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RESEARCH PAPER

Human atrial β_{1L}-adrenoceptor but not β_3-adrenoceptor activation increases force and Ca^{2+} current at physiological temperature

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BACKGROUND AND PURPOSE

It has been proposed that BRL37344, SR58611 and CGP12177 activate β_3-adrenoceptors in human atrium to increase contractility and L-type Ca^{2+} current ($I_{Ca\text{-}L}$). β_3-adrenoceptor agonists are potentially beneficial for the treatment of a variety of diseases but concomitant cardiostimulation would be potentially harmful. It has also been proposed that (–)-CGP12177 activates the low affinity binding site of the β_1-adrenoceptor in human atrium. We therefore used BRL37344, SR58611 and (–)-CGP12177 with selective β-adrenoceptor subtype antagonists to clarify cardiostimulant β-adrenoceptor subtypes in human atrium.

EXPERIMENTAL APPROACH

Human right atrium was obtained from patients without heart failure undergoing coronary artery bypass or valve surgery. Cardiomyocytes were prepared to test BRL37344, SR58611 and CGP12177 effects on $I_{Ca\text{-}L}$. Contractile effects were determined on right atrial trabeculae.

KEY RESULTS

BRL37344 increased force which was antagonized by blockade of β_1- and β_2-adrenoceptors but not by blockade of β_3-adrenoceptors with β_3-adrenoceptor-selective L-748,337 (1 μM). The β_3-adrenoceptor agonist SR58611 (1 nM–10 μM) did not affect atrial force. BRL37344 and SR58611 did not increase $I_{Ca\text{-}L}$ at 37°C, but did at 24°C which was prevented by L-748,337. (–)-CGP12177 increased force and $I_{Ca\text{-}L}$ at both 24°C and 37°C which was prevented by (–)-bupranolol (1–10 μM), but not L-748,337.

CONCLUSIONS AND IMPLICATIONS

We conclude that the inotropic responses to BRL37344 are mediated through β_1- and β_2-adrenoceptors. The inotropic and $I_{Ca\text{-}L}$ responses to (–)-CGP12177 are mediated through the low affinity site β_{1L}-adrenoceptor of the β_1-adrenoceptor. β_3-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ are restricted to low temperatures. Human atrial β_3-adrenoceptors do not change contractility and $I_{Ca\text{-}L}$ at physiological temperature.

LINKED ARTICLE

This article is commented on by Michel *et al.*, pp. 817–822 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2010.01005.x

Abbreviations

BRL37344, (RR + SS)[4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino]propyl]phenoxy]acetic acid; (–)-CGP12177, (7)-4-(3-tertiarybutylamino-2-hydroxypropoxy) benzimidazol-2-one; CGP20712A, (2-hydroxy-S-[2-[[2-hydroxy-3-[4-[methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-benzamide); IBMX, 3-isobutyl-1-methylxanthine; $I_{Ca\text{-}L}$, L-type Ca^{2+} current; ICI118,551, 1-[2,3-dihydro-7-methyl-1H-inden-4-yl] oxy-3-[(1-methylethyl) amino-2-butanol)]; L-748,337, N-(3-[3-[2-(4-benzenesulphonylamino phenyl)ethylamino]-2-hydroxypropoxy]benzyl acetamide; PDE, phosphodiesterase enzyme; PKA, cAMP-dependent protein kinase; SR58611, ethyl{(7S)-7-[(2R)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphtyl2-yloxy} acetate hydrochloride

Introduction

There is evidence that β_3-adrenoceptors are expressed in human heart (Krief *et al.*, 1993; Moniotte *et al.*, 2001a,b) but the relevance to contractile function is controversial. Agonists for the β_3-adrenoceptor have been reported to cause cardiodepressant effects on human ventricle obtained from endomyocardial biopsies (Gauthier *et al.*, 1996; Rozec and Gauthier, 2006), possibly by release of nitric oxide from endothelial and/or endocardial cells. In contrast, β_3-adrenoceptor agonists did not produce cardiostimulant or depressant effects in human ventricular trabeculae (Molenaar *et al.*, 1997; Kaumann and Molenaar, 2008) or human ventricular myocytes (Harding, 1997). However, Napp *et al.* (2009) recently reported a negative inotropic effect of (RR + SS)[4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino]propyl]phenoxy]acetic acid (BRL37344) that was prevented by the NO antagonist L-N-methyl-L-arginine. We have previously reported that β_3-adrenoceptor agonists are devoid of cardiostimulant or cardiodepressant effects in human atrium in the presence of (–)-propranolol (200 nM) (Kaumann *et al.*, 1997), a concentration that antagonizes the effects of (–)-noradrenaline and (–)-adrenaline through human atrial β_1- and β_2-adrenoceptors (Gille *et al.*, 1985) but is not expected to block β_3-adrenoceptors (Cohen *et al.*, 1999; Hoffmann *et al.*, 2004). Modest cardiostimulant effects of additional β_3-adrenoceptor agonists (Sennitt *et al.*, 1998), including BRL37344 (Arch and Kaumann, 1993; Pott *et al.*, 2003), were antagonized by (–)-propranolol (200–300 nM), consistent with their mediation through β_1- and β_2-adrenoceptors but not β_3-adrenoceptors. Taken together, the information published is inconsistent with regard to a cardiostimulant or cardiodepressant role of human atrial β_3-adrenoceptors.

Some β-blockers with high affinity for both β_1- and β_2-adrenoceptors cause cardiostimulation at concentrations considerably greater than those needed to significantly antagonize the effects of catecholamines. Their agonist effects are smaller and more resistant to antagonism by β-blockers [e.g. propranolol, nadolol (Kaumann and Molenaar, 2008)] than the effects of catecholamines. Accumulating evidence from cardiac and recombinant receptors indicates that these non-conventional partial agonists induce their agonist effects through a β_1-adrenoceptor site [β_{1L}-adrenoceptor, low affinity β_1-adrenoceptor] for which they have lower affinity than for the site [β_{1H}-adrenoceptor, high affinity β_1-adrenoceptor] through which they antagonize the effects of catecholamines (reviewed, Kaumann and Molenaar, 2008). The hydrophilic compound (7)-4-(3-tertiarybutylamino-2-hydroxypropoxy) benzimidazol-2-one (CGP12177), introduced as a high-affinity β-adrenoceptor radioligand by Staehelin *et al.* (1983) and soon thereafter discovered to exert cardiostimulant properties (Kaumann, 1983), has since been extensively used as an experimental non-conventional partial agonist (Kaumann and Molenaar, 2008). The cardiostimulant effects of (–)-CGP12177 mediated through the β_{1L}-adrenoceptor, are antagonized by (–)-bupranolol (Kaumann and Molenaar, 2008). Although CGP12177 does bind to β_3-adrenoceptors (Cohen *et al.*, 1999; Hoffmann *et al.*, 2004), it is only a weak lipolytic partial agonist in murine adipocytes (Preitner *et al.*, 1998) through β_3-adrenoceptors but hardly in human adipocytes (Tavernier *et al.*, 1996), and there is no evidence that its cardiostimulant effects are mediated through β_3-adrenoceptors (Cohen *et al.*, 1999; Kaumann and Molenaar, 2008). However, when human β_3-adrenoceptors are overexpressed in the murine heart they mediate cardiostimulation (Kohout *et al.*, 2001).

Surprisingly, Skeberdis *et al.* (2008) recently postulated that β_3-adrenoceptors mediate increases in human atrial contractility. They demonstrated, in human atrial myocytes at room temperature, that the agonists BRL37344, ethyl{(7S)-7-[(2R)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphtyl2-yloxy}acetate hydrochloride (SR58611) and CGP12177 increase the L-type Ca^{2+} current ($I_{Ca\text{-}L}$) and these responses were reversed by the β_3-adrenoceptor-selective antagonist N-(3-[3-[2-(4-benzenesulphonylaminophenyl)ethylamino]-

2-hydroxypropoxy]benzyl acetamide (L-748,337) (Candelore *et al.*, 1999). They reported agonist-evoked increases in contractility of atrial tissues at 37°C. These modest human atrial responses to CGP12177 (Kaumann, 1996; Skeberdis *et al.*, 2008) can be enhanced by the non-selective phosphodiesterase enzyme (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Kaumann and Molenaar, 1997) and PDE3 inhibitor cilostamide (Kaumann *et al.*, 2007). In addition, Skeberdis *et al.* (2008) observed marked increases in the contractile responses to CGP12177 and BRL37344 in the presence of IBMX in human atrium at 37°C. Importantly, however, they did not investigate whether these effects could be antagonized by L-748,337, as one would expect from an interaction through β_3-adrenoceptors.

Activation of β_3-adrenoceptors has been proven as a therapeutic target for the treatment of overactive bladder disorder (Yamaguchi, 2002; Chapple *et al.*, 2008; Michel *et al.*, 2010), and could plausibly be beneficial in type 2 diabetes (Sawa and Harada, 2006; Arch, 2008) as well as for disorders such as anxiety and depression (Stemmelin *et al.*, 2008). However, if human atrial β_3-adrenoceptors mediate cardiostimulation, this could be associated with tachycardia and potentially deleterious arrhythmias, including atrial fibrillation, thus limiting or even excluding their use in humans. Further research is therefore needed to confirm whether or not β_3-adrenoceptors mediate an increase in human atrial contractility.

The interpretation of Skeberdis *et al.* (2008) also challenges the concept that the positive inotropic effects of CGP12177 and other non-conventional partial agonists on human atrial myocardium are mediated through β_{1L}-adrenoceptors (Joseph *et al.*, 2003; Sarsero *et al.*, 2003; Kaumann and Molenaar, 2008). We previously found in human atrial trabeculae that L-748,337 failed to antagonize the positive inotropic effects of the non-conventional partial agonist (–)-pindolol, mediated through β_{1L}-adrenoceptors, as confirmed with recombinant β_1-adrenoceptors (Joseph *et al.*, 2003). Our aim was therefore to investigate whether L-748,337 could antagonize the inotropic effects of BRL37344, SR58611 and (–)-CGP12177 on human atrial preparations, using IBMX, at 37°C. However, we did not observe inotropic effects with SR58611. To investigate whether different receptors could be activated by BRL37344, SR58611 and (–)-CGP12177 as a function of temperature, we compared their $I_{Ca\text{-}L}$ responses at 24°C and 37°C. Finally, to investigate whether the $I_{Ca\text{-}L}$ responses to these agonists at 24°C couple to atrial contractile force, we also determined their effects on contractile force at 24°C.

Table 1

Characteristics of the patients

n	139
Gender, m/f	104/35
Age, years	68 ± 0.8
BMI, $kg \cdot m^{-2}$	27.6 ± 1
CAD, *n*	110
AVD/MVD, *n*	29
CAD + AVD/MVD, *n*	20
Hypertension, *n*	99
Diabetes, *n*	34
Hyperlipidaemia, *n*	73
LVEF, %	52 ± 1.2
Cardiovascular medication, *n*	
Digitalis	6
ACE inhibitors/AT_1-blockers	79
β-blockers	104
Metoprolol	67
Bisoprolol	27
Carvedilol	5
Nebivolol	1
Calcium channel blockers	29
Diuretics	15
Nitrates	28
Lipid-lowering drugs	67

BMI, body mass index; CAD, coronary artery disease; AVD, aortic valve disease; MVD, mitral valve disease; LVEF, left ventricular ejection fraction; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.

Methods

Patients

Right atrial appendages were obtained from patients undergoing coronary artery bypass surgery at The Prince Charles Hospital, Brisbane, who had provided written informed consent (The Prince Charles Hospital Ethics Committee EC27133; QUT human ethics committee 0800000066) and Dresden University of Technology (Medical Faculty Ethics committee document EK790799). Patient characteristics are outlined in Table 1.

Contractility studies

After excision, the appendages for the experiments of Figures 1, 3 and 4 (carried out in Brisbane) were immediately placed in modified oxygenated ice-cold Krebs solution containing (mM): Na^+ 125, K^+ 5, Ca^{2+} 2.25, Mg^{2+} 0.5, Cl^- 98.5, SO_4^{2-} 0.5, HCO_3^- 34, HPO_4^{2-} 1, ethylenediaminetetraacetic acid 0.04, and

equilibrated with 95% O_2/5% CO_2. Trabeculae were dissected and set up, on occasion, in pairs to contract at 1 Hz in an apparatus with a 50-mL organ bath in the solution above supplemented with (mM): Na^+ 15, fumarate 5, pyruvate 5, L-glutamate 5, glucose 10 at 37°C, as described (Gille *et al.*, 1985; Molenaar *et al.*, 2007). The tissues were attached to Swema SG4-45 strain gauge transducers (SWEMA, Stockholm, Sweden) and force recorded on a Watanabe polygraph (Graphtec Corporation, Yokohama, Japan). The tissues were driven with square-wave pulses of 5 ms duration and just over threshold voltage. After determination of a length–tension curve, the length of each trabeculum was set to obtain 50% of the resting tension associated with maximum developed force.

Experiments depicted in Figures 2 and 8 were performed in Tyrode's solution (mM): Na^+ 149.12, K^+ 5.4, Ca^{2+} 1.8, Mg^{2+} 1.05, Cl^- 137.8, HCO_3^- 22, HPO_4^{2-} 0.42, ethylenediaminetetraacetic acid 0.04, ascorbate 0.2, glucose 5, and equilibrated with 95% O_2/5% CO_2.

The conditions of Skeberdis *et al.* (2008) were used. Experiments were carried out in the presence of nadolol (200 nM), a concentration expected to block both β_{1H}- and β_2-adrenoceptors but hardly affect β_3-adrenoceptors (pK_D = 6.2; Baker, 2005) or β_{1L}-adrenoceptors (pK_B = 6.2; Joseph *et al.*, 2004a), and IBMX (10 μM) to boost the response to (–)-CGP12177 (Kaumann and Molenaar, 1997; Kaumann *et al.*, 2007).

Protocols

Cumulative concentration-effect curves for BRL37344 and SR58611 were carried out in the absence and presence of L-748,337 (1 μM). Tissues were pre-incubated with L-748,337 15 min after the addition of nadolol (200 nM), followed by the administration of IBMX (10 μM) 30 min later, and finally the curve for an agonist begun 15 min later. At least two trabeculae from each patient were used and curves for (–)-CGP12177 in the absence and presence of L-748,337 were time-matched. In other experiments concentration-effect curves for BRL37344 were carried out in the absence or presence of nadolol (200 nM), 1-[2,3-dihydro-7-methyl-1H-inden-4-yl] oxy-3-[(1-methylethyl)amino-2-butanol)] (ICI118,551) (50 nM) or 2-hydroxy-S-[2-[[2-hydroxy-3-[4-[methyl-4-(trifluoromethyl)-1H-imidazol-2-yl] phenoxy] propyl] amino] ethoxy]-benzamide (CGP20712A) (300 nM) or the combination of ICI118,551 and CGP20712A, in the presence of IBMX (10 μM). The effects of BRL37344 are expressed as a function of the response to (–)-isoprenaline (200 μM), administered after the highest concentration of BRL37344. Some experiments were carried out at 24°C and results with the agonists expressed as % changes of the response to IBMX in order to more easily detect small changes.

Kinetic experiments were used to investigate the influence of L-748,337 on the contractile responses to (–)-CGP12177. When four or more atrial trabeculae were available, a time-matched procedure was used comprising four experimental groups (as shown in the representative experiment of Figure 3A). Trabeculae were incubated with IBMX (10 μM), which remained in contact with the trabeculae for the remainder of the experiment. By the 30th min, three groups of trabeculae were incubated with (–)-CGP12177 (200 nM). Twenty minutes later, upon establishment of the cardiostimulant effect of (–)-CGP12177 one group received L-748,337 (1 μM), another received (–)-bupranolol (1 μM), while the third group was used as a time-matched control to establish the time course of the cardiostimulant effect of (–)-CGP12177.

All experiments were concluded by the administration of a receptor-saturating concentration of (–)-isoprenaline (200 μM) and, after an equilibrium response to (–)-isoprenaline was established, by raising the Ca^{2+} concentration to 9.25 mM (total bath concentration).

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

Human atrial myocytes were enzymatically dissociated as described previously (Christ *et al.*, 2001). Myocytes were stored at room temperature until use in a solution containing (mM): K^+ 100, Cl^- 20, $H_2PO_4^{2-}$ 10, glutamic acid 70, taurine 10, β-hydroxybutyrate 10, EGTA 10, HEPES 10, albumin 1%, glucose 10, pH 7.4. The single electrode patch clamp technique was used to measure $I_{Ca\text{-}L}$ at 37°C. Holding potential was –80 mV. The K^+ currents were blocked by replacing K^+ with Cs^+. The external perfusing solution contained (mM): tetraethylammonium 120, Cs^+ 10, Ca^{2+} 2, Mg^{2+} 1, Cl^- 136, HEPES 10 and glucose 10 with pH adjusted with CsOH. The pipette solution contained (mM): Cs^+ 110, Ca^{2+} 3, Mg^{2+} 4, Cl^- 26, methanesulphonate$^-$ 90, HEPES 10, ATP 4, Tris-GTP 0.4 and EGTA 10 with a calculated free Ca^{2+} concentration of 60 nM (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 from a holding potential of –80 mV. Myocytes were exposed only once to the different agonists in the absence or presence of IBMX (10 μM), nadolol (200 nM), L-748,337 (1 μM), ICI118,551 (50 nM) or (–)-bupranolol (10 μM).

Statistics and assessment of antagonism

Results from the kinetic experiments are expressed in mN force. The $-\log EC_{50}M$ values for BRL37344 and (–)-CGP12177 in the absence and presence of L-748,337 were calculated from the corresponding curves. Data comparisons were made with ANOVA and non-paired or paired *t*-tests as appropriate using GraphPad Prism® (GraphPad Software, Inc., La Jolla, CA, USA). Expected concentration ratios (CR) of BRL37344 in the presence and absence of an antagonist concentration ([B]) were calculated from $CR = 1 + [B]/K_B$, where K_B is the equilibrium dissociation constant. Results are presented as mean ± s.e.mean, where *n* values refer to the number of trabeculae or myocytes.

Drugs

(–)-CGP12177 [(7)-4-(3-tertiarybutylamino-2-hydroxypropoxy) benzimidazol-2-one] was a gift from Dr Jonathan Arch (GlaxoSmithKline, Harlow, UK), (–)-bupranolol was a gift from Dr Klaus Sandrock (Sanol-Schwarz, Monheim, Germany), SR58611 (ethyl{(7S)-7-[(2R)-2-(3-chlorophenyl)-2-hydroxyethylamino]-5,6,7,8-tetrahydronaphtyl2-yloxy}acetate hydrochloride) was a gift from Dr Luciano Manara (Sanofi, Milan, Italy); L-748,337 (N-(3-[3-[2-(4-benzenesulphonylamino phenyl) ethylamino]-2-hydroxypropoxy]benzyl acetamide) was from Tocris (Bristol, UK), BRL37344 [(RR + SS)[4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino] propyl]phenoxy]acetic acid] was from Tocris or Sigma (Castle Hill, Australia). IBMX (3-isobutyl-1-methylxanthine) and (–)-isoprenaline hydrochloride were from Sigma (Castle Hill, Australia or Poole Dorset, UK).

Results

Antagonism of the inotropic effects of BRL37344 by β-adrenoceptor subtype-selective antagonists in atrial trabeculae

To reduce cAMP hydrolysis and enhance inotropic responses, experiments were carried out in the presence of the non-selective PDE inhibitor IBMX (10 μM). IBMX increased force from 5.86 ± 0.50 mN to 8.70 ± 0.70 mN (n = 116 trabeculae from 29 patients) (Figures 1, 2A and 4). There was no difference between the groups represented in Figures 1, 2A and 4 (ANOVA $P = 0.18$).

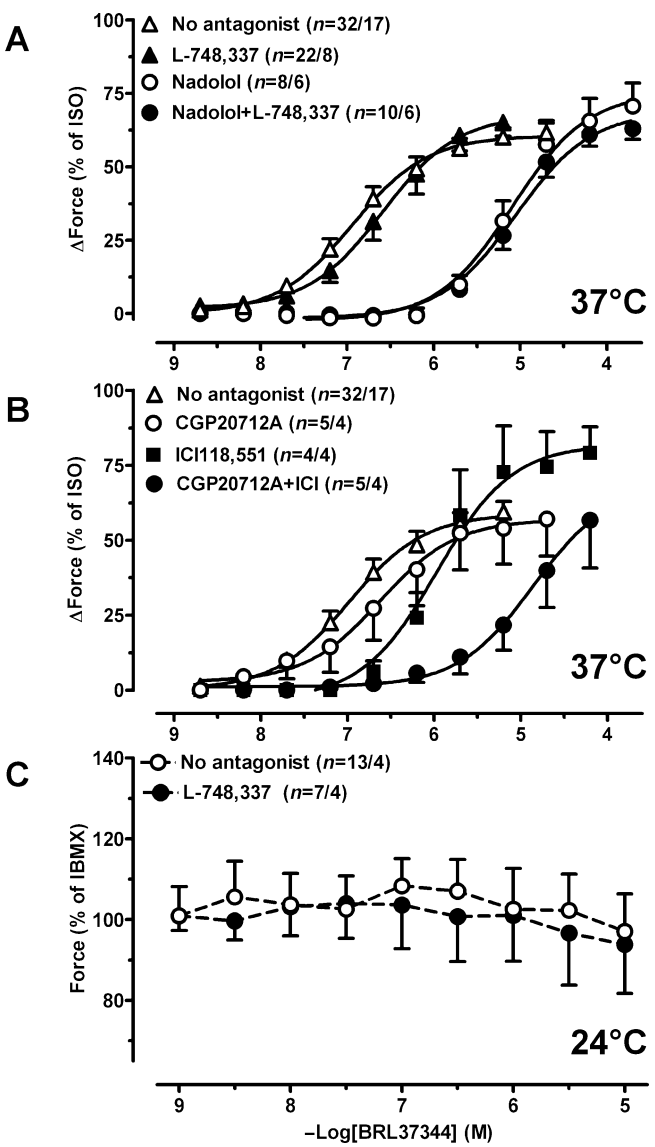

Figure 1

Mediation of the positive inotropic effects of BRL37344 through β_1- and β_2-adrenoceptors but not β_3-adrenoceptors in human atrial trabeculae. (A) Cumulative concentration-effect curves were carried out in the presence of IBMX (10 μM) for BRL37344 in the absence and presence of nadolol (200 nM, high affinity β_1-adrenoceptor/β_2-adrenoceptor antagonist), L-748,337 (1 μM, β_3-adrenoceptor antagonist) or nadolol + L-748,337. (–)-Isoprenaline (200 μM), administered after a steady state response to 200 μM BRL37344, increased force (% over IBMX) by 284 ± 42.7%. (B) Curves for BRL37344 were also determined in the absence and presence of ICI118,551 (50 nM, β_2-adrenoceptor-selective antagonist, ICI) or CGP20712A (300 nM, β_{1H}-adrenoceptor-selective antagonist) or co-administration of ICI118,551 + CGP20712A. (–)-Isoprenaline (200 μM), administered after a steady state response to 60 μM BRL37344, increased force (% over IBMX) by 312 ± 63.5%. Antagonists were incubated for at least 45 min before a curve for an agonist was started. Nadolol was incubated for 15 min, followed by the additional administration of L-748,337 for 30 min, followed by the administration of IBMX (10 μM) for 15 min before a curve was begun. (C) Lack of inotropic effects of BRL37344 at 24°C. At 24°C (–)-isoprenaline and Ca^{2+} (8 mM) increased force (mN) from a basal of 5.2 ± 0.9 to 10.8 ± 1.5 and 13.3 ± 2.1, respectively (n = 28/4). At 37°C (–)-isoprenaline and Ca^{2+} increased force (mN) from a basal level of 7.6 ± 0.6 to 12.7 ± 0.7 and 13.5 ± 0.7, respectively (n = 86/17). ISO, (–)-isoprenaline.

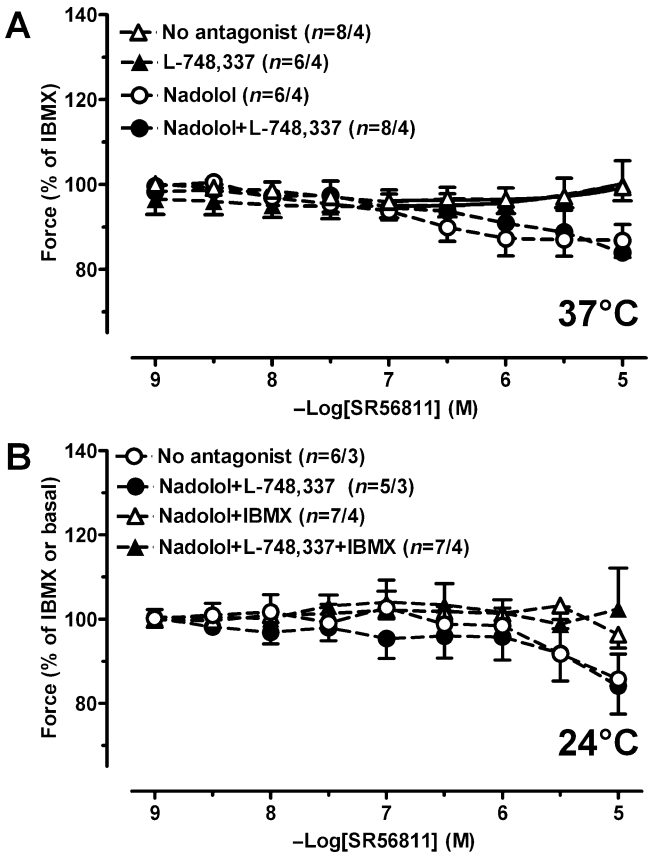

Figure 2

Lack of positive inotropic effects of SR58611 at 37°C and 24°C in human atrial trabeculae. (A) Cumulative concentration-effect curves at 37°C were carried out for SR58611 in the absence and presence of nadolol (200 nM, high affinity β_1- and β_2-adrenoceptor antagonist) or L-748,337 (1 μM, antagonist) or nadolol + L-748,337, each curve in the presence of IBMX (10 μM). (B) Curves for SR58611 at 24°C carried out in the absence or presence of nadolol, nadolol + L-748,337, or nadolol + IBMX, or IBMX + nadolol + L-748,337. (–)-Isoprenaline (200 μM), administered after a steady state response to 10 μM of SR58611, increased force (% over IBMX) at 37°C by 162 ± 13.9 (% over IBMX, $n = 30$). At 24°C the increase in force induced by (–)-isoprenaline (% over basal) in the absence and presence of IBMX was 767 ± 152%, $n = 14/4$ and 198 ± 24%, $n = 30/4$, respectively. Numbers in parentheses represent trabeculae/patient. At 24°C (–)-isoprenaline and Ca^{2+} (8 mM) increased force (mN) from a basal value of 2.6 ± 0.5 to 6.3 ± 0.8 and 6.8 ± 0.7, respectively ($n = 28/4$). At 37°C (–)-isoprenaline and Ca^{2+} increase force (mN) from a basal value of 5.5 ± 1.0 to 10.6 ± 1.5 and 11.3 ± 1.5, respectively ($n = 28/4$).

BRL37344 caused concentration-dependent increases in contractile force (Figure 1A,B). The intrinsic activity of BRL37344 with respect to (–)-isoprenaline (200 μM) in the groups of Figure 1 did not differ significantly (ANOVA, $P = 0.27$) with an average of 0.66 ± 0.02 ($n = 34$ trabeculae from 17 patients). The β_{1H}- and β_2-adrenoceptor antagonist nadolol (200 nM) and β_2-adrenoceptor antagonist ICI118,551 (50 nM) produced rightward shifts of the curve for BRL37344 (Figure 1A,B) by 1.9 log units and 0.9 log units (Table 2), respectively. The β_3-adrenoceptor antagonist L-748,337 (1 μM) caused a 0.3 log rightward shift of the curve for BRL37344 (Figure 1A, Table 2). L-748,337 in the presence of nadolol did not cause further antagonism of the effects of BRL37344. The β_1-adrenoceptor antagonist CGP20712A tended to cause a rightward shift of the curve for BRL37344 (Figure 1B) but it did not reach statistical significance (Table 2). Co-administration of ICI118,551 and CGP20712A caused a greater shift of the curve to BRL37344 than that induced by ICI118551 alone (Figure 1B, Table 2).

The β_3-adrenoceptor agonist SR58611 does not increase contractile force

Inotropic results with SR58611 are shown in Figure 2A. The β_3-adrenoceptor agonist SR58611 (1 nM–10 μM) did not produce positive inotropic effects in the absence or presence of nadolol or L-748,337 at 37°C (Figure 2A). High SR58611 concentrations tended to reduce force in the presence of nadolol but the effects of the highest concentration used (10^{-5} M) were not significant ($P = 0.14$ IBMX vs. nadolol; $P = 0.12$ IBMX + L748337 vs. IBMX + nadolol + L748337).

Stable (–)-CGP12177-evoked contractions are decreased by (–)-bupranolol but not by L-748,337

Human right atrial trabeculae were incubated with nadolol to block β_{1H}- and β_2-adrenoceptors, and IBMX was used to enhance the contractile effects of (–)-CGP12177 (Skeberdis *et al.*, 2008). Figure 3A illustrates the results from a representative experiment and Figure 3B shows a summary of the results. IBMX (10 μM) slightly increased trabecular force after 30 min (basal 4.13 ± 0.49 mN, IBMX 5.92 ± 0.79 mN, $n = 24$ trabeculae, four patients, $P < 0.0001$). The effect of IBMX remained stable for 1 h but faded by the 90th min of exposure. (–)-CGP12177 (200 nM) caused a stable increase in force which was significantly reduced by the addition of (–)-bupranolol (1 μM) but not affected by the addition of L-748,337 (1 μM) ($P = 0.001$, one-way ANOVA, Figure 3B). Results of trabeculae obtained from four patients demonstrate that L-748,337 did not affect the response to (–)-CGP12177 ($P = 0.12$), inconsistent with the effect of (–)-CGP12177 being mediated through β_3-adrenoceptors. In contrast (–)-bupranolol reduced the response to (–)-CGP12177 by 91 ± 16%, $n = 4$, $P = 0.002$ (Figure 3B), consistent with it being mediated through β_{1L}-adrenoceptors, as observed before on human atrium (Kaumann,

Table 2

Effects of antagonists on the inotropic potency of BRL37344 in the presence of IBMX (10 μM)

	n	$-\text{LogEC}_{50}$ (M)	LogCR Observed	Expected
Control (no antagonist)	32/17	6.82 ± 0.11	–	–
CGP20712A (300 nM)	5/4	6.44 ± 0.10	0.38	2.28[a] (β_{1H})
ICI118,551 (50 nM)	4/4	5.96 ± 0.18**	0.86	1.72[a] (β_2)
CGP20712A + ICI118,551	5/4	4.61 ± 0.28**	2.21	– (β_{1H} + β_2)
L-748,337 (μM)	22/8	6.49 ± 0.11**	0.33	0.55[b] (β_{1H})
				0.77[b] (β_2)
				1.67[c] ($\beta_{3)}$
				2.55[b] (β_3)
Nadolol (200 nM)	8/6	5.13 ± 0.10***	1.69	1.02[d] (β_{1H})
				0.64[e] (β_{1H})
				1.91[e] (β_2)
Nadolol + L-748,337	10/6	5.10 ± 0.09***	1.72	–

n = Trabeculae/patients.
CR, concentration ratios.
**$P < 0.01$, ***$P < 0.001$ compared with control.
[a]Kaumann and Lemoine, 1987.
[b]Candelore *et al.*, 1999.
[c]Wuest *et al.*, 2009.
[d]Joseph *et al.*, 2004a.
[e]Baker, 2005.

1996) and with recombinant β_1-adrenoceptors (Joseph *et al.*, 2004a).

L-748,337 does not modify the contractile potency of (–)-CGP12177

Conceivably, the lack of antagonism by L-748,337 of the inotropic response to (–)-CGP12177 could be due to firm binding of (–)-CGP12177 to β_3-adrenoceptors. We therefore investigated whether the β_3-adrenoceptor occupancy caused by a 30 min pre-incubation of the atrial trabeculae with L-748,337 could prevent the inotropic effects of (–)-CGP12177. However, cumulative concentration-effect curves for (–)-CGP12177 in the presence of nadolol (200 nM) and IBMX (10 μM) were not shifted to the right by pre-incubation with L-748,337 (1 μM) (Figure 4), indicating the response is not mediated through β_3-adrenoceptors. The $-\text{logEC}_{50}$M values of (–)-CGP12177 in the absence and presence of L-748,337 were 7.21 ± 0.09 and 7.41 ± 0.13, respectively (data from 25 trabeculae from eight patients, $P = 0.2$).

$I_{Ca\text{-}L}$ responses to BRL37344, SR58611 and (–)-CGP12177 at 24°C

$I_{Ca\text{-}L}$ responses to the agonists in the presence of nadolol (200 nM) at 24°C are shown in Figure 5. Similar to the findings of Skeberdis *et al.* (2008), BRL37344 and SR58611 increased $I_{Ca\text{-}L}$ and the responses were prevented by L-748,337, consistent with mediation through β_3-adrenoceptors. The responses to BRL37344 (1 μM) were (% over basal): 29.4 ± 9.1% (range –10.7 to 94.7%), n = 14/9 myocytes/patients (P = 0.01, paired t-test). The responses to SR58611 (1 μM) were: 21.1 ± 10.0% (range: from –4.8 to 217.6%), n = 22/8 (P = 0.034).

(–)-CGP12177 (1 μM) also increased $I_{Ca\text{-}L}$. This effect was not observed when β_{1L}-adrenoceptors were blocked with (–)-bupranolol (Figure 5). However, L-748,337 did not prevent the response to (–)-CGP12177. Our results therefore indicate mediation of the response to (–)-CGP12177 through β_{1L}-adrenoceptors but not β_3-adrenoceptors. The response to (–)-CGP12177 was 34.5 ± 10.8% (–3.9 to 151.9%) n = 18/7 (P = 0.002). For comparison, the response to (–)-isoprenaline (1 μM) was 289.6 ± 57.9% (90.0–705.0%) n = 10/5 (P = 0.00002).

BRL37344 and SR58611 do not increase $I_{Ca\text{-}L}$ at 37°C

BRL37344 and SR58611 did not affect $I_{Ca\text{-}L}$ responses at 37°C in the absence or presence of IBMX (Figure 6). (–)-Isoprenaline (1 μM) in the absence of

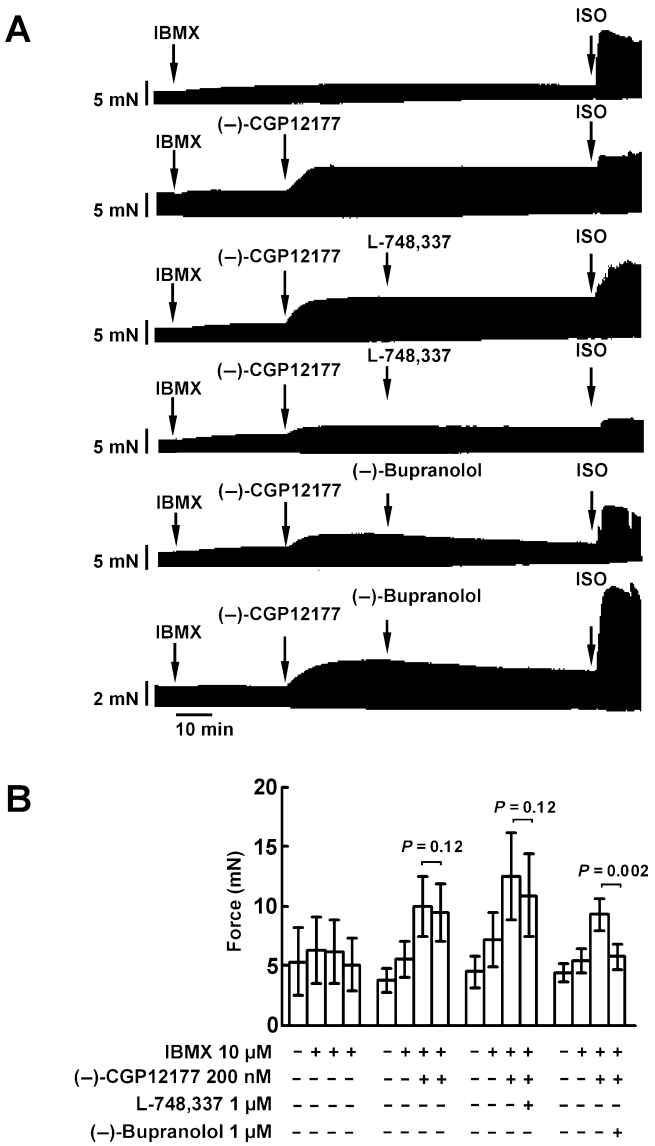

Figure 3

(A) Mediation of the positive inotropic effects of (–)-CGP12177 through β_{1L}-adrenoceptor but not β_3-adrenoceptor. (–)-Bupranolol, but not L-748,337 reduced the increase in contractile force elicited by (–)-CGP12177 (200 nM) in the presence of IBMX (10 µM) and nadolol (200 nM). Representative and time-matched experiments in six trabeculae from the right atrial appendage of a 51-year-old male patient undergoing coronary artery bypass grafting depicting the time course of the effects of IBMX (top trabeculum), of (–)-CGP12177 (second tracing), lack of antagonism by L-748,337 (1 µM) (third and fourth tracings) and blunting effect of (–)-bupranolol (1 µM) (bottom two tracings). Experiments were concluded by the addition of 200 µM (–)-isoprenaline (Iso) and 7 mM Ca^{2+} (final bath concentration 9.25 mM). Arrows indicate the addition of drugs. Bars indicate forces and time scale. (B) A summary of data from 24 trabeculae of four patients. A comparison of the contractile force obtained in the presence of 200 nM (–)-CGP12177 and after incubation of L-748,337, (–)-bupranolol or control was carried out by one-way ANOVA which indicated differences in cardiodepression ($P = 0.001$). *Post hoc* tests revealed that (–)-bupranolol caused a reduction in contractile force, while there was no difference between control (marginal fade of (–)-CGP12177 contractile force over 50 min corresponding to the time of L-748,337 exposure) and L-748,337 (statistics indicated on the figure).

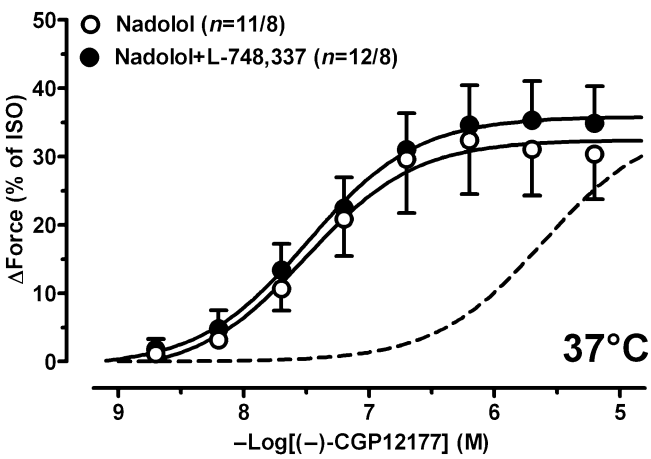

Figure 4

L-748,337 does not change the inotropic potency of (–)-CGP12177 in the presence of nadolol (200 nM) and IBMX (10 µM). Concentration-effect curves for (–)-CGP12177 in the absence and presence of L-748,337 (1 µM). Dotted concentration-effect curve shows a theoretical curve for (–)-CGP12177 in the presence of L-748,337, expected to occur if L-748,337 antagonized the effects of (–)-CGP12177 at β_3-adrenoceptors with $pK_B = 7.65$. $-LogEC_{50}$ values for (–)-CGP12177 in the absence (control) and presence for L-748,337 were 7.21 ± 0.09 (13 trabeculae) and 7.41 ± 0.13 (12 trabeculae), respectively, from eight patients. (–)-Isoprenaline (200 µM) increased force by 511 ± 103% over IBMX, n = 25/8. Numbers in parentheses represent trabeculae/patients. ISO, (–)-isoprenaline.

IBMX increased $I_{Ca\text{-}L}$ to 344 ± 34.3 (160–530%) over basal (n = 13/9 P = 0.00002).

Antagonism of (–)-CGP12177-evoked increase in $I_{Ca\text{-}L}$ by (–)-bupranolol but not by L-748,337 at 37°C

The effects of (–)-CGP12177 on $I_{Ca\text{-}L}$ responses at 37°C are shown in Figure 7. (–)-CGP12177 (1 µM) did not affect $I_{Ca\text{-}L}$ while (–)-isoprenaline caused a fourfold increase. However, in the presence of IBMX, which caused a small increase of $I_{Ca\text{-}L}$, (–)-CGP12177 elicited a further increase in $I_{Ca\text{-}L}$. The response to IBMX (% over basal) was 21.8 ± 9.4% (–12.5 to 131.3%) n = 20/9 (P = 0.037) and that to (–)-CGP12177 (as % of IBMX) was 31.4 ± 10.0% (–14.2 to 124.1%) (P = 0.015). The response to (–)-CGP12177 was resistant to blockade by ICI118,551 and L-748,337 but prevented by (–)-bupranolol. During blockade of β_{1H}- and β_2-adrenoceptors with nadolol (200 nM) (–)-CGP12177 caused an increase of $I_{Ca\text{-}L}$ in the presence of IBMX that was prevented by (–)-bupranolol but resistant to blockade by L-748,337. The response

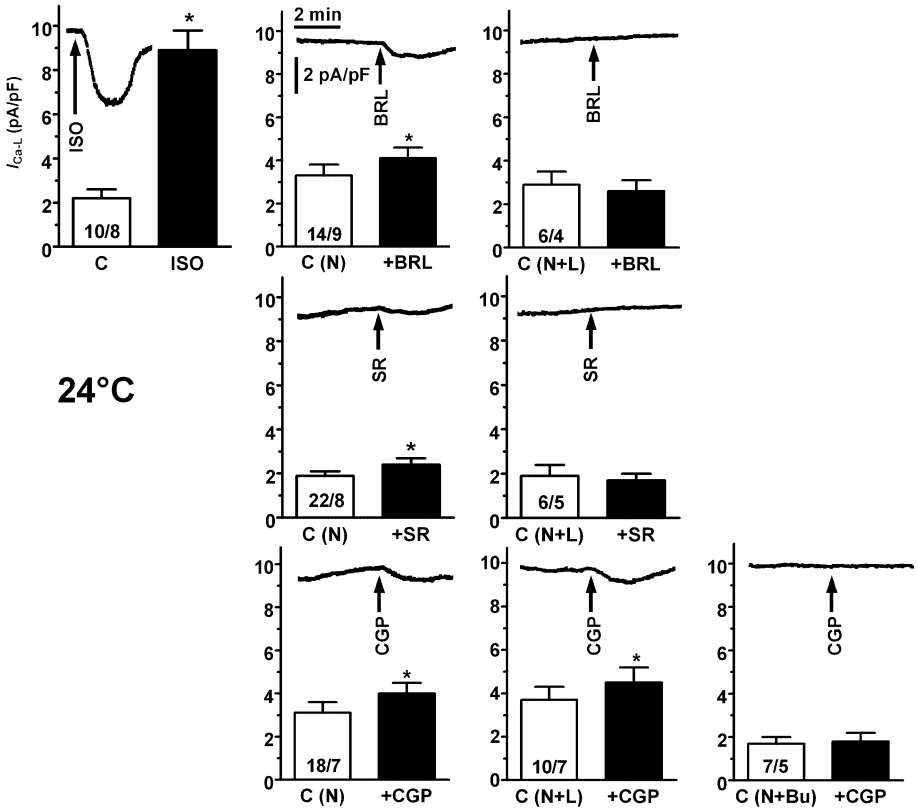

Figure 5

L-748,337 (1 μM, L) prevents small $I_{Ca\text{-}L}$ responses to SR58611 (1 μM, SR) and BRL37344 (1 μM, BRL) but not to (–)-CGP12177 (1 μM, CGP) at 24°C in the presence of nadolol (200 nM, N). The responses to (–)-CGP12177 were prevented by (–)-bupranolol (10 μM, Bu). The $I_{Ca\text{-}L}$ response to (–)-isoprenaline (1 μM, ISO) in the absence of nadolol is shown for comparison. Numbers in columns represent myocytes/patients. *$P < 0.05$ compared with control (C).

to IBMX in the presence of nadolol (200 nM) was 9.3 ± 4.8% over basal (–16.4 to 39.1%) $n = 13/5$ ($P = 0.04$). The response to (–)-CGP12177 in the presence of both nadolol and IBMX (as % over IBMX) was 16.8 ± 8.2% (0 to 103.1%) ($P = 0.027$). The responses to (–)-CGP12177 in the presence of IBMX were not different in the absence and presence of nadolol ($P = 0.3$). Responses to (–)-CGP12177 in the presence of IBMX did not significantly differ from the corresponding responses in the presence of ICI118,551 or L-748,337. Taken together, these results are consistent with mediation of the $I_{Ca\text{-}L}$ responses to (–)-CGP12177 through β_{1L}-adrenoceptors, but not β_{1H}-, β_2- or β_3-adrenoceptors.

BRL37344 and SR58611 do not increase atrial force at 24°C

To investigate whether the small increases in $I_{Ca\text{-}L}$, evoked by BRL37344 and SR58611, that can be inhibited by L-748,337, are inotropically relevant, we studied their effects at 24°C in the presence of nadolol (200 nM), in the absence and presence of IBMX. BRL37344 (Figure 1C) and SR58611 (Figure 2B) failed to increase contractility under these conditions. In the presence of nadolol, high SR58611 concentrations tended to reduce force in the absence but not presence of IBMX, but the effects of the highest concentration used (10^{-5} M) were not significant ($P = 0.13$).

(–)-CGP12177-evoked increases in atrial force at 24°C are antagonized by (–)-bupranolol but not by L-748,337.

We also investigated whether the increase in $I_{Ca\text{-}L}$ observed with (–)-CGP12177 in the presence of IBMX at 24°C is also translated into a positive inotropic effect (Figure 8). Nadolol (200 nM), used to block β_{1H}- and β_2-adrenoceptors, decreased contractile force, probably as an inverse agonist. Since the positive inotropic effects to (–)-CGP12177 tend to fade (Kaumann, 1996; Kaumann *et al.*, 2007) due to phosphodiesterase activity, and to amplify possible force responses (Kaumann and Molenaar, 1997; Kaumann *et al.*, 2007), IBMX (10 μM) was administered. IBMX increased force until a plateau ensued, on which cumulatively increasing (–)-CGP12177

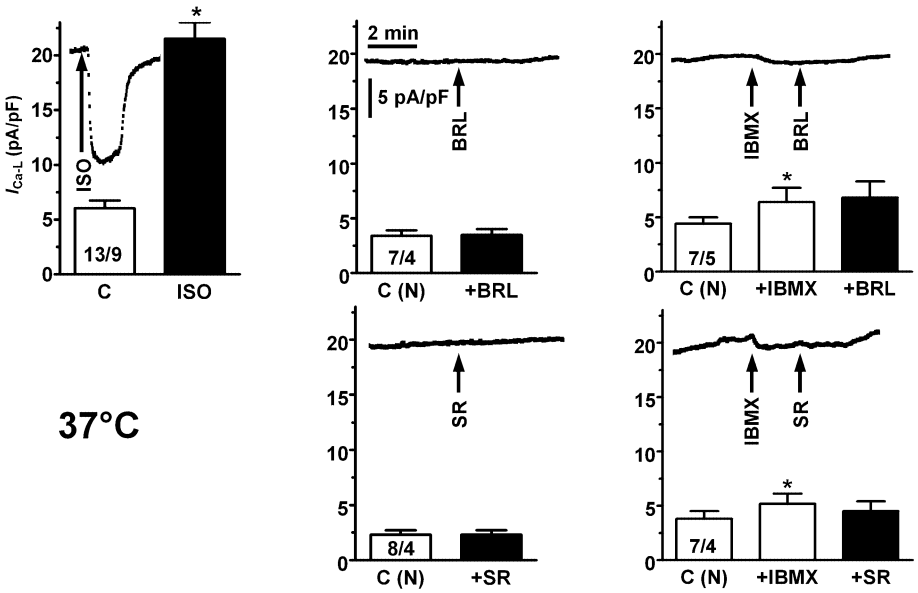

Figure 6

SR58611 (1 μM, SR) and BRL37344 (1 μM, BRL), in the presence of nadolol (200 nM, N) fail to increase I_{Ca-L} in the absence and presence of IBMX (10 μM) at 37°C. The I_{Ca-L} response to (–)-isoprenaline (1 μM, ISO) in the absence of IBMX is shown for comparison. Numbers in columns represent myocytes/patients.*$P < 0.05$ compared with control (C).

concentrations were administered. (–)-CGP12177 caused concentration-dependent decreases of force at low concentrations followed by marginal increases in force at high concentrations. Pre-incubation with L-748,337 (1 μM) for 30 min before the IBMX administration did not affect the cardiodepressant or cardiostimulant effects of (–)-CGP12177 (Figure 8A).

The full inverse agonist (–)-propranolol (Chidiac *et al.*, 1994) abolishes the inverse agonist activity of (–)-CGP12177 at I_{Ca-L} in the presence of IBMX in murine ventricular myocytes (Freestone *et al.*, 1999). Therefore, to prevent the inverse agonist activity and uncover agonist activity of (–)-CGP12177, we used (–)-propranolol. As expected from a robust inverse agonist, (–)-propranolol decreased contractile force significantly more than nadolol (Figure 8). (–)-CGP12177 produced positive inotropic effects in the presence of both IBMX and (–)-propranolol. L-748,337 did not antagonize the effects of (–)-CGP12177 but (–)-bupranolol (1 μM) caused a log unit rightward shift of the concentration-effect curve for (–)-CGP12177 (Figure 8B), consistent with mediation through β_{1L}-adrenoceptors, but not β_3-adrenoceptors.

Discussion

In the present work we provide evidence against the hypothesis that β_3-adrenoceptor activation enhances human atrial contractility. The inotropic effects of BRL37344 were not mediated through β_3-adrenoceptors but through β_1- and β_2-adrenoceptors. BRL37344 and SR58611 caused small increases of I_{Ca-L} through β_3-adrenoceptors at 24°C but not 37°C. The β_3-adrenoceptor agonist SR58611 did not modify atrial force. The increases of I_{Ca-L} at 24°C caused by BRL37344 and SR58611 were uncoupled from contractility. (–)-CGP12177 increased force and I_{Ca-L} at both 24°C and 37°C through β_{1L}-adrenoceptors but not β_3-adrenoceptors.

Unlikelihood of positive inotropy through human atrial β_3-adrenoceptors

We confirmed the findings of Skeberdis *et al.* (2008) that BRL37344 increases atrial force in the presence of IBMX and nadolol. The β_3-adrenoceptor-selective antagonist L-748,337 (1 μM) caused a small twofold rightward shift of the concentration-effect curve for BRL37344 (Figure 1A, Table 2). For the following reasons, this shift is consistent with mediation of the effects of BRL37344 through β_1-adrenoceptors and/or β_2-adrenoceptors but not β_3-adrenoceptors. The affinity (K_D values) of L-748,337 for β_1-, β_2- and β_3-adrenoceptors have been found to be (nM) 390, 204 and 2.8, respectively (Candelore *et al.*, 1999). The expected log concentration ratios (logCR) caused by the antagonist concentrations of receptor subtype-selective blockers used are compared with the theoretically expected logCR through the specific receptors (Table 1). The small antagonism of

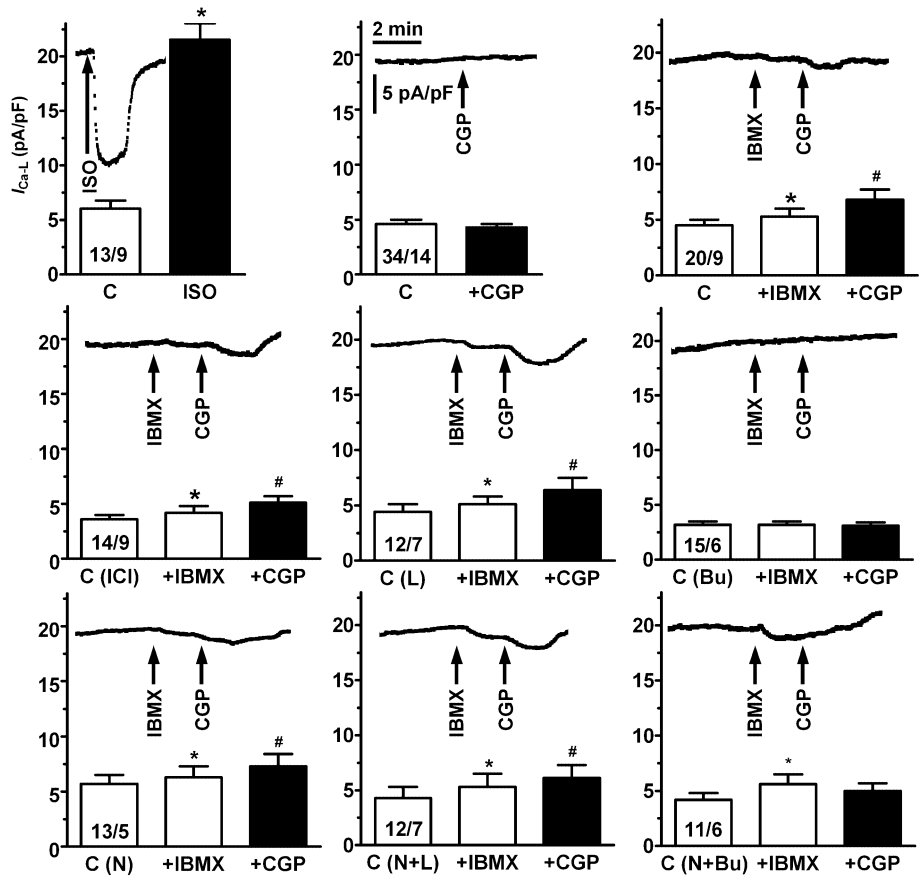

Figure 7

(–)-CGP12177 (1 μM, CGP) causes small increases of L-type Ca^{2+} current ($I_{Ca\text{-}L}$) at 37°C, compared with (–)-isoprenaline (1 μM, ISO), in the presence but not absence of IBMX (10 μM) that are prevented by (–)-bupranolol (10 μM, BU) but not by L-748,337 (1 μM, L), ICI118,551 (50 nM, ICI) or nadolol (200 nM, N). *P < 0.05 compared with the absence of IBMX. #P < 0.05 compared with IBMX alone. Numbers in columns represent myocytes/patients. One arrow indicates agonist addition, two arrows indicate addition of IBMX followed by addition of (–)-CGP12177. C = control.

the effects of BRL37344 by L-748,337 is expected to result from an interaction with β_1-adrenoceptors or β_2-adrenoceptors, but not β_3-adrenoceptors.

The positive inotropic effects of BRL37344 in human atrial myocardium are antagonized by propranolol (Arch and Kaumann, 1993; Pott *et al.*, 2003) and it has been suggested that BRL37344 acts through both β_1- and β_2-adrenoceptors, plausibly mainly through the latter receptor (Arch and Kaumann, 1993). Nadolol caused a rightward shift of the concentration-effect curve for BRL37344 (Figure 1A, Table 2), indicating that its effects are mediated through β_1- and β_2-adrenoceptors. The inotropic effects of BRL37344 shown by Skeberdis *et al.* (2008) in the presence of nadolol are consistent with the agonist surmounting the blockade of β_1- and β_2-adrenoceptors by nadolol. The affinity estimate (dissociation equilibrium constant K_i) of nadolol of 22 nM from recombinant β_1-adrenoceptors, expressed at physiological density (Joseph *et al.*, 2004b) and 59 nM for the receptors expressed at higher density (Baker, 2005), allows the prediction of a 0.6–1.0 log unit rightward shift with the nadolol concentration of 200 nM (Table 2) used by Skeberdis *et al.* (2008). As expected, our experiments depicted in Figure 1A demonstrate that the effects of BRL37344 are surmountably antagonized by 200 nM nadolol. However, the nadolol-induced shift of the concentration-effect curve was 1.0–1.3 log units greater than the expected 0.6–1.0 log unit shift (Table 2). This could be related to preferential activation of β_2-adrenoceptors by BRL37344 in human atrium (Arch and Kaumann, 1993) and is supported by the 20-fold higher affinity of BRL37344 for recombinant β_2-adrenoceptors compared with β_1-adrenoceptors (Sennitt *et al.*, 1998). Affinity estimates for nadolol for recombinant receptors, expressed at high density, reveal a 23-fold higher affinity of nadolol for β_2-adrenoceptors than for β_1-adrenoceptors (Baker, 2005). With a K_i = 2.5 nM for nadolol at β_2-adrenoceptors (Baker, 2005) a 1.9 log unit shift of the concentration-effect curve for

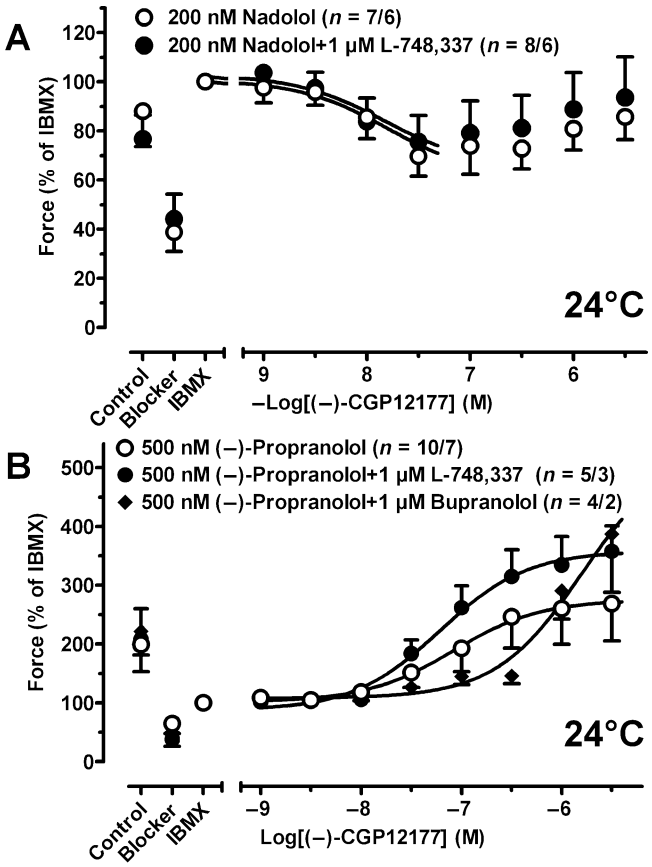

Figure 8

Inotropic effects of (–)-CGP12177 at 24°C. (–)-CGP12177 is a moderate inverse agonist in the presence of both nadolol (200 nM) and IBMX (10 µM) (A) but causes positive inotropic effects in the presence of both (–)-propranolol (200 nM) and IBMX (10 µM) through β_{1L}-adrenoceptors but not β_3-adrenoceptors (B). The effects of (–)-CGP12177 were resistant to blockade by L-748,337 (1 µM) but antagonized by (–)-bupranolol (1 µM). Antagonists were incubated for 45 min, followed by an additional administration of IBMX for 15 min before a concentration-effect curve for (–)-CGP12177 was carried out. Numbers in parentheses represent trabeculae/patients. (–)-Isoprenaline (200 µM), administered after a steady state response to the highest agonist concentration had been observed, increased force (% over IBMX) by 340 ± 79%, n = 15/6 in (A) and 947 ± 202%, n = 19/9 in (B). (–)-Isoprenaline and Ca^{2+} (8 mM) increased force (mN) from a basal value of 2.4 ± 0.5 to 6.8 ± 0.8 and 6.9 ± 0.9, respectively (n = 34/8).

BRL37344 was expected and was experimentally observed, indicating interaction with β_2-adrenoceptors (Table 2). A preferential activation of β_2-adrenoceptors by BRL37344 is also supported by the greater antagonism of its inotropic effects in human atrial trabeculae by the β_2-adrenoceptor-selective ICI118,551 compared with that induced by the β_1-adrenoceptor-selective CGP20712A (Table 2). The greater antagonism induced by co-administration of ICI118,551 + CGP20712A than by ICI118,551 alone (Figure 1B), suggests that BRL37344 activates β_2-adrenoceptors at low concentrations and β_1-adrenoceptors at high concentrations.

SR58611 causes colon relaxation (Bianchetti and Manara, 1990; Kaumann and Molenaar, 1996) and lipolytic effects (Langin *et al.*, 1991) through native β_3-adrenoceptors, as well as marked activation of adenylyl cyclase through recombinant β_3-adrenoceptors (Blin *et al.*, 1994). Skeberdis *et al.* (2008) reported a marginal increase in atrial force with SR58611 in the absence of IBMX but did not investigate this effect in the presence of IBMX. We did not observe any inotropic effects of SR58611 (1 nM–10 µM) in human atrium despite the presence of IBMX, which should have boosted the inotropic responses mediated through a cAMP-dependent pathway following β-adrenoceptor activation. This finding is in agreement with those of Bianchetti and Manara (1990) and Kaumann and Molenaar (1996) who were unable to show that SR58611 caused cardiostimulation in guinea pig and rat atria, respectively.

β_{1L}-adrenoceptors but not β_3-adrenoceptors mediate the positive inotropic effects and $I_{Ca\text{-}L}$ responses to (–)-CGP12177 in human atrium at 37°C

The β_3-adrenoceptor-selective blocker L-748,337 failed to antagonize the positive inotropic and $I_{Ca\text{-}L}$ responses to (–)-CGP12177, which is inconsistent with their mediation through β_3-adrenoceptors (Skeberdis *et al.*, 2008). This together with our finding that (–)-bupranolol blocks both the increases in $I_{Ca\text{-}L}$ and contractile force by (–)-CGP12177 in human atrial myocardium suggests that (–)-CGP12177 mediates these effects through β_{1L}-adrenoceptors.

Affinity estimates for the L-748,337–β_3-adrenoceptor complex ($-\log K_L = pK_L$) are available from recombinant human β_3-adrenoceptors, transfected into CHO cells [pK_L = 8.40 (Candelore *et al.*, 1999)] and from human β_3-adrenoceptors of detrusor muscle [pK_L = 7.65 (Wuest *et al.*, 2009)]. Knowing the K_L of L-748,337, the CR of equieffective (–)-CGP12177 concentrations in the presence and absence of L-748,337 can be calculated from $CR = 1 + [\text{L-748,337}]/K_L$, where K_L is the equilibrium dissociation constant of L-748,337 for the β_3-adrenoceptor. Using 22 nM (from the pK_L = 7.65) as the equilibrium dissociation constant (Wuest *et al.*, 2009) and assuming that L-748,337 and (–)-CGP12177 compete for the same β_3-adrenoceptor population, 1 µM L-748,337 would cause a CR of 46.5, equivalent to a 1.67 log unit rightward shift of the concentration-effect curve for (–)-CGP12177 (broken line of Figure 4). However, 1 µM L-748,337 did not change the concentration-effect curve and

potency (–logEC_{50}M) of (–)-CGP12177, ruling out mediation through β_3-adrenoceptors. Moreover, L-748,337 did not significantly reduce the effects of (–)-CGP12177 in our kinetic experiments (Figure 3). Instead (–)-bupranolol antagonized the effects of (–)-CGP12177 (Figure 3), as previously observed with human atrial (Kaumann, 1996; Kaumann and Molenaar, 2008) and recombinant β_1-adrenoceptors (Joseph *et al.*, 2004a), indicating mediation through β_{1L}-adrenoceptors (Kaumann and Molenaar, 2008). Furthermore (–)-bupranolol also prevented the small increase in $I_{Ca\text{-}L}$ caused by (–)-CGP12177, as expected from mediation through β_{1L}-adrenoceptors. The consistency of moderate inotropic and $I_{Ca\text{-}L}$ responses to (–)-CGP12177 suggests that the inotropic effect is triggered by increased Ca^{2+} release from the sarcoplasmic reticulum as a result of increased $I_{Ca\text{-}L}$ (Bers, 2002). Our results agree with the persistence of cardiostimulant effects of (–)-CGP12177 and blockade by (–)-bupranolol in β_3-adrenoceptor-knockout mice (Kaumann *et al.*, 1998).

(–)-CGP12177-evoked increases in $I_{Ca\text{-}L}$ are mediated through β_{1L}-adrenoceptors at both 24°C and 37°C

Skeberdis *et al.* (2008) demonstrated, in human atrial cardiomyocytes at room temperature (19–25°C), an increase in $I_{Ca\text{-}L}$ by BRL3744, SR58611 and CGP12177, which was antagonized by 1 μM L-748,337, suggesting mediation through β_3-adrenoceptors. We confirmed that the 3 agonists increased $I_{Ca\text{-}L}$ at 24°C and that L-748,337 antagonized the effects of BRL37344 and SR58611. However, we found that the (–)-CGP12177-evoked $I_{Ca\text{-}L}$ responses were resistant to blockade by L-748,337 but antagonized by (–)-bupranolol, ruling out the involvement of β_3-adrenoceptors, but indicating mediation through β_{1L}-adrenoceptors. We do not know the reason for the discrepancy between our results and those of Skeberdis *et al.* (2008). Inspection of figure 2 of Skeberdis *et al.* (2008), which compares the blockade by L-748,337 of the $I_{Ca\text{-}L}$ responses to BRL37344 and CGP12177, reveals that the onset kinetic of the response to BRL37344 after washout of L-748,337 was conspicuously slower than the onset kinetic of BRL37344 before the L-748,337 exposure. The slow onset response to BRL37344 after washout of L-748,337 is presumably due to the time it takes for the antagonist to dissociate from the β_3-adrenoceptor. One would expect a similar delay for the onset kinetics of CGP12177 after washout of L-748,337. Surprisingly, however, for unknown reasons, the onset kinetics of CGP12177, before and after L-748,337, were virtually identical. Unfortunately, these puzzling kinetic differences between BRL37344 and CGP12177 and the antagonism by L-748,337 were not discussed (Skeberdis *et al.*, 2008).

We did not detect $I_{Ca\text{-}L}$ responses to (–)-CGP12177 at 37°C but uncovered small significant increases of $I_{Ca\text{-}L}$ in the presence of IBMX. L-748,337 (1 μM) did not prevent these responses to (–)-CGP12177, rejecting an involvement of β_3-adrenoceptors, but (–)-bupranolol blocked the responses, confirming their mediation through β_{1L}-adrenoceptors (Figure 7). These results suggest that at physiological temperature phosphodiesterases hydrolyse sufficient cAMP, generated through β_{1L}-adrenoceptor stimulation, to blunt cAMP-dependent protein kinase (PKA)-catalysed phosphorylation of the L-type Ca^{2+} channel. We reported previously that inhibition of phosphodiesterases with IBMX facilitates the appearance of small (–)-CGP12177-evoked $I_{Ca\text{-}L}$ responses (equivalent to 19% of the response to (–)-isoprenaline) on murine ventricular cardiomyocytes (Freestone *et al.*, 1999).

Human atrial β_2-adrenoceptors mediate robust increases in $I_{Ca\text{-}L}$ at room temperature (Skeberdis *et al.*, 1997) and there is evidence for agonist effects of racemic CGP12177, mediated through recombinant β_2-adrenoceptors (Pak and Fishman, 1996) and (–)-CGP12177 in human β_2-adrenoceptors overexpressed in the hearts of TG4 mice (Heubach *et al.*, 2003). However, the β_2-adrenoceptor-selective antagonist ICI118,551 failed to antagonize the (–)-CGP12177-evoked $I_{Ca\text{-}L}$ response, therefore ruling out a contribution of β_2-adrenoceptors under our conditions at 37°C.

The β_3-adrenoceptor-mediated $I_{Ca\text{-}L}$ responses to BRL37344 and SR58611 at 24°C are uncoupled from the contractile machinery

We confirmed that at 24°C BRL37344 and SR58611 evoke a β_3-adrenoceptor-mediated increase of $I_{Ca\text{-}L}$ in myocytes. These β_3-adrenoceptor-mediated increases of $I_{Ca\text{-}L}$ were surprisingly small compared with the effect of non-selective β-adrenoceptor-stimulation with (–)-isoprenaline (Figure 5). It should be noted that the size of the agonist effects on $I_{Ca\text{-}L}$ reported by Skeberdis *et al.* (2008) differed widely between 136 ± 21% and 45 ± 6% increase over basal, of which we could only confirm the latter estimate. We therefore investigated whether the small increases in $I_{Ca\text{-}L}$ are translated into contractile responses at 24°C. Our results with atrial trabeculae at 24°C in the presence of nadolol failed to uncover any inotropic effects of BRL37344 and SR58611 (1 nM–10 μM, Figure 2B), not even in the presence of the phosphodiesterase inhibitor IBMX. The lack of inotropic response to BRL37344 and SR58611 could be related to the small magnitude of

the $I_{Ca\text{-}L}$ response at 24°C (10% and 7%, respectively, of the (–)-isoprenaline response), or to an inherent inability of the β_3-adrenoceptor to produce the coupling messages necessary to activate the contractile machinery. For either situation, the enhanced activation of $I_{Ca\text{-}L}$ through β_3-adrenoceptors appears to be unable to stimulate Ca^{2+}-induced Ca^{2+} release from the RyR2 channels of the sarcoplasmic reticulum (Fabiato, 1983; Bers, 2002), thereby preventing an increase in atrial contractility.

It could be argued that phylogenetically, β_3-adrenoceptors may have had a particular role in brown adipose tissue to generate heat under cold circumstances in rodents but largely lost in humans (reviewed in Arch, 2008). Human β_3-adrenoceptors share only 40–50% of the amino acids with β_1- and β_2-adrenoceptors, consistent with early divergence during evolution (Granneman *et al.*, 1993). Interestingly, increased mRNA of β_1-adrenoceptors, β_2-adrenoceptors and especially β_3-adrenoceptors, as well as increased left ventricular ejection responses to isoprenaline have been reported in the hearts of hibernating bears compared with active bears (Nelson *et al.*, 2010). We therefore also investigated the inotropic effects of BRL37344 at 24°C. However, as found with SR58611, BRL37344 also did not reveal a vestige of β_3-adrenoceptor-mediated contractile responses at 24°C.

In contrast to the effects at 37°C, at 24°C BRL37344 did not augment force through β_{1H}- and β_2-adrenoceptors but (–)-isoprenaline and (–)-CGP12177 still increased force at 24°C. These findings suggest that the low temperature blunts the inotropic message caused by the partial agonist BRL37344 but still allows the full agonist (–)-isoprenaline to produce robust increases in force through β_{1H}- and β_2-adrenoceptors. Since (–)-CGP12177 still increased force at 24°C, it appears that positive inotropic responses are less attenuated by low temperature through β_{1L}-adrenoceptors than the responses to the partial agonist BRL37344 through β_{1H}- and β_2-adrenoceptors.

Relationship between $I_{Ca\text{-}L}$ responses and inotropic responses to (–)-CGP12177 at 24°C

We have demonstrated that at 37°C, the small $I_{Ca\text{-}L}$ responses correlate with the moderate inotropic responses to (–)-CGP12177, both mediated through β_{1L}-adrenoceptors under our conditions of PDE inhibition with IBMX. The inotropic relevance of the β_{1L}-adrenoceptor-mediated increase in $I_{Ca\text{-}L}$ at 24°C is, however, not clear. (–)-CGP12177 only produced marginal increases of contractile force in the presence of nadolol. We attempted to uncover (–)-CGP12177-evoked increases in force by inhibiting phosphodiesterase with IBMX. However, low (–)-CGP12177 concentrations in the presence of both nadolol and IBMX caused concentration-dependent negative inotropic effects, higher concentrations tended to increase contractile force (Figure 8). These cardiodepressant effects and marginal cardiostimulant effects of (–)-CGP12177 were not significantly modified by L-748,337 and appear therefore unrelated to β_3-adrenoceptors (Figure 8). We attribute the negative inotropic effects of (–)-CGP12177 to inverse agonism via β_1-adrenoceptors, as previously observed on murine ventricular $I_{Ca\text{-}L}$ at room temperature. Under these conditions (–)-CGP12177 was an inverse agonist in the presence of IBMX, but increased $I_{Ca\text{-}L}$ (Freestone *et al.*, 1999) in the presence of the efficacious inverse agonist (–)-propranolol (Chidiac *et al.*, 1994). We suspected that the marginal positive inotropic responses to higher (–)-CGP12177 concentrations were probably mediated through β_{1L}-adrenoceptors. As previously observed with murine $I_{Ca\text{-}L}$ responses to (–)-CGP12177 (Freestone *et al.*, 1999), (–)-propranolol abolished the negative inotropic effects and facilitated positive inotropic effects of (–)-CGP12177. These positive inotropic effects of (–)-CGP12177 in the presence of (–)-propranolol and IBMX were antagonized by (–)-bupranolol but not L-748,337, consistent with the $I_{Ca\text{-}L}$ responses to (–)-CGP12177 being mediated through β_{1L}-adrenoceptors but not β_3-adrenoceptors (Figure 8).

Although the small $I_{Ca\text{-}L}$ responses to (–)-CGP12177 through β_{1L}-adrenoceptors as well as BRL37344 and SR58611 through β_3-adrenoceptors at 24°C were not significantly different ($P = 0.39$), only activation of the β_{1L}-adrenoceptor, but not β_3-adrenoceptor, enhanced contractility. However there is a caveat, unlike the $I_{Ca\text{-}L}$ responses to the three agonists, which could be elicited in the absence of IBMX, a special condition containing not only IBMX but also (–)-propranolol (to prevent (–)-CGP12177-evoked inverse agonism) was necessary to demonstrate small concentration-dependent increases in atrial contractility with (–)-CGP12177. Nevertheless at 24°C, β_{1L}-adrenoceptors, but not β_3-adrenoceptors, appear to activate mechanisms leading to enhanced contractility. Thus, excitation-contraction coupling was receptor-dependent but not temperature-dependent.

Conclusions

We provide strong evidence against the hypothesis that β_3-adrenoceptor activation increases human atrial contractility. Increases in human atrial force by BRL37344 and (–)-CGP12177 are mediated through β_2-adrenoceptors > β_1-adrenoceptors and β_{1L}-adrenoceptors, respectively. The β_3-adrenoceptor

agonist SR58611 did not increase atrial force. Small β_3-adrenoceptor-mediated increases in $I_{Ca\text{-}L}$ by BRL37344 and SR58611 become apparent only at low non-physiological temperatures but appear uncoupled from contractility. The inotropic and $I_{Ca\text{-}L}$ responses to (–)-CGP12177 are mediated through the low affinity site of the β_1-adrenoceptor, β_{1L}-adrenoceptor, at both 24°C and 37°C. The lack of human atrial responses through β_3-adrenoceptors demonstrated with (–)-CGP12177, BRL37344 and SR58611, suggests that therapeutically beneficial β_3-adrenoceptor agonists, such as mirabegron for overactive bladder (Michel *et al.*, 2010), do not pose a risk to cardiac function.

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Conflicts of interests

The authors state no conflicts of interests.

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