



Characterization of β_1 -selectivity, adrenoceptor- G_s -protein interaction and inverse agonism of nebivolol in human myocardium

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1 Intrinsic activity and β_1 -selectivity are important features of β -blockers in the treatment of patients with coronary syndromes and heart failure. In human myocardium, intrinsic activity and β_1 -selectivity of the novel β -adrenoceptor antagonist nebivolol have not yet been determined.

2 The study examines intrinsic activity, β -adrenoceptor-G-protein coupling and β_1 -selectivity of nebivolol and bisoprolol in human ventricular myocardium. Furthermore, intrinsic activity of both compounds is compared to the one of bucindolol, carvedilol and metoprolol in human atrial myocardium.

3 Radioligand binding studies ($[^{125}\text{I}]$ -Iodocyanopindolol) were performed on membrane preparations of human failing and nonfailing myocardium and on COS-7 cells transfected with human β_1 - and β_2 -adrenoceptors, respectively. Functional experiments were carried out on isolated muscle preparations of human left ventricular and right atrial myocardium from failing and nonfailing hearts.

4 Radioligand binding studies reveal 3–4 fold β_1 -selectivity for nebivolol and 16–20 fold β_1 -selectivity for bisoprolol in human myocardium. In COS-7-cells, β_1 -selectivity is 3 fold for nebivolol and 15 fold for bisoprolol.

5 Neither the binding of nebivolol nor of bisoprolol is affected by the presence of guanylylimidodiphosphate (Gpp(NH)p).

6 Nebivolol and bisoprolol exert similar inverse agonist activity in human ventricular as well as atrial myocardium. In atrial myocardium, inverse agonism of both compounds is higher compared to bucindolol, equal to carvedilol and lower compared to metoprolol.

7 Favourable haemodynamic effects of nebivolol in humans are not due to β_1 -selectivity or partial agonist activity of this agent. Other mechanisms, i.e. the production of nitric oxide, may thus be responsible for its unique haemodynamic profile.

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Abbreviations: β -AR, β -adrenergic receptor; EC_{50} , concentration achieving 50% of maximum effect; FOC, force of contraction; Gpp(NH)p, guanylylimidodiphosphate; $G_{S(\alpha)}$, stimulatory G-protein (α -subunit); ICYP, $[^{125}\text{I}]$ -Iodocyanopindolol; ISA, intrinsic sympathomimetic activity; K_d , K_i , dissociation constant; NF, nonfailing; NO, nitric oxide; n_H , pseudo Hill coefficient (slope factor); NYHA, New York Heart Association; R_H , receptors in a high-affinity state; K_H , K_L , dissociation constant of receptors in a high (K_H) and low affinity (K_L) state

Introduction

In patients with chronic heart failure, β -blockers have favourable effects on ventricular function and survival. These benefits are provided by second-generation β_1 -selective (bisoprolol, CIBIS II, 1999; metoprolol, MERIT-HF, 1999) as well as third-generation non-selective agents (carvedilol, Packer *et al.*, 1996). In contrast, xamoterol, a partial agonist, has led to an increase of mortality (Nicholas, 1990). A recent study with bucindolol in patients with heart failure (BEST-trial) had to be terminated due to a lack of benefit (Bristow, 2000). It is currently discussed whether this negative result may be due to comparable high intrinsic activity of bucindolol that has been observed in animal models (Willette *et al.*, 1998) and also human myocardium (Maack *et al.*,

2000). These data indicate that intrinsic activity of β -blockers has to be considered for therapeutic reasons.

Nebivolol is a novel third-generation β -blocker, being a racemic mixture of equal amounts of d-(SRRR-) nebivolol and l-(RSSS-) nebivolol. The d-isomer is reported to be highly selective for β_1 -adrenoceptors in several animal and cell models (Pauwels *et al.*, 1989; 1991), whereas the l-isomer accounts for vasodilatory effects that are mediated by endothelial generation of nitric oxide (Mangrella *et al.*, 1998). A special feature of nebivolol is that, in contrast to classical β -blockers, its acute administration increases ejection fraction and maintains cardiac output despite lowering of blood pressure in patients with cardiovascular diseases (Stoleru *et al.*, 1993). From these clinical results it cannot be excluded that besides vasodilation a positive inotropic or lack of negative inotropic effect of nebivolol may contribute to the acute increase of ejection fraction.

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Inotropic effects of β -adrenoceptor ligands are generally related to their intrinsic activity. To date, no determination of intrinsic activity of nebivolol in human myocardium has been performed.

In its unoccupied condition, the β -adrenoceptor exists in an equilibrium between its active (R^*) and inactive conformation (R). The binding of an agonist induces a conformational change towards R^* , whereas the binding of an inverse agonist stabilizes the inactive conformation of the receptor (Bond *et al.*, 1995). The R^* conformation allows the interaction of the receptor with the stimulatory G-protein (G_S). Subsequent dissociation of the α -subunit from the $\beta\gamma$ -subunits of G_S leads to stimulation of adenylate cyclase. Due to the influence of receptor systems and experimental conditions (i.e. different stoichiometry of receptors/G-proteins, different sensitization state of receptors etc.), the same ligand may behave as an inverse agonist, a neutral antagonist or even a partial agonist (de Ligt *et al.*, 2000; Chidiac *et al.*, 1996). In the present study, β -adrenoceptor-G-protein interaction of the novel β -blocker nebivolol is evaluated in comparison to bisoprolol by radioligand binding experiments with [125 I]-iodocyanopindolol in the absence and presence of guanylylimidodiphosphate (Gpp(NH)p). Furthermore, intrinsic activity of nebivolol is compared to bisoprolol, metoprolol, carvedilol and bucindolol by functional experiments on human myocardium.

In animal studies and cell systems, nebivolol was reported to be highly β_1 -selective (Pauwels *et al.*, 1989; 1991). However, up to date, no direct assessment of binding characteristics of nebivolol to human myocardial β_1 - and β_2 -adrenoceptors has been performed. In the present study, affinity of nebivolol and bisoprolol to β_1 - and β_2 -adrenoceptors is determined by radioligand binding experiments in the absence and presence of ICI 118.551 and CGP 207.12A, respectively. Furthermore, the results of these experiments on human myocardium are confirmed by binding studies on COS-7-cells that have been transfected with human β_1 - and β_2 -adrenoceptors, respectively.

Methods

Myocardial tissue

Experiments were performed on human right atrial trabeculae obtained during open-heart bypass surgery and on left ventricular muscle strip preparations or cell membrane preparations from failing and nonfailing human hearts obtained during cardiac transplantations. Tissue was brought to the laboratory and prepared for functional experiments immediately. Tissue samples for binding experiments were stored at -80°C until assay. Failing hearts were taken from 17 patients with end-stage heart failure (New York Heart Association (NYHA) class IV), resulting from either idiopathic dilated ($n=7$) or ischemic ($n=10$) cardiomyopathy (15 male, two female; age, 55 ± 1 years). Nonfailing hearts were taken from five organ donors whose hearts could not be used for transplantation (four male, one female; age 45 ± 4 years). For the latter group, echocardiography revealed normal left ventricular contractility. Bypass patients (mean age, 67 ± 2 years, 17 male, six female) had no signs of atrial dilation or arrhythmias or of left ventricular dysfunction. All

patients gave written informed consent before surgery. Since nebivolol and bisoprolol were mostly compared in tissue samples from the same hearts (radioligand binding and functional experiments), there were no significant differences between these two groups concerning i.e. age, sex and medication. Tissue preparation for functional experiments and membrane preparation for binding experiments has been described elsewhere (Maack *et al.*, 2000; Böhm *et al.*, 1990b).

Isolated cardiac muscle strip preparation and measurement of force of contraction

Isometric force of contraction was determined on isolated, electrically driven muscle preparations as described previously (Böhm *et al.*, 1990b). Bathing solution was maintained at 37°C , pH 7.4, and aerated with 95% O_2 and 5% CO_2 . Muscles of uniform size (length, 5–6 mm; thickness, 1–2 mm) were stretched to the length at which force of contraction was maximal (5–8 mN). In experiments with inotropic prestimulation, muscle strips were pre-exposed to isoprenaline ($1 \mu\text{mol l}^{-1}$, $\cong \text{EC}_{90}$) for 30 min and to forskolin ($0.3 \mu\text{mol l}^{-1}$, $\cong \text{EC}_{50}$) for at least 45 min. The β -adrenoceptor antagonists nebivolol (1 – $10,000 \text{ nmol l}^{-1}$), bisoprolol (1 – $10,000 \text{ nmol l}^{-1}$), bucindolol (0.1 – 1000 nmol l^{-1}), carvedilol (0.1 – 1000 nmol l^{-1}) and metoprolol (1 – $100,000 \text{ nmol l}^{-1}$) were applied cumulatively to the organ bath for 30 min at each concentration. When concentrations of β -blockers are indicated as K_i or $100 \times K_i$ (bar graphs), the results are also derived from these cumulative dose-response curves. In atrial myocardium, the normal decline of force of contraction was determined by a control group without β -blocker treatment. The decrease of contractility by the respective β -blocker was related to the decline in the control group. In ventricular myocardium, force of contraction in the control group remained constant.

Cell culture and transfection

COS-7 cells were cultured and transfected using the Transfectam reagent (Promega, Heidelberg, Germany) as described previously (Schnabel *et al.*, 1993). Membrane preparations were performed as described (Schnabel *et al.*, 1993), except that the pellet was resuspended in a buffer containing 50 mmol l^{-1} Tris/HCl, pH 7.4 and 10 mmol l^{-1} MgCl_2 .

β -adrenoceptor binding studies

β -adrenoceptors in cardiac tissue were investigated using [125 I]-iodocyanopindolol (ICYP) as the radiolabelled ligand. Cold ligand binding affinity was measured by ligand-ICYP competition curves using 37.5 pmol l^{-1} of ICYP to maintain the radioligand concentration at approximate K_d . The assay was performed in a total volume of $250 \mu\text{l}$. The total amount of protein used per assay was 20 – $30 \mu\text{g}$. Protein concentrations were determined according to the method of Lowry *et al.* (1951). The incubation at 25°C for 60 min allowed complete equilibration of the β -adrenoceptors with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters (Whatman Inc., Clifton, NJ, U.S.A.). The filters were washed immediately three times with 6 ml of ice-cold incubation buffer. All experiments were performed in triplicate.

Statistical analysis

Regression analysis was performed with the computer program GraphPadPrism (GraphPad Software, San Diego, CA, U.S.A.). Competition curve slope (pseudo-Hill coefficient, n_H), the concentration at which 50% of receptors are occupied (EC_{50}) and the percentage of receptors in a high affinity ($\%R_H$) or low affinity ($\%R_L$) state were determined by nonlinear regression analysis, comparing the fitting of the curve to either one or two receptor states by F-test analysis. Cold ligand dissociation constants for a high affinity (K_H) or a low affinity receptor state (K_L) were calculated according to the method of Cheng & Prusoff (1973). K_i values were calculated from EC_{50} values that were determined by fitting the results of competition experiments with a nonlinear regression analysis assuming only one receptor state, regardless if they actually reflect one or two affinity states.

Unless indicated, the data shown are mean \pm s.e.mean. For multiple comparisons, ANOVA analysis was performed. Otherwise, statistical significance was analysed with the Mann-Whitney- or Wilcoxon-test. A value of $P < 0.05$ was considered significant.

Materials

Nebivolol (a racemic mixture of equal parts of (\pm)-SRRR- and RSSS-nebivolol) was donated from Berlin Chemie, Berlin, Germany. (\pm)-Bisoprolol was purchased from Biotrend, Cologne, Germany. Carvedilol and bucindolol are from Smith Kline Beecham (King of Prussia, U.S.A.). Metoprolol was provided from Astra Chemicals (Hamburg, Germany). [125 I]-ICYP was produced by Amersham-Buchler (Freiburg i.Br., Germany). All other chemicals were of analytic grade or the best commercially available.

Results

Radioligand binding studies

Figure 1 shows representative results from competition experiments of the β -adrenoceptor ligands ICI 118.551, CGP 207.12A and isoprenaline on human nonfailing myocardium.

The highly selective ligands ICI 118.551 (β_2 -selective, Figure 1A) and CGP 207.12A (β_1 -selective, Figure 1B) exerted biphasic binding curves, allowing the discrimination of the total β -adrenoceptor population into approximately 70% β_1 - and 30% β_2 -adrenoceptors. When performing binding experiments in the presence of the indicated concentrations of ICI 118.551 and CGP 207.12A, respectively, binding characteristics of the studied ligand (nebivolol or bisoprolol) can be determined selectively on β_1 - and β_2 -adrenoceptors.

On myocardial membranes of the same heart, the agonist isoprenaline exerted a biphasic binding curve with the identification of a high- and a low-affinity binding site (Figure 1C). In the presence of guanylimidodiphosphate (Gpp(NH)p), a non-hydrolyzable guanine nucleotide, binding became monophasic with the detection of only one (low-affinity) binding site. As a consequence, the slope factor (n_H) steepened from 0.43 to 0.67. This indicates that in the examined heart, a fraction of 30% of β -adrenoceptors were in

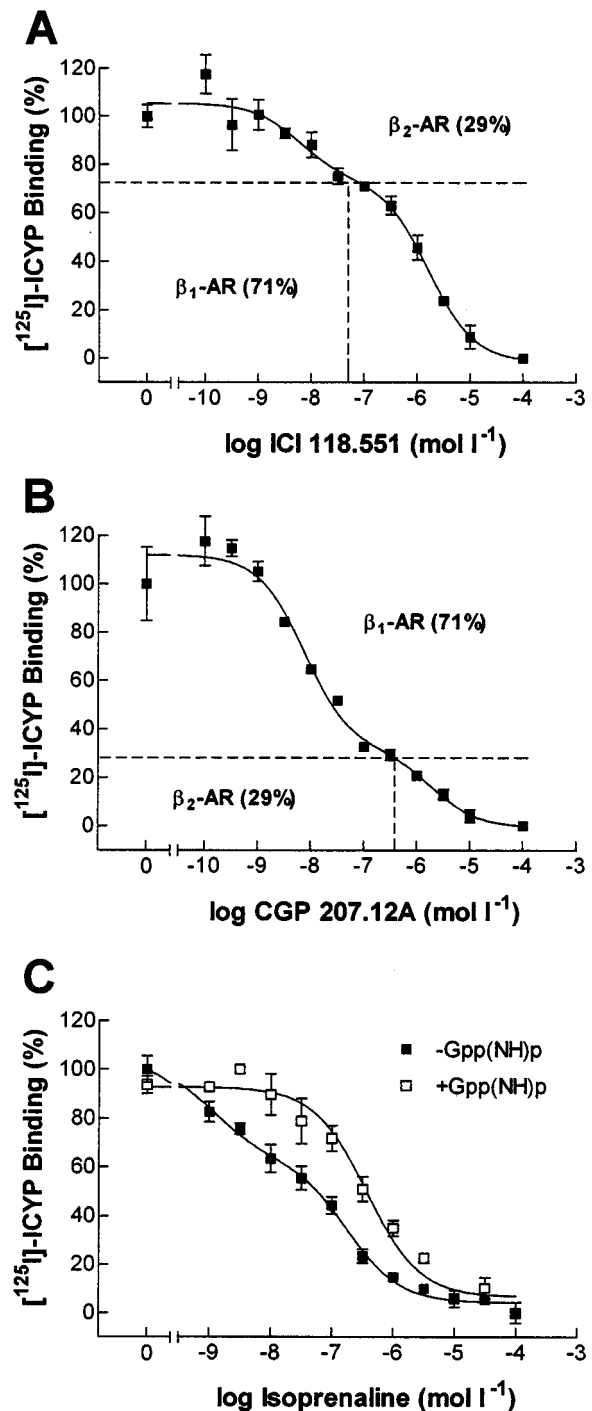


Figure 1 [125 I]-ICYP competition curves in human left ventricular myocardium from a nonfailing heart. (A) ICYP-ICI 118.511 competition. The identification of two affinity sites allows the discrimination into 29% β_2 - and 71% β_1 -adrenoceptors. (B) ICYP-CGP 207.12A competition. 71% β_1 - and 29% β_2 -adrenoceptors are identified. The indicated concentrations (dashed lines) are used to determine binding properties of β -adrenoceptor ligands at β_1 - and β_2 -adrenoceptors specifically. (C) ICYP-isoprenaline competition in the absence and presence of 100 $\mu\text{mol l}^{-1}$ of Gpp(NH)p. In the absence of Gpp(NH)p, 30% of adrenoceptors are in a high affinity state. This high affinity state is eliminated in the presence of Gpp(NH)p.

a high affinity state and thus sensitive for the detection of eventual agonist-like binding properties of the examined β -

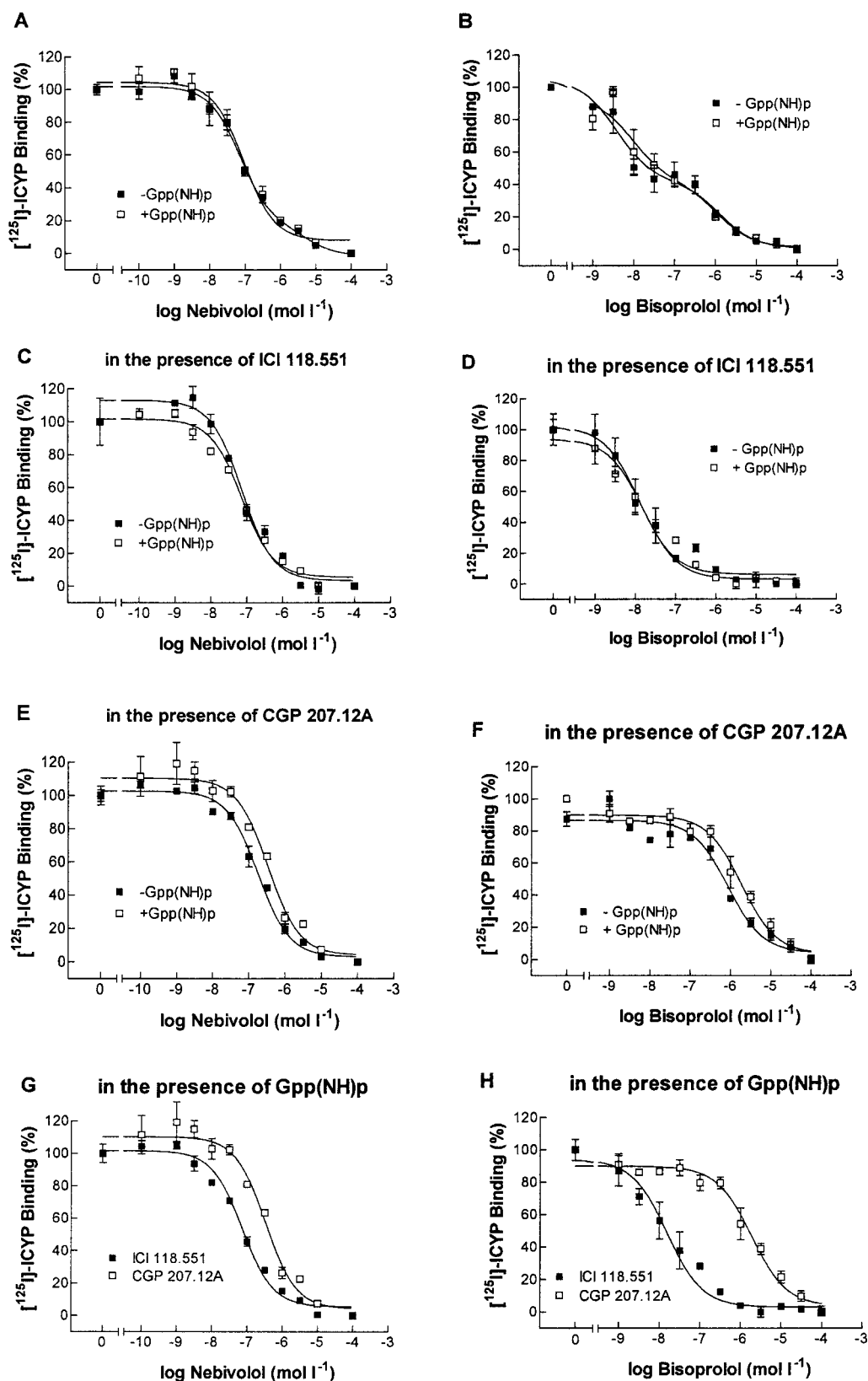


Figure 2 $[^{125}\text{I}]\text{-ICYP}$ competition curves in human left ventricular myocardium from a nonfailing heart. (A, C, E) ICYP-nebivolol competition in the absence and presence of $100 \mu\text{mol l}^{-1}$ of Gpp(NH)p. (B, D, F) ICYP-bisoprolol competition in the absence and presence of Gpp(NH)p. (A, B) competition experiments in a mixed β_1 - and β_2 -adrenoceptor population. (C, D) Experiments in the presence of ICI 118.551 (50 nmol l^{-1}), determining the affinity of the respective β -blocker to β_1 -adrenoceptors. (E, F) Experiments in the presence of CGP 207.12A (300 nmol l^{-1}), determining the affinity of the respective β -blocker to β_2 -adrenoceptors. (G, H) All experiments in the presence of Gpp(NH)p, in the presence of ICI 118.551 or CGP 207.12A, respectively, indicating β_1 -selectivity of the β -blockers.

blockers. The average amount of β -adrenoceptors in a high affinity state was $16 \pm 5\%$ (not shown).

In Figure 2, binding characteristics of nebivolol (left panels) and bisoprolol (right panels) are compared on a representative nonfailing heart. In Table 1, the cumulative results are listed. In the majority of experiments (11 of 16), nebivolol modelled for a one-site binding curve (Figure 2A). The slope factor of 0.90 ± 0.07 approached unity, indicating similar dissociation constants of nebivolol for β_1 - and β_2 -adrenoceptors. In the presence of Gpp(NH)p, in two experiments the two-affinity site binding was converted into monophasic curves (Table 1). However, neither the slope factor nor the pK_i -value were significantly affected by the presence of Gpp(NH)p. Table 2 indicates that there were no differences in the binding of nebivolol to β -adrenoceptors on nonfailing and failing myocardium.

In contrast to nebivolol, bisoprolol modelled for two distinct affinity sites in nine out of 10 experiments in the absence and in eight out of 10 experiments in the presence of Gpp(NH)p (Figure 2B, Table 1). The slope factors in both cases were significantly different from unity and also from the slope factors of nebivolol. Neither pK_i -values nor slope factors of bisoprolol were affected in the presence of Gpp(NH)p. Also, binding characteristics did not differ between nonfailing and failing myocardium (Table 2).

In the presence of ICI 118.551 and CGP 207.12A, respectively, binding curves revealed slightly higher pK_i -values for nebivolol on β_1 - compared to β_2 -adrenoceptors (Figure 2C,E, Table 1). The calculated β_2/β_1 -ratio, expressing β_1 -selectivity, was 2.8 ± 0.8 in the absence and 4.5 ± 2.2 in the presence of Gpp(NH)p (Table 1). Slope factors were not affected by the presence of ICI 118.551 or CGP 207.12A, respectively. In contrast, in the majority of the experiments with bisoprolol in the presence of ICI 118.551 or CGP 207.12A, respectively, binding curves were converted into monophasic curves. The slope factors steepened significantly towards unity and the pK_i -values were significantly different between β_1 - and β_2 -adrenoceptors (Table 1). The calculated β_2/β_1 -ratio was 19.7 ± 9.7 in the absence and 15.6 ± 5.8 in the presence of Gpp(NH)p and significantly higher compared to nebivolol. In the presence of ICI 118.551 or CGP 207.12A, respectively, Gpp(NH)p neither affected binding characteristics of nebivolol nor of bisoprolol.

Another way of calculating β_1 -selectivity of a compound is to determine the dissociation constants of the high- and the

low-affinity site in two-site fitted binding curves. Table 3 gives the results discriminated into experiments with the detection of two and experiments with the detection of only one affinity site. According to the calculation of β_1 -selectivity from the high- and low-affinity binding site, the β_1 -selectivity was substantially higher for both compounds compared to the calculation in Table 1. Again, bisoprolol displayed higher β_1 -selectivity in the absence (143 vs 76) as well as in the presence of Gpp(NH)p (242 vs 66) compared to nebivolol. However, due to the limited number of experiments in the nebivolol group, this difference was statistically not significant. Also, it has to be considered that nebivolol modelled for two-site binding in the minority and bisoprolol in the majority of experiments. Thus, these selectivity data do not represent the cumulative results of all experiments.

Table 4 displays the pK_i -values from binding experiments in COS-7-cells transfected with human β_1 - and β_2 -adrenoceptors, respectively. The β_2/β_1 -ratios for nebivolol (3.0) and bisoprolol (15.1) were in agreement with the selectivity ratios derived from experiments on human myocardium (Table 1).

Functional studies

In order to estimate the implications of β -adrenoceptor binding properties of nebivolol and bisoprolol on myocardial

Table 2 Radioligand binding experiments in failing and nonfailing myocardium

Agent	GppNHp	n (2-s-f/total)	pK_i (mol l ⁻¹)	n_H	
Nebivolol	Nonfailing	–	2 / 5	7.23 ± 0.12	0.83 ± 0.13
		+	1 / 5	7.24 ± 0.10	0.75 ± 0.06
	Failing	–	3 / 11	7.53 ± 0.17	0.93 ± 0.09
		+	2 / 11	7.43 ± 0.16	0.80 ± 0.09
Bisoprolol	Nonfailing	–	3 / 3	6.91 ± 0.48	0.51 ± 0.14
		+	3 / 3	6.71 ± 0.33	0.56 ± 0.13
	Failing	–	6 / 7	6.83 ± 0.06	0.48 ± 0.05
		+	5 / 7	6.85 ± 0.14	0.57 ± 0.09

GppNHp, in the absence (–) and presence (+) of Gpp(NH)p; n (2-s-f/total), number of experiments modelled for a two-affinity site fit/number of all experiments; n_H , pseudo Hill coefficient (slope factor).

Table 1 Radioligand binding experiments in human ventricular myocardium

Agent	GppNHp	n (2-s-f/total)	pK_i (mol l ⁻¹)	n_H	Selectivity	
Nebivolol	–	5 / 16	7.44 ± 0.13	0.90 ± 0.07		
	+	3 / 16	7.37 ± 0.11	0.79 ± 0.06		
	+ ICI	–	3 / 15	7.32 ± 0.28	0.81 ± 0.10	
	+	3 / 15	7.29 ± 0.24	0.76 ± 0.08		
	+ CGP	–	1 / 15	7.08 ± 0.12	0.84 ± 0.06	2.77 ± 0.81
Bisoprolol	+	0 / 15	6.98 ± 0.20	1.01 ± 0.08	4.46 ± 2.19	
	–	9 / 10	6.87 ± 0.13	0.49 ± 0.05***		
	+	8 / 10	6.81 ± 0.13	0.57 ± 0.07*		
	+ ICI	–	2 / 7	7.11 ± 0.25	0.76 ± 0.05*	
	+	1 / 7	7.18 ± 0.31	0.78 ± 0.13		
	+ CGP	–	0 / 7	6.38 ± 0.10	1.00 ± 0.15*	19.7 ± 9.67*
	+	0 / 7	6.16 ± 0.15	0.82 ± 0.07**	15.6 ± 5.75*	

* $P < 0.05$; ** $P < 0.005$ vs –ICI/-CGP; *** $P < 0.001$ vs Nebivolol. GppNHp, in the absence (–) and presence (+) of Gpp(NH)p; n (2-s-f/total), number of experiments modelled for a two-affinity site fit/number of all experiments; n_H , pseudo Hill coefficient (slope factor).

Table 3 Radioligand binding experiments – subgroup analysis

Agent	G	fit	n	f_H (%)	K_H (nmol l ⁻¹)	K_L (nmol l ⁻¹)	pK_H (mol l ⁻¹)	pK_L (mol l ⁻¹)	n_H	β_2/β_1
Nebi	–	2	5	57.6±9.7	22.3±9.2	1473±719	7.89±0.28	6.03±0.24	0.61±0.05	76±13
		1	11	—	—	87.2±59.1	—	7.56±0.17	1.03±0.07	—
	+	2	3	47.0±27.6	30.2±15.8	1557±1144	7.71±0.35	6.12±0.40	0.55±0.06	66±25
Biso	–	1	13	—	—	74.6±34.4	—	7.47±0.11	0.84±0.07	—
		2	9	43.8±3.8	23.7±14.9	856±218	8.13±0.22	6.15±0.09	0.47±0.05	143±41
	1	1	—	—	171	—	6.77	0.72	—	
	+	2	8	51.5±4.8	41.7±32.9	1474±600	8.01±0.35	6.12±0.20	0.51±0.06	242±113
	1	2	—	—	210.5±48.5	—	6.69±0.10	0.83±0.18	—	

G, in the absence (–) and presence (+) of Gpp(NH)p; fit, experiments modelled for a two- (2) or one- (1) affinity site fit; f_H , fraction of receptors in a high affinity state; K_H , dissociation constant of high affinity state receptors; K_L , dissociation constant of low affinity state receptors; n_H , pseudo Hill coefficient (slope factor); β_2/β_1 , β_1 -selectivity; Nebi/Biso, nebivolol/bisoprolol.

Table 4 Radioligand binding studies on COS-7-cells

Agent	AR	n	pK_i (mol l ⁻¹)	β_2/β_1
Nebivolol	β_1	4	8.20±0.12	3.0
	β_2	7	7.79±0.15	
Bisoprolol	β_1	3	7.82±0.08	15.1
	β_2	3	6.69±0.17	

AR, adrenergic receptor.

Table 5 Force of contraction of ventricular myocardium at baseline and after treatment

Stimulation	Agent	n	Baseline (mN)	Treatment (mN)
Isoprenaline	Nebivolol	4	2.2±0.3	5.6±0.4
	Bisoprolol	7	2.3±0.6	4.7±1.1
Forskolin	Nebivolol	10	2.1±0.4	3.3±0.6
	Bisoprolol	6	2.1±0.9	3.5±0.9

contractile function, experiments on left ventricular and right atrial muscle strips from failing and nonfailing hearts were performed.

In contraction experiments, muscles were stretched to the length at which force of contraction was maximal. In all experiments on ventricular as well as atrial myocardium, diastolic tension did not differ between different treatment groups (not shown). In human ventricular myocardium, the application of 1 $\mu\text{mol l}^{-1}$ of isoprenaline enhanced basal force of contraction by 123±16% ($n=11$). After equilibration, cumulative concentrations of nebivolol or bisoprolol were added to the organ bath. There were no significant differences among the three groups in basal as well as in isoprenaline-enhanced force of contraction (Table 5). At concentrations occupying 50% (K_i) and 100% ($100 \times K_i$) of β -adrenoceptors, both β -blockers significantly antagonized isoprenaline enhanced force of contraction (Figure 3A,B).

In order to determine the intrinsic activity of both agents, the coupling of β -adrenoceptors to adenylate cyclase was facilitated by forskolin (0.3 $\mu\text{mol l}^{-1}$). By this method, both partial agonist (Böhm *et al.*, 1990b) as well as inverse agonist activity (Mewes *et al.*, 1993) of ligands can be detected. Forskolin increased force of contraction by 81±20% ($n=16$). Nebivolol and bisoprolol induced similar decreases of contractility at the cumulative concentrations (Figure 3C). Accordingly, when relating the decrease of contractility to the respective K_i -values, the decrease of contractility was not different between both agents (Figure 3D). Also in myocardium not pretreated with forskolin or isoprenaline, force of contraction was significantly reduced by both β -blockers (Figure 4).

When examining intrinsic activity of β -adrenoceptor antagonists, it is of importance to compare various compounds within the same system, since intrinsic activity of ligands is strongly dependent on the underlying tissue type (de Ligt *et al.*, 2000). Thus, we compared the intrinsic activity

of nebivolol and bisoprolol to the one of bucindolol, carvedilol and metoprolol in human atrial myocardium. In Figure 5, the effects of all agents on contractility are compared at concentrations occupying 50% (Figure 5A) and 100% (Figure 5B) of β -adrenoceptors. At both conditions, bisoprolol and nebivolol displayed significantly higher amounts of inverse agonism compared to bucindolol and lower amounts compared to metoprolol. Carvedilol exerted slightly lower inverse agonism compared to nebivolol and bisoprolol. In general, β -blockers with Gpp(NH)p-modulatable binding properties, like bucindolol and carvedilol (Yoshikawa *et al.*, 1996; Maack *et al.*, 2000), displayed less inverse agonist activity than agents without these properties. A typical feature of inverse agonists is that their negative inotropic effect can be antagonized by a ligand with lower inverse agonist activity (ideally a neutral antagonist). In Figure 6 it is illustrated that in human myocardium pretreated with forskolin, the negative inotropic effect of nebivolol could be antagonized in the presence of bucindolol.

Discussion

The main findings of the present study are that in human myocardium, nebivolol and bisoprolol behave as inverse agonists. The rank order of inverse agonism in human myocardium is metoprolol >>bisoprolol = nebivolol \geq carvedilol > bucindolol. Furthermore, nebivolol is nonselective for myocardial β_1 - and β_2 -adrenoceptors, while bisoprolol is 16–20 fold β_1 -selective.

Intrinsic activity

Determination of intrinsic activity in intact human myocardium is complicated by several mechanisms. In myocardium from patients with heart failure, β -adrenoceptor density is

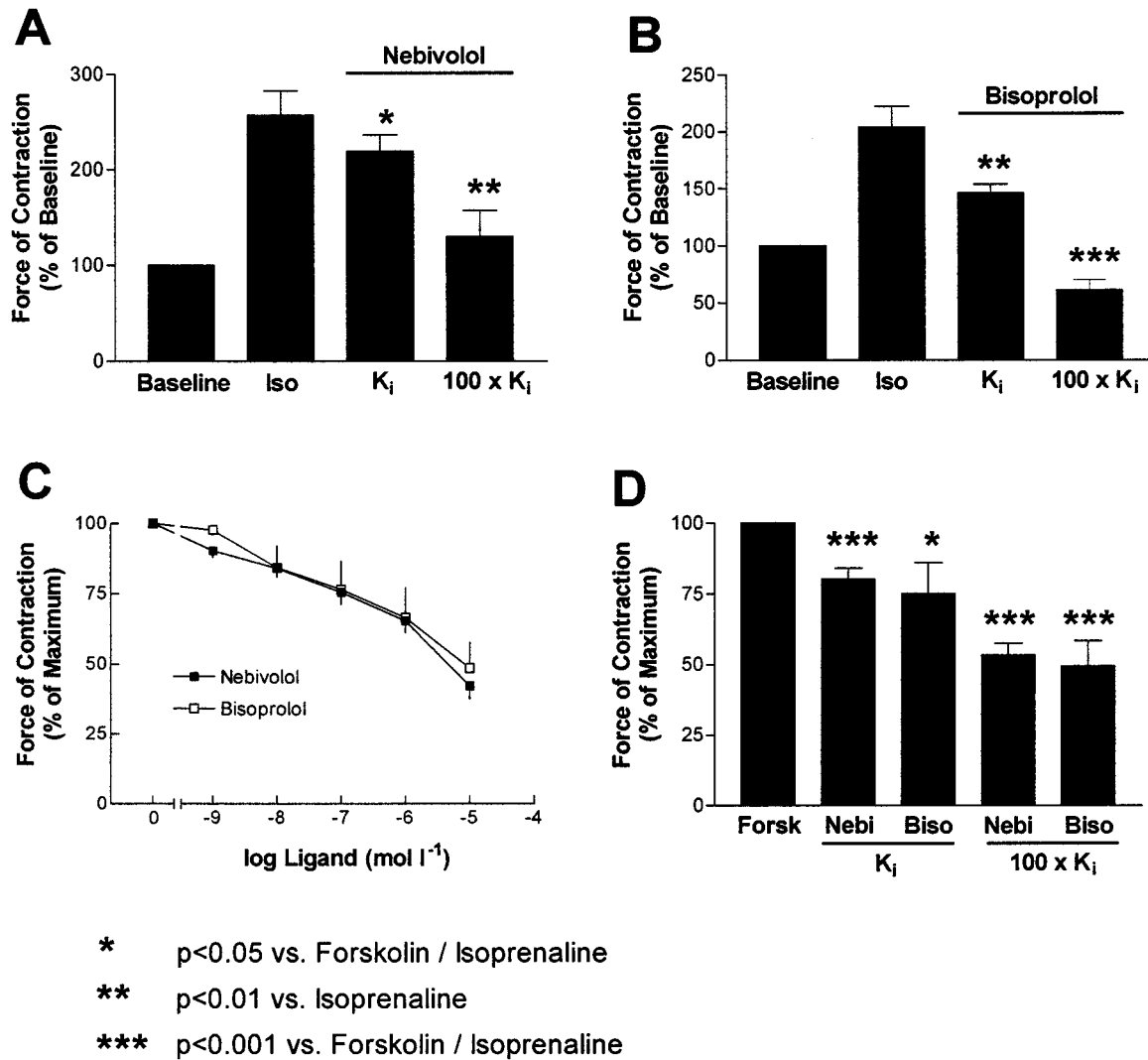


Figure 3 (A, B) Effects of nebivolol ($n=4$) and bisoprolol ($n=7$) on isoprenaline ($1 \mu\text{mol l}^{-1}$) prestimulated force of contraction in human ventricular myocardium. Both β -blockers significantly inhibit the isoprenaline induced increase in force of contraction. (C) Cumulative dose-response curves of nebivolol ($n=10$) and bisoprolol ($n=6$) in the presence of forskolin ($0.3 \mu\text{mol l}^{-1}$). (D) Inotropic effect of nebivolol and bisoprolol at concentrations occupying 50% (K_i) and 100% ($100 \times K_i$) of β -adrenoceptors. Data are derived from cumulative dose-response curves (C), related to the respective dissociation constants determined in radioligand binding experiments (Table 1). Concentrations are as follows: nebivolol, 0.03 (K_i) and $3 \mu\text{mol l}^{-1}$ ($100 \times K_i$); bisoprolol, 0.1 (K_i) and $10 \mu\text{mol l}^{-1}$ ($100 \times K_i$).

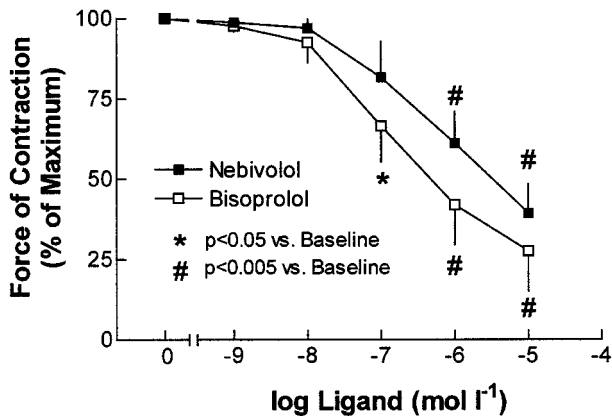


Figure 4 Effect of cumulative doses of nebivolol ($n=10$) and bisoprolol ($n=6$) on force of contraction in human atrial myocardium.

reduced (Bristow *et al.*, 1982), and remaining receptors are uncoupled from G_s (Hausdorff *et al.*, 1990). In addition, increased membrane concentrations of inhibitory G-protein α -subunits (Böhm *et al.*, 1990a) might also reduce partial agonist or inverse agonist responses. Therefore, contraction experiments were performed in the presence of forskolin, a diterpene that facilitates the coupling of $G_{s\alpha}$ to adenylate cyclase. By this method, effects of partial agonists and also inverse agonists become detectable (Böhm *et al.*, 1990b).

With this approach, in a recent study we determined intrinsic activity of bucindolol, carvedilol and metoprolol (Maack *et al.*, 2000). In the mentioned study, metoprolol behaved as a strong inverse agonist, while bucindolol and carvedilol displayed $G_{pp(NH)p}$ -modulatable binding and significantly lesser amounts of inverse agonist activity compared to metoprolol. In a part of the experiments, bucindolol even behaved as a partial agonist. Since in some

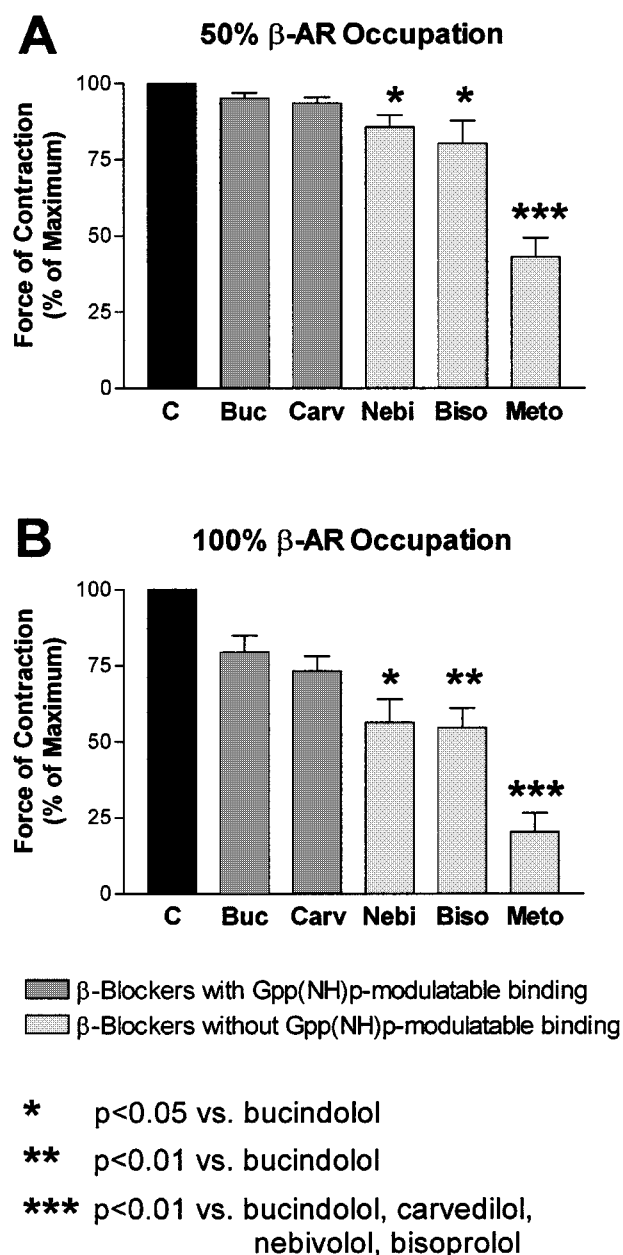


Figure 5 Inotropic effects of bucindolol ($n=15$), carvedilol ($n=8$), nebivolol ($n=16$), bisoprolol ($n=12$) and metoprolol ($n=6$) on human atrial myocardium in the presence of forskolin ($0.3 \mu\text{mol l}^{-1}$) at concentrations occupying 50% (K_i , panel A) and 100% ($100 \times K_i$, panel B) of β -adrenoceptors. K_i -values are derived from radioligand binding studies of this study (Table 1, nebivolol and bisoprolol, see also legend to Figure 3) and from Maack *et al.* (2000) for bucindolol (0.01 (K_i) and $1 \mu\text{mol l}^{-1}$ ($100 \times K_i$)), carvedilol (0.001 (K_i) and $0.1 \mu\text{mol l}^{-1}$ ($100 \times K_i$)) and metoprolol (1 (K_i) and $100 \mu\text{mol l}^{-1}$ ($100 \times K_i$)). Force of contraction is related to the respective vehicle-control groups.

studies (Willette *et al.*, 1998; 1999; Trochu *et al.*, 1999; Maack *et al.*, 2000) bucindolol had revealed partial agonist effects, yet not in other studies (Hershberger *et al.*, 1990; Bristow *et al.*, 1992), intrinsic activity of β -blockers can be system-dependent.

For nebivolol, in a study on reserpinized dogs and spontaneously hypertensive rats, no partial agonist activity could be detected (Janssens *et al.*, 1989). In the pithed rat

model of normal and heart failure rats, bisoprolol also had no partial agonist activity (Willette *et al.*, 1999). In the present study, contraction experiments in human myocardium were performed in the presence of forskolin. Stimulation of adenylate cyclase by forskolin is dependent on the presence of $G_{S\alpha}$ (Darfler *et al.*, 1982), but the dissociation of $G_{S\alpha}$ from the $\beta\gamma$ subunits of the G-protein only occurs when the receptor is in an activated conformation (R^*). The negative inotropic actions of nebivolol and bisoprolol in the presence of forskolin may reflect a reduced G_S -mediated stimulation of adenylate cyclase and thus indicate that both β -blockers are inverse agonists that primarily stabilize the inactive conformation of the β -adrenoceptor (R). Accordingly, neither nebivolol nor bisoprolol induce a dissociation of $G_{S\alpha}$ from the $\beta\gamma$ -subunits as estimated by radioligand binding experiments in the absence and presence of Gpp(NH)p.

When examining inverse agonist activity of β -blockers in intact tissue, it is of importance to rule out that the negative inotropic effects of the compounds are due to the presence of contaminating endogenous catecholamines. This can be done by performing contraction experiments in the presence of a rather weak inverse agonist (ideally a 'neutral antagonist'). In our experiments, the negative inotropic effect of nebivolol could be specifically antagonized in the presence of bucindolol. This further indicates that the negative inotropic effect of nebivolol can be related to its action on β -adrenoceptors and is not due to a decrease of myofibrillar calcium sensitivity (Zeit *et al.*, 2000) or non-specific effects.

The activation state of β -adrenoceptors has an impact on receptor regulation. Benovic *et al.* (1988) observed a correlation of the intrinsic activity of partial and full agonists with the degree of receptor phosphorylation by β -adrenergic receptor kinase (β -ARK), which is a prerequisite for receptor desensitization and downregulation (Hausdorff *et al.*, 1990). In mice transfected with a constitutively active mutant of the β_2 -adrenoceptor, treatment with the inverse agonist ICI 118.551 led to a 50 fold upregulation of myocardial β -adrenoceptor density (Samana *et al.*, 1997). According to these data, it may be hypothesized that in patients with heart failure, phosphorylation of the β -adrenoceptor by β -ARK and subsequent desensitization or even down-regulation might be effectively prevented by nebivolol and bisoprolol. In fact, in patients undergoing coronary artery bypass grafting, chronic treatment with bisoprolol increased myocardial β_1 -adrenoceptor density (Motomura *et al.*, 1990). For nebivolol, no data concerning its effects on β -adrenoceptor regulation exist.

β_1 -selectivity

Another important feature of β -blockers is selectivity for β_1 -adrenoceptors. Nebivolol is frequently termed a β_1 -selective compound. In fact, in a study of van de Water *et al.* (1988) nebivolol revealed a 293 fold β_1 -selectivity. However, in that study dissociation constants were determined by Schild plot analysis of inhibitory effects of nebivolol on β -adrenergic responsiveness of right atrial (β_1) vs tracheal (β_2) tissue in guinea-pigs. Pauwels *et al.* (1989) performed radioligand binding studies on rabbit lung (β_1 -) and rat lung (β_2 -) adrenoceptors and observed a 48–55 fold β_1 -selectivity of nebivolol. In CHO-cells transfected with human β_1 - and β_2 -adrenoceptors, the same group (Pauwels *et al.*, 1991) found

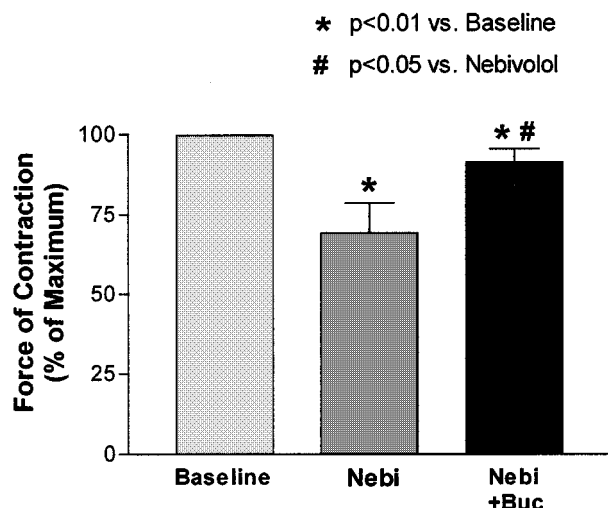


Figure 6 The negative inotropic effect of nebivolol (Nebi, $1 \mu\text{mol l}^{-1}$) in human atrial myocardium was antagonized in the presence of bucindolol (Buc, $0.1 \mu\text{mol l}^{-1}$). Experiments ($n=5$) were carried out in the presence of forskolin ($0.3 \mu\text{mol l}^{-1}$).

Table 6 Force of contraction of atrial myocardium at baseline and after treatment

Stimulation	Agent	n	Baseline (mN)	Treatment (mN)
Isoprenaline	Nebivolol	11	3.3 ± 0.4	9.0 ± 0.7
	Bisoprolol	12	3.6 ± 0.4	9.1 ± 1.0
Forskolin	Nebivolol	16	3.5 ± 0.6	5.6 ± 0.7
	Bisoprolol	12	3.4 ± 0.6	5.7 ± 1.1
	Bucindolol	15	3.9 ± 0.6	6.9 ± 0.8
	Carvedilol	8	2.6 ± 0.5	3.9 ± 0.6
	Metoprolol	6	4.3 ± 1.3	7.5 ± 1.7
No pretreatment	Nebivolol	10	6.4 ± 0.8	—
	Bisoprolol	6	6.1 ± 0.9	—

only a 10 fold β_1 -selectivity of nebivolol assessed by radioligand binding studies. In the present study, in human ventricular myocardium β_1 -selectivity of nebivolol was 2–4 fold, which is more or less non-selective. Also in COS-7 cells transfected with human β_1 - and β_2 -adrenoceptors, selectivity was only 3 fold. In contrast, the β_1 -selectivity of bisoprolol was 16–20 fold in human myocardium and 15 fold in COS-7 cells.

The importance of β_1 -selectivity in the treatment of patients with heart failure is controversial. Both the β_1 -

selective agents bisoprolol (CIBIS II, 1999) and metoprolol (MERIT-HF, 1999) as well as the non-selective compound carvedilol (Packer *et al.*, 1996) have improved survival in heart failure. However, since carvedilol has also α -blocking (Bristow *et al.*, 1992) and antioxidative effects (Yue *et al.*, 1992), it is difficult to discriminate which pharmacological properties lead to its beneficial effects in the treatment of patients with heart failure. Accumulated evidence from experimental *in vivo* and *in vitro* studies indicate that a blockade of all adrenergic signal transduction pathways may be of particular importance in the treatment of heart failure (Bristow, 2000). In two meta-analyses of the effects of β -blockers on mortality in patients after myocardial infarction, both selective and non-selective compounds reduced mortality (Soriano *et al.*, 1997; Freemantle *et al.*, 1999). In contrast, agents with intrinsic sympathomimetic activity (ISA) were associated with a lower risk-reduction compared to agents without ISA. These data indicate that among different features of β -blockers, especially intrinsic activity, but not β_1 -selectivity is important in terms of prognosis in cardiovascular diseases.

Besides β -adrenoceptor antagonistic effects, nebivolol has vasodilatory effects mediated by the endothelial release of nitric oxide (Bowman *et al.*, 1994; Cockcroft *et al.*, 1995). A recent study indicates that these effects are at least in part due to active metabolites of nebivolol that may act *via* the stimulation of endothelial β_2 -adrenergic receptors (Broeders *et al.*, 2000). Thus, *in vivo* effects of nebivolol may be slightly different compared to the *in vitro* effects. However, the present study investigates intrinsic activity of β -blockers independent of haemodynamically confounding effects on the vasculature. In this respect, these experiments might possess some advantage compared with *in vivo* experiments.

The present data for the first time characterize the β -adrenergic effects of nebivolol in human myocardium. The lack of G-protein interaction as well as inverse agonist activity are desirable effects of a β -blocker in the treatment of heart failure. The observation that in human myocardium, nebivolol is non-selective sheds new light on the pharmacological actions of this agent. Further studies are needed to clarify the special haemodynamic profile of nebivolol.

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References

- BENOVIC, J.L., STANISZEWSKI, C., MAYOR, F., CARON, M.G. & LEFKOWITZ, R.J. (1988). β -Adrenergic receptor kinase. *J. Biol. Chem.*, **263**, 3893–3897.
- BÖHM, M., GIERSCHIK, P., JAKOBS, K.-H., PIESKE, B., SCHNABEL, P., UNGERER, M. & ERDMANN, E. (1990a). Increase of G_{i2} in human hearts with dilated but not ischemic cardiomyopathy. *Circulation*, **82**, 1249–1265.
- BÖHM, M., MITTMANN, C., SCHWINGER, R.H.G. & ERDMANN, E. (1990b). Effects of xamoterol on inotropic and lusitropic properties of the human myocardium and on adenylate cyclase activity. *Am. Heart J.*, **120**, 1381–1392.
- BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMAN, H.A., MCMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2 -adrenoceptor. *Nature*, **374**, 272–276.
- BOWMAN, A.J., CHEN, C.P. & FORD, G.A. (1994). Nitric oxide mediated venodilator effects of nebivolol. *Br. J. Clin. Pharmacol.*, **38**, 199–204.
- BRISTOW, M.R. (2000). β -Adrenergic receptor blockade in chronic heart failure. *Circulation*, **101**, 558–569.

- BRISTOW, M.R., GINSBURG, R., MINOBE, W., CUBICCIOTTI, R.S., SAGEMAN, W.S., LURIE, K., BILLINGHAM, M.E., HARRISON, D.E. & STINSON, E.B. (1982). Decreased catecholamine sensitivity and β -adrenergic receptor density in failing human hearts. *N. Engl. J. Med.*, **307**, 205–211.
- BRISTOW, M.R., LARRABEE, P., MINOBE, W., RODEN, R., SKERL, L., KLEIN, J., HANDWERGER, D., PORT, J.D. & MÜLLER-BECKMANN, B. (1992). Receptor pharmacology of carvedilol in the human heart. *J. Cardiovasc. Pharmacol.*, **19** (Suppl. 1), S68–S80.
- BROEDERS, M.A.W., DOEVEDANS, P.A., BEKKERS, B.C.A.M., BRONSAER, R., VAN GORSEL, E., HEEMSKERK, J.W.M., OUDE EGBRINK, M.G.A., VAN BREDA, E., RENEMAN, R.S. & VAN DER ZEE, R. (2000). Nebivolol: A third-generation β -blocker that augments vascular nitric oxide release. *Circulation*, **102**, 677–684.
- CHENG, Y.C. & PRUSSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzyme reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- CHIDIAC, P., NOUET, S. & BOUVIER, M. (1996). Agonist-induced modulation of inverse agonist efficacy at the β_2 -adrenergic receptor. *Mol. Pharmacol.*, **50**, 662–669.
- CIBIS II INVESTIGATORS AND COMMITTEES (1999). The Cardiac Insufficiency Bisoprolol Study (CIBIS-II): a randomized trial. *Lancet*, **353**, 9–13.
- COCKCROFT, J.R., CHOWIENCZYK, P.J., BRETT, S.E., CHEN, C.P., DUPONT, A.G., VAN NUETEN, L., WOODING, S.J. & RITTER, J.M. (1995). Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. *J. Pharmacol. Exp. Ther.*, **274**, 1067–1071.
- DARFLER, F.J., MAHAN, L.C., KOACHMAN, A.M. & INSEL, P.A. (1982). Stimulation of forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. *J. Biol. Chem.*, **257**, 11901–11907.
- DE LIGHT, R.A.F., KOUROUNAKIS, A.P. & IJZERMAN, A.P. (2000). Inverse agonism at G-protein-coupled receptors: (patho)-physiological relevance and implications for drug discovery. *Br. J. Pharmacol.*, **130**, 1–12.
- FREEMANTLE, N., CLELAND, J., YOUNG, P., MASON, J. & HARRISON, J. (1999). β -Blockade after myocardial infarction: systematic review and meta regression analysis. *Br. J. Med.*, **318**, 1730–1737.
- HAUSDORFF, W.P., CARON, M.G. & LEFKOWITZ, R.J. (1990). Turning off the signal: Desensitization of β -adrenergic receptor function. *FASEB J.*, **4**, 2881–2889.
- HERSHBERGER, R.E., WYNN, J.R., SUNDBERG, L. & BRISTOW, M.R. (1990). Mechanisms of action of bucindolol in human ventricular myocardium. *J. Cardiovasc. Pharmacol.*, **15**, 959–967.
- JANSSENS, W.J., VAN DE WATER, A., XHONNEUX, R., RENEMAN, R.S., VAN NUETEN, J.M. & JANSSEN, P.A. (1989). Nebivolol is devoid of intrinsic sympathomimetic activity. *Eur. J. Pharmacol.*, **159**, 89–95.
- LOWRY, O.H., ROSENBOUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- MAACK, C., CREMERS, B., FLESCH, M., HÖPER, A., SÜDKAMP, M. & BÖHM, M. (2000). Different intrinsic activities of bucindolol, carvedilol and metoprolol in human failing myocardium. *Br. J. Pharmacol.*, **130**, 1131–1139.
- MANGRELLA, M., ROSSI, F., FICI, F. & ROSSI, F. (1998). Pharmacology of nebivolol. *Pharmacol. Res.*, **38**, 419–431.
- MERIT-HF STUDY GROUP (1999). Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). *Lancet*, **353**, 2001–2007.
- MEWES, T., DUTZ, S., RAVENS, U. & JAKOBS, K.H. (1993). Activation of calcium currents in cardiac myocytes by empty beta-adrenoceptors. *Circulation*, **88**, 2916–2922.
- MOTOMURA, S., DEIGHTON, N.M., ZERKOWSKI, H.R., KHAMSSI, M. & BRODDE, O.E. (1990). Differential regulation of human cardiac beta-adrenergic and muscarinic receptors by chronic beta-adrenoceptor antagonist treatment. *Br. J. Clin. Pharmacol.*, **30** (Suppl. 1), 112S–114S.
- NICHOLAS, G., OAKLEY, C., POULEUR, H., ROUSSEAU, M.F., RYDÉN, L.E. & WELLENS, H. for The Xamoterol In Heart Failure Study Group. (1990). Xamoterol in severe heart failure. *Lancet*, **336**, 1–6.
- PACKER, M., BRISTOW, M.R., COHN, J.N., COLUCCI, W., FOWLER, M.B., GILBERT, E.M. & SHUSTERMAN, N.H. for the US Carvedilol Heart Failure Study Group. (1996). The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N. Engl. J. Med.*, **334**, 1349–1355.
- PAUWELS, P.J., GOMMEREN, W., VAN LOMMEN, G., JANSSEN, P.A.J. & LEYSEN, J.E. (1989). The receptor binding profile of the new antihypertensive agent nebivolol and its stereoisomers compared with various β -adrenergic blockers. *Mol. Pharmacol.*, **34**, 843–851.
- PAUWELS, P.J., VAN GOMPEL, P. & LEYSEN, J.E. (1991). Human β_1 - and β_2 -adrenergic receptor binding and mediated accumulation of cAMP in transfected chinese hamster ovary cells. *Biochem. Pharmacol.*, **42**, 1683–1689.
- SAMANA, P., COTECCHIA, S., COSTA, T. & LEFKOWITZ, R.J. (1993). A mutation-induced activated state of the β_2 -adrenergic receptor. *J. Biol. Chem.*, **268**, 4625–4636.
- SCHNABEL, P., CAMPS, M., CAROZZI, A., PARKER, P.J. & GIERSCHIK, P. (1993). Mutational analysis of phospholipase C- β 2: Identification of regions required for membrane association and stimulation by G-protein $\beta\gamma$ subunits. *Eur. J. Biochem.*, **217**, 1109–1115.
- SORIANO, J.B., HOES, A.W., MEEMS, L. & GROBBEE, D.E. (1997). Increased survival with β -blockers: importance of ancillary properties. *Prog. Cardiovasc. Dis.*, **39**, 445–456.
- STOLERU, L., WIJNS, W., VAN EYLL, C., BOUVY, T., VAN NUETEN, L. & POULEUR, H. (1993). Effects of d-nebivolol and l-nebivolol on left ventricular systolic and diastolic function: comparison with d-l-nebivolol and atenolol. *J. Cardiovasc. Pharmacol.*, **22**, 183–190.
- TROCHU, J.N., ERFANIAN, M., KHANDOUDI, N., BARON, O., BRIL, A. & GAUTHIER, C. (1999). Carvedilol produces contractile effects different from those of bucindolol in human atria. *Circulation*, **100**, 1–439 (abstract).
- VAN DE WATER, A., JANSSENS, W., VAN NEUTEN, J., XHONNEUX, R., DE CREE, J., VERHAEGEN, H., RENEMAN, R.S. & JANSSEN, P.A. (1988). Pharmacological and hemodynamic profile of nebivolol, a chemically novel, potent, and selective β_1 -adrenergic receptor antagonist. *J. Cardiovasc. Pharmacol.*, **11**, 552–563.
- WILLETTE, R.N., AIYAR, N., YUE, T.L., MITCHELL, M.P., DISA, J., STORER, B.L., NASELSKY, D.P., STADEL, J.M., OHLSTEIN, E.H. & RUFFOLO, Jr. R.R. (1999). In vitro and in vivo characterization of intrinsic sympathomimetic activity in normal and heart failure rats. *J. Pharmacol. Exper. Therap.*, **289**, 48–53.
- WILLETTE, R.N., MITCHELL, M.P., OHLSTEIN, E.H., LUKAS, M.A. & RUFFOLO, JR. R.R. (1998). Evaluation of intrinsic sympathomimetic activity of bucindolol and carvedilol in rat heart. *Pharmacology*, **56**, 30–36.
- YOSHIKAWA, T., PORT, J.O., ASANO, K., CHIDIAC, P., BOUVIER, M., DUTCHER, D., RODEN, R.L., MINABE, W., TREMMEL, K.O. & BRISTOW, M.R. (1996). Cardiac adrenergic receptor effects of carvedilol. *Eur. Heart J.*, **17**, (Suppl. B): 8–16.
- YUE, T.L., CHENG, H.Y., LYSKO, P.G., MCKENNA, P.J., FEUERSTEIN, R., GU, J.L., LYSKO, K.A., DAVIS, L.L. & FEUERSTEIN, G. (1992). Carvedilol, a new vasodilator and beta adrenoceptor antagonist, is an antioxidant and free radical scavenger. *J. Pharmacol. Exp. Ther.*, **263**, 92–98.
- ZEITZ, O., RAHMAN, A., HASENFUSS, G. & JANSSEN, P.M. (2000). Impact of beta-adrenoceptor antagonists on myofilament calcium sensitivity of rabbit and human myocardium. *J. Cardiovasc. Pharmacol.*, **36**, 126–131.

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