

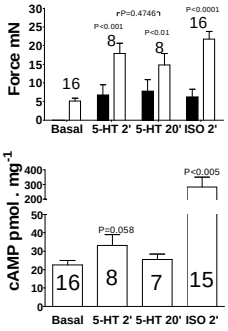


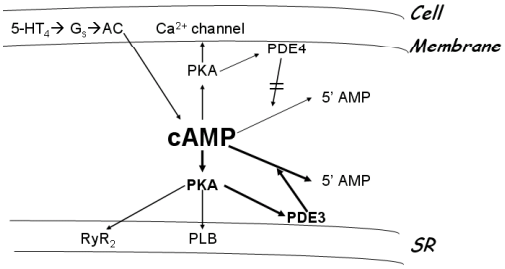
No PDE inhibitor



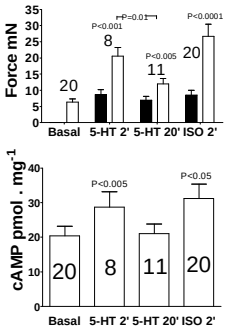


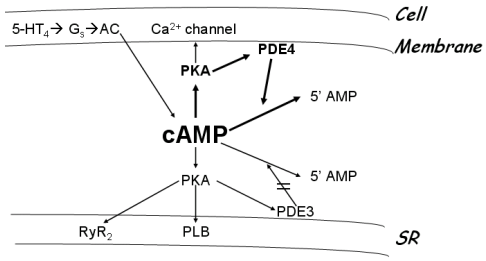
Rolipram 1µM



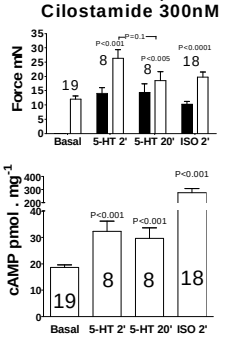


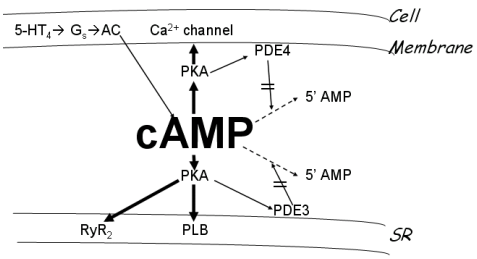
Cilostamide 300nM





Rolipram 1µM + Cilostamide 300nM





Supported by FIS PI 052338, Ministerio de Sanidad y Consumo, and Seneca Foundation Murcia, Spain.

SCP008

Modulation of 5-HT responses through porcine atrial and ventricular 5-HT_4 receptors by phosphodiesterases PDE3 and PDE4; plausible ontogenic changes in ventricle

[1]Galindo-Tovar A, [1]Vargas ML, [1]Escudero E & [2]Kaumann AK. *[1]Hospital Universitario Virgen de la Arrixaca and Department of Pharmacology, University of Murcia, Spain and [2]Department of Physiology, Development and Neuroscience, University of Cambridge, UK*

Responses to 5-HT, mediated through 5-HT_4 receptors, are facilitated by phosphodiesterase (PDE) inhibition in human and porcine myocardium (Kaumann and Levy, 2006). 5-HT-evoked increases in human atrial contractility tend to fade (Sanders and Kaumann, 1992) and non-selective inhibitor 3-isobutyl-1- methyl-xanthine abolishes fade of 5-HT-evoked inotropic response in porcine atrial trabeculae (DeMaeyer et al., 2006) but the PDE isoenzymes involved have not been characterized. We investigated the effects of the the PDE3-selective inhibitor cilostamide (300 nM) and PDE4 inhibitor rolipram (1 μM) on the fade to the inotropic responses to 5-HT (10 μM) in left atrial trabeculae, as well as the ability of these PDE inhibitors to uncover inotropic 5-HT responses in right ventricular trabeculae, obtained from adolescent pigs (2 months either sex). Pigs were anaesthetised with pentobarbital (70 $mg.kg^{-1}$), the hearts rapidly removed and tissues dissected and paced at 1 Hz at 37°C. Atrial 5-HT responses faded continuously and disappeared completely (n=6) after 30 min. Fade of the 5-HT response was completely prevented by concomitant cilostamide+rolipram (104±13% n=5). Cilostamide and rolipram preserved the 5-HT response by 52±13% (n=6) and 27±4% (n=5) % respectively by the 30^{th} min. Similar results were obtained from left atria of newborn piglets. Cilostamide but not rolipram tended to uncover ventricular 5-HT responses from newborn piglets (P=0.045 n=9). Neither cilostamide nor rolipram revealed 5-HT responses in adolescents. Concomittant cilostamide+rolipram unconcealed 5-HT responses in both piglets (P=0.025 n=8) and adolescent (P=0.006 n=7). We conclude that both PDE3 and PDE4 blunt atrial 5-HT responses. In newborn piglets PDE3 appears to selectively prevent 5-HT responses while in the ventricle of adolescents PDE3 and PDE4, acting in concert, abolish the 5-HT response.

References:

DeMaeyer *et al*. Br J Pharmacol. 2006; 147: 140-157.
Kaumann and Levy Pharmacol Ther. 2006; 111: 647-706.
Sanders and Kaumann Arch Pharmacol. 1992; 345: 382-386

Modulation of 5-HT responses through porcine atrial and ventricular 5-HT$_4$ receptors by phosphodiesterases PDE3 and PDE4; plausible ontogenic changes in ventricle

[1]Alejandro Galindo-Tovar, [1]María Luisa Vargas, [1]Elisa Escudero & [2]Alberto J Kaumann.

[1]Department of Pharmacology, University of Murcia, Research Unit of the Universitary Virgen Arrixaca Hospital, Murcia, Spain and [2]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, UK.

Introduction:

Responses to 5-HT, mediated through 5-HT$_4$ receptors, are facilitated by phosphodiesterase (PDE) inhibition in human and porcine myocardium (Kaumann & Levy 2006 Pharmacol Ther 111:674-706). 5-HT-evoked increases in human atrial contractility tend to fade (Sanders & Kaumann 1992 Arch Pharmacol 345:382-386) and the non-selective PDE inhibitor 3-isobutyl-1- methyl-xanthine abolishes fade of 5-HT-evoked inotropic response in porcine atrial trabeculae (De Maeyer et al 2006 Br J Pharmacol 147:140-157) but the PDE isoenzymes involved have not been characterized.

Methods:

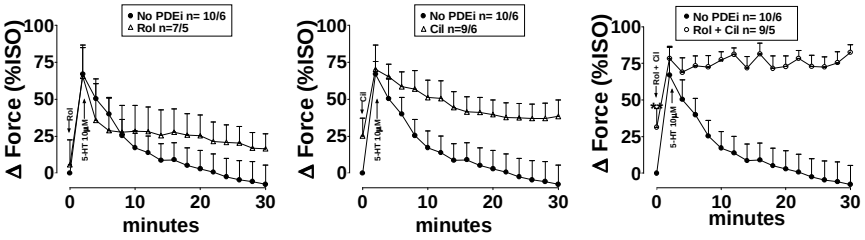
We investigated the effects of the the PDE3-selective inhibitor cilostamide (300 nM) and PDE4 inhibitor rolipram (1 μM) on the fade of the inotropic responses to 5-HT (1-10 μM) in left atrial strip and trabeculae, as well as the ability of these PDE inhibitors to uncover inotropic responses to 5-HT in right ventricular trabeculae, obtained from new born piglets (first postnatal day) and adolescent pigs (2 months either sex). Pigs were anaesthetised with pentobarbital (70 mg.kg^{-1}), the hearts rapidly removed and tissues dissected and paced at 1 Hz at 37°C.

Results:

Left Atria

Atrial responses to 5-HT completely or partially faded by the 20th min in adolescent and newborn piglets respectively. In newborn rolipram but not cilostamide reduced fade (P<0.01). In adolescent both, rolipram and cilostamide, reduced fade. Fade of the 5-HT response was completely prevented by concurrent cilostamide + rolipram.

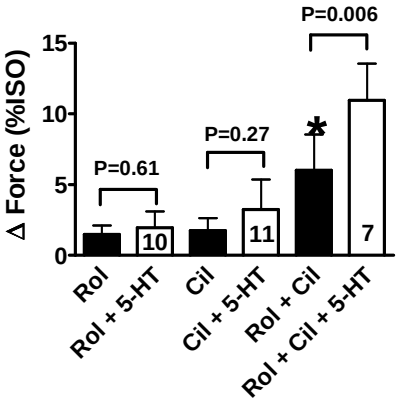
Left atria from adolescent pigs



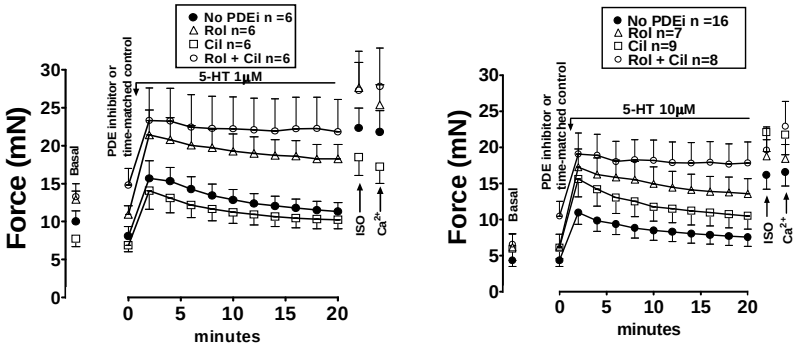
Right ventricular trabeculae

Cilostamide but not rolipram uncovered ventricular responses to 5-HT in newborn piglets. Neither cilostamide nor rolipram revealed responses to 5-HT in adolescents. Concurrent cilostamide+rolipram unconcealed responses to 5-HT in both newborn piglets and adolescent pigs.

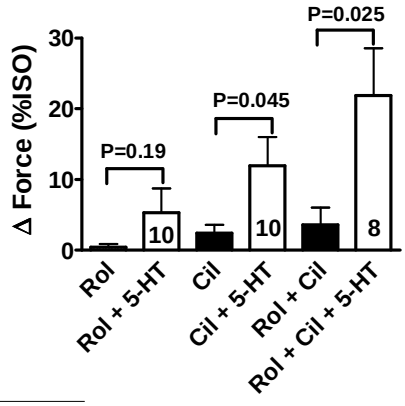
Right ventricular trabeculae from adolescent pigs



Left atria from newborn piglets



Right ventricular trabeculae from newborn piglets



Conclusions:

1. *In adolescent pigs, both PDE3 and PDE4 blunt atrial inotropic responses to 5-HT and jointly prevent ventricular responses to 5-HT.*
2. *In newborn piglets PDE4, but not PDE3, reduces atrial responses to 5-HT but PDE3 prevents ventricular responses to 5-HT.*
3. *Both PDE3 and PDE4 control 5-HT$_4$ receptor-mediated responses in porcine heart, but their relative contribution to limit 5-HT responeses differs between atrium and ventricle and changes with the age.*

Supported by FIS PI 052338, Ministerio de Sanidad y Consumo, Spain

Phosphodiesterases3- and 4–controlled compartments, activated by β_1- and β_2-adrenoceptors, differ for L-type Ca^{2+} current and inotropy in rat heart

T. Christ[1], A. Galindo-Tovar[1], M. Thoms[1], U. Ravens[1], A. Kaumann[2]

[1]Department of Pharmacology and Toxicology, Medical Faculty Carl Gustav Carus, Dresden University of Technology

[2]Department of Physiology, University of Cambridge

Background

(-)-Noradrenaline and (-)-adrenaline increase force of the mammalian heart through co-existing β_1- and β_2-adrenoceptors (β_1AR, β_2AR) coupled to cAMP-dependent pathways. Hydrolysis of cAMP by phosphodiesterases limits stimulation by the sympathetic nerve system but there are differences between βAR subtypes and between PDE isoforms.

(-)-Isoprenaline increase cAMP in subsarcolemmal pools through β_1AR or β_2AR and these effects are enhanced by inhibition of PDE3 or PDE4 in rat heart.

Here we have investigated how these β_1AR and β_2AR-mediated events in the membrane microdomains translate into contractility.

Results and Conclusion

- Increases of $I_{Ca,L}$ through β_1AR activation were augmented by inhibition of PDE3 or 4, whereas force generation was blunted by PDE4 only.
- Inhibition of PDE3 but not 4 facilitated ventricular $I_{Ca,L}$ through β_2AR activation, however concomitant inhibition of PDE3 and 4 was needed to uncover clear inotropic effects.
- Our findings are consistent with an important control by PDE3 of cAMP generated in the subsarcolemmal space ($I_{Ca,L}$), but a more strict control of contractility by both PDE3 and PDE4 near the contractile machinery.

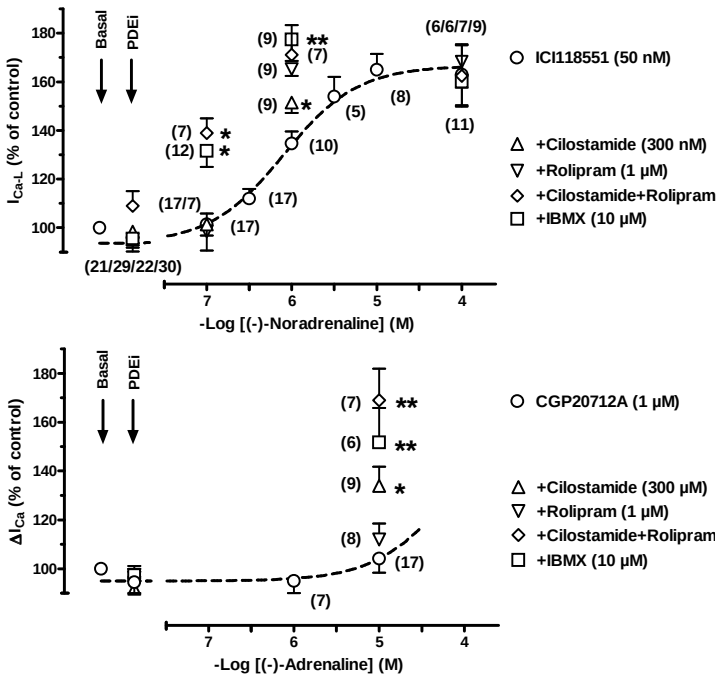


Fig. 1: Influence of PDE inhibitors on the increase in $I_{Ca,L}$

β_1AR and β_2AR receptors were stimulated by noradrenaline and adrenaline. Concentration-dependent effects on $I_{Ca,L}$ in ventricular cells. Mean values ± S.E.M. numbers of cells are given in parenthesis, * p<0.05, **p<0.01 vs. respective control; PDEi, PDE inhibitor

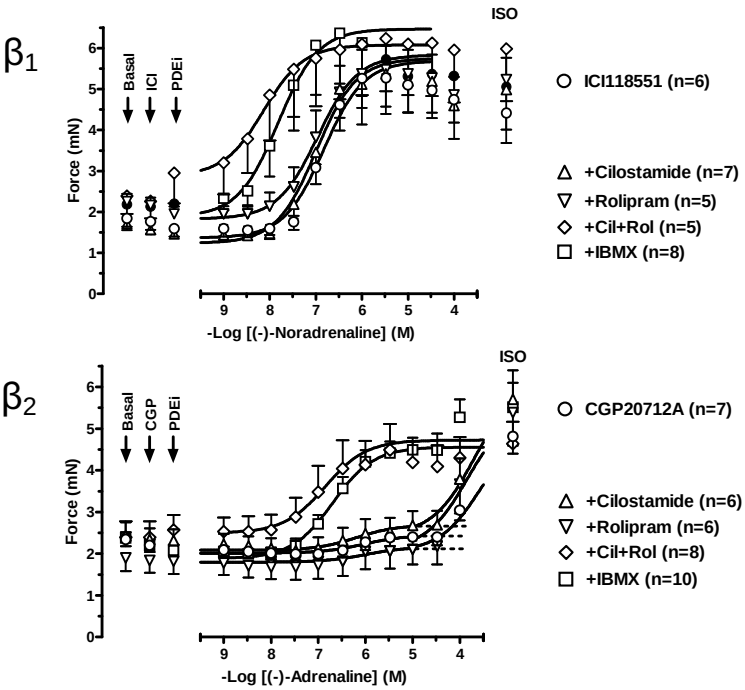


Fig. 2: Influence of PDE inhibitors on the positive inotropic effects

Fits of some biphasic curves were constrained by using the effect of (-)-isoprenaline as maximum. Broken lines depict a small β_2AR-mediated component. Means ± S.E.M., numbers of right ventricles are given in parenthesis. ISO, (-)-isoprenaline (200 µM); PDEi, PDE inhibitor

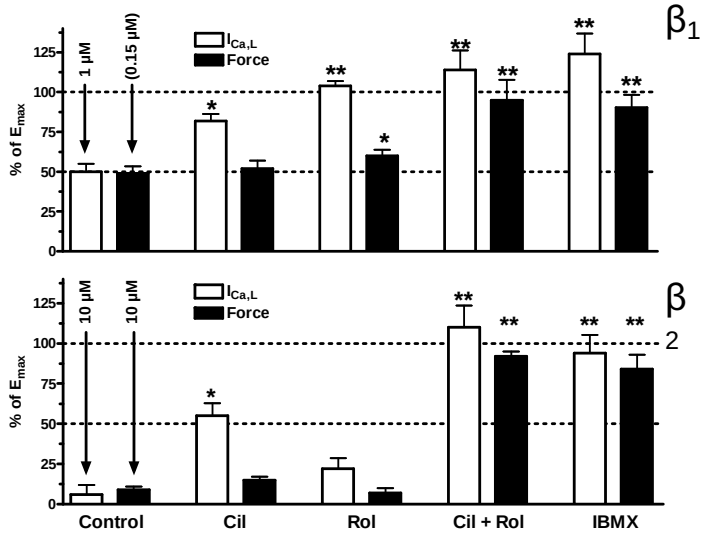


Fig. 3: Comparison of of PDE3 and 4 effects on $I_{Ca,L}$ and force

Results are taken from figure 2 and 3 and are expressed as % of maximum effect. Noradrenalin effects were given for concentrations giving half-maximum effect on $I_{Ca,L}$ and force respectively, adrenalin effects for only 10 µM . * p<0.05, **p<0.01 vs. respective control.

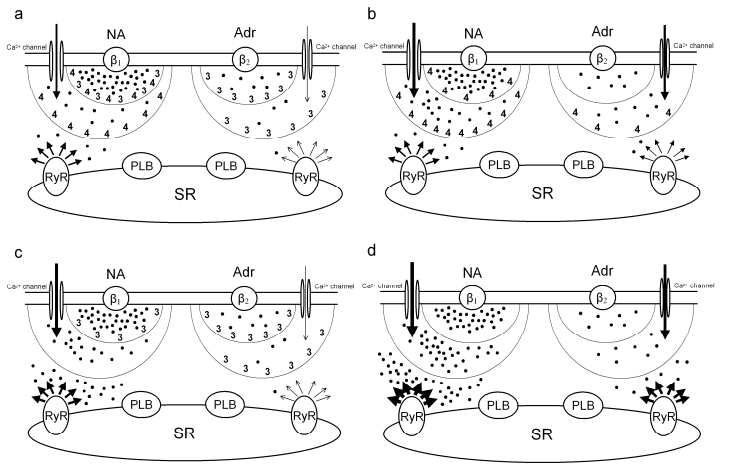


Fig. 4: Regulation of $I_{Ca,L}$ and SR responses by PDE

Hypothetical regulation of cAMP (black dots) levels by activated PDE3 (3) and PDE4 (4) in compartments at different distances from β_1AR and β_2AR in rat ventricle. a. Physiological situation (no inhibition of PDE). b. Inhibition of PDE3 (Please note activation of PDE4!). c. Inhibition of PDE4. d. Inhibition of both PDE3 and PDE4.

Small potentiation of the inotropic effects of catecholamines by PDE inhibitors is accompanied by dramatic increases of arrhythmias in rat right ventricular myocardium

Catecholamines can induce ventricular arrhythmias. PDE3 and PDE4 differently blunt the effects of β_1- and β_2-adrenoceptor (β_1AR, β_2AR) stimulation on L-type Ca^{2+} current and force generation, suggesting that PDEs not only limit overall cAMP but also generate compartmentation of cAMP. Accordingly inhibition of PDE activity is expected to abolish functional compartments. However, the relevance of PDE inhibition on electrical stability of the myocardium is unclear. Here we investigated the effects of PDE3 and PDE4 inhibitors on electrical stability in right ventricular strips paced at 1 Hz. The preparations were challenged with noradrenaline (NA) and adrenaline (Adr) to stimulate β_1AR and β_2AR respectively. Under control conditions NA provoked arrhythmias in 8 out of 33 preparations (24%), which occurred at high concentrations that produced maximum inotropic responses (-logEC_{50}(M) 5.2 for arrhythmia vs. 6.9 for inotropy). In the presence of the PDE4 inhibitor rolipram (1 µM) the incidence of NA-induced arrhythmias was dramatically increased of 19 out of 22 preparations (80%). The -logEC_{50} values for arrhythmia induction and inotropic response were no longer different (~6.9 for both). In contrast stimulation of β_2AR by Adr elicited robust inotropic effects only in the concomitant presence of rolipram and cilostamide (300nM) to inhibit PDE4 and PDE3, but 11 out of 12 preparations (92%) became arrhythmic although maximum force generation tended to be smaller than with NA. This suggests that catecholamine-induced arrhythmias facilitated by PDE-inhibition are the result of loss of compartmentation for cAMP rather than caused by an increase in overall cAMP. Compartmented hydrolysis cAMP by PDE3 and PDE4 has an important impact on the electrical stability during catecholamine challenge in rat ventricular myocardium under in-vitro condition. We speculate that abolishing PDE-mediated compartments may lead to harmful arrhythmias.

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Small potentiation of the inotropic effects of catecholamines by PDE inhibitors is accompanied by dramatic increases of arrhythmias in rat right ventricular myocardium

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Background

Catecholamines can induce ventricular arrhythmias via cAMP-mediated cardiostimulation. PDE3 and PDE4 differently blunt the effects of β_1- and β_2-adrenoceptor (β_1AR, β_2AR) stimulation on L-type Ca^{2+} current and force generation, suggesting that PDEs not only limit overall cAMP but also generate compartmentation of cAMP[1]. Accordingly inhibition of PDE activity is expected to abolish functional compartments. However, the relevance of PDE inhibition on electrical stability of the myocardium is unclear. Here we investigated the effects of PDE3 and PDE4 inhibitors on electrical stability in right ventricular strips challenged with noradrenalin and adrenalin in order to stimulate β_1AR and β_2AR.

Methods

Right ventricular strips were dissected and placed in oxygenated, modified Tyrode's solution at room temperature containing (mM): NaCl 126.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 22, NaH_2PO_4 0.45, 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.

Positive inotropic effects of (-)-catecholamines were only measured from non-arrhythmic ventricular preparations or during periods of stable non-arrhythmic contractions. Arrhythmias were defined as sustained spontaneous activity (more than 10 following beats) faster than stimulation rate (1 Hz).

Agents used:

- Phenoxybenzamine (5 µM) for 90 min followed by washout, to irreversibly block α-adrenoceptors and tissue uptake of the catecholamines (all experiments),
- ICI118551 (50 nM) to block β_2-AR (experiments with (-)-noradrenaline),
- CGP20712A (300 nM) to block β_1AR (experiments with (-)-adrenaline),
- Cilostamide (300 nM) to inhibit PDE3 (approximately 86% of PDE3 would be inhibited with negligible (<0.4%) inhibiton of PDE4)
- Rolipram 1 µM (PDE4 was likely to be inhibited by approximately 50% with negligible inhibition (0.4%) of PDE3)

Results

- About 25% of right ventricular strips developed spontaneous activity (arrhythmias?), however only at very high concentrations of catecholamines.
- Inhibition of PDE4 did not only increase maximum effect but also sensitized the strips for arrhythmiainduction.
- Sensitization for arhythmias was larger than for inotropic responses.
- Even small cAMP-signals (β_2AR) can provoke arrhythmias when PDE is inhibited.
- PDE-inhibiton mediated arrhythmias are probably not related to increased $I_{Ca,L}$.

Conclusion

Compartmented hydrolysis cAMP by PDE3 and PDE4 has an important impact on the electrical stability during catecholamine challenge in rat ventricular myocardium under in-vitro condition. We speculate that abolishing PDE-mediated compartments may lead to harmful arrhythmias.

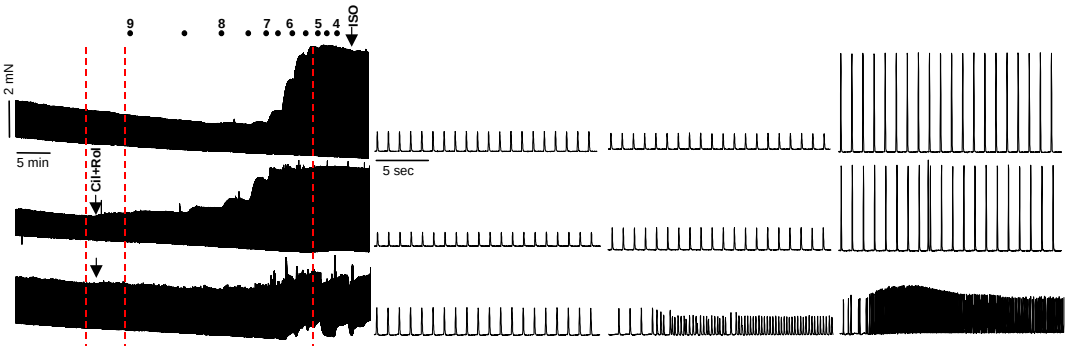

Fig. 1: Effect of PDE-Inhibition and catecholamines on contractility and electrical stability in rat right ventricles

Original registrations of force of contraction of three different muscle strips challenged with (-)-noradrenaline under control conditions (top) and in the concomitant presence of cilostamide and rolipram (middle, bottom). Addition of cilostamide and rolipram is indicated PDE-I, numbers indicating the –log (M) value for (-)-noradrenaline. ISO stands for 200 µM (-)-isoprenaline. Right: contractions at an expanded time scale are given. Please note the occurrence of fast spontaneous activity The respective time points are indicated by red dotted lines.

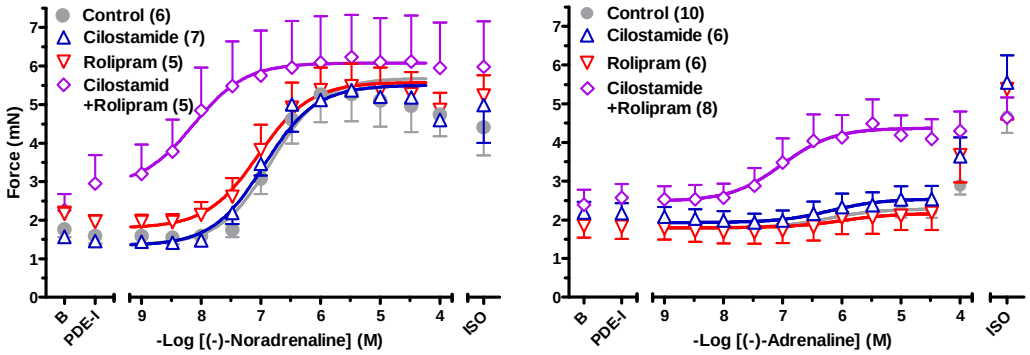

Fig. 2: Potentiation of βAR mediated-inotropy by PDE-inhibition

Concentration-dependency for the positive inotropic effect of (-)-noradrenaline through β_1AR (left) and adrenaline through β_2AR (right). B indicates basal force in the presence of ICI118551 (50nM) or CGP20712A (300nM) respectively; PDE-I indicates force after PDE-Inhibition. At the end of each experiments 200 µM (-)-isoprenaline was given to activate both β_1AR Data are taken from Christ et al. BJP 2009; 156:62-83

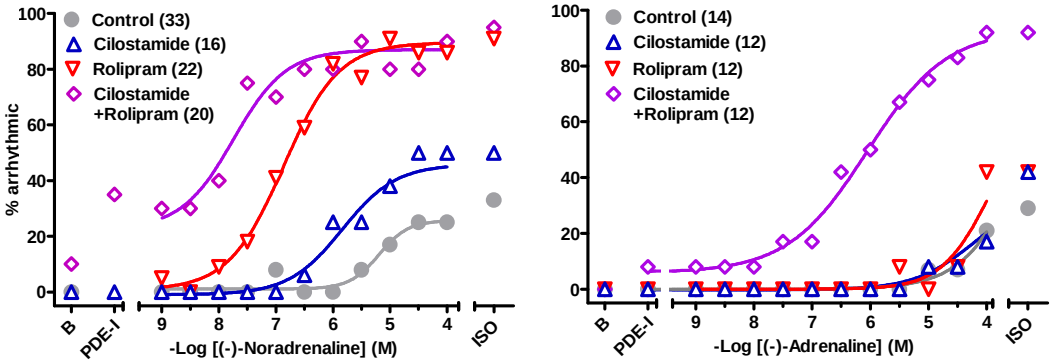

Fig. 3: Effect of PDE-Inhibition on catecholamine-provoked arhythmias

Arrhythmias provoked by (-)-noradrenaline through β_1AR (left) and by (-)-adrenaline through β_2AR (right). Data are given as percentage of muscles, with spontaneous activity; numbers in the brackets indicate total number of experiments. Symbols as in fig. 1.

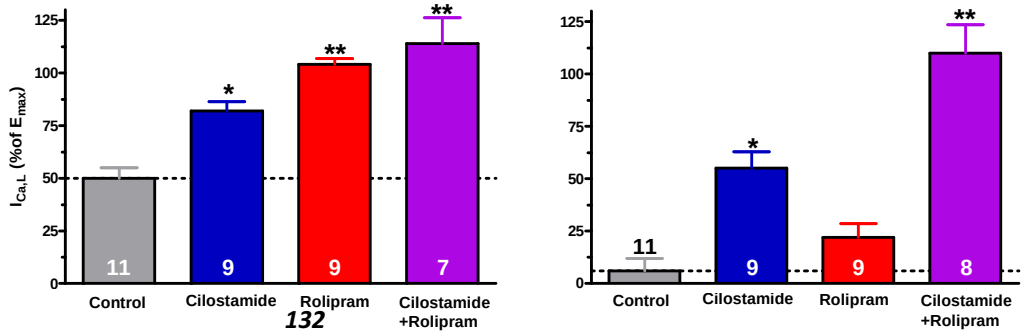

Fig. 4: Effect of PDE-Inhibition on catecholamine-stimulated $I_{Ca,L}$

Effect of 1 µM (-)-noradrenaline through β_1AR (left) and of 10 µM (-)-adrenaline through β_2AR (right) under control condition and after inhibition of PDE3, PDE4 or both. Increase in $I_{Ca,L}$ is defined as % of maximum effect in control cells. Dashed line indicates control level. * p<0.05, ** p<0.01 vs. respective control.

11. Anexo III

Factor de Impacto

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☐	1	BRIT J PHARMACOL	0007-1188	23987	4.902	4.214	1.297	536	7.8	0.05425	1.211

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Journals 1 - 1 (of 1) [1] **Page 1 of 1**

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				Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life	*Eigenfactor*™ Score	*Article Influence*™ Score
☐	1	N-S ARCH PHARMACOL	0028-1298	4579	2.830	2.389	0.362	130	9.6	0.00765	0.631

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Journals 1 - 1 (of 1) [1] **Page 1 of 1**

Los abajo firmantes Alberto J Kaumann, Torsten Christ, Marcus Thoms, Ursula Ravens, M.Luisa Vargas y Elisa Escudero, coautores de los trabajos presentados por Alejandro Galindo-Tovar para solicitar la defensa de su tesis doctoral como compendio de publicaciones

DECLARAN

Su conformidad con la presentación de los siguientes artículos: **1**. Phosphodiesterase-4 blunts inotropic and arrhytmias but not sinoatrial tachycardia of (-)-adrenaline mediated through mouse cardiac β_1-adrenoceptors (A Galindo-Tovar, AJ Kaumann); **2.** Ontogenis changes of the control by phosphodiesterase 3 and 4 of the 5-HT responses in porcine heart and relevance to human atrial 5-HT_4 receptors (A Galindo- Tovar, M L Vargas, E Escudero , AJ Kaumann); **3**. Inotropy and L-type Ca^{2+} current, activated by β_1- and β_2-adrenoceptors are differently controlled by phosphodiesterases 3 and 4 in rat heart (T Chist, A Galindo-Tovar, M Thoms, U Ravens, AJ Kaumann); **4**. Phosphodiesterase PDE 3 and PDE 4 jointly control the inotropic effects but not chronotropic effects of (-)-CGP12177 despite PDE4- evoked sinoatrial bradycardia in rat atrium (A Galino-Tovar, ML Vargas, AJ Kaumann) y su compromiso de no presentar estos artículos como parte de otra Tesis Doctoral.

Y para que así conste donde proceda firmamos la presente en Murcia y Dresden (Alemania) a 5 de noviembre de 2008

AJ Kaumann T Christ ML Vargas E Escudero

M Thoms U Ravens