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The preferential β_3-adrenoceptor agonist BRL 37344 increases force *via* β_1-/β_2-adrenoceptors and induces endothelial nitric oxide synthase *via* β_3-adrenoceptors in human atrial myocardium

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1 The present study investigated the effects of the preferential β_3-AR agonist BRL 37344 (BRL) on force of contraction (FOC), Ca^{2+}-transient and eNOS-activity in human right atrial myocardium.

2 BRL concentration-dependently caused an increase in FOC that was paralleled by an increase in Ca^{2+}-transient and a shortening of time to half peak relaxation (T0.5T). These effects were abolished in the presence of propranolol (0.3 μM).

3 BRL acted as a competitive antagonist towards isoprenaline and in binding experiments it was shown to have a distinct affinity towards $\beta_{1/2}$-AR.

4 In immunohistochemical experiments BRL (10 μM) increased detection of activated eNOS. This effect remained constant in the presence of propranolol (0.3 μM).

5 BRL increased directly detected NO in DAF-staining experiments. This increase was significantly smaller in the presence of the NO-inhibitor L-NAME.

6 The inotropic effects of BRL were not changed in the presence of L-NMA.

7 These results suggest that the inotropic effects of BRL in human atrium are mediated *via* $\beta_{1/2}$-AR, whereas the increase of atrial eNOS-activity is due to β_3- adrenergic stimulation. This increase in eNOS-activity did not influence atrial myocardial contractility. In conclusion, this study shows that β_3-adrenergic stimulation is present in human atrium, but may not be functionally as significant as in the left ventricle.

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Abbreviations: AR, adrenoceptor; BRL, BRL 37344; cGMP, cyclic guanosine monophosphate; DAF, diaminofluorescein; eNOS, endothelial nitric oxide synthase; FOC, force of contraction; L-NMA, N-nitro-L-arginine; L-NAME, N-Nitro-L-arginine methylester hydrochloride; NO, nitric oxide; T0.5T, time to half peak relaxation

Introduction

BRL 37344 (BRL, Figure 1), a preferential β_3-AR agonist (Arch *et al*., 1984; Arch & Wilson, 1996; Balligand *et al*., 2000), has widely been used to characterize the human and animal β_3-adrenergic system. BRL was first referred to in 1984 when it was described to mediate thermogenesis in brown adipocytes *via* the β_3-AR (Arch *et al*., 1984). Since then it has been described to mediate lipolysis in human and animal adipose tissue (Zaagsma, 1990; Langin *et al*., 1991; Lowell & Flier, 1995; Tavernier *et al*., 1996), and it has furthermore been shown to cause relaxation of vasculature (Berlan *et al*., 1994; Shen *et al*., 1994) and intestinal smooth muscle (Bond and Clarke, 1988; Manara & Bianchetti, 1990; Koike *et al*., 1994; Pietri-Rouxel & Strosberg, 1995) *via* the β_3-AR.

Only recently the protein expression of β_3-AR was shown in human cardiac tissue (Moniotte *et al*., 2001). In human left ventricular myocardium BRL and other β_3-AR agonists have been described to decrease FOC (force of contraction) *via* the β_3-AR (Gauthier *et al*., 1996; 1999; Moniotte *et al*., 2001). The observation, that these effects were blunted in the presence of NO-antagonists, led to the suggestion of an NO dependent pathway mediating β_3-adrenergic negative inotropic effects in human myocardium (Gauthier *et al*., 1998).

Both functional and binding studies have shown that the affinity of standard of β_1- and β_2-AR antagonists such as propranolol towards the β_3-AR is 100–1000 fold lower than towards β_1- and β_2-AR (Arch & Kaumann, 1993). Accordingly in human left ventricular myocardium (Gauthier *et al*., 1996) as well as in vascular tissue (Trochu *et al*., 1999) and intestinal smooth muscle (Roberts *et al*., 1997; Sennit *et al*., 1998) β_3-adrenergic stimulation has been described to remain unchanged in the presence of β_1- and β_2-AR antagonists. Arch & Kaumann (1993) even established this aspect as an obligatory criterion to define β_3-adrenergic stimulation.

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BRL 37344

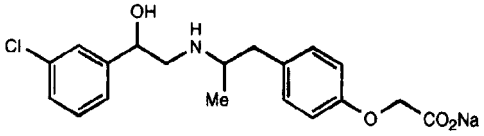

Figure 1 Chemical structure of the preferential β_3-agonist BRL 37344 ((RR+SS)- (±)-4-[2-(2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl]phenoxyacetate).

Although Chamberlain *et al*. (1999) were able to detect β_3-AR in human right atrium, functional results in human atrium remain quite unclear. In contrast to left ventricular myocardium, specific β_3-AR agonists, among them BRL, produce positive inotropic effects in isolated right atrium (Arch & Kaumann, 1993; Sennit *et al*., 1998), as well as an increase of sinual heart rate during *in vivo* experiments (Wheeldon *et al*., 1994). Consistently a baroreflex mechanism was suggested to be responsible for the *in vivo* increase of heart rate, due to β_3-adrenergic mediated vasorelaxation (Tavernier *et al*., 1992), but this, however, does not explain the increase of inotropy during *in vitro* experiments with isolated atrial myocardial tissue.

In addition, the increase of FOC in human atrial myocardium is prevented in the presence of β_1- and β_2-antagonists, such as propranolol (Kaumann & Sanders, quoted in Arch & Kaumann, 1993), however it is unknown by which receptor system these effects are mediated. Furthermore it is unclear whether BRL causes β_3-adrenergic 'hidden' effects on the enzymatic level that do not directly influence FOC and are therefore not detectable by functional methods.

It is therefore unclear whether β_3-adrenergic stimulation is existent in human atrial myocardium, by which intracellular messenger it is mediated and which influence it may have on atrial myocardial function.

The present study therefore investigates the inotropic effects of BRL in human right atrium by simultaneous measurements of intracellular Ca^{2+}-transient and FOC. Furthermore, it characterizes the affinity of BRL towards classical AR by binding experiments in human right atrium and investigates BRL induced activation of eNOS.

Methods

Tissue Collection

Tissue from patients was taken during sternotomy from 45 individuals (33 male, 12 female; age 68.9±1.2 years) with either coronary heart disease ($n=32$) or valvular disease ($n=13$). Medical treatment consisted of diuretics, nitrates, ACE inhibitors, cardiac glycosides and β-adrenoceptor antagonists. Drugs used for general anaesthesia were flunitrazepam, fentanyl and pancuronium bromide with propofol. Immediately after excision the tissue was placed in ice-cold cardioplegic solution containing (in mmol l^{-1}) NaCl 15, KCl 10, $MgCl_2$ 4, histidine HCl 180, tryptophane 2, mannitol 30 and potassium dihydrogen oxoglutarate 1, and was delivered to the laboratory within 15 min. The study was approved by the local ethics committee.

Electrically stimulated human right atrial trabeculae

During preparation trabeculae of less than 0.5 mm thickness and 3–6 mm in length, with the muscle fibres running approximately parallel to the length of the trabeculae, were isolated from right atrial tissue. Force of contraction (FOC) and time to half peak relaxation (T0.5T) were measured as described previously (Schwinger *et al*., 1990b; 1992).

Concentration-response curves for BRL (0.01–0.1 mmol) or isoprenaline (1.0–0.01 mmol) were determined by adding the drugs cumulatively to the organ bath after apparent equilibration of the effects of the previous concentration. When dose response curves with BRL were performed in presence of NO-inhibition, the NO-antagonist L-NMA was added to the organ bath 90 min before experiments were conducted.

To investigate the effects of BRL in presence of propranolol, propranolol was applied to the organ bath at least 45 min before a single dose of BRL (10 μM) was added.

Ca^{2+}- and force measurements

Intracellular Ca^{2+}-transient was measured *via* fura-2 loading in isolated, electrically driven right atrial trabeculae. Experiments included parallel measurements of FOC and were performed as described previously (Brixius *et al*., 1997).

Membrane preparation and binding experiments

Preparation of right atrial membranes and detection of radioactivity were performed as described before (Schwinger *et al*., 1991; Brixius *et al*., 2001).

To investigate the affinity of BRL towards atrial β-adrenoceptors, the assays were incubated with BRL in rising concentrations (10 fM–1 mM). ^{3}H-CGP 12177 (0.6 nm; specific activity 50 Ci $mmol^{-1}$), a $\beta_{1/2/3}$-AR radiolabelling ligand was then given to the membrane preparations for 90 min at 37°C. These conditions allowed complete equilibration of the receptors with the radioligand. The reaction was terminated by rapid vacuum filtration, and then radioactivity was determined. Thus, the displacement of ^{3}H-CGP 12177 from $\beta_{1/2/3}$-AR by rising concentrations of BRL could be documented. Nonspecific binding of ^{3}H-CGP 12177 was measured in the presence of propranolol (10 μmol l^{-1}). All experiments were performed in triplicate.

Immunohistochemistry

Tissue pretreatment Investigation of β_3-AR mediated changes in eNOS-activity by use of an eNOS-antibody, which only detected the activated eNOS (Bloch *et al*., 2001), made it necessary to conduct preincubation procedures with freshly obtained tissue. Therefore, two pieces of myocardial tissue, obtained from one patient were suspended in separated organ baths for at least 45 min. Then, one of the pieces was taken from the bath and was immediately placed in 4% paraformaldehyde, whereas the remaining piece of myocardium was incubated with BRL (10 μM) within the organ bath for another 5–10 min and then also fixed in 4% paraformaldehyde. When experiments were performed in the presence of $\beta_{1/2}$-AR inhibition, propranolol (0.3 μM) was

added to the organ bath at least 45 min before experiments were conducted.

Fixation procedures The pretreated tissue was kept in 4% paraformaldehyde for 4 h and then rinsed in 0.1 M phosphate-buffered saline (PBS) for 24 h. Tissues were then stored for 12 h in PBS solution with 18% sucrose for cryoprotection and frozen at −80°C.

Immunocytochemistry Prior to immunohistochemical examination 20 μM slices from pretreated human tissue were placed in a bathing solution of 3% H_2O_2 and 60% methanol PBS for 30 min, then permeabilized with 0.2% TritonX-100 in 0.1 M PBS. Thereafter, specimens were treated with 5% normal goat serum (NGS) and 5% bovine serum (BSA) solution in PBS. Prior to each step the sections were rinsed in PBS buffer three times. Incubation with primary antibody was performed in a PBS-based solution of 0.8% BSA and 20 mM NaN_3 for 12 h at 4°C. The polyclonal rabbit anti-eNOS antibody (Biomol) was applied at a dilution of 1 : 1500. After rinsing with PBS the sections were incubated with the corresponding secondary biotinylated goat anti-rabbit antibody for 1 h at room temperature. A streptavidin-horseradish peroxidase complex was then applied as a detection system (1 : 100 dilution) for 1 h. Finally, staining was developed for 3–5 min with 3,3-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M TRIS-HCl buffer and 0.1% H_2O_2. Negative control sections were incubated without the primary antibody.

Semi-quantitative analysis For semi-quantitative analysis of the human cardiac tissue, all slices were incubated and stored under identical conditions. An individual, semi-quantitative score was used to differentiate between negative (−), slightly positive (+), and clearly positive (++). All specimens were judged by two independent investigators in a double blinded fashion. Data were only accepted as altered if both investigators agreed upon the score.

TV-densitometry For intensity analysis of immunostaining in cardiomyocytes we measured the grey values of 30 cardiomyocytes from three randomly selected areas of each slice. The intensity of immunostaining was reported as the mean of measured cardiomyocyte grey value minus background grey value. The background grey value was measured at a cell free area of the slice. For staining intensity detection a Zeiss Axiophot microscope coupled to a 3-chip CCD-camera was used and the analysis was performed using the Optimas 6.01 image analysis program.

DAF-Fluorometry

Diaminofluorescein (DAF-FM) is converted *via* an NO-specific mechanism to an intensely fluorescent triazole derivative (Kojima *et al*., 1998; Itoh *et al*., 2000). We used DAF-FM DA to detect changes of the NO level induced by BRL. Therefore right atrial myocardial tissue was collected as described above. It was then shock frozen and stored at−80°C. For experiments the tissue was equilibrated at −20°C for at least 1 h and sliced to 25 μM thickness. Slices were fixed to a plastic scale which had been coated with 15% gelatine. Incubation medium was added immediately containing $CaCl_2$, $MgCl_2$, KCl, NaCl, NaH_2PO_4, Glucose, $NaHCO_3$ and L-Arginine 1 mM.

For measurements DAF (10 μM), BRL (10 μM), NONOate (10 μM) and L-NAME (100 μM) were added for the relevant experiment. In the following intensity of DAF-FM fluorescence was measured each 10 s for 10 min. The intensity of DAF-FM in absence of any of the agents listed above was set 100% as reference value for each timepoint. The effects of BRL and of BRL + L-NAME on the NO-level were then investigated in relation to the DAF-FM fluorescence intensity. For negative and positive controls we repeated experiments with L-NAME or the NO donor NONOate instead of BRL.

Materials

The β_3-AR agonist BRL 37344 ((RR + SS)-(±)-4-[2-(2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl]phenoxyacetate) was obtained by Tocris (Bristol, U.K.). Further substances used were isoprenaline and propranolol (Sigma, St. Louis, U.S.A.), the radioligand ^{3}H-CGP 12177 (Amersham, Braunschweig, Germany), forskolin (Sigma-Aldrich, St. Louis, U.S.A.), the NO-donor NONOate (Alexis), DAF (Alexis) and the NO inhibitor N-Nitro-L-Arginine (L-NMA) (Buchs, Switzerland). Rabbit anti-eNOS antibody against the bovine eNOS peptide (599-613) plus additional C-terminal Cys conjugated to KLH (PYNSSPREQHKSYKC) was obtained from Biomol (Hamburg, Germany), the secondary biotinylated goat anti-rabbit antibody was ordered from Vector Laboratories (Burlingame, CA, U.S.A.).

BSA and normal goat serum as well as chemicals required for staining with the avidin-biotin-peroxidase complex were purchased from Sigma (Deisenhofen, Germany). All other chemicals were of analytical grade or the best grade commercially available. For studies with isolated myocardium and trabeculae, stock solutions were prepared and added to the organ bath. All compounds were dissolved in twice distilled water and did not change the pH of the medium.

Statistical analysis

All data are presented as mean ± s.e.mean. Data analysis was performed using Student's *t*-test for paired and unpaired data, where appropriate. Significance was considered at a P value <0.05. Data obtained *via* DAF-fluorometry was analysed using Mann–Whitney-test with statistical significance considered at $P<0.05$.

Results

Effects of BRL on force of contraction and on intracellular calcium-transient

The present study investigates the inotropic effects of the preferential β_3-AR agonist BRL 37344 (BRL) in human atrial myocardium (Figure 2). Figure 2a shows original tracings of force of contraction under cumulative application of BRL (0.01 μM–100 μM). Figure 2b summarizes the results. BRL increased FOC concentration-dependently (0 μM BRL: 13.6 ± 2.28 mN/mm^2; + BRL (100 μM): 20.8 ± 3.24 mN/mm^2;

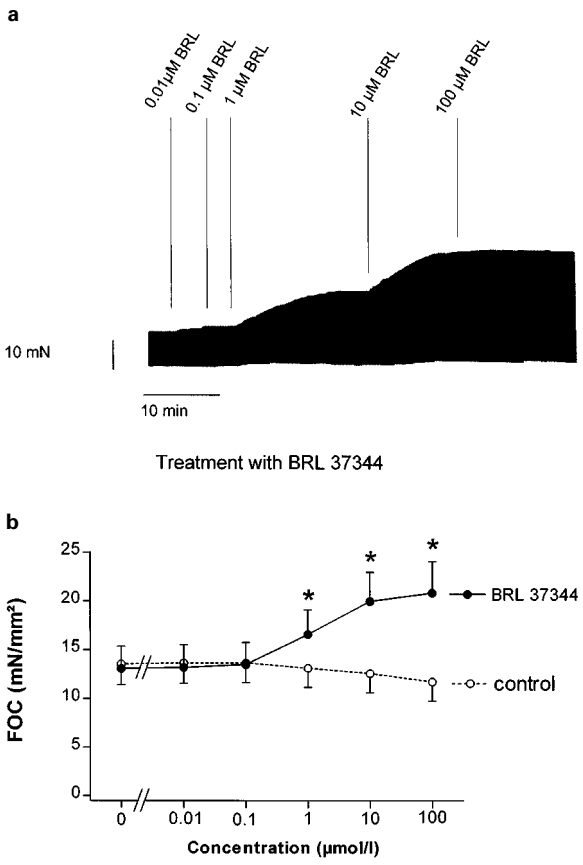

Figure 2 Effects of BRL 37344 (BRL) on the twitch tension of human right atrial trabeculae. Dose-response curve and controls for the effects of BRL on peak tension. (a) Original tracings of the effects of BRL in human right atrial trabeculae. Figure 2a is representative for experiments shown in Figure 2b. (b) Values are the means ± s.e.mean of 19 experiments for BRL and six for controls. The response is expressed as absolute values of peak tension in mN per mm^2 of trabecula diameter. *=Significant statistical difference ($P<0.01$) from basal peak tension.

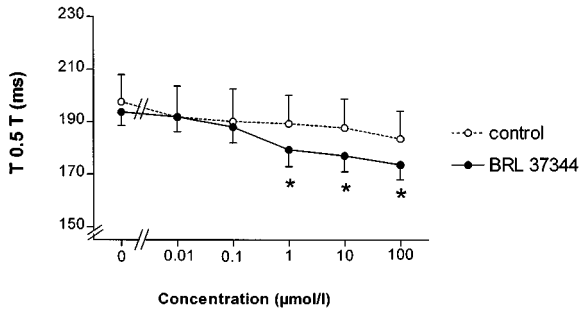

Figure 3 Effects of BRL on time to half peak relaxation (T0.5T) in human right atrial trabeculae. Values are the means ± s.e.mean of 19 experiments for BRL and six for controls. The response is expressed as absolute values of T0.5T in ms. *: $P<0.01$ vs basal T0.5T.

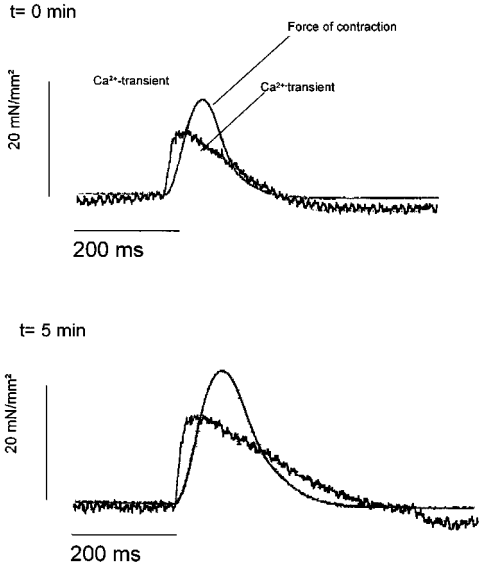

Figure 4 Ca^{2+} -transient and force of contraction. Ca^{2+}-transient was measured by fura-2 fluorescence. Original tracings of basal contraction and Ca^{2+}-transient (above) and after treatment with BRL (below).

$+54.6\pm16.1\%$; $P<0.01$; $n=19$) compared to control ($n=6$). This positive inotropic effect was associated with an abbreviation of time to half peak relaxation (0 μM BRL: 193.7 ± 5.24 ms; +BRL (100 μM): 173.4 ± 5.66 ms; $-10.22\pm2.33\%$; $P<0.01$; $n=19$; Figure 3) compared to control ($n=6$).

Figure 4 gives representative original tracings of force of contraction and Ca^{2+}-transient under basal conditions and after 5 min of incubation with BRL. Growth of contractile force induced by BRL (0 μM BRL: 11.25 ± 3.75 mN/mm^2; +BRL (10 μM): 13.85 ± 3.65 mN/mm^2; $+26.3\pm9.7\%$) was accompanied by an increase of intracellular Ca^{2+}-transient ($+86.0\pm32.2\%$) in right atrial trabeculae.

Effects of BRL in the presence of propranolol

To investigate whether the positive inotropic effect of BRL is due to $\beta_{1/2}$-adrenergic stimulation, experiments were performed in the presence of propranolol. After application of propranolol, FOC declined in right atrial trabeculae (0 μM propranolol: 17.6 ± 3.59 mN/mm^2; +propranolol (0.3 μM): 13.71 ± 2.98 mN/mm^2; $-22.3\pm4.37\%$; $P<0.05$; $n=7$). Incubation with propranolol continued (at least 45 min) until a steady state in force of contraction was reached. The following application of BRL (10 μM) did not change isometric force of contraction (0 μM BRL: 13.71 ± 2.98 mN/mm^2; +BRL (10 μM): 13.77 ± 2.86 mN/mm^2; $+1.02\pm1.28\%$; $P>0.05$; $n=7$). Trabeculae obtained from the same patients showed a distinct increase in FOC, when not pretreated with propranolol (0 μM BRL: 9.07 ± 3.39 mN/mm^2; +BRL (10 μM): 17.47 ± 6.56 mN/mm^2; $+96.7\pm14.42\%$; $P<0.05$; $n=6$). Figure 5 shows original tracings of BRL induced effects on FOC in the absence and presence of propranolol. These results indicate that the positive inotropic effect of BRL measured in right auricular trabeculae may be mediated *via* β_1- and β_2-AR.

Influence of BRL on isoprenaline induced inotropic effects

To investigate whether BRL acts as a competitive antagonist towards isoprenaline, concentration response curves of

isoprenaline (isoprenaline: 10^{-10} – 10^{-5} M) were measured in isolated, electrically stimulated right atrial trabeculae in the absence ($n=11$) and in the presence ($n=11$) of BRL (10 μM). In presence of BRL the maximum isoprenaline-induced increase in force of contraction was slightly decreased (isoprenaline: (10^{-5} M): $+11.16\pm2.35$ mN/mm^2; isoprenaline (10^{-5}M)+BRL (10^{-5}M): $+8.58\pm1.78$ mN/mm^2; $P=0.132$; $n=11$; Figure 6). Yet, in the presence of BRL, the concentration of isoprenaline, needed to achieve a 50% increase of the maximum isoprenaline-induced positive inotropic effect (EC_{50} isoprenaline) was significantly shifted to the right (EC_{50} isoprenaline: control : 28.4 ± 8.2 nM, +BRL (10 μM): 144.7 ± 53.6 nM; $P<0.05$; $n=11$).

Affinity of BRL towards β_1- and β_2-AR

To study possible affinities of the preferential β_3-adrenergic agonist BRL towards classical AR, competition experiments to the $\beta_{1/2/3}$-AR ligand ^{3}H-CGP 12.177 were performed in human right atrial membrane preparations (three experiments; triple measurements each). Membranes were incubated with cumulative concentrations of BRL (10 fM – 1 mM) and ^{3}H-CGP 12.177 (0.6 nM) was added. Figure 7 shows the competition curve obtained with BRL in percentage of basal specific ^{3}H-CGP 12.177 binding $\pm$ s.e.mean. In concentrations from 1 μM – 1 mM BRL was able to displace ^{3}H-CGP 12.177 from 96.3% of all labelled β-AR (i.e. $\beta_{1/2}$- and β_3-AR). Since only a small fraction of all labelled β-AR can account for β_3-AR we have to assume that BRL largely displaced the radioligand from $\beta_{1/2}$-AR indicating a distinct affinity towards β_1- and β_2-adrenoceptors.

Activation of the endothelial NO-synthase (eNOS)

There is evidence that cardiac effects of the β_3-adrenoceptor are mediated by activation of the endothelial NO-Synthase (Gauthier *et al*., 1998; Moniotte *et al*., 2000). To investigate whether the preferential β_3-adrenoceptor agonist BRL induces an activation of the eNOS, that may be functionally overruled by its agonism at the β_1-/β_2-adrenoceptors in human right atrium, immunohistochemical stainings of activated eNOS were performed in isolated right atrial trabeculae in the absence and presence of BRL (10 μM), using an antibody which only detect eNOS after activation of the enzyme (Bloch *et al*., 2001). Figure 8 presents pictures taken from original immunostainings. We observed a distinct cytosolic increase in eNOS immunoreaction in myocardial tissue preincubated with BRL (Figure 8b) compared to non pretreated tissue (Figure 8a). Besides atrial cardiomyocytes, atrial vascular tissue also showed an increase in eNOS, when treated with BRL (data not shown). This is consistent with reports on β_3-adrenergic vasorelaxation and increase of cGMP (Trochu *et al*., 1999) and can in this context be evaluated as a further control for a factual stimulation *via* β_3-AR.

To investigate whether these effects were indeed mediated by β_3-AR and not by $\beta_{1/2}$-AR, we repeated experiments in presence of propranolol (0.3 μM). Increase of eNOS staining detection was unchanged in presence of propranolol (see Figure 8c (basal) and d (+BRL)).

Positive inotropic substances have also been observed to stimulate eNOS (Tsukahara *et al*., 1994). To exclude that the observed activation of eNOS is due to Ca^{2+}-increase or myocardial share-stress induced by the $\beta_{1/2}$-adrenergic component of BRL, we performed stainings of activated eNOS in atrial trabeculae in the absence (see Figure 8e) and

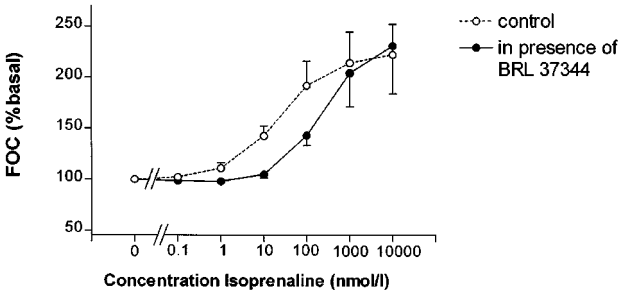

Figure 6 Dose-response-curves for the positive inotropic effects of isoprenaline in non-pretreated trabeculae (11 experiments; control) and trabeculae pretreated with BRL (10 μM; 11 experiments). Values are the means $\pm$ s.e.mean and are expressed in percentage of basal peak tension.

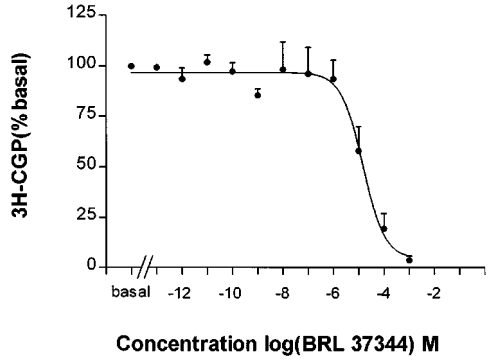

Figure 7 Binding: Displacement of ^{3}H-CGP 12.177 by BRL 37344. Values are the means $\pm$ s.e.mean of three experiments (triple measurements each). Atrial membrane preparations were saturated with increasing concentrations of BRL and incubated with the β_1-, β_2- and β_3-radioligand ^{3}H-CGP 12·177. Note that displacement of ^{3}HCGP 12.177 takes place within the same range of concentrations in which significant change in force of contraction is caused (Figure 2).

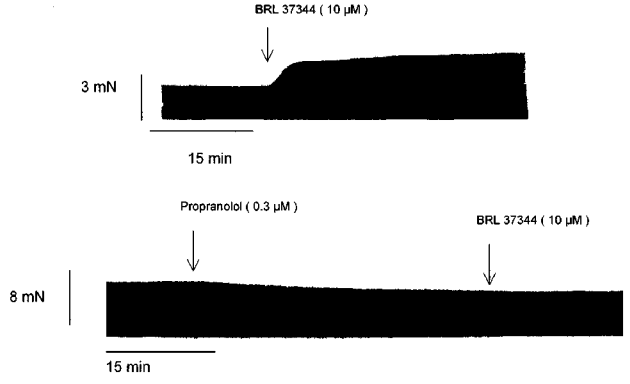

Figure 5 Effects of BRL on the twitch tension in presence of propranolol. Original tracings on the effects of BRL (10 μM) on right atrial trabeculum in the absence (above) and in the presence of propranolol (0.3 μM; below). This figure is representative for six experiments in the absence and seven in the presence of propranolol.

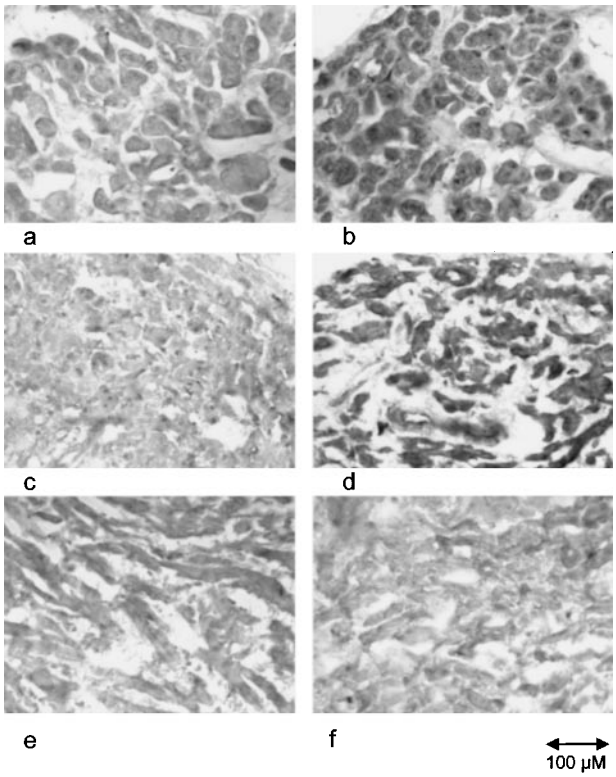

Figure 8 Influence of BRL on intensity of immunostaining for eNOS proteins in human right atrial myocardium. Cytosolic staining is observed in myocytes under basal conditions (a) and is increased after 5 min of incubation with BRL (b). Experiments were repeated in the presence of propranolol (0.3 μM). Staining again increased from basal (c) to pretreatment with BRL (10 μM) (d). Incubation with forskolin (0.3 μM; 5 min; (f)) did not increase detection of activated eNOS compared to basal level (e). Pictures are representative for 11 experiments with BRL (five in absence and six in presence of propranolol) and three experiments with forskolin.

presence (see Figure 8f) of forskolin (0.3 μM), a potent positive inotrope. In contrast to BRL forskolin did not cause an increase of eNOS.

Microscopic slides were evaluated *via* TV densitometry and increase of staining intensity was documented in per cent of basal densitometric units for non-pretreated tissue (+BRL (10 μM); $+53.52\pm15.21\%$; $P<0.05$; $n=5$) and tissue pre-incubated with propranolol (+BRL (10 μM); $+75.08\pm17.6\%$; $P<0.05$; $n=6$).

The evaluation of atrial tissue in the presence and absence of forskolin was performed equally (+forskolin (0.33 μM); $-20.2\pm12.3\%$; $P>0.05$; $n=3$).

Effects of BRL on NO-release

We measured NO-detection in right atrial myocardium *via* DAF-staining method. Figure 9 shows fluorescence images taken after reaction time of 150 μM) that are representative for these experiments.

Change in NO release in control time courses in absence of BRL was taken as reference value (Figure 9a and b; reaction time 300 s: $100\%\pm0.00$; $n=8$). Compared to controls NO-detection significantly increased in presence of 10 μM BRL (Figure 9c and d; reaction time 300 s: $216\pm36\%$; $n=7$). This BRL induced increase in NO-release was significantly diminished in the presence of the NO-antagonist L-NAME (100 μM; Figure 9e and f; reaction time 300 s: $121\pm20\%$; $n=7$).

To demonstrate the reliability of these findings we performed DAF control curves without BRL in the presence of L-NAME (100 μM) and in the presence of the NO-donor NONOate (10 μM). As expected NO-release was decreased in the presence of L-NAME ($89\pm8\%$; $n=4$) and distinctly increased by NONOate ($218\pm59\%$; $n=3$).

BRL in presence of NO inhibition

The dose response curves with BRL were now repeated in the presence of L-NMA, a potent inhibitor of the NO synthesis. Figure 10 shows mean values in percentage of maximum force $\pm$ s.e.mean. Maximum increase in FOC induced by BRL in presence of L-NMA was not changed (0 μM BRL: 10.30 ± 1.91 mN/mm^2; +BRL (100 μM): 14.9 ± 2.58 mN/mm^2; $+53.43\pm32.95\%$; $n=4$) compared to maximum increase in FOC in absence of L-NMA (0 μM BRL: 13.84 ± 2.40 mN/mm^2; +BRL (100 μM): 20.14 ± 2.95 mN/mm^2; $+55.80\pm16.99\%$; $n=18$).

Discussion and conclusions

The present study investigated the cardiac effects of the preferential β_3-adrenoceptor agonist BRL 37344 in human

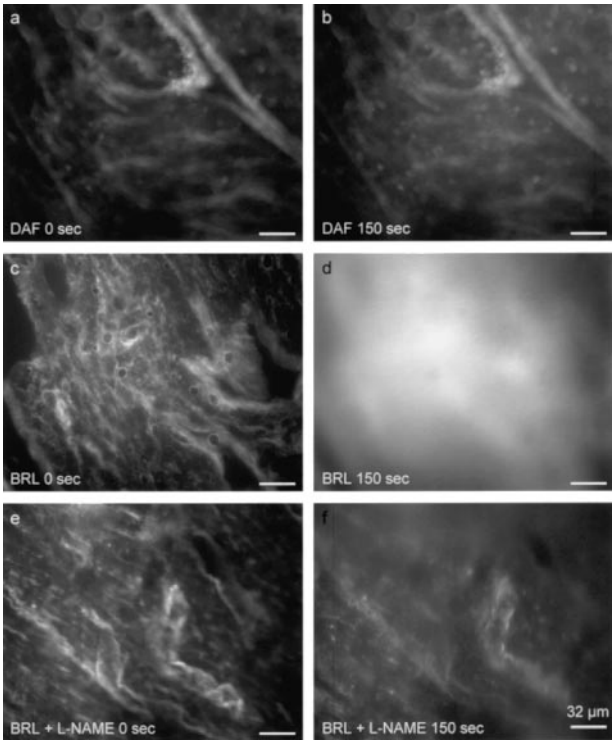

Figure 9 Bioimaging of BRL induced changes in NO release in right atrial myocardium *via* DAF fluorometry. (a–b): Controls: NO-detection *via* fluorescence intensity basal (a) and after 150 s of reaction time (b) in absence of BRL. (c–d): NO-Detection in presence of BRL (10 μM) basal (c) and after 150 s (d). (e–f): NO-Detection in presence of BRL (10 μM) and L-NMA basal (e) and after 150 s (f).

right atrial myocardium. It was shown that BRL induces a positive inotropic effect in human atrial myocardium *via* stimulation of the β_1- and β_2-adrenoceptors. Nevertheless, BRL-application was paralleled by an increase in eNOS-activity, that remained unchanged in the presence of propranolol, a β_1-/β_2-antagonist. Thus, BRL induces an increase in eNOS activity, that is most likely due to β_3-adrenergic stimulation. Atrial functional effects due to β_3-adrenergic stimulation of eNOS through BRL could not be detected.

β_1-/β_2-adrenoceptor mediated increase in force of contraction by BRL

Various studies have shown that β_3-adrenergic agonistic substances cause positive inotropic and chronotropic effects in human atrium (Arch & Kaumann, 1993; Wheeldon *et al*., 1994; Sennit *et al*., 1998). *In vivo* increase of heart rate induced by BRL has often been attributed to baroreflex mechanisms, due to a β_3-adrenergic vasodilatation (Tavernier *et al*., 1992; Takayama *et al*., 1993). However this does not explain the positive inotropic effects of BRL detected in isolated atrial myocardium as shown in this study. These findings are supported by previous reports as well (Arch & Kaumann, 1993; Sennit *et al*., 1998).

In the present study the positive inotropic effect of BRL was abolished after pretreatment with the β_1- and β_2-AR antagonist propranolol, indicating that the BRL-mediated positive inotropic effect may be due to β_1-/β_2-adrenergic stimulation. In agreement, the binding experiments of the present study provided evidence that BRL has a distinct affinity towards $\beta_{1/2}$-AR and the range of concentration in which BRL acted as an agonist towards classical AR was exactly the same in which it was able to cause significant cardiostimulant effects. Consistently, we could show that in human atrial myocardium BRL acts as a competitive antagonist towards isoprenaline at $\beta_{1/2}$-AR. The shortening of time to half peak relaxation (T0.5T) and the increase of intracellular Ca^{2+}-transient induced by BRL are as well consistent with $\beta_{1/2}$-adrenergic myocardial stimulation.

From these findings, we conclude that the above described effects were caused by stimulation of atrial β_1- and β_2-AR. In agreement, β_3-adrenergic stimulation has in a variety of tissues been described to be resistant against antagonists possessing only high affinity towards β_1- and β_2-AR such as propranolol (Kaumann & Molenaar, 1996; Gauthier *et al*., 1996; Sennit *et al*., 1998; Trochu *et al*., 1999).

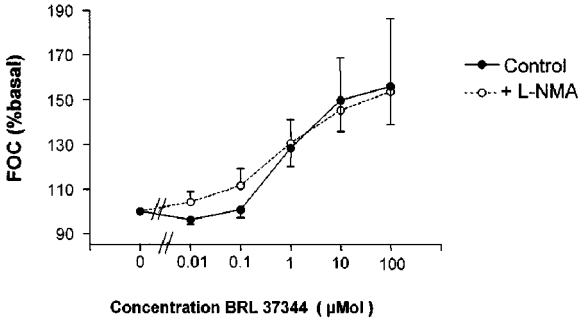

Figure 10 Effects of BRL on trabeculae preincubated with the NO-inhibitor L-NMA (0.1 mM; four experiments). Controls are non-pretreated trabeculae (18 experiments). Dose-response-curves for BRL in the presence and in the absence of L-NMA are shown in percent of basal peak tension.

Yet we observed that the maximum inotropic effect reached by BRL in right atrium is significantly lower than the one reached by isoprenaline (+BRL (100 μM): $+57.01 \pm 15.77\%$; $n=16$; +Isoprenaline (10 μM): $+122.09 \pm 98.8$; $n=11$ ($P<0.05$ (1 : 2)). Both isoprenaline and BRL are known to stimulate β_3-AR in cardiac tissue, however isoprenaline has a much lower affinity towards β_3-AR than BRL (Gauthier *et al*., 1996). Thus, the minor positive inotropic effect observed after application of BRL in comparison to the application of isoprenaline may be attributed to a BRL-induced stimulation of the β_3-AR diminishing its β_1- and β_2-adrenergic effects. On the other hand this observation may indicate that BRL, although it significantly causes β_1- and β_2-adrenergic effects in right atrium, still has a lower affinity or a lower intrinsic activity towards β_1- and β_2-AR than isoprenaline.

eNOS activation by BRL in human myocardium

This study is the first to show a direct stimulation of eNOS by the β_3-AR agonist BRL followed by an increase of NO in human atrial myocardium. The existence of eNOS in human cardiovascular tissue and its involvement in β_3-adrenergic mediation have been evaluated in earlier studies (Balligand *et al*., 1995; Gauthier *et al*., 1998; Trochu *et al*., 1999), however evidence for a direct influence of β_3-AR agonists on the activity level of the eNOS enzyme has still been lacking. We detected a distinct increase in eNOS immunoreaction and in directly detected NO in presence of BRL, and we related this effect to β_3-adrenergic stimulation. This assumption was secured by the observation that forskolin as a positive inotrope could not induce similar effects and that BRL induced effects on eNOS remained unchanged in the presence of propranolol. Therefore we conclude that the effects of BRL on eNOS are, in contrast to its inotropic effects, mediated *via* atrial β_3-AR.

Functional implication of atrial β_3-adrenergic stimulation

This study shows that in human atrium BRL increases eNOS-activity and in the following NO *via* the β_3-AR, but fails to directly induce negative inotropic effects as it does in the left ventricle (Gauthier *et al*., 1996; Moniotte *et al*., 2000). Even in the presence of propranolol, a potent $\beta_{1/2}$-antagonist, a negative inotropic effect of BRL could not be detected. Consistently, after inhibition of the β_3-adrenergic second messenger NO by the NO-inhibitor L-NMA the dose response curve of BRL remained unchanged, indicating that β_3-adrenergic stimulation may not directly influence contraction in human atrial myocardium. It is therefore possible that α_3- adrenergic stimulation in human atrium is not of the same functional significance as in left ventriculum. These regional differences may be attributed by the different kind of β-adrenoceptor coupling in right atrial and left ventricular myocardium (Schwinger *et al*., 1991). Another possible explanation is that the atrial β3-AR might be expressed in much lower numbers compared to the left ventricle. In addition, an overexpression of the eNOS has been described for atrial myocardium compared to the left ventricle (Bloch *et al*., 1999). Thus, β_3-adrenergic stimulation may not be

sufficient to elicit the already high basal atrial eNOS activity significantly over its basal level, thereby failing to increase the cGMP-level and to elicit measurable negative inotropic effects.

Thus, the role of eNOS as a second messenger for β_3-adrenergic stimulation may be blunted in human atrium, compared to left ventriculum. However, from our findings we cannot exclude that atrial β_3-adrenergic stimulation may oppose $\beta_{1/2}$-adrenergic chronotropic and dromotropic effects via an increase of NO and by this way possibly decreases sinual frequency. Thus, the atrial β_3-adrenergic system might provide a protective negative feed back mechanism against atrial arrhythmia and sinual tachycardia, resulting from excessive $\beta_{1/2}$-adrenergic stimulation. Furthermore atrial β_3-adrenergic stimulation might become functionally significant in the setting of heart failure, where increased expression of β_3-AR has recently been reported, at least for the ventricle (Moniotte *et al*., 2000). β_3-adrenergic stimulation may as well influence cell growth and differentiation.

In conclusion, this work shows that NO-mediated β_3-adrenergic stimulation is present in human atrium, but, at least under normal conditions, does not directly influence atrial contractility. In addition we could provide further information about the β_3-adrenergic intracellular mediation pathway by showing that β_3-adrenergic stimulation directly and time dependently activates eNOS which again induces a release of NO.

Limitation of the study

The present study was performed with right atrial tissue from patients with either valvular or coronary disease. Furthermore the patients received medical and anaesthetical treatment. It therefore cannot be excluded that the observed effects may have been influenced by disease state or medication.

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