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ORIGINAL ARTICLES

β_3-Adrenergic Regulation of L-Type Ca^{2+} Current and Force of Contraction in Human Ventricle

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Abstract β_3-Adrenergic receptor (β_3-AR) is expressed in human atrial and ventricular tissues. Recently, we have demonstrated that it was involved in the activation of L-type Ca^{2+} current ($I_{Ca,L}$) in human atrial myocytes and the force of contraction of human atrial *trabeculae*. In the present study, we examined the effect of β_3-AR agonist CGP12177 which also is a β_1-AR/β_2-AR antagonist on $I_{Ca,L}$ in human ventricular myocytes (HVMs) and the force of contraction of human ventricular *trabeculae*. CGP12177 stimulated $I_{Ca,L}$ in HVMs with high potency but much lower efficacy than isoprenaline. The β_3-AR antagonist L-748,337 inhibited the effect of CGP12177. CGP12177 and L748,337 competed selectively on β_3-ARs because L748,337 had no effect on isoprenaline-induced stimulation of $I_{Ca,L}$, while CGP12177 completely blocked the effect of isoprenaline. The activation of β_3-ARs by CGP12177 does not involve the activation of G_i proteins because CGP12177 had no effect on forskolin-induced stimulation of $I_{Ca,L}$. CGP12177 had no effect on the force of contraction of human ventricular *trabeculae*. L-NMMA, an inhibitor of NO synthase, and IBMX, a nonselective inhibitor of phosphodiesterases, did not potentiate the effect of CGP12177 either on contraction of human ventricular *trabeculae* or on $I_{Ca,L}$ in HVMs. We conclude that in human ventricles β_3-AR activation has no inotropic effect, while it slightly increases $I_{Ca,L}$. In contrast to human atrium, the activation of β_3-ARs in human ventricle is not accompanied by increased activity of phosphodiesterases.

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Keywords β_3-Adrenergic receptors · Human ventricle · L-type Ca^{2+} channel current · Contraction force

Abbreviations

β-AR	β-Adrenergic receptor
$I_{Ca,L}$	L-type Ca^{2+} channel current
HAM	Human atrial myocyte
HVM	Human ventricular myocyte
IBMX	Isobutylmethylxanthine
L-NMMA	NG-monomethyl-L-arginine
CFTR	Cystic fibrosis transmembrane conductance regulator
PDE	Phosphodiesterase
NOS	Nitric oxide synthase

Introduction

The sympathetic nervous system plays a major role in the regulation of the function of cardiovascular system. In cardiac myocytes, catecholamines acting on the β-ARs stimulate Ca^{2+} current through the L-type Ca^{2+} channels ($I_{Ca,L}$) which provide Ca^{2+} for the activation of the contractile apparatus (Bers 2002) and determine inotropic responses of the heart (Bers 2002; Brette et al. 2006). By now three types of β-adrenergic receptors (β-ARs) have been cloned, respectively, β_1-, β_2-, and β_3-ARs. Classically, β_1- and β_2-ARs were considered as mediating most of the cardiac contractile responses; however, during last two decades the expression β_3-AR in the human heart has been demonstrated at the mRNA (Gauthier et al. 1996; Moniotte et al. 2001b) and protein (Chamberlain et al. 1999; De Matteis et al. 2002; Moniotte et al. 2001a; Moniotte et al. 2001b; Napp et al. 2009) level and numerous, often controversial studies confirmed their

functional significance. For instance, in human endomyocardial biopsies from transplanted hearts (Gauthier et al. 1996; Rozec and Gauthier 2006) and in left ventricular samples from failing and nonfailing explanted hearts (Moniotte et al. 2001a) β_3-AR agonists have been shown to exert negative inotropic effects, while other authors did not detect any cardiodepressant effect (Christ et al. 2011; Kaumann and Molenaar 1997) or observed stimulation of $I_{Ca,L}$ and contractility in human atrium (Skeberdis et al. 2008). In human ventricles, negative inotropy of β_3-AR agonists was linked with activation of NO/cGMP pathway, whereas in atria stimulation of $I_{Ca,L}$ and contraction force was associated with cAMP-dependent phosphorylation (reviewed in (Dessy and Balligand 2010)). The amount of β_3-AR proteins was shown to increase up to threefold in different models of heart failure (Chen et al. 2010; Cheng et al. 2001; Kulandavelu and Hare 2012; Moniotte et al. 2001a; Morimoto et al. 2004; Sheng et al. 2012; Zhao et al. 2012), but whether this up-regulation is a protective mechanism against adrenergic overactivity in ischemia (Li et al. 2010; Niu et al. 2012; Rasmussen et al. 2009) or contributes to heart failure (Chen et al. 2010; Kong et al. 2010; Sheng et al. 2012) is not completely understood.

In this study, our aim was to get further insights into the role of β_3-ARs in regulation of human ventricular function at the single cell level. These effects were compared with those obtained on the contractility of human ventricular strips.

Materials and Methods

Our investigations were performed in accordance with the principles outlined in the Declaration of Helsinki and approved by Kaunas Regional Bioethics Committee (BE-2-18, 2006). Tissue samples were acquired via Lithuanian University of Health Sciences (LUHS) Institutional Review Board-approved protocol, and informed consent was obtained before cardiac surgery, patient identifiers were removed to ensure anonymity.

Human Ventricular Myocytes

Specimens of left ventricular *trabeculae* were obtained from patients undergoing heart surgery for congenital defects, valve replacement, or heart transplantation at the Department of Cardiothoracic and Vascular Surgery, LUHS. Most patients received a pharmacological pretreatment that was stopped 24 h before surgery. In addition, all patients received sedatives, anesthesia, and antibiotics. Details regarding the clinical characteristics of the patients and their drug regimens are shown in Table 1.

Table 1 Patient data, clinical diagnosis, and treatment

Patient data	
Age range (years)	14–81
Mean age (years)	59
Female, *n*	11
Male, *n*	22
Total, *n*	33
Ischemic versus nonischemic cardiac diseases, *n*	13 vs 20
Surgical intervention	
Aortic valve surgery[a], *n*	11/33
Mitral valve surgery[a], *n*	16/33
CABG[a], *n*	14/33
Cardiac transplant, *n*	1
Bentall operation[b], *n*	7/33
Treatment regimens	
β-Blockers	21/33
Calcium antagonists	1/33
ACE inhibitors/angiotensin receptor blockers	18/33
Antiarrhythmic agents	2/33
Diuretics	15/33
Nitrates	10/33

[a] Some patients underwent both valve surgery and coronary artery bypass graft (CABG) surgery

[b] Two patients underwent Bentall operation and CABG

Dissociation of the cells was performed immediately after the surgery as described previously (Puceat et al. 1990; Rucker-Martin et al. 1993). The cell suspension was filtered, centrifuged, and the pellet resuspended in Dulbecco minimal essential medium supplemented with 10 % fetal calf serum, nonessential amino acids, 1 nM insulin, and antibiotics (100 IU/ml penicillin and 0.1 μg/ml streptomycin).

Solutions

For electrophysiology, the control external solution contained (in mM): NaCl 107; HEPES 10; CsCl 40; $NaHCO_3$ 4; NaH_2PO_4 0.8; $MgCl_2$ 1.8; $CaCl_2$ 1.8; D-glucose 5; sodium pyruvate 5; tetrodotoxin 6×10^{-3}; pH 7.4 adjusted with NaOH. Patch electrodes (0.6–1.5 M Ω) were filled with the control internal solution which contained (in mM): CsCl 119.8; EGTA (acid form) 5; $MgCl_2$ 4; creatine phosphate disodium salt 5; Na_2ATP 3.1; Na_2GTP 0.42; $CaCl_2$ 0.062 (pCa 8.5); HEPES 10; pH 7.3 adjusted with CsOH. Collagenase type 2 was purchased from Worthington Biochemicals (NJ, USA). DMEM was obtained from Gibco-BRL. Tetrodotoxin (TTX) was from Latoxan (Rosans, France). All other drugs were from Sigma-Aldrich Co. (St. Louis, MO, USA). All drugs tested in patch-clamp experiments were solubilized in experimental solutions just

before application onto the cell studied, i.e., only fresh solutions were tested.

Whole-Cell Current Recording

The whole-cell configuration of the patch-clamp technique was used to record the high-threshold L-type Ca^{2+} current $I_{Ca,L}$ in Ca^{2+}-tolerant rat and human ventricular myocytes (HVMs). In the routine protocols, the cells were depolarized every 8 s from a holding potential of −80 mV by a short pre-pulse (50 ms) to −50 mV and then to 0 mV for 400 ms. The pre-pulse and/or the application of TTX were used to eliminate fast sodium currents. K^+ currents were blocked by replacing all K^+ ions with intracellular and extracellular Cs^+. Voltage-clamp pulses were generated and currents were recorded using VP-500 (Bio-Logic, Claix, France) patch-clamp amplifier. Visual-Patch v.1.30 (Bio-Logic) was used to control all experimental parameters, cell stimulation, and current recording. Recordings were low-pass filtered at 2 kHz and stored on the hard disk of an IBM-compatible computer. Control and drug containing solutions were applied to the exterior of the cell by placing the cell at the opening of 300-μm inner diameter capillary tubes flowing at the rate of about 50 μl/min. Changes in extracellular solutions were automatically achieved using a rapid solution changer (RSC-200, Bio-Logic). All experiments were done at room temperature (19–25 °C), and the temperature did not vary by more than 1 °C in a given experiment.

Mechanoelectrical Measurements

Experiments were performed on human *trabeculae* isolated from the left ventricle. During transport from hospital to the laboratory, ventricular tissues were placed in a cold (10 °C) St. Thomas cardioplegic solution composed of (in mM): NaCl 110, KCl 16, $CaCl_2$ 1.2, $MgCl_2$ 16, glucose 5, HEPES 10, and pH 7.4 adjusted with NaOH. *Trabeculae* were then placed in an experimental chamber and superfused at the flow rate of 6 ml/min with oxygenated (100 % O_2) Tyrode solution (pO_2 580–600 mmHg) composed of (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.9, glucose 5, and HEPES 10 at 36.0 ± 0.5 °C, pH 7.4. *Trabeculae* were subjected to field stimulation with the following characteristics: the stimulus pulse width was 5 ms, stimulation rate 1.0 Hz, and the amplitude was twice the diastolic threshold. Isometric contraction was recorded using a mechanoelectrical force transducer. The β_3-AR agonists and other drugs were applied after steady-state conditions were established, i.e., after 40–50 min perfusion with control Tyrode solution. All experiments were done at 37 °C.

Data Analysis

The maximal amplitude of whole-cell $I_{Ca,L}$ was measured as previously described (Kirstein et al. 1995). Currents were not compensated for capacitive and leak currents. On-line analysis of the recordings was done for each membrane depolarization, peak, and steady-state current values. The changes in contractile force were expressed as percentage variation versus control. The results are expressed as mean ± SEM. For statistical evaluation, the paired and unpaired Student's t test were used, and a difference was considered statistically significant when p was <0.05. ANOVA followed by post-hoc Bonferroni's test was used for contractile experiments when comparing the effects of the β_3-AR agonists in the absence or presence of IBMX and L-NMMA.

Results

β_3-AR-Dependent Stimulation of $I_{Ca,L}$ in HVMs

Since β_3-AR agonists were reported to reduce the force of contraction in human ventricular samples (Gauthier et al. 1996; Moniotte et al. 2001a), we examined whether β_3-AR activation had different effects on $I_{Ca,L}$ in HVMs versus human atrial myocytes (HAMs). The examination of β_3-ARs in the human heart is hampered because atria as well as ventricles express all three known types of β-ARs, β_1-, β_2-, and β_3-ARs, which lack selective agonists and antagonists. Therefore in this study, we chose to use β_3-AR agonist CGP12177 which also is a β_1/β_2-AR antagonist. As shown in Fig. 1a, CGP12177 stimulated $I_{Ca,L}$ in HVMs in a dose-dependent manner.

From the cumulative dose-response curve (Fig. 1b), EC_{50} was 18.0 ± 6.5 nM and E_{max} was 65 ± 9 % ($n = 5$). It is necessary to note that despite no differences found in β_3-AR expression levels across different layers of the human ventricular myocardium (Moniotte et al. 2001b) the maximal responses of $I_{Ca,L}$ to CGP12177 varied from 8 to 78 % over control ($n = 17$) and in faintly responding myocytes it was impossible to discriminate between the effects of four increasing concentrations of CGP12177 for calculation of EC_{50}; however, we used these experiments for estimation of E_{max} which otherwise would have been largely overestimated. Averaged from 17 experiments, E_{max} for CGP12177 was 29 ± 5 %, while that of isoprenaline was 232 ± 23 % (E_{max} varied from 68 to 484 % over control; $n = 20$) (Fig. 1c and summary in Fig. 4). Thus, CGP12177 had the same potency but much lower efficacy in HVMs as compared to HAMs where its maximal effect was similar to that produced by isoprenaline (Skeberdis et al. 2008).

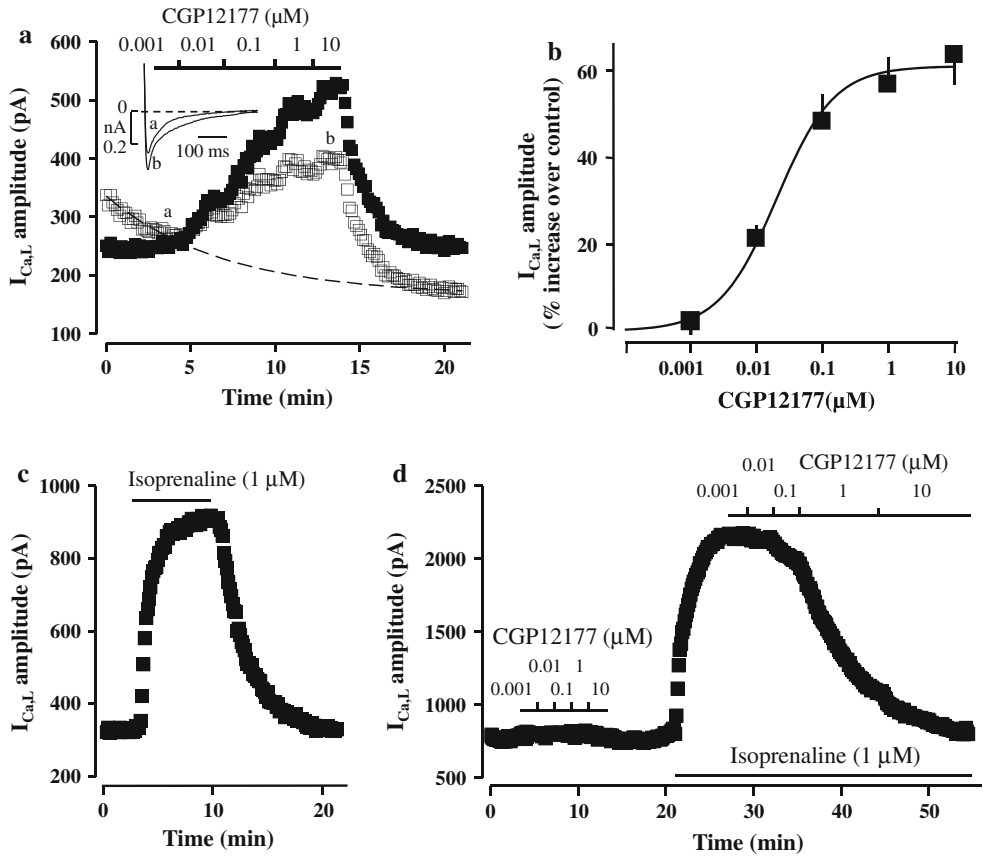

Fig. 1 β_3-AR-dependent stimulation of $I_{Ca,L}$ in HVMs. **a** Each experiment shows the time course of $I_{Ca,L}$ amplitude recorded in single HAM that was exposed to increasing concentrations of CGP12177. *Each symbol* indicates a net amplitude of $I_{Ca,L}$ measured every 8 s at 0 mV membrane potential. Original $I_{Ca,L}$ amplitude (*open squares*) was subjected to spontaneous run-down (indicated by *dotted line*) which was eliminated for a precise measurement of EC_{50} and E_{max} of the compound (*filled squares*). The individual current traces were obtained at the times indicated by the corresponding *letters* on the main graph. **b** The concentration-response curve for the effect of CGP12177 on $I_{Ca,L}$. The *points* show the mean ± SEM ($n = 5$). **c** Isoprenaline stimulates $I_{Ca,L}$ with much higher efficacy than CGP12177. **d** In rat ventricular myocytes, CGP12177 has no effect of $I_{Ca,L}$, but completely blocks isoprenaline-induced stimulation of $I_{Ca,L}$

Since the stimulation of contraction force of the cardiac muscle directly depends on stimulation of $I_{Ca,L}$ in single myocytes, our results are inconsistent with the negative inotropic effect of CGP12177 observed by Gauthier in failing human ventricle (Gauthier et al. 1996; Rozec and Gauthier 2006). Likewise, Kaumann et al. have demonstrated that CGP12177 exerts a positive inotropic effect in rat ventricle and raised a hypothesis that this effect was obtained through the activation of the putative β_4-AR (Sarsero et al. 1999). Unfortunately, the existence of β_4-AR has so far not been confirmed by molecular cloning. In other studies, it has been demonstrated by the same group that in the right atrium of non-failing and failing human heart CGP12177 increases the force of contraction and hastens relaxation (Sarsero et al. 2003). These effects were attributed not to the β_3- or β_4-ARs, but to the putative low-affinity site of β_1-ARs (Joseph et al. 2004) which was discovered in human β_1-AR expressing CHO cells labeled with (−)-[^{3}H]-CGP12177. We have tested the functionality of the low-affinity site of β_1-ARs in rat ventricular myocytes in which β_1-ARs dominate and are functionally coupled to $I_{Ca,L}$ (Kuznetsov et al. 1995). However, in rat ventricular myocytes CGP12177 had no effect on the basal $I_{Ca,L}$, but completely blocked isoprenaline-dependent stimulation of the current as it was expected to do being β_1/β_2-AR antagonist [see Fig. 1d and our previous publication (Skeberdis et al. 2008)]. These experiments confirm that even though rat and human β_1-ARs may exhibit some functional differences, in human ventricles CGP12177 most probably did not act through β_1-ARs. Moreover, this effect was achieved through β_3-ARs, since it was blocked by β_3-AR antagonist L-748,337 (1 μM, $n = 4$, Fig. 2a). In addition, in several HVMs faintly responding to β_3-AR stimulation, CGP12177 (1 μM, $n = 5$, Fig. 2c), but not L-748,337 (1 μM, $n = 3$, Fig. 2b), antagonized the stimulatory effect of isoprenaline (1 μM), confirming its β_1/β_2-AR antagonistic properties. The relatively low efficacy of CGP12177 (Fig. 1a) compared to that of isoprenaline

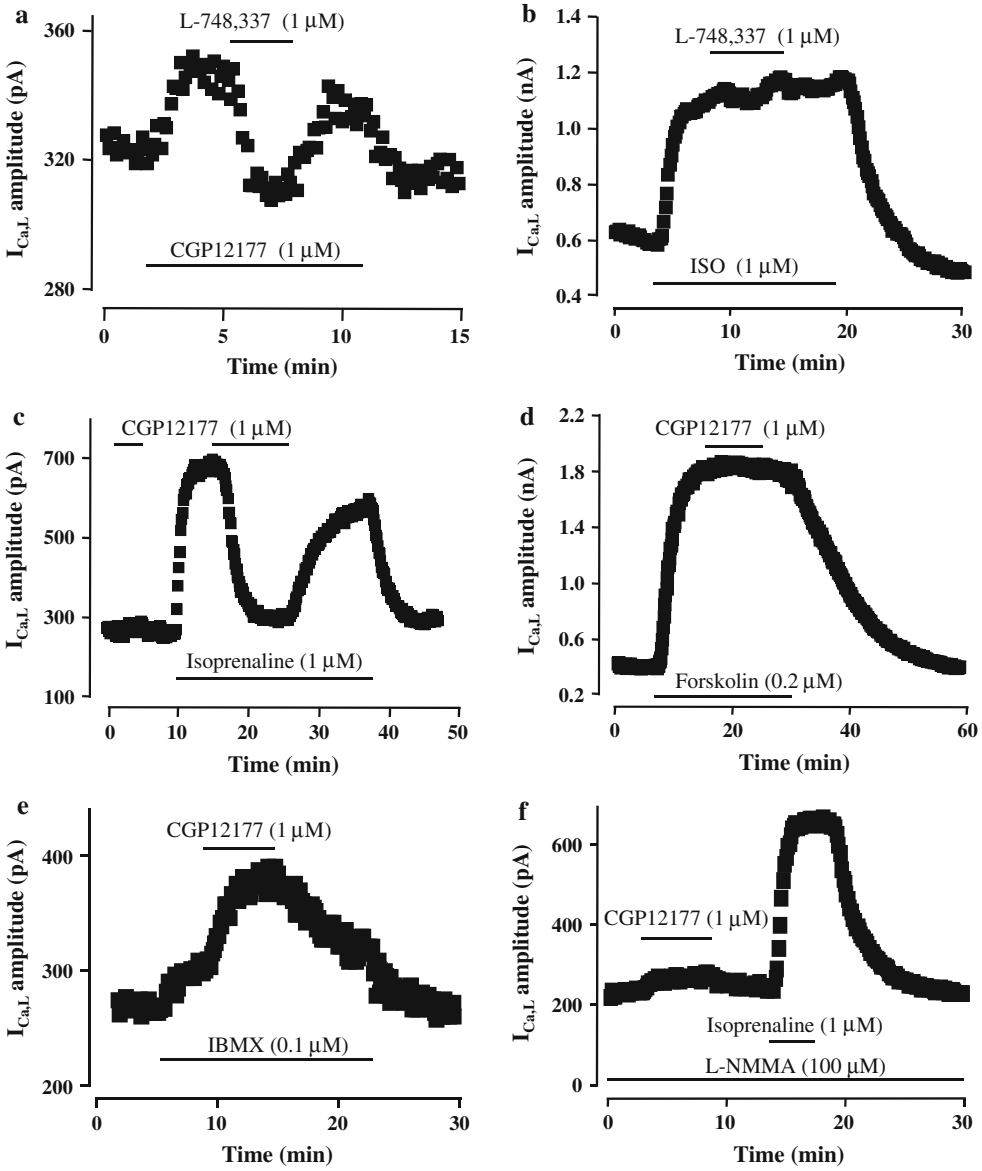

Fig. 2 CGP12177 stimulates $I_{Ca,L}$ in HVMs through β_3-ARs. In presented typical experiments, $I_{Ca,L}$ was recorded in a single HAM that was exposed to the respective compound during the periods indicated by *horizontal lines*. **a** β_3-AR antagonist L748,337 completely blocked the effect of GP12177. **b** In contrast, L748,337 has no effect on $I_{Ca,L}$ stimulated by isoprenaline through β_1/β_2-ARs. **c** CGP12177, as β_1/β_2-AR antagonist, completely blocked the effect of isoprenaline on $I_{Ca,L}$. **d** CGP12177 has no effect on the stimulation of $I_{Ca,L}$ induced by forskolin. **e** IBMX did not potentiate the stimulatory effect of CGP12177 on $I_{Ca,L}$. **f** L-NMMA added into the external and internal solutions did not modify the stimulatory effects of CGP12177 and isoprenaline on $I_{Ca,L}$

(Fig. 1c) was not due to the involvement of the secondary inhibitory signaling pathway related to the activation of G_i proteins, since CGP12177 had no effect on the stimulation of $I_{Ca,L}$ induced by the direct activator of adenylyl cyclase forskolin (200 nM, $n = 4$, Fig. 2d).

Altogether, these results indicate that CGP12177 behaves as a β_1/β_2-AR antagonist and a β_3-AR agonist which is positively coupled to $I_{Ca,L}$ in HVMs.

The Role of Phosphodiesterases in β_3-AR-Dependent Activation of $I_{Ca,L}$ in HVMs

We have demonstrated in human atrium that despite the potent and efficient action of β_3-AR agonists on $I_{Ca,L}$ in HAMs, their action on the force of contraction of the atrial *trabeculae* was much weaker (Skeberdis et al. 2008). This discrepancy could be eliminated by the pretreatment of *trabeculae* by isobutylmethylxanthine (IBMX), a non-selective inhibitor of phosphodiesterases. To verify the possible implication of phosphodiesterases in low response of $I_{Ca,L}$ to CGP12177 in HVMs, we pre-stimulated $I_{Ca,L}$ by low concentration of IBMX and then applied CGP12177. However, in contrast to its effect on the contraction force of human atrial *trabeculae*, IBMX did not potentiate the effect of CGP12177 on $I_{Ca,L}$ in HVMs (Fig. 2e and summary in Fig. 4).

The Role of Nitric Oxide Synthase in β_3-AR-Dependent Regulation of $I_{Ca,L}$ in HVMs

The involvement of the nitric oxide synthase (NOS) in the signaling pathway of β_3-ARs was suggested in human

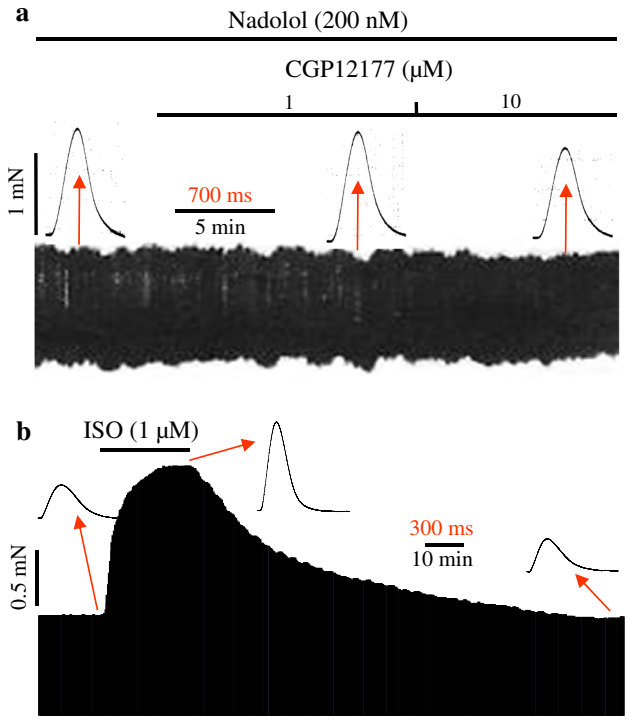

Fig. 3 Activation of β_3-ARs has no effect on the contraction force of human ventricular *trabeculae*. **a** CGP12177 was applied to human ventricular preparations at the concentrations indicated, after 15 min of perfusion with 200 nM nadolol and in the continuous presence of nadolol. **b** Isoprenaline was applied alone at the concentration indicated. The individual contractile traces were obtained in each experimental condition at the times indicated by the corresponding *arrows*

ventricles (Gauthier et al. 1998). To verify the involvement of NO/cGMP in the effect of CGP12177, we used a cell permeant potent NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) (Derici et al. 2012; Gauthier et al. 1998; Zima et al. 2000). L-NMMA (100 μM) added to the extracellular and intracellular solutions did not modify the effects neither of CGP12177 nor of isoprenaline on $I_{Ca,L}$ in HVMs (Fig. 2f).

The Effects of β_3-AR Activation on the Force of Contraction of Human Ventricular *Trabeculae*

In cardiac myocytes, Ca^{2+} current through the L-type Ca^{2+} channels provides Ca^{2+} for the activation of the contractile proteins and is a crucial determinant of the cardiac contractile activity. Therefore, any modulation of $I_{Ca,L}$ amplitude might be expected to induce parallel changes in the contraction amplitude. Since our results indicate that β_3-ARs are positively coupled to $I_{Ca,L}$ in HVMs, the experiments were performed to evaluate the effects of β_3-AR activation on the force of contraction in the *trabeculae* isolated from human ventricular tissues. To eliminate possible overlapping effects of CGP12177, i.e., concomitant inhibition of β_1/β_2-ARs and stimulation of β_3-ARs, experiments were performed in the presence of another β_1/β_2-AR antagonist nadolol (200 nM) which alone reduced ventricular basal contractile amplitude to 82 ± 3.5 % ($n = 18$) of the control level. Then CGP12177 (10 μM, Fig. 3a) had no effect on the force of contraction, while isoprenaline induced a large positive inotropic effect (1 μM; 253 ± 54 % relatively to control; $n = 5$; Fig. 3b).

The Role of Phosphodiesterases and NO Synthase in β_3-AR-Dependent Regulation of the Force of Contraction in Human Ventricle

Recently, we have shown in human atrium that β_3-AR-dependent stimulation of the force of contraction was very modest compared with stimulation of $I_{Ca,L}$, and the inhibition of phosphodiesterases by IBMX strongly potentiated contractile responses to β_3-AR agonists (Skeberdis et al. 2008). Therefore, we tested the possible involvement of phosphodiesterases in β_3-AR-dependent stimulation of the force of contraction of human ventricular *trabeculae*. The experiments were performed in the presence of nadolol (200 nM). Since complete inhibition of phosphodiesterases may cause maximal stimulation of $I_{Ca,L}$ in human cardiac myocytes, we used low concentration of IBMX (10 μM) which we found as a threshold concentration for stimulation of the force of contraction (106 ± 4 % relatively to control; $n = 13$; $p > 0.05$). Then, CGP12177 (10 μM) added to the perfusion solution stimulated the force of contraction respectively to 118 ± 14 % ($n = 4$; $p > 0.05$) of control level (Fig. 4b). The potentiation of β_3-AR contractile responses by IBMX was much weaker than in human atrium (Skeberdis et al. 2008); however, it is necessary to note that $I_{Ca,L}$ responses to β_3-AR stimulation in HVMs (see above) also were much weaker than in HAMs (Skeberdis et al. 2008). Naturally, the stimulation of $I_{Ca,L}$ and the force of contraction in human ventricles by β_3-AR agonist was much weaker than by isoprenaline, a non-selective activator of β_1/β_2-ARs. Gauthier with colleagues has demonstrated in several publications that β_3-ARs in human ventricles produced a negative inotropic effect due to NOS3 activation [reviewed in (Rozec and Gauthier 2006)]. Through the activation of soluble guanylyl cyclase and accumulation of intracellular cGMP, NO can lead to opposite effects on $I_{Ca,L}$: a stimulation at low concentrations due to inhibition of the cGMP-inhibited phosphodiesterase (PDE3) and an inhibition at higher concentrations due to the activation of the cGMP-stimulated phosphodiesterase (PDE2) (Kirstein et al. 1995; Vandecasteele et al. 2001). To evaluate the contribution of the NO/cGMP pathway in the regulation of the force of contraction by β_3-ARs, we examined the effect of CGP12177 in the presence of L-NMMA. L-NMMA (100 μM) added to the extracellular solution did not modify the effect of CGP12177

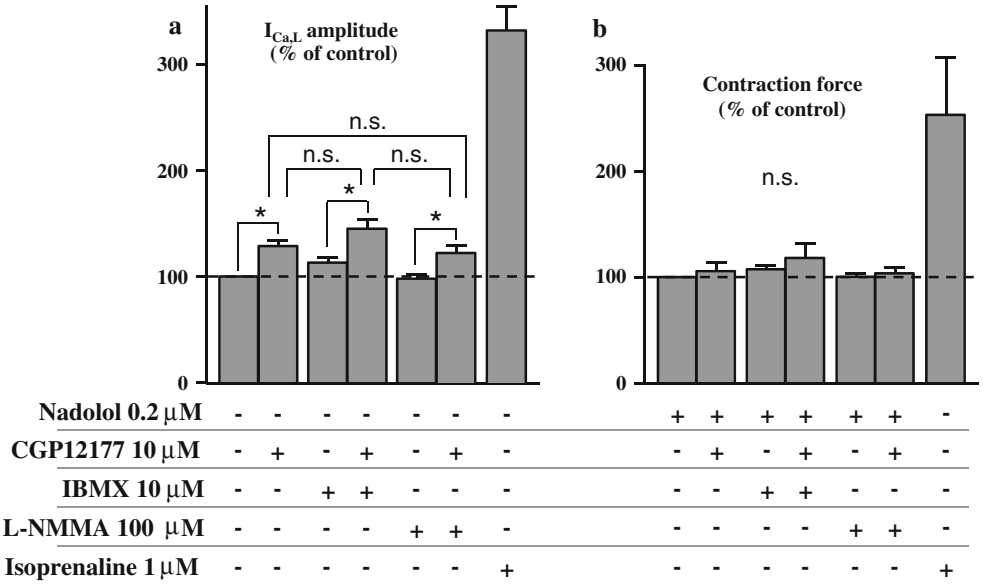

Fig. 4 Summary of the effects of the CGP12177 on $I_{Ca,L}$ in HVMs and contractility of human ventricular *trabeculae*. **a** The effect of CGP12177 (10 μM) on $I_{Ca,L}$ respectively was 129 ± 5 % ($n = 17$) in control; 127 ± 6 % ($n = 6$) after IBMX (10 μM); 123 ± 8 % ($n = 4$) after L-NMMA (100 μM). The effects of IBMX, L-NMMA, and isoprenaline alone were 113 ± 5, 98 ± 3, and 323 ± 23 %, respectively. **b** The effect of CGP12177 (10 μM) on the contraction amplitude respectively was 106 ± 8 % ($n = 8$) in control; 118 ± 14 % ($n = 4$) after IBMX (10 μM); 104 ± 6 % ($n = 4$) after L-NMMA (100 μM). The effects of IBMX, L-NMMA, and isoprenaline alone were 106 ± 4, 100 ± 1, and 253 ± 54 %, respectively. In **b**, all effects except that of isoprenaline were nonsignificant. *$p < 0.05$; *n.s.* nonsignificant

(1 μM) on the force of contraction (Fig. 4b). This indicates that the NO/cGMP pathway is not involved in the regulation of the contraction force by β_3-ARs in human ventricles.

Discussion

In the present study, we have demonstrated that β_3-AR agonist CGP12177, which is also a β_1/β_2-AR antagonist, stimulated the L-type Ca^{2+} current in HVMs, but the activation of β_3-ARs was not coupled to contractile activity in the ventricular tissue. In HVMs, the effect of CGP12177 on $I_{Ca,L}$ was reversible and involved β_3-AR stimulation, rather than the low-affinity site of β_1-ARs, because: (i) EC_{50} for CGP12177 was similar to adenylyl cyclase activating constant for human β_3-ARs expressed in CHO cells (Baker 2005; Emorine et al. 1989) and to $I_{Ca,L}$ stimulating constant in HAMs (Skeberdis et al. 2008); (ii) in rat ventricular myocytes in which β_1-AR dominate and $I_{Ca,L}$ is functionally coupled only to β_1-ARs, CGP12177 had no effect on basal $I_{Ca,L}$, and completely blocked isoprenaline stimulated $I_{Ca,L}$; (iii) in HVMs faintly responding to CGP12177, it completely antagonized the stimulatory effect of β_1/β_2-AR agonist isoprenaline; (iv) the stimulatory effect of CGP12177 on $I_{Ca,L}$ in HVMs was blocked by the β_3-AR antagonist L-748,337, while L-748,337 had no effect on isoprenaline-induced stimulation of $I_{Ca,L}$. Although a number of studies have demonstrated the expression of β_3-ARs in the human heart (Chamberlain et al. 1999; De Matteis et al. 2002; Moniotte et al. 2001a, b), our recent study (Skeberdis et al. 2008) was the first to explore their function in human atrium at the single cell level and the current study is the first such on human ventricle. Several studies at the single cell level in other species are summarized in our previous publication (Skeberdis et al. 2008), while a more recent study in part repeating our experiments in human atrium (Christ et al. 2011) presents an intriguing observation that while β_3-ARs are positively coupled to $I_{Ca,L}$ at room temperature at which patch-clamp experiments are usually performed, at body temperature they lose their functionality. However, this observation needs to find a physiological relevance for why human myocardium expresses nonfunctional receptors which switch to the functional state at temperatures incompatible with mammalian life.

In our experiments, the stimulatory effect of β_3-AR agonists on $I_{Ca,L}$ was not due to NOS activation, because the effect persisted in the presence of L-NMMA. The effect was not mediated by $G_{i/o}$ proteins since CGP12177 had no effect on $I_{Ca,L}$ pre-stimulated by forskolin, while $G_{i/o}$ protein activation via muscarinic M2 receptors inhibited the current under similar conditions (Vandecasteele et al. 1998). Thus, the β_3-AR-dependent responses differ not only among species but also in different compartments of the human heart (see Table 2).

Table 2 Comparison of CGP12177 effect on $I_{Ca,L}$ and force of contraction (P) in human atria and ventricles (% over control)

Tissue:	Human atrium[b]		Human ventricle	
Compound	$I_{Ca,L}$	P	$I_{Ca,L}$	P
CGP12177 (1 μM)	136 ± 21 (8)*	12 ± 4 (4)*	29 ± 5 (17)*	6 ± 8 (8)
CGP12177 (1 μM) after pretreatment with IBMX (10 μM)	118 ± 10 (5)	165 ± 36 (4)*	27 ± 6 (6)	18 ± 14 (4)
CGP12177 (1 μM) after pretreatment with L-NMMA (100 μM)	65 ± 11 (4) vs 58 ± 4 (4)[a]	25 ± 7 (4)*	23 ± 8 (4)	4 ± 6 (4)
Isoprenaline (1 μM)	241 ± 47 (5)*	277 ± 53 (5)*	223 ± 23 (20)*	153 ± 54 (5)*

[a] Separate control experiments were performed due to lower efficacy of CGP12177 alone in this series of experiment

[b] Data from Skeberdis et al. (2008)

* $p < 0.05$ (the effects of single compounds were compared with basal $I_{Ca,L}$ or P, respectively, and the effects of CGP12177 after pretreatment with IBMX or L-NMMA were compared with the effects of CGP12177 alone)

In part these differences can be explained by different roles of phosphodiesterases in β_3-AR signaling in human atrium and ventricle. β_3-AR activation modestly increased $I_{Ca,L}$ in HVMs and had no inotropic effect in human ventricular *trabeculae*. This indicates that in the human heart stimulation of the force of contraction through β_3-ARs is not directly related to the increase in $I_{Ca,L}$, unlike in the case of β_1/β_2-AR stimulation by isoprenaline (Figs. 1c, 3b). In human atrium CGP12177 stimulated $I_{Ca,L}$ more than two-fold over control but was more than ten times less efficient on the force of contraction (Skeberdis et al. 2008). Logically, ~4 times smaller increase of $I_{Ca,L}$ in human ventricles could be expected to initiate a proportionally smaller increase in the force of contraction which consequently in our experiments stayed insignificant (Fig. 3a). If the β_3-AR signaling mechanisms in both compartments were the same and proportional differences in responses were defined only by different expression levels of functional β_3-ARs [for instance, one study using high-affinity monoclonal antibody in contrast to human atrium was even unable to detect β_3-AR protein in human ventricles (Chamberlain et al. 1999)], both $I_{Ca,L}$ and the contractile responses to CGP12177 in the ventricles would have been potentiated by IBMX; however, the differences between effects of CGP12177 alone and in the presence of IBMX (or L-NMMA) were insignificant (Fig. 4). Thus, even though in our experiments the effects of β_3-AR stimulation in human atrium and ventricle were not obverse, their downstream signaling pathways differed as it is known for NOS signaling between two cell types (Kirstein et al. 1995) or the serotonin 5-HT4 (Jahnel et al. 1992; Schoemaker et al. 1993) and the angiotensin II AT1 receptors (Holubarsch et al. 1994), which are coupled to the increases in the force of contraction in atria but not in ventricles. The same NO/cGMP signaling pathway may cause different effects on $I_{Ca,L}$, depending on which isoform of cAMP-targeted phosphodiesterases, cGMP-stimulated PDE2, or cGMP-inhibited PDE3, dominate in the specific tissue or its subcellular compartments (Perera and Nikolaev 2013). Usually biopsies for the electrophysiological experiments come from a diseased human heart therefore of note is that depending on the age or pathological state, the balance of PDE or NOS isoforms in the heart may change as it has been shown in the rat (Abi-Gerges et al. 2009; Amour et al. 2007; Birenbaum et al. 2008). Moreover, in patch-clamp experiments we deal with β_3-ARs expressed only on myocytes, while in cardiac tissues β_3-ARs are expressed both on the endothelial cells of the coronary microvasculature and on the myocytes. Thus, even if NOS is not involved in β_3-AR signaling in the myocytes, NO generated in the endothelial cells could paracrinally modulate contractile responses of the myocardium. Finally, β_3-ARs can modulate other than Ca^{2+} ion channels which differently contribute to the formation of the action potential in the atria and ventricles. At least in the human ventricles the negative inotropic effect of β_3-ARs was accompanied by the shortening of the action potential (Gauthier et al. 1996; Leblais et al. 1999).

The absence of inotropic responses to β_3-AR stimulation suggests the existence of other not yet identified roles for this receptor. L-type calcium channels recently have been shown to be linked to the transcription of genes. Ca^{2+} entering through L-type Ca^{2+} channels can directly activate nuclear Ca^{2+}-dependent enzymes, such as calmodulin kinase IV, that regulate the activity of transcription factors and co-regulators or activate molecules that in turn transmit signals to nucleus. In addition, C-terminus of $Ca_v1.2$ subunit of L-type Ca^{2+} channel may function as transcriptional regulator (Gomez-Ospina et al. 2006) providing the negative feedback mechanism to regulate the expression of $Ca_v1.2$ in neurons and cardiac myocytes (Gomez-Ospina et al. 2013; Schroder et al. 2009). Being positively coupled to $I_{Ca,L}$, β_3-ARs may contribute to the regulation of transcriptional activity of L-type Ca^{2+} channels, especially in

failing human heart where the amount of β_3-AR proteins was shown to increase up to threefold (Moniotte et al. 2001a). Interestingly, the up-regulation of β_3-ARs is accompanied by down-regulation of β_1-ARs, uncoupling of β_2-ARs from G_s-adenylyl cyclase pathway and up-regulation of G_i proteins (Brodde et al. 2006). These processes may represent the intrinsic regulatory mechanisms aimed at sparing the heart of excessive oxygen demand, reducing myocardial overload and preventing cardiac hypertrophy during chronic adrenergic stimulation in heart failure.

In summary, despite earlier reports of negative inotropic effects in failing human ventricles, our study demonstrates that β_3-AR activation has no inotropic effect in human ventricular muscle samples, while it increases $I_{Ca,L}$ in HVMs but with much lower efficacy than in HAMs (Skeberdis et al. 2008). β_3-ARs are positively coupled to L-type Ca^{2+} channels in both atrial and ventricular chambers, and different signaling efficacy may be related to the variation of β_3-AR densities in both tissues. We did not detect any inhibitory pathway involved what strongly suggests that β_3-ARs share a common cAMP signaling pathway with other G_s-coupled receptors in human ventricles as well as in atria.

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