

# Beta Adrenoceptor Subtype Binding Activity in Plasma and Beta Blockade by Propranolol and Beta-1 Selective Bisoprolol in Humans. Evaluation with Schild-Plots<sup>1</sup>

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## ABSTRACT

In the present study we investigated whether the *beta* adrenoceptor subtype binding activity in plasma samples can predict selective and nonselective *beta* blockade in humans. From the right shifts of isoprenaline dose-response curves 0 to 84 hr after administration of propranolol and the *beta*-1 selective bisoprolol, *in vivo beta* blockade was assessed. In an *in vitro* radioreceptor assay with membrane preparations of *beta*-1 or *beta*-2 adrenoceptors, plasma samples were assayed for subtype selective blocking activity. After *propranolol* administration, *in vitro beta*-1 and *beta*-2 adrenoceptor occupancy declined from initially 97% to < 10% within 48 hr. An isoprenaline dose ratio (DR)-1 of 1 coincided with a 50% occupancy of the *beta*-1 or the *beta*-2 subtype *in vitro*. In Schild-plots using plasma concentrations (radioreceptor assay) and the isoprenaline DR-1 for heart rate, diastolic blood pressure and inotropy (QS<sub>2</sub>c), slopes of unity

were observed. After *bisoprolol* administration, *in vitro beta*-1 occupancy shifted from initially 95% to < 10% within 72 hr. For the *beta*-2 subtype, an occupancy of > 10% was detectable only within the first 12 hr. An isoprenaline DR-1 of 1 coincided with a 50% occupancy of *beta*-1 adrenoceptors. The bisoprolol Schild-plots yielded a slope of unity for inotropy, but less than unity for the heart rate and diastolic blood pressure. From an extended analysis of subtype selective antagonism in Schild-plots, the fractions of the *beta*-2 adrenoceptor subtype participating in the isoprenaline response were calculated: heart rate  $0.45 \pm 0.12$  and diastolic blood pressure  $0.23 \pm 0.13$ . It is concluded that *in vitro* receptor occupancy can predict *beta* blockade in humans for propranolol. *Beta* adrenoceptor subtype-mediated effects in humans can be evaluated with a selective antagonist and a refined analysis of Schild-plot data.

In isolated human tissues, pA<sub>2</sub> values of nonselective *beta* blocking agents derived from the classical Schild-technique have been in good agreement with pK<sub>d</sub> values obtained from receptor binding studies using membrane preparations (Brodde *et al.*, 1986; Gille *et al.*, 1985). This also was valid for subtype-selective antagonists, when using an extended evaluation technique of curvilinear Schild-plots (see Kenakin, 1984, 1985; Lemoine and Kaumann, 1983). Thus, as expected from the standard theory for nonselective antagonists (see Gaddum, 1957), receptor occupancy detected in ligand binding studies indicates the right shifts of an agonist dose-response curve *in vitro*. For a subtype-selective antagonist, one should expect deviations from this standard relationship, if different fractions of receptor subtypes are involved with the dose-response of a nonselective agonist (see Kenakin, 1985). In the present studies, we wanted to apply these theories to evaluate selective and nonselective *beta* blockade in humans.

Isoprenaline infusions were used to titrate *beta* blockade brought about by propranolol and the *beta*-1 selective bisoprolol (Kaumann and Lemoine, 1985; Tattersfield *et al.*, 1984). The approach was adopted from the time-dose-response design of Tallarida *et al.* (1978). At various time intervals after administration of a single dose of antagonist to healthy volunteers, DR-1 values were derived from the agonist dose-response curves. *In vitro* receptor occupancy by antagonist present in plasma sampled in parallel with the effect measurements was derived from a subtype-selective radioreceptor assay with a *beta*-1 or a *beta*-2 subtype population (rat salivary gland or reticulocytes, respectively; see Wellstein *et al.*, 1986a,b). The rationale for the use of *plasma samples* to detect receptor occupancy was delineated from studies of Sriwatanakul and Nahorski (1980) in rats. In *ex vivo* receptor binding studies with various tissues, a direct relationship between the plasma concentrations of propranolol or atenolol and *in vivo* receptor occupancy had been shown.

Thus, the aim of our study was to investigate whether the *in vitro* assay of *beta* adrenoceptor occupancy by antagonist pres-

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ent in plasma samples can predict the right shift of isoprenaline dose-response curves in humans after the administration of propranolol. In addition to this, we hoped to get an estimate of the receptor subtype fractions involved with different cardiovascular effects of isoprenaline by applying *beta*-1 selective bisoprolol.

## Methods

**Receptor binding studies.** Receptor binding studies were performed using the hydrophilic *beta* adrenoceptor radioligand (–)-[<sup>3</sup>H] CGP 12177 {[4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on]hydrochloride} (Staehelin *et al.*, 1983; specific activity, 35–45 Ci/mmol). Submandibular salivary glands were obtained from Wistar rats (200–250 g b.w.). Membranes were prepared after homogenization of the glands (1 g/10 ml) in 310 mOsM sodium phosphate buffer (pH 7.4 at 4°C = buffer A). They were washed 3 times by means of 15 min of centrifugation at 45,000 × *g* and resuspension of the pellet (see also: Wellstein *et al.*, 1984a). Reticulocyte rich blood was obtained 7 days after a 3-day treatment period of female Wistar rats (80–100 g b.w.) with 40 mg/kg/day of acetylphenylhydrazide i.p. Cells were freed from plasma by washing them 3 times in buffer A (1/1 v/v) and centrifugation for 10 min at 1500 × *g*. White cells were removed by aspirating the supernatant buffy coat; 70 to 90% of the red blood cells were identified as reticulocytes. This preparation is thus referred to later on as reticulocytes (Wiemer *et al.*, 1982). After hypotonic lysis of the cells (1 volume of cells/10 volumes of 17 mOsM of buffer A) and centrifugation at 20,000 × *g*, the resulting membrane fraction was washed twice in the original buffer A. Both membrane preparations from salivary glands and reticulocytes were stored at –70°C in buffer A at a protein concentration of 2 to 4 mg/ml until use.

After the membranes had thawed, they were again washed and resuspended in buffer A. Fifty-microliter aliquots of this suspension (100–300 μg of protein) were incubated in a total volume of 300 μl with 20 μl of the radioligand (0.1–10 nM in saturation experiments and 1 nM in competition studies), 30 μl of buffer A or 1 μM of (±)-carteolol (nonspecific binding) and 200 μl of human plasma obtained after placebo or verum treatment. After the samples were incubated in microtest plates (NUNC; Weisbaden, FRG) at 25°C for 90 min, they were filtered through glass-fiber filters (AP 15; Millipore, Dreieich, FRG) and washed twice with 10 ml of buffer A using a BRANDEL R12 filtration manifold (Dunn Laboratories, Arnsbach, FRG). The retained radioactivity on the filters was detected by liquid scintillation counting after the addition of 10 ml of Optifluor (Packard Instruments, Frankfurt/M, FRG). Samples were run in triplicates for each data point of each volunteer. Nonspecific binding was below 5% at the *K<sub>d</sub>* concentration of the radioligand.

**Studies in healthy volunteers.** Eighteen male medical students with normal body weight were enrolled after a physical and laboratory examination and after having given written informed consent. In this double blind randomized study they received either a single p.o. dose of placebo (*n* = 6) or propranolol, 240 mg (Dociton tablets; *n* = 6) or bisoprolol, 100 mg (Concor tablets; *n* = 6) after overnight fasting. At 0, 3, 6, 9, 12, 24, 36, 48, 60, 72 and 84 hr after drug ingestion, the following study protocol was observed: a venous blood sample was drawn into heparinized syringes and plasma was separated by centrifugation and frozen (–20°C) for further use in the receptor binding studies (see above). After 3 base-line recordings, isoprenaline (Aludrin; Boehringer Ingelheim, Ingelheim, FRG) was infused in increasing doses (0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 μg/min) for 3 min at each dose step until the heart rate increased by at least 25 beats/min. Infusion time (3 min) was sufficient to achieve a steady state of agonist effects independent of the presence of the antagonist (unpublished observations).

At each isoprenaline dose step, systolic and diastolic blood pressure (phase I and IV according to Korotkoff) were monitored by the same person using an ordinary cuff mercury manometer. Heart rate and heart rate corrected electromechanical systole (QS<sub>2c</sub>) were derived

using standard methods (Belz *et al.*, 1981; Weissler *et al.*, 1968). QS<sub>2c</sub> reflects changes in inotropy and is only slightly influenced by changes in load conditions (Lewis *et al.*, 1977; Stern *et al.*, 1984). After termination of the isoprenaline infusion, the volunteers rested for another 5 min until their rate had returned to base-line values. Therefore, 4 min of bicycle ergometry (horizontal position with the head elevated to 15°) was run at a work load, which in a preliminary test had induced a tachycardia of 135 beats/min.

**Data evaluation.** Data evaluation was performed on a Hewlett-Packard series 200 (HP 9836) desk top calculator using the Gießen Iteration procedure software package for nonlinear, weighted least-squares curve fitting (Wiemer *et al.*, 1982). The following equations were used.

## Receptor Binding Studies

$$B_{Li} = \frac{B_{max} \times L}{L + K_d \times (1 + i/K_i)} + nsb \times L \quad (1)$$

where *B<sub>Li</sub>* is radioligand bound at concentration *L* and concentration *i* of antagonist *B<sub>max</sub>* is the binding capacity and *K<sub>d</sub>* the dissociation constant of the radioligand and *nsb* its nonspecific binding. *K<sub>i</sub>* is the dissociation constant of the antagonist.

To measure the unknown antagonist concentrations in the plasma sampled after drug administration, a saturation isotherm of the radioligand was run with drug-free human plasma. From this data *B<sub>max</sub>*, *K<sub>d</sub>* and *nsb* of the system were calculated. In a parallel assay, plasma obtained after verum administration was incubated with a fixed concentration of radioligand (1 nM) and the amount of receptor binding (*B<sub>Li</sub>*) was determined. Equation 1 was solved for *i/K<sub>i</sub>* and unknown antagonist concentrations in the samples were calculated from these data:

$$i/K_i = \left( \frac{B_{max} \times L}{B_{Li} - L \times nsb} - L \right) \times \frac{1}{K_d} - 1 \quad (2)$$

In contrast to the usual processing of radioreceptor assays (see *e.g.* Wellstein *et al.*, 1984b, 1986b), calibration curves with unlabeled antagonist were thus avoided. Furthermore, the receptor occupancy of the respective subtype present in the membrane preparation can be calculated from

$$\text{Fractional occupancy} = 1 - [1 / (1 + i/K_i)] \quad (3)$$

## Agonist Dose-Response Curves in the Volunteers

Isoprenaline dose-response curves at different time points after antagonist administration (or after placebo) were described by a *simultaneous fit* of the following equation to the data:

$$E_A = \frac{E_{max}}{1 + \frac{(DR_n \times K_A)^h}{A^h}} + b_n \quad (4)$$

In these dose-response curves, *E<sub>A</sub>* is the effect of the respective dose of the agonist (*A*), *K<sub>A</sub>* the apparent ED<sub>50</sub>, *h* the slope of the curves, *E<sub>max</sub>* the maximum capacity above the lower asymptote *b<sub>n</sub>*; *n* indicates the *n<sup>th</sup>* time point and DR<sub>*n*</sub> the respective right shift of the agonist curve expressed as the dose ratio. DR values from the placebo group different from 1 were used to correct the respective values in the verum groups. The *E<sub>max</sub>* estimates were more than 20-fold of the changes in the base-line values due to antagonist treatment or circadian variations in the placebo group. Thus, a bias of DR-1 due to changes in the base-line values can be ruled out (see also Belz *et al.*, 1987; Wellstein *et al.*, 1987).

## Schild-plots

Linear Schild-plots were evaluated using

$$\log (DR - 1) = m \times \log i - \log K_i \quad (5)$$

as originally suggested by Arunlakshana and Schild (1959). DR denotes

the dose ratio of the agonist,  $m$  the slope of a straight line in the double log plot,  $i$  the concentration of antagonist and  $K_i$  the dissociation constant.

If the slope  $m$  of the curves was smaller than 1, the data were submitted for further analysis assuming that two subtypes were responsible for the antagonist effects (Kenakin, 1985; Lemoine and Kaumann, 1983). In this case, the fractional receptor occupancy ( $s$ ) was split between the subtype 1 and 2, where  $s_1 = (1 - s_2)$ . The fractional occupancies by the antagonist can thus be written as follows:

$$\frac{1}{1 + i/K_i} = (1 - s_2) \times [1/(1 + i/K_{i1})] + s_2 \times [1/(1 + i/K_{i2})] \quad (6)$$

Resolving this equation for  $i/K_i$ , and thus also for DR-1, equation 7 is then obtained:

$$\log(\text{DR} - 1) = \log\left(\frac{(1 + i/K_{i1}) \times (1 + i/K_{i2})}{(1 - s_2) \times (1 + i/K_{i2}) + s_2 \times (1 + i/K_{i1})}\right) \quad (7)$$

This equation is identical to equation 5 of Lemoine and Kaumann (1983) which was used in a similar mathematical approach. Figure 2A shows the various functions resulting from this equation.

**Statistics.** Parameters of the above mentioned equations were calculated from individual experiments (or volunteers) and then pooled to give the mean  $\pm$  S.E.M. (Tallarida and Murray, 1981). A  $t$  test was used to test for differences.

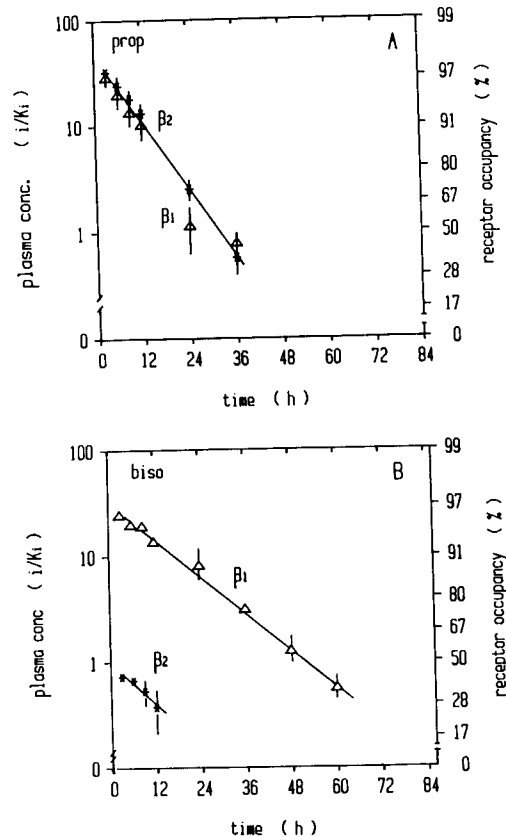
Alternatively, mean parameters were calculated from the pooled data. Upper and lower confidence intervals (e.g. 95%) of each parameter were estimated using the following procedure: the residual sum of squared deviations (SSQ) from the best fit of a function ( $\text{SSQ}_{\text{min}}$ ) was compared to the SSQ resulting from an increase or decrease of the respective parameter away from its optimum value ( $\text{SSQ}_{\text{new}}$ ). The iterative shift of the respective parameter was allowed to proceed until the ratio of  $\text{SSQ}_{\text{new}}/\text{SSQ}_{\text{min}}$  ( $F$  value) reached the tabulated  $F$  value for the respective probability level (usually 0.05; degree of freedom = number of data points minus 1). Confidence intervals calculated by the standard procedure did not differ from those derived from the iterative procedure. The iterative procedure, however, is much less time consuming, as pooled data can be used (see also Belz *et al.*, 1987; Wellstein *et al.*, 1987).

**Materials used.** Experimental animals were generously donated by the Hoechst AG (Frankfurt/M, FRG) animal breeding facilities. (-)-[<sup>3</sup>H]CGP 12177 was purchased from Amersham-Buchler (Braunschweig, FRG). Tablets of ( $\pm$ )-propranolol hydrochloride (DOCITON 80) were obtained from ICI-Pharma (Plankstadt, FRG). Tablets of ( $\pm$ )-bisoprolol hemifumarate (CONCOR 10) were gifts from E. Merck (Darmstadt, FRG) and ( $\pm$ )-carteolol hydrochloride from Madaus (Köln, FRG). Other substances were purchased from E. Merck.

## Results

### Plasma Concentrations and *In Vitro* Receptor Occupancy

In the membrane preparations used in the *in vitro* part of our studies, the radioligand [<sup>3</sup>H]CGP 12177 binds with similar  $K_d$  values:  $0.52 \pm 0.05$  nM ( $n = 13$ ) in rat salivary gland and  $0.59 \pm 0.06$  nM ( $n = 12$ ) in rat reticulocyte membranes, respectively (not depicted). The time course of *in vitro* receptor occupancy detected from plasma samples after propranolol or bisoprolol administration is shown in figure 1 (right ordinates). After propranolol administration, a similar pattern was observed with both preparations. Receptor occupancy declined within 48 hr after administration from initially 97% to below the detection limit. In the bisoprolol group, a comparable initial receptor occupancy was observed only with salivary gland membranes (95%). With this system, receptor occupancy was detectable until 72 hr after administration. With reticulocyte membranes, receptor occupancy was only detectable within the



**Fig. 1.** Receptor subtype occupancy *in vitro* (right ordinates) and plasma concentration (conc.) kinetics (left ordinates) after propranolol (prop) (A) or bisoprolol (biso) administration (B). Two groups of six healthy volunteers received either p.o. propranolol (240 mg) or bisoprolol (100 mg) and plasma was sampled at different time intervals after administration. A  $\beta$  adrenoceptor subtype selective radioreceptor assay with rat salivary gland ( $\beta$ -1) and rat reticulocyte membranes ( $\beta$ -2) was used. The inhibition of radioligand binding [ $(-)$  [<sup>3</sup>H]CGP 12177; 1 nM] by the antagonist present in the plasma samples was detected. Receptor occupancy was calculated from parallel saturation isotherms of the radioligand with plasma obtained after placebo treatment. From the respective occupancy of  $\beta$ -1 or  $\beta$ -2 adrenoceptors, plasma concs. were calculated as multiples of the dissociation constant ( $i/K_i$ ; see equation 2 under "Methods"). Kinetic parameters are given in table 1.

first 12 hr of sampling after administering bisoprolol. The respective values were below 50%. These findings are in agreement with both the  $\beta$  adrenoceptor subtype populations present in the *in vitro* systems ( $\beta$ -1, rat salivary gland;  $\beta$ -2, rat reticulocytes; see Wellstein *et al.*, 1986a) and with the selectivity profiles of propranolol (rather nonselective) and bisoprolol  $\beta$ -1 selective; see e.g. Kaumann and Lemoine, 1985).

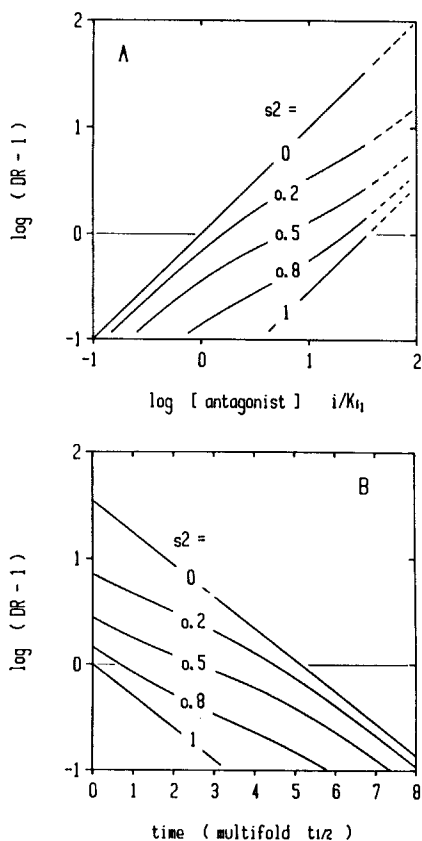
Plasma concentrations after antagonist administration were obtained from the inhibition of radioligand binding at the  $\beta$  adrenoceptor subtype present in the respective membrane preparation. Saturation isotherms of the radioligand were used as calibration curves. The application of this approach yields concentrations in the samples in  $K_i$  units of the respective receptor subtype ( $i/K_i$ ; see equation 2 under "Methods"). After propranolol administration (fig. 1A; left ordinate), plasma concentrations declined monoexponentially with a half-life of  $5.4 \pm 0.4$  hr. Initial concentrations (time = 0) of  $40 \pm 1$   $i/K_i$  ( $\beta$ -1) and  $49 \pm 3$   $i/K_i$  ( $\beta$ -2) indicate a slight  $\beta$ -2 selectivity of propranolol. Initial bisoprolol plasma concentrations (fig. 1B; left ordinate) detected in the  $\beta$ -1 system were  $30 \pm 1.5$   $i/K_i$ .

The respective antagonist concentrations derived from the *beta*-2 system were below 1. A  $34.5 \pm 1.6$ -fold *beta*-1 selectivity was calculated from the parallel measurements in both subtype systems. The elimination half-life of bisoprolol obtained from the data of the *beta*-1 system was  $10.5 \pm 0.5$  hr.

### DR Studies in Humans

To quantitate antagonist effects in humans, isoprenaline was used as an extrinsic *beta* adrenergic agonist. Computer simulations of isoprenaline DR-1 expected from the above *in vitro* measurements are depicted in the Schild-plot in figure 2A. The simulated functions are shown for various receptor subtype fractions participating in the agonist response (see equation 7 under "Methods"). For propranolol, we expected data points close to the identity line between isoprenaline DR-1 and plasma concentrations given in  $K_i$  units. Deviations from the identity line should become apparent with the 35-fold *beta*-1 selective bisoprolol, if significant fractions of *beta*-2 adrenoceptors participated in the isoprenaline response. Figure 2B shows the time course simulations of DR-1 expected from the elimination of the respective antagonist. In order to allow a comparison independent of the various pharmacokinetic profiles, the x-axis in this plot is scaled in multiples of the elimination half-life.

In the following section, the *in vivo* measurements are compared with the above *in vitro* data shown in figure 1. Parameters of the functions are summarized in tables 1 and 2.



**Fig. 2.** A, simulations of Schild-plots of a 35-fold subtype selective antagonist expected for different fractions of subtype 2 ( $s_2 = 0-1$ ; see equation 7 under "Methods"). Abscissa: antagonist concentrations in multiples of the dissociation constant at subtype 1 ( $i/K_{i1}$ ; log scale). The dashed lines are the extrapolation beyond the antagonist concentrations detected in the present study (see fig. 1). B, time course of DR-1 resulting from A assuming a monoexponential elimination of the antagonist. Abscissa: time in multiples of the elimination half-life ( $T_{1/2}$ ).

**Heart rate (fig. 3).** At the highest dose administered, isoprenaline increased maximally the heart rate in the placebo group by about 45 beats/min. Repeated isoprenaline infusions showed only minor variations in the response during the observation period: The DRs of isoprenaline calculated from the dose-response curves at different time points during the observation period were close to 1 (see inset of fig. 3A).

Administration of propranolol or bisoprolol induced a pronounced shift to the right of the isoprenaline dose-response curves, with propranolol being more effective than bisoprolol 3 hr after drug administration (fig. 3B). The decline in the baseline values was comparable to changes in the placebo group (see fig. 3A). The DR-1 calculated from the right shifts at various time intervals after drug administration is shown in the inset of figure 3C. From these data, together with the parallel assay of antagonist concentrations in plasma (see above), Schild-plots were constructed. The data from the propranolol group are well described by the identity line in this Schild-plot. The data after bisoprolol administration, however, yielded slopes of less than unity ( $0.67 \pm 0.16$ ; table 2). Thus, an extended model assuming two subtypes to participate in the antagonist effects was used to describe the data (equation 7 under "Methods"). From this model, a *beta*-2 adrenoceptor fraction of  $0.45 \pm 0.12$  was estimated to participate in isoprenaline-induced tachycardia.

**Diastolic blood pressure (fig. 4).** Isoprenaline lowered the diastolic blood pressure by 40 mm Hg at the maximum dose (fig. 4A). The antagonism brought about by propranolol in comparison to bisoprolol showed a pattern similar to the heart rate (fig. 4, B and C): A linear Schild-plot not different from the identity line was derived for the propranolol group. For the bisoprolol group, a slope of  $0.68 (\pm 0.19)$  was calculated from the Schild-plot. Thus, the subtype model already described for the heart rate data was used to describe these data. A *beta*-2 adrenoceptor fraction of  $0.23 \pm 0.12$  was estimated to participate in the isoprenaline response.

**Electromechanical systole ( $QS_{2c}$ ; fig. 5).** This parameter was chosen for further analysis, as it is an indicator of changes in inotropy (see "Methods" and "Discussion"). As shown in figure 5A, isoprenaline induces a marked shortening of the electromechanical systole (note that the data have been corrected for the increase in heart rate). The right shift of the isoprenaline dose-response curves 3 hr after propranolol and bisoprolol administration were identical (fig. 5B). In the Schild-

TABLE 1

#### Half-lives of antagonist elimination and decline of isoprenaline DR-1

Apparent half-lives ( $T_{1/2}$ ) were calculated using log-linear regression analysis. Mean parameters  $\pm$  S.E.M. ( $n = 6$ ) are given.

		Propranolol $T_{1/2}$	Bisoprolol $T_{1/2}$
		hr	hr
Antagonist elimination	<i>Beta</i> -1	$5.4 \pm 0.4$	$10.5 \pm 0.5$
from plasma	(fig. 1A)		
	<i>Beta</i> -2	$5.4 \pm 0.4$	— <sup>a</sup>
	(fig. 1B)		
Decline of isoprenaline	Heart rate	$5.1 \pm 0.5$	$17.1 \pm 0.8$
DR-1	(Fig. 3C)		
	Diastolic blood pressure	$3.9 \pm 0.5$	$12.6 \pm 0.7$
	(fig. 4C)		
	Electromechanical systole	$3.9 \pm 0.6$	$10.8 \pm 0.6$
	( $QS_{2c}$ ) (fig. 5C)		

<sup>a</sup> Not calculated.

TABLE 2  
Parameters calculated from the Schild-plots

The slopes (meters) and apparent  $K_i$  values were derived using linear regression analysis with equation 5. Apparent  $K_i$  values calculated from the Schild-plots are given in multiples of  $K_i$  (see fig. 1). The propranolol data can be explained by the identity line ( $P < .01$ ). The slopes in the Schild-plots of bisoprolol for heart rate and diastolic blood pressure were  $< 1$  ( $P < .05$ ). Therefore, from an extended model, the fraction of subtype 2 ( $s_2$ ) was calculated with equation 7. Mean parameters and 95% confidence intervals are given.

	Propranolol		Bisoprolol		
	Slope	Apparent $K_i$ value ( $K_i/K_{i1}$ )	Slope	Apparent $K_i$ value ( $K_i/K_{i1}$ )	$s_2$ (subtype 2 fraction)
	<i>m</i>		<i>m</i>		
Heart rate (fig. 3C)	1.16 (0.96–1.29)	1.16 (0.87–1.79)	0.67 (0.17–0.89)	1.12 (0.54–5.11)	0.45 (0.30–0.84)
Diastolic blood pressure (fig. 4C)	1.44 (0.81–1.69)	1.83 (1.27–4.16)	0.68 (0.10–0.91)	1.08 (0.48–8.01)	0.23 (0.11–0.70)
Electromechanical systole (QS <sub>2</sub> c) (fig. 5C)	1.11 (0.71–1.36)	1.75 (1.16–3.79)	0.90 (0.71–1.01)	0.72 (0.47–1.41)	0 — <sup>a</sup>

<sup>a</sup> Not calculated.

plot, data from both groups were explained adequately by the identity line with a slope of unity and an apparent  $K_i$  value not differing from 1.

**Antagonism vs. extrinsic and endogenous agonistic stimulus (fig. 6).** Exercise-induced tachycardia is the most common parameter for detecting  $\beta$  blockade in humans. In the present study the inhibition of exercise-induced tachycardia was monitored parallel to the above *in vitro* and *in vivo* measurements. From parallel time points after the administration of antagonist, the inhibition of exercise-induced tachycardia and the isoprenaline DR-1 data are shown in figure 6. A saturation isotherm precisely described the data (see equation 1 under "Methods"). Half-maximal reduction of exercise-induced tachycardia was observed at DR-1 values of the isoprenaline-induced tachycardia of 1.5 (propranolol) and 1.2 (bisoprolol). These parameters were not different from unity.

## Discussion

Numerous studies have been performed to quantify agonist effects at  $\beta$  adrenoceptors and relate such effects to receptor subtypes (for a review see e.g. Broadley, 1982; Brodde, 1987). The quantitative evaluation of the action of subtype selective antagonists using the Schild-plot technique have been described in the elaborate *in vitro* studies of Kaumann and co-workers (Kaumann and Lemoine, 1985; Kaumann and Marano, 1982; Lemoine and Kaumann, 1983; Lemoine et al., 1985) and reviewed by Kenakin (1984, 1985). In contrast to the *in vitro* studies, studies in humans are, however, characterized by poor quantitative evaluations. Only a few authors have described the effects of a wide range of antagonist doses or related the effects measured in humans to a wide range of plasma concentrations (e.g., Chidsey et al., 1976; Coltart and Shand, 1970; DeLeve et al., 1985; McDevitt and Shand, 1975). However, there seemed to be an insurmountable gap between the procedures of *in vitro* pharmacology and the approaches used in humans.

The purpose of the present study was to investigate whether or not a quantitative relationship between *in vitro* receptor occupancy and  $\beta$  blockade in humans can be established. Furthermore, we hoped to obtain a measure of  $\beta$  adrenoceptor subtype-mediated effects by using the rather nonselective propranolol (Fitzgerald, 1980) in comparison to the  $\beta$ -1 selective bisoprolol (Kaumann and Lemoine, 1985; Schliep and Harting, 1984; Schliep et al., 1986; Wang et al., 1985).

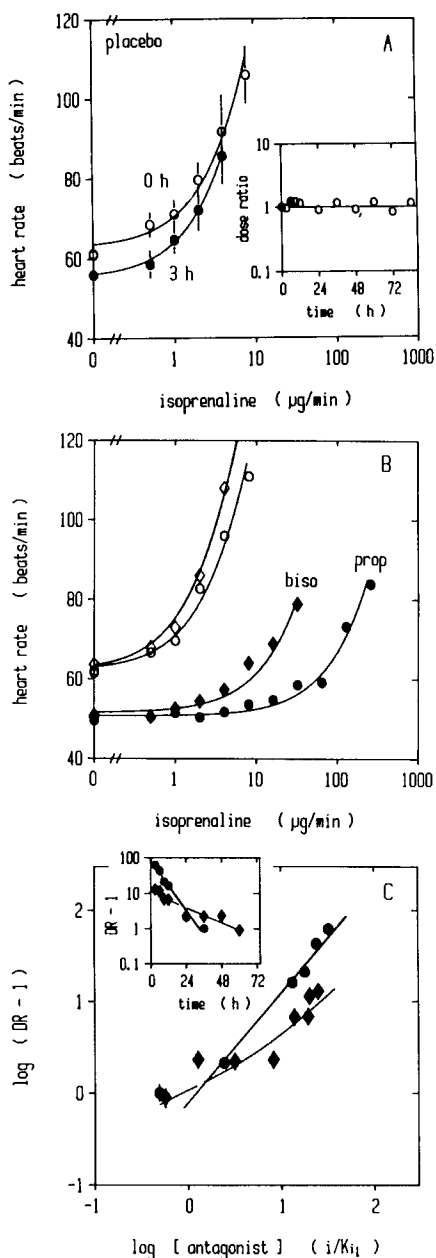
In the receptor binding studies, the hydrophilic radioligand

[<sup>3</sup>H]CGP 12177 was used in order to avoid interference of plasma protein binding (see Wellstein et al., 1984b). As a source of  $\beta$  adrenoceptors we used two preparations which contain either  $\beta$ -1 or the  $\beta$ -2 subtype only (rat salivary glands and reticulocytes, respectively; see Wellstein et al., 1986a). Monophasic competition isotherms of subtype-selective ligands in both membrane preparations indicated a homogeneous subtype population in both preparations. In these studies, bisoprolol had shown a 30- to 80-fold  $\beta$ -1 selectivity, propranolol a 1.5- to 3-fold  $\beta$ -2 selectivity and the investigational ligand ICI 118,551 a 100- to 300-fold  $\beta$ -2 selectivity. The selectivity ratios of the unlabeled ligands concurred with data from the literature using different experimental approaches (see e.g. Bilski et al., 1983; Gille et al., 1985; Harms, 1976; Hiatt et al., 1984; Lemoine et al., 1985; O'Donnell and Wanstall, 1980; Wang et al., 1985).

As expected from these selectivity profiles, receptor occupancy detected from plasma samples after propranolol administration shows a similar pattern for both  $\beta$  adrenoceptor subtypes. After bisoprolol administration,  $\beta$ -1-subtype occupancy is higher than occupancy of the  $\beta$ -2-subtype with respect to extent and duration (see fig. 1; right ordinates).

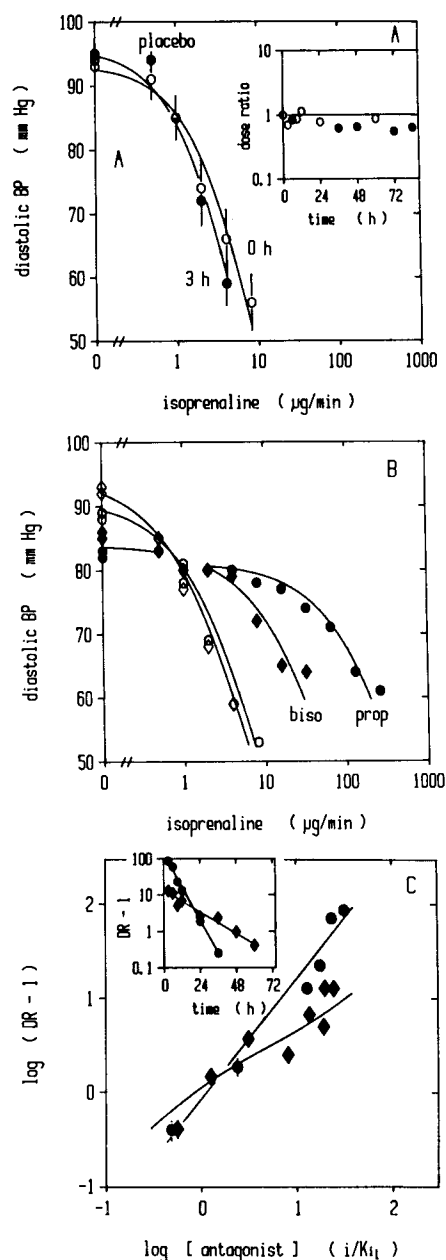
From the standard receptor theory, one would expect an agonist DR-1 of 1, if 50% of the receptors are occupied by the respective competitive antagonist (see Gaddum, 1957). In the propranolol group, the time interval with a 50% *in vitro* receptor occupancy (fig. 1A) coincides with the time intervals of an isoprenaline DR-1 of 1 (insets of figs 3C, 4C and 5C). The respective comparison of the bisoprolol data indicates agreement between agonist response and  $\beta$ -1-subtype occupancy. However, such comparisons are only partly valid, if a subtype selective antagonist is used to block the response of a nonselective agonist (see Kenakin, 1985). As shown by the simulations in figure 2, a deviation from linearity is expected in the Schild-plots with a subtype selective antagonist if both subtypes participate in the agonist response. To avoid the bias perhaps encountered with the single point comparison between 50% receptor occupancy and the respective DR-1, we submitted our data to a further Schild-plot analysis.

For the x-axis in the Schild-plots, plasma concentration data derived from the subtype selective radioreceptor assays were used. The assays were carried out with native plasma samples and thus respond to unbound  $\beta$  blocking agent only. This is irrespective of the presence of parent drug, active metabolites or perhaps enantioselective kinetics of the racemic drug mix-



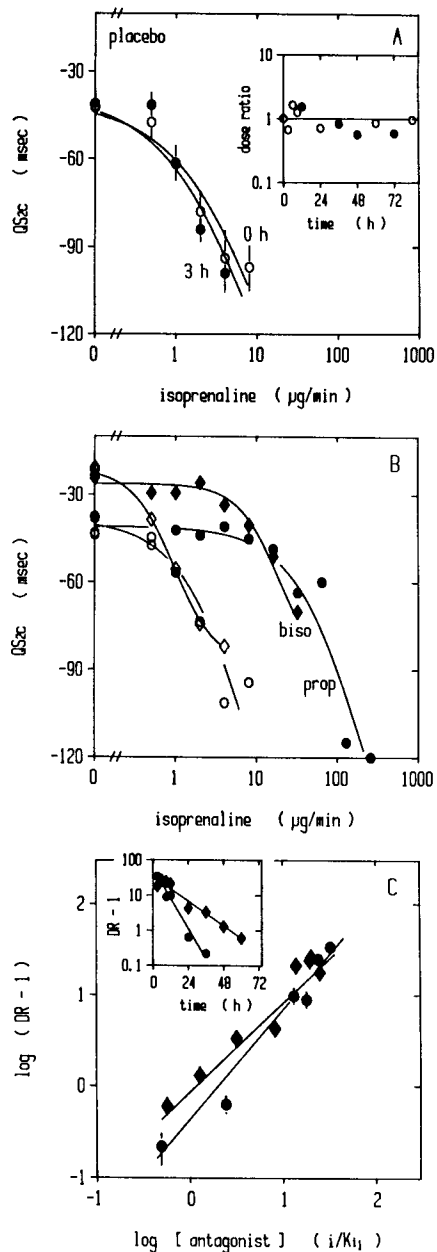
**Fig. 3.** Isoprenaline dose-response curves of the *heart rate* and the effects after placebo, propranolol (prop) or bisoprolol (biso) administration ( $n = 6$  in each group). A, Placebo: effect before (open symbols) and 3 hr after administration (closed symbols; means  $\pm$  S.E.M.). Inset: DR of isoprenaline at different time points after administration. Filled symbols indicate a DR different from unity ( $P < .05$ ). B, effect before (open symbols) and 3 hr after (closed symbols) administration of 240 mg of prop or 100 mg of biso. Only mean values are shown. C, Schild plot. Ordinate: isoprenaline DR-1 at different time points (see inset) after administration of prop ( $\bullet$ ) or biso ( $\blacklozenge$ ). Shown are mean values  $\pm$  S.E.M. Abscissa: antagonist concentration in plasma ( $i/K_{i1}$ ) at the respective time points (see fig. 1). Parameters of the functions are given in table 2 (inset, see table 1).

tures administered in the present study. Evaluation of the assays may therefore be biased, if competition isotherms of unlabeled *parent* drug are used as a standard (see Wellstein *et al.*, 1984b). We have tried to avoid the bias encountered with this standard evaluation method of the assays by using an alternative approach. Saturation isotherms of the radioligand were used to calculate unknown antagonist plasma concentra-



**Fig. 4.** Isoprenaline dose-response curves of the *diastolic blood pressure* (BP) and the effects after placebo, propranolol (prop) or bisoprolol (biso) administration ( $n = 6$  in each group). A, placebo: effect before (open symbols) and 3 hr after administration (closed symbols; means  $\pm$  S.E.M.). Inset: DR of isoprenaline at different time points after administration. Filled symbols indicate a DR different from unity ( $P < .05$ ). B, Effect before (open symbols) and 3 hr after (closed symbols) administration of 240 mg of prop or 100 mg of biso. Only mean values are shown. C, Schild plot. Ordinate: isoprenaline DR-1 at different time points (see inset) after administration of prop ( $\bullet$ ) or biso ( $\blacklozenge$ ). Shown are mean values  $\pm$  S.E.M. Abscissa: antagonist concentration in plasma ( $i/K_{i1}$ ) at the respective time points (see fig. 1). Parameters of the functions are given in table 2 (inset, see table 1).

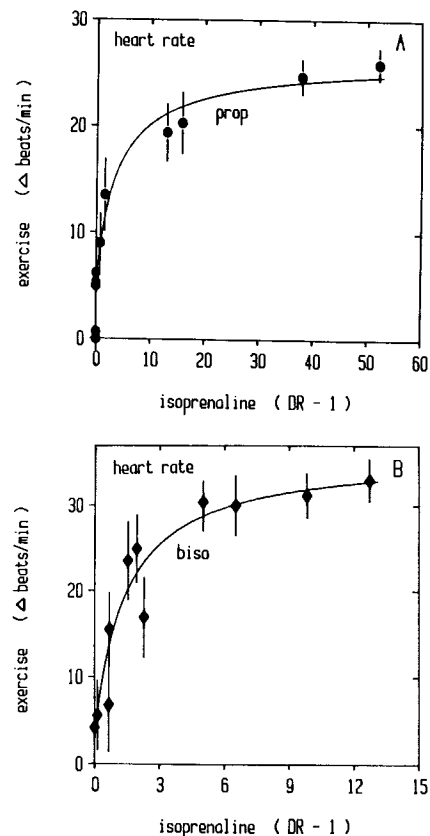
tions (equation 2 under "Methods"). Concentrations in plasma are then derived relative to the dissociation constant at the respective receptor subtype preparation used ( $=i/K_{i1}$  or  $i/K_{i2}$ ). A value of *e.g.*  $1/i/K_{i1}$  indicates that the concentration of unknown antagonist (or the mixture of parent drug and metabolites) present in the sample is sufficient to occupy 50% of  $\beta$ -1 adrenoceptors (see equation 3). Thus, a direct comparison of



**Fig. 5.** Isoprenaline dose-response curves of the *electromechanical systole* (QS<sub>2c</sub>) and the effects after placebo, propranolol (prop) or bisoprolol (biso) administration ( $n = 6$  in each group). A, placebo: effect before (open symbols) and 3 hr after administration (closed symbols; means  $\pm$  S.E.M.). Inset: DR of isoprenaline at different time points after administration. Filled symbols indicate a DR different from unity ( $P < .05$ ). B, effect before (open symbols) and 3 hr after (closed symbols) administration of 240 mg of prop or 100 mg of biso. Only mean values are shown. C, Schild plot. Ordinate: isoprenaline DR-1 at different time points (see inset) after administration of prop (●) or biso (◆). Shown are mean values  $\pm$  S.E.M. Abscissa: antagonist concentration in plasma ( $i/K_i$ ) at the respective time points (see fig. 1). Parameters of the functions are given in table 2 (inset, see table 1).

antagonist plasma concentrations after propranolol and bisoprolol administration can be carried out with respect to the *in vitro* pharmacologic activity at *beta* adrenoceptor subtypes.

From the left ordinates in figure 1, it is apparent that 3 hr after administration, 100 mg of bisoprolol are almost equipotent to 240 mg of propranolol with respect to *beta*-1-subtype blockade, but 50-fold less potent with respect to *beta*-2 blockade.



**Fig. 6.** Antagonism of propranolol (prop) (A) and bisoprolol (biso) (B) at heart rate obtained from extrinsic (isoprenaline) and endogenous (exercise) tachycardia. Ordinate: reduction of exercise-induced tachycardia (4 min of ergometry). Abscissa: DR-1 of isoprenaline effects on the heart rate. Data are taken from parallel measurements at different time points of drug administration (c.f., inset fig. 3C). The functions shown are based on a saturation isotherm (equation 1) giving the capacity ( $E_{max}$ ) in beats per minute and the apparent  $EC_{50}$  in multiples of DR-1. prop:  $E_{max} = 25.4$ ,  $EC_{50} = 1.5$ ; biso:  $E_{max} = 35.9$ ,  $EC_{50} = 1.2$ . Both  $EC_{50}$  values were not different from 1 ( $P < .05$ ).

Furthermore, about two decades of pharmacologically active antagonist concentrations with respect to *in vitro beta*-1 and *beta*-2 blockade are covered during the time course of propranolol elimination from the plasma compartment. For bisoprolol, this holds true for the *beta*-1-subtype. The *beta*-1 selectivity of bisoprolol calculated from the *in vitro* assay of the plasma samples is 35-fold and thus in the lower range of data from the literature (see above). Still, the question remains open, whether or not these plasma concentrations (despite the sophisticated detection method) have any predictive value in the Schild-plots with the *in vivo* data. This aspect will thus be discussed below.

From the Schild-plots of the *propranolol* data, a good agreement between the *in vitro* assay of plasma concentrations (in  $i/K_i$  units) and DR-1 values of isoprenaline in humans is observed (figs. 3C, 4C and 5C). DR-1 data for the heart rate, diastolic blood pressure and the electromechanical systole can be described by an identity line between *in vitro* and *in vivo* measurements ( $P < .01$ ; see table 2). This finding allows for several conclusions: 1) from the radioreceptor assays used, the *in vivo* antagonism titrated with isoprenaline can be predicted accurately. Thus, *in vitro* receptor occupancy of propranolol detected by these assays is indeed a direct measure for pharmacologic activity in humans. 2) Unbound propranolol present in the plasma compartment represents the concentration pres-

ent at *beta* adrenoceptors in humans. Distribution of this lipophilic drug beyond the plasma compartment is obviously irrelevant for its *beta* blocking agent activity. 3) Saturable uptake of isoprenaline (Ebner, 1981), saturable inactivation of the antagonist (Kenakin and Beek, 1987) or a decrease in isoprenaline clearance after propranolol (Ziegler *et al.*, 1986) do not considerably affect the *in vivo* data. 4) Equilibrium between agonist and antagonist receptor occupancy was obviously achieved (Kenakin, 1980). This is in agreement with the rapid steady state of response achieved during the isoprenaline infusion in humans and a dissociation half-life below 1 min detected in receptor binding studies with [<sup>3</sup>H]propranolol (A. Wellstein, unpublished results).

The Schild-plots of the *bisoprolol* data give a more complicated pattern as expected for a subtype-selective antagonist (see fig. 2). For the electromechanical systole (QS<sub>2c</sub>), the DR-1 data points are predicted 1/1 by the *in vitro* detection of plasma concentrations from the *beta*-1 subtype assay (see fig. 5C). For the heart rate and diastolic blood pressure, a slope below unity was observed (see figs. 3C and 4C; table 2). This result indicates that isoprenaline-induced effects at the electromechanical systole are due to a *beta*-1 adrenoceptor stimulation only: isoprenaline DR-1 can be predicted from the *in vitro beta*-1 adrenoceptor occupancy of the antagonist. For the heart rate and diastolic blood pressure, a participation of the *beta*-2 subtype is obvious. How do these findings correlate with the data from the literature?

QS<sub>2c</sub> is an elegant noninvasive parameter for quantifying inotropic responses in humans (see Lewis *et al.*, 1977; Stern *et al.*, 1984). Cardiac glycosides, as prototype inotropic drugs, induced a dose- and plasma concentration-dependent shortening of QS<sub>2c</sub> (Belz *et al.*, 1978, 1981). QS<sub>2c</sub> was not affected by the afterload reducing drugs urapidil and dihydralazine, despite a decrease in diastolic blood pressure comparable to the maximum isoprenaline response in the present study (Belz *et al.*, 1985; Stern *et al.*, 1984). The tachycardia after the administration of vasodilators was similar to the effects after isoprenaline. Thus, one can attribute the isoprenaline-induced shortening of QS<sub>2c</sub> to a rate- and load-independent inotropic effect of *beta* adrenergic stimulation. Furthermore, the isoprenaline-induced inotropic response in humans is due to a stimulation of *beta*-1 adrenoceptors only (see above). This conclusion is supported by studies of Corea *et al.* (1984) with subtype selective agonists in humans. Furthermore, *in vitro* studies with intact human tissues have related ventricular inotropy to *beta*-1 adrenoceptors (Gille *et al.*, 1985; Kaumann *et al.*, 1982), atrial contractile response, however, to both *beta* adrenoceptor subtypes (Zerkowski *et al.*, 1986).

In contrast to inotropy, isoprenaline-induced tachycardia obviously involves *beta*-1 and *beta*-2 adrenoceptors (see fig. 3C; table 2). From the analysis of the respective Schild-plots, the *beta*-2 subtype fraction participating in the agonist response was 0.45. A subtype-selectivity of isoprenaline can be ruled out from the data of Brick *et al.* (1968). These authors have reported that the chronotropic response of isoprenaline and adrenaline are similarly antagonized by the *beta*-1 selective practolol (ICI 50,172) and by propranolol. Furthermore, studies by Arnold *et al.* (1985) with the *beta*-2 selective ICI 118,551 have shown an antagonism *vs.* the isoprenaline-induced tachycardia at doses not affecting the exercise-induced tachycardia (see also: Tattersfield *et al.*, 1983). From these data, one can thus conclude that isoprenaline indeed stimulates the heart rate *via* both *beta*

adrenoceptor subtypes (see also: Brown *et al.*, 1986; Stene-Larsen *et al.*, 1986). The present study gives the first quantitative estimate of the participation of subtypes *in vivo*. Our studies with 200 mg of p.o. atenolol failed to give this information due the low *beta*-1 adrenoceptor occupancy achieved after this dose and the lesser degree of selectivity in comparison to bisoprolol (A. Wellstein, unpublished results).

For diastolic blood pressure, a participation of both *beta* adrenoceptor subtypes in the isoprenaline response also was observed from the bisoprolol data (see fig. 4C; table 2). A *beta*-2 fraction of 0.23 was calculated from the respective Schild-plot. Data on peripheral circulation (Hiatt *et al.*, 1984) also have shown the involvement of both *beta* adrenoceptor subtypes. The interdependence between diastolic blood pressure and peripheral resistance has been shown in studies with angiotensin as a vasoconstrictor (Belz *et al.*, 1987; Wellstein *et al.*, 1987) and with the vasodilators urapidil or dihydralazine (Belz *et al.*, 1985; Stern *et al.*, 1984). Thus, one should expect that the isoprenaline-induced decrease in peripheral resistance is modulated by a *beta*-2 fraction similar to the fraction involved in diastolic blood pressure. In the present study, only a rough estimate of the *beta* fraction participating in the isoprenaline-induced decrease of peripheral resistance was possible due to the scattering in the respective data ( $0.49 \pm 0.43$ ; not depicted).

Independent from the detection of antagonist, the estimate of subtype fractions calculated from the Schild-plots can be tested by the time-dependent decline of the isoprenaline DR-1 data (insets of figs. 3C, 4C and 5C; table 1). The simulated functions in figure 2B indicate for a nonselective drug or for only one subtype participating in the agonist response, that the decline of DR-1 data should be parallel to the elimination of antagonist (see also DeLeve *et al.*, 1985). After propranolol administration, DR-1 values indeed decline with apparent half-lives in agreement with antagonist elimination. After bisoprolol administration, the respective agreement is only apparent for the QS<sub>2c</sub> data, whereas for diastolic blood pressure a 1.2-fold and for heart rate a 1.7-fold overestimate is observed. The simulated functions in figure 2B can explain this result: for DR-1 values between 1 and 30 of a 35-fold selective antagonist, a log-linear decline with time approximates the functions. From this approximation one would expect increasing apparent half-lives of DR-1 (relative to the half-life of antagonist elimination), if the fraction of subtype 2 approaches 0.5. Although this analysis is rather qualitative, it supports the subtype fractions calculated from the Schild-plots (table 2).

The antagonism of isoprenaline-induced tachycardia in comparison to exercise tachycardia should allow us to estimate the fractional agonistic stimulus of the *beta* adrenergic system induced by the exercise procedure: it can be assumed that during the exercise procedure a fixed endogenous *beta* adrenergic stimulus is induced. This assumption is based on earlier studies with propranolol (Wellstein *et al.*, 1985a,b). In these studies, the concentration- and dose-dependent inhibition of exercise tachycardia by propranolol obeyed a simple isotherm for a competitive antagonist (Hill-slope of unity). In the present study, this result is confirmed inasmuch as the heart rate DR-1 data of isoprenaline and the reduction of exercise tachycardia can be described by a saturation isotherm according to the law of mass action (see fig. 6; equation 1 under "Methods"). If the exercise procedure induces a fractional stimulus below 0.1 (*i.e.*, below 10% of the maximum capacity of the *beta* adrenergic system), a 50% antagonist receptor occupancy should inhibit



the exercise tachycardia by about 50% (see Gaddum, 1957). As shown in figure 6, 50% inhibition of exercise tachycardia by propranolol and by bisoprolol coincide with isoprenaline DR-1 values of 1.5 and 1.2, respectively. These parameters were not different from 1. Thus, it seems likely to assume that the functional  $\beta$  adrenergic stimulus due to the exercise procedure is below 0.1.

In conclusion, the extent and time course (Druckrey and K pfm ller, 1949) of  $\beta$  adrenoceptor blockade in humans by propranolol can be predicted from *in vitro* receptor binding studies with plasma samples. With a subtype-selective antagonist, the application of a selective *in vitro* assay of plasma samples indicates the antagonism of *in vivo* effects mediated only by one subtype. With two subtypes participating in the agonist response, a refined analysis of Schild-plots can quantify the subtype fractions. Thus, the approaches established in basic pharmacology, *i.e.*, Schild-plots and receptor binding studies, can be recommended as very useful tools, as well as for studies in humans.

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